Playing hide-and-seek:

Duration, detectability and transmissibility of asymptomatic malaria infections in Ethiopia

Surafel Kefyalew Tebeje

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The research presented in this thesis was performed at the Department of Medical Microbiology at the Radboud University Medical Center in collaboration with The Armauer Hansen Research Institute in Ethiopia

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by

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CHAPTER 1

General Introduction

Malaria: Discovery and brief historical background

Throughout historical records and narratives, there is no infectious disease that shapes human life and even human evolution like malaria.

Malaria is caused by a unicellular protozoan parasite that was discovered by Charles Alphonse Laveran in 1880 while observing a patient's blood through a microscope [1]. The parasite is classified under the phylum *Apicomplexa* and genus *Plasmodium* [2]. More than 200 species of *Plasmodium* have been discovered of which four species commonly infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* (*P. ovale curtis*i and *P. ovale wallikeri*) and *Plasmodium malariae* [3]. A fifth species that causes morbidity in humans, *Plasmodium knowlesi* that naturally infects long-tailed macaque monkeys, was first reported as public health concern in Southeast Asia in 2009 [4]. For *P. knowlesi,* human-vector-human transmission is not yet confirmed. *P. falciparum* and *P. vivax* are the most prevalent human-infecting species and account for the majority of all malaria illness globally.

In 1897, Ronald Ross discovered that *Culicine* mosquitoes are vectors of avian malaria parasites; this led to the discovery of *Anopheles* mosquitoes as human malaria vectors by the Italian scientists Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Angelo Celli, Camillo Golgi and Ettore Marchiafava between the years 1898 and 1900 [1]. Female *Anopheles* mosquitoes serve as vectors to transmit the parasite to humans while looking for mammalian blood to complete the process of oogenesis [5]. While different *Plasmodium* species are transmitted by different *Anopheles* vectors, *P. falciparum* and *P. vivax* are mainly transmitted by *An. gambiae s.l* and *An. funestus* in Africa and by several other species of *Anopheles* mosquitoes in Asia-Pacific regions [6].

Life cycle of *P. falciparum* and *P. vivax*

The parasite life cycle in a human host is initiated when a female *Anopheles* mosquito discharges sporozoites from salivary glands into the skin during the process of probing to find a blood meal (**Fig.1**). While a large portion of these sporozoites are either drained by the lymphatic system [7] or phagocytosed by mucosal phagocytes [8], others find their way to the nearest capillaries to enter the circulation and migrate to liver where they infect hepatocytes.

In the liver, sporozoites multiply by several thousands in a process called exo-erythrocytic schizogony, to form the next developmental stages called merozoites [9]. A secondary dormant schizogony formation during *P. vivax* infections produces quiescent stages, called hypnozoites, that persist in the liver to allow the parasite's periodic release from the liver and invasion of erythrocytes [10]. The average duration from sporozoite entry to release of first batch merozoites into the blood circulation is estimated to be 6 - 8 days for *P. falciparum* [11] and 8 - 10 days for *P. vivax* [12].

Merozoites are then released back into blood circulation to infect red blood cells (RBCs) initiating an erythrocytic cycle. *P. falciparum* merozoites infect all stages of erythrocytes, whereas *P. vivax* prefers young RBCs called reticulocytes. In a RBC, a merozoite enters into a ~48 hour multiplication cycle while feeding on hemoglobin as the main source of nutrients with the formation of hemozoin as disposal product [13]. During the multiplication cycle, merozoites are vacuolated to become ring-stage parasites that further develop into trophozoites. Trophozoites are the most active parasite life stages in terms of metabolism, with a high rate of glycolysis and ~100 times faster glucose usage than normal RBCs [14]. *Plasmodium* trophozoites also lead to major deformations in the RBC, taking up nearly the entirety of the cell volume features at some time-point during the life cycle. *P. falciparum* remodels membranes of parasitized cells, and exports proteins to the outer surface of the RBC to facilitate attachment with endothelial cells of blood vessels [15] to prevent splenic clearance of parasitized cells. Trophozoites differentiate further and multiply, producing young merozoites in a process called erythrocytic schizogony [16, 17]. At the end of each 48-hour multiplication cycle, new progeny of merozoites are produced and released into the circulation by bursting infected RBCs. This is the step that initiates disease symptoms such as fever, headache, chill and anemia [18]. A portion of these merozoites (typically 1% – 5 %) differentiate and commit to become sexual stages called gametocytes; forming either male or female gametocytes [19]. Gametocytes are the only forms transmissible from humans to mosquitoes [20]. *P. falciparum* parasites produce gametocytes during a prolonged process. Immature gametocytes, termed stage I - IV early gametocytes (**Fig.1**), sequester mainly to the bone marrow before being released back into the circulation as mature stage V gametocytes [21] 8 to 10 days later [22, 23]. These mature gametocytes may remain in circulation for a mean duration of 3.4 - 6.5 days per gametocyte [24, 25] until naturally cleared or ingested by mosquitoes during a blood meal. In contrast to this long process

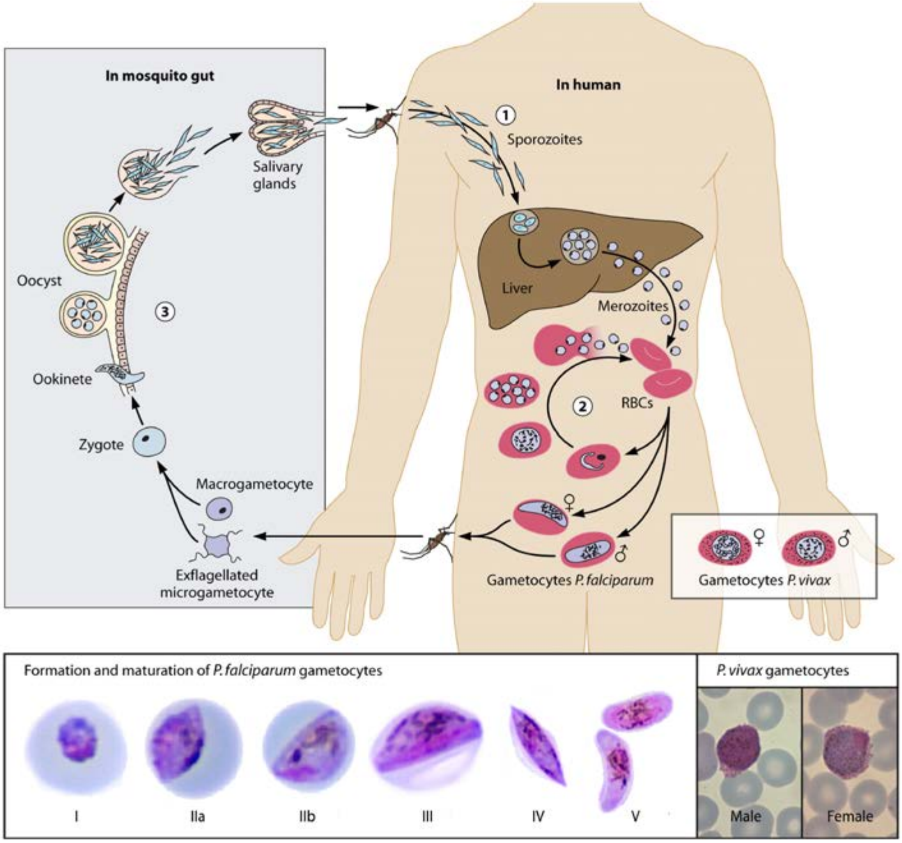

Figure 1. Life cycle of *P. falciparum* and *P. vivax.* [Top] Malaria parasites enter the human bloodstream in the form of sporozoites that are injected by infected female *Anopheles* mosquitoes taking a blood meal. Small portion of the sporozoites migrate to the liver, where they invade hepatocytes and multiply. Merozoites are formed that are released into the bloodstream, where they invade red blood cells, initiating the asexual multiplication cycle. A fraction of merozoites that invade red blood cells form gametocytes, the transmissible parasite form. For *P. falciparum*, the formation and maturation of gametocytes take place in five morphologically recognizable stages. Early-stage gametocytes are sequestered, and only mature stage V gametocytes circulate in the peripheral blood, where they can be taken up by mosquitoes. Once ingested by mosquitoes, gametocytes are activated to egress from RBCs, and each individual gametocyte forms 1 female macrogamete or up to 8 male microgametes. In the mosquito midgut, the fusion of gametes results in the formation of a zygote that develops into a motile ookinete that can penetrate the midgut wall to form oocysts. The oocysts enlarge over time and burst to release sporozoites that migrate to the mosquito salivary gland, rendering the mosquito infectious to human beings. [Bottom] The five developmental stages of *P. falciparum* gametocytes and mature *P. vivax* gametocytes. (Adapted with permission from Bousema T and Drakeley C. "Epidemiology and Infectivity of *Plasmodium falciparum* and *Plasmodium vivax* Gametocytes in Relation to Malaria Control and Elimination", Clin Microbiol Rev. 2011: 24 (2):377-410. The *P. falciparum* gametocyte photographs are reprinted from (Sharp, S. 2007. Ph.D. thesis. University of London, London, United Kingdom.) with permission; the *P. vivax* gametocyte photographs are courtesy of Debbie Nolder, Malaria Reference Laboratory, London School of Hygiene and Tropical Medicine, United Kingdom, reproduced with permission.)

for *P. falciparum*, *P. vivax* produces mature gametocytes as soon as erythrocyte infection is established, and they disappear from circulation within 2 to 3 days after asexual parasites are cleared [26]; whether they are sequestered in human tissues is not well established.

The mosquito phase of the life cycle starts when mosquitoes ingest gametocytes during blood meal. In a mosquito gut, male and female gametocytes egress from the infected RBCs following gut-related cues such as lower temperature [27] and acidic pH (xanthurenic acid) [28, 29], and form male micro- and female macro-gametes during a process called gametogenesis. Then, micro- and macro-gametes fuse into a diploid zygote which later elongates to form a motile ookinete [30]. The ookinetes penetrate and reside in the basal gut layer [31] and further develop into oocysts [32]. The oocysts undergo meiotic division which gets the parasite back to haploid phase [33]. When oocysts mature, parasites are released into the haemocoel as sporozoites 10 to 12 days post-infection depending on the temperature mosquitoes experience and their nutritional status; these sporozoites migrate to the salivary gland where they stay until they are discharged into the next host upon a bloodmeal.

Pathogenesis and burden of *P. falciparum* and *P. vivax* infections

P. falciparum and *P. vivax* parasite densities can vary considerably between and during infections. Higher parasitemia during infections are typically associated with clinical illness – termed symptomatic malaria. Malaria symptoms are prompted when merozoites are released into the circulation by bursting infected erythrocytes, resulting in hemolysis. Phagocytosis of merozoites by macrophages or dendritic cells in circulation or spleen initiates the production of TNFα, which along with other cytokines induces fever [34, 35]. Patients may also show other mild (or sometimes severe) symptoms including headache, nausea, chill, anemia and night sweats and experience uncomplicated malaria [36]. If untreated, symptoms may intensify and parasites, particularly of *P. falciparum*, can proceed to impair vital organs including kidneys, liver, spleen and the brain leading to severe malaria that can be fatal.

While *P. falciparum* and *P. vivax* have many shared characteristics, their main differences include severity of symptoms and parasite preference for specific erythrocytes. During *P. falciparum* infections, parasites infect RBCs and remodel their surface membranes [37] to express adhesive proteins such as PfEMP-1 (*P. falciparum* Erythrocyte Membrane Protein-1) [38]. PfEMP-1 mediates attachment of parasite infected erythrocytes with endothelial surfaces, platelets and other uninfected erythrocytes; this process can lead to the blockade of vascular vessels and intensification of illness severity. This enables the parasite to disappear from the circulation for nearly half of the asexual cycle and avoids phagocytic and splenic clearance [39]. In contrast to the ability of *P. falciparum* to infect mature RBCs and remodel RBC surfaces, *P. vivax* prefers to infect reticulocytes and does not generate an adhesive character of the RBC surface. In part because of the lower parasite densities – reticulocytes only comprise 0.5 - 2.5% of all RBCs in adults, allowing fewer RBCs to be infected by *P. vivax* – and absence of adherence of infected RBCs, symptoms associated with *P. vivax* infections are often not life-threatening. Nevertheless, severe infections have also been reported, particularly in young children [40, 41].

Clinical malaria patients represent small portion of all infections that are present in communities; the largest proportion of infections may pass without acute symptoms and are commonly called asymptomatic malaria infections. Although without acute symptoms and sometimes chronic, pathologic features in these 'asymptomatic infections' may in fact cause serious health conditions such as anemia [42], leukopenia and thrombocytopenia [43]. In addition, asymptomatic malaria infections are associated with increased levels of inflammation along with endothelial and platelet activation [44] which suggests that the term 'asymptomatic' could be misleading.

The World Malaria Report 2021 [45], reported that there were 227 million cases of clinical malaria and 558,000 attributed deaths in 85 malaria endemic countries in 2019, which increased to 241 million cases and 627,000 deaths in 2020, partially attributed to health system failures during the COVID-19 pandemic. Most of these cases and deaths occurred in children under five years in Sub-Saharan African countries. Due to the high prevalence of *P. falciparum* in Africa and *P. vivax* being the most common malaria in endemic areas outside Africa, these two species are the main etiologic agents of pathogenesis and burden of malaria globally [36]. The global morbidity and mortality estimates provide an important picture of the burden of malaria; however, they do not take non-clinical asymptomatic infections into account. This needs to be considered to get a more comprehensive picture of the burden of malaria infections.

Parasite detection methods

Microscopic slide examination of peripheral blood has long been the standard detection method for malaria parasites in endemic areas. This examination depends on stained thin and thick blood smears. The fixed thin smear is used to identify parasite species, whereas the thick smear is for quantification of parasites at increased sensitivity by not fixing cells but examining multiple layers of lysed red blood cells. Microscopy can detect parasite densities as low as ~5 parasites/μL under perfect conditions but in practice has a limit of detection around 100 parasites/μL [46]. The difference between the theoretical and practical threshold can be due to technical challenges in the field such as loss of parasites during washing and staining steps, staining quality, time-spent on slide reading and due to personal errors. Therefore, although a useful tool that remains a vital component of clinical decision making, microscopy readings can misdiagnose or miss malaria parasites. Microscopy also requires trained personnel and laboratory equipment and reagents, which make it un-deployable in some low-resource field settings.

Rapid diagnostic test strips (RDTs) are low-cost, easy-to-use, rapid and field-deployable diagnostics that were developed to meet the challenges of microscopy [47]. These strips detect parasites based on immunochromatography of antigen-antibody reactions where strips are coated with dye-labelled antibodies that recognize specific antigens of lysed parasites in a blood sample of suspected patient. Most of these RDTs have a detection limit of 50 -100 parasites/μL [48], and have advantages over microscopy in that they require less training and are applicable in resource limited settings such as in many parts of Africa where over 75% of all malaria diagnoses are made by RDTs [49, 50].

Currently, three parasite antigens are being used in conventional RDTs: *P. falciparum* histidine-rich protein (PfHRP), *Plasmodial* lactate dehydrogenase (pLDH) and *Plasmodial* aldolase [51]. HRPs are specific to *P. falciparum* which are functionally important to facilitate the process of polymerizing toxic heme to hemozoin [52]. Three distinct proteins of PfHRP, PfHRP-1, PfHRP-2 and PfHRP-3, have been identified [53-55]. PfHRP-2 is the most common target protein and it accounts for about 90% of all RDT-based parasite detections [56]. PfHRP-2 RDTs are coated with anti-PfHRP-2 antibodies that may also detect the closely-related protein PfHRP-3 [57]. In Ethiopia, PfHRP-2 RDT based malaria diagnosis was deployed in 2005 for the first time [58] mainly in resource limited areas of the country. This enables early diagnosis and treatment of malaria patients, and improves disease burden estimates of malaria surveys in the country. However, false-negative results associated with low parasite densities, antigen variation and HRP-2/-3 gene deletions are being reported [59].

HRP-2/-3 gene deletions have become a genuine diagnostic concern. PfHRP-2/-3 negative parasites are increasingly being detected in different malaria endemic regions since the first evidence of PfHRP-2/-3 gene deleted *P. falciparum* parasites was reported by Gamboa *et al.* in the Peruvian part of The Amazon river basin in 2010 [60]. In Ethiopia, heterogeneous prevalence levels of PfHRP-2/-3 gene deletions have been reported in different regions [61-64]. The highest prevalence of PfHRP-2 gene deletion, 15%, was reported from Tigray region by Feleke *et al.* [63]. The Tigray region shares borders with Eritrea that recorded a 62% prevalence of the gene deletions [65]. In addition, high levels of PfHRP-2/-3 gene deletions have been reported in Djibouti [66]. This may reflect the concentration of PfHRP-2 gene deleted malaria parasites in Eastern Africa. The WHO recommends a change to non-HRP-2 RDTs when the local prevalence of PfHRP-2/-3 gene deleted malaria parasites is >5% [67]. Therefore, the suitability of PfHRP-2 RDTs in Ethiopia and other Eastern African countries and alternative diagnostic targets need to be considered. Contrasting with PfHRP-2/-3 gene deletions, the interpretation of positive RDTs is affected by the persistence of PfHRP-2 antigens in peripheral blood after parasite clearance [68, 69]. Although advantageous to (indirectly) detect sequestered parasites, this characteristic can lead to false-positive results where tests remain positive for several weeks after parasites are cleared from the circulation.

As an alternative, pLDH and plasmodial aldolase have been tested as target antigens. Both proteins are enzymes involved in the parasite’s glycolytic pathway. Although not as commonly used as PfHRP-2, several studies evaluated the ability of pLDH based RDTs in detecting malaria parasites [70-73]. pLDH is a well conserved protein with an epitope that is expressed in all human infecting *plasmodia* [74]. However, pLDH RDTs were reported to perform poorly at low parasitemia [75, 76]. Compared to PfHRP-2 and pLDH, the performance of aldolase based RDTs in detecting malaria parasites is not well investigated. As the aldolase gene is highly conserved across the different human malaria parasite species [77], aldolase based RDTs could be promising diagnostic tools in areas where multiple species co-exist. Nevertheless, some studies [78, 79] reported low sensitivity of aldolase based RDTs which may be due to low expression levels of the antigen during infection or related to the RDT manufacturing processes [80, 81].

Thus, despite concerns such as their poor sensitivity for detecting low-density parasites and false-negative results associated with antigen variation and/or HRP-2/-3 gene deletions, RDTs remain important diagnostic tools as alternative to microscopy in resource limited field settings.

Nucleic acid amplification tests such as (quantitative) polymerase chain reaction (PCR or qPCR) enables detection of low-density malaria parasites that are below the detection limit of microscopy/RDT [82]. The limit of parasite detection depends on sample input and the selected targets. In microscopy/RDT, only small amount (0.025 - 0.0625 µL) of blood is examined which can reliably detect parasite densities in the range of 50 – 100 parasites/µL [83]. Most qPCRs allow examination of much larger blood volume compared to microscopy and RDT, and target the most abundant parasite's 18S ribosomal RNA (rRNA) to amplify and quantify parasites [84-86]. As a result, qPCRs have the potential of detecting parasite densities between 0.02 – 1 parasites/µL, depending on the blood volume used, extraction efficiency, etc. [87]. However, the requirement of expert personnel and well-established laboratory resources hamper their application as routine diagnostic methods in endemic areas.

Treatment of malaria and elimination efforts in Ethiopia

Treatment of malaria should strictly be preceded by standard clinical and laboratory diagnosis as malaria shares many of its symptoms with other infectious diseases. For the treatment of *P. falciparum* malaria, the WHO malaria treatment guideline [36] recommends combination therapy with an artemisinin component to ensure high efficacy of treatment. Accordingly, in Ethiopia, the current first line of drugs for treating uncomplicated *P. falciparum* infections is Artemether-Lumefantrine (AL) [88]. In addition to clearing asexual parasites, AL can also kill early-stage gametocytes [89] while being less effective against mature gametocytes that are capable to transmit to mosquitoes [20].To prevent transmission of parasites to mosquitoes, a single dose of primaquine along with AL can be prescribed except for pregnant and breast feeding women and infants <6 months. For complicated *P. falciparum* infections, intravenous Artesunate or quinine is recommended [90]. For malaria caused by *P. vivax*, where drug resistance to non-artemisinins is currently less of a concern, a combination of Chloroquine and Primaquine radical cure is the first line of treatment. In the case of mixed infections of the two species, AL is given [90]. To prevent development of drug-resistant parasites, accurate diagnosis and efficacious drug prescriptions and continuous surveillance of drug resistance is required.

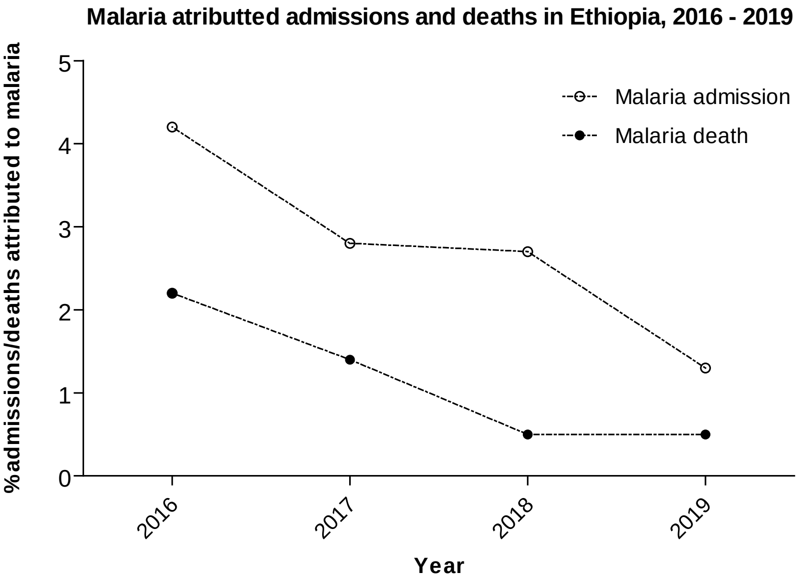

Figure 2. Malaria attributed admissions and deaths in Ethiopia, 2016 – 2019. Percentages were calculated as malaria attributed hospital admissions and/or deaths out of all hospital admissions and/or deaths for each year. (Adapted from "Ethiopian Malaria Elimination Strategic Plan: 2021-2025", Ministry of Health, Addis Ababa, Ethiopia, 2020)

The progressive reduction and control of malaria transmission in the past few years motivated the government of Ethiopia to aim for elimination by 2030 [91] (**Fig.2**). The country's malaria elimination strategic plan 2021-2025 aims to decrease malaria morbidity and mortality by 50% from baseline 2020, and achieve zero indigenous malaria in districts with annual parasite index [92] <10, and prevent reintroduction [91]. API is defined as the number of confirmed new malaria cases in a specific year and geographical area, expressed per 1,000 individuals under surveillance. To achieve zero indigenous malaria in low-API settings, the current concerted efforts of the country towards achieving this objective are centered around detecting and treating all (not only clinical) infections and implementing effective vector control strategies. The country implements regular nationwide malaria epidemiological surveys to detect and treat malaria infections, and to update its strategic plan in controlling transmission [93]. These surveys are crucial in identifying malaria transmission hot spots and reservoirs of high parasite carriage which in turn helps to rationally allocate resources to intensify the elimination activities.

In parallel, the country is focused on vector control activities such as indoor (IRS) and outdoor spraying of long lasting insecticides in malarious areas to reduce the vector population [91]. In addition, concerted national activities in distributing long lasting insecticide treated bed nets (LLINs), along with monitoring their appropriate use, are a central component of control and elimination effort [91].

Epidemiology and detectability of asymptomatic *P. falciparum* and *P. vivax* infections

Asymptomatic *P. falciparum* and *P. vivax* infections are more common across endemic areas than previously thought and may harbor varying parasite densities. Parasite densities in these infections can be partially controlled by effective anti-blood stage immunity [94] which is acquired after repeated infections [95]. The age at which effective anti-blood stage immunity is acquired (if at all) is thus dependent on transmission intensity in different settings. Due to this immunity, asymptomatic infections may persist with low parasite densities for several days, weeks, months or even years without causing symptoms. In areas where malaria transmission is seasonal, infections may persist during the long dry months and serve as infection sources for the next rainy season [96]. This suggests that asymptomatic infections have a substantial contribution for sustaining malaria transmission and have clear relevance for malaria elimination efforts that may need to detect and target all infections to drive malaria down to zero.

The advent of molecular methods, such as PCR, that have higher sensitivity than microscopy/RDT increased our understanding of the extent to which asymptomatic infections are prevalent and detectable. A previous meta-analysis of 106 surveys from different endemic settings estimated that microscopy misses on average 54.1% of all *P. falciparum* detected by PCR [97]. RDTs can also miss ~41% of asymptomatic *P. falciparum* that are detected by PCR [98]. This could be a significant challenge for the malaria elimination efforts if these missed infections are relevant for onward transmission. Importantly, the detectability of parasites by either microscopy or RDT varies among surveys [97] which may depend on local factors. In high endemic areas where malaria prevalence is $\geq$75% by microscopy, only ~20% of asymptomatic *P. falciparum* infections are submicroscopic; whereas in low endemic areas where malaria prevalence is <10%, the proportion of asymptomatic infections that is submicroscopic increases to 80-90% [99]. Further strengthening these previous estimates, a recent analysis estimated that the detectability by microscopy/RDT of all PCR-positive *P. falciparum* infections decreases from 49% in medium-high transmission areas to 14% in low transmission areas [100]. Though insightful, these estimates define the broad global prevalence and detectability of asymptomatic *P. falciparum* infections and typically involve multiple countries and lack detailed comparisons within a locally defined region. In addition, while several studies reported that asymptomatic *P. vivax* infections are prevalent, and large proportions are undetectable by microscopy/RDT [101-103], there is still a scarcity of data on the size of the submicroscopic reservoir of *P. vivax* infections.

Similarly, factors that may be related to differences in detectability of asymptomatic malaria by microscopy/RDT in different settings are not well characterized. Parasite exposure and the level of acquired immunity may explain global patterns partially. In high endemic areas where there is repeated parasite exposure, parasite densities may remain low as a result of immunity that allows infections to occur without causing clinical symptoms but at densities that may still be detectable by microscopy. In contrary, in low endemic areas that were previously of higher endemicity but where recent malaria control or elimination efforts were successful, ongoing exposure is less but lingering acquired immunity could limit parasite densities to a level that microscopy cannot detect. Alternatively, individuals who may reside in pockets of high malaria transmission [104] within a larger geographical area that historically has been low endemic can develop immunity and harbor parasites undetectable by microscopy. Another factor that may influence detectability of these infections in low endemic areas is the presence of low parasite genetic variation that allows a rapid acquisition of immunity to genetically similar parasites and keep parasite densities below the detection threshold [105, 106].

In Ethiopia, community surveys of *Plasmodium* infections involving molecular methods emerged very recently. Implementation of molecular methods demonstrated the presence of a large reservoir of asymptomatic malaria infections [107-110], and a substantial number of infections that are missed by microscopy/RDT [108, 111]. This enabled to landscape the nationwide malaria epidemiology and risk stratification map of the country (**Fig.3**) This could be a significant hurdle for the country's malaria elimination effort that relies on microscopy/RDT as routine diagnostic methods during community surveillance. Though insightful, these studies were performed separately by different teams in different epidemiological settings. This contributes to the current lack of nationwide data that directly and consistently investigates and compares performance of microscopy/RDT in describing the epidemiology and detectability of asymptomatic malaria infections in different settings in Ethiopia.

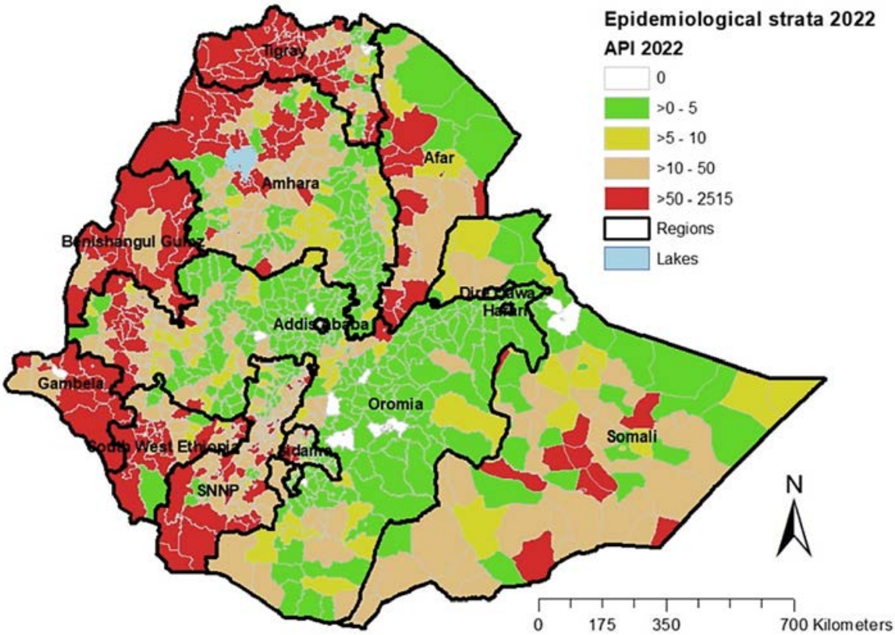

Figure 3. Map of malaria epidemiology in Ethiopia, 2022 (Adapted from the U.S. President's Malaria Initiative (PMI) Ethiopia malaria profile 2023). API = Annual Parasite Index.

Parasite density oscillations, gametocyte production and transmissibility of asymptomatic *P. falciparum* and *P. vivax* infections

As described above, asymptomatic infections of *P. falciparum* and *P. vivax* are prevalent in all malaria endemic settings, and especially in low endemic areas where parasite densities may be low. Parasite densities in these infections may fluctuate over time due to unknown factors. Some infections that initially are of low parasite density may increase over time and become symptomatic [112] and/or microscopy detectable [113, 114]. Some other low-density asymptomatic infections may be spontaneously cleared from blood circulation within weeks [115-117] while some others can persist for many months without causing apparent symptoms [118]. Therefore, the duration of asymptomatic malaria infections can range from days to months due to unidentified factors [117, 119]. The duration of asymptomatic *P. falciparum* and *P. vivax* infections may also vary depending on transmission settings. The average duration of particular genotypes of *P. falciparum* infections has been estimated at 6 months in high [94, 118, 120] and 2 months in low endemic settings [68, 119], although a decade-long *P. falciparum* infection has also been reported in incidental infections during blood transfusion [121]. In *P. vivax* infections, the average duration of infection was estimated to be up to six months in a low endemic setting in Asia [119]. Currently, our understanding of oscillations and durations of asymptomatic malaria infections mainly comes from the above few studies that primarily focused on *P. falciparum* infections. Thus, this calls for more longitudinal studies with frequent sampling that can adequately capture the fluctuation of parasite densities and duration of asymptomatic malaria infections.

It is currently unclear to what extent parasite density oscillations have implications for gametocyte production and infectiousness to mosquitoes. Time since infection appears to be an important factor for gametocyte production [112]. In general, the longer the persistence of asymptomatic infections, the higher the chance that parasites commit to gametocytes. However, detecting gametocytes in circulation does not guarantee parasite transmission to mosquitoes [122-124] indicating the role of other factors such as gametocyte sex ratio, gametocyte maturity and anti-gametocyte immunity. There is a clear and predictable positive association between *P. vivax* asexual parasites and gametocytes densities [125] suggesting a well synchronized production of gametocytes following asexual blood stage establishment. This association is not always observable during *P. falciparum* infections[94, 126].

Transmission of gametocytes from humans to mosquitoes is commonly measured using two methods: direct skin feeding (DSF) and ex vivo direct membrane feeding assays (DMFAs) [127, 128] (**Fig. 3**). In DSF assays, female *Anopheles* mosquitoes are allowed to feed on the arms, inner thighs or calves of a volunteer individual [129, 130] (**Fig. 3**). During DMFA, venous blood of a volunteer individual that is kept warm on a glass feeder is offered to female *Anopheles* mosquitoes (**Fig. 3**). In both methods, after blood meal, mosquitoes are maintained for a required period of time at optimal temperature and humidity. To assess transmission of parasites, mosquitoes are dissected at 10 to 12 or 7 to 9 days post-feeding for *P. falciparum* and *P. vivax* infections, respectively, by staining midguts with mercurochrome to detect oocysts [131, 132]. DMFAs are often challenged by technical constraints such as keeping blood temperature constant between the time of collection and mosquito feeding. Comparative studies reported contradicting results between the two methods regarding mosquito feeding efficiency and infectivity which could be related to the technical constraints in DMFAs. While some studies reported that DSF had higher mosquito feeding and infectivity rates than DMFAs [127, 133, 134], others reported similar measurements in both methods [130, 135]. Such contradicting results would not allow comparisons between studies in different settings. Therefore, currently, there is an effort to identify sources of variation between the two methods that may lead to the optimization and standardization of the mosquito infectivity measurement methods [133, 136]. Factors such as mosquito age, duration of mosquito starvation prior to feeding, and time of day for direct skin feeding (dawn or dusk) were found to be associated with mosquito feeding and infectivity rates [136]. These findings, however, await confirmation from other studies. Although challenging, DMFAs are important tools serving as surrogate assays for DSF that often cause discomfort in participating individuals and are ethically debated.

Several studies reported that asymptomatic low density infections can produce gametocytes and infect mosquitoes using DMFAs [113, 124, 125, 137, 138], although their contribution for the onward malaria transmission at population level is not well established and not without debate. Findings on a relevant transmission role of asymptomatic infections were contradicted by a small number of studies that observed hardly any infected mosquitoes after feeding on blood of asymptomatic parasite carriers [139, 140]. There is also uncertainty about the relative importance of microscopic and submicroscopic asymptomatic infections for transmission. A meta-analysis of eight studies with mosquito feeding assays of asymptomatic *P. falciparum* infections estimated that an individual with subpatent infections on average can be a third (0.348 times) as infectious as a microscopy positive individual [100]. Considering the high prevalence of subpatent infections in low-endemic areas, their contribution to the infectious reservoir can be considerable. Similarly, a recent meta-analysis predicted that submicroscopic *P. falciparum* infections account for 17.5% to 68% of onward malaria transmission in areas which are (and were) of low transmission; but their contribution becomes less important in areas where transmission declined recently [141]. In Ethiopia, one study by Tadesse and colleagues [113] reported that asymptomatic microscopy-detected *P. vivax* infections were responsible for 76.2% of the infectious reservoir in low malaria endemic area while PCR-detected asymptomatic infections were responsible for 15.8%. While these studies valuably suggest a substantial contribution of asymptomatic sub-patent malaria infections to the infectious reservoir, at their cross-sectional design means that they lack temporal information with regard to infection dynamics and infection duration in relation to infectiousness. Therefore, cohort follow-up studies with frequent sampling for parasite quantification, complemented with DMFAs [142], will increase our understanding of the relevance of asymptomatic infections for transmission.

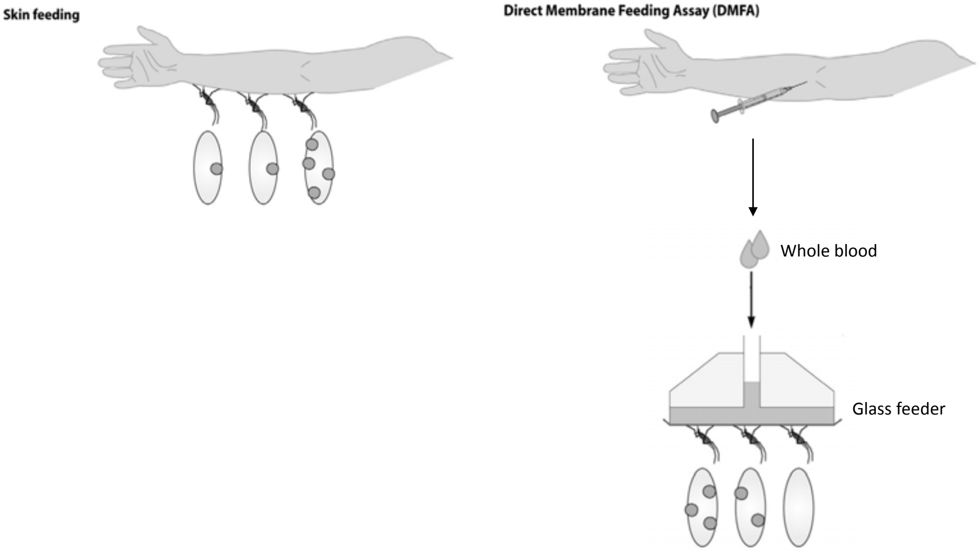

Figure 4. Direct skin and membrane mosquito feeding assays. In direct skin feeding assays, female Anopheles mosquitoes are allowed to feed on the skin of a volunteer individual for 10 minutes. During DMFA, venous blood of a volunteer individual is immediately placed on a special warm-water-jacketed glass feeder that is covered by a membrane at the bottom. The warm-water-jacket helps to continuously keep the blood temperature at ~37°C preventing ex-flagellation of male gametocytes. Then, the warm blood on glass feeder is supplied to female Anopheles mosquitoes. In both methods, after blood meal, mosquitoes are maintained for a required period of time in optimum room temperature and humidity. To confirm transmission of parasites, mosquitoes are dissected at 10 to 12 or 7 to 9 days post-feeding for *P. falciparum* and *P. vivax* infections, respectively by staining midguts with mercurochrome to detect oocysts. (Adapted with permission from Bousema T and Drakeley C. "Epidemiology and Infectivity of *Plasmodium falciparum* and *Plasmodium vivax* Gametocytes in Relation to Malaria Control and Elimination", Clin Microbiol Rev. 2011: 24 (2):377-410)

Naturally acquired immunity to sexual stages of *P. falciparum* and *P. vivax*

Naturally acquired immunity against malaria parasites can control parasitemia in the circulation which in turn may impact clinical outcomes, detectability of parasites by routine diagnostic methods, parasite density oscillations and gametocyte production. This immunity is developed after repeated exposure to infections in endemic areas. As a consequence young children are generally most at risk of severe infections [143]. Because of their higher parasite densities, young children were also considered as important contributors of parasite transmission as they commonly harbor (high densities of) gametocytes [144, 145].

Naturally acquired immunity against sexual stage parasites is a well-documented biological feature and has been associated with reduction of human-to-mosquito transmission of parasites. The best studied antigens in this context are the gametocyte/gamete antigens of *P. falciparum* Pfs48/45 and Pfs230 that were identified as targets of transmission blocking antibodies in rodents that were immunized with *P. falciparum* parasites [146-148]. The prevalence of naturally acquired antibodies against Pfs48/45 and Pfs230 is associated with reduced transmission of malaria parasites from humans to mosquitoes in various endemic settings [149-153]. The empirical evidence for the functional reduction of parasite transmission from humans to mosquitoes due to these antibodies, however, came from a study by Stone et. al. [154]. The *P. vivax* orthologues of these antigens, Pvs48/45 and Pvs230, can also induce antibody responses in vaccinated animals that reduce mosquito infections [155, 156], but information about naturally acquired antibodies against these *P. vivax* targets in humans is lacking. Immunity against other sexual stage antigens and the potential in reducing/blocking parasite transmission to mosquitoes is under investigation. A detailed review of naturally acquired immunity against sexual stages of *P. falciparum* and *P. vivax* is presented in Chapter 4.

Thesis outline and objectives

The overall characteristics of malaria have, for long, been described based on symptomatic infections that are commonly accompanied with fever, chill and nausea. Extensive data that emerged in the past decades, however, show that symptomatic infections are just the tip of the iceberg, and that asymptomatic malaria infections often comprise the largest portion of malaria infection burden. Asymptomatic infections caused by *P. falciparum* and *P. vivax* are more prevalent than previously thought and are prevalent in all transmission settings. Many countries, including Ethiopia, are currently aiming for elimination of malaria in the coming few years, and whether asymptomatic malaria infections should be included as targets for control efforts is increasingly becoming a center of scientific discussions. We need concrete data to make decisions on these front. Scientific information regarding the epidemiology of asymptomatic malaria infections in different transmission settings is piling up, despite the fact that we still need more data to strengthen our understanding of these infections related to their clinical features, prevalence, duration, parasite density oscillations, gametocyte production and transmissibility to mosquitoes, and their importance for the onward transmission of malaria. In addition, information regarding the intrinsic and extrinsic factors that influence the parasite's biology is essential.

Therefore, this thesis aims to contribute insights regarding the epidemiological, laboratory and field information on asymptomatic *P. falciparum* and *P. vivax* infections in Ethiopia relative to their symptomatic counterparts.

In **Chapter-2, "Epidemiology and detectability of asymptomatic *P. falciparum* and *P. vivax* infections in low, moderate and high transmission settings in Ethiopia"**, we described and directly compared the epidemiology and detectability of asymptomatic *P. falciparum* and *P. vivax* infections in low, moderate and high transmission settings of selected sites in Ethiopia by three diagnostic methods: microscopy, RDT and qPCR. Although with vital information, this study and several of the previous investigations on asymptomatic malaria infections are cross-sectional in design, thus only providing a snap-shot of insights by lacking temporal information. Therefore, we extended our investigation to the temporal characteristics of asymptomatic *P. falciparum* and *P. vivax* infections in a low-endemic area in Ethiopia in **Chapter-3, "Dynamics of asymptomatic *Plasmodium falciparum* and *Plasmodium vivax* infections and their infectiousness to mosquitoes in a low transmission setting of Ethiopia: a longitudinal observational study"**. Here, more detailed longitudinal information is provided such as parasite density oscillations, detectability, duration of infections, gametocyte production and infectivity to mosquitoes of asymptomatic *P. falciparum* and *P. vivax* infections in a low-endemic area of Ethiopia, Adama.

Naturally acquired immunity against malaria parasites are among the main factors that influence parasite density and their temporal oscillation, detectability, duration of infections, gametocyte production and infectivity to mosquitoes. Naturally acquired immunity against sexual stages, in particular, can be responsible for limiting gametocyte transmissibility to mosquito vectors. In **Chapter-4, "Naturally acquired immunity to sexual stage of *P. falciparum* and *P. vivax* infections**", we extensively reviewed the existent literature regarding the evidence for naturally acquired immunity against *P. falciparum* and *P. vivax* sexual stages, and their relevance in modulating the human-to-mosquito transmission. Although a large portion of the evidence is for *P. falciparum*, which justifiably received more focus due to its severity of infections, naturally acquired immunity against *P. vivax* sexual stages was also evidently demonstrated. In **Chapter-5, "Naturally acquired antibodies to gametocyte antigens are associated with reduced transmission of *Plasmodium vivax* gametocytes to *Anopheles arabiensis* mosquitoes"**, we investigated the existence of naturally acquired immunity against *P. vivax* gametocyte antigens in an Ethiopian population for the first time, and described the association between the prevalence of naturally acquired antibodies against selected *P. vivax* gametocyte antigens and infectivity to mosquitoes in two moderate transmission settings of Ethiopia.

Finally, all findings and their interpretation are discussed and placed into the context of other works in **Chapter-6, "General discussion**".

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CHAPTER 2

The Epidemiology and Detectability of Asymptomatic *Plasmodium vivax* and *Plasmodium falciparum* Infections in Low, Moderate and High Transmission Settings in Ethiopia

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Abstract

Background: As countries move to malaria elimination, detecting and targeting asymptomatic malaria infections might be needed. Here, we investigated the epidemiology and detectability of asymptomatic *Plasmodium falciparum and P. vivax* infections in different transmission settings in Ethiopia.

Method: A total of 1093 dried blood spot (DBS) samples were collected from afebrile and apparently healthy individuals across ten study sites in Ethiopia from 2016 to 2020. Of these, 862 were from community and 231 from school based cross-sectional surveys. Malaria infection status was determined by microscopy or rapid diagnostics tests (RDT) and 18S rRNA based nested PCR (nPCR). The annual parasite index (API) was used to classify endemicity as low (API>0 and<5), moderate (API ≥5 and <100) and high transmission (API≥100) and detectability of infections was assessed in these settings.

Results: In community surveys, the overall prevalence of asymptomatic *Plasmodium* infections by microscopy/RDT, nPCR and all methods combined was 12.2% (105/860), 21.6% (183/846) and 24.1% (208/862), respectively. The proportion of nPCR positive infections that was detectable by microscopy/RDT was 48.7% (73/150) for *P. falciparum* and 4.6% (2/44) for *P. vivax*. Compared to low transmission settings, the likelihood of detecting infections by microscopy/RDT was increased in moderate (Adjusted odds ratio [AOR]: 3.4; 95% confidence interval [95%CI]:1.6-7.2, P=0.002) and high endemic settings (AOR=5.1; 95%CI=2.6-9.9, P<0.001). After adjustment for site and correlation between observations from the same survey, the likelihood of detecting asymptomatic infections by microscopy/RDT (AOR per year increase = 0.95, 95%CI=0.9-1.0, P=0.013) declined with age.

Conclusion: Conventional diagnostics missed nearly half of the asymptomatic *Plasmodium* reservoir detected by nPCR. The detectability of infections was particularly low in older age groups and low transmission settings. These findings highlight the need for sensitive diagnostic tools to detect the entire parasite reservoir and potential infection transmitters.

Keywords: *Plasmodium* infection, elimination, asymptomatic, transmission, nPCR, detectability, density distribution

Introduction

Following considerable successes in the control of malaria in the last two decades, progress plateaued or stalled in many settings in Africa [1]. Ethiopia runs a successful malaria control program [2] that makes it one of the four countries (together with India, Rwanda, and Pakistan) that continues to maintain the declining trend in malaria burden [3]. As a result, the country is on track for a 40% reduction in incidence (together with Rwanda, Zambia, and Zimbabwe) and malaria mortality rates (together with Zambia) by 2020 [1]. To guide elimination efforts that currently targets 239 selected districts, the National Malaria Control Program (NMCP) of Ethiopia stratified the country into four strata using district level annual parasite index (API) data from 2017 [4] as malaria-free (API, 0), low (API, 0–5), moderate (API, 5–100), and high (API, ≥100) [4]. Despite its value, the adopted stratification lacks granularity and is not able to capture relevant spatial and temporal heterogeneities in low endemic settings [5, 6]. The unique epidemiology of malaria transmission in Ethiopia; the presence of strictly seasonal transmission in some settings and perennial transmission elsewhere, as well as different levels of co-endemicity of *Plasmodium falciparum* and *P. vivax* [2], calls for the use of tailored approaches to characterize the epidemiology of malaria.

District level stratification that relies on malaria incidence data has limitations in settings where case numbers are extremely low. Incidence data are also sensitive to changes in care seeking behavior, rates of testing of suspected cases, and reporting completeness [7]. Screening approaches to determine the prevalence of (often asymptomatic) infections that are present in communities have great potential to define transmission intensity [8]. However, parasite prevalence estimates are greatly affected by parasite density distributions in communities that determine the detectability of infections by different diagnostics. Malaria elimination efforts may benefit from targeting all infections present in communities, irrespective of clinical presentation [9-11]. There is a growing body of evidence on the public health importance of asymptomatic malaria infections and their contribution to onwards malaria transmission in high [12, 13] and low transmission settings [13, 14]. Importantly, most asymptomatic infections detected in community surveys are of low parasite density and the proportion of all infections that are submicroscopic varies between settings [15]. Previous studies in Ethiopia detected a significant burden of asymptomatic *P. falciparum* and *P. vivax* infections [16-19]. These studies used different diagnostic techniques and sampling designs, making it difficult to compare parasite prevalence estimates or diagnostic performance indicators across settings. The aim of the present study was to understand the epidemiology of asymptomatic *Plasmodium* infections in different settings in Ethiopia and their detectability by microscopy, rapid diagnostics test (RDT) and molecular methods.

Methods and Materials

Study areas

The study was conducted in ten districts (woredas) encompassing different transmission settings (**Fig. 1**). Malaria transmission is highly heterogeneous in Ethiopia and transmission intensity varies spatially and temporally [20]. Study sites representing low (n=2), moderate (n=4), and high (n=4) transmission settings as per the national stratification were selected from five administrative regions (**Fig. 1**). Low transmission settings include Gomma and Babile districts from Oromia region. Moderate transmission settings include Bahir Dar Zuria and North Achefer districts from Amhara region and Arba Minch Zuria from the Southern region and Mao Komo from Benishangul region. High transmission districts were from Gambela (Lare and Abobo), Amhara (Jawi), and Benishangul (Meng) regions.

Study population and sample collection

Samples were collected in community and school-based cross-sectional surveys from 2016–2020. Specifically, community-based surveys were conducted at Abobo, Lare, Mao-komo, Menge, and Gomma districts in 2016, Babile district in 2018, and Arba Minch Zuria district in 2020. School based surveys were conducted at North Achefer, Bahir Dar Zuria, and Jawi districts in 2017. For the school-based surveys, students were randomly selected from elementary school students between the ages of 5 and 15, as described before [21] following protocols developed by Brooker and colleagues [22].

Prior to recruitment of participants for community surveys, sensitization was undertaken by teams that involve study team members, village-based health extension workers, malaria focal person of the district, local administrators, and elderly. The study purpose, procedure, risk, and benefit were explained in local language. After this first step, volunteer community members were invited to join the study upon obtaining informed written consent and enrolled in the study on first come, first served basis.

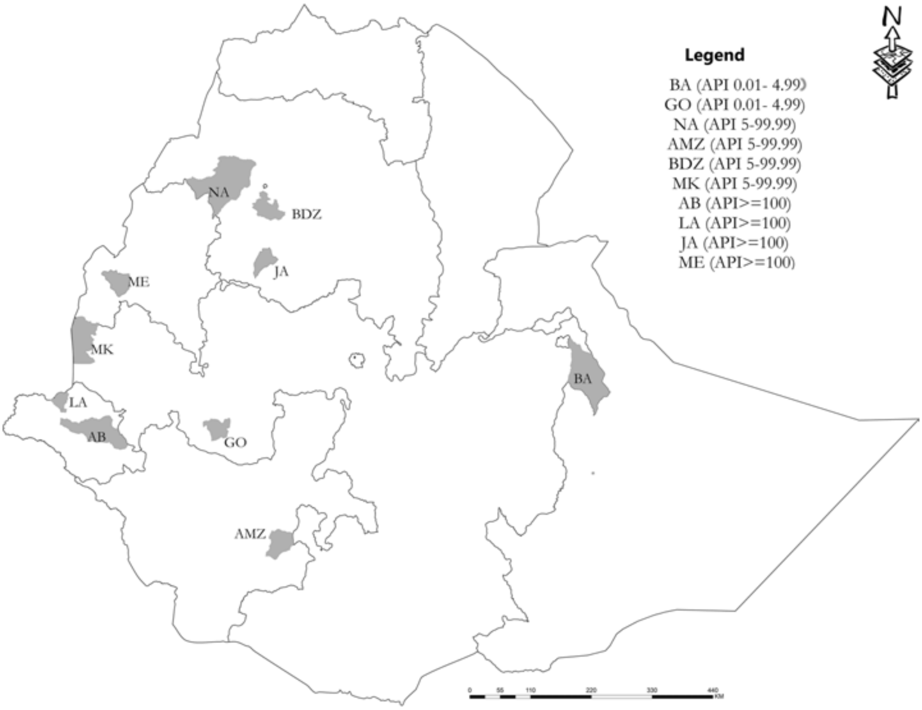

Figure 1. Location of study sites and their Annual Parasite Index (API) as per the stratification by National Malaria Control Program based on 2017 data: BA = Babile, GO = Gomma, NA = North Achefer, AMZ = ArbaMinch Zuria, BDZ = Bahir Dar Zuria, MK = Mao-komo, AB = Abobo, LA = Lare, JA = Jawi, ME = Meng

Finger prick blood samples (~300μL) collected from all participants were used to diagnose malaria using RDT (First Response® malaria Antigen pLDH/HRP2 P.f and Pan Combo Card Test, Premier Medical Corporation Ltd, Dist. Valsad, India) or thin and thick blood films, and to prepare dried blood spots (DBS) on 3MM Whatman filter papers (Whatman, Maidstone, UK). Malaria was diagnosed using RDT at Abobo, Lare, Mao-Komo, Menge, and Gomma districts whilst microscopy was used at the school surveys, Arba Minch Zuria and Babile districts. Detailed clinical and socio-demographic data were captured using a pretested semi-structured interview-based questionnaire. Axillary body temperature was measured for all participants. If a participant was found febrile (axillary temperature ≥37.5°C) or reports history of fever in the past 48 hours, malaria status was checked using RDT and treated immediately when found positive following the national treatment guideline [23]. DBS were air dried, protected from direct sunlight, and enclosed in zip locked plastic bags individually with self-indicating silica gel (Loba Chemie, Mumbai, India). Samples were transported at ambient temperature and stored at -20˚C until further use. Giemsa-stained thick and thin smears were read independently by two experienced malaria microscopists. A third expert microscopist was consulted in case of discordant results. Thick smear slides were declared negative if no parasites were detected after observing 100 fields under oil immersion (100X magnification).

Species specific detection of Plasmodium parasites by 18S rRNA based nested polymerase chain reaction

Genomic DNA was extracted from 6mm diameter DBS punches using Chelex-Saponin extraction method [24]. In brief, DNA was eluted after an overnight lysis in 0.5% saponin (SIGMA)/PBS (SIGMA) buffer and washing step followed by boiling at 97 ˚C in 150 µL of 6% Chelex (Bio Rad) in DNase/RNase free water (SIGMA). From the final eluate, 80 µL was transferred into a new plate and stored at -20°C until further use. *Plasmodium* species identification was done by nested polymerase chain reaction (nPCR) that targeted the small subunit 18S rRNA gene as described before [25]. A positive control (for *P. falciparum* NF54 culture from Radboudumc, Nijmegen, The Netherlands; for *P. vivax* the malaria reference laboratory positive controls from the London School of Hygiene and Tropical Medicine, London, UK) and negative controls (PCR grade water) were run in every reaction plate. Amplified products were visualized using UV transilluminator (Bio Rad, USA) after electrophoresis using 2% agarose gels (SIGMA, ALDRICH) stained with Ethidium Bromide (Promega, Madison, USA).

Statistical analysis

For the school surveys, sample size was calculated based on protocols by Brooker and colleagues [22] for the original study that aimed at assessing longitudinal evaluation of parasite prevalence in school children [21]. For this study, 70.0% (231/330) of the students were successfully sampled. For the community surveys, we expected an overall prevalence of 6.8% asymptomatic *Plasmodium* infections based on previous observations [26-37] with a precision of 5%. Based on previous experience, a minimum of 75 samples for the school surveys and 114 for the community samples was targeted across the study sites [21]. Data was double entered into excel, compiled, checked for consistency, and analyzed using Stata version 15 (Stata corporation; College Station, TX, USA) and GraphPad Prism 5.3 (GraphPad Software Inc., CA, USA). Proportions of parasite infections, by microscopy/RDT and nPCR, were compared between categories using Fisher's exact test and Pearson's chi-squared test. Equality tests on unmatched data such as age between school and community surveys were tested by two-sample Wilcoxon rank-sum (Mann-Whitney) test. Generalized Estimating Equation (GEE) was used to determine the association between parasite detectability by microscopy/RDT or nPCR and transmission intensity by allowing exchangeable correlation matrix and robust standard errors to adjust for clustering of observations from the same study site. Sample characteristics such as age, gender, and transmission intensity were tested in the model for their association with infection prevalence and roles as potential confounders. A 5% level of significance was considered in all cases.

Results

Characteristics of study participants

A total of 1093 individuals, 231 from school (3 schools; 75–80 per school survey) and 862 from community surveys (7 surveys; 114–161 per study site) participated in the study. None of the participants was febrile at the time of sampling. Female participants constituted 43.5% (372/855) of community and 51.8% (118/228) of school surveys (P= 0.026). The overall median age of the participants was 16 years (Interquartile range [IQR]: 11–35). As expected, participants from the school surveys were younger (median age, 12; IQR, 11–14) than community surveys (median age, 23; IQR, 10–38; P<0.001). Results are presented separately for community and school surveys, focusing on community surveys for the main comparisons (**Table 1**). Within the community surveys, participants from low (median age, 30; IQR, 18–45; n=232) and moderate (median age, 30; IQR, 12–42; n = 272) endemic settings were older than participants from high endemic settings (median age, 13; IQR, 8-28; n = 318; P < 0.001).

Prevalence of asymptomatic malaria infection across the study sites

In the community surveys, the overall prevalence of asymptomatic *Plasmodium* infections was 12.2% (105/860) by microscopy/RDT and 21.6% (183/846) by nPCR (**Table 1**); 24.1% (208/862) of participants were parasite positive by either nPCR and/or microscopy/RDT. When considering infecting *Plasmodium* species by nPCR, 16.4% (139/846) of samples were *P. falciparum* positive; 3.7% (31/846) were *P. vivax* and 1.5% (13/846) were mixed *P. vivax* and *P. falciparum*. Although the school surveys were from high and moderate transmission sites, there was overall lower *Plasmodium* infection prevalence in the school surveys than in the community surveys as measured by all methods combined (11.3% vs 24.1%; χ^2= 17.9, P < 0.001)

Among the school surveys, the overall prevalence of asymptomatic malaria was 0.4% (1/231) by microscopy/RDT whilst 11.3% (26/231) were parasite positive either by nPCR or both methods combined. Of these nPCR positive malaria infections from the school surveys, 2.6% (6/231) were due to *P. falciparum*, 5.2% (12/231) were due to *P. vivax,* and 3.5% (8/231) were due to mixed *P. falciparum* and *P. vivax* species infections (Additional **Table 1** and **Table 2**).

Across the community surveys, in high transmission settings, nPCR-based prevalence of malaria infection ranged from 17.6% (21/119) at Meng to 46.1% (47/102) at Lare district. In the moderate transmission sites, the nPCR-based prevalence was 29.3% (34/116) at Mao-Komo and 12.4% (20/161) at Arbaminch Zuria district. In low transmission sites, the overall nPCR infection prevalence was 9.4% (22/234). The overall microscopy/RDT based prevalence was 23.1% (81/351), 7.9% (22/277), and 0.9% (2/232), in high, moderate, and low transmission settings, respectively (**Table 1**).

Among the community samples, the prevalence of *Plasmodium* infections detected by all methods combined was substantially higher for the high transmission settings (36.7%, 129/351; 95% CI, 31.9–41.9; P<0.001) compared to moderate (20.6%, 57/277; 95%CI, 16.2–25.8) and low transmission settings (9.4%, 22/234; 95%CI, 6.3–13.9). Moreover, the burden of asymptomatic *Plasmodium* infection was higher in the 5–15 age groups as measured by microscopy/RDT (20.7%, 50/241, P < 0.001) and nPCR (26.7%, 62/232, P = 0.008) (**Table 1**) as compared to under-five children and adults older than 15 years (**Table 1**).

Detectability of asymptomatic *Plasmodium* infections in different endemicities

Among community samples, microscopy/RDT detected 44.2% (80/181) of nPCR detected *Plasmodium* infections (Agreement=86.9%, κ=0.526, **Table 2**). All, but 8 RDT positive *P. falciparum* and 1 microscopy positive *P. vivax* sample, were also nPCR positive (**Additional Table 2**). The likelihood that *Plasmodium* infected individuals (i.e. individuals who were parasite positive by any diagnostic method) were detected by RDT was increased for individuals living in higher transmission settings (AOR= 5.1, 95%CI = 2.6–9.9, P < 0.001) and individuals living in moderate transmission (AOR: 3.4, 95% CI=1.6-7.2, P = 0.002) compared to low transmission settings (**Additional table 3**, **Fig.2**). Age was an important predictor of asymptomatic malaria positivity by microscopy/RDT. After adjusting for site and correlation between observations from the same survey, a 5% decline in detection using microscopy/RDT was observed for every year increase of age from those that tested positive by all methods (AOR = 0.95, 95%CI = 0.9–1.0, P = 0.013).

Table 1. Community-based prevalence of asymptomatic *Plasmodium* infection using nPCR and microscopy/RDT

Attributes	**Category**	**Parasite prevalence by nPCR, % (n/N) [95% CI]**	***P-value***	**Parasite prevalence by microscopy/RDT,% (n/N) [95% CI]**	***P-value***
Gender	Male	22.5 (106/472) [18.9–26.5]	0.442	13.5(65/483) [10.7–16.8]	0.232
	Female	20.2 (74/367) [16.4–24.6]		10.7(40/372) [7.9–14.3]	
Age group (years)	≤ 5	21.5 (14/65) [13.1–33.3]	0.008	10.6 (7/66) [5.1–20.7]	< 0.001
	5–15	26.7 (62/232) [21.4–32.8]		20.7 (50/241) [16.1–26.3]	
	≥ 15	16.9 (87/513) [13.9–20.5]		5.6 (29/515) [3.9–7.9]	
Study sites (n/N) High transmission	Lare	46.1 (47/102) [36.6–55.8]	< 0.001	35.9 (41/114) [27.6–45.2]	< 0.001
	Abobo	34.2 (39/114) [26.1–43.4]		23.7 (28/118) [16.9–32.3]	
	Meng	17.6 (21/119) [11.8–25.6]		10.1 (12/119) [5.8–16.9]	
Moderate transmission	Mamo-Komo	29.3 (34/116) [21.7–38.3]		16.4 (19/116) [10.7–24.3]	
	Arba Minch zuria	12.4 (20/161) [8.1–18.5]		1.8 (3/161) [0.6–5.6]	
Low transmission	Babile	15.4 (18/117) [9.9–23.2]		1.7 (2/117) [0.4–6.6]	
	Gomma	3.4 (4/117) [1.3–8.8]		0.0 (0/115 [NA]	
Transmission intensity	High	31.9 (107/335) [6.3–13.9]	< 0.001	23.1(81/351) [18.9–27.8]	< 0.001
	Moderate	19.5 (54/277) [15.2–24.6]		7.9 (22/277) [5.3–11.8]	
	Low	9.4 (22/234) [6.3–13.9]		0.9 (2/232) [0.2–3.4]	
Overall Prevalence (n/N)	–	21.6 (183/846) [18.9–24.5]		12.2 (105/860) [10.1–14.6]	

Age was missed for 40 samples. Gender was missed for seven samples
CI confidence interval, *API* Annual Parasite Index /1000 people

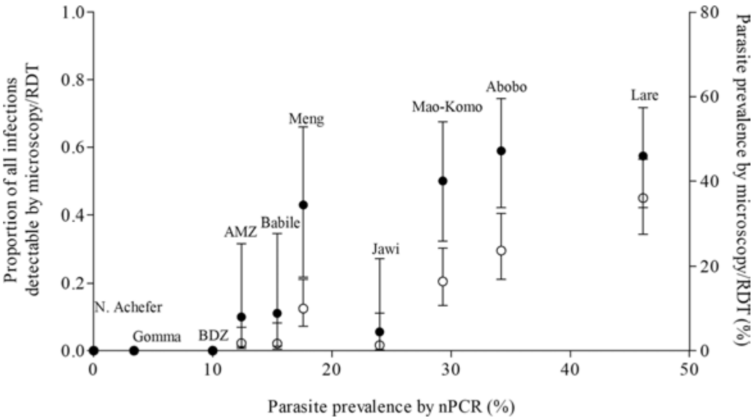

Figure 2. Community and school- based surveys asymptomatic malaria infection prevalence and detectability using nPCR and microscopy/RDT: black circles represent parasite prevalence by nPCR (x-axis) and proportion all infections detected by microscopy/RDT (left y-axis); white circles indicate parasite prevalence by microscopy/RDT (right y-axis). School surveys were (N. Achefer) North Achefer, BDZ (Bahir Dar Zuria) and Jawi

The parasite species composition and detectability varied between transmission settings (**Fig. 2**). Among the *Plasmodium* species detected in the community samples, the majority were attributable to *P. falciparum* (77.4%, 161/208) when all samples were combined. Of the nPCR detected *P. falciparum*-mono species infections (n=139) and mixed-species infections (n=13), microscopy/RDT successfully detected *P. falciparum* in 48.7% (73/150) of infections (**Table 2**). Of the nPCR detected *P. vivax*-mono infections (n = 31) and mixed-species infections (n = 13), microscopy/RDT successfully detected *P. vivax* in 4.6% (2/44) of infections (**Table 2**).

Table 2. Species specific asymptomatic malaria infection prevalence and detectability across the study sites using nPCR and microscopy/RDT from 2016-2020

Sample source and API	Study sites	Microscopy/RDT % [n/N]				nPCR % [n/N]				Proportion of infections detected by microscopy/RDT (%)		
		Pf	Pv	Mixed	Proportion (%) of Pv	Pf	Pv	Mixed	Proportion (%) of Pv	Any	Pf	Pv
Community- (API ≥100)	Lare*	33.3 [38/114]	0.0 [0/114]	2.6 [3/114]	7.3 [3/41]	41.2 [42/102]	0.9 [1/102]	3.9 [4/102]	10.6 [5/47]	57.4 [27/47]	56.5 [26/46]	0.00 [0/5]
	Abobo¥	17.8 [21/118]	0.0 [0/118]	5.9 [7/118]	25.0 [7/28]	28.1 [32/114]	3.5 [4/114]	2.6 [3/114]	17.9 [7/39]	58.9 [23/39]	57.1 [20/35]	14.3 [1/7]
	Meng	10.1 [12/119]	0.0 [0/119]	0.0 [0/119]	0.0 [0/12]	13.5 [16/119]	3.4 [4/119]	0.8 [1/119]	23.8 [5/21]	42.9 [9/21]	47.1 [8/17]	0.0 [0/5]
	Total	20.2 [71/351]	0.0 [0/351]	2.8 [10/351]	12.3 [10/81]	26.9 [90/335]	2.7 [9/335]	2.4 [8/335]	15.9 [17/107]	55.1 [59/107]	55.1 [54/98]	5.8 [1/17]
Community – (API≥5&<100)	Mao-komo	16.4 [19/116]	0.0 [0/116]	0.0 [0/116]	0.0 [0/19]	18.9 [22/116]	9.5 [11/116]	0.8 [1/116]	35.3 [12/34]	50.0 [17/34]	65.2 [15/23]	0.0 [0/12]
	AMZ	0.0 [0/161]	0.6 [1/161]	1.2 [2/161]	50.0 [3/6]	6.8 [11/161]	3.1 [5/161]	2.5 [4/161]	45.0 [9/20]	10.0 [2/20]	13.3 [2/15]	11.1 [1/9]
	Total	6.8 [19/277]	0.4 [1/277]	0.72 [2/277]	13.6 [3/22]	11.9 [33/277]	5.8 [16/277]	1.8 [5/277]	38.9 [21/54]	35.2 [19/54]	44.7 [17/38]	4.8 [1/21]
Low (API>0 &<5)	Gomma§	0.00 [0/115]	0.0 [0/115]	0.0 [0/115]	0.0 [0/115]	2.6 [3/117]	0.8 [1/117]	0.0 [0/117]	25.0 [1/4]	0.0 [0/2]	0.0 [0/1]	0.0 [0/1]
	Babile	1.7 [2/117]	0.0 [0/117]	0.0 [0/117]	0.0 [0/2]	11.1 [13/117]	4.3 [5/117]	0.0 [0/117]	27.8 [5/18]	15.4 [2/18]	15.4 [2/13]	0.0 [0/5]
	Total	0.9 [2/232]	0.0 [0/232	0.0 [0/232]	0.0 [0/2]	6.8 [16/234]	2.6 [6/234]	0.0 [0/234]	27.3 [6/22]	10.0 [2/20]	14.3 [2/14]	0.0 [0/6]
Community	Grand Total	10.7 [92/860]	0.1 [1/860]	1.4 [12/860]	12.4 [13/105]	16.4 [139/846]	3.7 [31/846]	1.5 [13/846]	24.0 [44/183]	44.2 [80/181]	48.7 [73/150]	4.6 [2/44]
School (API ≥100)	Jawi	1.3 [1/75]	0.0 [0/75]	0.0 [0/75]	0.0 [0/1]	5.3 [4/75]	10.7 [8/75]	8.00 [6/75]	77.8 [14/18]	5.6 [1/18]	10.0 [1/10]	0.0 [0/14]

Table 2. Continued

Sample source and API	Study sites	Microscopy/RDT % [n/N]				nPCR % [n/N]				Proportion of infections detected by microscopy/RDT (%)		
		Pf	**Pv**	**Mixed**	**Proportion (%) of Pv**	**Pf**	**Pv**	**Mixed**	**Proportion (%) of Pv**	**Any**	**Pf**	**Pv**
School (API≥5&<100)	BDZ	0.0 [0/80]	0.0 [0/80]	0.0 [0/80]	0.0 [0/80]	2.50 [2/80]	5.00 [4/80]	2.5 [2/80]	75.0 [6/8]	0.0 [0/8]	0.0 [0/4]	0.0 [0/6]
	N. Achefer	0.0 [0/76]	0.0 [0/76]	0.0 [0/76]	0.0 NA	0.0 [0/76]	0.0 [0/76]	0.0 NA	0.0 [0/76]	0.0 NA	0.0 NA	0.0 NA
School	Total	0.4 [1/231]	0.0 [0/231]	0.0 [0/231]	0.0 [0/1]	2.6 [6/231]	5.2 [12/231]	3.5 [8/231]	76.9 [20/26]	3.8 [1/26]	7.1 [1/14]	0.0 [0/20]

AMZ Arba Minch Zuria, BDZ Bahir Dar Zuria, N. Achefer North Achefer, Pf *P. falciparum*, Pv *P. vivax*, mixed Pf Pv, Any all species detected. Proportion of Pv is calculated together with mixed infections. API annual parasite index /1000 people. * 12 DBS samples (11 Pf and 1 mixed infection positive by RDT) were missed from Lare for PCR. [¥] 4 DBS samples (3 Pf and 1 mixed infection positive by RDT) were missed from Abobo for PCR. [§] microscopy was not done for 2 samples that were Pf positive by PCR

Discussion

This study describes the prevalence and detectability of asymptomatic *Plasmodium* infections in ten different transmission settings by nPCR and conventional diagnostics (i.e. microscopy/RDT). More asymptomatic infections were detected in high transmission settings by both methods. The detectability of asymptomatic *Plasmodium* infections using microscopy/RDT relative to nPCR increased as transmission intensity increases. As a result, most infections in low transmission settings were not detectable by microscopy/RDT.

In Ethiopia, several cross-sectional studies have documented asymptomatic parasite carriage using conventional and molecular methods [33-35, 38, 39]. The current multi-site study allowed an assessment of factors influencing the prevalence of infections as well as their detectability by microscopy-RDT. The prevalence of asymptomatic *Plasmodium* infections in the current study was in the same range as other reports from high [34, 38] and moderate [27] transmission settings in Ethiopia and elsewhere [29, 35, 40, 41].

Consistent with other studies [39, 42, 43], the current study observed that microscopy/RDT detected fewer asymptomatic infections as compared to PCR. The proportion of *Plasmodium* infections that was detectable by microscopy/RDT increased with increasing in transmission intensity. Whilst this trend has been reported in meta-analyses for *P. falciparum* [40, 44, 45], it is striking that this trend is also apparent in the current study within one country affected by both *P. falciparum* and *P. vivax*. Moreover, the effect size was comparatively large with approximately 5-fold higher detectability of infections in high endemic settings compared to low endemic settings. The trend of increasing detectability with increasing transmission intensity may be attributable to the fact that asymptomatically infected individuals have higher average parasite densities in high transmission settings [44, 46]. Moreover, in low endemic settings individuals will receive fewer infectious bites with, due to the absence of super-infections, lower parasitemia over the course of infection [40, 47]. Low genetic diversity of the parasite population in low transmission settings may also contribute to rapidly acquired immunity to the specific clones [48], further limiting parasite density. An impact of immunity on parasite density and the detectability of infections is also illustrated by the negative impact of increasing age on the detectability on infections in line with the current study [49].

Lower parasite densities in *P. vivax* compared to *P. falciparum* [50, 51] also results in a low detectability of *P. vivax* infections by microscopy/RDT. This low density in *P. vivax* is mainly attributable to the parasite's preference to infect reticulocytes [52, 53] that typically constitute less than 1% of the total erythrocyte population [54] and also to the early acquisition of immunity [53]. These findings have implications for estimates of the relative burden of *P. falciparum* and *P. vivax* infections. The introduction of sensitive molecular tools may thus improve the detection of *P. vivax* infections substantially. Since treatment strategies differ for *P. falciparum* and *P. vivax*, this is relevant for public health interventions.

Although RDT and microscopy were used separately in the study sites due to logistics reasons, the prevalence measured by conventional RDT and microscopy was assumed to be comparable [41].

Nine samples that were declared microscopy/RDT positive were negative by nPCR while seven samples that were detected *P. falciparum* positive by RDT were *P. vivax* positive by nPCR. False RDT positivity might be due to the presence of parasite antigens after adequate clearance of parasites which might explain the variation between RDT positivity and PCR negative detection among asymptomatic malaria infections [55, 56]. Hence, there is a possibility that RDT can be positive for lingering antigens of *P. falciparum* while missing the low-density *P. vivax* infection from the same patient.

Conclusion

Conventional diagnostics missed nearly half of the asymptomatic malaria reservoir detected by nPCR. Moreover, the detectability of asymptomatic *Plasmodium* infections in all endemic sites might reflect the long persistence of these infections from weeks up to months in high [57] as well as in low transmission settings [58, 59] even in the presence of effective control and elimination interventions. As these infections can have relevance for onward malaria transmission [13, 44, 60], a detailed understanding of the distribution, detectability, and contribution to the infectious reservoir of asymptomatic infections will greatly improve our ability to target all relevant infections. The wide scale presence of low-density infections calls for more in-depth studies on understanding parasite density oscillations, their relevance for malaria symptoms, and onward transmission to mosquitoes.

Declarations

Ethical statement

The study protocol was approved by the Ethiopian National Research Ethics Review Committee (3.10/016/20), and the institutional ethics review boards of the Department of Biochemistry (Ref.No.SOM/DRERC/BCH005/2009) and the College of Natural Sciences (Ref.No. SOM/DRERC/BCH005/2009) at Addis Ababa University, and the Armauer Hansen Research Institute (P035/17, P024/17 and P032/18).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declared that they have no competing interests.

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Author’s contributions

FGT, EH, EG, CD and TB conceived the study, contributed to data analysis and critically commented on the manuscript. EH analyzed the data. EH and SKT drafted the manuscript. EH, SK, WC, GS and MK conducted the laboratory study. WC, MK, EH, SKT, AG, GS, TA collected blood samples. All authors read and approved the final manuscript.

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Supplementary Information

Table S1. School-based prevalence of asymptomatic malaria in selected sites from different transmission settings using nPCR and microscopy/RDT from 2016- 2018, Ethiopia

Attributes	Category	Parasite prevalence by nPCR, % (n/N) [95% CI]	P-value	Parasite prevalence by microscopy/RDT, % (n/N) [95% CI]	P-value
		(N= 231)		(N= 231)	
Gender	Male	12.7(14/110) [7.6-20.4]	0. 544	0.0 (0/110) [NA]	0.333
	Female	10.2(12/118) [5.8-17.1]		0.85 (1/118) [0.01-5.8]	
Agegroup (years)	<5	-		-	
	5-15	10.2 (21/206) [6.7-15.2]	0.182	0.5 (1/206) [0.07-3.4]	0.755
	>15	20.0 (4/20) [7.5-43.6]		0.0 (0/20) [NA]	
Study sites (n/N)					
High transmission (API ≥100)	Jawi	24.0 (18/75) [15.62-35.01]	<0.001	1.3 (1/75) [0.18-8.98]	0.352
Moderate transmission (API≥5&<100)	BDZ	10.0 (8/80) [5.05-18.83]		0.0 (0/80) [NA]	
	N. Achefer	0.0 (0/76) [NA]		0.0 (0/76) [NA]	
Overall Prevalence (n/N)	-------	11.3 (26/231) [7.5-16.0]		0.4(1/231) [0.01-2.3]	

Table S2. Concordance of RDT and Microscopy detected samples compared to nPCR among the study participants, 2016-2020

		18S nPCR			
		P. falciparum		*P. vivax*	
		Positive	Negative	Positive	Negative
RDT	Positive	68	8	8	0
	Negative	52	302	20	302
Microscopy	Positive	4	0	1	1
	Negative	38	557	34	557

NB: the mixed species infections are added with the *P. falciparum* and *P. vivax* infections

Table S3. GEE model for association of malaria infection prevalence using all methods combined (nPCR and/or microscopy/RDT) among community survey samples with sample characteristics such as gender, age category, level of endemicity from 2016-2020, Ethiopia

Sample characteristics		**Total malaria infection prevalence**			
		COR (95% CI)	**P- value**	**AOR (95% CI)**	**P-value**
Gender	Female	1 (Ref)		1(Ref)	-
	Male	1.2 (0.8-2.0)	0.385	-	-
Age as continuous variable (years)		0.95(0.9-1.0)	0.001	0.95(0.9-1.0)	0.013
Level of endemicity	Low (API >0 &<5)	1(Ref)	-	1(Ref)	-
	Moderate (API ≥5 & <100)	5.5 (1.6-19.1)	0.008	3.4 (1.6-7.2)	0.002
	High (API≥100)	15.0 (9.0-24.1)	<0.001	5.1 (2.6-10.0)	<0.001

CHAPTER 3

Dynamics of asymptomatic *Plasmodium falciparum* and *Plasmodium vivax* infections and their infectiousness to mosquitoes in a low transmission setting of Ethiopia: a longitudinal observational study

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Abstract

Objective: A 15-month longitudinal study was conducted to determine the duration and infectivity of asymptomatic qPCR-detected *P. falciparum* and *P. vivax* infections in Ethiopia.

Method: Total parasite and gametocyte kinetics were determined by molecular methods; infectivity to *Anopheles arabiensis* mosquitoes by repeated membrane feeding assays. Infectivity results were contrasted with passively recruited symptomatic malaria cases.

Results: For *P. falciparum* and *P. vivax* infections detected at enrolment, median durations of infection were 37 days (95% confidence interval [CI], 15-93) and 60 days (95% CI, 18-213), respectively. *P. falciparum* and *P. vivax* parasite densities declined over the course of infections. From 47 feeding assays on 22 asymptomatic *P. falciparum* infections, 6.4% (3/47) were infectious and these infected 1.8% (29/1579) of mosquitoes. No transmission was observed in feeding assays on asymptomatic *P. vivax* mono-infections (0/56); one mixed-species infection was highly infectious. Among the symptomatic cases, 4.3% (2/47) of *P. falciparum* and 73.3% (53/86) of *P. vivax* patients were infectious to mosquitoes.

Conclusion: The majority of asymptomatic infections were of short duration and low parasite density. Only a minority of asymptomatic individuals were infectious to mosquitoes. This contrasts with earlier findings and is plausibly due to the low parasite densities in this population.

Keywords: asymptomatic, longitudinal, membrane feeding assay, infectiousness, transmission, *P. falciparum*, *P.* vivax

Introduction

Recent progress in malaria control has stalled in many endemic settings and gains are threatened by emerging biological, social and economic challenges [1]. Despite challenges, malaria control in Ethiopia remained on track with an aspirational target for elimination in selected low transmission settings by 2030 [2]. There is increased awareness of the widespread presence of *Plasmodium* infections that neither elicit acute symptoms nor treatment seeking behavior in low transmission settings [3-5]. Whether these asymptomatic infections form a hurdle for malaria elimination depends on their relative importance for sustaining malaria transmission [4, 6], which is in turn dependent on infection duration and the production of transmissible gametocytes [3]. The duration of asymptomatic infections varies from days [7, 8] to months or even years [9]. Notable differences were observed in infection dynamics and duration of infections between *P. falciparum* and *P. vivax* infections [10]. In natural infections, *P. vivax* has lower peripheral blood parasitemia density compared to *P. falciparum* [11, 12] due to its strong preference to infect reticulocytes [13] and rapid acquisition of immunity [14]. Uniquely, *P. vivax* has liver stage hypnozoites that can cause relapses within weeks to months attributed to over 80% of blood stage infections and sustaining transmission [15]. Both species show marked fluctuations in parasite and gametocyte densities over the course of infections [3, 6, 10].

Gametocytes are typically highly prevalent in asymptomatic infections and can be detected with high sensitivity by molecular methods that target gametocyte-specific mRNA transcripts [11, 16]. Gametocyte density is positively associated with the asexual parasite biomass [5, 11] but this association is weaker for *P. falciparum* where gametocytes develop over a prolonged 10-12 day period [17] than where gametocytes appear within 2-3 days [11]. Importantly, there is considerable debate about the relative infectivity of asymptomatic infections and especially low-density asymptomatic infections as these predominate in malaria endemic populations. The likelihood of mosquito infection increases rapidly when densities in the human blood exceed ~5 gametocytes/µL for *P. falciparum* [18] or ~100 *Pvs25* gametocyte transcripts/µL for *P. vivax* [19]. A study in Africa estimated that >95% of all *P. falciparum* mosquito infections find their source in asymptomatic infections [6]; ~15% of mosquito infections may be attributed to submicroscopic parasite carriage in humans [4, 20], while a study from Southeast Asia reported that symptomatic infections with high gametocytemia are major drivers of transmission [21]. For

P. vivax, studies on the human infectious reservoir give similarly contradicting results on the transmission importance of asymptomatic versus symptomatic infections [4, 19, 21]. From a public-health perspective, it is important to understand the duration, detectability and transmission-potential of asymptomatic infections.

Here, we examined infection duration and gametocyte production in longitudinally monitored asymptomatic *P. falciparum* and *P. vivax* infections in a low-endemic setting where both species are co-endemic in Ethiopia. The transmissibility of infections to *Anopheles arabiensis* mosquitoes (a locally relevant vector) was investigated and contrasted with that of symptomatic malaria patients recruited from the same setting and period.

Methods and materials

Study area and participant selection

A longitudinal, observational study was conducted from September 2018 – March 2020 in Adama district, Ethiopia. The district is characterized by low and seasonal malaria transmission following the long and primary (mid-June to mid-September) and short and sporadic (February to May) rainy seasons [22]. Both *P. falciparum* and *P. vivax* are endemic in the district [4]. Following community meetings, inhabitants of the villages Batu Degaga, Hurufa Kurfa, Mermersa, Dibibisa, Dongore Furda and Guraja Furda were invited for screening for *P. falciparum* and *P. vivax* infections by 18S based quantitative PCR (qPCR). Individuals were eligible for participation if they were parasite positive, $\geq$ 2 years old, permanent residents of the area, had no measured fever (axillary temperature: range 32-37.4°C) or reported fever in the past 48 hours, and no known chronic illness and/or acute illness requiring immediate clinical care. Eligible participants or, in case of minors, parents/guardians provided written informed consent; additional assent was obtained for participants aged 12-17 years.

Participant recruitment, blood sampling and follow-up

Individuals who tested positive for either *P. falciparum* or *P. vivax* parasites were enrolled and followed twice weekly for 2.5 months and monthly for the subsequent 12 months. Finger prick blood samples (~0.3mL) were collected in microtainer EDTA tubes (K2E EDTA Vacutainers, BD) on all days except for days 3, 18, 33, 48 and 63 when venous blood samples (5mL) were collected using Heparin tubes (Lithium Heparin Vacutainers, BD) for mosquito feeding assays. A separate population of symptomatic malaria cases with measured fever (axillary temperature ≥ 37.5°C) or history of fever and microscopy-confirmed *P. vivax* and *P. falciparum* infections were passively recruited at Adama malaria control center. Symptomatic cases provided a single venous blood sample, after which they received treatment following the national malaria treatment guidelines [23].

Parasite quantification

Parasite and gametocyte prevalence was determined by microscopy by screening 100 microscopic fields. Parasite quantification was done by molecular methods following total nucleic acid extraction by MagNAPure LC automated extractor (Total Nucleic Acid Isolation Kit High Performance; Roche Applied Sciences, Indianapolis, IN, USA) from 100µL whole blood stored in RNAProtect (Qiagen cell reagent). *P. falciparum* and *P. vivax* parasites were quantified by quantitative PCR (qPCR) targeting the 18S small rRNA subunit gene [24, 25]. Gene copies were translated to parasite densities by assuming 5 and 3 gene copies per *P. falciparum* and *P. vivax* parasite, respectively. When assessing parasite clearance or the duration of infection, all qPCR positive signals were considered true positives, acknowledging that parasite densities may fluctuate around the threshold density for detection [6]. To classify as a new infection during follow-up and avoid over-interpreting very low qPCR signals, estimated parasite densities had to exceed 0.01 parasite/µL to be considered positive [5]. Gametocyte density was quantified in *P. falciparum* 18S positive samples by quantitative reverse transcriptase PCR (qRT-PCR) targeting the male *PfMGET* and female *CCp4* gametocyte mRNA transcripts [26]; *Pvs25* mRNA transcripts were quantified as transcripts/µL as an indicator of female *P. vivax* gametocyte density [16]. Primer and probe sequences are described elsewhere [6].

Mosquito infectivity assessment

Membrane feeding assays (MFA) using locally reared *An. arabiensis* mosquitoes were performed as described previously [4]. MFA were conducted at enrolment for clinical malaria cases (prior to treatment), and on days 3, 18, 33, 48 and 63 for asymptomatic parasite carriers. Briefly, three aliquots of, ~400µL each, whole blood collected in Lithium Heparin tubes (Lithium Heparin Vacutainers, BD) were fed to 3 cups with ~40 female mosquitoes in each cup (~120 mosquitoes per experiment) that were 4 - 7 days old, using water jacketed glass feeders maintained at 37°C using a circulating water bath. Fully fed mosquitoes were selected and maintained on 10% sucrose solution at 26°C ± 2 and 60% ± 10 humidity until dissection on day 7-10. Following staining in 1% mercurochrome, mosquito guts were examined by two microscopists to detect and quantify oocysts.

Statistical analysis

The association between parasite density and gametocyte density was determined on a log10 scale using Pearson's correlation. We estimated, using the Kaplan-Meier method, the proportion of individuals that remained infected over time. This is different from the observed proportions, in that the observed proportions exclude right-censored observations entirely after the time in which they are right-censored. These right-censored observations were infections that did not clear by the end of their follow-up. The gametocyte fraction was defined as the proportion of the total parasite biomass of parasites that was gametocyte for *P. falciparum* and the number of *Pvs25* mRNA copies per parasite for *P. vivax*. The trajectories of parasite density, gametocyte density, and gametocyte fraction over time were modelled using the generalized additive mixed effects models [17] where random intercepts were used for each individual to account for intra individual correlations. The slope, indicative of a decline in densities over time, was tested by running linear models with log10 parasite densities. Full details and mathematical description are described in [5]. R (v 4.2.2) and Rstudio (v 2023.03.1) with packages survival, survminer, and mgcv were used for the statistical analyses.

Results

A total of 2373 individuals were screened between September 2018 and March 2020. Microscopy detected *P. falciparum* in 0.47% (10/2107), *P. vivax* in 0.14% (3/2107) and mixed-species infections in 0.05% (1/2017) of the population. By qPCR, infection prevalence was 3.5% (83/2373) for *P. falciparum,* 4.0% (95/2373) for *P. vivax* and 0.08% (2/2373) for mixed-species infections. Seventy individuals with *P. falciparum* (n=35), *P. vivax* (n=34) or mixed-species infection (n=1) were enrolled into the longitudinal study; for the other 92 parasite positive individuals, follow-up was not possible due to COVID-19 lockdown measures. At enrolment, geometric mean parasite densities were 0.96 (95% confidence interval [CI], 0.33-2.75) parasites/µL for *P. falciparum* and 1.04 (95% CI, 0.54-1.99) parasites/µL for *P. vivax* mono-infections (**Table 1**).

Table 1. Baseline characteristics of study participants

Attributes	*P. falciparum*	*P. vivax*	Mixed *(P. falciparum* and *P. vivax)*
N	35	34	1
Sex, % male (n/N)	68.6 (24/35)	52.9 (18/34)	0.0 (0/1)
Age, median (IQR)	22 (12-40)	15 (9-28)	23
% microscopy positive	18.2 (6/33)	3.3 (1/30)	0.0 (0/1)
P. falciparum parasite density/µL, GM (95% CI)	0.96 (0.33-2.75)	-	1.54 (-)
P. vivax parasite density/µL, GM (95% CI)	-	1.04 (0.54 -1.99)	1.74 (-)

IQR = interquartile range (25th - 75th percentile); GM = geometric mean; 95% CI=95% confidence interval.

Parasite persistence and densities during asymptomatic *P. falciparum* and *P. vivax* infections

The 70 participants in the longitudinal study contributed a total of 835 observations (median 12; interquartile range (IQR), 3-18 observations per individual) after enrollment. For 18 individuals (13 *P. falciparum* and 5 *P. vivax*), their infection was resolved immediately and no subsequent parasite positive samples were observed despite initially being parasite positive. 12 individuals (7 *P. falciparum* and 5 *P. vivax*) became symptomatic with the pre-symptomatic period ranging from 7-243 days for *P. falciparum* and 3-243 days for *P. vivax* and received treatment by the study team. At baseline, only one of the *P. vivax* infected individuals was microscopy positive; 18.2% (6/33) of individuals with submicroscopic qPCR-detected infections at enrolment became microscopy positive during follow-up. From all *P. vivax* infected individuals, 38.2% (13/34) had qPCR-detected *P. falciparum* infections at least once during follow-up **(Supplementary Table 1).** Among participants recruited with asymptomatic *P. falciparum* infections six were microscopy detectable at baseline. Of the remaining 29 qPCR-detected-submicroscopic infections at baseline, 31.0% (9/29) became microscopically detectable for *P. falciparum* during follow-up. Importantly, 14.2% (5/35) of individuals who were initially positive for *P. falciparum* later became positive for *P. vivax* on at least one time point during the follow-up (**Supplementary Table 1**).

When we only included individuals who were *P. falciparum* parasite positive at enrolment and accepted low qPCR signals as evidence for persisting infections, 21.7% of infections (95% CI, 10.3%- 46.0%) persisted for ≥3 months and the median duration of infection was 37 days (95% CI, 15-93 days) (**Fig. 1**). When we also included *P. falciparum* infections that were acquired during follow-up (giving a total of 54 *P. falciparum* infections), the median duration of infection was 18 days (95% CI, 11–49 days). For *P. vivax*, including only infections that were present at baseline resulted in 39.8% (95% CI, 25.5%-61.9%) of infections persisting for ≥3 months and an estimated median duration of blood-stage infection of 60 days (95% CI, 18-213) (**Fig. 1**). Including all infections (n=43) gave a median infection duration of 45 days (95% CI, 22-123 days) (**Fig. S1**). A log-rank test showed no statistically significant difference in the Kaplan-Meier curves when restricting to infections detected at baseline (P=0.23) or including all infections (P=0.12). Parasite densities at enrolment were variable and we observed faster clearance for infections with low parasite density at enrolment compared to infections with higher parasite densities, a pattern that was statistically significant for *P. falciparum* (P<0.0001) but not for *P. vivax* (P=0.24; **Fig. S2**).

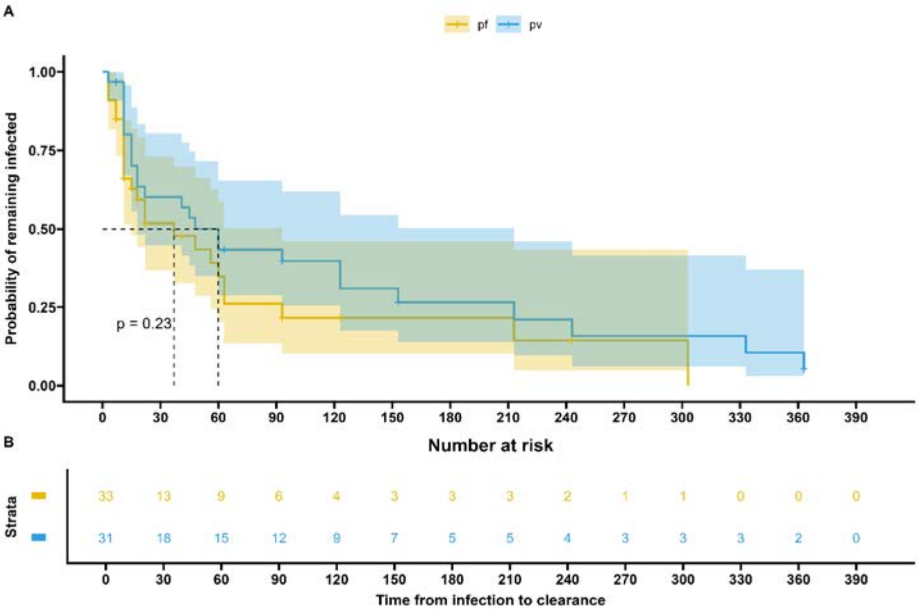

Figure 1. Kaplan-Meier plot showing the probability of remaining infected over time. Probability of remaining infected (**A**) is shown with numbers remaining infected (**B**). Indicated on the X-axes are days since recruitment in the study. pf, P. falciparum; pv, P. vivax; n. censor, number censored. from a total of 33 P. falciparum infections that were present at baseline, 24 were observed to clear (9 right-censored); From a total of 31 P. vivax infections that were present at baseline, 25 were observed to clear (6 right-censored). A log-rank test showed no significant difference in the Kaplan-Meier curves (P=0.23).

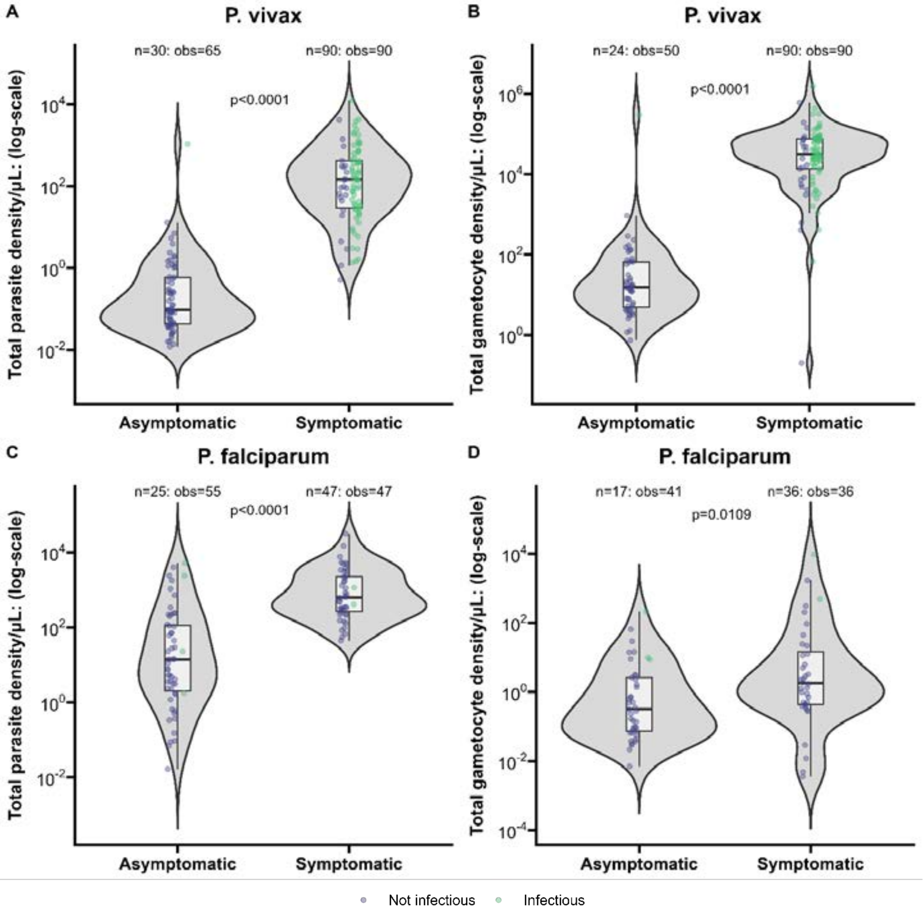

Figure 2. Distribution of parasite and gametocyte densities for infectious and non-infectious feeds. Violin plots showing the distribution of parasite densities and gametocyte densities by symptomatic status for *P. falciparum* and *P. vivax* infections. In each panel, n indicates the number of individuals and obs indicates the number of observations used in the violin plots. The boxplots indicate the quartiles of the densities by symptomatic status. Purple dots indicate observations that were not infectious while green dots indicate observations that were infectious i.e. resulted in at least one infected mosquito.

P. falciparum gametocytes were detected by qRT-PCR in 62.0% (160/258) of *P. falciparum* qPCR positive samples; gametocyte prevalence by microscopy was only 10.5% (27/258) in this population. *P. falciparum* total parasite density was positively associated with concurrent gametocyte density (r=0.71, P<0.001; **Fig. S3 A**). *P. vivax* gametocytes were detected by qRT-PCR in 75.0% (213/284) of qPCR positive samples and by microscopy in 1.1% (3/284) of this population. *P. vivax* parasitemia was strongly associated with concurrent *Pvs25* transcript density (r=0.87, P<0.001; **Fig. S3 B**).

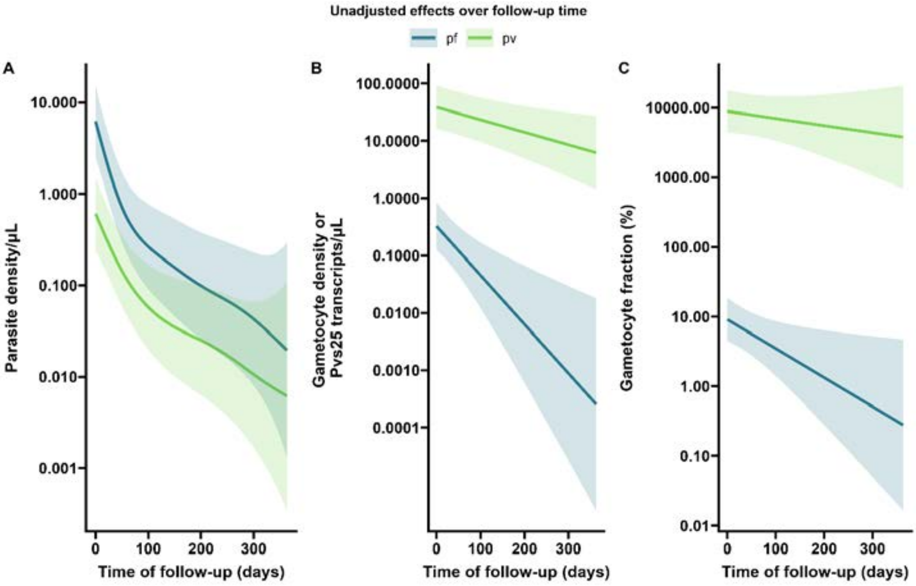

Figure 3. Estimated trends for parasite and gametocyte densities and fractions over time. Estimated trends for parasite density (**A**), gametocyte density of *Pvs25* transcript density (**B**) and gametocyte fraction (**C**) over time. For parasite density 868 *P. falciparum* observations and 866 *P. vivax* observations from 70 individuals were used in the model. Of these observations, there were 258 *P. falciparum* parasite positive observations from 54 individuals and 284 *P. vivax* parasite positive observations from 43 individuals, together making up samples from 69 individuals. From these parasite positive observations, 224 *P. falciparum* and 253 *P. vivax* observations from 43 and 36 individuals, respectively had available gametocyte densities for the modelling of gametocyte density trajectories (**B**) and gametocyte fraction trajectories (**C**) over time. From these available gametocyte densities there were 160 *P. falciparum* gametocyte positive densities from 24 individuals and 216 *P. vivax* gametocyte positive densities from 28 individuals. Here we used a generalized additive mixed effects model with a negative binomial distribution to model trajectories of parasite density and gametocyte density and gametocyte fraction (all on natural log (ln) scale) over time while accounting for correlation between measures from the same individual through subject-specific random effects.

The estimated geometric mean parasite density over follow-up was 1.21 parasites/µL (95% CI, 0.54-2.71) for *P. falciparum* and 0.19 parasites/µL (95% CI, 0.08-0.47) for *P. vivax* (P<0.001) (**Fig. 2**). The estimated geometric mean gametocyte density over follow-up (amongst positive samples) was 0.13 gametocytes/µL (95% CI, 0.05-0.34) for *P. falciparum* and 30.68 (95% CI, 9.15-102.87) *Pvs25* mRNA transcripts/µL. In *P. falciparum* infections, parasite density (P<0.001), gametocyte density (P=0.001) and gametocyte fraction (P=0.018) all declined during follow-up. For *P. vivax* only parasite density was statistically significantly declining over time (P<0.001) while no statistically significant decline was observed for gametocyte transcript density (P=0.568) or the ratio of gametocyte transcripts over total parasite density (P=0.706) (**Fig. 3**).

Infectivity of asymptomatic and symptomatic infections to mosquitoes

When including only those experiments that were conducted at time-points when parasites were detected in blood samples, a total of 114 membrane feeding experiments were performed on 56 asymptomatic participants. Three *P. falciparum*-infected individuals were infectious, each at one time-point only, and infected a total of 29 mosquitoes (1.8% of all dissected mosquitoes; **Table 2**). None of the asymptomatic individuals with *P. vivax* mono-infections infected mosquitoes while a single individual with a mixed infection infected 40 mosquitoes 83.3% (40/48) of this single feed. This person had 23.0 *P. falciparum* and 1066.7 *P. vivax* parasites per µL at the time of mosquito feeding experiment; while no *P. falciparum* gametocytes were observed, the number of *P. vivax Pvs25* mRNA transcripts was estimated at 3200/µL.

Table 2. Mosquito feeding results from asymptomatic parasite carriers and symptomatic malaria cases

	Experiments (donors)	Proportion infectious donors (n/N)	Proportion infectious feeds (n/N)	Proportion infected mosquitoes (n/N)
Asymptomatic infections				
P. falciparum	47 (22)	13.6 (3/22)	6.4 (3/47)	1.8 (29/1579)
P. vivax	56 (24)	0.0 (0/24)	0.0 (0/56)	0.0 (0/1800)
Mixed species	11 (10)	10.0 (1/10)	9.1 (1/11)	12.2 (40/328)
Clinical malaria cases				
P. falciparum	47 (47)	4.3 (2/47)	4.3 (2/47)	2.7 (38/1389)
P. vivax	86 (86)	73.3 (63/86)	73.3 (63/86)	42.4 (1068/2519)
mixed species	4 (4)	100% (4/4)	100% (4/4)	32.8 (39/119)

Note: The number of experiments is identical to the number of donors for clinical malaria cases while asymptomatically infected individuals could contribute multiple observations. Of note: parasite status was determined at the moment of feeding and some individuals lost their infection prior to feeding. Mixed species = *P. falciparum* and *P. vivax* co-infection.

Infectious individuals were 7, 10, 13 and 42 years of age. Whilst three infectious individuals harbored submicroscopic infections at enrolment, at the time they infected mosquitoes 2 were microscopy positive for *P. falciparum*, 1 was microscopy positive for *P. vivax* and 1 was qPCR-positive but microscopy-negative for *P.* falciparum.

To compare infectivity in the cohort to that observed among clinically symptomatic individuals, 86 patients with clinical *P. vivax* malaria, 47 patients with clinical *P. falciparum* malaria and 4 patients who had mixed species infection were recruited for MFA (**Supplementary Table 2**). Fever was measured in 39.7% (52/131) of these symptomatic cases. All *P. vivax* symptomatic cases (n=86) were gametocyte positive by qRT-PCR. Of these, 73.3% (63/86) resulted in mosquito infections with 42.4% (1068/2519) infected mosquitoes. Gametocyte positivity in *P. falciparum* symptomatic cases was 80.9% (38/47) by qRT-PCR, with 4.3% (2/47) infectious feeds and 2.7% (38/1389) of dissected mosquitoes being infected. All four mixed species infections were infectious to mosquitoes with 32.8% (39/119) of mosquitoes becoming infected (**Table 2**). Gametocyte densities were statistically significantly higher in symptomatic individuals for both species although numbers were very small for *P. falciparum,* and also higher in infectious feeds (**Fig. 2**).

Discussion

We performed the first longitudinal assessment of parasite longevity, gametocyte production and infectivity in asymptomatic *P. vivax* and *P. falciparum* infections in a co-endemic setting in Africa. When restricting our analyses to those infections detected at enrolment, the median duration of infections was 37 days for *P. falciparum* and 60 days for *P. vivax*; new infections that were first detected during the follow-up period were of considerably shorter duration. Four of 70 asymptomatically infected individuals were infectious to mosquitoes at any time-point during follow-up.

Asymptomatic infections are increasingly considered as reservoirs for onward transmission that need to be targeted to accelerate malaria elimination efforts. However, for infections to contribute to transmission, their infection duration should be sufficiently long to produce gametocytes [5] and gametocyte densities should be sufficiently high to be transmissible [5, 6, 19, 21]. Infection duration is a major determinant of transmission potential, yet there is limited information on the duration of natural infections, especially for infections that are submicroscopic. In our study, most asymptomatic infections were submicroscopic at enrolment. A number of *P. falciparum* (31.0%, 9/29) and *P. vivax* (18.2%, 6/33) infections became microscopically detectable on at least one time-point during follow-up. Nevertheless, there was a general trend of declining parasite densities over time and majority of infections became undetectable and were considered cleared early during follow-up. In our cohort, 22% of *P. falciparum* infections were persisting for at least 3 months and the median duration of infections already detected at baseline was 37 days. Infection clearance appeared to take longer for *P. vivax* with 40% persisting for at least 3 months and a median duration of infection of already detected at baseline of 60 days. Whilst very long infection durations have been reported for *P. falciparum* [9], many studies indicate that the majority of infections are of short duration. In Vietnam, median carriage times of 2 months for *P. falciparum* and 6 months for *P. vivax* were reported [10]; only 13% of *P. falciparum*-infected Cambodian adults carried these infections for ≥2 months, whereas 35% of *P. vivax* were of long duration [27]. The analysis of monthly blood samples in cohort studies indicated a median duration of blood-stage *P. falciparum* infection of 36 days in Papua New Guinea and 135 days in Thailand whilst the duration of *P. vivax* infections in these settings was in the range of 24-29 days [28]. Interestingly, a large proportion of individuals who were initially detected with either *P. vivax* or *P. falciparum* mono-infections later tested positive for the other species. These findings are in line with findings from Southeast Asia where both *Plasmodium* species were commonly observed in the same individuals [12, 27] suggesting that individuals at high risk of being infected by one species are also at elevated risk for other species. It should be noted that *P. falciparum* infections that were detected during follow-up were of considerably shorter duration and often detected only once. This is in line with findings from Uganda [5] and supported by earlier studies on spontaneous clearance of incident infections [29].

Given the very low gametocyte densities we observed during follow-up, it is unsurprising that only a minority of asymptomatic infections were infectious to mosquitoes. To put our mosquito findings in perspective, we also assessed infectivity of clinical cases that, as predicted, were highly infectious for *P. vivax* [4, 19]. Parasite and gametocyte densities were higher among clinical cases. Although this is commonly observed for *P. vivax* [4, 11, 19], *P. falciparum* gametocyte densities are sometimes lower in clinical cases compared to asymptomatic infections [6, 8]. The observed very low transmission from asymptomatic *P. vivax* infections is different from our previous estimates from the same setting where 32% of asymptomatic microscopy-positive infections were infectious to mosquitoes [4]. Of note, a single highly infectious asymptomatic individual who was co-infected with both *P. vivax* and *P. falciparum* probably infected mosquitoes with *P. vivax* since high gametocyte densities were observed for this species but no *P. falciparum* gametocytes were observed. Nevertheless, the overall pattern of low infectivity is striking. Since conducting the prior study in this setting, malaria declined substantially following massive bed nets distribution, indoor residual spraying and the roll out of single dose primaquine since 2017 in addition to Artemether-Lumefantrine treatment [2] (**Fig. S4**). In line with this, parasite densities among asymptomatic *P. vivax* infections were considerably lower with on average 0.19 *P. vivax* parasites/µL during asymptomatic infections in this study compared to ~22 parasites/µL reported previously [4]. We consider it likely that the lower average parasite density resulted in a lower gametocyte density that explains the considerably lower infectiousness of asymptomatic *P. vivax* infections [10, 30]. Whilst speculative, it is likely that some of the infections that we detected at baseline were older infections that were already past their peak parasite density, which occurs early in infections [5].

The strength of the current study was that we directly quantified infectiousness to mosquitoes and observe mosquito infections from both symptomatic and asymptomatic donors. Extrapolating results from feeding experiments to population-wide transmission dynamics needs to be done with caution and requires adjustment for factors like mosquito exposure and biting preferences that we did not assess in this study [20]. While *An. arabiensis* is a principal vector in Ethiopia [31], other native (*An. coustani complex, An. gambiae s.l.* and *An. pharoensis*) [32] and invasive species (*An. stephensi*) [33] also contribute to transmission; these might differ in vector competence but were not examined in the current study.

Our study has several other limitations. Firstly, COVID-19 lockdown measures resulted in a smaller study population than initially anticipated. Secondly, we have no information on the duration of infection prior to enrolment, resulting in underestimates of the duration of infections [10]. Thirdly, we did not perform genotyping that would have allowed us to distinguish between persisting and newly acquired clones acquisition of new clones; our focus was on the duration of blood-stage parasitemia and not on clonal dynamics. Fourthly, infection duration estimates are influenced by the frequency of sampling and diagnostic sensitivity [29]; our frequency of sampling after the first 10 weeks was only once per month, limiting the precision of longer infection durations. Our intensive monitoring during the first phase of the study makes it unlikely that unreported self-treatment for malaria played a relevant role in infection clearance.

In conclusion, in our longitudinally followed cohort of individuals living in an area where both *P. falciparum* and *P. vivax* are endemic, total parasite densities fluctuate but show a general decline in densities over time with the majority of infections for both species being cleared within 2-3 months. Related to the low parasite and gametocyte densities, a very low proportion of asymptomatic infections were infectious to mosquitoes.

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Potential conflict of interest

All authors declared that they have no conflict of interest.

Author contributions

FGT, TB, CD, EH and SKT conceived the study. EH and SKT drafted the manuscript. FGT, TB, CD, EG, BP and HM critically commented on the manuscript. EH, TB, SKT and JR analyzed the data. TA, AG, EH, SKT, WC, EE, TT, T.Tsegaye, TE were involved in mosquito membrane feeding assay. EH, TA, AG, EE, TT and SK were involved in mosquito rearing. WC, EH, SKT, GS conducted mosquito midgut dissection. EH, SKT, TE and T. Tsegaye, GS, HA and MG collected follow-up data and blood samples from community participants. EH, FGT, SKT, SWB and KJ conducted the molecular laboratory work.

Ethical approval

The study protocol was approved by ethics review committees of the Armauer Hansen Research Institute and ALERT Hospital (PO35/17 and PO32/18), the London School of Hygiene and Tropical Medicine (15811), and the National Research Ethics Review Committee (SHE/S.M./14.4/708/19) of Ethiopia.

Data sharing

Data from this study can be obtained upon formal request from the corresponding authors.

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Supplementary information

Supplementary table 1. Summary of parasite species oscillation patterns during follow-up

Species at baseline by 18s qPCR	Changes in parasite status during follow-up	Number of participants % (n/N)
P. vivax(n=34)	From Pv-sub-microscopic infection to Pv-microscopy positive	18.2 (6/33)
	From Pv-infected to Pv-Pf mixed infection	26.5 (9/34)
	From Pv-infected to Pf infected	38.2 (13/34)
P. falciparum (n=35)	From Pf-sub-microscopic infection to Pf-microscopy positive	31.0 (9/29)
	From Pf-infected to Pf-Pv mixed infection	17.1 (6/35)
	From Pf-infected to Pv infected	14.2 (5/35)

Note: 1 *P vivax* and 6 *P. falciparum* infections were microscopy detectable at baseline. Pf= *P. falciparum*, Pv= *P.* vivax.

Supplementary table 2. Characteristics of clinical malaria study participants

Attributes	*P. falciparum*	*P. vivax*	Mixed *(P. falciparum* and *P. vivax)*
N	47	86	4
Sex, % male (n/N)	79.6 (35/44)	78.8 (67/85)	50.0 (2/4)
Age, median (IQR)	28.5(21.5-39)	26 (19-32)	13.5(10.5-22.5)
P. falciparum parasite density/μL, GM (95% CI)	445.0 (228.2-867.6)		-
P. vivax parasite density/μL, GM (95% CI)	-	111.2 (71.2-173.7)	-

IQR = interquartile range (25th - 75th percentile); GM = geometric mean; 95% CI=95% confidence interval.

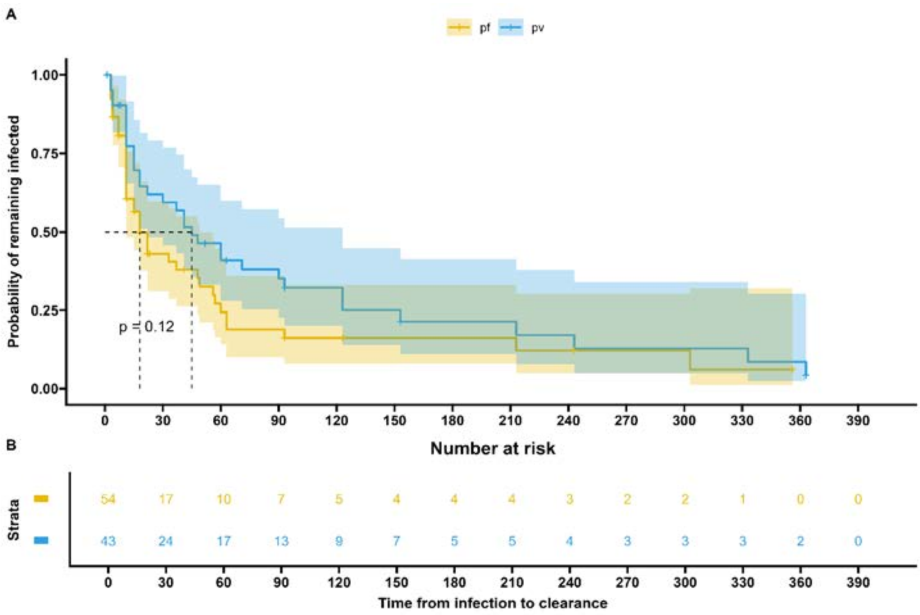
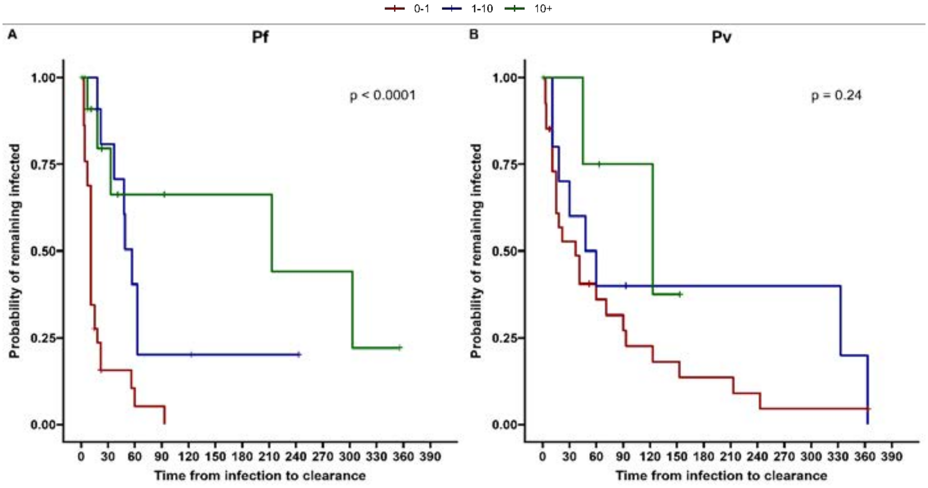


Figure S1. Kaplan-Meier plot showing the probability of remaining infected (i.e. not cleared) over the duration of follow-up. From the 54 pf infections, 33 were observed at baseline, and 40 were observed to clear (14 were right-censored) with a median time of 18 days (95% CI, 11 days – 49 days). From the 43 pv infections, 31 were observed at baseline, and 33 were observed to clear (10 were right-censored) with a median time of 45 days (95% CI, 22 days-123 days). A log-rank test showed no significant difference in the Kaplan-Meier curves (P=0.12).



For Pf:	Hazard ratio (95% CI, P-value)	
Reference group	(0, 1] vs ref	(1, 10] vs ref
(1,10]	4.0 (1.7 – 9.0, P=0.00010)	-
(10, 10000]	7.8 (2.6 – 24.0, P=0.0003)	2.0 (0.6 – 6.7, P=0.277)

For Pv:	Hazard ratio (95% CI, P-value)	
Reference group	(0, 1] vs ref	(1, 10] vs ref
(1,10]	1.6 (0.7 – 3.6, P=0.2690)	-
(10, 10000]	2.7 (0.6 – 11.6, P=0.1770)	1.7 (0.4 – 8.2, P=0.4990)

Figure S2. Kaplan Meier graphs showing that lower parasite density infections at baseline tend to have fastest parasite clearance. Here, persistence is indicated for three infection classes: <1 parasite/uL (red), 1-10 parasites/uL (blue) and >10 parasites/uL (green) for *P. falciparum* (left) and *P. vivax* (right) separately. Infections with the lower parasite density at baseline have the fastest parasite clearance although this is not stastically significant for *P. vivax* due to a small number of long-persisters in the population with the lowest parasite densities at baseline.

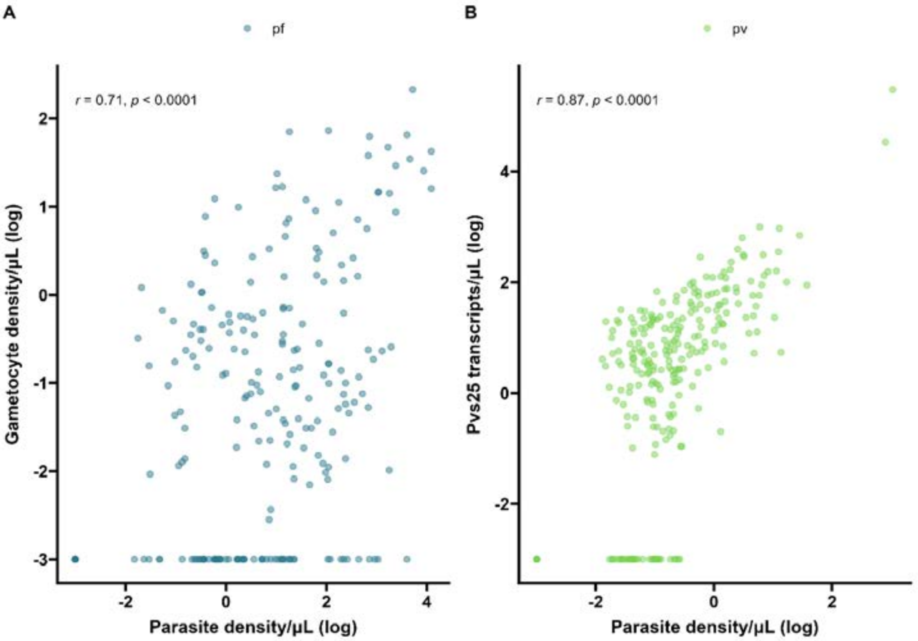


Figure S3. Association between parasite density and gametocyte density. Scatter plots for parasite density versus gametocyte density with Pearson's correlation coefficients on log10 transformed values and their corresponding p-values. These plots include 55 individuals with 465 follow-up pairs of parasite density and gametocyte density. For *P. falciparum,* 25 individuals contributed 160 pairs of parasite and gametocyte positive samples, and for *P. vivax* 29 individuals contributed 213 pairs of parasite positive and gametocyte positive samples.

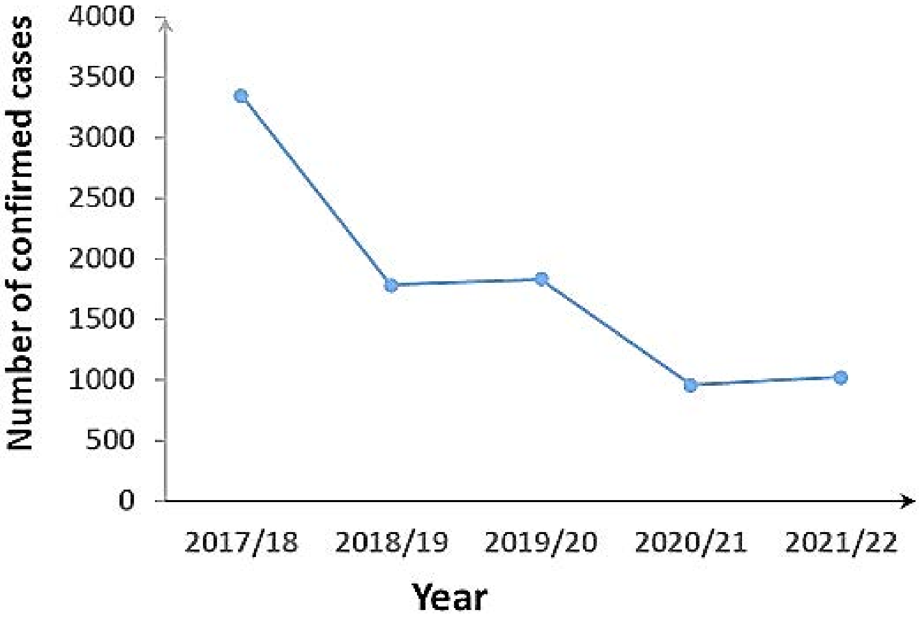


Figure S4. Trends of malaria transmission in the study area (Adama) from 2017-2022. The x-axis represents dates of the year and the y-axis represents the number of clinical malaria cases reported in routine data. The earlier study where asymptomatic infections were of relatively high parasite density took place in 2016, the current study in 2018-2020.

CHAPTER 4

Immunity against sexual stage *Plasmodium falciparum* and *Plasmodium vivax* parasites

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Abstract

The efficient spread of malaria from infected humans to mosquitoes is a major challenge for malaria elimination initiatives. Gametocytes are the only *Plasmodium* life stage infectious to mosquitoes. Here, we summarize evidence for naturally acquired anti-gametocyte immunity and the current state of transmission blocking vaccines. Although gametocytes are intra-erythrocytic when present in infected humans, developing *Plasmodium falciparum* gametocytes may express proteins on the surface of red blood cells that elicit immune responses in naturally exposed individuals. This immune response may reduce the burden of circulating gametocytes. For both *P. falciparum* and *Plasmodium vivax*, there is a solid evidence that antibodies against antigens present on the gametocyte surface, when co-ingested with gametocytes, can influence transmission to mosquitoes. Transmission reducing immunity, reducing the burden of infection in mosquitoes, is a well-acknowledged but poorly quantified phenomenon that forms the basis for the development of transmission blocking vaccines. Transmission enhancing immunity, increasing the likelihood or intensity of transmission to mosquitoes, is more speculative in nature but is convincingly demonstrated for *P. vivax*. With the increased interest in malaria elimination, transmission blocking vaccines and monoclonal antibodies have moved to the center stage of malaria vaccine development. Methodologies to prioritize and evaluate products are urgently needed.

Keywords: *Plasmodium falciparum, Plasmodium vivax,* gametocytes, transmission, immunity, vaccines

Introduction

Malaria is one of the few infectious diseases earmarked for worldwide eradication by the World Health Organization (WHO) [1, 2]. The majority of the malaria cases are caused by infection with *Plasmodium falciparum* or *Plasmodium vivax.* Whilst *P. falciparum* is the dominant *Plasmodium* species in most of Africa and is associated with the most severe morbidity and mortality, *P. vivax* is more widely distributed and is increasingly recognized as an important source of morbidity and restrained economic productivity [3]. Malaria control efforts in recent decades, including improved access to efficacious treatment and vector control, were followed by significant reductions in malaria burden [4] and stimulated malaria elimination initiatives. Despite these successes, the WHO estimates that there were 219 million new malaria cases and 435,000 malaria related deaths in 2017 [5]. This figure has remained fairly stable since 2015 indicating that progress has plateaued; some countries even experience recent increases in malaria burden and several more are off track in their elimination efforts [5]. The emergence of parasite resistance to antimalarials [6, 7] and mosquito resistance to insecticides [8] are important threats to recent gains. One of the major challenges for malaria elimination initiatives is the very efficient spread of malaria from infected humans to mosquitoes [1]. Interventions that target this process and interrupt transmission to mosquitoes may be crucial to achieve elimination in many areas [9].

Gametocytes are the only *Plasmodium* life stages that are infectious to mosquitoes, so the uptake of these specialized forms by blood-feeding female *Anopheles* mosquitoes is essential for human-to-mosquito transmission. *P. falciparum* gametocytes form when asexual schizonts become committed to produce sexual progeny by the activation and expression of the Apatella2-g gene (*AP2-G*) [10, 11]. The expression of AP2-G is under tight epigenetic control by *P. falciparum* heterochromatin protein 1 (PfHP1) [12]. The interplay between histone deacetylases [13] and gametocyte development 1 (GDV1) [14] in turn determines the binding or release of PfHP1 and thus the expression of AP2-G. AP2-G is a highly conserved member of the apicomplexan AP2 (APiAP2) family of DNA binding proteins whereby its DNA binding domains are highly conserved across all *Plasmodium* species; all *P. falciparum* ApiAP2 proteins have syntenic homologues in *P. vivax* and are expressed at a similar stage of development [15]. For *P. falciparum*, gametocyte formation is a 10-12 day process during which the parasite passes through five morphologically distinct forms (stages I-V) (**Fig. 1**). Immature gametocytes (stages I-IV) sequester outside the peripheral circulation, primarily in the bone marrow and spleen [16], and are released in the circulation to complete their final maturation steps [17]. The mature stage V gametocytes then become accessible in the peripheral blood for uptake by blood-feeding mosquitoes [18]. The development of *P. vivax* gametocytes is markedly faster than *P. falciparum* and only ~48 hours are required for maturation [19] that may also involve a bone marrow phase [20]. The circulation time of *P. falciparum* and *P. vivax* gametocytes differs significantly. Whilst mature *P. falciparum* gametocytes can be detected for several weeks after clearance of asexual parasites [21, 22], *P. vivax* gametocyte half-life is very short [23], with microscopically detectable gametocytes and gametocyte-specific mRNA disappearing within days of asexual stage clearance [23, 24]. Stage V *P. falciparum* gametocytes can be morphologically recognized by their characteristic crescent shape, while mature *P. vivax* gametocytes display a round shape and almost fill the entire red blood cell (RBC) [19] (**Fig. 1**).

In the mosquito midgut, *Plasmodium* gametocytes rapidly egress from the host erythrocyte and develop into gametes. Gametogenesis is induced by a reduction in temperature, increase in pH and exposure to xanthurenic acid [25, 26]. Male gametocytes exflagellate producing up to eight motile microgametes; whereas, female gametocytes ‘round-up’ to form one immotile macrogamete [27, 28]. Fertilization of a macrogamete by a microgamete results in the formation of a zygote, which then develops into an intermediate ‘retort’ leading to the formation of a mature motile ookinete that traverses the midgut wall and forms an oocyst. Approximately 10-12 days after blood meal ingestion the rupture of oocysts results in the release of sporozoites, which will invade the mosquito salivary glands completing the mosquito stage of the *Plasmodium* life cycle [29].

Many factors influence the likelihood of gametocytes being transmitted to mosquitoes and establishing a successful mosquito stage infection [30]. Considerably more work on gametocyte biology and infectivity has been performed for *P. falciparum* than for *P. vivax*, although it is likely that many factors are shared between *Plasmodium* species. General parasite characteristics that have been associated with differences in transmission potential and infectivity include gametocyte density [31-34] (**Fig. 2**), concurrent asexual parasite density [35, 36], ratio of male and female gametocytes [31, 37], duration of infection [35, 38], and level of gametocyte maturity [39]. Host factors such as anaemia, age, mosquito factors, and importantly, human immunity are also known to affect gametocyte infectiousness [40, 41].

The first empirical evidence that human immune responses to gametocytes could affect their infectiousness to mosquitoes came from immunization studies in birds [42-44], following earlier observations that gametocyte infectivity *per capita* appeared to change the course of an infection [45, 46]. These experiments led to the identification of a small number of proteins expressed by gametocytes, gametes or ookinetes, which for decades have been the focus of gametocyte research and formed the basis of malaria transmission blocking vaccine (TBV) development [47, 48]. Research on gametocyte immunobiology has been outweighed by research on the life stages leading to human infection (the pre-erythrocytic stages) and clinical disease (the asexual blood stages), but as TBV development has gained pace our understanding of gametocyte biology has improved dramatically. In 2002, the *P. falciparum* genome [49] and proteome [50, 51] were first published. These and many subsequent investigations have revealed biology that is unique to gametocytes (reviewed by Beri et al. [52]), the gametocyte sexes [53, 54], and different stages of their development [55]. An integrated approach using proteomic and transcriptomic data from 18 studies predicted 602 proteins to be enriched in *P. falciparum* gametocytes [56]; transcriptome analysis in *P. vivax* revealed that the expression of 1613 genes was correlated with the expression of known gametocyte genes [57]. Many of the proteins produced specifically by gametocytes remodel the human host cell to support their morphological development [58], while others have roles during gametogenesis and fertilization in the mosquito. These proteins represent potential targets of gametocyte specific immunity.

This review will discuss the evidence for the existence of naturally acquired human immune responses against the sexual parasite stages of *P. falciparum* and *P. vivax,* discuss the effect of these responses on transmission, and propose strategies for transmission blocking interventions. Immature and mature gametocytes have markedly different biology, morphology, and preferential localization in human tissues. Immune responses to early and late gametocytes therefore have the potential to affect transmission differently; early gametocyte immunity could reduce the number of gametocytes achieving maturity in the peripheral blood, while late gametocyte immunity may affect gametocyte number and their likelihood of undergoing successful sporogonic development in the mosquito. Whilst this review will focus on *P. falciparum*, we will also summarize the current state of knowledge for the less-studied *P. vivax* and indicate major knowledge gaps with regard to anti-gametocyte immunity, implications for transmission dynamics and potential vaccine strategies.

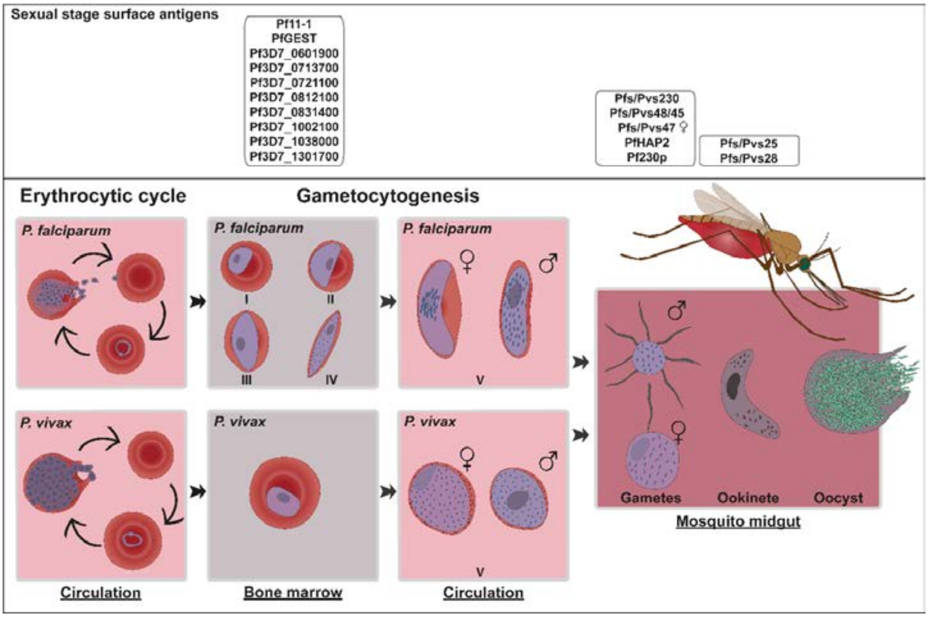

Figure 1. The sexual stage development of *P. falciparum* and *P. vivax* parasites. Schematic illustration of the development of intra-erythrocytic gametocytes and post-transmission development in the mosquito midgut.

Merozoites that are released from infected liver cells invade red blood cells (RBCs) to initiate the erythrocytic cycle. The sexual development is initiated by a subset of parasites that are committed to produce gametocytes. Immature *P. falciparum* gametocytes sequester outside the peripheral circulation, primarily in the bone marrow and spleen. Their maturation (10-12 days) involves five distinct developmental stages (I-V); mature stage V are released in the peripheral circulation. *P. vivax* gametocytogenesis may also involve a blood marrow phase, and only takes 48 hours. After ingestion by a blood-feeding *Anopheles* mosquito, gametocytes rapidly egress from the host RBC and develop into gametes. Male gametocytes exflagellate to form eight microgametes that subsequently fertilizes a ‘round-up’ microgamete to form a zygote leading to the formation of a motile ookinete. The ookinete penetrates the midgut wall and forms an oocyst that produces hundreds to thousands of sporozoites. Upon oocyst rupture, sporozoites are released and migrate to the salivary glands from where they can be transmitted to a new human host. Boxes represent surface antigens that are under consideration for vaccine development.

Immune responses affecting gametocytes in the human circulation

Immune responses targeting gametocyte sequestration:

Both the asexual and sexual intra-erythrocytic forms of *P. falciparum* sequester to avoid prolonged circulation in the blood. Asexual parasites sequester in the tissue microvasculature through well-defined ligand-receptor interactions; Knob-associated histidine rich protein (KAHRP) is critical to the formation of 'knobs' on the infected erythrocyte surface [59], while members of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family accumulate on these knobs and mediate cytoadherence [60]. The first stages of *P. falciparum* gametocyte development are marked by changes in gene expression. *P. falciparum* gametocyte exported protein 5 (PfGEXP5) is detectable from 14h after RBC invasion by a sexually committed merozoite [61]; Pfs16 [62] and Pfg27 [63] are detectable from 24-30h post invasion. At the same time, proteins associated with asexual stage cytoadherence are down regulated; stage I gametocytes have a smooth surface, without knobs [64]. By stage II, gametocytes are morphologically distinguishable from asexual trophozoites, KAHRP protein is undetectable, and PfEMP1 protein is present only at low levels [64]. Unlike the asexual blood stages, *P. falciparum* gametocytes sequester primarily in the bone marrow and spleen [16, 17, 65, 66]. The role of PfEMP1 in early gametocyte infected RBC (giRBC) adhesion to the bone marrow vasculature is unclear. Adherence of giRBC to either C32 melanoma cells or human bone marrow endothelial cells has been demonstrated [67, 68], but a later study did not detect any adherence of gametocyte stages later than I and IIa to C32 cells [69], and Silvestrini et al. demonstrated only limited adhesion to a variety of endothelial cells [70]. Recently, adhesion experiments using bone marrow mesenchymal stromal cells demonstrated that immature gametocytes were able to adhere to this cell type via unknown ligands on the giRBC surface [71].

A study using intravital imaging of *P. berghei* parasites in mice demonstrated direct evidence of homing by merozoites to the extravascular niche of the bone marrow and spleen. Using specific inhibitors, de Niz et al. demonstrated that this extravasation (movement from the bone marrow/spleen vasculature to the organ's extravascular space) is mediated via a receptor-ligand interaction [72]; blocking of P-selectin alone reduced the accumulation of gametocytes in the bone marrow by 60%. They also provide evidence for invasion of red blood cell precursor cells in the bone marrow by sexual merozoites. Several theories have been proposed to explain the enrichment of gametocytes in the extravascular space of the bone marrow; e.g. sexually committed merozoites translocate and gametocytes develop in that space, non-sexually committed merozoites translocate there and commitment occurs in the extravascular space, or young gametocytes translocate there directly [73]. The *P. berghei* study supports the hypothesis that a subset of sexual merozoites bearing specific surface ligands home to the bone marrow and spleen, bind the epithelium, move into the extravascular space, and invade erythrocyte precursors (abundant in this niche) to become young gametocytes [72]. Comparative experiments in the same study using human autopsy material indicate that there is a similar phenomenon possible for *P. falciparum.* These data provide evidence for the presence of surface molecules on sexually committed merozoites (or schizonts containing merozoites) that are involved in cytoadhesion during the process of homing and retention in the bone marrow. During the acute phase of infection, asexual and sexual infected erythrocytes also accumulate in the bone marrow, indicating that early gametocytes can form in the blood and may specifically home to the bone marrow/spleen. The targeting of any parasite ligands that mediate gametocyte sequestration by immune factors could possibly inhibit transmission potential.

Numerous studies have shown that immature giRBCs are rigid and that a change in host cell deformability occurs in the transition to maturity [58, 74]. Interestingly, the study by de Niz et al. showed that mature *P. berghei* gametocytes pass freely into and out of the vascular spaces of the bone marrow, and that a switch in host cell deformability (here tested by blocking the signal cascade leading to host cell deformability) underlies this freedom of movement [72]. This supports the hypothesis that gametocyte sequestration is maintained not by receptor-ligand interactions, but by mechanical retention. Additionally, the flexibility of mature gametocytes allows them to transit the splenic endothelial slits and thereby escape clearance. Members of the STEVOR protein family are associated with the erythrocyte membrane of immature gametocytes. Accompanying the shift in deformability as gametocytes mature STEVOR disappears, indicating a possible role for this protein family in this process [58].

Naturally induced antibodies may affect gametocyte morphology and fitness. In one study, serum antibodies from Thai malaria patients were incubated with stage I gametocytes, and were observed to reduce their numbers, interfere with maturation, distort their morphology and reduce the number of oocysts developing in subsequent mosquito feeding assays [75]. Here, the binding of antibodies to the surface of immature forms from stage II onwards was described as a possible mechanism for the observed transmission reduction. Immune responses against antigens on the immature giRBC could affect gametocyte development or circulation time by interfering with sequestration or mediating direct clearance. An epidemiological study performed in Indonesia in the early 1990s compared two groups living in an hyperendemic area; native residents and transmigrants with no (or limited) history of malaria. Lower gametocyte densities in native residents were attributed to specific immune responses [76], giving rise to the hypothesis that naturally acquired antibodies against surface antigens on giRBC may directly affect gametocyte densities in circulation independent of a reduction in the asexual parasite biomass.

Several studies have aimed to identify the erythrocyte surface antigens of immature gametocytes that could be involved in sequestration. The first study used a flow cytometry-based method with purified *P. falciparum* 3D7 gametocytes, and observed reactivity of immune sera from Gambian children with the surface of mature giRBCs, but not with immature stages [77]. No association was observed in antibody recognition of asexual parasites and mature gametocytes, indicating that a distinct antigen panel is displayed on giRBCs. Follow-up data on gametocytaemia also suggested that antibodies against giRBCs might be able to control gametocyte densities. A more thorough study over a five-week period in a Ghanaian cohort also demonstrated the presence of antibodies against mature 3D7 giRBCs using flow cytometry [78]. These findings were confirmed by repeating the experiments using two clinical Kenyan isolates. In antibody staining experiments analyzed by microscopy, no antibody reactivity was observed against immature giRBCs.

Chan et al. quantified antibody reactivity to erythrocytes infected with gametocytes and asexual stages using microscopy in order to better understand the difference in humoral response against these two life stages [79]. Among two Kenyan cohorts, low antibody reactivity was observed against stage II to V giRBCs from the 3D7 strain. This low reactivity is contrasted with high antibody responses to the surface of trophozoite infected erythrocytes. To confirm their hypothesis that these high responses are the result of reactivity to PfEMP1 they repeated the experiment with two transgenic parasite strains with repressed PfEMP1 expression. In the absence of PfEMP1, surface reactivity of erythrocytes infected with asexual parasites was equal to giRBCs, indicating that PfEMP1 is the major asexual stage erythrocyte surface antigen. The low levels of PfEMP1 expression and the absence of other immunogenic antigens on the surface of giRBCs would explain the observed low antibody reactivity.

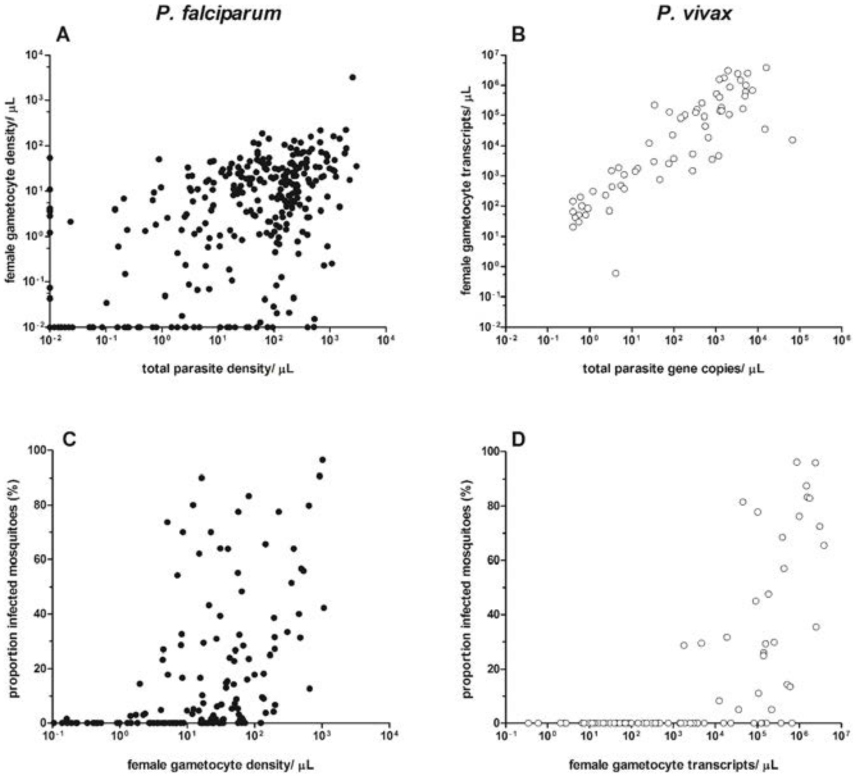

Figure 2. Parasite and gametocyte densities in relation to each other and the proportion of infected mosquitoes. Log_{10} transformed parasite (X-axes) and gametocyte (Y-axes) quantities are indicated for *P. falciparum* (**A**) and *P. vivax* (**B**). Total parasite density is measured by 18S based quantitative polymerase chain reaction (qPCR) and female gametocytes were quantified in a reverse transcription-based qPCR assays that targeted Pfs25 for *P. falciparum* and Pvs25 for *P. vivax*. Indicated are parasite and gametocyte densities/µL for *P. falciparum* and gene copies/µL for *P. vivax*. Parasite and gametocyte culture of NF54 was used for quantification for *P. falciparum*. For *P. vivax* gene copies were quantified from recombinant plasmids containing the respective genes. Log_{10} transformed *P. falciparum* gametocyte density/µL (**C**) and *P. vivax* transcript copies/µL (**D**) are indicated in the X-axes with respect to the percent of infected mosquitoes (Y-axes). Data points are indicated in filled circles for *P. falciparum* and unfilled circles for *P.* vivax.

Interestingly, a recent study demonstrated that surface recognition by naturally acquired antibodies was only present on erythrocytes infected with immature forms (I to III) of NF54 and a genetic strain with a Pf2004 background. In this study, no measurable reactivity with the surface of giRBC infected with mature stage V gametocytes was observed [80]. The authors emphasized that their contrasting findings could be due to the more stringent conditions in obtaining the different developmental gametocytes forms, with which RBC integrity and the activation of mature gametocytes into gametes were controlled for by counterstaining with antibodies specific to proteins on the gametocyte (not giRBC) surface. The authors used a transgenic parasite line and flow cytometry to demonstrate reactivity of Malawian immune sera to antigens on the surface of immature giRBC, that are mostly shared with asexual infected erythrocytes. Subsequently, they used three complementary approaches to identify the antigenic targets. First, they probed giRBC membranes (stage I to III) with Malawian immune plasma to identify differential protein bands between surface-intact and surface-depleted samples by mass-spectrometry. Additionally, they probed sera of mice immunized with the membranes used for mass-spectrometry on a protein microarray consisting of gametocyte proteins [81]. Lastly, they used the same protein microarray to construct an immune profile for a selection of the plasma samples that showed a range of membrane reactivity as identified by flow cytometry. Combining data of these three approaches and an initial filtering resulted in an overlapping list of 30 proteins of which 26 are predicted to be exported. The vast majority of these hits were shared with asexual life stages. Responses to giRBC were associated with increased phagocytosis of erythrocytes infected with both asexuals and gametocytes. This suggests a possible mechanism of increased clearance of giRBC by antibody-mediated phagocytosis. This study provides evidence for the presence of antigens on the surface of erythrocytes infected with immature gametocytes that are targeted by functional naturally acquired responses.

The functional phenotype of giRBC immunity may be related to interference with gametocyte sequestration or clearance of developing gametocytes. Based on the *P. berghei* model it seems likely that a subset of merozoites directly translocate into the bone marrow, spleen, and possibly other areas of low vascular flow, but it is also possible that some degree of homing occurs by early gametocytes [72]. Inside the bone marrow, adhesion of giRBCs to mesenchymal cells is observed [71], indicating the presence of early giRBC surface antigens. For inhibition of sequestration, targeting the initial homing of these tissues is a plausible transmission blocking strategy. Alternatively, antibodies may neutralize gametocytes that are developing in the bone marrow. Both interference with sequestration and neutralization of developing gametocytes would result in a reduced release of mature gametocytes into the circulation. As such, giRBC immunity may contribute to the variation that is observed among natural infections in the production of *P. falciparum* mature gametocytes (**Fig. 2A**), although this association remains to be established. Despite remarkable differences in gametocyte development between

P. falciparum and *P. vivax*, it has been demonstrated that there are similarities in sexual stage gene expression dynamics [20], suggesting conservation of pathways involved in sexual development. Erythrocytes infected with *P. vivax* parasites lack knob structures, so it was thought that ligand-receptor mediated sequestration was not possible for this species. However, it has been shown in several *in vitro* studies that *P. vivax* infected erythrocytes can adhere to a variety of cells including lung and brain endothelial cells [82, 83]. Furthermore, it has been demonstrated in human [84] and non-human primate [20] biopsies that *P. vivax* gametocytes sequester in the parenchyma of the bone marrow. Whether the *P. berghei* model of invasion followed by gametocyte development in the extravascular niche [72] also applies to *P. vivax* remains to be seen. In contrast to *P. falciparum, P. vivax* infected erythrocytes are deformable throughout all stages [85]. This suggests that homing and retention are not mediated by membrane flexibility, but via ligand-receptor interactions. Interestingly, *P. vivax* parasites lack a homologue of PfEMP1, although they express a group of variable proteins (VIR) [86] that have been implicated in tissue adhesion [82, 87]. So far there have been no reports of naturally acquired immune responses against these proteins that potentially inhibit sequestration. The tight association between *P. vivax* total parasite density and gametocyte density (**Fig. 2B**) may argue against an important role of giRBC immunity affecting gametocyte production among naturally infected individuals.

Immune responses influencing gametocyte tropism:

Mature gametocytes are ingested during a mosquito blood meal from the sub-dermal capillaries. Convincing evidence that gametocytes accumulate preferentially in capillary beds is lacking [88]; however, one hypothesis is that mature giRBC surface antigens bind these tissues specifically ('tropins') or facilitate their release from sequestration or visceral circulation at times when mosquitoes are feeding ('circadins') [89].

STEVORs, RIFINS, and SURFINs are all hypothetical mature giRBC surface antigens, but as yet none have been shown to mediate gametocyte tropism in the sub-dermal capillaries [89]. Though there is limited consensus in prior studies (66-69), recent work indicates that there is a progressive loss of giRBC surface recognition by immune sera during gametocyte maturation [80]. The lack of antigens on the surface of erythrocytes infected with mature gametocytes is a plausible mechanism of immune evasion, and there is a hypothesis that the specific crescent shape of *P. falciparum* mature gametocytes may be sufficient to result in their disproportionate accumulate in the capillary beds [90]. Obviously, this cannot be the case for *P. vivax* gametocytes that are transmitted efficiently despite their spherical morphology. At present, there is therefore no evidence for the existence of 'tropins' or 'circadins' or associated immune responses that could affect gametocyte densities in the skin.

Cellular immunity affecting circulating gametocytes:

Although to a limited extent, anti-malaria cellular and innate immunity might play a role in reducing malaria transmission to mosquitoes. It is well established that merozoites and erythrocytes infected with asexual parasites are phagocytosed by monocytes and neutrophils [91-94]. The internalization of non-opsonized infected erythrocytes by monocytes and culture-derived macrophages is mediated by interactions of CD36 and the parasite ligand PfEMP1 [93]. There is evidence that erythrocytes infected with stage I and stage IIa *P. falciparum* gametocytes are phagocytosed in a similar way [95]. Phagocytosis of giRBCs later than stage II has not been demonstrated [95, 96].

The role of cellular immune mechanisms in the clearance of circulating gametocytes is contentious. As erythrocytes lack major histocompatibility complex molecules, direct targeting of giRBCs by T lymphocytes is not possible. However, CD4+ T cells clearly respond to gametocyte antigens [97-99] and appear capable of inducing long lasting gametocytocidal immunity in rodent models [100]. Serum factors from splenectomised macaques (infected with *P. cynomolgi*) taken at the point of infection 'crisis' or paroxysm can kill gametocytes, and this appears to be mediated by inflammatory cytokines including tumour necrosis factor-α (TNF-α) which stimulate leukocytes to produce toxic nitric oxides [101, 102]. In semi-immune *P. vivax* infected humans, cytokine concentrations were insufficient to induce killing factors during paroxysm [103], and it is unclear how this may differ in non-immune humans. Interestingly, gametocyte killing factors appear non-specific to species or parasite stage; that is, supernatant from peripheral blood mononuclear cells stimulated with *P. vivax* schizont extract was able to kill *P. falciparum* gametocytes, and *vice versa* [102]. T cell responses seem similarly non-specific to parasite stage [104]. These data present an exciting avenue for whole parasite vaccine development [105], but it remains unclear whether infection crisis in humans leads to meaningful levels of gametocyte death.

Immune responses against intra-erythrocytic gametocytes and exoerythrocytic gametes

In contrast to the sparse and partially conflicting evidence for immune responses against intact giRBCs that may reduce circulating gametocyte density, there is a large and cohesive body of data demonstrating that humoral responses to intra-erythrocytic gametocyte proteins can inhibit parasite development inside mosquitoes [44, 81, 106-122]. The antigens responsible are not present on the erythrocyte surface but are expressed on the intra-erythrocytic gametocyte during their maturation in humans. Key gametocyte surface antigens shared by gametes are involved in processes necessary for colonization of the mosquito midgut: egress from the red blood cell; male gamete exflagellation and exflagellation center formation; fertilization and ookinete invasion of the gut epithelium. As gametocytes die in the human host they are cleared by the spleen and the immune system is exposed to these antigens that are shared by the human and mosquito parasite stages. The resulting antibodies circulate in humans, but their functional consequence only becomes apparent in the mosquito, where they can interfere with parasites in the mosquito midgut and cause inhibition or total arrest of the mosquito infection. Though the effect is likely to be transitory and quantitatively less profound compared to transmission inhibition, there is also some evidence that immune factors may lead to enhancement of gamete infectivity in certain conditions [123].

The proof of principle for the existence of transmission reducing (TR) antibody responses comes from experiments in which birds were immunized with whole inactivated gametocytes or gametes in the 1950's and 1970's [42-44, 106, 124]. Evidence for naturally acquired TR immunity acting to prevent mosquito stage parasite development comes from cross-sectional studies using mosquito feeding assays. Mendis et al. showed that Sri Lankan individuals with acute *P. vivax* infections produced gamete specific antibodies, and that antibodies from these patients inhibited transmission in direct membrane feeding assays [109]. Shortly after, Graves et al. showed that similar mechanisms prevented *P. falciparum* gamete viability using the standard membrane feeding assay (SMFA), in which the effect of sera from patients in Papua New Guinea was tested on cultured gametocytes [108]. These experiments demonstrated that the dominant immune mediator of gametocyte infectivity appears to be the humoral immune response. Antibodies directed to surface antigens on the gamete surface may prevent fertilization via direct lysis of gametes by activation of the complement system [125, 126], opsonization resulting in immune cell-mediated lysis [127] or agglutination of gametes [128, 129].

Parasite antigens that can be targeted by antibodies to inhibit transmission can be divided into two broad classes; pre-fertilization and the post-fertilization antigens. Pre-fertilization antigens are expressed during gametocyte development and contribute to the viability of mosquito stage gametes, zygotes and/or ookinetes. Antibodies to pre-fertilization antigens are naturally acquired in the human host only because the majority of gametocytes die in circulation, releasing their intra-erythrocytic proteins. Post-fertilization antigens are expressed solely in the mosquito vector; though transcription may occur in circulating gametocytes, the resulting mRNA is held in translational repression until the gametocytes activate in the mosquito midgut [130]. Consequently, humans do not acquire humoral responses to these antigens. This second group of antigens are beyond the scope of this review, since no naturally acquired humoral responses are observed to these antigens.

The most studied pre-fertilization antigens are P48/45 and P230, which belong to the 6-cysteine protein family [131] and play crucial roles in fertilization [132, 133]. These proteins were first identified as targets of transmission blocking monoclonal antibodies (mAbs) isolated from mice immunized with gametocyte/gamete preparations [48, 107]. The first evidence of naturally acquired antibody responses to these proteins was shown in sera from individuals from Papua New Guinea [108]. P48/45 is attached on the surface of both female and male gametes via a glycophosphatidylinositol-anchor and forms a stable complex with P230 [134-136]. Male gametes of Pfs48/45 and Pbs48/45 knock-out lines are unable to adhere and penetrate female gametes, which results in a dramatic reduction of oocyst number [132]. Although P48/45 is expressed on the surface of macrogametes, its disruption in female gametes does not seem to affect their fertility. Replacing the endogenous *Pb48/45* by its vivax orthologue seemed to reduce oocyst development, but did not abolish it, indicating that there is probably functional conservation of P48/45 in these two parasite strains since fertilization was not affected [137]. In *P. falciparum* gametocytes, Pfs230 is present on the gametocyte surface, and a 50kDa fragment is proteolytically cleaved from the surface-bound protein after the parasites emergence from the RBC in the mosquito gut [138, 139]. Transgenic *P. falciparum* Pfs48/45 knock-outs (KO) produce Pfs230, but it is not retained on the gametocyte surface, indicating that Pfs48/45 mediates Pfs230 retention [133]. Male gametes lacking Pfs230 are still able to undergo host cell egress and exflagellate but are unable to bind uninfected RBCs to form exflagellation centres. The importance of this protein in fertilization is reflected by the significantly reduced oocyst numbers of the Pfs230 KO parasites [133].

In alignment with their exposure on the surface of gametes and importance in gamete fertility, studies have shown that the presence and titer of naturally acquired antibody responses to Pfs48/45 and Pfs230 are statistically associated with serum transmission reducing activity (TRA) [108, 112, 113, 118, 120, 140]. Though the TRA of mAbs against Pfs48/45 and Pfs230 was demonstrated decades ago [47, 48, 107], it was only recently shown that naturally acquired antibodies against Pfs48/45 and Pfs230 are functionally involved in natural TRA [81]. Antibodies against Pfs48/45 and Pfs230 were separately purified from six individuals whose antibodies (total IgG) showed high TRA in the SMFA. The purified antigen specific IgGs were reconstituted to the original serum volume and tested in the SMFA. The α-Pfs48/45 antibodies of one individual blocked transmission independently, whereas α-Pfs230 antibodies from another donor reduced transmission significantly. Concentration of antibodies resulted in higher TRA for several donors. This was the first direct evidence of the reducing potential of naturally acquired antibodies against Pfs48/45 and Pfs230.

Besides P230, rodent and human parasites also encode a paralog P230p. This protein is only expressed by mature male gametocytes [141]. Disruption of the gene in *P. berghei* did not result in any defects throughout the life cycle, indicating that the protein is dispensable [142]. The *p230p* locus in *P. berghei* [143-145] and *P. knowlesi [146]* parasites has been commonly used as a neutral insertion cassette to generate transgenic parasites. On the other hand, *P. falciparum* mutants lacking Pf230p have a strongly reduced ability to bind erythrocytes to form exflagellation centers, similar to the observed phenotype in Pfs230 KO parasites [147]. Pf230p KO resulted in a dramatic reduction in oocyst density in mosquitoes, indicating an important role in fertilization. The misconception that Pfs230p is dispensable has resulted in the disregard of this protein as a target of TR immunity. To our knowledge mAbs targeting P230p in *P. falciparum* or *P. vivax* have neither been tested, nor have proteins been produced for immunization studies with a view to TBV testing. Naturally acquired Pfs230p antibody responses have been assessed in one study, which linked the TRA of serum antibodies from individuals living in malaria endemic areas with the same individual's antibody responses to 315 gametocyte enriched proteins [81]. Pfs230p was not the focus of this study, but the microarray data generated are publicly accessible (https://doi.org/10.5061/dryad.8bp05). The magnitude of α-Pfs230p responses was not significantly different between individuals with evidence of blocking transmission in the SMFA and individuals whose antibodies had no notable transmission reduction activity. However, the proportion of individuals deemed 'sero-reactive' in a mixture model was borderline significantly higher in blockers (13.6%) than in non-blockers (3.9%) for one of the two Pfs230p peptides tested (PF3D7_0208900.e1s2, p=0.054). It should be noted that this analysis was not adjusted for false discovery from multiple comparisons, and overall prevalence of responses was low (13/276). Further serological studies with Pfs230p in its native conformation will be valuable.

Pfs47 is another member of the 6-cysteine protein family [131] and is a paralog of Pfs48/45. It is specifically expressed in female gametocytes and present on the surface of female gametes, zygotes and ookinetes [148, 149]. The protein is known to protect the ookinete from the mosquito complement system by disrupting the c-Jun N-terminal kinase pathway in *A. gambiae* [150]. However, Pfs47's role appears unessential as disruption of the gene does not result in a reduction in oocyst numbers in *A. stephensi* [148, 151]. Interestingly, in *P. berghei* the opposite has been demonstrated, with gene disruption resulting in a significant reduction of oocyst numbers [132, 152]. Although Pb47 and Pfs47 are clear paralogs, there is only limited sequence conservation [153], which could explain the observed differences in function. There has been contrasting evidence for the effect of mAbs against Pfs47. A study by van Schaijk et al. showed that transmission was not affected by any of three different Pfs47 specific mAbs in the SMFA [148]. However, recent data suggests that antibodies specific to the central region have the ability to reduce the number of ookinetes in the mosquito midgut and thereby reduce transmission to both *A. gambiae and A. stephensi* [154]. It remains unresolved whether Pfs47 has an essential role in either fertilization or ookinete protection. The magnitude and prevalence of naturally acquired antibodies to Pfs47 appear nearly identical in individuals with antibodies that block transmission in the SMFA and those without such antibodies [81].

The observed differences in phenotype between *P. berghei* and *P. falciparum* after disruption of *P230p* and *P47* suggest that there are functional differences in these proteins between these two *Plasmodium* spp. There are no data available with respect to the function of these proteins in *P. vivax,* and it remains unknown whether they have an essential role in mosquito stage development.

The male-specific sterility gene (*HAP2*) was first identified in *Arabidopsis thaliana* [155], and HAP2 homologues were later identified in higher plants and protists, including *P. berghei* [156]. In *P. berghei* HAP2 is expressed in gametocytes and present on the surface of intra-erythrocytic gametocytes and microgametes. Disruption of the gene results in reduced transmission by blocking gamete fertilization [157]. Using membrane dyes this report demonstrated that HAP2 is not involved in adhesion of gametes but has a role in membrane fusion during zygote formation. Serum from rodents immunized with recombinant PbHAP2 [158] or PfHAP2 [159] inhibited oocyst development in the SMFA. Naturally acquired antibodies against the recombinant PfHAP2 were identified in sera from Malian adults [159]; however, it remains unclear if these functionally contribute to TRA in the field. No significant differences in PfHAP2 specific antibody magnitude or prevalence were observed between SMFA blockers and non-blockers in microarray analyses [81]. However, it has been recently demonstrated that sera from mice immunized with peptides targeting the fusion loop of PfHAP2 inhibit the transmission of *P. falciparum* gametocytes sourced from naturally infected donors [160]. These data make HAP2 an interesting antigen for more extensive study. To our knowledge there are currently no data testing HAP2 function in *P. vivax,* though it seems likely that the function is conserved in *P. vivax* based on the observed functional conservation of HAP2 throughout species [156].

Antibody mediated transmission reduction has been observed in the absence of Pfs48/45 and Pfs230 antibodies in serum [41, 108, 110, 114], and after active depletion of these antibodies from purified total IgG fractions [81]. These observations have led to the hypothesis that antibody responses to other sexual stage pre-fertilization surface-associated may contribute to naturally acquired TRA. In a recent microarray analysis of antibody profiles against gametocytes and TRA, antibody responses to 13 novel proteins were associated with TRA and displayed features that suggest surface expression. This includes Pf11-1 and PfGEST, which have both been implicated in the process of gamete egress from the erythrocyte [161-163]. Monoclonal antibodies against Pf11-1 can reduce transmission possibly by interfering with egress [162]. PbGEST KO gametes show a clear defect in host cell egress [163]; however, there are no data on the *P.* falciparum orthologue. Future work should include the evaluation of these antigens in rodent immunization studies to determine their potential to induce functional antibody responses. The remainder of the TRA associated antibody specificities in this analysis were largely conserved proteins with an unknown function, so it remains to be seen if these have the potential to induce functional transmission blocking immunity.

Though antibodies targeting gametocytes has been the focus of epidemiological and vaccine focused research, other human immune factors are ingested by mosquitoes when they feed and co-circulate with parasites as they activate and develop in the mosquito gut. Human phagocytes are present in the pre-digested blood meal and gametes (after RBC egress) are potentially vulnerable to direct phagocytosis. It has been shown *in vitro* that extracellular gametes can be phagocytosed and that the addition of immune serum leads to an increase in phagocytosis [94]. However, these processes were inefficient in the environment of the mosquito midgut, probably due to the reduced temperature (26°C rather than 37°C). The role of human cellular immunity on mosquito stage parasites is likely to be limited.

Modulation of transmission by anti-gametocyte immune responses

Read-outs and methods of assessment:

Immune modulation of malaria transmission can be assessed directly using mosquito feeding assays [108, 123], which can determine both the direct transmission potential of naturally infected hosts and the effect of host immune factors. The SMFA is the most controlled of these assays, in which plasma, serum or their purified components are added to a blood source containing cultured gametocytes [164]. Multiple SMFA feeds can be performed in parallel with the same infective material, and all experiments are performed with a relevant control (e.g. the same infective material with plasma from non-malaria endemic areas) allowing results to be combined and compared reliably between experiments. Transgenic parasites can be used to increase the scalability of the SMFA for population cross sectional screening, using luminescence as the assay read-out rather than dissection and oocyst counting [165]. Alternatively, the direct membrane feeding assay (DMFA) allows the impact of immune factors to be assessed on locally circulating parasite strains with more natural gametocyte characteristics (i.e. density, sex ratio, maturity). In its most basic form, the DMFA involves feeding colony adapted mosquitoes blood collected from naturally infected individuals to determine their transmission potential; as in the SMFA, the traditional read-out is the number of midgut oocysts or salivary gland sporozoites. To assess immune modulation, the plasma component of the blood sample (i.e. its autologous plasma) can be replaced with naïve plasma, revealing the transmission potential of gametocytes in the absence of immune factors from the host. In this 'serum-replacement' version of the assay (so called because anticoagulants were not used when the assay was developed [109]) transmission modulation due to the autologous plasma is determined by comparison to a feed where autologous plasma is removed and then replaced as a control for the methodological disturbance [166].

The read-out of the SMFA and serum-replacement DMFA is generally reported as percent TRA. This is the percent inhibition of oocyst density (or sometimes prevalence) in test mosquitoes relative to the experimental control. For example, if mosquitoes fed a blood meal containing test plasma have a mean oocyst load of ten, and control mosquitoes a mean oocyst load of 100, the percent TRA would be 90%. Transmission inhibition by immune factors is referred to as either transmission reducing (TR) immunity or transmission blocking (TB) immunity. Specifically, 'blocking' should refer only to the total annulment of mosquito infection. Transmission enhancing (TE) immunity is less commonly reported than TR immunity, and though random variation of mosquito infection rate around the baseline (control) is likely to be the cause of many observations of low level TE, there is substantial evidence for immune mediated enhancement of immunity [167]. TE would be reported as a negative TRA, or a relative infectivity >100%. As with all biological systems, the results of feeding assays require confirmation; replicable TRA of ≥90% in the SMFA is viewed as high-level reduction (equivalent to blocking in natural infections) [168]. The aim of transmission blocking vaccine development is to induce TB immunity, which will reduce the number of infected mosquitoes feeding on a vaccinee. TR and TE immunity are a continuum (thus transmission modulation) while TB immunity is likely to be uncommon in nature, so we use the former terms.

P. falciparum transmission modulating immunity:

In experiments with sera from Papua New Guineans performed by Graves et al., serum TRA in SMFA experiments varied from -124% TRA (TE immunity) to 99.4% (TR immunity). TE immunity was apparent in 10/41 sera (5/33 tested in duplicate) [108]. Subsequent studies have shown that TR immunity against *P. falciparum* develops rapidly after malaria exposure [119] and is short lived [120, 122]. Cross sectional analyses show wide variations in the frequency and intensity of *P. falciparum* TR immunity [110, 114, 121], which is likely related to differences in sampling strategy and transmission intensity. Van der Kolk et al. performed a rigorous assessment of transmission modulation in 642 sera from Cameroonian, Indonesian, and Tanzanian *P. falciparum* gametocyte carriers [121]. SMFA showed that TR immunity was present in 48% of sera, while TE immunity was present in 7%. The reproducibility of these results was variable, but significant numbers retained their TE and TR activity in repeat feeds. For *P. falciparum*, studies with serum-replacement DMFA generally show increases in mosquito infection rates between 14-66% in the absence of host immune factors [114, 116, 169, 170], corroborating the results of individual studies that TR immunity is more common and has a greater effect size than TE immunity.

There is evidence that TR immunity is associated with the presence or titre of antibodies against mature *P. falciparum* gametocyte antigens [81, 108, 110-122, 171-173]. A synthesis of data from six studies which measured antibody mediated TRA in the SMFA and measured α-Pfs48/45 and α-Pfs230 responses by enzyme-linked immunosorbent assay (ELISA) [115, 116, 118-121] showed that there was a significantly increased likelihood of strong TRA (≥90%) for individuals seropositive for either antigen (combined odds ratio (OR) = 3.72 [1.96-7.15, $p<0.0001$]) [41]. Despite the general consensus, several individual studies showed no association between α-Pfs230 and/or α-Pfs48/45 and TR immunity [108, 110, 114, 121]. TR immunity has been observed in the absence of Pfs48/45 or Pfs230 antibodies, and vice versa [108, 113-115, 120, 121]. These somewhat inconsistent findings suggest that TRA due to α-Pfs48/45 and α-Pfs230 antibodies is incomplete in most individuals, may be synergistic, and that responses to unknown gametocyte surface proteins may also affect transmission. With an agent based model combining DMFA, antibody, and parasite density data, Ouédraogo et al. recently demonstrated that antibodies against Pfs48/45 and Pfs230 were associated with up to 44% reduction in the proportion of infected mosquitoes and up to 70% reduction in oocyst density, among individuals with and without observable gametocytaemia in Burkina Faso [171]. Stone et al. [81] used plasma from numerous cross-sectional studies [32, 35, 116, 174-179] to assess antibody mediated transmission modulation. As in previous studies, antibody mediated TRA (quantified in SMFA) was statistically associated with the presence of Pfs48/45 and Pfs230 antibodies (combined OR 5.90 [95% CI: 2.1–16.7], $p=0.001$). These and other antibody correlates of TR immunity (including α-Pf11-1 and α-PfGEST) were also associated with reduced infectivity in field based DMFA. *Plasmodium falciparum* gametocyte immune responses may therefore contribute to the substantial variation in mosquito infectivity from natural gametocyte donors (**Figure 2C, 2D**).

P. vivax transmission modulating immunity:

In 1987, Mendis et al. showed that naturally acquired antibody mediated immune responses to the sexual stages of *P. vivax* (confirmed by immunofluorescence) were able to block *P. vivax* gametocyte transmission in the DMFA [109]. In this study, two-thirds of serum samples from acutely infected individuals in Sri Lanka (n=40) mediated mid to high level reduction of autologous parasite transmission, while three samples showed oocyst intensities in the presence of the test sera 'considerably greater than in controls'. In a subsequent study, the authors showed that the effects of this immunity were short-lived; reductions were associated with an interval of <4 months between a first and second infection [180]. Also, testing individuals with acute *P. vivax* infection, Carter and Mendis reported complete suppression of mosquito infection by 22% of sera from Sri Lankans (n=196), and enhancement of infectivity in 12% of sera [181]. A technically similar study by Ramsey et al. showed that Mexican individuals with secondary *P. vivax* infections either completely blocked transmission or showed varying levels of reduction (n=41). Individuals with primary infections produced a similar proportion of enhancing and reducing effects in the serum-replacement DMFA (n=63) [182]. TR immunity has been substantiated by numerous other studies [183, 184] and is often accompanied by observations of enhancement.

Few studies assessed the presence of antigen-specific *P. vivax* sexual stage antibodies [185, 186], and to our knowledge, none have associated specific antibody responses with TR immunity. This is likely due to difficulties expressing the proteins in significant quantities in correct conformation, and because the focus of sexual stage protein production efforts has been on *P. falciparum*. Various studies however have assessed *P. vivax* gamete recognition using immunofluorescence assays; Mendis [109] showed that there is a negative correlation between TRA and anti-gamete antibody titer, while Ranakawa [180] showed this correlation only when mosquitoes were fed directly on patient blood, but not on the same blood source through a membrane feeder. A comparatively study of *P. vivax* infected (n=105) and uninfected individuals (n=44) from Colombia also examined antibody titer and TRA using serum-replacement DMFAs. Among infected individuals, 44.6% had 50-89% TRA and 35.2% had ≥90% TRA. Among exposed, currently uninfected individuals, the correlation between anti-gamete antibody titer and TRA was clear; individuals with low titers tended to enhance transmission, and individuals with higher titers had no effect or blocked transmission [184].

Evidence for transmission enhancing immunity:

We recently reviewed the evidence for immune enhancement of *Plasmodium* transmission [167]. In longitudinal assessments of *P. cynomolgi* infection in macaques, anti-gamete antibody titre was shown to increase steadily from baseline. The peak level coinciding with a period of serum TR immunity [187, 188]. When antibody concentration was lower (in the early phase of the infection, and after the peak during convalescence) TE immunity was observed, before eventually both antibody titre and relative infectivity returned to baseline. These findings mirrored observations that gametocytes appeared most infectious to mosquitoes at the very start of a blood stage infection [189, 190]. A key study by Peiris et al. showed that dilution of *P. vivax* anti-gamete antibodies in *P. vivax* infected human blood led to enhanced oocyst infection in mosquitoes compared to controls; highly dilute immune serum and mAbs (which caused significant TRA at higher concentrations) promoted infection in experiments where gametocyte density was insufficient to cause infection without additional factors [123]. In the aforementioned study of *P. vivax* exposed individuals from Colombia, sera with varying levels of TRA were titrated to study the effect of dilution on transmission modulation [184]. Dilution of sera with low TRA showed enhancement (-200%) when diluted, while dilution of sera with no TRA did not have the same effect, indicating a possible role for low levels of blocking antibodies in TE.

These studies collectively promote the hypothesis that ingestion of low sexual stage antibody titers may lead to enhanced mosquito stage infection. The work of Peiris et al. shows that for *P. vivax*, enhancing antibodies have the same specificity as reducing antibodies [123]. Carter and Mendis suggested that antibody-mediated TE is the result of both low antibody titers and low inherent gametocyte infectivity (i.e. low gametocyte densities) [181]. This was apparent in the time-course studies of *P. cynomolgi* [187], and stands in contrast to TR immunity that appears to follow intensive, recent gametocyte exposure. The exact mechanisms of enhancement are unknown, but it may be that at low concentration, transmission reducing antibodies targeting shared surface antigens of both male and female gametes, though unable to neutralize the gametes or promote opsonization, may promote fertilization [123]. In *P. falciparum*, the evidence for enhancement is less clear. Although multiple studies show low level immune mediated enhancement for *P. falciparum* [108, 113, 121, 164, 172], Ponnudurai et al. reported minimal enhancement due to Pfs48/45 and Pfs25 mAbs at low titer, and concluded that the phenomenon was due to experimental variation [164]. Recent studies add weight to the enhancement hypothesis for *P. falciparum*; mAbs against the central peptides of the D2 region of Pfs47 (present on the gametocyte, gamete, zygote and ookinete surface) blocked transmission to mosquitoes, while mAbs against proteins at the regions N-terminus gave rise to twice the number of midgut oocysts [154]. The existence and relevance of TE presents an important knowledge gap in our understanding of how anti-gametocyte immunity influences transmission dynamics.

Malaria transmission blocking vaccine development

The conserved nature of gametocyte antigens:

The conservation of a protein across parasite isolates and species is an indicator for its specialization and functional preservation. Antigenic variation of *P. falciparum* RBC surface antigens results from the constant re-organization and variable expression of *var* genes [191]. This gene family encodes the PfEMP1 surface protein that mediates immune evasion and sequestration. Though the *var* gene family is the most intensively studied, non-*var* gene variation is increasingly appreciated, in large part due to recent sequencing efforts and the availability of sequence data in open access databases (PlasmoDB [192], PlasmoView [193] and Pf3k [194]). A common observation is that gametocyte-specific genes show higher sequence conservation than asexual blood stage-expressed genes.

Assuming positive selection for variant genes is part of an "arms race" between the human host and the parasite, there are several reasons why gametocyte proteins might be more conserved than blood stage proteins. The human host immune response will be targeted more towards the asexual blood stages as they vastly outnumber gametocytes during acute infection; <5% of microscopically detectable parasites are gametocytes in most endemic settings [195]. However, this proportion appears to change with host age and immunity, transmission intensity, and duration of infection [30]. Furthermore, asexual parasites actively remodel the RBC surface protein structure, while mature gametocytes do so to lesser extent. This difference has implications for the antigenic targets of immunity because cytoplasmic, mitochondrial and nuclear proteins are more conserved than exported or apicoplast- and membrane-targeted proteins [196]. Recently, the duality between internal and, potentially, secreted proteins was confirmed for immature gametocytes. It was shown that even a subset of surface-associated early gametocyte proteins had very little sequence variation indicating limited selection by host immunity [80]. The limited genetic variation in gametocyte-specific genes is illustrated by challenges to discriminate the gametocytes from multiple clones. The sequences of *Pfs48/45* and *Pfs16* in lab-adapted strains and isolates from Papua New Guinea showed few polymorphisms, while diversity in *Pfs230* fragments allowed for some discrimination between samples [197]. Genetic variation among *P. falciparum* TBV candidates and their orthologues in *P. vivax* is lower compared to key vaccine targets for the pre-erythrocytic or asexual blood stage (*P. falciparum* reviewed in [198], *P. vivax* reviewed in [199]) (**Fig. 3**). The high conservation of gametocyte-specific genes makes these attractive targets for vaccination, increasing the chances of being effective in a strain-transcendent manner.

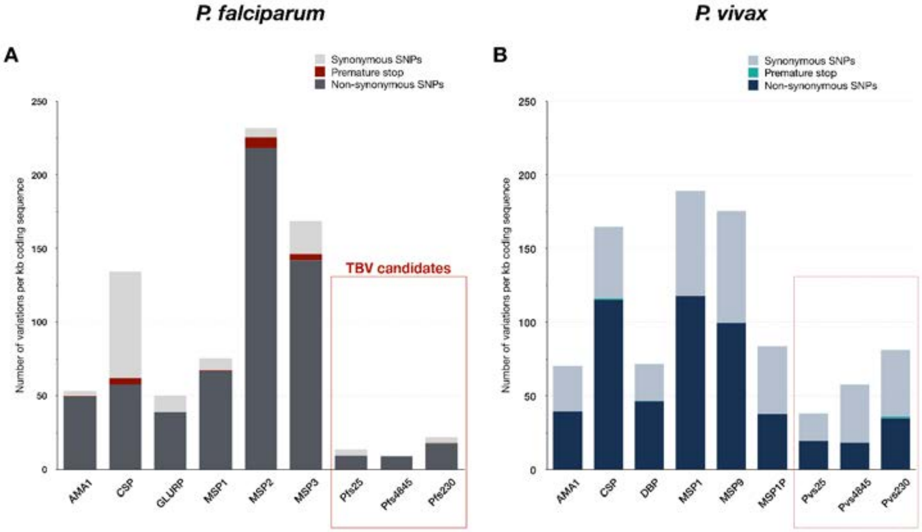

Figure 3. Reported variations in *P. falciparum* and *P. vivax* vaccine candidates. All plasmoDB (v44) listed variations in *P. falciparum* (**A**) and *P. vivax* (**B**) vaccine candidates normalized for gene length. Transmission blocking vaccine (TBV) candidates in *P. falciparum* and their orthologues in *P. vivax* are indicated with a red box and dotted box, respectively. The displayed TBV candidates for *P. falciparum* harbour an average non-synonymous SNP density of 11.98 SNPs per kb coding sequence, while the pre-erythrocytic and blood stage candidates have significantly more non-synonymous SNPs (95.54, p=0.017, Welch's t-test). For *P. vivax*, the orthologues of *P. falciparum* TBV candidates have an average of 24.34 non-synonymous SNPs/kb, significantly less than the targets of asexual stage vaccine candidates (76.01 non-synonymous SNPs/kb, p=0.011, Welch's t-test). Premature stopcodon is introduced by a non-synonymous SNP and as a result the stability and function of transcripts and proteins can be altered.

Leading transmission blocking vaccine candidates:

It is conceptually attractive to develop a vaccine that affects transmission to mosquitoes by inducing immune responses that prevent sequestration, interfere with gametocyte maturation or target mature circulating gametocytes in the human host. Recent findings that antibodies against antigenic targets expressed on the giRBC surface are negatively associated with both asexual and gametocyte load in Malawians [80] provide a lead for further investigation that should demonstrate whether giRBCs are a viable target for immunization.

By comparison, there is a much longer history of TBV development targeting antigens present on the surface of intra-erythrocytic gametocytes and exoerythrocytic gametes. The rationale for the development of these TBVs comes from above-mentioned animal studies where transmission reducing immunity was induced by vaccination [44, 106] and paved the way for the identification of P48/45, P230 and P25 as important TBV candidates [48, 107].

The post-fertilization antigen P25 is the most extensively studied TBV candidate to date with a variety of vaccine constructs evaluated in both pre-clinical studies and clinical trials (reviewed by Chaturvedi et al. [200] and Mueller et al. [201] (**Fig. 4A**). P25 contains four EGF-like domains with 22 cysteines [202, 203] and is anchored on the surface of zygotes and ookinetes [48]. The first in-human trials with full-length Pfs25 showed modest immunogenicity [204], stimulating efforts to develop more potent vaccine products, such as fusion with and coupling to carrier proteins (Pfs25-EPA [205], Pfs25-IMX313 [206], Pfs25-GPI [207]), expression on virus-like particles [208] or combining Pfs25 with other adjuvants [209, 210]. Administration of virus-like particles comprising Pfs25 in healthy US adults was safe but TRA of the induced antibodies was weak [211]. Pfs25 conjugated to *Pseudomonas aeruginosa* ExoProtein A (EPA) has been tested in malaria naïve US adults, and is the only Pfs25 construct tested in malaria-exposed individuals. Four doses were required to induce higher functional transmission reducing antibody responses in a subset of vaccinated Malian adults compared to the control group and antibody levels waned rapidly [212, 213]. The future of Pfs25 as a TB vaccine is uncertain given the limited efficacy and the availability of other candidates that may hold more promise, including Pfs48/45 and Pfs230.

The development of vaccines against P48/45 and P230 has been challenging due to their complex native conformation. Pfs48/45 is a 51.6 kDa protein and contains three domains with up to six conserved cysteines in each domain [131, 134, 214] (**Fig. 4B**). The correct formation of disulphide bridges within each domain is essential for proper protein folding, which is necessary for eliciting functional transmission blocking antibody responses [215]. Production of correctly folded full-length Pfs48/45 with sufficient yield has had highly variable success rates in different heterologous expression systems including baculovirus-insect cells [134], *E. coli* [216], Vaccinia virus [217], yeast [218], *Nicotiana benthaniana* [219, 220], green algae [221] and *D. melanogaster* S2 cells [222]. It has been demonstrated that the C-terminal fragment containing six cysteines (6C) (Epitope I) is the target of the most potent transmission blocking mAb (85RF45.1) [223]. Therefore, several truncated versions of Pfs48/45 were produced, both in *E. coli* and *Lactococcus lactis,* including a two-domain fragment containing ten cysteines (10C) [224-226] and the 6C fragment [227]. Alternative approaches, to overcome problems with recombinant protein expression of Pfs48/45 include the use of DNA vaccines, showing promising results in non-human primates [228, 229], and expression of Pfs48/45 as a transgene in *P. berghei* parasites [145]. After decades of limited success, TBV development of both full-length and a truncated version of Pfs48/45 now show considerable progress [222, 225-227, 230-233]; both full-length Pfs48/45 and R0.6C (Pfs48/45-6C fused to the N-terminal region of the glutamate rich protein GLURP-R0) are being prepared for clinical trials in the near future.

The expression of Pfs230 has been even more challenging and has long lagged behind Pfs48/45. Pfs230 is over 300kDa in size and contains 14 six cysteine motifs, which has hampered the expression of full-length protein (**Fig. 4C**). The first Pfs230-specific mAbs were generated by intraperitoneal injection of isolated *P. falciparum* macrogametes in mice [48]. The isolated mAbs against Pfs230 lacked the ability to reduce transmission, a finding that nearly eliminated Pfs230 as transmission blocking vaccine candidate. It was subsequently demonstrated that complement was required for the blocking activity of these mAbs to Pfs230 [234, 235], and that only mAbs of a complement fixing isotype blocked transmission [236].

The production of six fragments of Pfs230 allowed the identification of an N-terminal region of Pfs230 (C-region) that induces functional antibody response [237]. A subsequent study aimed to further define the region by expression of smaller fragments of this C-region [238]; None of the truncated fragments was able to induce a functional antibody response, while the anti-serum did recognize the native protein on gametes. However, Tachibana et al. more recently expressed the N-terminal C-region and several truncated versions thereof and demonstrated that, besides the whole C region, the truncated (C2, C1 and C0) fragments were also able to induce a functional antibody response, even in the absence of complement [239]. They emphasized that the minimal epitope required to induce functional antibodies is the N-terminal pro-domain which does not contain cysteines.

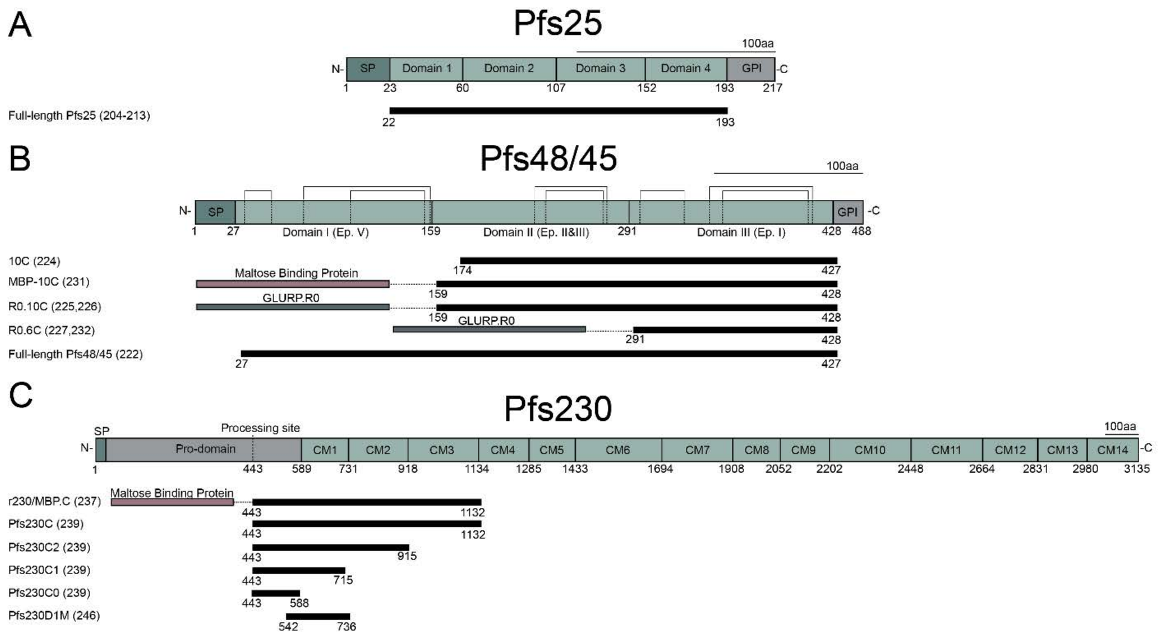

Figure 4. Native protein structure of Pfs25 (A), Pfs48/45 (B) and Pfs230 (C)

(**A**) Schematic representation of the four EGF-like domains of Pfs25 with 22 cysteines with, underneath, the full-length vaccine construct used in pre-clinical and clinical studies.

(**B**) Domain structure of Pfs48/45 with cysteines forming disulphide bridges (dotted lines) based on homology to other 6-cys domain proteins. Underneath, several vaccine constructs are presented that have been tested in pre-clinical studies.

(**C**) Schematic of Pfs230 with 14 cysteine motifs (CM). The processing site is the location where the protein is cleaved after gamete emergence from the red blood cell. Underneath, vaccine constructs that have been tested in pre-clinical studies; Pfs230D1M has been tested in clinical studies (ClinicalTrial.gov NCT02334462 and ClinicalTrial.gov NCT02942277). SP: Signal peptide; GPI: Glycosylphosphatidylinositol anchor·

Similar to Pfs48/45, many different expression systems have been used to overcome difficulties of expressing native epitopes of Pfs230. These include *E. coli* [237, 238, 240, 241], a wheat germ cell-free system [239], *Nicotiana benthamiana* [242], the baculovirus-insect cell system [243, 244] and DNA vaccination [245]. Importantly, all Pfs230 constructs that have been expressed in the recent years are based on the results of the first study demonstrating that region C is the only part that elicits functional antibodies [237]. Recently, a systematic approach by Tachibana et al. aiming to express 27 different protein fragments confirmed that the first cysteine motif is the main region for the induction of functional antibodies. Nevertheless, it cannot be excluded that other regions of Pfs230 are also targets of functional antibodies.

Currently, the most advanced Pfs230 construct in the clinical pipeline is the Pfs230D1M construct expressed in yeast. This construct comprises amino acids 542 to 736 and has been conjugated to EPA [246]. Sera of rabbits immunized with Pfs230D1M-EPA in Alhydrogel® were able to block transmission of cultured *P. falciparum* NF54 and two Thai patient isolates in the SMFA. Safety studies have been performed in US and Malian adults with this construct formulated in Alhydrogel® (ClinicalTrial.gov NCT02334462) and AS01 (ClinicalTrial.gov NCT02942277). Results of these trials have to date not been reported. Recruitment is ongoing for a phase 2 trial using the Pfs230D1M-EPA/AS01 vaccine. In this trial, groups of healthy Malian children of decreasing age will be recruited (ClinicalTrial.Gov NCTO03917654).

While clinical trials are ongoing, more potent Pfs230-based formulations are being developed, including the conjugation of Pfs230D1M to the Outer Membrane Protein Complex (OMPC) which induces higher TRA responses in mice than Pfs230D1M-EPA [247].

P. vivax TBV research has moved along the same path as that of *P. falciparum,* although the inability to culture *P. vivax* infected RBC *in vitro* (and thus gametocytes) has limited vaccine discovery and evaluation.

The best characterized *P. vivax* transmission blocking vaccine candidate to date is Pvs25 [203]. Early studies demonstrated that Pvs25 has a superior ability to induce potent transmission blocking antibodies compared to another ookinete surface protein Pvs28 [248, 249]. Phase I clinical trials in healthy US adults using full-length Pvs25 expressed in *Saccharomyces cerevisiae* (Pvs25H) [250] formulated in Alhydrogel® resulted in only modest induction of transmission blocking antibodies [251]. Pre-clinical adjuvant optimization studies demonstrated potent induction with Pvs25H formulated in Montanide [252-254], but a phase I clinical trial was terminated due to unexpected high reactogenicity related to the adjuvant [204]. This stimulated efforts to develop different vaccine antigen formulations with higher potency and lower undesirable reactivity, such as viral delivery systems [255, 256] or fusion to carrier molecules [257-259]. It remains to be investigated whether these formulations are safe and improve Pvs25 immunogenicity in humans.

Full-length Pvs48/45 has been recombinantly expressed in *E. coli* with intact native epitopes as demonstrated by reactivity of antibodies in sera from Colombian individuals [185]. Potent antibodies were induced in immunization studies using mice and monkeys as demonstrated by complete transmission blocking activity in DMFAs using three natural isolates. Similar to Pfs48/45, Pvs48/45 recombinant protein expression has been challenging; Tachibana et al. [260] used DNA immunizations to overcome problems with protein folding. They immunized rodents with both full-length *Pvs48/45* and the C-terminal cysteine rich domain equivalent to Pfs48/45 6C. Antibodies against full-length Pvs48/45 showed superior reactivity against protein lysates and reduced oocyst numbers. Whether antibodies against Pvs48/45 6C were able to reduce oocyst numbers has not been tested. Pvs47, orthologue of Pvs48/45, has also been tested in this study and antibodies induced after DNA immunizations were also able to reduce oocyst number. Using a similar approach, rodent immunizations with a fragment of Pvs230 based on the Pfs230C region (excluding the pro-domain) also demonstrated the induction of functional antibody responses [261]. Interestingly, the reduction of oocyst numbers in the DMFA was not dependent on the addition of complement. These preliminary data indicate a potential for these pre-fertilization antigens as TBV, but more extensive studies will be required to confirm feasibility.

Several TBV candidates (e.g. Pfs25 [204, 211-213], Pfs230 (Clinicaltrails.gov NCT02334462 and NCT029442277) and Pvs25 [204, 251]) have been tested in clinical studies in the last two decades and Pfs48/45 based vaccine constructs are currently being prepared for clinical testing. In addition, transmission blocking mAbs are increasingly being considered to reduce transmission in exceptional circumstances and support vaccine development.

Transmission blocking monoclonal antibodies

The importance of naturally acquired immunity in malaria was demonstrated in the 1960s by the passive transfer of polyclonal antibodies from adults to infected children, reducing parasitemia and alleviating (severe) clinical disease [262]. It is increasingly recognized that the B-cell repertoire of individuals who have been exposed to and are protected against an infectious disease can be a rich source of highly potent mAbs. For human use, mAbs should be potent, target conserved epitopes and preferably be of human origin.

Table 1. Selected transmission blocking monoclonal antibodies

Target	Name	Target epitope	Source	Isotype	Potency in SMFA
Pfs230	63F2A2.2a (236)	Unknown	Mouse	IgG2a	80% at 1 μg/ml, 100% at 4 μg/ml
	P5E2-2F7-2B4 (234)	Unknown	Mouse	IgG2a	72% at 10 μg/ml, 97% at 30 μg/ml
Pfs48/45	85RF45.5 (223)	Epitope V	Rat	IgG2a	79% at 25 μg/ml. 98% at 50 μg/ml,
	32F3 (48, 223)	Epitope I	Mouse	IgG2b	61% at 12.5 μg/ml. 99% at 25 μg/ml
	85RF45.1 (223, 267)	Epitope I	Rat	IgG1	IC_{80} = 1-2 μg/ml
	TB31F (267)	Epitope I	Humanised rat antibody	IgG1	IC_{80} = 0.5-1 μg/ml
Pfs25	4B7 (263, 269)	Site 1a/b, EGF3	Mouse		IC_{80} = 29 μg/ml
	32F81 (48, 311)	LDTSNPVKT peptide on EGF3	Mouse	IgG1	>80% at 10 μg/ml
	AB1245 (263)	Site 2, EGF1-4	Transgenic mouse	Generated as IgG1	IC_{80} = 263 μg/ml
	AB1269 (263)	Site 1a/b, EGF3	Transgenic mouse	Generated as IgG1	IC_{80} = 63 μg/ml
	2530 (264)	Site 3, mainly EGF2	Human		IC_{80} = 65 μg/ml
	2544 (264)	Site 1, EGF1/3/4	Human		IC_{80} = 16 μg/ml
Pf11-1 (Pfs2400)	mAb1A1 (162)	Nonamer repeat [PEE(L/V) VEEV(I/V)]2	Mouse	IgG1	70 & 80% at 93 μg/ml, 58% at 44 μg/ml and 57% at 9 μg/ml
Pfs47	IB2 (154)	Central region of domain 2	Mouse		66, 70, 84 & 88% at 200 μg/ml (4 feeds)
	BM2 (154)	Central region of domain 2	Mouse		74 & 94% at 200 μg/ml (2 feeds)

For each target the most potent and human(ised) monoclonal antibodies (mAbs) are given. Note that information about potency is limited for many mAbs since these have often been tested at few, unknown or unspecified concentrations. All mAbs against Pfs230 are complement dependent, unlike mAbs against other targets (235, 236).

The first human mAbs against a sexual stage antigen were derived from transgenic mice, i.e. mice expressing human immunoglobulins, that were immunized with Pfs25. Characterization of these mAbs revealed sites associated with transmission blocking activity but also demonstrated that these mAbs have low potency [263]. More recently, anti-Pfs25 mAbs were isolated from a human volunteer that was immunized with Pfs25. These mAbs target three different epitopes, two of which had been identified in the transgenic mice study. Interestingly, one of these human mAbs is the most potent anti-Pfs25 mAb described to date [264]. A large panel of (potent) rodent mAbs against other sexual stage targets is available and could be of interest for therapeutic use upon humanization [265]. Many of these mAbs were isolated after immunizations described above that contributed to the identification of P230 and P48/45 and showed high potency in the SMFA [48, 107, 234, 235] (**Table 1**).

Monoclonal antibody 63F2A2.2a is the most potent Pfs230 mAb to date. The most potent Pfs48/45 mAbs described to date target epitope I on the C-terminal 6-Cys domain [215, 223]. mAb 85RF45.1 achieves >80% TRA at 1-5 µg/ml [223, 266, 267] and is currently being developed for clinical testing. The variable sequence of the heavy and light chains were used to identify the closest human germline homologues and design a humanized antibody, TB31F, that had a similar affinity and potency as the parental mAb [267]. Furthermore, crystal structures of both 85RF45.1 and TB31F with the C-terminal 6-Cys domain revealed that the antibodies target a highly conserved site on Pfs48/45 [222, 267]. This suggests that TB31F will be effective against most *P. falciparum* strains, a finding that can be confirmed using DMFA with genetically diverse strains in field settings. Interestingly, TB31F appears more stable than 85RF45.1, with higher aggregation and melting temperatures [267], highlighting one of many possibilities to engineer antibodies to introduce desired characteristics [268]. Although transmission reducing mAbs against other sexual stage targets such as Pfs25 [48, 263, 269], Pf11-1 [162], Pfs47 [154] have been identified, mAbs against Pfs230 and Pfs48/45 appear to be the most potent and may therefore be prioritized (**Table 1**). Rodent mAbs with TRA against *P. vivax* have been described. Crystallization of one of these in complex with Pvs25 revealed its binding site; however, the potency of this mAb has not been established [270]. Monoclonal antibodies against *P. vivax* target unknown antigens and/or have very limited information on potency [123, 183, 271, 272]. Better characterization of these mAbs and identification of novel human mAbs will thus be required before considering clinical development of mAbs that target *P. vivax* transmission.

Isolation and characterization of human mAbs can also guide vaccine design, an approach that has been pioneered in the quest for a broadly neutralizing HIV vaccine [273]. Only recently have researchers started isolating human mAbs against malaria antigens. Two independent groups isolated B-cells from volunteers who were immunized with sporozoites and identified potent mAbs against Circumsporozoite Surface Protein (CSP) that are unique in binding both the NANP repeat as well as the junctional epitope [274, 275]. Not only are these good candidates for therapeutic antibodies, they also provide valuable information for vaccine design. Strikingly, the CSP targeting vaccine RTS,S (Mosquirix) lacks the junctional epitope. It is tempting to speculate whether including this epitope may increase vaccine efficacy [274-276]. Another example of how mAbs can inform vaccine design comes from the structural studies of 85RF45.1 in complex with Pfs48/45 that describe epitope I [222, 267]. Since antibodies against the epitope I are very potent, vaccine design should aim to direct antibody responses against this conserved epitope. This could for instance be achieved by either reducing immunogenicity of other regions on Pfs48/45 through amino acid mutations and glycosylation or coupling of the antigen to virus-like particles in such a way that epitope I is well presented to cross-link B-cell receptors.

It is anticipated that many human mAbs against sexual stage targets will become available in the near future, either from individuals who are naturally exposed to gametocytes and exhibit high levels of TRA [41, 80] or from individuals taking part in ongoing clinical trials with Pfs230 (ClinicalTrial.gov NCT02334462, NCT02942277, NCTO03917654) or forthcoming trials with Pfs48/45. These developments will plausibly support the development of next generation vaccines and may potentially warrant further clinical development of mAbs for passive immunization.

Evaluation of malaria transmission blocking vaccines

Pre-clinical testing

TBVs and mAbs are recognized as tools with great promise for malaria elimination initiatives. As a consequence of the increased interest in elimination, the pipeline of candidate TBVs and mAbs with transmission blocking properties has expanded considerably in recent years [178, 206, 232, 233, 257, 277-280], not only in terms of antigens but also in terms of adjuvant formulations and delivery platforms. Whilst TBVs are most likely to be used in combination with vaccines targeting other stages [281], guidelines of the US Food and Drug

Administration stipulate that vaccine components require efficacy assessments as stand-alone products. There is currently no consensus on protocol design and endpoints of clinical trials with transmission blocking interventions. Given the complexity and expense of phase III field evaluations that are likely to involve cluster-randomized trials (CRT), it is, however, evident that robust pipelines for candidate prioritization are needed [233]. The early evaluation of TBV candidates currently depends on the SMFA where reductions in oocyst density or prevalence are used as indicator of vaccine potency.

Importantly, these *in vitro* assays are only available for *P. falciparum* and not for *P. vivax* where parasite culture is currently not possible. In addition, SMFA typically relies on a single gametocyte-producing *P. falciparum* parasite line at unnaturally high gametocyte densities in combination with a single mosquito strain [282]. Whilst the value of the SMFA for early TBV evaluation is beyond dispute, the assay has clear limitations when estimating the public health impact of TBVs. The only *P. falciparum* TBV with published results from a field study to date (Pfs25-EPA as described above) showed excellent results in *in vitro* assays but failed to induce substantial transmission blocking activity when serum of vaccinated volunteers from the United States was offered to mosquitoes with cultured gametocytes [213]. The candidate proceeded to a clinical trial in Malian adults where it showed limited efficacy [212]. This experience illustrates the challenges with early evaluation assays and the urgent need for a model to accelerate vaccine development and identify early failures of vaccine candidates.

One approach that allows pre-clinical samples, e.g. serum samples from rodents, in a real-life context is to add these antibody samples to giRBCs of naturally infected individuals in the DMFA. These natural giRBC gametocyte donors can be infected with multiple gametocyte-producing clones [283, 284], thus allowing the testing of antibodies induced in pre-clinical studies against genetically diverse and complex gametocyte infections at natural gametocyte densities using locally relevant mosquito populations [282]. This approach has been utilized to assess the efficacy of pre-clinical and clinical samples for *P. falciparum* TBVs Pfs48/45, Pfs230 and Pfs25 [81, 178, 285] and for pre-clinical samples for *P. vivax* TBVs Pvs48/45, Pvs230 and Pvs47 as described above [185, 260, 261]. This *ex vivo* assessment of antibody efficacy may be used to support the interpretation of early immunogenicity trials and explore possible challenges with genetically diverse gametocytes and escape mutants

Early clinical testing

An early evaluation model has recently been developed that may allow a direct assessment of vaccine efficacy against gametocytes that are induced in the vaccinated donor.

Controlled human malaria infection (CHMI) models provide powerful tools for early evaluation of malaria vaccine candidates and have been used predominantly for pre-erythrocytic [286-288] and blood stage [289] vaccines. CHMI involve deliberate and controlled infection of malaria naïve individuals with either sporozoites (by mosquito bite or intravenous injection of cryopreserved sporozoites) or parasitized erythrocytes (iRBCs) [289-292]. The appearance and timing of blood stage parasites can be used as an endpoint for CHMI testing pre-erythrocytic vaccines. Parasite multiplication rate can be used as an endpoint for blood stage [286, 289] vaccines. For transmission blocking vaccines, CHMI-transmission models were recently developed using mosquito bite or iRBC inoculation with *P. falciparum* 3D7 parasites. Treatment of asexual parasites with a subcurative dose of piperaquine monotherapy allows the production of viable mature gametocytes whose infectivity can be assessed by DMFA or direct skin feeding experiments. Both mosquito bite [21] and iRBC inoculation [33] result in the production of male and female gametocytes in all volunteers. Gametocyte production is markedly higher following iRBC inoculation, for unknown reasons, and results in a higher likelihood of volunteers infecting mosquitoes by either DMFA or direct skin feeding. This model makes it possible to test the efficacy of highly efficacious TBVs for *P. falciparum* in small groups of 10-20 malaria naïve volunteers per study arm, all infected with a single well-characterized parasite line [293]. A CHMI-transmission model with similar efficiency in terms of induction of transmissible gametocytes has been developed for *P. vivax* [294]. At present, CHMI-transmission studies have only been conducted in malaria naïve volunteers, with the advance of CHMI studies in naturally exposed populations [295-297], it is only a matter of time until CHMI-transmission studies will also be conducted in endemic settings.

Conventional phase II trials in adult volunteers in endemic settings may complement the clinical development pipeline for TBVs. In addition to immunogenicity and activity of serum samples of vaccinees in the SMFA, such studies can examine the transmissibility of possible naturally acquired infections by DMFA or direct skin feeding [212]. Whilst incident infections in these studies, unlike CHMI-trans, are unpredictable and transmission endpoints may be underpowered unless sufficiently high gametocyte densities (e.g. >5 gametocytes/µL

[31, 33]) are observed in a considerable fraction of study participants, they allow preliminary efficacy assessments against local parasite strains.

Public health endpoint for TBVs

Definitive evidence on the public health endpoints of TBVs will require larger studies that are complex due to the intended outcome of vaccination that aims to reduce incident infections by reducing the reproductive number (R0) of malaria and overall exposure to infective mosquitoes [298]. As such, TBV confers a delayed individual benefit [299] that is achieved by reducing the number of mosquitoes that become infected when biting on vaccinated individuals [298] and thereby progressively reducing the force of infection over multiple transmission cycles [300]. The classic approach to phase III clinical trials with TBV, supported by regulatory agencies in consultation with the malaria research community, is to conduct CRT where clusters (e.g. villages) are randomized to receive either TBV vaccination or no TBV vaccination (control arm) [299]. A cohort of participants in each cluster may be followed for incident infections by molecular or conventional diagnostics as definitive evidence for a reduction in the force of infection [301]. Infectivity of vaccinated and unvaccinated individuals to mosquitoes and *Plasmodium* sporozoite rates in field-caught mosquitoes are among the obvious secondary endpoints, and should provide insight into the mechanism of actions of TBV. As surrogate endpoints, it has been debated whether these outcome measures would allow for accelerated approval [299], thereby allowing registration and postponing (but not annulling) the requirement to demonstrate the public health benefits of TBV until after vaccine implementation. No consensus has been reached in this discussion. The expectation that the largest effect size of TBV will be observed in low endemic settings (e.g. EIR <8 infectious bites/year [302]) or an incidence of infection below 0.2/person/year [303] and the large heterogeneity in malaria transmission intensity between and within clusters, implies that cluster-randomized trials will need to be large, involve many clusters and will be costly.

Concluding remarks

Early malaria research was dominated by investigations of the asexual blood stage that are the cause of clinical disease and mortality. Following the release of the first global malaria elimination framework in 2007 [304, 305], attention has shifted to include human-to- mosquito transmission as an important research interest. These efforts have improved our understanding of gametocyte biology as well as the epidemiology and infectivity of gametocytes. *P. falciparum* has been the primary focus of investigations to date, and our understanding of *P. vivax* gametocyte biology is limited. Several independent studies reported the presence of gametocyte antigens on the *P. falciparum* giRBC membrane surface. Whilst data are in part conflicting [77-80] giRBC immunity may contribute to the large variation in gametocyte production observed in natural infections. Whether *P. vivax* giRBC immunity exists remains unknown.

Whilst the existence of TRA is beyond dispute, and several key antigens are firmly established, the importance of TRA in determining natural transmission dynamics remains to be quantified [81, 171]. This is particularly true for *P. vivax* where very few studies have examined the occurrence of gametocyte immunity and TRA in endemic populations or whether the kinetics of TRA differs with respect to mosquito-derived infection versus relapsing infection.

Compared to TRA, the potential importance of TE for the transmissibility of natural infections is very poorly understood. Understanding the dynamics of TRA and TE following natural exposure and following vaccination may be particularly important for malaria elimination initiatives. Some evidence suggests that malaria transmission efficiency may increase as the burden of malaria decreases [306]; it is conceivable that waning gametocyte immunity may play a relevant role in this. Future research should quantify the acquisition and waning of TRA in relation to malaria exposure, prove or disprove the existence of TE and unravel the associated immune profiles that may include other targets than the well-established Pfs48/45 and Pfs230 [41, 81, 108, 110, 114].

TBVs may play important roles in the malaria endgame, preventing secondary infections from remaining pockets of transmission, preventing malaria outbreaks and protecting other interventions from escape mutants [281]. The discovery of giRBC immunity opens new avenues for TBV development. Great progress has also been achieved with conventional TBVs. Pfs25 has been the first vaccine to be tested in naturally exposed individuals [212], providing invaluable insights for future field trials, and great progress has been achieved with TBVs targeting pre-fertilization antigens. Several pre-fertilization TBV candidates are currently in, or approaching, clinical testing. Next-generation TBVs may include novel immunogens or a combination of known antigens. The future inclusion of multiple antigens may decrease the proportion of vaccinees that respond poorly [307], reduce the risk of escape mutant and potentially result in a synergistic effect [277, 308-311]. Currently, the most logical dual-antigen vaccine would include the top candidates Pfs48/45 and Pfs230 and preliminary data of a chimeric protein showed potential synergy [312]. The increased pipeline of vaccine candidates and mAbs that aim to reduce transmission is promising and generates a sense of urgency to reach consensus on study designs to prioritize candidates and accelerate testing and implementation processes of lead candidates. The expectation that cluster-randomized trials for TBV will be large and costly makes it unlikely that international community can afford such trials for many TBV. This is a strong argument to invest in early evaluation models, improve our understanding of transmission and continue to interrogate naturally acquired and vaccine-induced immunity against gametocyte antigens.

Author's contribution

TB initiated and supervised the literature review and drafting of the manuscript. WS supervised the literature review and drafting of the manuscript. RMJ and SKT performed the literature review and drafted the manuscript. LMK, FGT and MMJ enriched the manuscript by drafting selected sections and by providing constructive comments.

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CHAPTER 5

Naturally Acquired Antibodies to Gametocyte Antigens are Associated with Reduced Transmission of *Plasmodium vivax* Gametocytes to *Anopheles arabiensis* Mosquitoes

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Abstract

Naturally acquired antibodies may reduce the transmission of *Plasmodium* gametocytes to mosquitoes. Here, we investigated associations between antibody prevalence and *P. vivax* infectivity to mosquitoes. A total of 368 microscopy confirmed *P. vivax* symptomatic patients were passively recruited from health centers in Ethiopia and supplemented with 56 observations from asymptomatic *P. vivax* parasite carriers. Direct membrane feeding assays (DMFA) were performed to assess mosquito infectivity; for selected feeds these experiments were also performed after replacing autologous plasma with malaria naïve control serum (n=61). The prevalence of antibodies against 6 sexual stage antigens (Pvs47, Pvs48/45, Pvs230, PvsHAP2, Pvs25 and PvCelTOS) and an array of asexual antigens was determined by ELISA and multiplexed bead-based assays. Gametocyte (< 0.42; $p = 0.0001$) and parasite (ρ= 0.21; $p = 0.0001$) densities were positively associated with mosquito infection rates. Antibodies against Pvs47, Pvs230 and Pvs25 were associated with 23% - 34% reductions in mosquito infection rates ($p<0.0001$). Individuals who showed evidence of transmission blockade in serum-replacement DMFAs (n=8) were significantly more likely to have PvsHAP2 or Pvs47 antibodies. Further studies may demonstrate causality for the observed associations, improve our understanding of the natural transmission of *P. vivax* and support vaccine development.

Keywords: *Plasmodium vivax*, gametocyte, transmission, immunity, Anopheles, malaria, transmission-blocking, vaccine.

Introduction

The significant reduction in malaria burden in the first decade of this millennium has plateaued since 2015 and even reverted in some settings in recent years [1]. Current intervention strategies appear insufficient to achieve malaria elimination in the majority of endemic countries [2]. Whilst most malaria-attributed deaths are due to *Plasmodium falciparum*, *Plasmodium vivax* also has a high clinical burden with an estimated 4.5 million cases in 2020 [1]. *P. vivax* appears particularly hard to eliminate [3], principally due to its ability to form dormant liver stages that can result in relapsing infections unless treated with an effective radical cure. Another challenge in controlling and eliminating *P. vivax* infections is the rapid formation of gametocytes, the parasite life stage responsible for onward transmission to mosquitoes. Once taken up by blood-feeding *Anopheles* mosquitoes, gametocytes activate to become male and female gametes that fuse to form a motile ookinete. This ookinete penetrates the mosquito gut to develop into an oocyst that releases sporozoites that ultimately render the mosquito infectious upon its next bite. Whilst gametocyte maturation is a long process in *P. falciparum*, infectious *P. vivax* gametocytes appear in the bloodstream within 48 hours of blood stage infection [4]. The strong association between asexual parasite and gametocyte density [5] make clinical *P. vivax* malaria cases highly infectious to mosquitoes at the moment of clinical presentation [6; 7]. Given that *P. vivax* parasites are capable of infecting a wide variety of *Anopheles* mosquitoes [8; 9], it is unsurprising that *P. vivax* transmission is highly efficient in many settings.

Naturally acquired human immune responses can reduce or fully prevent the transmission of *Plasmodium* parasites from humans to mosquitoes (recently reviewed in [10]). For *P. vivax*, the first empirical evidence for naturally acquired transmission blocking activity (TBA) [5] came from studies conducted in the 1980s and 1990s in Sri Lanka [11; 12; 13]. In these studies, investigators fed mosquitoes with blood from acutely infected Sri Lankan individuals. In paired feeding experiments, gametocyte infectivity was compared between a condition where the patient's autologous plasma was present in the bloodmeal versus a condition where this plasma was replaced with malaria naïve control serum. In most of these paired experiments, transmission was reduced in the presence of autologous plasma (indicative of plasma derived TBA) although in some experiments the autologous plasma had the opposite effect and thus enhanced transmission. [12; 13]. In supporting experiments, indirect immunofluorescence tests showed that sera with TBA were more reactive to gamete surface antigens compared to sera without this functional activity. Moreover, purified immunoglobulins from these suppressive sera blocked transmission when added to a gametocytaemic bloodmeal, confirming this TBA to be antibody-mediated [12]. Subsequent studies in Mexico [14], and Colombia [15] similarly demonstrated the existence of naturally acquired *P. vivax* TBA. However, the antibody specificity or strength of the associations remain largely undescribed. Several *P. vivax* gametocyte antigens have been developed as transmission blocking vaccine candidates. Antibody responses that are elicited by these proteins can reduce *P. vivax* transmission, as demonstrated by ookinete culture experiments or by mosquito feeding experiments where serum from vaccinated animals was added to gametocytes from naturally infected malaria-positive blood donors and offered to mosquitoes. These experiments have confirmed the potency of several pre-fertilization antigens including Pvs48/45 [16; 17], Pvs230 [18], Pvs47 [16] and PvHAP2 [19] and post-fertilization antigens Pvs25 and Pvs28 [20]. Lastly, *P. vivax* Cell-traversal protein for ookinetes and sporozoites (PvCelTOS) [21; 22; 23] was identified as potential inducer of TBA [24]. Recognition of these recombinant proteins has so far not been systematically studied in malaria-endemic populations or related to *P. vivax* transmission efficiency.

Here, we therefore investigated the relationship between the infectivity of *P. vivax* infections to locally reared *An. arabiensis* mosquitoes and naturally acquired antibodies against *P. vivax* gametocyte antigens in symptomatic and asymptomatic infections in Ethiopia.

Methods

Study design and population

This study was conducted in Adama district and Metehara town of Oromia Regional State and Arba Minch in Southern Region, Ethiopia. Adama district is characterized by low and seasonal malaria transmission that primarily occurs during the long (July to September) and short (May to June) rainy seasons [25]. Metehara town has low perennial malaria transmission with peaks in incidence from September to November and March to May. Arba Minch is characterized by moderately intense perennial malaria transmission. *P. falciparum* and *P. vivax* are co-endemic in all study sites where *Anopheles arabiensis* is a primary malaria vector [26; 27; 28].

Across sites, microscopy confirmed symptomatic *P. vivax* patients were recruited at health centers from December 2017 to March 2022 (**Table 1**). Demographic data was collected from patients or their guardians (if minors) at the moment of presentation. Patients were included in the study if they had microscopy-confirmed *P. vivax* infections, had symptoms suggestive of malaria (defined as axillary temperature ≥37.5°C or history of fever, alone or in combination with nausea, chills and headaches) and were ≥2 years of age. Patients were excluded if they had a chronic and/or an acute illness that required immediate clinical care or bleeding disorders. Hemoglobin concentration was measured by HemoCue photometer (HemoCue 201+, Angelholm, Sweden).

Observations from clinical malaria cases were complemented with observations from asymptomatic infections. These comprised samples from qPCR confirmed *P. vivax* infections collected during the same study period from asymptomatic individuals who were enrolled in a longitudinal observational study on the dynamics of asymptomatic infections in Adama district (Hailemeskel, Tebeje *et al.* in preparation). Samples from these individuals were included in the current analysis to ensure coverage of a broad range of transmissible and non-transmissible gametocyte densities to allow more precise fitting of the associations between parasite and gametocyte densities and mosquito infection rates. For asymptomatic parasite carriers, afebrile community members were eligible if they were above the age of 2 years and positive for *P. vivax* parasites by qPCR, without symptoms suggestive of malaria in the past 48 hours and with no evidence of chronic and/or acute illness. Venous blood samples were collected every 14 days for 2-months and used in mosquito feeding assays and for plasma collection. For the current study, we randomly selected samples from this cohort without the intention to describe (longitudinal) patterns in parasite carriage but to obtain an informative population of low parasite and gametocyte densities for curve-fitting.

Ethics statements

All participants or their guardians (if minor) provided written informed consents to participate in the study. The study protocol was approved by the Ethics Review Committees of the Armauer Hansen Research Institute (AHRI) (protocol number: PO32/18 and PO35/17), the Ethics committee of the London School of Hygiene and Tropical Medicine (LSHTM Ethics ref: 15811), and the National Research Ethics Review Committee (SHE/S.M./14.4/708/19) at the Ministry of Science and Higher Education of The Federal Democratic Republic of Ethiopia.

Study procedures

Blood sampling

Venous blood was collected from each participant for mosquito feeding, molecular assays and serology. Three mL of blood was collected in either EDTA (BD K2E EDTA Vacutainers) or serum tubes (BD Vacutainer Plus Serum) for nucleic acid collection and plasma/serum collection. An additional 2mL was collected in Heparin tubes (BD Lithium Heparin Vacutainers) for mosquito feeding experiments (to measure mosquito infectivity) since other anticoagulants interfere with transmission efficiency [29]. Plasma (1:1 in 0.05% NaAz) and serum were stored at -70°C for serological investigations.

Parasite quantification

Asexual parasites and gametocytes were detected and quantified by microscopy [30]. For quantification of gametocytes with reverse transcriptase quantitative PCR (RT-qPCR), total nucleic acids were extracted from 100µL whole blood stored in 500µL RNAProtect (Qiagen) and extracted by MagNAPure LC automatic extractor (Roche applied Sciences). RT-qPCR targeted *Pvs25* mRNA, an established marker of female gametocytes [7; 31]. All primer and probe sequences and combinations are included in supplementary file (**Table S3**).

Infectivity assessment

Mosquito membrane feeding assays were performed as described previously [26; 32] to assess infectivity to locally reared *An. arabiensis* mosquitoes. Briefly, a heparinized whole blood sample was fed to ~ 40 (4 - 7 days old) female *An. arabiensis* mosquitoes per cup (three cups with a total of 120 mosquitoes per experiment) using water jacketed glass feeders (0.3mL capacity) maintained on a water bath to keep the temperature in the feeders at 37^{o}C. Mosquitoes were starved for ~12 hours prior to feeding. For the serum replacement mosquito feedings, heparinized whole blood (~833µL) was centrifuged at 1800 × g for 5 minutes in a pre-warmed (37°C) centrifuge to collect autologous plasma (~500µL). Pre-warmed (37°C) ~500µL naïve AB European serum (Sanquin, Nijmegen, the Netherlands) was used to replace the autologous plasma. Fully fed mosquitoes were maintained on 10% sucrose solution (g/mL) at 26°C ± 2 room temperature and 60% ± 10 humidity post feeding. Mosquitoes (30 per experiment) were dissected on day 7 post feeding, and midguts were stained with 1% mercurochrome for microscopic detection and quantification of oocysts.

Serological analyses

Antibody levels against *P. vivax* antigens, Pvs25, Pvs48/45, Pvs47, Pvs230 and PvCelTOS were assessed by Enzyme Linked Immunosorbent Assay (ELISA). Pvs25, Pvs48/45, Pvs47 and Pvs230 were expressed using a wheat germ cell-free (WGCF) system (CellFree Sciences, Matsuyama, Japan) [33]; an additional Pvs230 was expressed in *Pichia pastoris* (Pvs230D1M [34]); and the sporozoite/ookinete antigen PvCelTOS was expressed in *E. coli* [22; 23; 35; 36]. ELISA plates were coated with 1µg/mL (in PBS) of antigen overnight at 4°C. After thorough washing with home-made PBS-Tween (0.05%), plates were blocked with 5% skimmed milk in 0.05% PBS-Tween for 1 hour at room temperature (RT). Plates were then washed. Diluted test samples/controls in 1% skimmed milk (in 0.05% PBS-Tween) were added to the plates and incubated for 2 hours at RT. After a washing step, Goat anti-human IgG-HRP (1:50,000) (Pierce 31412) in PBS-Tween (0.05%) was added and plates were incubated at RT for 2hours. Following a final washing step, a colorizing substrate TMB (K-Blue substrate, Neogen, Sigma), was added and incubated for ~20 minutes. After ~20 minutes of incubation, a stopping solution of 0.2M Sulphuric acid ($H_2SO_{4,}$Merck KGaA cat 100731) was added to stop the reaction. Plates were immediately read by plate reader (Bio-Rad, iMark microplate reader) at 450 nm. OD values were used as measure of antibody density; antibody prevalence was assessed by a mixture model, taking the mean plus two standard deviations from the negative population as cut-off for positivity [37]. A more conservative approach with three standard deviations is sometimes used [38] but more appropriate for larger sample sizes. The availability of WGCF proteins was a limiting factor and not all samples could be processed by ELISA; samples with serum replacement observations were prioritized for a complete dataset.

Alongside ELISAs, we tested a panel of 7 *P. vivax* antigens from different life stages in a bead based multiplex immunoassay for comparison; one pre-erythrocytic stage (Circumsporozoite protein [VK210 CSP]), 5 asexual blood stage (Merozoite surface protein 1-19 [MSP1-19], Apical membrane antigen 1 [AMA1], Duffy binding protein [DBP RII], Reticulocyte binding protein [RBP 2b], and Erythrocyte binding protein [EBPII]), and one sexual stage (HAP2). Antibody responses were quantified using a Luminex MAGPIX© suspension bead array, as described previously [39]. Briefly, plasma/serum samples were assayed at a dilution of 1:400. Secondary antibody was an R-phycoerythrin conjugated goat anti-human IgG (Jackson Immuno Research, PA, USA; 109-116-098) diluted to 1:200. Data are presented as background adjusted median fluorescence intensities (MFI).

Data analyses

Statistical analyses were performed using STATA (version 14.2, StataCorp., TX, USA) and R (version 4.1.1). Proportions were compared using McNemar's test for paired observations, and Pearson χ2 test or Fisher exact test for independent observations. Spearman rank correlation coefficient (ρ) was used to assess associations between continuous variables. Continuous variables were presented as medians and interquartile ranges (IQRs). TBA was calculated as percent inhibition in oocyst density in naïve sera feeds compared to whole blood feeds [40]. A cut-off for antibody positivity was determined by first using a mixture model to distinguish two Gaussian distributions. The cut-off was determined as the estimated 97.5th percentile of the underlying Gaussian distribution for the negative antibodies (i.e. 2 standard deviations above the mean of the negative population). Logistic and Linear mixed effects regression models were used for multivariate analyses. Analysis with $p<0.05$ were considered statistically significant.

Results

Participant characteristics

In total, 368 symptomatic patients with a median age of 19 years (interquartile range (IQR), 13 – 28 years) were enrolled in the study between December 2017 and March 2022 (**Table 1**). Of these participants 47.8% (174/364) had an active fever and 0.6% (2/332) had hemoglobin levels <8g/dL. In these patients, the median asexual parasite density by microscopy was 4752.0 parasites/µL (IQR, 1547.0 - 10706.0) and 36.4% (134/368) had microscopically detected gametocytes. By Pvs25 RT-qPCR, *P. vivax* gametocytes were detected in 76.1% (280/368) of samples with median *Pvs25* transcript numbers of 22245.9 copies/µL (IQR, 3770.4 - 70980.8). These data from clinical malaria patients, who typically harbor high parasite and gametocyte densities [41; 42], were supplemented with a total of 56 follow-up observations from 24 asymptomatic parasite carriers who were for the most part microscopy negative for malaria parasites but qPCR positive for *P. vivax* and thus carried very low density infections [43] (**Table 1**). Asymptomatic parasite carriers had a median age of 14 years (IQR, 9 - 21.5). Microscopically quantified asexual parasites and molecularly quantified gametocyte densities differed markedly between symptomatic and asymptomatic populations (**Fig. 1A**). When combining observations from both symptomatic and asymptomatic populations, gametocyte density was positively associated with parasite density (**Fig. 1B**,= 0.43; $p < 0.0001$).

Table 1. Characteristics of study participants recruited between December 2017 and March 2022

Characteristics	**Symptomatic**	**Asymptomatic**
Age, median (IQR)	19 (13 - 28)	14 (9 - 21.5)
Asexual parasites/µl (microscopy), median (IQR)	4752.0 (1547.0 - 10706.0)	0.0 (0.0 - 0.0)
Gametocyte prevalence, microscopy (% (n/N))	36.4 (134/368)	0.0 (0.0/56)
Gametocyte prevalence (*Pvs25*), RT-qPCR (% (n/N))	76.1 (280/368)	80.4 (45/56)
Mosquito infectious individuals, % (n/N)	73.9 (272/368)	0.0 (0.0/56)*
Infected mosquitoes, % (n/N)	37.0 (4036/10,879)	0.0 (0.0/1,800)

*24 asymptomatic patients with 56 longitudinal data points were included.

Symptomatic (n= 368); Asymptomatic (n=24, with 56 longitudinal mosquito feeding moments); Total (n= 424). Abbreviations: IQR, inter quartile range
All data points selected for the asymptomatic individuals were *P. vivax* positive by RT-qPCR

Mosquito infection rates are associated with parasite and gametocyte density

For all clinical patients and asymptomatic parasite carriers, whole blood was offered to locally reared *An. arabiensis* mosquitoes that were examined 7 days later for infection status. For 61 clinical patients recruited between March 2020 and March 2022, these mosquito feeding experiments were also conducted after replacing autologous plasma with malaria-naïve serum. Asexual parasite density was positively associated with the proportion of mosquitoes that became infected when feeding on whole blood of parasite carriers (**Fig. 1C**); this association was stronger for molecularly quantified gametocyte density (**Fig. 1D**). Oocyst prevalence, or the proportion of infected mosquitoes, was strongly positively associated with mean oocyst density (= 0.63; $p < 0.0001$) (**Fig. 2A**).

Despite these statistically significant associations, many individuals with relatively high gametocyte densities did not infect mosquitoes. For the subset of participants whose blood was used in serum replacement experiments (n=61), we observed a strong positive association in mosquito infection rates between the whole blood and serum replacement feeds (**Fig. 2B**; = 0.92, $p<0.0001$). We observed higher mosquito infection rates for the control serum condition with a median increase of 13.2% (IQR, 0% - 25.0%) infected mosquitoes. We expressed the lower mosquito infection rates in the autologous plasma condition relative to that in the naïve serum condition as transmission blocking activity (TBA). This TBA ranged from -80% (enhancement) to 100% (complete blockade). For those showing higher infection rates in whole blood conditions (n=5), this 'transmission enhancement' was mostly very weak

(3.2 - 12.1%). Only 1 individual showed more than 80% enhancement with the proportion of infected mosquitoes being 15.6% for whole blood and 6.3% for serum replacement conditions. In contrast, 13.1% (8/61) of experiments showed greatly increased infection rates following serum replacement (>80% higher infection rates and thus TBA >80%). TBA estimates for five experiments were not interpretable since infection rates were zero in the whole blood and serum replacement conditions.

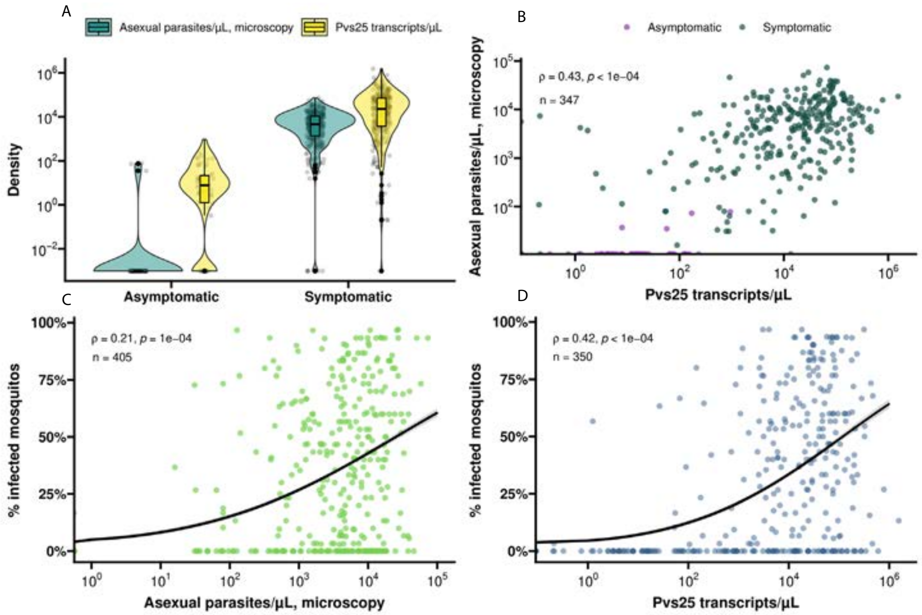

Figure 1. A. Violin and box plot for the distribution of molecularly determined gametocyte density (yellow) and microscopically detected asexual parasite density (turquoise) by symptom status. **B.** A scatter plot for the association between asexual parasite density and gametocyte density by symptom status (asymptomatic – red, symptomatic – green). Spearman's = 0.43 indicating a moderate positive monotonic association (p<0.0001). **C.** Scatter plot showing the association between asexual parasite density and mosquito infectivity. Spearman's = 0.21 indicating a weak positive monotonic association (p<0.0001). The black line shows the fit from a logistic regression model and the grey shading shows the 95% confidence interval. **D.** Scatter plot showing the association between gametocyte density and mosquito infectivity. Spearman's = 0.42 indicating a moderative positive monotonic association (p<0.0001). The black line shows the fit from a logistic regression model and the grey shading shows the 95% confidence interval.

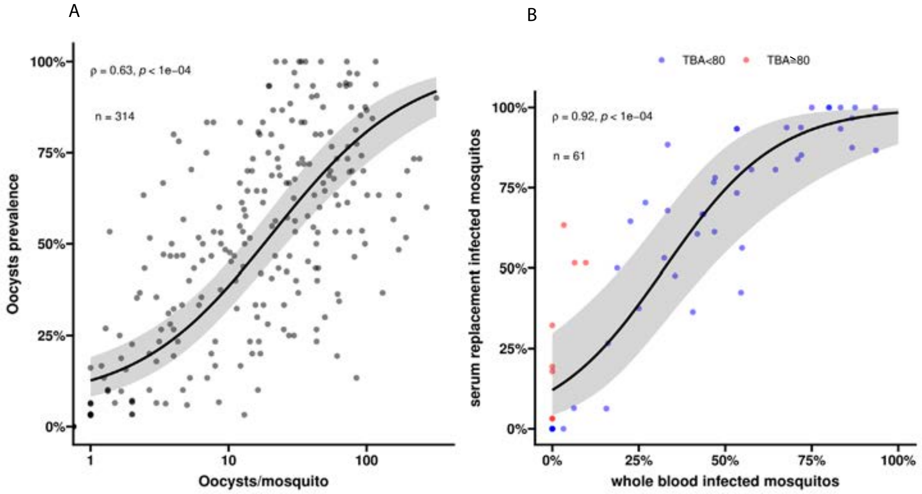

Figure 2. A. A scatter plot showing association between oocyst prevalence, or mosquito infection rates, and mean oocyst per mosquito. Spearman's =0.63 indicating a moderate positive monotonic association p<0.0001. The black line shows the fit from a logistic regression model and the grey shading shows the 95% confidence interval. **B.** A scatter plot indicating the association in mosquito infection rates between whole blood and serum replacement feeds. Observations with TBA<80% are indicated in blue and TBA ≥ 80% are indicated in red. Spearman's = 0.92 indicates a strong positive monotonic association (p<0.0001). The black line shows the fit from a logistic regression model and the grey shading shows the 95% confidence interval. The plot also indicates that infectivity in the serum replacement condition (median 60.6%, IQR 6.3% - 85.2%) was consistently higher than in the whole blood condition (median 33.3%; IQR 0% - 57.6%) (p<0.0001). This value is different, but not contradicting, the comparison in the main text where we calculated the pairwise differences and report the median and IQR for the pairwise differences.

Transmission efficiency is associated with gametocyte immune responses

To test a possible impact of anti-gametocyte immune responses on transmission efficiency, we determined antibody responses to sexual stage antigens Pvs47, Pvs48/45, Pvs230 (two variants), PvCelTOS and Pvs25 by ELISA. These assessments were done for 224 individuals, including all those participating in serum replacement mosquito feeding experiments. Due to antigen scarcity, not more samples were completed by ELISA. Additional antigens, including sexual-stage antigen PvHAP2, were measured by bead-based multiplex assay. Antibody positivity was defined based on a mixture model, assuming an antibody positive and antibody negative population for each antigen within the study population. The optical density values in ELISA, reflective of antibody densities, were strongly correlated between most of the antigens (**Fig. 3**). For instance, responses to the two Pvs230 variants were strongly correlated

(= 0.68, p<0.0001), as were responses to Pvs230 and Pvs25 (= 0.63, p<0.0001). Among individuals with microscopy-positive infections, antibody prevalence was weakly associated with a lower parasite density at the time of sampling for PvDPB-RII (p= 0.06) but not for any of the other antigens (**Table S1**).

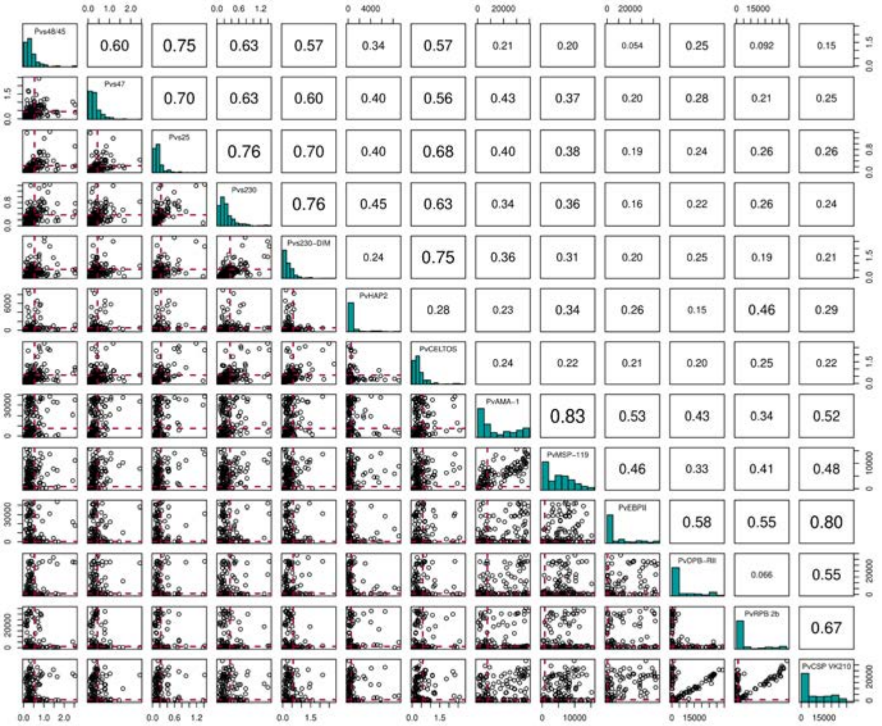

Figure 3. Correlation matrix plot for the 13 different antigens. Here the diagonal shows the histogram distribution for each of the antigens. The triangle above the diagonal shows the Pearson's correlation coefficient for every pair of antigens on the diagonal. The bottom triangle shows the scatter plots for every pair of antigens on the diagonal. Here, the red dashed lines indicate the cut-offs for antibody positivity.

Individuals with >80% TBA were more likely to be antibody positive for most gametocyte antigens (**Fig. 4**), although this difference only reached statistical significance for Pvs47 and PvHAP2 (**Table S2**). We did not observe any evidence of associations with >80% TBA for any of the asexual antigens (**Fig. 4**, **Table S2**). We next examined whether antibody prevalence was also associated with transmission efficiency in the larger dataset where whole blood samples were offered to mosquitoes and gametocytes were quantified by molecular methods.

We thus determined whether the proportion infected mosquitoes for a given gametocyte density was lower in the presence of gametocyte antibodies. The presence of antibodies against Pvs47 was associated with a relative reduction in mosquito infection rates of 34% ($p<0.0001$). Similarly, Pvs230 and Pvs25 antibody prevalence were associated with statistically significant relative reductions of 23 and 34%, respectively; for Pvs48/45 antibodies a smaller and non-significant reduction was observed (**Fig. 5**). For PvCelTOS and asexual stage antigens, highly heterogeneous effects were observed. The presence of antibodies against CSP and RBP-2b was associated with 61% and 20% increases in mosquito infection rates, respectively; while antibodies against AMA-1 were associated with a 26% decrease in mosquito infection rates ($p<0.0001$) (**Fig. S1**).

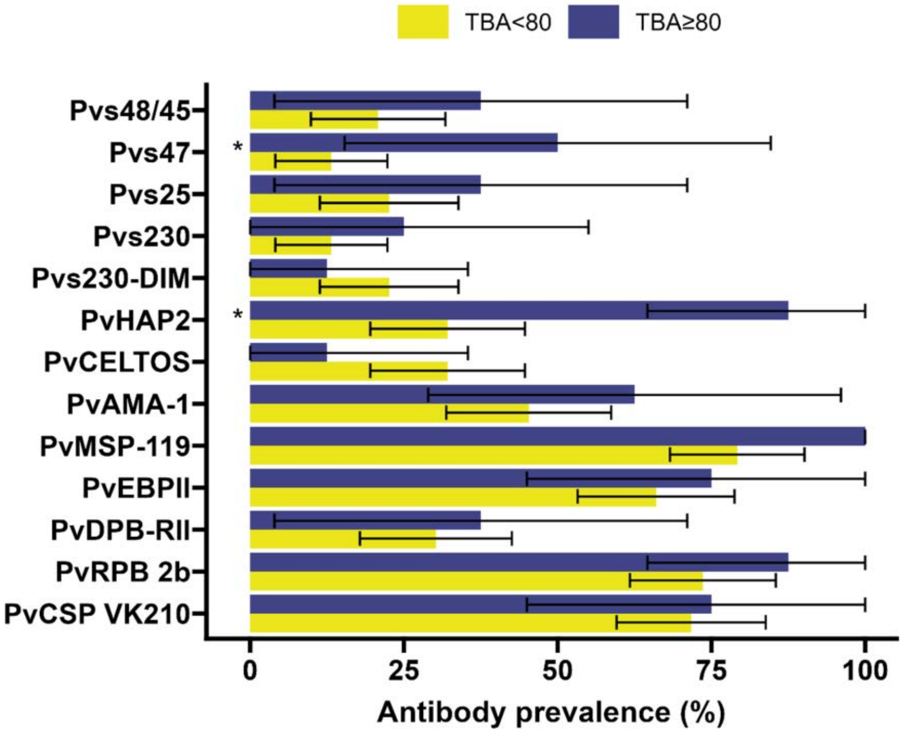

Figure 4. Antibody prevalence [50] for the 13 different antigens stratified for whether the transmission blocking activity (TBA) was at least 80% (blue) or less (yellow). 95% confidence intervals are shown by the black bars. Significant ($p<0.05$) differences in prevalence between TBA<80% and TBA≥80% are indicated by * where p-values were calculated from Fisher's exact tests.

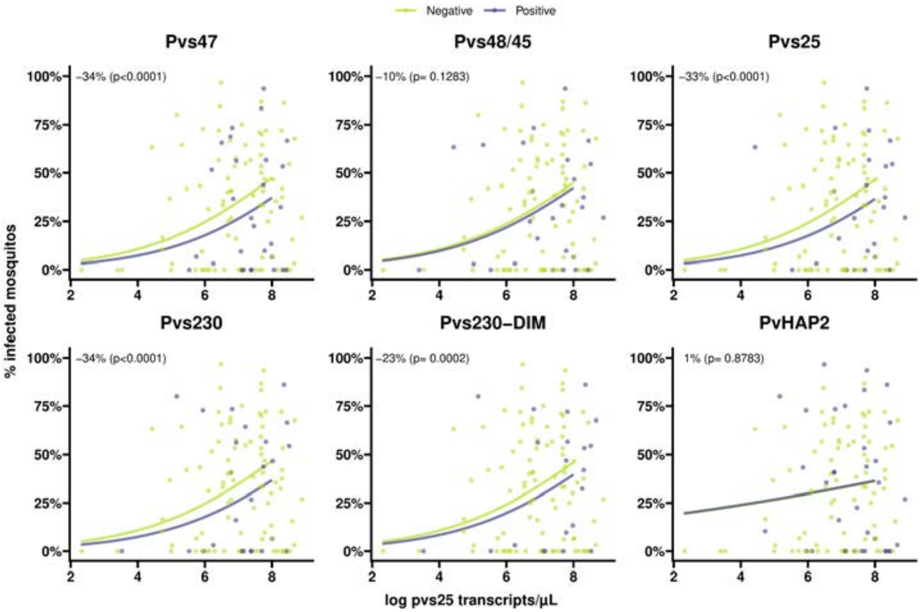

Figure 5. Scatter plots indicating the association between gametocyte density and antibody prevalence on mosquito infectivity for six different antigens. Positive antibodies are indicated in blue and negative antibodies are indicated in green. The blue and green lines shows the fit from logistic regression models for the association between gametocyte density and mosquito infectivity for positive and negative antibodies respectively. The numbers in the plot (estimated from logistic regression models) indicate the average difference in mosquito infectivity between positive and negative antibodies while accounting for gametocyte density, and the p-values indicate whether or not these differences are significantly different from 0%.

Discussion

Here, we described the association of antibody responses to a portfolio of gametocyte and non-gametocyte antigens with *P. vivax* transmission efficiency. We observed higher prevalences of antibodies against gametocyte and ookinete antigens Pvs47, Pvs48/45, Pvs230, PvHAP2 and Pvs25 among individuals with high levels of transmission blocking activity with statistically significant differences for Pvs47 and PvHAP2. No such association was found for antibodies against PvCelTOS and to a panel of asexual and sporozoite antigens. Antibody responses against Pv47 and Pvs25 were further associated with a lower per-gametocyte infectivity when whole blood samples were offered to mosquitoes.

The transmission of *P. vivax* parasites to mosquitoes is strongly associated with gametocyte density [7; 44; 45] that is in turn associated with asexual parasite density [7; 31; 45]. Whilst the positive association between mosquito infection rates and gametocyte density is also observed for *P. falciparum* [46; 47], for *P. vivax* this association is particularly strong and very high mosquito infection rates and infection intensities are commonly observed [7; 44; 45]. At present, *Pvs25* is the most widely used mRNA target to quantify circulating gametocytes although it may not accurately reflect gametocyte maturity or infectivity. These high mosquito infection rates and limited inter-person variation in per-gametocyte infectivity, suggest that host immune factors may play a relatively minor role in dictating transmission efficiency. Nevertheless, anti-gametocyte antibody responses have been observed for *P. vivax* and some of the most compelling evidence for immune-modulatory properties of serum factors comes from *P. vivax* infections [11; 12; 13] where antibodies may both reduce and enhance transmission (reviewed in [48]). In our cohort of patients with clinical *P. vivax* infections, 13.1% (8/61) individuals reduced transmission by more than 80%. We classified these individuals as individuals having high TBA. This arbitrary cut-off is commonly used in malaria transmission research although it is acknowledged that also lower levels of TBA may have a meaningful impact on transmission [49]. Too few individuals (n=5) enhanced transmission and the magnitude of transmission enhancement was very modest for most individuals. We concluded that this did not allow for meaningful analyses of immune responses associated with enhancement in our cohort. Also our number of transmission-blockers (n=8) was very modest but the effect was much larger with 80-100% reduction in the proportion of infected mosquitoes in whole blood compared to serum replacement conditions.We observed that the prevalence of antibodies against gametocyte antigens Pvs47 (OR=6.57; 95% CI: 1.33 – 32.48, p=0.0209) and PvHAP2 (OR=14.82; 95% CI: 1.69 – 130.25, p=0.015) were significantly higher among individuals with this strong TBA (>80%) compared to those with lower levels of TBA. Whilst the small number of observations warn that these findings have to be interpreted with caution, it is striking that none of the asexual antigens was associated with TBA>80%, suggesting that cumulative prior exposure to *P. vivax* may not be predictive of functional transmission-reducing immunity.

We detected antibodies against the ookinete protein Pvs25 in ~25% of individuals which is in line with a previous study [50] where 19.2% of naturally infected patients had detectable Pvs25 antibodies. *P. falciparum* Pfs25 mRNA is translationally repressed [51] and no or very low levels of antibodies are detected in naturally exposed individuals [52; 53; 54]. In rodent models, Pys25, an ortholog of Pvs25 and Pfs25 in *P. yoelii*, protein expression was detected in gametocytes [55]. Our findings suggest that translational repression could be incomplete (leaky expression) in *P. vivax* similar as in *P. yoelii* [55]. Strikingly, the presence of Pvs25 antibodies was associated with lower transmission-efficiency in our broader dataset. Similar associations were observed for Pvs47, where antibody prevalence was associated with an estimated 34% reduction in mosquito infection rates, and Pvs230. PvCelTOS can be expressed on surfaces of both sporozoites and ookinetes [24], and we speculated that antibodies against PvCelTOS expressed on sporozoites could influence ookinete development, and thus transmission. However, in our cohort, we observed no association between PvCelTOS antibodies and either TBA or general transmission efficiency.

Our study has several limitations. First of all, our findings were primarily based on clinical malaria cases who are highly infectious in *P. vivax* [44; 45] and were therefore considered an informative population to examine changes in transmission efficiency. However, asymptomatic infections may also be relevant for the human infectious reservoir in some *P. vivax* endemic settings [7] and will have a different infection history and therefore plausibly a different antibody profile. Our findings can thus not be extrapolated to asymptomatic populations. We also used two platforms to quantify antibody responses. After initial attempts to couple antigens to beads for Luminex assays which were unsuccessful for all antigens except for HAP2, we used ELISA for all antigens other than HAP2. Most importantly, we describe epidemiological associations and our findings do not provide conclusive evidence for a causal role of anti-gametocyte antibodies in dictating *P. vivax* gametocyte infectivity. Whilst our findings for several antigens with an established role in gametocyte fertilization are biologically plausible and supported by pre-clinical studies where vaccine-induced antibodies were causally associated with TRA and also broadly corroborate findings in *P. falciparum*, causality would require a different set of experiments. For *P. falciparum*, antibodies specific for Pfs48/45 and Pfs230 were purified from large-volume plasma samples and offered to mosquitoes in the presence of cultured gametocytes [53]. Similar experiments would allow demonstrating causality for antibodies against *P. vivax* gametocyte antigens but would have to rely on natural gametocyte donors since continuous culture of *P. vivax* has not been established. In addition, we did not collect the volumes of plasma (up to 9mL) that was used to demonstrate the functionality of antibodies against Pfs48/45 and Pfs230 in *P. falciparum* [53]. Furthermore, our study did not test these associations for different concentrations of antibodies or dilutions of autologous plasma. We also did not examine TBA after depleting plasma of antibodies using the recombinant proteins; this would have allowed us to explore TBA associated with antibodies to antigens other than the panel examined here or antibody-independent TBA. It is therefore important to interpret the described associations with caution, also demonstrated by positive and negative associations of mosquito infection rates with antibodies against several asexual antigens.

In conclusion, our mosquito feeding experiments on naturally infected Ethiopians provide evidence for a plausible role of antibodies against *P. vivax* sexual stage antigens in determining the transmission efficiency of *P. vivax* gametocytes to locally relevant *An. arabiensis* mosquitoes. These findings, that require confirmation in other populations, can help understand the natural transmission of *P. vivax* and support the development of transmission-blocking vaccine candidates.

Conflict of Interest

N.H.T. is listed as an inventor on patent US20190276506A1 related to CelTOS.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Conceived, designed and supervised the study: TB, FGT. Wrote manuscript: SKT TB FGT. Edited manuscript: CD BW AV MJ WS IH WC EH NDS NHT DN[6]. Performed experiments: SKT WC EH AG TA DN[1] EE TE TT KT KL IH WS. Analysed data: JR TB SKT. Provided validated antigens: ET TT NDS NHT

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Supplementary Information

Table S1. Parasite density ratio between antibody positive and negative samples

Antigens	Density ratio	Lower CI	Upper CI	p-value
Pvs47	1.03376551	0.555177543	1.92491779	0.9167441
Pvs48/45	0.970252518	0.495599809	1.899496191	0.9298983
Pvs25	0.595658352	0.282660158	1.255248973	0.1749864
Pvs230	0.775207773	0.410002812	1.465714559	0.4344639
Pvs230-DIM	0.852875156	0.432412687	1.682180133	0.646683
PvCELTOS	1.0036775	0.523660802	1.923704275	0.9911899
PvAMA-1	0.890743838	0.423333609	1.874230081	0.761311
PvEBPII	0.816628842	0.388470056	1.716690015	0.5946083
PvHAP2	1.08067882	0.487100072	2.397590925	0.8491537
PvMSP-119	1.072617334	0.45735181	2.515586293	0.8723655
PvDPB-RII	0.488550347	0.236505021	1.009202429	0.0566313
PvRPB 2b	1.423115233	0.658281188	3.076583387	0.3725003
PvCSP VK210	0.737979624	0.34484648	1.579293855	0.4361784

Table S2. TBA of blocker and non-blocker sera

Antibody	Blocker (TBA>80%)	Non-blocker	OR (95% CI), p
Pvs47	50.0 (4/8)	13.2 (7/53)	6.57 (1.33, 32.48), p=0.0209
Pvs48/45	37.5 (3/8)	20.8 (11/53)	2.29 (0.47, 11.1), p=0.3031
Pvs25	37.5 (3/8)	22.6 (12/53)	2.05 (0.43, 9.85), p=0.3700
Pvs230	25.0 (2/8)	13.2 (7/53)	2.19 (0.37, 13.08), p=0.3898
Pvs230-DIM	12.5 (1/8)	22.6 (12/53)	0.49 (0.05, 4.37), p=0.5213
PvCELTOS	12.5 (1/8)	32.1 (17/53)	0.3 (0.03, 2.66), p=0.2809
PvHAP2	87.5 (7/8)	32.1 (17/53)	14.82 (1.69, 130.25), p=0.015
PvAMA-1	62.5 (5/8)	45.3 (24/53)	2.01 (0.44, 9.3), p=0.3699
PvEBPII	75.0 (6/8)	66.0 (35/53)	1.54 (0.28, 8.43), p=0.6168
PvMSP-119	100.0 (8/8)	79.2 (42/53)	8103773.78 (0, Infty), p=0.9894
PvDPB-RII	37.5 (3/8)	30.2 (16/53)	1.39 (0.3, 6.52), p=0.6782
PvRPB 2b	87.5 (7/8)	73.6 (39/53)	2.51 (0.28, 22.28), p=0.408
PvCSP VK210	75.0 (6/8)	71.7 (38/53)	1.18 (0.21, 6.54), p=0.8462

Table S3. Primers and Probes combinations

Primer type	***P. falciparum* (18S)**	***P. vivax* (Pvs25)**
Forward	5′-GTAATTGGAATGATAGGAATTTACAAGGT-3′	5'-ACA CTT GTG TGC TTG ATG TAT GTC-3'
Reverse	5′-TCAACTACGAACGTTTTAACTGCAAC-3′	5'-ACT TTG CCA ATA GCA CAT GAG CAA-3'
probe	5'-6FAM-AACAATTGGAGGGCAAG-MGBNFQ-3'	5'-FAM-TGC ATT GTT GAG TAC CTC TCG GAA-BHQ1-3'

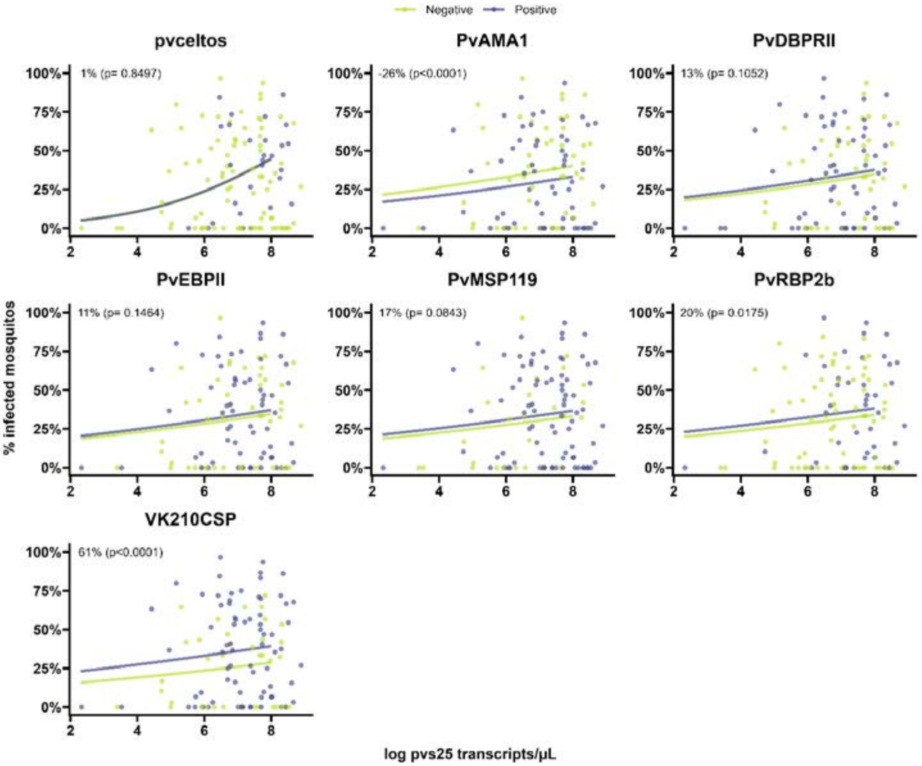

Figure S1. Scatter plots indicating the association between gametocyte density and antibody prevalence on mosquito infectivity for seven asexual stage antigens. Positive antibodies are indicated in light blue and negative antibodies are indicated in green. The light blue and green lines show the fit from logistic regression models for the association between gametocyte density and mosquito infectivity for positive and negative antibodies respectively. The numbers in the plot (estimated from logistic regression models) indicate the average difference in mosquito infectivity between positive and negative antibodies while accounting for gametocyte density and the p-values indicate whether or not these differences are significantly different from 0%.

CHAPTER-6

General Discussion

General discussion

With a disease as devastating as malaria, it is understandable that there has historically been a large focus on understanding the occurrence and pathophysiology of symptomatic malaria. However, a large proportion of malaria infections can remain asymptomatic and do not elicit treatment seeking behavior. These asymptomatic infections are more prevalent across transmission settings than previously assumed [1]. Although not symptomatic in the traditional way (i.e. not associated with fever), these infections may nevertheless impose considerable health threats including low-grade anemia [2], leukopenia and thrombocytopenia [3]. Furthermore, asymptomatic malaria infections may predispose to increased levels of inflammation along with endothelial and platelet activation [4], suggesting that the term 'asymptomatic' could be misleading.

Asymptomatic malaria infections harbor fluctuating parasite densities during their lifespan. At times their parasite density reaches the threshold that allows detection by routine diagnostic methods while at other times they become sub-patent. The longer the duration of these infections, the higher the chance that they produce gametocytes and are transmissible to mosquitoes. Under these conditions, they can become important drivers of malaria transmission. Currently, there is a scientific debate on whether asymptomatic malaria infections need to be targeted to achieve elimination. This is mainly due to the lack of conclusive scientific data that enable us to fully understand their relevance for onward malaria transmission. Therefore, the aim of this thesis is to provide insights in the biology, epidemiology and field characteristics of asymptomatic malaria infections in Ethiopia.

Epidemiology and detectability of asymptomatic *P. falciparum* and *P. vivax* infections in Ethiopia

Combining data collected in the previous decade, the epidemiology and detectability of asymptomatic malaria infections were estimated in a large meta-analysis [5]. That study combined a multitude of surveys from different sites in different endemic countries, each with their own study and laboratory procedures. This approach did not allow direct comparison of parameters among different transmission intensity sites. In our study in **Chapter-2,** we determined the prevalence and detectability of asymptomatic *P. falciparum* and *P. vivax* infections in low, moderate and high transmission settings in Ethiopia using three diagnostic methods: microscopy, rapid diagnostic test (RDT) and polymerase chain reaction (PCR). Our study was uniquely designed to allow direct comparison of parameters between the three transmission intensity areas. Our findings indicate that asymptomatic *P. falciparum* and *P. vivax* infections are prevalent in all of the study sites while being more prevalent in high endemic areas compared to moderate and low transmission areas. This indicates a positive association between malaria transmission intensity and the prevalence of asymptomatic malaria infections. This could be due to the fact that in high malaria transmission areas, there is a high number of repeated infectious mosquito bites which keeps malaria prevalence high. Therefore, individuals residing in these areas can develop immunity against the parasite due to repeated infections; this immunity allows the infected human to control parasite densities and prevents symptomatic illness. In low transmission areas, there are a lower number of infectious mosquito bites constituting low infection prevalence. If malaria transmission intensity declined, there may still be lingering immunity that may control parasite density [6]. Importantly, even in low endemic settings some level of immunity may be acquired after (limited) malaria exposure [7].

With regard to their detectability, we observed that asymptomatic malaria infections are more detectable by microscopy/RDT in high transmission areas compared to moderate and low transmission areas. The likelihood that asymptomatic malaria infections were detectable by microscopy/RDT as ~5-fold higher in the high transmission area and ~3-fold higher in the moderate transmission area compared to the low-transmission area. One critical factor that is likely to explain this, is parasite density where high parasite density asymptomatic infections are more prevalent in high endemic sites and, obviously more detectable [8, 9]. Previously, Okell et al [5] reported that microscopy may miss more than half (54.1%) of the *P. falciparum* infections detected by PCR. Similarly, in our study, infection detectability of all study sites together showed that microscopy/RDT missed more than half (~52%) of *P. falciparum* and ~95% of *P. vivax* that are detected by nPCR. This constitutes a considerable difference in detectability between the two *Plasmodium* species. This could partly be explained by the unique biology of *P. vivax* including its preference to infect reticulocytes, which account for 0.5 - 2.5% of all red blood cells (RBCs) in adults, allowing fewer RBCs to be infected by *P. vivax*. Parasite densities are thus lower for *P. vivax* and it is therefore intuitive that fewer infections are detectable by less sensitive diagnostics like microscopy [10]. In addition, it is possible that *P. vivax* has a unique ability to persist as a low density infection for long periods of time. It was previously estimated that the number of asymptomatic malaria infections that could be missed by microscopy/RDT could even be higher in low transmission sites [8]. This low parasite density could result from the low number of infectious mosquito bites experienced by the human population, which limits parasite genetic diversity and infection prevalence. Low genetic diversity in low transmission areas may allow the development of immunity to be faster [11], which enables the infected host to control parasite density which in turn limits infection detectability. Another factor that could influence detectability of malaria parasites, specifically by RDT, is genetic variation and/or deletion of the HRP2/3 gene [12-14]. As 90% of RDT based parasite detection is targeting HRP-2/3 [15], the increasing prevalence of HRP-2/3 negative parasites will make parasite detection even more challenging in the future. Also in our studies, we used HRP2-based RDTs for the detection of *P. falciparum* infections. *P. vivax* infections were detected based on the pLDH protein, for which no known gene-deletions are described but that is less abundant than the *P. falciparum*-specific HRP-2 [16].

The occurrence and rise in prevalence of HRP2 deletions is a great concern for clinical case management as well as malaria elimination efforts. Future studies need to explore the extent to which HRP-2/3 negative parasites are prevalent in different transmission settings, and bring forward upgraded diagnostic methods for these parasites. For our studies, it is unclear to what extent HRP-2/3 deletions contributed to the high prevalence of undetectable *P. falciparum* infections; given that infections that are undetectable by microscopy also occur in areas like The Gambia [17]. without known HRP-2 deletions, we consider it likely that our findings are primarily explained by low parasite densities and not by HRP-2/3 deletions. This important role for parasite density in determining detectability is definitely the case for *P.* vivax.

We further observed that age is an important factor in parasite detectability. After adjusting for site and correlation between observations from the same survey, a 5% decline in detection using microscopy/RDT was observed for every year increase in age. This suggests that microscopy/RDT can miss a particularly substantial number of malaria infections in older individuals, particularly in low transmission areas. This has been observed before [18, 19] and could be due to the naturally acquired immunity that older individuals develop through years of repetitive infectious mosquito bites and keeps parasite densities low [20].

Taken together, our data in **Chapter-2** show that asymptomatic *P. falciparum* and *P. vivax* infections are more prevalent, and more detectable by microscopy/RDT in high endemic areas compared to moderate and low-endemic sites. This may indicate that microscopy/RDT can still be used in resource limited settings in high and moderate-endemic areas while in low endemic sites more sensitive detection methods such as molecular diagnostic methods, including qPCR, need to be involved if the aim is to detect all (or the majority of) infections.

Our study has several limitations. The first is its cross-sectional nature. Cross-sectional studies provide only a snapshot of information, leaving room for speculation regarding temporal infection dynamics. The prevalence and detectability of parasites change over time depending on the trend of malaria transmission in a particular site. For example, the more time elapsed since the transmission started to decline, the more existing infections are relatively old (i.e. acquired when transmission was still high). With older infections, parasite densities are likely to decline [8, 21, 22], resulting in infections becoming less detectable. A second shortcoming is that we used nPCR that does not allow quantitative comparison of parasite density between transmission settings. Therefore, future studies that survey prevalence and detectability should be done periodically to have updated information on parasite carriage and ideally be complemented with quantitative data.

Duration, parasite dynamics and infectiousness of asymptomatic *P. falciparum* and *P. vivax* infections in low endemic area in Ethiopia

The cross-sectional nature of our study in **Chapter-2** did not allow us to unravel the temporal dynamics of asymptomatic malaria infections and associated infectiousness to mosquitoes. Therefore, we explored the duration, temporal infection dynamics and infectiousness to mosquitoes of asymptomatic *P. falciparum* and *P. vivax* infections by performing a longitudinal study in a low transmission site in Ethiopia. This information is provided in **Chapter-3** of this thesis. The life span of asymptomatic malaria infections can range from few weeks [23-25] to several years [26]. In our study, conducted in a low transmission area, our findings indicate that asymptomatic *P. falciparum* and *P. vivax* infections are short lived. The median duration of asymptomatic *P. falciparum* infections was 37 days (95% CI, 15-93) while the median duration of asymptomatic *P. vivax* infections was 60 days (95% CI, 18-213). These results are in congruent with the previous studies that were conducted in areas of low malaria transmission [27, 28]. Despite their short life span, we observed fluctuation in parasite and gametocyte densities in these infections that generally followed a trend of declining densities over the follow-up period. Similar declining patterns of parasite densities in asymptomatic infections have observed in previous studies as well [29-31]. We mainly observed three trends of parasitaemia over the follow up period. Some individuals that were parasite positive at enrolment immediately became parasite negative and continued to be parasite-negative for the remaining follow up time. Some other individuals that harboured low parasite density at enrolment became highly parasitic with densities that resulted in symptomatic illness. The remaining individuals that carried low parasite density infections at enrolment remained positive and infections regularly persisted for more than half of the follow up times. Furthermore, we also observed changes with respect to parasite species over the follow-up period. Some individuals that were *P. falciparum* positive at enrolment became positive for mixed infections with *P. vivax* during the follow up time points, while some others had only detectable *P. vivax* infections. These observations could be due to new incident infections that were acquired during the follow up period. Alternatively, it could also be partially explained by co-infections of *P. falciparum* and *P. vivax* where the undetectable dormant stages (Hypnozoites) of *P. vivax* were present at the start of the follow up period that caused relapse infections at a later time point. Co-infections are particularly common in areas where malaria parasites cluster and co-infected mosquitoes are abundantly available [32]. Similar patterns with changes in parasite species over time were observed for individuals initially only positive for *P. vivax*. This has important implications for cross-sectional surveys that are commonly used to estimate malaria epidemiology. Based on our observations, cross-sectional surveys could miss a considerable amount of data due to their one-time-point sampling method. Therefore, future studies or surveys may need to be complemented with longitudinal information to capture the full picture of asymptomatic malaria infections.

Over the course of an infection, we also observed that a large proportion of follow up time points were gametocyte positive for both species. While 62% of *P. falciparum* qPCR positive time points harboured gametocytes, this was 73% for *P. vivax*. Moreover, the gametocyte density was positively associated with total parasite density for both species with a stronger association for *P. vivax*. These findings are similar to the strong positive association between parasite density and gametocyte density that was observed in *P. vivax* infections in our study in **Chapter-5**. Previous studies also reported a positive association between parasite and gametocytes densities in *P. falciparum* [33] and *P. vivax* [34, 35] infections. This may suggest that gametocytes can be produced right after the establishment of blood stage infection, particularly, for *P.* vivax.

As a result of the high number of gametocyte positive moments, one may expect a large number of time-points when infections are transmissible to mosquitoes.

However, only 4 individuals with asymptomatic infections – 3 with *P. falciparum* mono-infection and 1 with mixed species infections – were infectious to mosquitoes and they were infectious only once during their follow up period. None of the *P. vivax* mono-infected individuals were infectious to mosquitoes. This could be influenced by several factors including gametocyte maturity, gametocyte sex ratio and immunity against sexual stages. Based on the low number of mosquito infectious bites, one may underestimate the importance of asymptomatic malaria infections in sustaining malaria transmission. However, only few infected individuals could be enough to be sources of mosquito infections to forward malaria transmission. For instance, in our study, a single individual that was infected with mixed (*P. falciparum* and *P. vivax*) infections and was able to infect 83.3% (40/48) of the mosquitoes that were dissected. Considering the population at large, the number of infectious individuals could be high enough to be important infection reservoirs, and that could make asymptomatic malaria infections to be important targets of malaria elimination programs.

Naturally acquired immunity against sexual stages of *P. vivax* and malaria transmission

Naturally acquired immunity against malaria parasites is one of the important factors that influence parasite density and thus detectability, infection duration and transmissibility to mosquitoes. Antibodies induced against sexual stage malaria parasites can limit parasite transmission to mosquitoes [36-38], which serves as an important basis for the development of transmission blocking vaccines (TBVs). As part of this thesis, an extensive literature review on the naturally acquired immunity against sexual stages of *P. falciparum* and *P. vivax* and its impact on parasite transmission from human to mosquito is provided in **Chapter-4**.

Most of the field and laboratory studies that examined naturally induced transmission-blocking immunity focused on *P. falciparum*, and only few on *P. vivax*. Even when writing this discussion section (October 2023), there were over 4-fold more articles on gametocyte/gamete immunity for *P. falciparum* (n=454) than *P. vivax* (n=113) in the search engine PubMed (search terms: “Plasmodium vivax AND immunity AND (gametocyte OR gamete OR sexual stage)”).

Are anti-gametocyte antibody responses responsible for transmission reduction?

For the first time, we reported insightful data in the existence of naturally acquired immunity against gametocytes of *P. vivax* in an Ethiopian population and its potential to reduce/block parasite transmission from humans to mosquitoes. The data are included in **Chapter-5** of this thesis. We conducted 368 Direct Membrane Feeding Assays (DMFA) using blood samples of passively recruited symptomatic patients with *P. vivax*. For these samples, we measured antibody responses to *P. vivax* gametocyte antigens (Pvs47, Pvs48/45, two variants of Pvs230, PvCelTOS, Pvs25 and PvHAP2). Combining our DMFA and antibody data, our analysis showed a negative association between the presence of antibodies against most of the sexual stage antigens and mosquito infection rate; in particular antibodies against Pvs47, Pvs230 and Pvs25 were statistically significantly associated with lower mosquito infection rates. Next, to evaluate the impact of antibodies on the intensity of mosquito infection, we conducted serum replacement mosquito feeding assays for a selection of donors. This assay directly assessed gametocyte infectivity in the presence and in the absence of donor serum. We observed a positive association between the prevalence of most of the antibodies and transmission blocking activity (TBA). Particularly, sera with high (>80%) TBA had higher antibody prevalence for Pvs47 and PvHAP2 antigens. To rule out the impact of antibodies against other parasite stages in our observation, we also measured antibody responses against a panel of asexual stage antigens using bead-based multiplexed immuno-assay (Luminex MAGPIX©). Our analysis showed highly heterogeneous antibody effects; while the presence of antibodies against some asexual stage antigens was associated with enhanced mosquito infection rates, the presence of antibodies against other asexual antigens was associated with reduction. The heterogenous effect that we observed with antibodies against asexual antigens, however, warns against simplistic interpretations; it is unlikely that these antibodies really play a causal role in transmission reduction and it is likely that they merely reflect a strong correlation between antibody responses to different malaria antigens.

Nevertheless, associations found for antibodies against sexual stage antigens may imply that a population with high prevalence of antibodies against *P. vivax* gametocytes that results from natural infections could be associated with reduced mosquito infection rate and intensity. This is of considerable importance for the malaria elimination efforts that aim to reduce malaria transmission with TBVs, as these could boost natural immunity, or be boosted by natural exposure. When the number of infectious individuals to mosquitoes decreases in a population, for instance after implementing TBVs, malaria transmission decreases as well, and boosting effects due to natural immunity may decrease.

Strikingly, several sera (~25%) were positive for Pvs25 antibodies which is in line with a previous study by Kim et al [39] where 19.2% of naturally infected patients had detectable Pvs25 antibodies. Pvs25 is a post-fertilization protein expressed in zygote and ookinete stages of the parasite in the mosquito midgut and therefore not exposed to the human immune system. mRNA encoding Pfs25, the P25 orthologue in *P. falciparum*, is translationally repressed in gametocytes [40, 41] and there are no, or very low levels of, antibodies against the Pfs25 protein in endemic populations [42, 43]. Therefore, we expected low level or no antibody responses to our recombinant Pvs25 protein. Whilst we cannot rule out cross-reactivity, it is also possible that there is (low level) Pvs25 protein expression in parasites in circulation. Indeed, protein expression of Pys25, an ortholog of Pvs25 and Pfs25 in the rodent parasite *P. yoelii*, was detected in gametocytes [44]. It is therefore conceivable that there may be leaky translation of Pvs25 proteins, similar to Pys25 in *P. yoelli* [44]. Whether there is expression of Pvs25 in circulating gametocytes could be assessed by Western blots, immunofluorescent staining of gametocytes or mass spectrometry.

Overall, our findings suggest that antibodies against the sexual stage antigens potentially block the transmission of *P. vivax* parasites to mosquitoes. In our DMFA experiments we used sera from immune volunteers, so we cannot rule out that there could be serum factors other than antibodies that block transmission to mosquitoes. Future experiments should therefore be carried out with purified antibodies, instead of serum, to definitely demonstrate that antibodies reduce (or enhance) human-to-mosquito parasite transmission. However, which antibody specificities and to what extent each of these subpopulations contribute to the reduction in parasite transmission is still unclear and therefore polyclonal antibodies should be studied in more detail to appreciate their causal role in TBA. This has previously been done for *P. falciparum* where affinity-purified polyclonal IgGs against Pfs48/45 and Pfs230 were shown to block transmission of *P. falciparum* gametocytes to mosquitoes in standard mosquito feeding assay (SMFA) [38]. A possible experiment that can thus prove causality and move beyond the statistical associations in our setting in Ethiopia would be to perform DMFA where mosquitoes are fed with blood meals with *P. vivax* gametocytes with and without purified antigen-specific polyclonal antibodies. In these experiments, gametocytes should be obtained from symptomatic patients who have high gametocyte density. Antigen-specific antibodies should be obtained from donors with high antigen-specific responses, and reduced transmission rates for whole blood compared to blood that had serum replaced. Our findings show that antibodies against antigens such as Pvs230, Pvs47 and Pvs25 were associated with significant reduction in mosquito infection rate and therefore these antigens could be the first in line to be tested in this kind of experiments. This would demonstrate whether the antibodies against specific antigens are responsible for transmission blockage. Previously, the transmission blockage of *P. falciparum* parasites to mosquitoes due to antibodies against Pfs230 were shown to be dependent on the presence of active human complement [45]. Therefore, it is also important to check if transmission-blocking of *P. vivax* is complement dependent. Moreover, to examine the potency of antigen-specific polyclonal antibodies, different dilutions of the antibodies can be tested. Furthermore, to examine if the antibodies can mediate cross-strain blockage of parasite transmission, gametocytes from different strains of the parasite can be tested, as previously done for *P. falciparum* [46]. Unlike for *P. falciparum*, it is currently not possible to acquire a continuous laboratory culture for *P. vivax*, let alone different isolates of *P. vivax*. Therefore one is restricted to using *P. vivax* gametocytes from naturally infected individuals. To cover larger natural diversity, one could use gametocytes from different geographical sites in Ethiopia, or even from Asia. In these experiments parasites should be genotyped to determine if antibodies are similarly active against different genotypes. Together, these experiments would conclusively demonstrate the role of antibodies against sexual stages of *P. vivax* in parasite transmission from humans to mosquitoes and whether this activity is cross-strain protective.

When polyclonal antibodies against specific antigen targets result in blockage of parasite transmission, then it would be possible to extend the study at monoclonal antibody level. This would allow to identify (potent) monoclonal antibodies and unravel functional and non-functional epitopes that can be important for vaccine design. This has recently been done for *P. falciparum* target antigens Pfs48/45 [47] and Pfs230 [48, 49]. In these studies, peripheral blood mononuclear cells (PBMCs) were used from naturally infected and immunized individuals that had high antigen-specific antibody levels and whose total IgG blocked transmission in SMFA. Single B cells were sorted and stimulated with cytokines to produce antibodies, or sorted with fluorescently labelled antigen. Then, B cells that were reactive to the recombinant antigens were cloned and recombinant monoclonal antibodies produced. Functional activity of the monoclonal antibodies was evaluated with SMFA. Finally, structural analysis enabled identification of the target epitopes of the monoclonal antibodies. These results provide clues on which epitopes should be, and which epitopes should not be, included in future vaccine constructs. In case of *P. vivax*, similar studies can be performed to identify monoclonal antibodies against target antigens and unravel (non-) functional epitopes.

Reducing infection burden in mosquitoes: Does it matter?

The burden of mosquito infection by malaria parasites can be measured in two ways: infection prevalence (TBA) and intensity (TRA). TBA measures percent inhibition in the prevalence of mosquito infection while TRA evaluates percent inhibition in the mean oocyst density per mosquito [50]. In our study, we observed that most of the antibodies against *P. vivax* gametocyte antigens have considerable impact on both TBA and TRA. This was in line with previous studies that reported that TBA and TRA are associated [51] [52]. Comparatively, TRA is easier to achieve and measure while TBA is more challenging. First, lower antibody levels are required to achieve TRA compared to TBA. Second, TRA, unlike TBA, is easier to quantify as it is not influenced by parasite density, as shown in a previous study by Bompard et al [53]. Currently, there is a scientific effort to answer the question whether achieving high TRA is sufficient to control malaria transmission or TBA is essential. In other words, is reducing the oocyst intensity in mosquitoes sufficient to reduce malaria transmission or do we really need to reduce the number of infected mosquitoes which may be more challenging to achieve? A recent study in our group [54] showed that the load of ruptured oocysts in the mosquito gut is positively correlated with the sporozoite load in the salivary gland. Furthermore, the expelled sporozoite density in artificial skin was positively associated with the sporozoite load in the salivary gland. Together these data indicate that a reduced oocyst burden in mosquitoes may result in reduced mosquito infectiousness to humans. However, the question whether mosquitoes with low and high number of oocysts are indeed differentially infectious when biting humans still remains to be answered. On the other hand, TBA reduces the prevalence of mosquito infection and it is clear that this could have an impact on transmission to humans in field settings. TBA may therefore be the preferred target for malaria transmission control programs.

Concluding remarks:

Asymptomatic malaria infections have long been neglected by the malaria research community. Whilst there was some logic to this, given asymptomatic malaria infections do not cause immediate health threats, insights from this thesis and recent research by others indicate that these infections are highly prevalent in all malaria transmission settings and may in fact have clinically relevant consequences for the infected host. Furthermore, there is increasing scientific evidence that asymptomatic malaria infections are important drivers of malaria transmission. The work in this thesis helped understand the epidemiology of asymptomatic infections and their importance for malaria transmission that will ultimately help to decide whether they need to be targeted in interventions aiming for control or elimination.

In **Chapter-2**, we investigated the epidemiology and detectability of asymptomatic *P. falciparum* and *P. vivax* infections in high, moderate and low transmission settings in Ethiopia. We demonstrated that asymptomatic malaria infections are prevalent in all study sites and their detectability by routine diagnostic methods (Microscopy and RDT) varies depending on transmission settings. After these relevant cross-sectional insights, we continued our investigation in the form of a longitudinal study in a low endemic site in Ethiopia; **Chapter-3**. Here, we could determine the duration of infection and temporal patterns in parasite and gametocyte densities, as well as transmissibility to mosquitoes. We could also compare the parasite and gametocyte density, and transmissibility to mosquitoes of asymptomatic malaria infections with their symptomatic counterparts. There is well-documented scientific evidence of host and parasite factors that may influence our observations in **Chapter-2** and **Chapter-3** of this thesis. These factors include naturally acquired immunity to malaria parasites, gametocyte sex ratio and gametocyte maturity. Among these factors, we extensively reviewed the evidence and extent of impact of naturally acquired immunity to sexual stages of *P. falciparum* and *P. vivax* on transmission efficiency in **Chapter-4**. Finally, in **Chapter-5**, we provided the first evidence on the existence of naturally acquired immunity against *P. vivax* gametocytes in an Ethiopian population and its impact on transmissibility of malaria parasites to mosquitoes.

Taken together, this thesis provides significant insights related to asymptomatic malaria infections and the presence and impact of naturally acquired immunity to malaria parasites in Ethiopia. Our work sets the stage for interventions that aim to reduce community-wide malaria transmission by targeting (the infectivity of) asymptomatic malaria infections in Ethiopia.

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CHAPTER 7

Appendix

Summary

Malaria is an infectious disease caused by *Plasmodium* parasites that are transmitted through infectious mosquito bites. There are over 200 species of *Plasmodium* of which four species commonly infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* (*P. ovale curtis*i and *P.* ovale *wallikeri*) and *Plasmodium malariae*. *P. falciparum* and *P. vivax* are the most prevalent human-infecting species and account for the majority of all malaria illness globally. The clinical and biological features of malaria infections have long been defined based on symptomatic malaria cases. However, a large proportion of malaria infections remain asymptomatic without eliciting treatment seeking behavior. These are less well studied. Although 'asymptomatic' and not associated with acute symptoms suggestive of febrile disease, these infections may nevertheless have considerable health consequences that have been neglected by the malaria research community. These health consequences include low-grade inflammation and mild anaemia. Currently, there is also increasing scientific evidence that indicates that asymptomatic malaria infections are more prevalent than previously thought in any transmission settings and can be important drivers of malaria transmission. However, whether these asymptomatic malaria infections need to be targeted for intervention to achieve malaria elimination is still debatable due to lack of conclusive data. Therefore, the aim of this thesis is to provide insights in the biology, epidemiology and field characteristics of asymptomatic malaria infections with a focus on Ethiopia.

In **Chapter-2**, we determined the prevalence and detectability of asymptomatic *P. falciparum* and *P. vivax* infections in low, moderate and high transmission settings in Ethiopia using three diagnostic methods: microscopy, rapid diagnostic test (RDT) and polymerase chain reaction (PCR). The latter, PCR, is considerably more sensitive than microscopy or RDT. Our study was uniquely designed to allow a direct comparison of parameters between the three transmission intensity areas. We found out that asymptomatic malaria infections were prevalent in all study sites. In addition, their detectability by microscopy /RDT decreases with decreasing transmission intensity. In other words: although infections are uncommon in low endemic settings, the infections that are present are likely to be missed by conventional diagnostics. This is likely to be caused by very low parasite densities among infections in low endemic settings.

Although insightful, our cross-sectional study (**Chapter-2**) did not allow us to unravel the temporal dynamics of asymptomatic malaria infections and associated infectiousness to mosquitoes. Therefore, we explored the duration, temporal infection dynamics and infectiousness to mosquitoes of asymptomatic *P. falciparum* and *P. vivax* infections. This was achieved by performing a longitudinal study in a low transmission site in Ethiopia where infections were monitored but, if asymptomatic, not treated (**Chapter-3**). Here, we found out that asymptomatic malaria infections were often short-lived and their parasite/gametocyte densities fluctuated over time showing an overall decline over the period of follow-up. We also observed that only small portion of these infections infected mosquitoes. By comparing parasite and gametocyte density, as well as mosquito infectiousness, between asymptomatic malaria infections and their symptomatic counterparts, we could determine their relative infectivity. We observed that especially for *P. vivax*, clinical patients can be highly infectious to mosquitoes. The importance of this finding will depend on how common symptomatic and asymptomatic infections are in populations.

Naturally acquired immunity against malaria parasites is one of the important factors that influence parasite density and thus detectability, infection duration and transmissibility to mosquitoes. Antibodies induced against sexual stage malaria parasites can limit parasite transmission to mosquitoes which serves as an important basis for the development of transmission blocking vaccines (TBVs). TBVs are considered a highly valuable intervention to support elimination efforts. By reducing the spread of malaria, TBVs result in fewer infected mosquitoes and thereby indirectly reduce the burden of malaria. As part of this thesis, an extensive literature review on the naturally acquired immunity against sexual stages of *P. falciparum* and *P. vivax* and its impact on parasite transmission from human to mosquito is provided in **Chapter-4**. From our literature review we understood that most of the previous research regarding naturally acquired immunity against malaria parasites was focused on *P. falciparum*, leaving a significant gap for *P. vivax*. Therefore, we investigated the existence of naturally acquired immunity against sexual stages of *P. vivax* in an Ethiopian population and its impact on human-to-mosquito transmission in **Chapter-5**. We found out that a considerable number of participants had acquired antibodies against multiple *P. vivax* gametocyte antigens. Responses to several of these antigens were significantly associated with reduced or sometimes completely arrested parasite transmission to mosquitoes.

Finally, in **Chapter-6**, we discussed our findings in reference with the previous studies, and forwarded recommendation for future studies. Taken together, this thesis provides significant insights related to asymptomatic malaria infections and the presence and impact of naturally acquired immunity to malaria parasites in Ethiopia. Our work sets the stage for interventions that aim to reduce community-wide malaria transmission by targeting (the infectivity of) asymptomatic malaria infections in Ethiopia.

Samenvatting

Malaria is een infectieziekte die wordt veroorzaakt door *Plasmodium*-parasieten die worden overgedragen door infectieuze muggenbeten. Er zijn meer dan 200 soorten *Plasmodium*, waarvan vier veelvuldig mensen infecteren: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* (*P. ovale curtisi* en *P. ovale wallikeri*) en *Plasmodium malariae*. *P. falciparum* en *P. vivax* zijn de meest voorkomende soorten die mensen infecteren en zijn verantwoordelijk voor het grootste deel van alle malariaziekten wereldwijd. De klinische en biologische kenmerken van malaria-infecties zijn vooral gebaseerd op symptomatische malariagevallen. Een groot deel van de malaria-infecties blijft echter asymptomatisch zonder dat die leiden tot ziekteverschijnselen die ernstig genoeg zijn om behandeling te zoeken. Deze asymptomatische gevallen zijn minder goed bestudeerd. Hoewel deze infecties 'asymptomatisch' zijn en niet gepaard gaan met acute symptomen die wijzen op koorts, kunnen ze toch aanzienlijke gevolgen hebben voor de gezondheid. Deze gezondheidsgevolgen zijn lange tijd door de malaria-onderzoeksgemeenschap verwaarloosd. Gevolgen zijn onder meer laaggradige ontstekingen en milde bloedarmoede. Er komt steeds meer wetenschappelijk bewijs dat aantoont dat asymptomatische malaria-infecties vaak voorkomen, veel vaker dan eerder werd gedacht. Bovendien kunnen asymptomatische infecties belangrijke aanjagers zijn van malaria-overdracht naar muggen. Of deze asymptomatische malaria-infecties behandeld moeten worden om malaria-eliminatie te bereiken, is nog steeds onderwerp van veel discussie. Daarom probeert dit proefschrift inzicht te geven in de biologie en epidemiologie van asymptomatische malaria-infecties met een focus op Ethiopië.

In **hoofdstuk 2** onderzochten we de prevalentie en detecteerbaarheid van asymptomatische *P. falciparum*- en *P. vivax*-infecties in lage, matige en hoge transmissiegebieden in Ethiopië met behulp van drie diagnostische methoden: microscopie, diagnostische sneltesten (RDTs) en polymerasekettingreactie (PCR). De laatste, PCR, is aanzienlijk gevoeliger dan microscopie en RDT. Onze studie was speciaal ontworpen om een directe vergelijking van parameters tussen de drie transmissie-intensiteitsgebieden mogelijk te maken. We ontdekten dat asymptomatische malaria-infecties veel voorkwamen op alle onderzochte locaties. Bovendien vonden we dat hun detecteerbaarheid door microscopie/RDT afneemt met afnemende transmissie-intensiteit. Met andere woorden: hoewel infecties weinig voorkomend zijn in lage endemische omgevingen, zijn de infecties die aanwezig zijn moeilijk te detecteren met conventionele diagnostiek. Dit wordt waarschijnlijk veroorzaakt door zeer lage parasietdichtheden van infecties die voorkomen in laag-endemische gebieden. Hoewel inzichtelijk, stond ons dwarssnede onderzoek (**hoofdstuk 2**) ons niet toe om het infectieverloop over tijd te bepalen en de daarbij passende besmettelijkheid voor muggen. Daarom onderzochten we de duur, het verloop van parasietdichtheden over tijd en besmettelijkheid voor muggen van asymptomatische *P. falciparum*- en *P. vivax*-infecties in een aparte studie. Dit is gedaan middels een longitudinale studie in een gebied met lage transmissiedruk in Ethiopië. Daar werden infecties gemonitord maar, indien asymptomatisch, niet behandeld met malariamedicijnen (**hoofdstuk 3**). Hiermee ontdekten we dat asymptomatische malaria-infecties vaak van korte duur zijn en dat hun parasiet- en gametocytdichtheid in de loop van de tijd fluctueerde en afnam tijdens de infectie. We zagen ook dat slechts een klein deel van deze infecties besmettelijk was voor muggen. Door de dichtheid van parasieten en gametocyten en ook de besmettelijkheid voor muggen te vergelijken tussen asymptomatische malaria-infecties en hun symptomatische tegenhangers, konden we hun relatieve besmettelijkheid bepalen. We ontdekten dat vooral patiënten met klinische (symptomatische) *P. vivax* infecties zeer besmettelijk kunnen zijn voor muggen. Het belang van deze bevinding zal afhangen van hoe vaak symptomatische en asymptomatische infecties voorkomen in populaties.

Natuurlijk verworven immuniteit tegen malariaparasieten is een van de belangrijke factoren die de dichtheid van parasieten beïnvloed. Daarmee is immuniteit een belangrijke factor in het bepalen van de detecteerbaarheid van infecties, de infectieduur en mogelijk ook de overdraagbaarheid op muggen. Antilichamen geïnduceerd tegen malariaparasieten in het seksuele stadium kunnen de overdracht van parasieten naar muggen beperken. Dit fenomeen vormt een belangrijke basis voor de ontwikkeling van transmissieblokkerende vaccins (TBV's). TBV's worden beschouwd als zeer waardevolle interventies om eliminatiepogingen te ondersteunen. Door de verspreiding van malaria te verminderen, resulteren TBV's in minder geïnfecteerde muggen en verlichten daarmee indirect de ziektelast van malaria. Als onderdeel van dit proefschrift wordt in **hoofdstuk 4** een uitgebreid literatuuroverzicht gegeven van de natuurlijk verworven immuniteit tegen seksuele stadia van *P. falciparum* en *P. vivax* en hun invloed op de overdracht van parasieten van mens op mug.

Uit ons literatuuronderzoek bleek dat het overgrote deel van eerder onderzoek naar natuurlijk verworven immuniteit tegen malariaparasieten gericht was op

P. falciparum. Hierdoor bestaat er een aanzienlijk 'kennisgat' voor *P. vivax*. Daarom onderzochten we het bestaan van natuurlijk verworven immuniteit tegen seksuele stadia van *P. vivax* in een Ethiopische populatie en kwantificeerden we de impact van deze natuurlijke immuniteit op de overdracht van malariaparasieten van mens op mug in **hoofdstuk 5**. We ontdekten dat een aanzienlijk aantal deelnemers antilichamen had verworven tegen meerdere *P. vivax*-gametocyt eiwitten. Reacties tegen verschillende van deze antigenen waren significant geassocieerd met verminderde of soms volledig geblokkeerde overdracht van parasieten naar muggen. Ten slotte bespraken we in **hoofdstuk 6** onze bevindingen in de context van eerdere studies en formuleerden we aanbevelingen voor toekomstige studies. Alles bij elkaar biedt dit proefschrift belangrijke inzichten op het vlak van asymptomatische malaria-infecties en de aanwezigheid en impact van natuurlijk verworven immuniteit tegen malariaparasieten in Ethiopië. Ons werk vormt de basis voor interventies die gericht zijn op het verminderen van de overdracht van malaria in de hele gemeenschap door zich te richten op (de besmettelijkheid van) asymptomatische malaria-infecties in Ethiopië.

ማጠቃለያ

ወባ በፕላዝሞዲየም ጥገኛ ተውሳኮች የሚመጣ ተላላፊ በሽታ ሲሆን በወባ ትንኝ ንክሻ የሚተላለፍ ነው። ከ200 በላይ የፕላዝሞዲየም ዝርያዎች ሲኖሩ ከእነዚህም ውስጥ አራቱ ዝርያዎች ማለትም ፕላዝሞዲየም ፋልሲፓረም፣ ፕላዝሞዲየም ቫይቫክስ፣ ፕላዝሞዲየም ኦቫሌ (ፒ. ኦቫሌ ኩርቲሲ እና ፒ. ኦቫሌ ዋሊኬሪ) እና ፕላዝሞዲየም ማሌሪ በተለምዶ ሰዎችን ያጠቃሉ። የፕላዝሞዲየም ፋልሲፓረም እና የፕላዝሞዲየም ቫይቫክስ ስርጭት መጠነ ሰፊ ሲሆን በዓለም አቀፍ ደረጃ አብዛኛውን የወባ በሽታ ያስከትላሉ። የወባ በሽታ ባህሪያት ለረጂም ጊዜ ሲገለጽ የነበረው ምልክት በሚያሳዩ የወባ ኢንፌክሽኖች ላይ ብቻ ተመስርቶ ነበር። ነገር ግን፣ የቅርብ ጊዜ ጥናቶች እንደሚያሳዩት ከሆነ ከፍተኛ መጠን ያለው የወባ ኢንፌክሽን ምንም አይነት ምልክት ሳይኖረው እንደሚቀር ነው። በእነዚህ ምልክት በማያሳዩ የወባ ኢንፌክሽኖች ላይ እስካሁን የተደረገው ጥናት አመርቂ አይደለም። ምንም እንኳን ምልክት ባያሳዩ እና የሰውነት ትኩሳት ባያስከትሉ ፣ እነዚህ ኢንፌክሽኖች ተዛማች የሆኑ የጤና ችግሮች ሊፈጥሩ ይችላሉ። ለምሳሌም ዝቅተኛ ደረጃ ያለው የሰውነት መቆጣት እና ቀላል የደም ማነስ ሊያስከትሉ ይችላሉ። ምልክት የማያሳዩ የወባ ኢንፌክሽኖች በየትኛውም ወባማ በሆኑ ቦታዎች ላይ ከዚህ ቀደም ከሚታሰበው በላይ የተስፋፉ መሆንቸውን እና ለወባ ስርጭት ወሳኝ አንቀሳቃሾች መሆናቸውን የሚያሳዩ ሳይንሳዊ መረጃዎች እየጨመሩ መጥተዋል። ስለዚህ እነዚህ ምልክት የማየሳዩ የወባ ኢንፌክሽኖች ፤ የወባ በሽታን ለማጥፋት በሚደረገው የተቀናጀ ጥረት ውስጥ ዒላማ መሆን አለባቸው? የሚለውን ጥያቄ ለመመለስ ከፍተኛ እና ተጨማሪ ጥናቶች ያስፈልጋሉ። በመሆኑም፤ የዚህ ጥናታዊ ጽሑፍ ዓላማ በኢትዮጵያ ላይ በማተኮር ምልክት የማያሳዩ የወባ ኢንፌክሽኖችን ኤፒዲሚዮሎጂ፤ እንዲሁም ባዮሎጂካዊ፣ እና የመስክ ባህሪያት መርምሮ ግንዛቤን መስጠት ነው።

በ**ምዕራፍ-2**፣ በኢትዮጵያ ውስጥ ዝቅተኛ፣ መካከለኛ እና ከፍተኛ የወባ ስርጭት ባለባቸው ቦታዎች ውስጥ ምልክት የማያሳዩ የፕላዝሞዲየም ፋልሲፓረም እና የፕላዝሞዲየም ቫይቫክስ ተውሳኮች በሶስት የመመርመሪያ ዘዴዎች ማለትም በማይክሮስኮፕ፣ በራፒድ ዲያግኖስቲክ ቴስት (አር.ዲ.ቲ) እና በፖሊመሬዝ ቼይን ሪአክሽን (ፒ.ሲ.አር) ያላቸውን የመለየት ባህሪያት ገልፀናል። ከነዚህ የመመርመሪያ ዘዴዎች ውስጥ ፒ.ሲ.አር በተሻለ ሁኔታ ተውሳኮችን እንደሚለይ ይታወቃል። ከዚህ በፊት ከተሰሩ ጥናቶች የእኛን ጥናት ልዩ የሚያደርገው ሶስት የመመርመሪያ ዘዴዎችን ተጠቅመን በሶስት የተለያዩ የወባ ስርጭት ያለባቸው ቦታዎች መካከል ያለውን የተውሳኮች የመለየት ቀጥተኛ ንፅፅር ስለሚያሳይ ነው። የጥናታችን ውጤት እንደሚያሳየው፤ በሁሉም የጥናት ቦታዎች ላይ ምልክት የማያሳዩ የወባ ኢንፌክሽኖች በብዛት የተስፋፉ እንደሆኑ ደርሰንበታል። በተጨማሪም ፣ ማይክሮስኮፕ እና አር.ዲ.ቲ በከፍተኛ ደረጃ ምልክት የማያሳዩ የወባ ተውሳኮችን መለየት እንደማይችሉ ለመገንዘብ ችለናል። ይህም ደግሞ ዝቅተኛ የወባ ስርጭት ባለባቸው አካባቢዎች ላይ ይጎላል። ይህም ማለት የወባ ስርጭት እየቀነሰ በሄደ ቁጥር የማይክሮስኮፕ እና የአር.ዲ.ቲ የመለየት ችሎታ እየቀነሰ ይመጣል።

ምንም እንኳን ዓይን ገላጭ ቢሆንም፣ በ**ምዕራፍ-2** ያደረግነው ጥናት ምልክት የማያሳዩ የወባ ኢንፌክሽኖች በጊዜ ሂደት ያላቸውን ተለዋዋጭ ባህሪያት፣ ለምን ያህል ጊዜ እንደሚቆዩ እና ወደ ወባ ትንኝ እንዴት እና ወቼ እንደሚተላለፉ አይገልፅም። ስለዚህ ምልክት የማያሳዩ የፕላዝሞዲየም ፋልሲፓረም እና የፕላዝሞዲየም ቫይቫክስ ኢንፌክሽኖችን የቆይታ ጊዜ፣ጊዜያዊ የኢንፌክሽን ተለዋዋጭ ባህሪያት እና ወደ ትንኞች የመተላለፍ አቅም መርምረናል። የዚህ ጥናት ውጤት በ**ምዕራፍ-3** ተገልጿል። በጥናቱ ውጤት መሰረት ምልክት የማያሳዩ የፕላዝሞዲየም ፋልሲፓረም እና የፕላዝሞዲየም ቫይቫክስ ኢንፌክሽኖች ለአጭር ጊዜ የሚቆዩ ሲሆኑ የእሴክሽዋል ጥገኛ ተውሳኮች እና የጋሜቶሳይቶች ብዛት ከጊዜ ወደ ጊዜ እየተለዋወጠ የሚሄድ ሆኖ በጠቅላላው ግን እያሽቆለቆለ የሚሄድ መሆኑን ተረድተናል። ከእነዚህ ኢንፌክሽኖች መካከልም ጥቂቶቹ ብቻ ወደ ወባ ትንኞች

እንደተላለፉም ተገንዝብናል። በተጨማሪም፤ ምልክት በማያሳዩት እና ምልክት በሚያሳዩት የወባ እንፌክሽኖች መካከል ያለውን የእሴክሽዋል ጥገኛ ተውሳኮች እና የጋሜቶሳይቶች ብዛት ልዩነት እንዲሁም ወደ ወባ ትንኝ የመተላለፍ አቅም ለማነፃፀር ችለናል። በተለይ ምልክት የሚያሳዩት የፕላዝሞዲየም ቫይቫክስ ኢንፌክሽኖች ወደ ወባ ትንኝ የመተላለፍ አቅማቸው ከፍተኛ ነው። በአጠቃላይ፤ የትኛውም የወባ ኢንፌክሽን፤ በወባ ስርጭት ላይ ተፅእኖ ለማድረግ ያለው አስተዋፅኦ በህብረተሰቡ ውስጥ ባለው ብዛት እና ስርጭት ይወሰናል።

በተፈጥሮ የሚገኝ የበሽታ መከላከያ የወባ ጥገኛ ተውሳኮች ላይ ከፍተኛ ተፅህኖ ያሳድራል። ይህም ጥገኛው በደም ውስጥ የሚኖረውን ብዛት፣ ቆይታ እንዲሁም ወደ ወባ ትንኞች የመተላለፍ አቅሙን ይወስናል። ፀረ-ጋሜቶሳይት የሆኑ አንቲቦዲዎች ጥገኛው ተውሳክ ወደ ወባ ትንኞች እንዳይተላለፍ የማድረግ አቅም እንዳላቸው ጥናቶች ያሳያሉ። ይህም የወባ ስርጭት እንዲቀንስ የሚረዳ ክትባት ለመስራት የንድፈ ሀሳብ መሰረት ጥሏል። ስለዚህ በተፍጥሮ ስለሚገኝ የሰውነት የበሽታ መካላከያ እና በፕላዝሞዲየም ፋልሲፓረም እና በፕላዝሞዲየም ቫይቫክስ ተውሳኮች ላይ ያለውን ተፅህኖ ከዚህ በፊት የተደረጉ ጥናቶችን ዋቢ በማድረግ አጠቃላይ መግለጫ በ**ምዕራፍ-4** አቅርበናል። ከዚህ እንደምንረዳው፤ ከዚህ ቀደም የተደረጉት አብዛኛው የምርምር ጥናቶች የሚያዘነብሉት በፕላዝሞዲየም ፋልሲፓረም ተውሳክ ላይ ነው። ይህም በፕላዝሞዲየም ቫይቫክስ ላይ ያለውን ግንዛቤ በእጅጉ ውስን አድርጎታል። ስለዚህም፤ በተፈጥሮ የሚገኙ ፀረ-ጋሜቶሳይት አንቲቦዲዎች በኢትዮጵያ ማህበረሰብ ውስጥ ያላቸውን ስርጭት እንዲሁም እነዚህ አንቲቦዲዎች በፕላዝሞዲየም ቫይቫክስ ወደ ወባ ትንኝ የመተላለፍ አቅም ላይ ያላቸውን ተፅህኖ መርምረናል (**ምዕራፍ-5**)። ይህም ጥናት በኢትዮጵያ ውስጥ በዓይነቱ የመጀመሪያ ነው። በዚህም ጥናት በርካታ ቁጥር ያላቸው የጥናቱ ተሳታፊዎች የፕላዝሞዲየም ቫይቫክስ ፀረ-ጋሜቶሳይት አንቲቦዲዎች በደማቸው ውስጥ እንደሚግኝ ደርሰንበታል። እንዲሁም እነዚህ አንቲቦዲዎች የፕላዝሞዲየም ቫይቫክስ ጋሜቶሳይቶች ወደ ወባ ትንኞች የመተላለፍ አቅማቸውን እንደሚቀንሱ ተገንዝበናል።

በመጨረሻ፣ በ**ምዕራፍ-6**፣ ግኝቶቻችንን ከቀደምት ጥናቶች ጋር በማጣቀስ በዝርዝር አቅርበናል። ለወደፊት ጥናቶች መሰረት የሚሆኑ ሳይንሳዊ ምክሮችንም አስተላልፈናል። በአጠቃላይ፤ ይህ ጥናታዊ ፅሁፍ በኢትዮጵያ ውስጥ የሚገኙ ምልክት የማያሳዩ የወባ ኢንፌክሽኖች ያላቸውን ባህሪያት እና በተፈጥሮ የሚገኘው የሰውነት መከላከያ በወባ ጥገኛ ተህዋሲያን ላይ ያለውን ተፅህኖ የሚግልፅ ነው። ከዚህ ጥናት የተገኙት ግንዛቤዎች በኢትዮጵያ ብሎም በሌሎች ሀገራት ለሚደረገው ወባን የማጥፋት ዘመቻ ግብአት ይሆናል ብለን እናምናለን።

Research data management

The research data in this thesis have been obtained during my PhD study at the department of Medical Microbiology, Radboud University Medical Center (Netherlands) and the Armauer Hansen Research Institute (Ethiopia). The data were collected, cleaned, analyzed and stored following the scientific integrity guidelines and principles of Radboud University, Radboud University Medical Center and the Armauer Hansen Research Institute. Field primary data were collected on paper which were then copied to computers in a double entry method. To maintain confidentiality, while the paper documents were kept in locked cabinets with only authorized access, the soft copies of the primary data in computers were kept on excel files with access passwords with, where appropriate, identifying information removed. The completeness and consistency of the data was checked by research team members. In all studies, privacy of participants were maintained by the use of unique codes for each participant. Laboratory data were collected electronically in computers using excel files with access passwords. The primary and laboratory data were then cleaned and merged for analysis. Chapters 2, 3 and 5 were based on human studies that were conducted in accordance with the Declaration of Helsinki. Informed consents were collected from all participants or from their legal guardians prior to enrolment to the study. Ethical approval were obtained from the Armauer Hansen Research Institute (PO32/18), London School of Hygiene and Tropical Medicine (15811) and the National Research Ethics Review Committee of Ethiopia (SHE/S.M./14.4/708/19). The primary and secondary data obtained in Chapters 2, 3, and 5 are stored at the Radboud University Medical Center, department of medical microbiology H-drive folder: (H:\ MMBdata$(\\umcfs083) and the knowledge management department of the Armauer Hansen Research Institute. Additionally, all published data are available upon reasonable request from the corresponding author and are stored on the H-drive under the name of the first author and journal details (H:\MMB NCMLS\Datasets published articles).

List of publications

Included in this thesis

1. **Naturally acquired antibodies to gametocyte antigens are associated with reduced transmission of *Plasmodium vivax* gametocytes to *Anopheles arabiensis* mosquitoes.**
   Surafel K. Tebeje,...Eizo Takashima, Takafumi Tsuboi,...Chris Drakeley,... Matthijs M. Jore,...Fitsum G. Tadesse and Teun Bousema. *Front. Cell Infect. Microbiol. 2023; 16:12*. doi:https://doi.org/10.3389/fcimb.2022.1106369

2. **Dynamics of asymptomatic *Plasmodium falciparum* and *Plasmodium vivax* infections and their infectiousness to mosquitoes in a low transmission setting of Ethiopia: a longitudinal observational study**
   Elifaged Hailemeskel, **Surafel K. Tebeje**,...Teun Bousema, Fitsum Tadesse. *IJID. 2024; 143.* DOI:https://doi.org/10.1016/j.ijid.2024.107010

3. **The epidemiology and detectability of asymptomatic *plasmodium vivax* and *plasmodium falciparum* infections in low, moderate and high transmission settings in Ethiopia.**
   Hailemeskel E, **Tebeje SK**, Behaksra SW, Shumie G, Shitaye G, Keffale M, Chali W, Gashaw A, Ashine T, Drakeley C, Bousema T, Gadisa E, Tadesse FG. *MalarJ. 2021;20:59*. doi: 10.1186/s12936-021-03587-4. PMID: 33482841

4. **Immunity against sexual stage *Plasmodium falciparum* and *Plasmodium vivax* parasites.**
   de Jong RM, **Tebeje SK**, Meerstein-Kessel L,...Teun Bousema. *Immunol. Rev. 2020; 293:190-215*. https://doi.org/10.1111/imr.12828

Not included in this thesis

5. ***Anopheles stephensi* Mosquitoes as Vectors of *Plasmodium vivax* and *falciparum*, Horn of Africa, 2019.**
   Tadesse FG, Ashine T... **Tebeje SK**... Drakeley C, Gadisa E, Bousema T. *Emerg Infect Dis.* 2021; *27*:603-607. doi: 10.3201/eid2702.200019. PMID: 33496217

About the Author

Surafel K. Tebeje was born in Addis Ababa, Ethiopia on April 05, 1983. He grew up in the comfortable care of his parents along with his brothers and sisters. He spent his childhood days playing and having fun all the time with his childhood friends.

He got his bachelor of sciences degree in applied biology (microbiology and parasitology) from Ambo University in Ethiopia in 2009. Then, he became an assistant lecturer in the same University for three years until 2012. In 2012, he got two scholarships one from Radboud University Medical Center (RadboudUMC study fund) and another from Radboud Scholarship Program (RSP) to pursue the most prestigious master program in the Netherlands: Molecular Mechanisms of Disease. During his master study, he did two research internships that resulted in two master theses. The first internship was in RadboudUMC, laboratory of hematology under the supervision of Dr. Bert van Der Reijden and Dr. Anne Marneth. The second was in RadboudUMC, laboratory of paediatric infectious diseases under the supervision of Dr. Dimitri Diavatopoulos and Dr. Marrit Habets. Obtaining his master degree in Molecular Mechanisms of Disease, he returned to Ambo University where he worked as a lecturer and research associate for four years. In 2018 he got an opportunity to pursue a PhD study in RadboudUMC in the department of medical microbiology under the supervision of Prof. Teun Bousema and Dr. Matthijs Jore in collaboration with the Armauer Hansen research Institute in Ethiopia under the supervision of Dr. Fitsum Tadesse and oversight of Dr. Endalamaw Gadisa.

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“እግዚአብሔር ብርሀኔና መድሀኒቴ ነው!”

በመጀመሪያ በመንገዴ ሁሉ አብሮኝ ለነበረው እና ሳይለየኝ አስከዘለዓለም ለሚዘልቀው አምላኬ ለ**እግዚአብሔር** ፤ ለ**ስላሴ** እጅጉን የተከበረ ምስጋናዬን አቀርባለሁ! አሜን!

በመቀጠል ሁልጊዜም ስለምናት ፈጥና ለምታማልደኝ **እመብርሀን ድንግል ማሪያም** የተመሰገነች ትሁን! አሜን!

እንዲሁም ቅዱሳን **መላእክት**፤ **ሀዋሪያት** ፤ **ነቢያት** እና **ሰማእታት** የትመሰገኑ ይሁኑ! አሜን!

This thesis was a result of many years of thinking, planning, experimenting and researching. Through these years I have been challenged to the fullest. I wouldn’t be able to obtain my PhD degree if it wasn’t for all the support and help that I got from many different people. To these people, I would like to forward my acknowledgement as follows.

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PhD portfolio of Surafel Tebeje

Department: **Medical Microbiology**
PhD period: **01/02/2018 – 31/12/2024**
PhD Supervisor(s): **Prof. dr.J.T.Bousema**
PhD Co-supervisor(s): **Dr. M.M. Jore, Dr. F.G.Tadesse**

Training activities	**Hours**
Courses	
– Advanced Course in Epidemiological Analysis (London School of Hygiene and Tropical Medicine, 2019)	67.20
– Scientific Integrity for PhD candidates (RIHS, 2020)	56.00
– RIHS - Introduction course for PhD candidates (2020)	36.00
– Cutting edge Immunology: from Chemistry to Metabolism (Leiden University, 2020)	56.00
– Ethical and Regulatory Aspects of Clinical Research (NIH, 2020)	56.00
– Scientific Writing for PhD candidates (2021)	84.00
Seminars	
– Radboudumc Research Rounds (2018 - 2021)	20.00
– Journal Club (2018 - 2022) Radboudumc/AHRI	30.00
– Meet the expert: How to unravel the secrets of protein 3D structures (Structural bioinformatics) (2020)	2.80
– Research Integrity Round: Sex and gender and scientific integrity: a tale of how and who (2020)	2.80
Conferences	
– Biology and Pathology of the Malaria Parasite (EMBL virtual conference 2020)	14.00
– Webinar: Spotlight on Immunity and Vaccines in Malaria (London School of Hygiene and Tropical Medicine, 2020)	2.80
– Webinar: World Mosquito Day, *Anopheles stephensi* a future threat in the horn of Africa? (London School of Hygiene and Tropical Medicine, 2020)	2.80
– ASTMH 2020 Annual Meeting	6.00
– ASTMH 2022 Annual Meeting	56.00
Other	
– Weekly work meetings (2018 - 2022) Radboudumc/ AHRI	60.00
Total	**552.40**

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