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PLASMODIUM GAMETOCYTE BIOLOGY AND THE INTERRELATION BETWEEN HOST AND MOSQUITO INFECTIVITY

by

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Ashleigh N. Roberds
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This research protocol was reviewed and approved by the Uniformed Services University of the Health Sciences and Walter Reed Army Institute of Research institutional review board (IRB) and Navy Medical Research Unit – Six institutional animal care and use committee (IACUC) in accordance with all applicable Federal regulations governing the protection of animals in research.

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13 August 2022

ABSTRACT

Plasmodium Gametocyte Biology and the Interrelation Between Host and Mosquito Infectivity

Ashleigh N. Roberds, Doctor of Philosophy, 2022

Thesis directed by: V. Ann Stewart, DVM, PhD, Professor, Department of Preventive Medicine and Biostatistics, Division of Global Public Health

Despite significant advancements over the past two centuries, nearly half the world's population remain at risk for malaria, a vector-borne disease caused by the *Plasmodium* parasite. *Plasmodium falciparum* and *Plasmodium vivax* cause the majority of global malaria disease burden and mortality, infecting over 600,000 people globally. As public health campaigns make continued strides towards malaria eradication, particular focus on transmission dynamics of these two species is critical. Within the human host, gametocytes are the sexual form of parasites responsible for transmission to the mosquito vector. The research described in this dissertation aims to improve our overall understanding of *P. falciparum* and *P. vivax* gametocyte biology and the impact that understudied host populations have on continued parasite transmission. Two different sets of novel molecular assays were developed using genetic orthologs of *Plasmodium* gametocyte-specific RNA transcripts. These assays used different

biotechnologies with high levels of sensitivity to detect sub-microscopic gametocyte prevalence in two endemic regions of the world.

First, we developed a digital droplet PCR (ddPCR) assay to detect and quantify male and female *P. falciparum* gametocyte transcripts in adults with asymptomatic malaria infections from the endemic Kisumu region of Kenya. Study participants were followed over the course of six months and categorized based on their HIV-1 status at enrollment. Using our novel ddPCR gametocyte assays, 51.1% of malaria-positive individuals had detectable gametocyte-specific transcripts throughout the six-month follow-up. HIV-1 positive volunteers who were treated for malaria with artemether/lumefantrine (AL) and initiated antiretroviral therapy (ART) and trimethoprim/sulfamethoxazole (TS) prophylaxis saw a significant reduction in gametocyte prevalence when compared to AL treatment alone. Additionally, we confirmed that mosquito infectivity occurs even in the absence of detectable parasites and gametocytes by highly sensitive molecular assays such as ddPCR. These data demonstrate the importance of combining sensitive gametocyte detection methods with subsequent mosquito infectivity data when assessing transmission potential and success. Additionally, our results also suggest that malaria control and elimination programs need to consider unique populations, such as individuals with HIV-1 malaria co-infection, when making recommendations for universal chemoprophylaxis and treatment.

Using *P. vivax* orthologs of the same gametocyte specific molecular markers from the *P. falciparum* study, we developed a novel panel of qPCR assays to detect and analyze *P. vivax* gametocyte transcript expression. These assays were used to characterize *P. vivax* gametocyte sex-ratio, gametocytemia, and gametocytogenesis in a

sporozoite induced *P. vivax* infection in *Aotus nancymaae* monkeys. Observations included early sub-microscopic detection of female gametocyte-specific transcripts as well as an increase in gametocyte transcript abundance immediately following mefloquine treatment. Orthologs for detection of possible early-stage gametocytes and gametocytogenesis proved to be inefficient, contributing to perceived differences between *P. falciparum* and *P. vivax* such as duration of gametocyte development. Furthermore, the utility of the assays was assessed in a subset of samples from febrile volunteers from the endemic Loreto region of Peru. These assays were able to detect gametocytes in both controlled monkey infections and natural human infections with high sensitivity and specificity. Our *P. vivax* assays can be used for future *in vivo* and epidemiological studies to further characterize understudied *P. vivax* gametocyte biology and kinetics.

In conclusion, the research presented in this dissertation provides new tools for sensitive molecular detection of gametocytes in two dominant malaria species. Additionally, this research highlights the complexities surrounding gametocyte biology and malaria parasite transmission and the need for continued research in understudied populations as part of the global progress towards malaria eradication.

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CHAPTER 1: General Introduction

GLOBAL VECTOR-BORNE DISEASES OF PUBLIC HEALTH IMPORTANCE

Vector-borne diseases account for more than 17% of all global infectious diseases, resulting in more than 700,000 deaths annually (175). With more than half of the world at risk, the burden of diseases is highest in tropical and subtropical areas of the world where vectors thrive in warm, moist climates. Most of the known vector-borne diseases are caused by pathogenic parasites, bacteria, or viruses and lead to high rates of mortality, morbidity, and consequences to economic and social development. Because these diseases disproportionally affect underdeveloped nations, the lack of public health infrastructures make it difficult to make significant changes despite significant funding and global health campaigns. Additionally, these diseases thrive among communities with poor living conditions, particularly areas that lack access to adequate housing, safe drinking water, and sanitation.

Vector-borne diseases distinguish themselves from other zoonotic diseases, such as rabies, Ebola, or anthrax, by requiring an intermediary living organism to convey infectious pathogens between hosts rather than transmitting directly to a host by mechanisms such as cutaneous contact, inhalation, or ingestion. The modes and routes of biological transmission of vector-borne diseases vary by pathogen. Some pathogens undergo replication and/or cyclical changes within the vector (biological - propagative, cyclo-developmental, and cyclo-propagative) and other pathogens simply use the vector solely for transmission to the next host (mechanical) (98).

Most vectors are bloodsucking insects such as mosquitoes, ticks, fleas, and flies. They have unique habitats that are changing with urbanization, globalization, global warming, and resistance to insecticide vector control programs (90).

Environmental changes are causing an increase in vectors by expanding their geographical range. Because many pathogens of vector-borne diseases also infect different non-human primate or other animal reservoirs, disease eradication and transmission reduction efforts are difficult.

Common vector-borne diseases caused by bacterial pathogens include but are not limited to plague, typhus, Lyme disease, various rickettsial diseases, and tularaemia. Common vector-borne diseases caused by viral pathogens include but are not limited to dengue, chikungunya, Zika, yellow fever, Japanese encephalitis, and Crimean-Congo hemorrhagic fever. Finally, common diseases caused by parasitic pathogens include but are not limited to malaria, lymphatic filariasis, schistosomiasis, onchocerciasis, leishmaniasis, and Chagas disease (175).

PARASITIC PATHOGENS OF VECTOR-BORNE DISEASES

Parasitic pathogens are eukaryotic organisms with a defined nucleus and other subcellular organelles. As the term parasite loosely describes, they use another living organism as a resource for development or replication. Parasites are genetically and biologically highly complex. They have evolved to overcome barriers to parasite transmission and reproduction such as evading host immunity, developing resistance to chemotherapies, and even deleting genes used in detection methods (160). Due to their complexity, there are not many successful vaccines for humans, and co-infections are an increasing threat.

Protozoa and metazoan organisms make up the most common medically important parasites. Metazoan parasites are broadly composed of helminths and arthropods while protozoan parasites have seven subgroups of medical concern. While the list of parasitic organisms is extensive, the most common parasitic

pathogens of vector-borne diseases are summarized in Table 1. Of this list, malaria has the highest global burden of infection and mortality rates with five species of *Plasmodium* capable of naturally infecting humans.

In order to control and/or prevent vector-borne parasitic disease, we must continue to better understand parasite kinetics, mechanisms of infection, and host and vector immune involvement. This includes improved diagnostics and detection methods as well as understanding transmission dynamics to and from the vector.

Table 1. Summary of Vector-Borne Diseases of Global Public Health Importance. Adapted from (160; 175; 177)

Organism	Vector	Disease	Global Distribution
Wuchereria bancrofti	Mosquito	Lymphatic filariasis/ Elephantiasis	Tropical and subtropical areas
Brugia malayi	Mosquito	Lymphatic filariasis	Tropical and subtropical areas
Loa Loa	*Chrysops* fly	Loaisis	Africa
Onchocerca volvulus	*Simulium* blackfly	Onchocerciasis (River Blindness)	Africa, Central and South America
Schistosoma	Snail	Schistosomiasis (Bilharziasis)	Africa, Asia, Latin America
Babesia spp.	*Ixodes* tick	Babesiosis	North America, Europe
Leishmania	*Phlebotomus* Sand-fly	Leishmaniasis	Tropical and Subtropical areas
Trypanosoma	Tsetse fly/ Triatomine bug	Sleeping sickness/ Chagas disease	Africa and Americas
Plasmodium spp.	Mosquito	Malaria	Global Tropical and subtropical areas

MALARIA

Since the turn of the century, international malaria eradication efforts have made significant progress in the control of malaria, with a global reduction in malaria incidence by 41% between 2000 and 2015 (176). Researchers from multiple organizations have made strides in the development of new vector control measures,

drugs for chemoprevention, and vaccine targets. However, public health efforts have stalled in recent years due to factors such as environment changes, globalization, political instability, and limited access to adequate health care. In fact, malaria cases and deaths flatlined between 2015 (224 million cases; 562,000 deaths) and 2019 (227 million cases; 558,000 deaths) followed by an increase to 241 million cases and 627,000 deaths in 2020 likely caused by disruption to services and access to care during the COVID-19 pandemic (176). There are two major human malaria species that make up most of the malaria burden and geographical distribution of the disease: *Plasmodium falciparum* and *Plasmodium vivax* (Table 2).

Table 2. Comparison of *Plasmodium falciparum* and *Plasmodium vivax*. Adapted from (245).

	Plasmodium falciparum	***Plasmodium vivax***
Geographic Distribution	Worldwide. + Sub Saharan Africa	Worldwide. + Americas, Eastern and Southern Africa, Western Pacific
Clinical Disease	Historically severe, High asymptomatic - Uncomplicated - Severe in young children, malaria-naïve individuals, immunocompromised - Asymptomatic (adults in holoendemic areas)	Generally mild, occasionally severe - Uncomplicated - Severe in young children and pregnant women
Parasitemia	Can be high	Low - Moderate
Sequestration-cytoadherence	Yes	Rarely
Earliest Appearance of Gametocytes	10 days	3 days
Hypnozoite stage	No	Yes
Age of RBC infected	All ages	Reticulocytes

Morphology	- High abundance of ring stages - Ring and gametocyte stages in peripheral blood - Gametocytes are crescent shaped	- Large - Round - Stippled
Challenges	- Asymptomatic carriers - Immunocompromised (high coinfection rates in SSA) - Drug resistance	- Early transmitters - Dormant infections - Drug resistance

In areas of high endemicity where *P. falciparum* partial protective immunity is present, clinical symptoms may be partially controlled in immune-experienced individuals, and asymptomatic reservoirs further challenge control programs as the malaria transmission cycle continues. The sexual stage responsible for onward parasite transmission to mosquitoes, the gametocyte stage, is often circulating in the bloodstream of infected individuals in the absence of clinical symptoms (42; 157).

This situation is further complicated by the disproportionate molecular research between *P. vivax* and *P. falciparum*. The disproportion is often attributed to the limited *P. vivax in vitro* culture model which makes it difficult to study parasite biology, behavior, and kinetics over a long-term continuous period. Understanding the unique biology of *P. vivax* is critical to elimination efforts as the species has the most widespread global distribution, uses a number of different tactically efficient mosquito species as vectors, and has unique aspects to the host-parasite relationship.

P. falciparum and *P. vivax* Malaria Epidemiology

Prevalence

P. falciparum malaria infections are found essentially worldwide in tropical regions, but 95% of all malaria cases in 2020 were in the WHO African Region

where *P. falciparum* clearly dominates (~95-99% of all cases are *P. falciparum*) (176). The actual prevalence number may be underreported due to the high density of asymptomatic individuals who are not presenting to clinics or health centers for testing.

Reduction in *P. vivax* infections has been significant in the past two decades where the proportion of global cases due to *P. vivax* infections changed from 8% to 4.5% (176). However, *P. vivax* is geographically the most widely distributed cause of human malaria and can still found throughout most major regions of the world. The proportion of cases in the WHO Western Pacific Region due to *P. vivax* has increased over the past two decades (17% to 33%) due to the strong focus on reducing *P. falciparum* burden (176). A similar shift was observed in the WHO Eastern Mediterranean and South-East Asia Regions. The political instability in the Bolivarian Republic of Venezuela led to an increase in malaria cases from 35,500 cases in 2000 to more than 467,000 cases in 2019. Unfortunately, many of the cases were from the ecological Amazonian region which extends into other countries of South America. Because the mosquito is not bound by geopolitical borders, infectious mosquitoes are introducing *P. vivax* malaria back into areas where significant progress has been made towards elimination (117; 155) .

Vector

Many factors contribute to the resurgence in malaria in many parts of the world. These include, but are not limited to, the effects from human behaviors such as migration, urbanization, deforestation, and irrigation that alter the distribution and abundance of the vector mosquitoes worldwide (198). The *Anopheles* genus of mosquitoes has been incriminated as containing all the malaria vectors of importance

in transmission to humans. *Anopheles* mosquitoes have a vast geographical distribution where different species dominate and interact with *Plasmodium* parasites, often with overlapping ranges and specialization into particular ecotypes.

In Sub-Saharan Africa (SSA), there are numerous *Anopheles* species acting as competent vectors for *P. falciparum* infections. The exact species is dependent upon climate, environment, and breeding preferences. The *Anopheles gambiae* complex are the most common vectors followed closely by vectors in the *An. funestus* and *An. arabiensis* groups (79). Even within the same species complex, each *Anopheles* species or subspecies can exhibit unique behaviors and/or breeding preferences, making vector surveillance and control challenging. For example, *An. arabiensis* is predominantly exophagic (feeds outdoor) and exophilic (rests outdoors) and bites during the evening hours, when humans at all socioeconomic levels are often meeting outdoors. *An. gambiae* and *An. funestus* have a higher affinity towards feeding and resting indoors (endophagic and endophilic) and have late-night biting patterns (58). These behaviors, coupled with the preference for human hosts (anthropophilic), contribute to the vector efficiency in SSA and expose vulnerable populations who socialize and sleep when the vectors are most active.

In areas of higher *P. vivax* transmission (i.e., Amazonian region of South America), primary vectors like *An. darlingi* are highly exophagic and exophilic and bite during the early night hours (189). *An. darlingi* is a riverine mosquito species that inhabits areas in and near forested areas with natural bodies of water as compared with some of the SSA vectors who tolerate urban and periurban locations with temporary, sometimes polluted, water sources (79; 220). However, the map of vector species remains highly complex and overlaps in most areas of active transmission. Because humans are the only known reservoirs of human malaria

species (except for *P. knowlesi*, with limited geographical distribution), understanding and controlling transmissible infections in humans is a primary public health goal.

Clinical Presentation/Disease

Clinical presentation of uncomplicated malaria in humans is historically characterized by acute febrile attacks, or malaria paroxysms, where an individual may alter between periods of classic malaria symptoms and no symptoms at all. The classic malaria paroxysm often follows a progression of worsening symptoms starting with non-specific prodromal symptoms such as myalgias, headaches, nausea, and fatigue followed by rising fever, rigors, and cold skin. This cold stage switches to a hot stage of high fever, vomiting, thirst, and rapid pulse. As the fever gradually subsides, individuals enter a stage of profuse sweating and exhaustion (245). In all *Plasmodium* malarias, pathogenesis is linked to the stages in the parasite lifecycle. Uncomplicated malaria is generally seen in older children and young adults who are progressing towards partial immunity in endemic settings. With increasing control and earlier diagnostic efforts, the classical paroxysmal presentation is less common; most modern diagnoses are made on patients with a current febrile disease.

In *P. falciparum* infections, symptoms may arise very quickly and may present with a more continuous fever (malignant tertian) rather than following a cyclical pattern. *P. vivax* (benign tertian) infections often worsen more gradually and thus may follow a more cyclical pattern. Severe *P. vivax* malaria accounts for less than 10% of all severe malaria disease and can present without high parasitemias. In young children, malaria-naïve individuals, pregnant women, and immunocompromised individuals, severe malaria caused by *P. falciparum* may

develop with signs of organ dysfunction and high parasitemias. Severe malaria in either species is often associated with high fever, anemia, hypoglycemia, acidosis, renal impairment, pulmonary dysfunction, and, for *P. falciparum*, highly morbid cerebral malaria (245).

While severe malaria is of the highest medical concern, the high prevalence of asymptomatic malaria in holoendemic regions should be of great public health concern in regard to transmission reduction. Asymptomatic malarias are most associated with individuals who experience an increase in protective immunity that can occur with repeat exposures and malaria infections in any species of malaria.

Organism Biology

All *Plasmodium spp.* are ameboid, contain pigmentation at some point in the life cycle, and have an asexual cycle in intermediate hosts (i.e., humans, non-human primates, rodents, birds, and reptiles) with a sexual cycle in definitive hosts (*Anopheles* mosquitoes in human malaria, and several genera of mosquitoes in malaria of other vertebrate hosts) (Figure 1). Forms of the parasite infectious to the intermediate hosts, called sporozoites, are injected by a mosquito into the subcutaneous tissues during blood feeding.

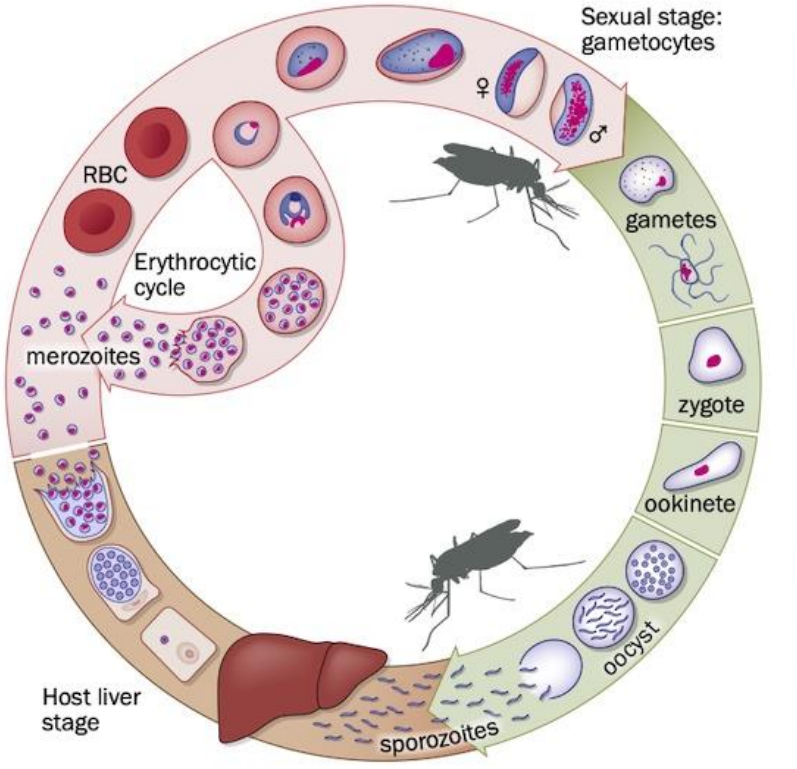

Figure 1. *Plasmodium falciparum* Life Cycle. Adapted from (203).

Most of what we know (and is described below) about the life cycle of *Plasmodium* species comes from extensive studies of *P. falciparum* and rodent-specific malaria species. Many rodent-specific malaria species, such as *P. berghei, P. yoelii, P. chabaudi*, and *P. vinckei* are closely related to *P. falciparum* and thus have been adapted to mimic *P. falciparum* human infections and provide a valuable *in vivo* model for *P. falciparum* research and development [reviewed in (72; 255)]. Additionally, in 1976, a successful continuous *in vitro* culture of *P. falciparum* parasites was published and remains the foundation for many protocols and research today (250). Unfortunately, due to many limitations in sustaining *in vivo* and *in vitro* models, there are still wide gaps in our knowledge of non-falciparum malarias, including the burden of disease and the biological parasite dynamics such as complete life-cycle specifications (158).

In humans, if the sporozoites survive initial immune defenses, and are motile enough, they migrate through the vascular system to the liver where they enter the hepatic cell. The nucleus undergoes repeated simultaneous division within the hepatocytes (schizogony) over the course of about a week until the schizonts rupture and uninucleate merozoites are released into the blood stream. These are infectious in turn for red blood cells (RBC). The approximately 48-hour merogony cycle (invasion, replication, and subsequent rupture) continues in RBCs as part of the erythrocytic cycle. A small portion of blood stage parasites develop into sexual stages that reach dermal capillaries or venules and can be taken up by another mosquito for sexual replication (sporogonic cycle) (245).

In *P. falciparum* only, infected red cells sequester in the postcapillary beds of various organs due to cytoadherence between endothelial cells and parasite proteins that are expressed on the RBC surface. During sequestration, asexual and sexual parasites progress towards maturity until they reenter the peripheral blood stream. Sequestration is responsible for both reduction in oxygenation and the induction of cytokine cascades that can contribute to the organ specificity of *P. falciparum* pathology (manifesting as cerebral, gastrointestinal, placental, etc. malaria). Asexual schizonts rupture and release merozoites at the end of the 48-hour erythrocytic cycle. However, the development of the sexual stages take approximately 10 to 14 days from commitment of each merozoite to infectious sexual parasites reentering the blood stream as stage V gametocytes after undergoing five distinct stages of development (260). Because partial sequestration occurs even in the gametogenic cycle, only the ring stage and stage IV or V gametocytes typically circulate in the periphery and are detectable by microscopy. Stage V gametocytes have distinguishable physical characteristics (crescent shape) that separate them from

sexual and asexual stages of all other *Plasmodium* species. Additionally, *P. falciparum* merozoites have a slight preference for younger red blood cells but are known to parasitize any aged RBC, thus allowing them to attain the high parasitemias often associated with these infections (245).

P. vivax parasites exhibit both similarities and differences in their asexual and sexual stage of malaria infections. *P. vivax* (and *P. ovale*) can form quiescent infections, called hypnozoites, after hepatocellular invasion. Essentially, the parasite begins to grow and develop slightly, but then halts its development prior to DNA replication for a variable period of time, apparently determined genetically by the parasite itself. Hypnozoites are associated with latency of infection and relapse up to at least five years (245); in malaria only, the term relapse is reserved for reactivation of disease from hypnozoites whereas reappearance of parasites from insufficient treatment is called recrudescence. Both *P. vivax* and *P. ovale* merozoites selectively invade reticulocytes, often resulting in more moderate peak parasitemias. Merozoites must bind with the Duffy antigen (Fy) receptor on the erythrocyte surface, resulting in certain Duffy-negative populations being resistant to *P. vivax* infections (157). As the infected red cells do not sequester like *P. falciparum*, all stages of the erythrocytic cycle of *P. vivax* parasites can be observed in the periphery and have distinguishable physical characteristics. Gametocytes more closely resemble their asexual counterparts: they are large, round, stippled, and nearly fill the entire host red blood cell (61). As compared to the late emergence of mature gametocytes in *P. falciparum* infections, *P. vivax* gametocytes appear to only take two to three days to mature after initial exoerythrocytic and erythrocytic schizont rupture (48) (see Chapter 4).

Gametocytes and Malaria Parasite Transmission[1]

Global efforts to control and eliminate malaria comprise multiple approaches, one of them being transmission reduction where gametocytes take center stage. As previously described, transmission occurs when the female *Anopheles* mosquito vector ingests both male and female gametocytes during a blood meal from an infected host. While the physical act of transmission seems simple, the molecular dynamics surrounding successful transmission are complex. Specific developmental and metabolic events must occur within the intermediate host before mosquito infection can occur. Similarly, intricate physiological events within the mosquito vector are necessary for onward transmission to a new host.

During the erythrocytic cycle, asexual replication occurs, and sexual differentiation is initiated, where less than 10% of parasites commit to forming the transmissible gamete precursors (gametocytes) (60; 82; 94). Numerous studies have expanded upon foundational work by Bruce et al. (52) to explain the mechanisms of sexual commitment and gametocytogenesis. Josling et al. (119) reviews the significant progress that has been made to identify molecular mechanisms surrounding parasite sexual differentiation. Foundational and novel studies have identified various factors that correlate with an increase in gametocytogenesis. These include, but are not limited to, host immunity (55; 96; 156; 222), regulation of the key parasite genes GDV1 and AP2-G (50; 66; 71; 83; 86; 118; 120), host lipid and biomolecule biosynthesis and metabolism (51; 99; 241; 246), fluctuations in host hormone production (142; 249), and chemotherapy (100; 114; 193; 204; 215; 247). *P. vivax*, as compared to *P. falciparum*, has a shorter gametocytogenesis which leads

[1] Excerpts taken from the review article: Roberds A, Ferraro E, Luckhart S, Stewart VA. 2021. HIV-1 Impact on Malaria Transmission: A Complex and Relevant Global Health Concern. *Front Cell Infect Microbiol* 11:656938

to the hypothesis that *P. vivax* may be able to successfully transmit the disease to mosquitoes before any presentation of clinical disease (157).

Sex-Ratio

Once sexual commitment occurs, each developing schizont produces either all female or all male gametocytes (218; 226). The quantitative balance between male and female gametocytes is hypothesized to contribute to the success of malaria parasite transmission (153). In 1979, Carter and Miller (60) described the gametocyte sex ratio for *P. falciparum* to be one male to every four females as determined by physiological differences observed microscopically. The use of molecular assays has confirmed that the ratio varies between one male to every 3-5 females in natural *P. falciparum* infections (42; 74). The female-dominant ratio is often described as a success mechanism for gamete fertilization post-ingestion by the mosquito because a single male gametocyte exflagellates in the mosquito intestine to produce eight gametes capable of independent fertilization events (89; 184). Male and female gametocytes are enriched in specific transcripts and proteins critical to their independent roles such as fertilization, DNA replication, and energy metabolism. Many of these proteins were identified by Lasonder et al. (138) and are the target of recent *P. falciparum* molecular studies focused on the relationship between sex ratio and transmission.

As reviewed by Henry et al. (106) several host and parasite factors can alter gametocyte sex ratio and ultimately impact transmission potential. Host factors include anemia and erythropoiesis (42; 185; 200; 227; 228), changing immunity (197; 199; 201; 223) and alterations in lipid profiles (51; 179), as well as antimalarial drug usage. Parasite factors include parasite density and gametocyte density (153;

205; 214; 240), as well as parasite diversity and competition (16; 38; 199; 228; 243; 258). Few studies have examined what causes these changes or how sex ratios differ in *P. vivax* infections.

Sexual Development in the Mosquito

P. falciparum gametocyte development takes approximately 10 days in the human host, at which point stage V gametocytes enter the peripheral circulation (105). The transmissible gametocytes remain in circulation for at least two to four days awaiting a mosquito blood meal (225). When the mosquito ingests at least one of each male and female gametocyte, sexual gametes emerge from the remnants of the RBC and begin fusion and ookinete formation in response to unique environmental stimuli in the mosquito midgut (reviewed in (29)). The male gametocyte produces eight flagellar microgametes, a process called exflagellation that was first observed by Laveran in 1880 and later described in more detail by Ross (1887) and Grassi (1900) (219). After exflagellation, the macrogamete (female) and microgamete (male) fuse in a process called syngamy to form a diploid zygote. Within 24 hours, the zygote transforms into a motile ookinete capable of traversing the mosquito peritrophic matrix and midgut epithelium before settling under the basal lamina to form an oocyst (29; 219). The oocyst remains at the interface of the epithelium and hemocoel for approximately 1 to 3 weeks (depending on environmental factors, such as temperature) while developing the infectious sporozoites. The oocyst wall deteriorates to release between 1,000 to 10,000 elongated sporozoites that migrate to the salivary glands awaiting onward transmission (22). The presence of one or more oocysts in the midgut is sufficient for transmission success.

Multiple studies have shown positive associations between expression of gametocyte-specific mRNA transcripts in host blood and oocyst counts in the mosquito [reviewed in (16; 23)]; revealing that even submicroscopic gametocyte densities are infectious and transmission to mosquitoes is likely [reviewed in (16; 42; 49)]. Many sexual stage-specific proteins are highly expressed during sporogony, the complex life cycle and developmental stages within the mosquito vector and have become prime candidates for transmission-blocking drugs and vaccines [reviewed in (22; 49; 136)].

Controlling Gametocyte Burden and Infectivity

Many mature gametocytes that reach peripheral circulation are not taken up by a mosquito and die in the human host thus stimulating the human host to develop antibodies derived from the release of parasite-produced, intraerythrocytic proteins (126). Some of these proteins/antigens are exposed on the surface of gametes after emerging from the RBC in the mosquito (45). When a host elicits a humoral immune response against these specific antigens, the antibodies produced are taken up during a mosquito blood meal, and these have been shown to inhibit fertilization and/or parasite development (anti-gamete immunity) with a concomitant decrease in mosquito infection (45; 126; 236). Evidence from multiple studies suggests that cellular immunity with cytokine production can also inactivate gametocytes and reduce infectivity (45; 92; 103; 165).

In addition to naturally acquired immunity, numerous antimalarial drugs and transmission blocking vaccine candidates have been developed to help control malaria. The advent of antimalarial drugs dates back to the 1600s when a Spanish countess was treated for intermittent fevers in Peru using bark from a tree (cinchona). In 1820, two French chemists isolated the compound known as quinine from the tree

bark and paved the way for 200 years of new drug discovery (115). Antimalarial drugs are often categorized based on parasite stage-specific effects with the primary focus often on stages found in the erythrocytic asexual cycle (blood schizontocides); very few drugs are specifically gametocytocidal or sporontocidal. Because the impacts on gametocytes and infectivity to mosquitoes are not often considered when antimalarials are approved for use in treatment or even prevention (2), the effect of most antimalarial drugs on transmission potential is incompletely understood. The 8-aminoquinolines including primaquine (PQ) have been reported to show the strongest activity against mature gametocytes and, therefore, have been labeled as gametocytocidal (56; 95). In 2012, the use of PQ as a gametocytocide was recommended by the World Health Organization (WHO) but not without caution that the drug has hemolytic toxicity in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency; such consequences have to be considered in malaria endemic areas where G6PD deficiency prevalence can be as high as 32.5% (1; 113).

Other antimalarials, such as artemisinin derivatives, indirectly reduce gametocyte burden but often only as a byproduct to the rapid killing of asexual parasites which disrupts gametocytogenesis [reviewed in (2; 42)]. In fact, some antimalarials such as sulfadoxine-pyrimethamine (SP) and chloroquine (CQ) can increase proportional gametocytemia in adults and children, although the subsequent infectivity to mosquitoes surrounding these observations is inconclusive (21; 55; 111; 132; 204; 215). Although the efficacy of both CQ and SP has been threatened by the development of parasite resistance (8; 97), the antifolate SP is still prescribed for Intermittent Preventive Therapy in Pregnancy (IPTp) (191) and as prophylaxis for HIV-1-associated opportunistic infections in malaria endemic areas. Antifolate

drugs have broad indications in both malaria and HIV/AIDS but due to high levels of resistance their ability to prevent malaria is reduced.

Extensive studies have shown that the many antimalarials recommended globally have little to no effect on gametocytes and may allow gametocytes to persist for more than one month after successful clearance of asexual parasites (40; 44). Many of the positive or negative effects that antimalarials have on blocking transmission are influenced by the high and continually emerging prevalence of drug resistant parasites globally [reviewed in (267)]. Furthermore, coinfections with other pathogens, such as HIV-1, are often associated with alterations to antimalarial drug efficacy [reviewed in (87)] and may contribute to enhanced malaria parasite transmission.

The biological complexity of parasites and their requirement to undergo multiple stages of development in a human host have made human malaria vaccine development a difficult task. Despite the challenges, significant progress has been made towards generating transmission blocking vaccine candidates that target gamete antigens as well as pre- and post-fertilization gametocyte antigens and antigens expressed by the mosquito [reviewed in (110)].

Physiological Associations with Transmission

The presence of gametocytes in a human host has not been associated with any distinct clinical findings. Gametocytes can be detected in acutely symptomatic malaria patients as well as asymptomatic carriers, especially in endemic areas where multiple infections are common and clinical immunity develops during childhood. The lack of clinical symptoms makes it challenging to identify patients capable of transmitting parasites without appropriate laboratory evaluation. However, there are several clinical parameters that could predict the likelihood of increasing

gametocytemia including anemia, immune regulation, and alterations in lipid profiles [reviewed in (42)]. A better understanding of these clinical conditions and their likely impacts on gametocytemia could aid in earlier identification of these patients and enhanced interventions to prevent transmission.

Asymptomatic infections pose a unique threat to malaria control as they represent a large reservoir of hosts capable of unknowingly transmitting parasites. Recent evidence (based on hypotheses from the early 1900s) indicates that many asymptomatic individuals harbor gametocytes at such low densities that they are not often detected by the microscopy [reviewed in (16; 166)]. Associations between asymptomatic infections with low parasite/gametocyte density and mosquito infectivity vary significantly in relation to parasite genetic diversity (complexity of infection), drug resistance, and host immunity/chronicity of infection (42; 254; 266). However, multiple studies have shown that individuals with submicroscopic gametocytemia and/or asymptomatic individuals infect mosquitoes just as frequently and/or more efficiently than those with higher, microscopically detectable gametocyte densities (64; 214; 239; 266). Those with asymptomatic infections have also been found to be more infectious to mosquitoes than clinically ill carriers (93) and are just as likely to carry gametocytes (65). These observations are highly concerning in the context of transmission reduction, especially considering the high prevalence of asymptomatics in varying endemnicities (7; 43; 212) and areas with seasonal transmission. Asymptomatic gametocyte carriers also are a likely source of seasonal epidemics as they harbor gametocytes throughout transmission-free seasons until the vector returns (14; 166).

Impact of Coinfections on Transmission

In many of the regions where malaria is endemic, coinfections with other neglected tropical diseases (NTDs) and non-NTDs such as HIV-1 and tuberculosis are common. Due to the intense pressure on the host immune system to control malaria parasite infection, any immune disruption from a competing infection could adversely affect clinical outcomes and gametocyte production/clearance. Amongst NTDs, polyparasitism can provide a minor protective role (162) but more often increases severe complications as well as prevalence, density, and infectivity of asexual parasites and gametocytes (11; 154; 161; 163; 167). Additionally, while many studies seek to explain how malaria impacts HIV-1 and HIV-1 transmission [reviewed in (87)], fewer studies have been designed to evaluate the converse.

Malaria Gametocyte Detection

Gametocyte cell biology and metabolism are significantly adaptable to multiple host environments through complex control of gene expression and protein synthesis (219). Many authors have sought to understand these specialized processes. The earliest method was based on microscopic enumeration of mature gametocytes on a thick or thin blood smear as described and adapted by Annecke in 1927 (9); this method, however, suffers from the same inaccuracies as malaria microscopy in general (32; 265), particularly for thick films (26; 80). In 1982, Ponnudurai et al. (188) described a protocol for successful continuous *in vitro P. falciparum* gametocyte culture, which has been adapted and used by many researchers to increase our understanding of gametocyte biology and kinetics. *P. vivax* infections are much more difficult to culture due to their requirement for reticulocytes for blood stage invasion and have not proven amenable for gametocyte studies (91) [reviewed in (30)].

The first molecular assay for indirect detection of gametocytes was described by Baker et al. (18) in which sexual stage-specific RNA expression was detected using *in situ* hybridization. Four years later, Babiker et al. (15) described the first use of reverse transcriptase polymerase chain reaction (rt-PCR) to detect *P. falciparum* gametocytes through the amplification of mRNA from different sexual stage-specific proteins. In a large-scale proteomic study, 315 of 1,289 malaria proteins identified were *P. falciparum* gametocyte-specific (138) and many of their genetic transcripts have been used in real-time quantitative gametocyte studies with technologies such as quantitative PCR (qPCR) and quantitative nucleic acid sequence-based amplification (QT-NASBA) [reviewed in (16; 42)]. Recently, transcriptomic studies have characterized transcriptomes of *P. vivax* gametocytes as well, opening the door for expanded *P. vivax* molecular research (37; 128; 207). New molecular technologies, such as digital droplet PCR (ddPCR) became commercially available within the past decade and offer advancements that can further lower the limit of gametocyte detection (244). ddPCR involves the partition of samples into thousands of distinct droplets, each being subject individually to end-point PCR and individual fluorescence measurements. The resulting measurement is an absolute quantitation of DNA. In a study looking at malaria *18S* rRNA in asymptomatic, low-density infections, ddPCR detected significantly more *P. falciparum* positive samples than qPCR and there was an observed reduction in differences between technical replicates (131). ddPCR is unique in that it does not require a standard curve, potentially enhancing accuracy and reproducibility of the assays.

Successful transmission occurs only after complete gametocyte maturation in the host followed by parasite fertilization and development in the mosquito vector. The results from Muirhead-Thompson (159) cautioned investigators that concluding

infectivity/transmissibility solely by gametocyte count may be misleading and encouraged combining gametocyte data with oocyst or sporozoite enumeration data from mosquitoes fed on infected blood.

RESEARCH GOALS, OBJECTIVES, RATIONALE

Research Goal

The overall goal of the research in this dissertation is to contribute to the overall understanding of malaria transmission by assessing the relationship between gametocyte sex ratio/density and mosquito infectivity relative to various host dynamics.

Research Objectives

The objectives of this dissertation are to 1) develop highly sensitive molecular assays that will detect gametocytes in low density infections and 2) assess the utility of the assays in unique human and primate populations.

Rationale

Enhanced Sensitivity of Molecular Methods to Quantify P. falciparum Male and Female Gametocyte Transcripts

When reviewing the most current literature, *P. falciparum* gametocyte markers have been described but have not been widely applied in field investigations of gametocytes in asymptomatic malaria and HIV positive subjects. High sensitivity is critical when studying asymptomatic patients with submicroscopic gametocyte detection levels; thus explaining our choice to apply the higher sensitivity and accuracy of ddPCR technology in many of our assays. The increased sensitivity is necessary when attempting to quantify male gametocyte gene expression that is shown to be expressed at lower transcript levels than females (264) and in an

asymptomatic population with low density gametocyte prevalence and documented female bias sex ratios by microscopy (238). Understanding the balance of male and female gametocytes in low-density infections and their relationship with malaria parasite transmission would be a useful contribution to designing elimination strategies focused on transmission reduction.

Application in Understanding HIV-1 and Malaria Co-infection

Evidence from Stiffler et. al. (231) suggests that positive HIV-1 co-infection status correlates with increased gametocytemia, specifically in individuals who are not receiving treatment for HIV-1. Additionally, studies have shown that gametocytemia is induced almost immediately after initiation of certain Antiretroviral Therapy (ART) to include the use of trimethoprim/sulfamethoxazole (TS) for prophylaxis against opportunistic infections such as bacterial infections and malaria (5; 39). However, little is known about the impact that prolonged use of ART/TS has on gametocyte carriage and further how HIV-1 status and ART/TS impacts gametocyte density, sex-ratio, and transmission to mosquitoes. Previous results from a short-term study showed an increase in gametocytemia immediately after antifolate treatment, however, the study was limited to only one month of follow-up (40). We were curious to study the dynamics surrounding this observed rise in gametocytemia due to the growing prevalence of antifolate drug resistance (20; 168). Additionally, studies have hypothesized that when parasites sense a threat to their survivability, they increase gametocyte production to increase transmission potential to the mosquito. Therefore, our hypothesis was that **the stage of HIV-1 infection and the use of HIV ART and TS affects overall gametocytemia and gametocyte sex-ratio. Upon initiation of ART and TS at first HIV-1 diagnosis, gametocyte density changes in favor of male gametocytes and contributes to**

increased gametocyte production. Ultimately, by observing gametocyte expression in asymptomatic HIV-1 malaria co-infected individuals coupled with infectivity rates from mosquito dissections we could enhance our overall understanding of how HIV-1 malaria co-infection affects malaria parasite transmission.

Use of Orthologous Genes to Quantify P. vivax Gametocyte Transcript Expression

Due to advanced genomic technologies, *P. vivax* and *P. falciparum* genomes have been described and highlight important *Plasmodium* genetic similarities and differences (57). Many genetically similar genes, orthologs, have been described for the primary gametocyte factors that we are interested in. Gametocyte densities and sex-ratios are important factors in the successful transmission of infection to mosquitoes and were the primary focus of this study. *P. vivax* gametocyte sex-ratios have rarely been described. Therefore, our hypothesis was that **orthologous gametocyte transcripts from *P. falciparum* could be used to develop a panel of assays that quantify *P. vivax* gametocyte expression and could be evaluated in the application of an induced malaria infection in *Aotus* monkeys and in natural human infections from volunteer samples in Peru.** We used multiple molecular markers from known *P. falciparum* orthologs to describe overall gametocytemia, gametocyte sex ratio, and indicators of gametocytogenesis at various time points during sporozoite induced *Aotus* monkey – *P. vivax* infections. Characterization could be confirmed and further evaluated in human samples from symptomatic malaria patients in Peru, contributing to our understanding of *P. vivax* gametocyte biology and kinetics.

AIM 1

Develop, apply, and evaluate a *Plasmodium falciparum* gametocyte molecular panel for sensitive detection in a longitudinal prevalence study of asymptomatic malaria patients with varying HIV-1 status.

AIM 2

Determine and evaluate molecular markers for sensitive detection of *Plasmodium vivax* gametocytes in monkey and human infections.

CHAPTER 2: HIV-1 Impact on Malaria Transmission: A Complex and Relevant Global Health Concern

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ABSTRACT

Malaria/HIV-1 co-infection has become a significant public health problem in the tropics where there is geographical overlap of the two diseases. It is well described that coinfection impacts clinical progression of both diseases; however, less is known about the impact of co-infection on disease transmission. Malaria transmission is dependent upon multiple critical factors, one of which is the presence and viability of the sexual-stage gametocyte. In this review, we summarize evidence surrounding gametocyte production in *Plasmodium falciparum* and the developmental factors and the consequential impact that HIV-1 has on malaria parasite transmission. Epidemiological and clinical evidence surrounding anemia, immune dysregulation, and chemotherapy as it pertains to co-infection and gametocyte transmission are reviewed. We discuss significant gaps in understanding that are often due to the biological complexities of both diseases as well as the lack of entomological data necessary to define transmission success. In particular, we highlight special epidemiological populations, such as co-infected asymptomatic gametocyte carriers, and the unique role these populations have in a future focused on malaria elimination and eradication.

INTRODUCTION

Over the past several decades, malaria/HIV-1 co-infection has become a significant global public health problem in co-endemic areas of the world. The geographical overlap between both diseases (Figures 2 and 3) combined with shared social determinants of health may explain the prevalence ofco-infection, especially in sub-Saharan Africa (SSA). The distribution of diseases throughout SSAvaries by country and localities, and can be explained by differing geographical, environmental,

andpopulation behaviors. However, some severely affected countries in SSA have an HIV-1 prevalencein adults above 10%, with more than 90% of the population exposed to malaria (174). Numerous models have tried to predict the impact that malaria/HIV-1 co-infection has on incidence and mortality of each disease (133) but the biologicaldynamics within and between the two diseases are highly complex.

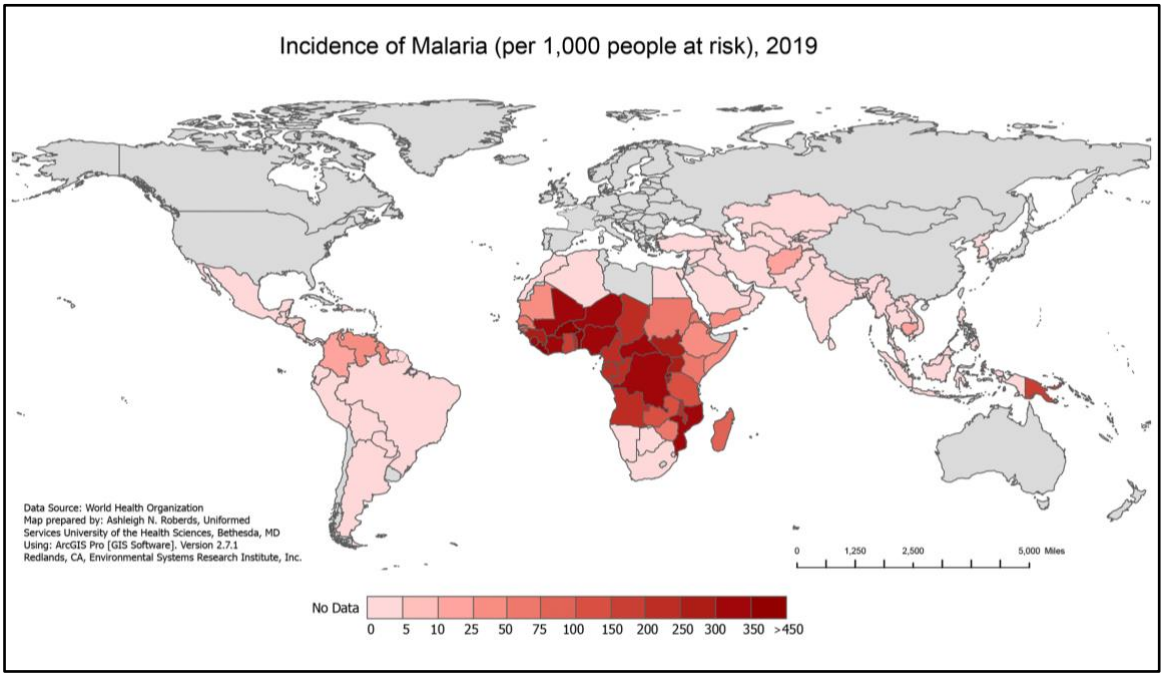


Figure 2. Incidence of Malaria, 2019. The incidence of all malaria cases per 1,000 people at risk in 2019, extracted from the World Health Organization (WHO) 2020 World Malaria Report.

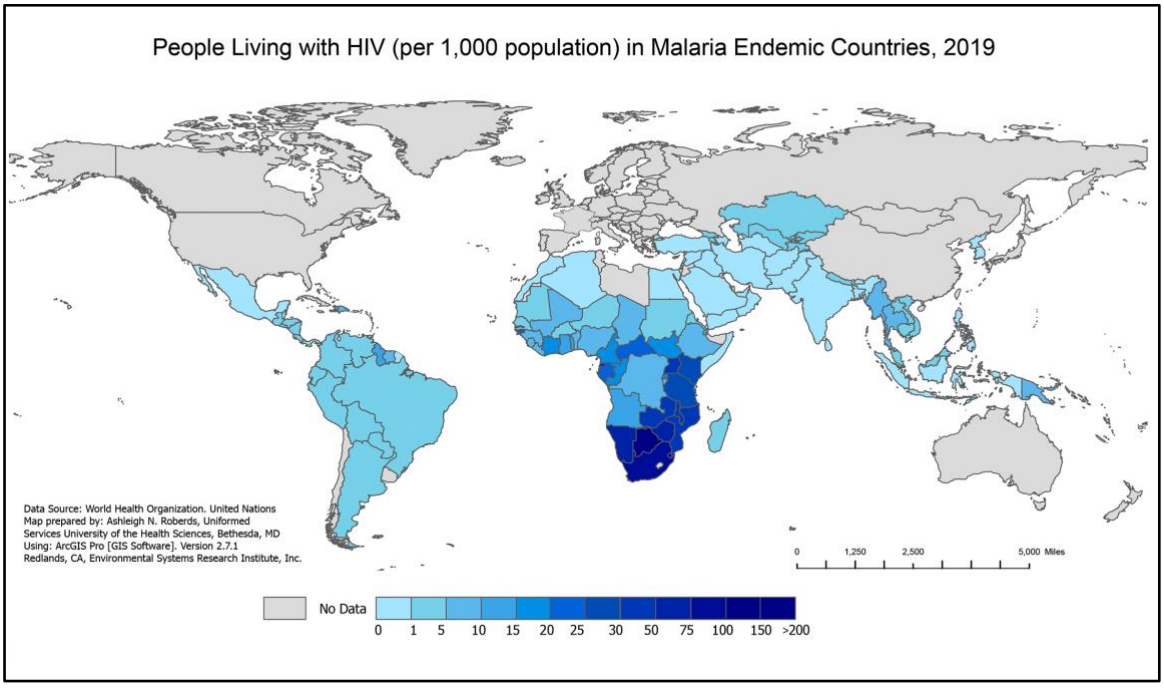


Figure 3. People Living with HIV in Malaria Endemic Countries, 2019.
The number of people living with HIV (per 1,000 population) in 2019 in malaria endemic countries from the WHO 2020 World Malaria Report. The 2019 HIV data are extracted from the Joint United Nations Programme on HIV/AIDS (UNAIDS) AIDS info data sheet. The 2019 country populations are extracted from the United Nations 2019 World Population Prospects.

Data across epidemiologic populations of interest have consistently shown HIV-1-mediated immune deficiency is associated with higher prevalence of clinical malaria and increased parasite density (87; 107). A smaller number of studies have shown an impact of malaria co-infection enabling the progression of HIV-1, as measured by increased viral load in co-infected patients compared to those who were not co-infected (129; 134). Critical factors associated with parasite transmission such as host anemia and gametocyte density are likely altered when the host is infected with HIV-1 as well. This review focuses on parasite and host factors that influence malaria parasite transmission and highlights the efforts being made to elucidate the impact that HIV-1 may have on malaria transmission potential. Consequently, this review acknowledges a substantial gap in research and understanding as the world continues to combat these two major global health priorities.

Background

Overview of Malaria Parasite Transmission

Significant advances have been made in reducing the burden of malaria through classic vector control, case detection and treatment strategies; however, in an era focused on malaria eradication, more attention is being diverted to reduction and prevention of transmission. For all *Plasmodium* species, transmission occurs when the female *Anopheles* mosquito vector ingests both male and female gametocytes during a blood meal from an infected host. While the physical act of transmission seems simple, the molecular dynamics surrounding successful transmission are complex. Specific developmental and metabolic events must occur within the mammalian host before mosquito infection can occur. Similarly, intricate physiological events within the mosquito vector are necessary for onward transmission to a new mammalian host.

Within the mammalian host, the parasite undergoes gametocytogenesis in which the parasite differentiates between asexual (associated with symptoms) and sexual (associated with transmission) replication. Although numerous studies have expanded upon foundational work by Bruce et al. (52) to explain the mechanisms of sexual commitment and gametocytogenesis, the entirety of the process is not completelyunderstood. Josling et al. (119) provides a recent review of the significant progress in identification of molecular mechanisms surrounding parasite sexual differentiation. Foundational andnovel studies have identified various factors that correlate with an increase in gametocytogenesis. These include, but are not limited to, host immunity (55; 96; 156; 222), regulation of the key parasite genes GDV1 and AP2-G (50; 66; 71; 83; 86; 118; 120), host lipid and biomolecule biosynthesis and metabolism (51; 99; 241; 246), fluctuations in host hormone production (142; 249),

and chemotherapy(100; 114; 193; 204; 215; 247). Each of these triggers may be altered in the context of a co-morbidity such as HIV-1.

Once sexual commitment occurs, individual developing schizonts produce either all female or all male gametocytes (218; 226). The quantitative balance between male and female gametocytes is hypothesized to contribute to the success of malaria parasite transmission (153). As reviewed by Henry et al. (106) several host and parasite factors can alter the gametocyte sex ratio and ultimately impact transmission potential. Host factors include anemia and erythropoiesis (45; 185; 200; 227; 228), changing immunity (197; 199; 201; 223) and alterations in lipid profiles, each of which are also factors associated with HIV-1 as discussed below. Parasite factors include parasite density and gametocyte density (153; 205; 214; 240), as well as parasite diversity and competition (16; 38; 199; 228; 243; 258). Very few studies have extensively and/or thoroughly included gametocyte and mosquito infectivity data. Successful transmission occurs only after complete gametocyte maturation in the host is followed by complete parasite fertilization and development in the mosquito vector. Results from Muirhead-Thompson (52) cautioned investigators that concluding infectivity/ transmissibility solely by gametocyte count may be misleading and encouraged combining gametocyte data with oocyst or sporozoite enumeration data from mosquito feeding. The presence of one or more oocysts in the midgut is sufficient for transmission success.

Other Factors That Influence Transmission

While numerous factors have the potential to influence malaria parasite transmission, immunogenetics and fluctuations in immune responses are widely studied topics. It is well described that in malaria-endemic regions, including SSA, residents are likely to have innate genetic adaptations and acquired resistance to

malaria (137). In 2010, Lawaly et al. reviewed the role that many of these human genetic factors play in transmission and highlighted genetic associations with asymptomatic gametocyte carriers (140).

A study in an endemic region in West Africa showed the ability to clear chloroquine-resistant parasites was most strongly associated with age, hematocrit, and ethnic background (76). Patients over four years of age had a dramatic increase in the ability to clear parasites, suggesting repeated exposure to malaria is a dominant factor in acquired immunity (76). Age as a surrogate for acquired protective immunity against asexual parasites densities is supported by numerous other studies (206). A cross-sectional study in Burkina Faso revealed that the prevalence of gametocyte-positive carriers decreased with age. However, this study also revealed that the median proportion of gametocytes relative to asexual parasites increased with age (180). These findings suggest that adults, especially those with acquired immunity and asymptomatic infections, may be important infectious reservoirs.

In many of the regions where malaria is endemic, co-infections with other neglected tropical diseases (NTDs) and non-NTDs such as HIV-1 and tuberculosis are common. Due to the intense pressure on the host immune system to control malaria parasite infection, any immune disruption from a competing infection could adversely affect clinical outcomes and gametocyte production/clearance. Amongst NTDs, polyparasitism with *Plasmodium spp*. provides a minor protective role (162) but more often increases severe complications as well as prevalence, density, and infectivity of asexual parasites and gametocytes (11; 154; 161; 163; 167). Additionally, while many studies seek to explain how malaria impacts HIV-1 disease progression and HIV-1 transmission (reviewed in (87)), fewer studies have been designed to evaluate the converse.

HIV Impact on Malaria Parasite Transmission

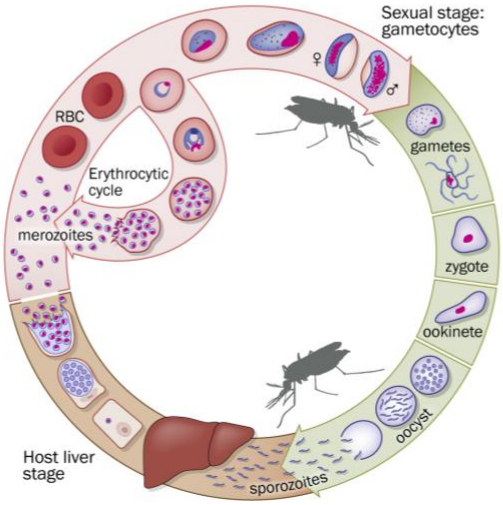
The impacts/effects of HIV-1 infection on malaria parasite transmission are often defined as direct or indirect, with the former being very difficult to identify due to the complexities of both diseases (Figure 4). An increase in parasitemia or parasite biomass is the most cited association with malaria/HIV-1 co- infection, but the direct relation to gametocytemia or transmission potential is often suggestive or speculative (3; 139; 173; 183; 256; 268). There have been various in vivo and epidemiological studies that seek to further explain the impact of HIV-1 on gametocytogenesis and gametocytemia, but most studies detail only indirect effects. Interesting data from a study of asymptomatic malaria among individuals who were either HIV-1 negative or positive suggests that HIV-1 positive individuals have a greater risk of carrying gametocytes than HIV- 1 negative individuals (232). This study is the first of its kind designed to evaluate the epidemiological impact of HIV-1 co-infection on the prevalence of asymptomatic gametocytemia in the field.

Figure 4. Malaria Life Cycle and Selected Factors Influencing Malaria Parasite Transmission and Suggested Impact During HIV-1 Malaria Co-infection. Example of selected factors that are known to be associated with malaria parasite transmission and the suggested impact (determined using Decision Matrix, Figure 5) with HIV-1 and/or malaria/HIV-1 co-infection. Suggestive impact is used due to the lack of available statistical evidence and the difficulty in ascertaining the biological relationship between malaria/HIV-1 co-infection and malaria parasite transmission success. Strong Direct Impact means that there is evidence, which includes mosquito transmission data, to suggest that HIV-1 malaria co-infection directly alters gametocyte conversion (GC), gametocyte development (GD), or mosquito infectivity (MI). Direct Impact means there is evidence, sans mosquito data, to suggest that HIV-1 malaria co-infection directly alters GC, GD, or MI. Indirect impact means there is evidence to suggest that HIV-1 or HIV-1 malaria co-infection indirectly alters factors that influence GC, GD, or MI. Possible Impact means there is evidence to suggest that HIV-1 or HIV-1 malaria co-infection may alter factors associated with GC, GD, or MI. Unknown Impact means there is currently not enough evidence to suggest that HIV-1 or HIV-1 malaria co-infection directly or indirectly alter GC, GD, MI, or the factors associated with GC, GD, or MI. The left table (gray, "Gametocyte Conversion") represents selected transmission factors that pertain to the gametocytogenesis and gametocyte commitment. The center

table (red, “Gametocyte Development”) represents selected transmission factors that pertain to the sexual stage development. The right table (green, “Mosquito Infectivity”) represents selected factors that may influence infectivity to mosquitoes and subsequent mosquito transmission success. In the middle is a representation of the life cycle of *Plasmodium falciparum* (adapted from NIAID/NIH, 2016, https://www.niaid.nih.gov/diseases-conditions/malaria-parasite) representing the exo-erythrocytic (liver) cycle, erythrocytic cycle (bloodstream), and sexual stage (gametocyte) development in the host, and the sporogonic cycle in the mosquito. [1](51); [2](241); [3](179); [4](81); [5](28); [6](47); [7] (268); [8](183); [9](3); [10](139); [11](173); [12](256); [13](231); [14](251); [15](130); [16](171); [17](145); [18](96); [19](166); [20](143) [21](45); [22](27); [23](83); [24](50); [25](118); [26](66); [27](120); [28](86); [29](108); [30](13); [31](55); [32](114); [33](116); [34](119); [35](209); [36](193); [37](215); [38](20); [39](46); [40](227); [41](195); [42](164); [43](242); [44](211); [45](169); [46](257); [47](236); [48](125); [49](214); [50](16); [51](77); [52](206); [53](239); [54](266); [55](254); [56](99); [57](36); [58](168); [59](248); [60](185); [61](204); [62](111); [63](21); [64](132); [65](121); [66](124); [67](150).

Selected Factors Influencing Malaria Parasite Transmission and Suggested Impact During HIV-1 Malaria Co-infection

Gametocyte Conversion	Suggested Impact*
Host Lipid Profiles	Possible[1-6]
Parasite Density	Direct[7-14]
Host Immunity	Possible[15-16]
Immune Dysregulation	Possible[7-8, 16-17]
Naturally Acquired Immunity	Possible[18-19]
Host Anemia	Strong Direct‡[14]/Direct[20-22]
Parasite Gene Regulation	Possible[23-28]
Use of ARTs	Strong Direct‡[29]/Indirect[30]
Antimalarial Use	Direct[30-33]
Gametocyte Sex-Ratio	Possible[25, 31, 34]



Life Cycle of the Malaria Parasite

Gametocyte Development	Suggested Impact*
Mature Gametocyte Presence	Strong Direct‡[14]/Direct[13]
Gametocyte Density	Strong Direct‡[14]/Direct[9, 13]
Parasite Species/Diversity	Unknown
Host Age/Genetics	Unknown
Use of ARTs	Strong Direct‡[29]/Indirect[35]
Parasitemia	Direct[7-13]
Antimalarial Use	Direct[29-30]/Indirect[36-39]
Host Anemia	Strong Direct‡[14]/Indirect[22, 40-46]
Host Immunity	Direct[15]/Indirect[21, 47]
Immune Dysregulation	Indirect[7, 15-17, 48]
Naturally Acquired Immunity	Possible[19, 49-55]
Host Lipid Profiles	Possible[1, 3-6, 56]
Recrudescence	Possible[57]
Parasite Drug Resistance	Direct[12, 58-59]

Mosquito Infectivity	Suggested Impact*
Gametocyte Sex Ratio	Possible[40, 60]
Complexity of Infection	Unknown
Parasite Drug Resistance	Direct[59, 61-64]
Chronicity of Infection	Direct[48, 54, 65]
Gametocytemia	Strong Direct[9, 13-14]
Use of Antifolates	Strong Direct‡[29]/Direct[30, 66-67]

*Suggested Impact determined from Decision Matrix (Supplementary Figure 1).
‡Evidence from studies using animal or *in vitro* models.

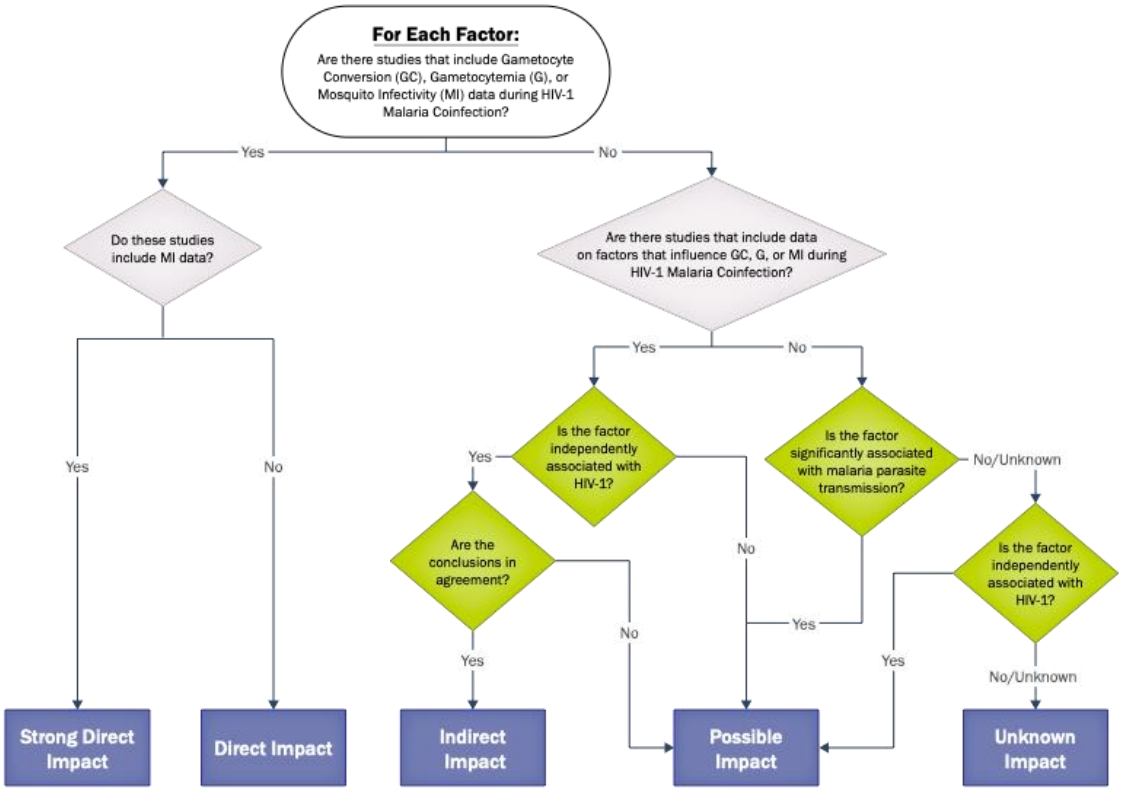


Figure 5. Decision Matrix for Determining Impact and Association with Malaria Parasite Transmission in Figure 4.

Although there have been advances in humanized mouse models and non-human primate models for HIV-1 and malaria co-infection studies (reviewed in (24; 104; 259)), there has been little research conducted in the laboratory to directly investigate co-infection and parasite transmission. In 2009, Koehler et al. described a non-human primate model for co-infection, but the study was not intended to provide any direct evidence related to malaria parasite transmission (130). In 2011, Trott et al. designed a study to evaluate effects of simian immunodeficiency virus (SIV) co-infection on malaria parasite transmission in a non-human primate model (251). The study modeled an underlying immunodeficiency virus infection prior to a subsequent malaria parasite infection by inoculating rhesus macaques with SIV followed by inoculation of *Plasmodium fragile*, approximately 28 days later. Results from the

study showed that co-infection was associated with a significantly increased risk of malaria parasite transmission and that SIV had a direct enhancing effect on parasitemia, gametocytemia and mosquito infectivity (251). The *P. fragile* model closely resembles human *P. falciparum* infections especially in the context of severe disease and parasite sequestration (70). Additional factors may have contributed to an indirect effect on increased transmission, such as immune regulation and reduced hematocrit levels. Additionally, it is difficult to extrapolate this acutely infected animal model with the more typical chronically infected situation in humans in malaria endemic areas.

Host Factors Affected by HIV That Could Perpetuate Malaria Parasite Transmission

To date, no clinical studies have looked directly at parasite transmission in HIV-1-infected patients. However, patients with progressive HIV-1 disease are known to have a number of disease-related sequelae, including immune deficiency, anemia and alterations of lipid profiles. Further, HIV-1-positive patients are commonly prescribed antiretroviral and antifolate medications. Numerous physiologic changes that occur during HIV-1 infection independently are known to affect malaria parasite transmission, some positively and some negatively. Here we present data from a variety of studies suggesting that HIV-1-infected patients are at increased risk of being reservoirs for malaria parasite transmission.

Immune Status

A review by Troye-Blomberg (252) highlighted the importance of both cell-mediated and humoral immunity to asexual blood stages of malaria. A later study by Goodier et al. found that gametocytes activate a CD4+ T-cell response (92). More recent reviews have confirmed these early findings, affirming that T-cells and

cytokines suppress gametocyte burden and decrease infectivity to mosquito vectors (126). When a host mounts a humoral immune response against gametocyte-specific antigens, the antibodies produced are taken up during a mosquito blood meal, and these have been shown to inhibit fertilization and/or parasite development (anti-gamete immunity) with a concomitant decrease in mosquito infection (42; 126; 235). These immunologic findings have been supported by field data on malaria parasite transmission. A study in Gambia found that the absence of high fever and of high parasite densities were independent risk factors for gametocytemia (261). The authors hypothesized that fever and high parasite densities in those with a history of infection represented acute infection with an active immune response. Lack of an acute infection could prevent gametocyte exposure to important damaging elements of the immune system, such as inflammatory cytokines (261). A more recent study in Ghana identified relatively lower prevalence of gametocytemia in patients with fever (75). *In vitro* findings demonstrated that increased levels of cytokines (including TNF-a and IFN-g) correlated with a lack of gametocyte infection of the mosquito vector (165). These data suggested that patients with clinical malaria may be less likely to develop gametocytemia, or to serve as good reservoirs for transmission.

Effects of immune deficiency were studied by Koehler et al. in a rhesus macaque model of SIV-*P. cynomolgi* co-infection (130). They found significant effects of co- infection on the immune response to malaria parasite infection, including a more rapid depletion of CD4+ T-cells, a failure to generate an appropriate CD4+ T-cell response to parasitemia, and decreased proliferative B-cell response (anti-parasite IgG) (130). This study did not directly examine effects on transmission to mosquitoes, but the data provided convincing evidence that co-

infection impacts the production of key immunological factors that control gametocytemia.

The inability of an HIV-infected host to mount a robust immune response against malaria infection is expected based on the depletion of CD4+ T cells during HIV-1 infection. Several clinical studies suggest a relationship between the severity of immune deficiency, specifically decreased CD4+ T-cell counts and repressed cytokine synthesis and worsening malarial disease. A long-term study in Uganda followed nearly 500 HIV-positive participants and controls over an eight-year period to determine the frequency of clinical malaria. The immune deficiencies of the HIV-1-positive patients were categorized according to the WHO hierarchical classification of CD4+ T-cell count. Results showed a positive association between increasing immunosuppression and increasing parasitemia. Further, the odds of having clinical malaria were six times higher in patients with CD4+ T-cell counts less than 200/ml as compared to those with counts higher than 500/ml (268). A second large cohort study explored the effects of HIV-1 co-infection on the outcomes of cerebral malaria in children, finding that children without HIV-1 had substantially increased levels of TNF-a and ICAM-1 during their clinical malaria episodes. Despite the varied inflammatory responses, time to parasite clearance was similar in HIV-1- infected and - uninfected groups (145). A third study by Laufer et al. investigated the impact of HIV-1-associated immunosuppression on malaria in patients in Malawi (139). The study showed that parasite density in symptomatic clinical infection was inversely related to CD4+ T-cell count, a relationship that was not seen in patients with asymptomatic infections. In contrast, a cross-sectional study in Nigeria observed that the prevalence of asymptomatic malaria parasitemia in children under five was significantly higher in HIV-1 co-infected children, was highest among HIV-

1 co-infected children who were severely immunosuppressed and was significantly associated with declining CD4+ T-cell counts (172). Each of these studies failed to measure gametocytemia specifically but given our current understanding of the importance of T-cells and cytokines in immunity to asexual parasites, it is possible that a similar association exists between decreasing CD4+ T-cell counts and blunted cytokine responses in HIV-infected patients and increasing gametocytemia. However, special consideration should be given to asymptomatic malaria carriers as they represent a unique and challenging demographic due to partial protective immunity acquired with age and exposure in endemic areas.

Hematocrit Levels

Malaria is known to cause anemia by multiple mechanisms, including destruction of infected erythrocytes, antibody- mediated bystander erythrocyte lysis, and impaired erythropoiesis (194). A large study by Nacher et al. examined host factors influencing *P. falciparum* gametocyte carriage and found that hemoglobin concentrations (as a measure for anemia) were negatively correlated with peak gametocyte counts and gametocyte carriage duration (164). This association between gametocytemia and anemia has been reported in other studies (75; 190; 230), suggesting that anemia, and thus perhaps tissue hypoxia, stimulates *P. falciparum* gametocytogenesis. A large Nigerian study of 1125 children attempted to discern the mechanism by which anemia influences gametocyte development and found the proportion of male to female gametocytes in anemic children was nearly two- fold higher than in non-anemic children. The authors suggested that anemia may prolong the half-life of male gametocytes and potentially their survival in circulation (227).

Possible mechanisms by which anemia triggers gametocyte development are well reviewed by Bousema and Drakeley (42). Anemia in a host is associated with

increases in both relative and absolute levels of the immature red blood cells called reticulocytes. Reticulocytes have high RNA content and increased hemoglobin synthesis and are stimulated by the glycoprotein cytokine erythropoietin (EPO). EPO has been implicated as a stimulant for gametocyte formation in in *P. chabaudi* and *P. vinckei* (200) and is thought to influence gametocyte sex allocation (185). These conditions in an anemic host are postulated to be ideal for gametocyte development (42), but cause-and-effect between reticulocytemia and gametocyte levels has not been elucidated.

Anemia is also a common clinical finding in HIV-1 infection, with prevalence up to 95% depending on patient factors such as stage of HIV-1 disease, sex, age, and pregnancy status (27). Because anemia is not unique to either disease, determining the effect of HIV-1-related anemia on malaria parasite transmission is challenging. Trott et al. examined the effects of immunodeficiency virus infection on malaria parasite transmission using SIV/*P. fragile* co-infection in rhesus macaques. These authors found an increase in the percentage of gametocytes during the initial drop in hematocrit in both parasite- infected and co-infected animals (251). The mechanism by which anemia in these animals induced gametocytemia is unclear, but these data suggested that immunodeficiency virus-induced anemia could be associated with higher risk of malaria parasite transmission.

Data from epidemiologic field studies examining this association are difficult to interpret, as most studies fail to associate the prevalence of anemia with increased risk of parasite transmission beyond measures of gametocytemia. Tay et al. studied 400 HIV-1 seropositive patients with and without malaria co-infection and found an overall anemia prevalence of 67%, with a prevalence of nearly 94% in HIV-malaria co-infected patients (242). A second study found median hemoglobin levels were

lower in HIV-1 positive patients with positive malaria blood smears than HIV-1 positive patients without malaria (211). In pregnant women in Nigeria, low hemoglobin levels were correlated with malaria/HIV-1 co-infection, but not with decreasing CD4+T-cell counts (170). Only one study in Zambia found HIV- 1-infection, not CD4+ T-cell count, to be an independent risk factor for a longer duration of anemia in co-infected patients (257). These studies clearly demonstrate that anemia is prevalent in co-infected patients but did not provide data to support that anemia in HIV-1-infected patients is a significant risk factor for increased malaria parasite transmission specifically. Additionally, it is difficult to discern if the primary etiology of anemia in these patients is HIV-1 infection or malaria, given that anemia is common in both diseases as well as other NTDs prevalent in these settings. More studies in co-infected patients are needed to determine if data from co-infected animal models are reproducible in humans.

Lipid Profiles

A recent in vitro study by Brancucci et al. (51) concluded that levels of host-derived lipids, specifically lysophosphatidylcholine (LysoPC), could act as an environmental stimulus for *P. falciparum* gametocyte differentiation. Specifically, *Plasmodium* parasites use LysoPC for phosphatidylcholine (PC) biosynthesis and the resultant depletion of LysoPC leads to dramatic induction of gametocytogenesis (51). This association between the host lipid profile and gametocytogenesis is clinically relevant given that systemic LysoPC levels are altered throughout the course of a malaria infection. The most common fluctuations are often associated with the host immune response to disease progression and parasitemia (81; 178). A study on lipid profiles in HIV-1 infection demonstrated increased levels of LysoPC in HIV-1-positive patients pre- and post-antiretroviral therapy (ART) compared to HIV-1-

negative patients (28). A recent study by Bowman et al. showed that the concentration and fatty acid composition of LysoPC differed between HIV-1-positive and HIV-1-negative patients. Although there were no significant differences between serum concentrations of total LysoPC, the species of LysoPCs showed differential enrichment. In particular, LysoPCs containing saturated fatty acids (SaFAs) were enriched, while LysoPCs containing polyunsaturated fatty acids (PUFAs) were reduced in HIV-1- positive patients. SaFA-enriched LysoPCs were also associated with immune activation in HIV-1-positive patients, consistent with elevated serum levels of interleukin-6 (IL-6) and markers of monocyte activation (47). Understanding the clinical relevance of these observations is in the early stages, but alterations in lipid profiles of HIV-1-infected patients could influence gametocyte production and transmission to mosquito vectors.

Impact of Therapeutic Agents

Antifolates

In populations most affected by malaria and HIV-1 co-infection, use of ART and antifolate prophylaxis are important to consider when discussing host factors that impact malaria parasite transmission. Excellent laboratory studies suggested direct effects of various antiretroviral agents, particularly cysteine protease inhibitors, on gametocytogenesis and malaria parasite transmission, which have been recently reviewed (12). Antifolate drugs, however, have been used both as primary antimalarial agents (e.g., Sulfadoxine-Pyrimethamine or SP) and as antibiotics adjuncts to ART (e.g., trimethoprim- sulfamethoxazole or TMP-SMX) for the prevention of opportunistic infections in HIV-positive patients.

Two early studies in the 1970's failed to show an effect of TMP-SMX on gametocytemia, though both studies were limited by a small sample of patients (101;

269). Research interest has grown dramatically over the last several decades and has revealed that SP monotherapy may increase gametocytemia in the early stages of treatment (20; 193; 215), but long-term TMP-SMX therapy could decrease parasite burden and transmission (108; 109; 124; 150).

Early studies in the 1980's began to associate growing resistance of *P. falciparum* to the use of SP (109, 110), though these data conflicted with a small study which failed to find a stimulating effect of SP on gametocytogenesis (116). In 1997, a study by Puta and Manyando (193) measured gametocytemia in patients treated with SP or chloroquine and found a significant difference between the two treatment groups. In 2006, Schneider et al. (215) convincingly showed the risk of gametocyte carriage and density in Kenyan children with falciparum malaria was significantly higher in patients treated with SP monotherapy compared to those treated with combination SP and artesunate (AS) therapy. Further, gametocyte prevalence and density decreased over time in patients with SP+AS therapy but not in SP-treated children.

These studies suggested increasing levels of *P. falciparum* resistance to SP, that was confirmed by Barnes et al. (20) in 2008. These authors conducted three therapeutic efficacy studies in *P. falciparum*-infected patients treated with SP over the course of five years. Notable findings included a significant increase in post-treatment gametocytemia with most significant increases in gametocyte positivity rates between days 14 and 21 of treatment. Over the course of the study, the mean maximum gametocyte density in patients increased seven-fold from 2000 to 2002, and this was attributed to a rapidly increasing frequency of Pf*dhfr/dhps* mutations, encoding SP resistance. Oesterhalt et al. (168) showed a relationship between submicroscopic gametocytemia and the presence of Pf*dhfr* mutations in areas of East

Africa that previously reported adequate response to SP treatment. These growing resistance patterns lead to the institution of artemisinin-based therapy as the primary treatment in 2006 (8; 97), though SP is still prescribed for intermittent preventive treatment in pregnancy (115) and as prophylaxis for HIV-1-associated opportunistic infections in malaria endemic areas.

While SP monotherapy has been associated with increased risk of gametocytemia, subsequent infectivity to mosquitoes surrounding these observations has been inconclusive (21; 55; 111; 132; 204; 215). In particular, TMP-SMX prophylaxis has been shown to reduce the risk of parasite transmission in malaria/HIV-1 co-infection. Hobbs et al. in 2012 (109) showed that TMP-SMX treatment of mice infected with *Plasmodium berghei* or *Plasmodium yoelii* significantly reduced liver stage parasite burden and peripheral parasitemia. These data were foundational for a second study Hobbs et al. in 2013 (108) focusing on the effect of ART and antifolates on two key aspects of parasite transmission - gametocyte burden and mosquito infectivity. In this study, strains of *P. falciparum* were exposed to TMP-SMX at concentrations equivalent to those in HIV-positive patients receiving prophylactic treatment. Unmetabolized TMP-SMX did not significantly reduce gametocyte viability or inhibit gametocyte exflagellation *in vitro*, but did reduce oocyst infection in mosquitoes. These findings suggested the possibility that TMP-SMX might impact mosquito infectivity directly, thus, potentially reducing transmission.

In HIV-1-malaria co-infected patients, numerous clinical studies have suggested that antifolate treatment has an inhibitory effect on parasite transmission. A large cohort study of 300 HIV-1-infected children in Uganda showed that TMP/SMX treatment and provision of insecticide-treated bed nets reduced malaria

incidence by 97%, despite geographically high levels of antifolate resistance(124). Similar effects were seen in a cohort study of over 1000 Ugandan adults, showing that TMP-SMX treatment led to an incidence of 9 clinical episodes of malaria per 100 person years compared to a baseline incidence of 50.8 clinical episodes per 100 person person years (150). TMP-SMX prophylaxis also showed promising effects in HIV-1 exposed infants, reducing malaria incidence by 39% relative to HIV-1-exposed infants who did not continue therapy (209).

A particularly intriguing study by Mermin et al. in 2005 (151) examined a large sample of HIV-infected patients and their HIV-1-negative family members and found that TMP-SMX prophylaxis in HIV-1-positive patients significantly reduced malaria disease burden in HIV-1-negative family members. While reductions in malaria-associated morbidity, infection rates, and hospitalization are unquestionably multifactorial, it is possible that antifolates are directly anti-parasitic. Further, although the study did not report malaria incidence for HIV-1-positive individuals, it could reasonably be surmised that malaria was prevalent in the HIV- 1-population given the known increased risk of malaria in HIV- 1-positive patients. Future studies similar in design but targeted at HIV-1-malaria co-infected patients and including mosquito infectivity studies, could further support a direct impact of antifolate therapy on malaria parasite transmission.

Malaria Treatment Failure

Malaria treatment failure, as defined by clinical failure, parasitological failure, or recrudescence, is a significant problem for efforts to mitigate clinical infection and control parasite burden. Though this issue is multi-faceted, encompassing vector biology, innate host immunity, drug resistance, and environmental considerations, this scope of challenges now extends to the impact of

HIV-1 on parasite clearance, including clearance remediated by antimalarial treatment. Birku et al.(35) investigated the effect of artemisinin on clearance of *P. falciparum* in patients with and without HIV-1 co-infection. Their data showed increased time for parasite clearance and inability to entirely clear parasitemia within a designated treatment period. Further, mean parasite density was 12-fold higher in HIV-1 seropositive patients than in seronegative controls. A second large retrospective study of 1965 patients in Uganda revealed similar findings, showing patients with HIV-1 co-infection had a greater than 3-fold increased risk of antimalarial treatment failure. Molecular analysis of these treatment failures indicated these failures were due to new infections rather than recrudescence (125). In a large cohort study of Ugandan children, Kakuru et al. (121) studied how different HIV-1 treatments and prophylaxis affected gametocytemia in children and found that dihydroartemisinin- piperaquine (DP) and TMP-SMX treatment were associated with an increased risk both of any gametocytemia and of failed gametocyte clearance during malaria follow-up. Additional studies have shown that many of the antimalarials recommended globally have little to no effect on gametocytes and may allow gametocytes to persist for more than 1 month after successful clearance of asexual parasites (40; 44). Beyond the clinical challenges of treatment failure, an inability to control parasitemia and gametocytemia may make HIV-1-infected patients an enhanced reservoir for transmission.

Population of Special Interest:Asymptomatic Individuals

The presence of gametocytes in a human host has not been associated with any distinct clinical findings. Gametocytes can be detected in acutely symptomatic malaria patients as well as asymptomatic carriers, especially in endemic areas where multiple infections are common and clinical immunity develops during childhood.

Asymptomatic infections pose a unique threat to malaria control as they represent a large reservoir of hosts capable of unknowingly harboring and transmitting parasites. Recent evidence (based on hypotheses from the early 1900s) indicates that many asymptomatic individuals harbor gametocytes at such low densities that they are not often detected by microscopy [reviewed in (16; 166)]. Associations between asymptomatic infections with low parasite/ gametocyte density and mosquito infectivity vary significantly in relation to parasite genetic diversity (complexity of infection), drug resistance, and host immunity/ chronicity of infection (42; 254; 266).

At this time, the prevalence of HIV-1 co-infected asymptomatics has not been fully described due to the difficulty in ascertaining asymptomatic malaria patients. Two large studies of adults in Africa showed conflicting results in overall prevalence of asymptomatic parasitemia, with HIV-1 positive patients showing significantly lower prevalence of asymptomatic parasitemia than HIV-1 negative controls. HIV-1 positive patients not using TMP-SMX prophylaxis had an increased risk of parasitemia, suggesting that antifolates reduce parasitemia (46; 123). These findings were proposed to result from an increased tendency of HIV-1 positive patients to seek medical care and use prophylactic measures (46). A cross-sectional study in Kenya revealed that more than 60% of malaria asymptomatic adults seeking HIV-1 testing in the Kisumu region are positive for malaria (127), but significant differences by HIV-1 status have not emerged. Recent evidence from Kamau et al. revealed that asymptomatic malaria infections were significantly associated with abnormal hematological outcomes in people living with HIV (123).

Contrary to parasitemia data above, asymptomatic individuals infected with HIV-1 have been shown to have a significantly higher risk of being gametocyte positive, and with a higher relative gametocyte density, compared to HIV-1-negative

individuals (232). Further, HIV-1 co-infection is associated with significant differences in *P. falciparum dhfr* and *dhps* haplotypes in the same patient population, suggesting that HIV-1 co-infection could impact the spread of drug resistance(248). These observations are concerning in the context of transmission reduction, especially considering the high prevalence of asymptomatics in varying endemnicities (7; 43; 212; 239) and areas with seasonal transmission. As reviewed by Babiker et al. (16), asymptomatic gametocyte carriers are a likely source of seasonal epidemics as they harbor gametocytes throughout transmission-free seasons until the vector returns (14; 166).

Conclusions

Although the current evidence is suggestive, the bidirectional impact of co-infection on the clinical progression of both diseases suggests that HIV-1/malaria co-infection may be a catalyst for increased parasite transmission. In order to elucidate how contributing co- infection factors, define malaria parasite transmission success, combined clinical and entomological studies must be undertaken. Importantly, the nature of the entomological studies depends on the question(s) to be answered. For example, if the question is whether circulating gametocytes are intrinsically infectious, the study design may include replacing serum from a volunteer blood sample with non-immune serum to eliminate circulating host factors that could obscure intrinsic infectivity of gametocytes to exposed mosquitoes. If, on the other hand, the question is whether a volunteer is infectious based on their current clinical or treatment status, direct mosquito feeding on whole blood would be preferable to account for circulating host factors and therapeutics that could impact parasite development in the mosquito host. Given that many of the intrinsic and extrinsic host factors noted above, some of which can fluctuate on a very short timescale, can affect

gametocytogenesis and gametocytemia, and that mean gametocyte circulation time in the periphery is 3.4-6.4 days (42), matched clinical samples collected at or very close to the time of mosquito feeding are essential to directly correlate volunteer clinical status to mosquito infection success. Even with the best coordination, however, infection of mosquitoes after direct skin feeding on infected falciparum gametocyte-positive volunteers is far from uniform; the average success rate of mosquito infection from a 2012 survey of 930 feeding experiments in a variety of endemic settings was 62% (41). Accordingly, longitudinal studies have particular value in that volunteers can be re-tested over time, perhaps multiple times per month for several months, to track both clinical and treatment profiles with mosquito infection success. Each volunteer, therefore, can provide both control (baseline) and temporal data on host factor correlates with mosquito infectivity. In this context, special consideration should be made to further understand the impact of asymptomatic gametocyte carriers, the duration and intensity of gametocyte carriage, the presence of HIV-1 co-infection and the potential effects of HIV-1 and malaria chemotherapies on experimental parasite transmission to competent mosquito vector species.

From a public health perspective, asymptomatic *Plasmodium*- infected individuals remain one of the biggest threats to malaria control and eradication programs. If HIV-1 co-infected individuals have increased prevalence, frequency, duration and/or intensity of gametocytemia, they are unwittingly and unintentionally more infectious reservoirs of parasite transmission for the rest of their community. As such, these individuals represent an ideal target for additional therapies in conjunction with ART for the protection of public health and for malaria elimination and eradication.

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Chapter 3: Longitudinal Impact of Asymptomatic Malaria/HIV-1 Co-infection on *Plasmodium falciparum* Gametocyte Transcript Expression and Transmission to *Anopheles* Mosquitoes

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Abstract

Despite significant developments towards malaria reduction, parasite transmission in the common context of HIV-1 co-infection and treatment for one or both infections has not been fully characterized. This is particularly important given that HIV-1 and malaria chemotherapies have the potential to alter gametocyte burden and mosquito infectivity. In this study, we examined 782 samples collected from a longitudinal cohort of 300 volunteers in the endemic region of Kisumu, Kenya, to define the impacts of HIV-1 co-infection, antiretroviral therapy (ART) plus trimethoprim-sulfamethoxazole (TS) and the antimalarials artemether/lumefantrine (AL) on *Plasmodium falciparum* gametocyte transcript prevalence and parasite transmission to the African malaria mosquito *Anopheles gambiae*. Using our highly sensitive digital droplet PCR (ddPCR) assay of three gametocyte specific transcript markers, we observed an overall gametocyte prevalence of 51.1% in *18S* positive volunteers across all study groups and time points. After correcting for multiple comparisons, the factors of HIV-1 status, time, CD4+ T-cell levels and hematocrit were not predictive of gametocyte prevalence or transmission. Among those volunteers who were newly diagnosed with HIV-1 at enrollment, however, the initiation of ART/TS and AL at enrollment was associated with a significant reduction in gametocyte transcript prevalence in the subsequent month when compared to HIV-1 negative volunteers. To assess gametocyte transmissibility, volunteer blood samples were used in standard membrane feeding assays with laboratory-reared *A. gambiae*, with evidence of transmission confirmed by at least one of 25 dissected mosquitoes per sample positive for at least one midgut oocyst. Although HIV-1 status, CD4+ T-cell levels and hematocrit were not significantly associated with successful transmission to *A. gambiae*, we observed that 50% of

transmission-positive blood samples failed to test positive by *Plasmodium*-specific *18S* ribosomal RNA quantitative PCR (qPCR) and 35% failed to test positive for any gametocyte specific transcript marker by droplet digital (ddPCR). Overall, these findings highlight the complexity of HIV-1 malaria co-infection and the need to further define the unpredictable role of asymptomatic parasitemia in transmission to mosquitoes.

Introduction

In areas of high endemicity for malaria, such as sub-Saharan Africa, high prevalence of asymptomatic malaria parasitemia poses a risk to malaria elimination efforts. Many of the same geographical areas represent a significant portion of the world's HIV-1 infections, leading to higher probability of HIV-1-malaria co-infection. It is well described that HIV-1-malaria confection increases clinical malaria and parasitemias in adults (107), (87). However, less is known about the impact that HIV-1 co-infection may have on gametocyte burden and malaria parasite transmission, specifically in asymptomatic populations [reviewed in (203)]. In a rhesus macaque model of simian immunodeficiency virus (SIV) malaria co-infection, there was a significant association with increased gametocytemia and parasite transmission to *Anopheles freeborni* mosquitoes (251). In 2020, an epidemiological study investigating the impact of HIV-1 co-infection on the prevalence of asymptomatic gametocytemia suggested that there is an increased risk for gametocyte prevalence amongst HIV-1 positive individuals (231). These observations prompted questions regarding the biological complexities surrounding both diseases and parasitic responses to the changes in intrinsic host factors and initiation of chemotherapies over time.

There are few studies that have examined the clinical parameters of HIV-1-malaria co-infection and their association with parasite transmission. Anemia is of particular interest in that it is observed in both the malaria and HIV-1 infection (27) and has been associated with altered gametocytogenesis and gametocyte development [reviewed in (42)]. Recent studies have shown that asymptomatic malarial parasitemias were significantly associated with abnormal hematological outcomes in people living with HIV-1 (122), suggesting that parasite transmission could be altered in the context of suppressed immunity and antiretroviral therapy (ART). The effects of immune deficiency on malarial parasitemia and clinical episodes have been well studied [reviewed in (87; 107)]. Due to the lack of clinical significance associated with gametocytes in endemic populations, however, there have been fewer studies that have investigated the effects of immunodeficiency on gametocytes and parasite transmission.

Antifolate therapy, such as trimethoprim-sulfamethoxazole (TS) is often prescribed to HIV-1 infected individuals for prophylaxis for opportunistic infections. Effects of TS on gametocytes and gametocytemia have been notable. For example, gametocytemia was observed to increase almost immediately after drug initiation and peaked around two weeks into therapy (40). Because TS does not kill gametocyte-committed rings, these results could be due to the continued development of early gametocytes. Hobbs et al. reported that TS reduced the risk of peripheral parasitemia and oocyst infection in exposed mosquitoes but did not reduce gametocyte viability or inhibit gametocyte exflagellation (108; 109). The contrasting results between gametocyte burden and transmission success highlights the need to include mosquito feeding assays when quantifying parasite transmission potential in epidemiological studies (41; 181) and clinical studies (213).

Results from our prior point-prevalence study (231) revealed that HIV-1 was associated with increased prevalence and abundance of *Plasmodium falciparum* gametocyte-specific transcripts in asymptomatic adults in western Kenya. Given that chronic asymptomatic *P. falciparum* parasitemia has been associated with long term intermittency of gametocytemia (224) and that asymptomatic infections have been associated with low quantities of gametocytes [reviewed in (203)], we sought to investigate the longitudinal impact of clinical parameters and initiation of chemotherapies on *P. falciparum* gametocyte burden and subsequent parasite transmission to the *Anopheles gambiae* using highly sensitive ddPCR and standard membrane feeding assays (SMFA).

Material and Methods

Sample Collection and Study Design

A total of 300 study participants were voluntarily enrolled from study sites in Kisumu County, Kenya, after seeking voluntary HIV testing at satellite HIV Testing and Counseling (HTC) Centers or the HTC at Kombewa Sub-County Hospital (KCH). All participants were apparently healthy adults, asymptomatic for *Plasmodium falciparum* malaria. Participants were screened and enrolled as previously described (Oyieko et al., 2022, manuscript submitted for publication) with a target of 100 participants in each of the target categories: HIV-1 negative, HIV-1 positive newly diagnosed, and HIV-1 positive on ART and TS. Patients stable on ART and TS were treated in accordance with guidelines from the Kenyan Ministry of Health (MoH). Whole blood samples were collected in accordance with Institutional Review Board protocols and with signed informed consent to include 6 months of follow-up visits. Rapid diagnostic tests (RDTs) were used for HIV-1 and malaria screening and confirmation followed by blood collection for further clinical

and molecular testing (Oyieko et al., 2022, manuscript submitted for publication). Any patients with positive malaria RDT results were treated with AL (Artemether-Lumefantrine) in accordance with Kenya MoH guidelines. Additional blood was collected for a Standard Membrane Feeding Assay (SMFA) in a subset of volunteers described below. Polymerase Chain Reaction (qPCR) assays were used for research-based questions to include the foundational testing for the presence of *Plasmodium*-specific transcripts using *18S* ribosomal RNA and quantitative PCR (qPCR) (127) (122) (Kifude et al., 2022, manuscript in preparation). Samples positive by *18S* qPCR were subjected to gametocyte transcript analysis.

Blood collected for molecular analysis was blotted onto Whatman® 903 Protein saver filter paper cards (GE Healthcare Life Sciences, Chicago, IL USA) and processed for RNA and DNA extraction. In brief, a single air-dried 50 μL blood spot (DBS) was minced and nucleic acids extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's standard protocol for RNA extraction. Genomic DNA was digested as part of this procedure prior to the reverse transcription of RNA. cDNA synthesis was conducted using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) and following the manufacturers standard protocol.

Selection of Gametocyte Specific Transcripts

Target genes *Pfs16, PfMGET,* and *Pfs25* were selected using documented transcript levels of genes from purified male and female *P. falciparum* gametocytes (138). *Pfs16* was selected as an early molecular marker of gametocytes (31; 53; 73) showing high transcript levels with relatively equal female to male ratio of 1.28:1.00 (138). *PfMGET* was shown to have more abundant gene transcripts than other previously used male markers (233), and *Pfs25* was selected as an abundantly

expressed female-specific marker in stage V gametocytes (138; 217). *Pfs16* and *Pfs25* primers and probes (Table 3) were derived and adapted for use with digital droplet PCR (ddPCR) from Stiffler et al. (231), and *PfMGET* primers and probe (Table 3) were derived and adapted for use with ddPCR from Wang et al. (264).

Table 3**.** ddPCR primers (Integrated DNA Technologies, Coralville, IA, US) and probe (Sigma-Aldrich, St. Louis, MO, US) sequences.
The 5' end of each probe is labeled with a fluorophore (FAM or HEX). The 3' end of each probe is labeled with a black hole quencher (BHQ-1).

Target	Forward Primer, Reverse Primer, and Probe Sequences		Adapted from:
pfs25 (Pf3D7_1031000)	**Forward**	5' TCTGAAATGTGACGAAAAGACTGT 3'	(231)
	Reverse	5' AGCGTATGAAACGGGATTTCC 3'	
	Probe	FAM 5' ATAAACCATGTGGAGATTT 3' BHQ-1	
pfMGET (Pf3D7_1469900)	**Forward**	5' AAAATTCGGTCCAAATATAAAATCCTG 3'	(264)
	Reverse	5' CTTCATCAATTAAAAATCCCTTTTTTGT 3'	
	Probe	HEX 5' CCTGGTAAAAAACAGCTCCAGCA 3' BHQ-1	
pfs16 (Pf3D7_0406200)	**Forward**	5' GGATCCCCTTCAACTTTGCA 3'	(231)
	Reverse	5' CCTTGAGATAGTCCACCTTGATTAGG 3'	
	Probe	FAM 5' TTCTTCAGGTGCCTCTCTTCATGCTGTTG 3' BHQ-1	

Digital Droplet PCR (ddPCR)

A duplex ddPCR assay was adapted using separate indicators for *PfMGET* and *Pfs25* markers in a single reaction, and *Pfs16* was quantified in a separate reaction (187). For both assays, each reaction included 11 µL Supermix for Probes (no dUTPs)(Bio-Rad Laboratories Inc., Hercules, CA, US), 2 µL cDNA template, nuclease free water as needed, 455 nM of each primer and 250 nM of each probe in a final reaction volume of 22 µL. Droplets were generated using the QX200 Droplet Generator (Bio-Rad Laboratories Inc., Hercules, CA, US) within a range of 10,000 to 20,000 droplets. Reactions were performed on the C1000 Touch Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, US) under the following programs (ramp rate 2°C/second): Duplex *PfMGET*/*Pfs25* - 95°C for 10 minutes, 40 cycles of 94°C for 30 seconds and 50°C for 1 minute, 98°C for 10 minutes; *Pfs16* - 95°C for 10 minutes, 40 cycles of 94°C for 30 seconds and 55°C for 1 minute, 98°C for 10

minutes. Droplets were analyzed and counted on a QX200 Droplet Reader (Bio-Rad Laboratories Inc., Hercules, CA, US).

ddPCR Assay Optimization and Validation

Each marker of interest was cloned into the PCR 2.1 TOPO TA Vector (Life Technologies, Carlsbad, CA, USA) in accordance with manufacturers' protocols (Invitrogen TOPO TA Cloning Kit with pCR 2.1-TOPO, One Shot TOP10 Chemically Competent *E. coli*). Plasmid DNA was purified using the QIAprep Spin MiniPrep kit (Qiagen, Hilden, Germany), quantified using a spectrophotometer (NanoDrop 2000C, ThermoFisher Scientific, Waltham, MA, USA), and verified by Sanger sequencing by GENEWIZ from Azenta Life Sciences (South Plainfield, NJ, USA). Ten-fold serial dilutions of each plasmid ranging from 10,000 copies per µL to 0.1 copies per µL were generated by diluting in water. Additional two-fold dilutions between 10 copies per µL and 0.3125 copies per µL were used to determine lower limits of detection and quantification. The limits of detection and quantification for *Pfs25*, *PfMGET*, and *Pfs16* were 3.125, 6.25, and 1.00 copies/µL respectively. Temperature gradient protocols were used to identify optimal annealing temperatures. Additional optimization was conducted to determine the greatest separation of relative fluorescence units (RFU) between control positive and negative droplets.

ddPCR Analysis

For the duplex assay, a data-driven approach was used to identify the threshold between positive and negative droplets using no-template-control (NTC) wells within each ddPCR plate. The ddpcRquant approach was adapted and used to determine the number of positive droplets in the duplex assay (253). For the *Pfs16*

single assay, droplets were considered positive if they were above 4000 RFU and the NTC negative droplets averaged between 1500 and 3000 RFU.

ddPCR concentration (as copies of target per microliter of final ddPCR reaction) was manually calculated using a Poisson algorithm, where [Concentration = - ln (Negative droplets/Total number of droplets) / Volume of Droplet] and the volume of droplet was always 0.85 nL. Concentration was further translated into total copies of target per microliter of starting sample using the following formula: [(Concentration x Ratio of total PCR volume (22 µL) to sample volume (2 µL)) x original sample volume (2 µL)] (34).

Sampling for Mosquito Infectivity Study

A subset of 100 volunteers was recruited and transported to the Kenya Medical Research Institute (KEMRI)/ U.S. Army Medical Research Directorate-Africa (USAMRD-A) Entomology Department laboratories in Kisian, Kenya. One mL of blood was collected from each volunteer at each visit for SMFA using laboratory-reared adult female *A. gambiae* (Oyieko et al., 2022, manuscript submitted for publication). Given that gametocytes circulate in the periphery for an average of 3.4-6.4 days (42), we included samples for clinical analysis that were within two days of SMFA. Accordingly, bloods samples for molecular analyses from each volunteer were collected on the date of the SFMA or within two days before or after the SMDA. Based on these parameters, blood samples from 44 HIV-1 positive volunteers and 39 HIV-1 negative volunteers were screened by qPCR, ddPCR, and transmission to *A. gambiae* by SMFA.

Insectary Procedures for Mosquito Rearing

SMFAs were conducting using laboratory-reared *A. gambiae s.s.*, originally obtained from Luanda village, Kisumu County Western Kenya. The mosquitoes were

reared in the USAMRD-A/K Insectary at KEMRI Centre for Global Health in Kisian, Kisumu. Adult mosquitoes were kept in 30x30x30 cm gauze-covered cages under ambient conditions. They were maintained on a 10% sterile sucrose solution and provided with water on cotton pads to increase relative humidity in the cages. Cow blood for colony mosquito feeding was obtained freely from local abattoirs; raw blood was collected from freshly slaughtered cow's jugular vein and anticoagulated with EDTA 60 mg/L for short-term (7-10 days) refrigerated storage. Mosquitoes were fed on cow blood through a Hemotek membrane feeder for 30 minutes and maintained under adult rearing conditions as previously described. After two days, an oviposition cup was placed in the previously blood-fed cage; any eggs laid on the moist filter paper were transferred into rearing pans with approximately 3 liters of old rainwater. The larvae were fed 2-3 times a day on Tetramin® fish food and Brewer's yeast; they were maintained at a temperature of 27±1°C until pupation. Water was replaced every other day to maintain good larval growth. Adult mosquitoes were kept inside a separate room, where temperatures were maintained at 27°C and relative humidity at 70–90% and were fed on 10% sterile sucrose solution. The insectary was set to a photoperiod of 12 hours darkness and 12 hours light.

Standard Membrane Feeding Assay (SMFA) and Mosquito Dissection

Laboratory-reared, 3–5-day old *A. gambiae* were partially starved for 3-5 hours prior to SMFA. They were subsequently allowed to feed for 20 minutes via an artificial membrane attached to a water-jacketed glass-bell parafilm membrane feeder maintained at 37°C. In accordance with the SMFA protocol (182), a 1 mL sample of the volunteer's blood was drawn into pre-heated glass feeders. After the SFMA, fully engorged mosquitoes were transferred to environmental chambers with automated control of temperature and humidity (27°C, 80% respectively) and a fixed

light-dark cycle (12h/12h). At 10 days post-feeding, samples of 25 mosquitoes per SFMA were moved from the environmental chambers to a freezer (-20°C) for immobilization prior to washing with 70% ethanol and rinsing with Phosphate Buffer Solution (PBS). Oocyst counting has been determined to be an accurate readout for transmissions success, even from low-intensity infections (234). Accordingly, mosquitoes were dissected as described by (182) for this purpose. After dissection and staining in 1% mercurochrome for 10 minutes, individual mosquito midguts were placed on a microscope slide with a cover slip and transferred to a compound microscope for enumeration of oocysts on the midgut. DBS corresponding to oocyst-positive mosquitoes were also included in gametocyte transcript analyses regardless of *18S* results.

Statistical Methods

Data were analyzed using R Studio (RStudio Team, Boston, MA, USA), SPSS (IBM Corp, Armonk, NY, USA) and GraphPad Prism 9 (GraphPad, San Diego, CA, USA). Samples were considered gametocyte positive if they were positive for at least one gametocyte-specific transcript. Samples tested for transmission were considered positive if at least one oocyst was detected in one midgut in a single group of mosquitoes used for a single SFMA. Gender, age, and differences between study groups at baseline were compared by Pearson's chi-square test. Proportional analyses were also conducted using Pearson's chi-square test throughout the study. When comparing markers from *18S* positive individuals with markers from those who tested negative for *18S* by qPCR, Mann Whitney non-parametric tests was used. Based on repeated measures in our design, generalized estimating equations (GEE) were used to quantify absolute differences across time and to generate adjusted odds ratios. In instances of log transformation of *18S* qPCR

data and gametocyte transcript ddPCR data, undetected samples were defined as "0.01" for transformation. Holm's method was used to adjust necessary p-values for multiple comparisons. The level of significance was set at alpha = 0.05.

Results

Study Profile

A total of 300 screened participants were enrolled for this study: 102 HIV-1-negative individuals and 198 HIV-1 positive individuals. HIV-1 positive individuals were further categorized as 106 HIV-1 positive newly diagnosed at enrollment and 92 HIV-1 positive and stable on ART and TS (Figure 6). A total of 782 *18S*-positive samples were collected and identified across all categories, participants, and time points with two lost in transport; *18S*-negative samples were not analyzed further unless blood fed mosquitoes became oocyst positive. Analyses of study parameters and *18S* prevalence by category over time are described in Kifude et al., (Kifude et al., 2022, manuscript in preparation) and presented in Figure 7A. Briefly, malaria prevalence by RDT at baseline (month 0, enrollment) was 17.3% (52/300) and overall malaria prevalence by *18S* qPCR was 42.7% (784/1835) with equal prevalence across monthly visits. A larger proportion of HIV-1 negative volunteers was malaria *18S* positive (61.43% *p=0.0156)* compared to the HIV-1 positive volunteers (newly diagnosed 36.45%; on treatment 31.51%). Of note, there was a significant drop in parasitemia, expressed as *18S* copy numbers per microliter, upon initiation of ART and TS from day of enrollment to one-month post-treatment in newly diagnosed HIV-1 positive volunteers (Kifude et al., 2022, manuscript in preparation). Parasitemias were significantly higher among HIV-1 negative individuals throughout the course of the study. As expected, baseline CD4+ T cell levels across all three study groups were consistent with known progression in HIV-1

infection under treatment; CD4+ T cell levels were highest in HIV-1 negative and lowest in the HIV-1 positive newly diagnosed (Oyieko et al., 2022, manuscript submitted for publication).

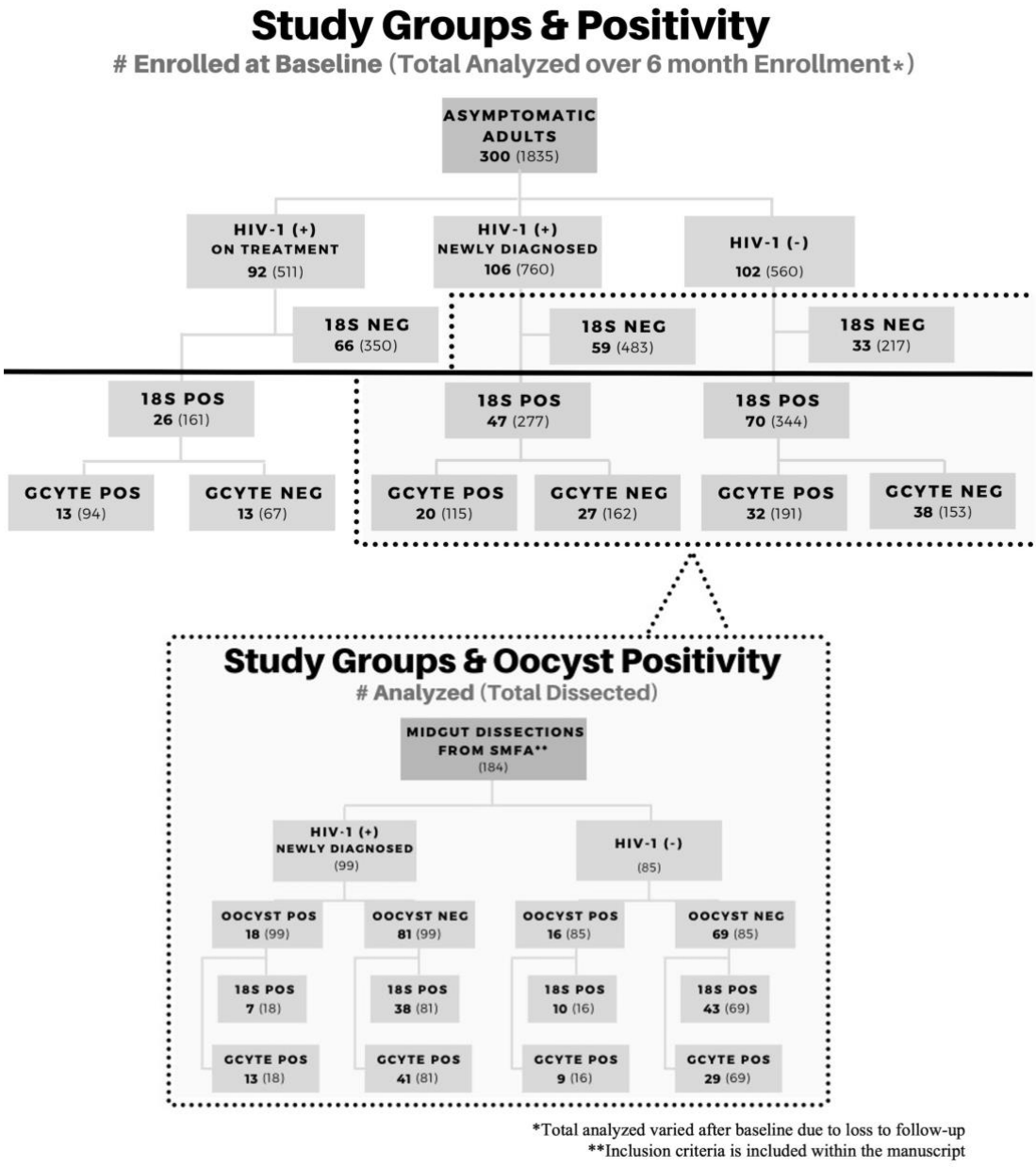

Figure 6. Study Profile Overview

Participant enrollment by study group and corresponding molecular data for asexual and sexual parasite biomarkers. Groups below the solid black line were included in this study for gametocyte analysis. A subset of volunteers was enrolled in a transmission study using the Standard Membrane Feed Assay (dotted line). Positive samples are abbreviated as (+) or POS. Negative samples are abbreviated as (-) or NEG. Full details of the study design and complete analyses of asexual stage biomarker data

are provided in Oyieko et al., 2022 (manuscript submitted for publication) and Kifude et al., 2022, (manuscript in preparation).

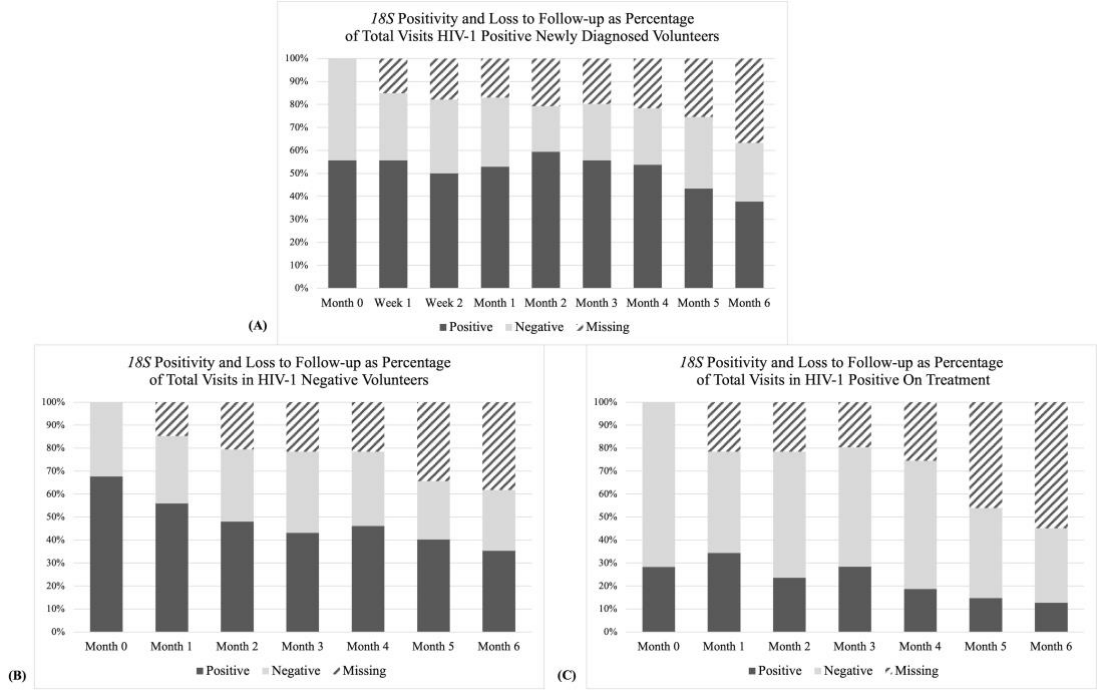

Figure 7. *18S* Positivity Over Time and Study Group.
The percentage of *18S* positive, *18S* negative, and missing samples (due to loss-to-follow up) for each study group over the course of the 6-month study. **(A)** HIV-1 positive newly diagnosed volunteers **(B)** HIV-1 positive volunteers on treatment **(C)** HIV-1 negative volunteers.

Baseline and Overall Characteristics

At baseline (month 0, enrollment), 65/143 (45.5%) *18S*-positive volunteers were positive for at least one gametocyte-specific marker (*Pfs25*, *PfMGET*, or *Pfs16*) by ddPCR. The impact of any seasonality could not be assessed as enrollment spanned 15 months and the study period crossed multiple seasons (Figure 8 and 9). Baseline prevalences of gametocyte positivity by study group were 46% in HIV-1 negative, 43% in HIV-1 positive newly diagnosed, and 50% in HIV-1 positive on ART and TS. Across all groups, study participants were divided into age brackets by interquartile ranges (18-23, 24-31, 32-36, 37-59). There were no significant differences in gametocyte prevalence among age groups, by gender, or among study groups at baseline (Pearson's Chi-square: $p = 0.699$, $p = 0.904$, $p = 0.827$ respectively). Of the 263 volunteers who were *18S*-positive at any time point, 192

were positive for at least one gametocyte-specific marker at any time point. Of these 192, 54.2% were female and 45.8% were male with a mean age of 32 (range, 18-56).

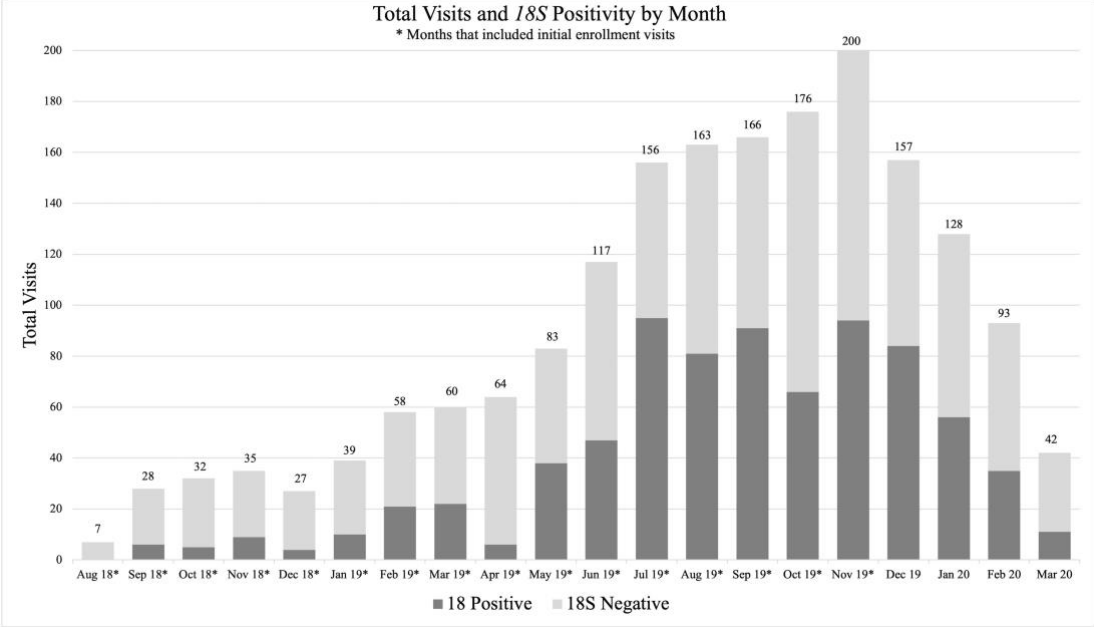

Figure 8. Distribution of *18S* Positive and Negative Samples by Calendar Month.

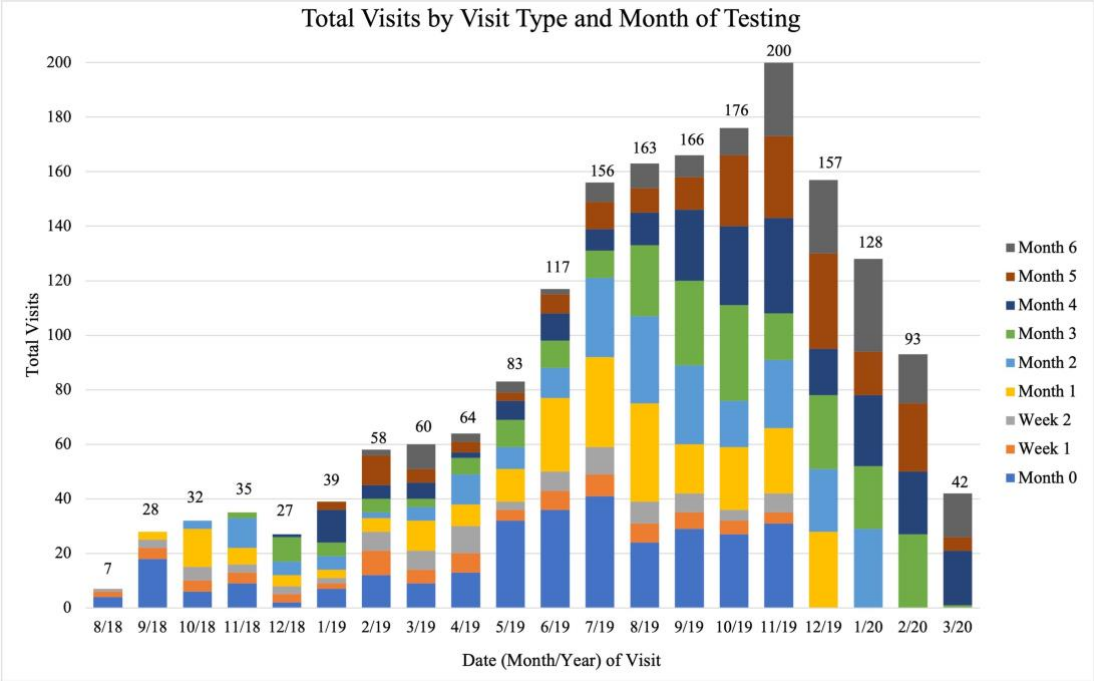

Figure 9. Study Visit by Calendar Month/Year.

GEE Analysis to Compare Longitudinal Gametocyte-Specific Transcript Prevalence Between and Within Study Groups

GEE analysis revealed that the odds of gametocyte specific transcript prevalence were significantly higher in month 1 for HIV-1 positive volunteers on treatment compared to HIV-1 positive newly diagnosed volunteers (aOR: 6.72, adjusted $p = 0.002$) and HIV-1 negative volunteers (aOR: 4.16, adjusted $p = 0.021$) (Figure 10). There were no significant differences among the study groups at any other time point.

GEE analysis also revealed no difference in the odds of gametocyte prevalence when compared to baseline or between any other visits for HIV-1 positive volunteers on treatment and HIV-1 positive newly diagnosed volunteers. For HIV-1 negative volunteers, the odds of being gametocyte positive were significantly higher for months 4 and 5 when compared to month 1 (month 4 aOR: 1.51, $p = 0.002$; month 5 aOR: 1.39, $p = 0.040$).

Overall, there was no significant interaction effect (adjusted $p = 0.552$) of study group and time. The main effect of study group (irrespective of time) was not a significant predictor of gametocyte positivity (adjusted $p = 0.088$). Additionally, the main effect of time (irrespective of study group) was also not a significant predictor of gametocyte positivity (adjusted $p = 0.112$).

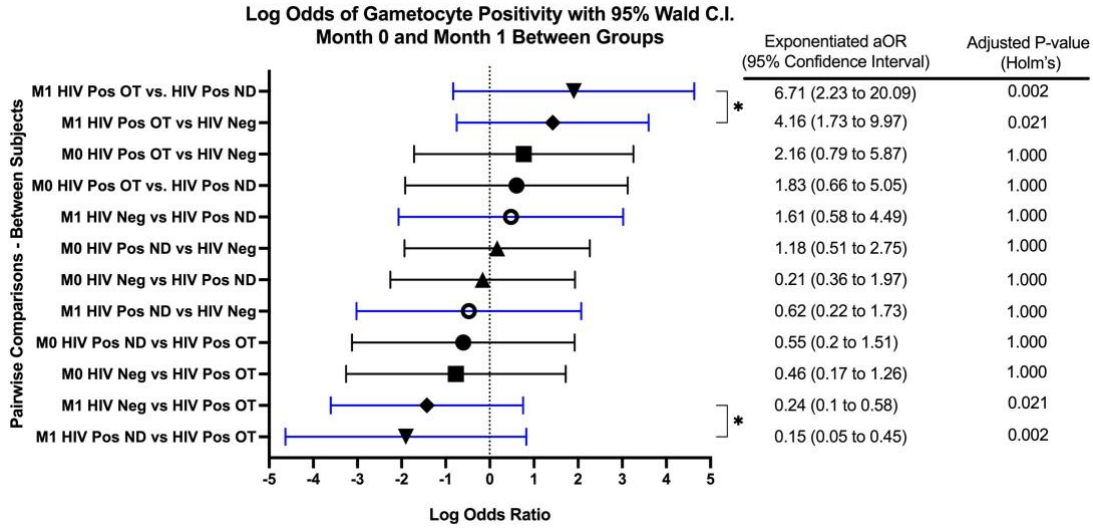

Figure 10. Forest Plot of the Log Odds of Gametocyte Positivity in Month 0 and Month 1.
The log odds and accompanying exponentiated adjusted odds ratio of being positive for at least one gametocyte specific transcript between each study group in month 0 (M0) and in month 1 (M1). "HIV Neg" represents HIV-1 negative volunteers. "HIV Pos OT" represents HIV-1 positive volunteers on treatment. "HIV Pos ND" represents HIV-1 positive newly diagnosed volunteers. P-values were adjusted using the Holm's method for multiple comparisons. Log odds are arranged in descending order. Blue confidence interval lines are comparisons within month 1 and black confidence interval lines are comparisons within month 0.

18S Copy Number is the Only Predictor of Gametocyte-Specific Transcript Prevalence

In an effort to predict overall gametocyte positivity, selected clinical and diagnostic predictors were used in the GEE analysis. Predictors included common indicators of altered immune systems based on white blood cell (WBC) (x 10^9/L) and CD4+ T-cell count, anemia based on red blood cell count (RBC) (x 10^{12}/L), age, gender, *18S* copy number/µL, *Pfs25* ddPCR concentration, and *PfMGET* ddPCR concentration. Log transformed *18S* copy number/µL was the only statistically significant predictor for gametocyte prevalence (aOR: 1.202, adjusted $p = 0.018$) (Table 4). For every 10-fold increase in *18S* copy numbers, the odds of gametocyte positivity increased by 20%.

Table 4. GEE Omnibus Results.
Predicting overall positivity using gender (male to female), time (visit month), study group, age, CD4+ T cell levels, white blood cell (WBC) counts (x 10^9/L), red blood cell (RBC) counts (x 10^{12}/L), and log *18S* copy numbers/µL. Time and study group were the main effects in the model, so a p-value only was provided for the Omnibus test. *When log transforming *18S* copy numbers/µL, undetermined results were defined as "0.01" for the transformation.

Predictor of Gametocyte positivity	Exponentiated aOR	95% Wald Confidence Interval	Adjusted p-value (Holm's)
Gender (Male:Female)	0.720	0.586 to 1.446	1.000
Time	-	-	0.112
Study Group	-	-	0.088
Age	0.991	0.966 to 1.017	1.000
CD4+ T cells	1.001	1.000 to 1.001	0.575

WBC	0.971	0.860 to 1.096	1.000
RBC	0.955	0.668 to 1.352	1.000
*Log *18S* Copy numbers/µL	1.202	1.069 to 1.352	0.018

Sex-Specific Gametocyte Transcripts in Each Study Group Over Time

Across all time points, 782 *18S*-positive samples were analyzed for gametocytes. 400 of 782 samples (51.1%) were positive for at least one gametocyte-specific marker with the majority positive for *Pfs25* (female) and *PfMGET* (male) (Figure 11). Across all groups, there was an increase in the prevalence of *Pfs25* and *PfMGET* transcripts at month 1, while *Pfs16* transcripts remained expressed at low levels (Figure 12).

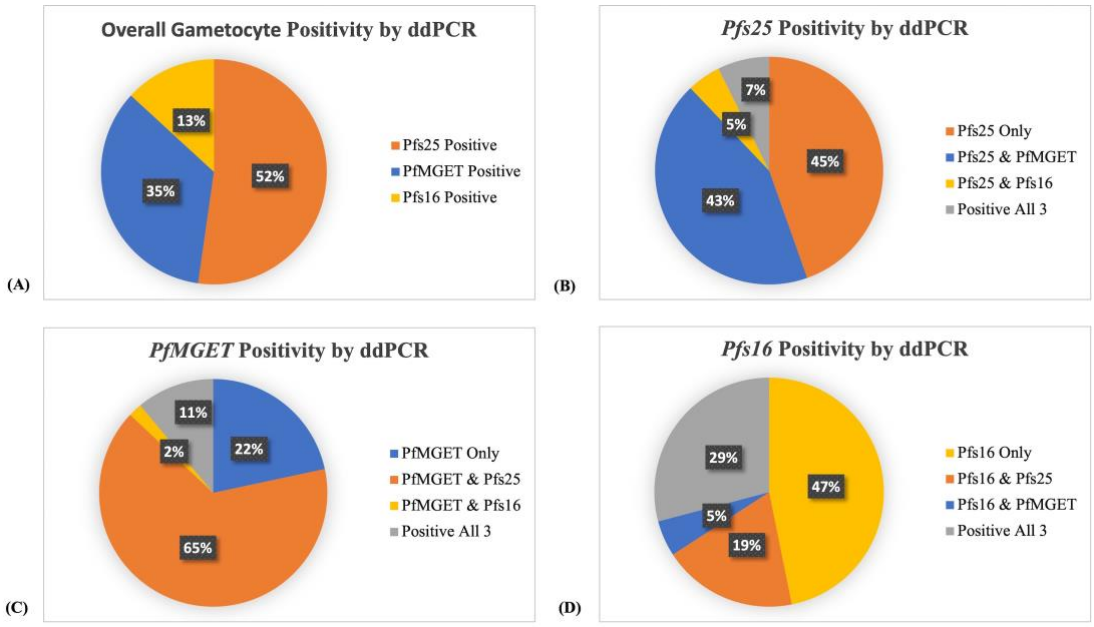

Figure 11. Percent Positivity by Gametocyte-Specicifc ddPCR Molecular Marker.
(A) Overall gametocyte positivity by ddPCR **(B)** *Pfs25* positivity by ddPCR
(C) *PfMGET* positivity by ddPCR and **(D)** *Pfs16* positivity by ddPCR

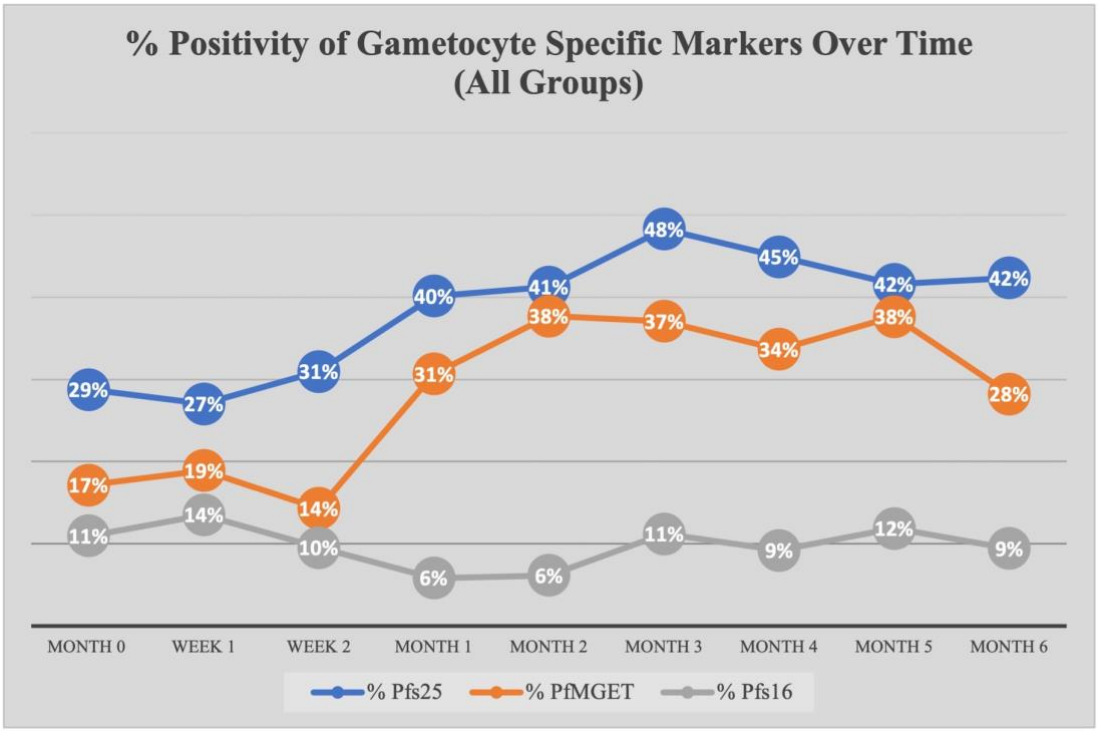

Figure 12. Percent Positivity of Each Gametocyte-Specific Marker Over Time for All Study Groups. The prevalence of each gametocyte-specific transcript biomarker (as a percentage of total analyzed) at each time point/visit.

Pfs25 Positive Transcripts and ddPCR Concentrations

At baseline, there were no differences in the odds of being *Pfs25* positive between study groups at any other month besides month 1 (GEE analysis). Similar to overall gametocyte prevalence, the odds of *Pfs25* positivity increased in month 1 for HIV-1 positive on treatment compared to HIV-1 negative and compared to HIV-1 positive newly diagnosed (aOR 5.00, adjusted p-value <0.001; aOR 5.93, adjusted p-value 0.005, respectively). As seen in Figure 13C, there was an increase from 42% positive for *Pfs25* in month 0 to 60% positive for *Pfs25* in month 1. *Pfs25* positivity unexpectedly dropped at month 4 for HIV-1 positive newly diagnosed, but the decreased odds were not significant after adjusting for multiple comparisons.

When comparing within each group over time, there were no differences in *Pfs25* positivity between timepoints for HIV-1 positive on treatment. HIV-1 positive newly diagnosed exhibited a tendency for increased positivity at months 3 and 5 when compared to baseline and month 1 (Figure 14). These observations were similar to those for HIV-1 negative volunteers who exhibited a tendency for increased positivity at months 3 and 4 when compared to baseline (Figure 15).

After adjusting for multiple comparisons there were no differences in *Pfs25* ddPCR concentration (Figure 16) between groups at each time point or over time within any group. Contrary to the prevalence analysis, log transformed *18S* copy number did not predict *Pfs25* ddPCR concentration (log transformed). There were no differences in *Pfs25* ddPCR concentrations by gender or age nor any predictive associations between WBC, RBC, or CD4+ T-cell levels.

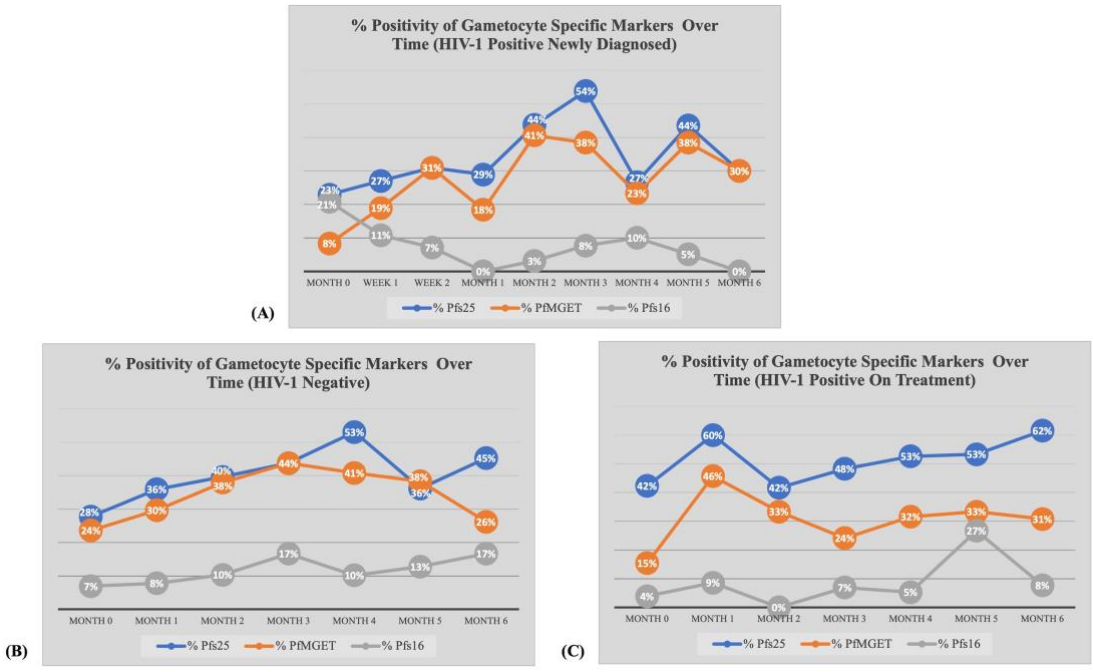

Figure 13. Percent Positivity of Each Gametocyte-Specific Marker Over Time for Each Study Group.
The prevalence of each gametocyte-specific transcript biomarker (as a percentage of total analyzed) at each time point for **(A)** HIV-1 positive newly diagnosed volunteers **(B)** HIV-1 negative volunteers and **(C)** HIV-1 positive volunteers on treatment.

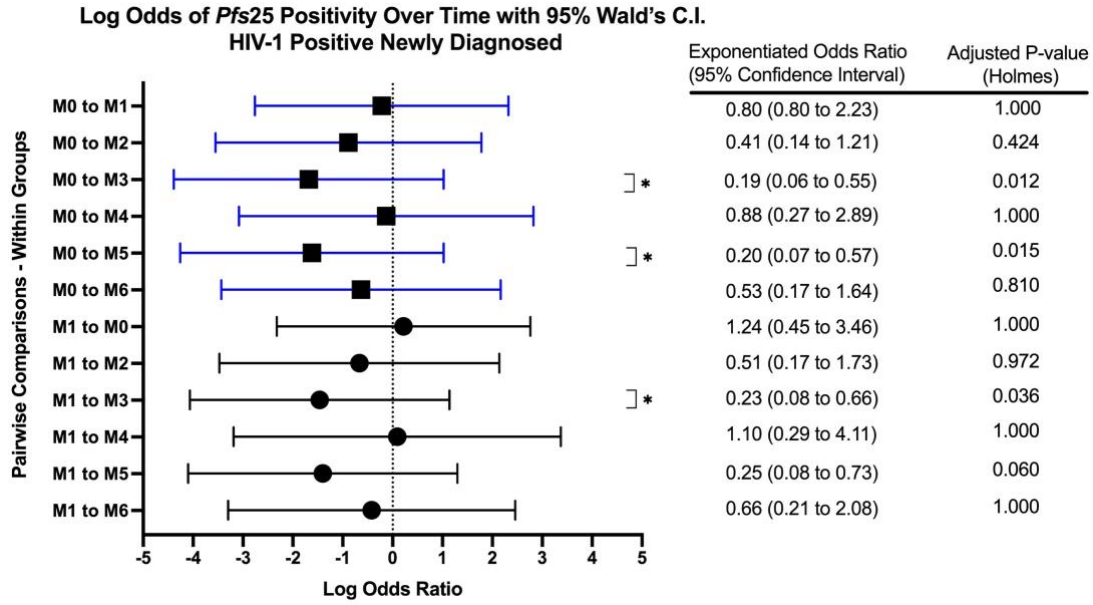

Figure 14. Forest Plot of the Log Odds of *Pfs25* Positivity in HIV-1 Positive Newly Diagnosed Volunteers.
The log odds and accompanying exponentiated adjusted odds ratio of being positive for at least one gametocyte-specific transcript within the HIV-1 positive newly diagnosed study group between month 0 (M0) and between month 1 (M1). P-values were adjusted using the Holm's method for multiple comparisons. Blue confidence interval lines are comparisons with month 0 and black confidence interval lines are comparisons with month 1.

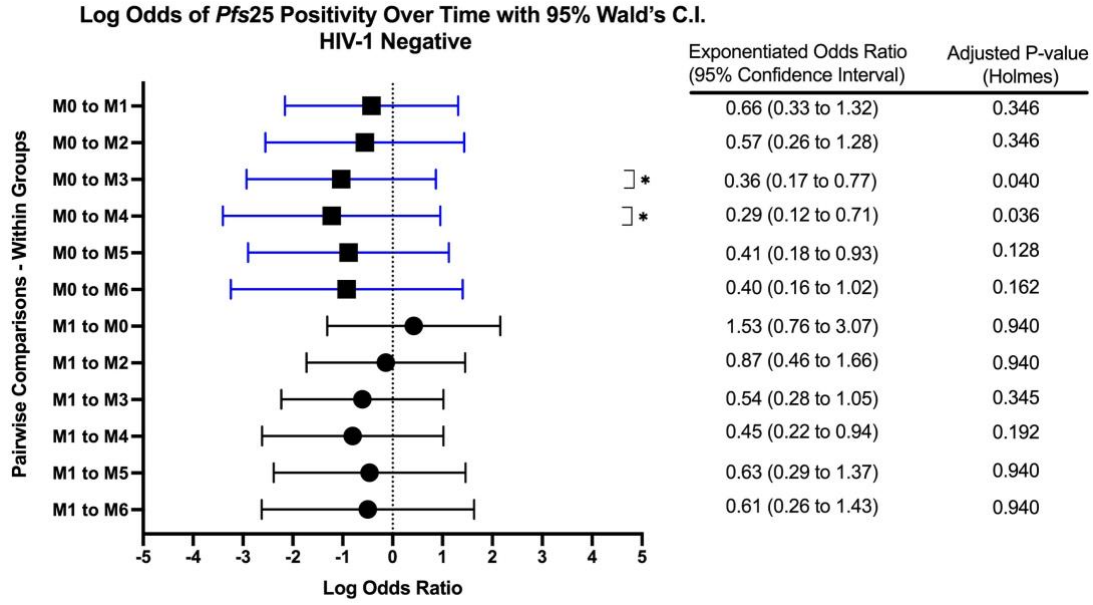

Figure 15. Forest Plot of the Log Odds of *Pfs25* Positivity in HIV-1 Negative Volunteers.
The log odds and accompanying exponentiated adjusted odds ratio of being positive for at least one gametocyte-specific transcript within the HIV-1 negative study group between month 0 (M0) and between month 1 (M1). P-values were adjusted using the Holm's method for multiple

comparisons. Blue confidence interval lines are comparisons with month 0 and black confidence interval lines are comparisons with month 1.

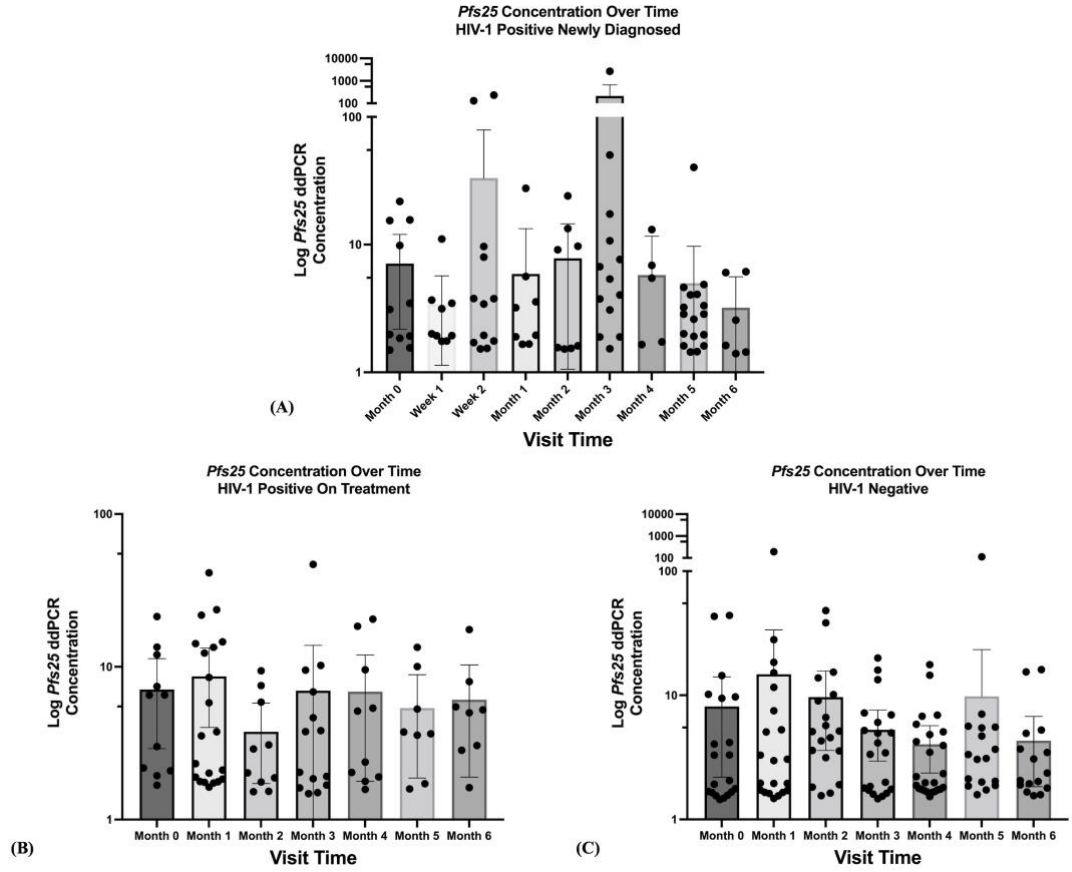

Figure 16. ddPCR Concentration of *Pfs25* by Study Group Over Time.
(A) HIV-1 positive newly diagnosed volunteers **(B)** HIV-1 positive volunteers on treatment **(C)** HIV-1 negative volunteers. Includes the mean bar and 95% confidence interval (above Log 1).

PfMGET Positive Transcripts and ddPCR Concentrations

At baseline, the odds of *PfMGET* positivity did not differ between study groups at any month except for month 1 (GEE Analysis). As with *Pfs25* transcript positivity, the odds of *PfMGET* transcript positivity in HIV-1 positive volunteers on treatment were higher at month 1 when compared to HIV-1 negative volunteers and HIV-1 positive newly diagnosed volunteers (aOR 3.08, adjusted p-value 0.046 and aOR: 7.33, adjusted p-value 0.009, respectively).

Within groups over time, there was a significant increase in *PfMGET* positivity for HIV-1 positive volunteers on treatment from month 0 to month 1 (aOR 5.43, adjusted p-value 0.024). HIV-1 positive newly diagnosed volunteers exhibited

a tendency for increased positivity at month 5 when compared to month 0 and at month 3 when compared to month 1 (Figure 17). HIV-1 negative volunteers exhibited an increase in positivity at months 3, 4, and 5 relative to month 0 (Figure 18).

After adjusting for multiple comparisons, *PfMGET* ddPCR concentrations (Figure 19) were not different between groups at each time point or over time within any group. Contrary to the prevalence analysis, the log transformed *18S* copy numbers did not predict *PfMGET* ddPCR concentrations (log transformed). There was no difference in *PfMGET* ddPCR concentrations by gender or age nor any predictive association between WBC, RBC, or CD4+ T-cell levels.

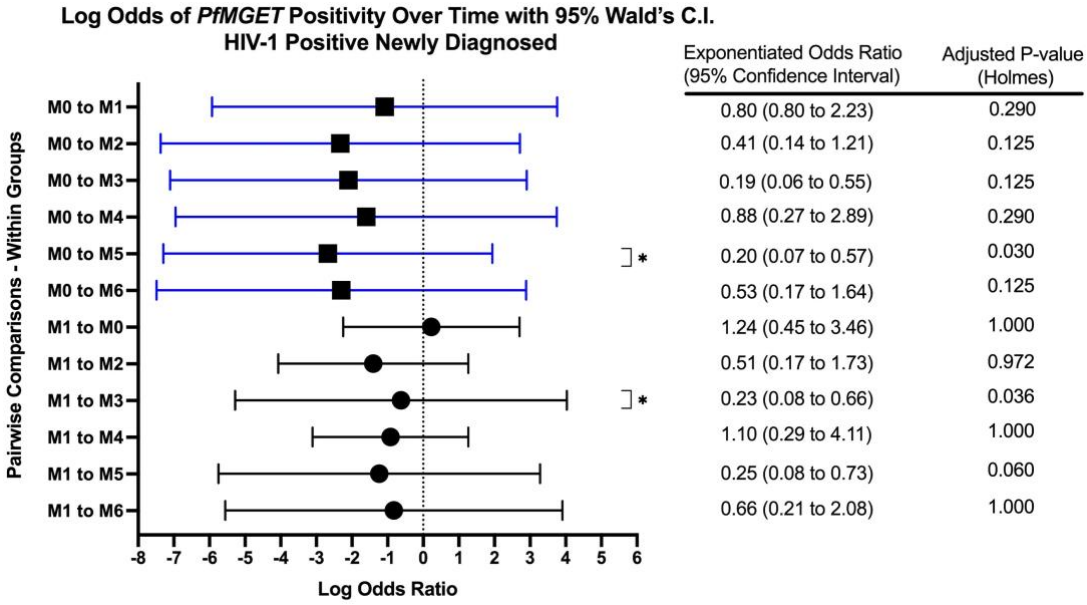

Figure 17. Forest Plot of the Log Odds of *PfMGET* Positivity in HIV-1 Positive Newly Diagnosed Volunteers.
The log odds and accompanying exponentiated adjusted odds ratio of being positive for at least one gametocyte specific transcript within the HIV-1 positive newly diagnosed study group between month 0 (M0) and between month 1 (M1). P-values were adjusted using the Holm's method for multiple comparisons. Blue confidence interval lines are comparisons with month 0 and black confidence interval lines are comparisons with month 1.

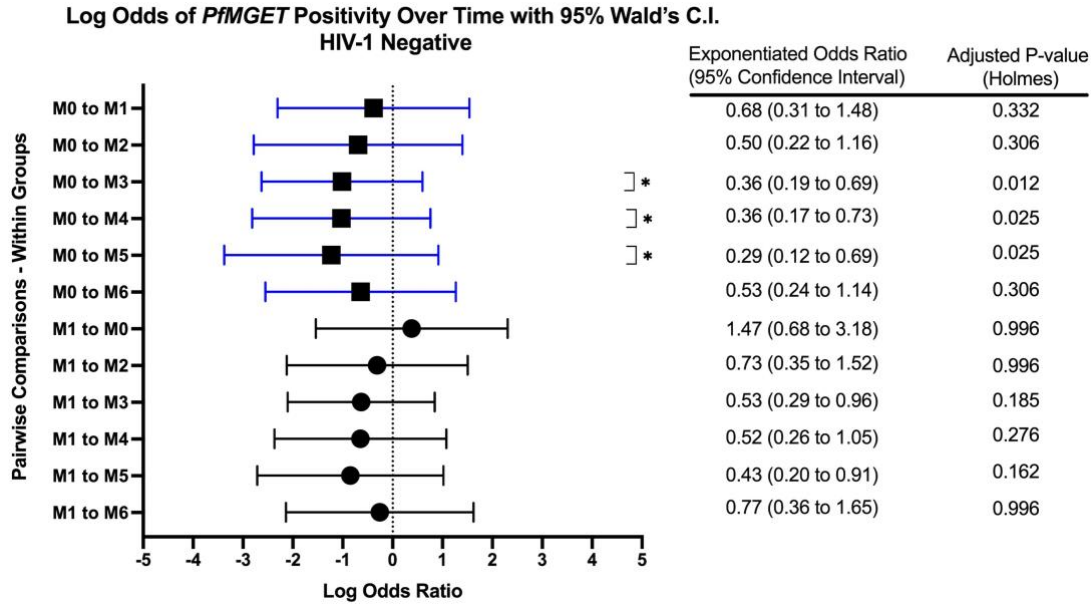

Figure 18. Forest Plot of the Log Odds of *PfMGET* Positivity in HIV-1 Negative Volunteers.

The log odds and accompanying exponentiated adjusted odds ratio of being positive for at least one gametocyte specific transcript within the HIV-1 negative study group between month 0 (M0) and between month 1 (M1). P-values were adjusted using the Holm's method for multiple comparisons. Blue confidence interval lines are comparisons with month 0 and black confidence interval lines are comparisons with month 1.

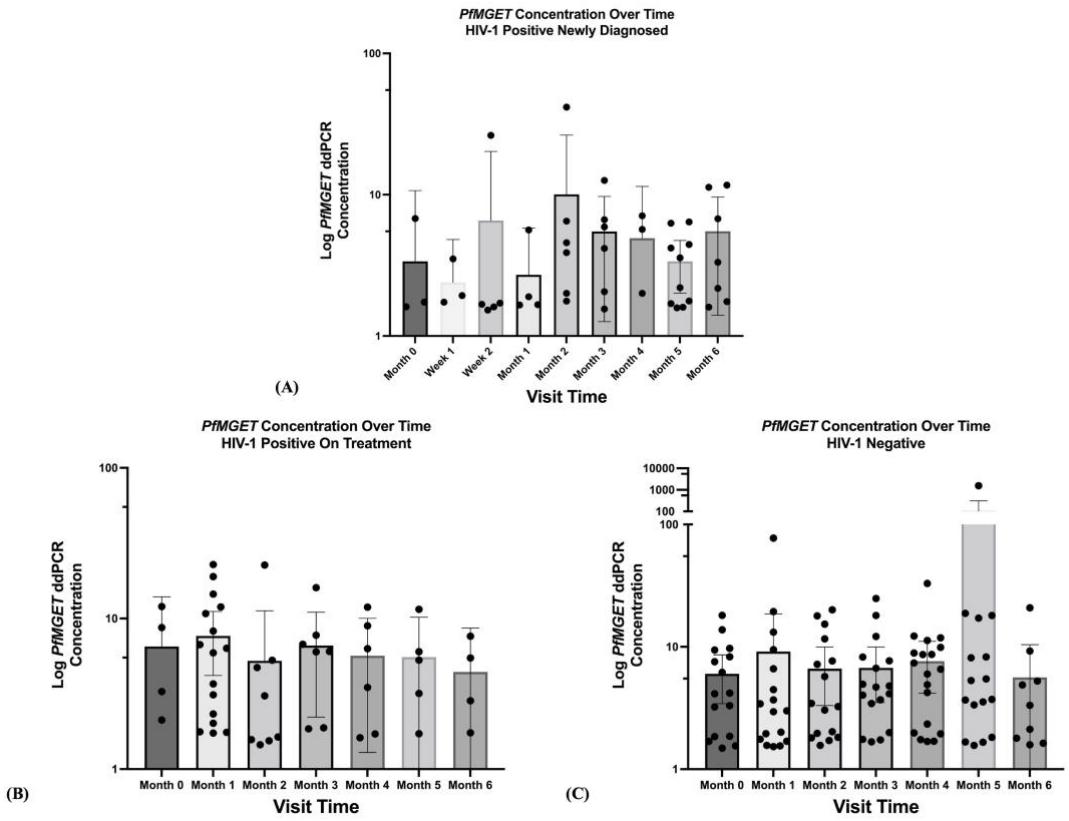

Figure 19. ddPCR Concentration of *PfMGET* by Study Group Over Time. **(A)** HIV-1 positive newly diagnosed volunteers **(B)** HIV-1 positive volunteers on treatment **(C)** HIV-1 negative volunteers. Includes the mean bar and 95% confidence interval (above Log 1).

Pfs16 Positive Transcripts

Due to low frequencies of *Pfs16* ddPCR positivity, GEE longitudinal analysis was not performed. However, we observed that *Pfs16* positivity trended downward from month 0 to month 1 in HIV-1 positive newly diagnosed volunteers (Figure 13).

Some Volunteers Remained 18S Positive Throughout the Entire Course of Study

Eight volunteers (all HIV-1 negative) were *18S* positive at every visit (two lost-to-follow up at month 6). We compared this group of eight volunteers to another group representing all volunteers who were not *18S* positive throughout the entire course of study to determine if they presented with any unique characteristics. While there were some sporadic positive associations with the defined variables (Table 5 and 6), there was no significant association between any predictor or visit. All eight

tested positive for malaria by RDT at some point during the study; therefore, they were treated with AL yet remained positive for asexual parasites.

Table 5. P-value chart comparing eight individuals who were 18S positive the entire study to all other individuals in the study who were not 18S positive the entire study (Mann-Whitney nonparametric test).
Green highlighted cells indicate significance (p-values) less than 0.05.
Predictors include *18S* copy numbers/µL, *Pfs25, PfMGET,* and *Pfs16* concentrations (Conc.), CD4+ T cell levels, white blood cell (WBC) counts (x 10^9/L), red blood cell (RBC) counts (x 10^{12}/L), hemoglobin (HGB) levels (g/L), neutrophils (NEU) (%), lymphocytes (LYM) (%), monocytes (MON) (%), platelets (PLT) (fL), and age.

	18S Copy # /uL	*Pfs25* Conc.	*PfMGET* Conc.	*Pfs16* Conc.	CD4+ T cells	WBC	RBC	HGB	NEU	LYM	MON	PLT	Age
Month 0	0.097	0.861	0.528	0.872	0.200	0.626	0.116	0.815	0.190	0.068	0.184	0.333	0.177
Month 1	0.011	0.873	0.920	0.416	0.025	0.835	0.242	0.827	0.416	0.847	0.063	0.051	
Month 2	0.035	0.580	0.049	0.613	0.230	0.664	0.401	0.802	0.140	0.494	0.834	0.468	
Month 3	0.070	0.592	0.625	0.255	0.125	0.275	0.068	0.653	0.386	0.106	0.939	0.110	
Month 4	0.026	0.492	0.719	0.396	0.058	0.043	0.080	0.779	0.212	0.420	0.584	0.100	
Month 5	0.002	0.475	0.833	0.270	0.180	0.585	0.027	0.305	0.971	0.758	0.045	0.178	
Month 6	0.068	0.201	0.616	0.128	0.101	0.328	0.233	0.646	0.412	0.266	0.029	0.049	

Table 6. P-value chart comparing eight individuals who were *18S* positive the entire study to all other HIV-1 negative individuals in the study who were not *18S* positive the entire study (Mann-Whitney nonparametric test).
Green highlighted cells indicate significance (p-values) less than 0.05.
Predictors include *18S* copy numbers/µL, *Pfs25, PfMGET,* and *Pfs16* concentrations (Conc.), CD4+ T cell levels, white blood cell (WBC) counts (x 10^9/L), red blood cell (RBC) counts (x 10^{12}/L), hemoglobin (HGB) levels (g/L), neutrophils (NEU) (%), lymphocytes (LYM) (%), monocytes (MON) (%), platelets (PLT) (fL), and age.

	18S Copy #/uL	*Pfs25* Conc.	*PfMGET* Conc.	*Pfs16* Conc.	CD4+ T cells	WBC	RBC	HGB	NEU	LYM	MON	PLT	Age
Month 0	0.150	0.926	0.960	0.482	0.378	0.248	0.249	0.881	0.157	0.139	0.080	0.344	0.733
Month 1	0.520	0.710	0.932	0.301	0.863	0.558	0.435	0.936	0.469	0.945	0.040	0.141	
Month 2	0.176	0.538	0.038	0.962	0.126	0.588	0.892	0.989	0.234	0.379	0.291	0.588	
Month 3	0.523	0.430	0.972	0.148	0.241	0.044	0.301	0.772	0.386	0.241	0.377	0.212	
Month 4	0.174	0.720	0.786	0.290	0.910	0.084	0.315	0.799	0.257	0.412	0.932	0.213	

Month 5	0.008	0.239	0.940	0.247	0.610	0.500	0.565	0.479	0.948	0.805	0.076	0.340
Month 6	0.391	0.233	0.569	0.491	0.511	0.051	0.726	0.677	0.381	0.484	0.069	0.222

Mosquito Infectivity was Not Associated with 18S Prevalence or Prevalence of Gametocyte-Specific Transcripts

A total of 184 blood samples used for SMFAs were analyzed for *18S* and all gametocyte markers regardless of *18S* positivity. A total of 34/184 groups of mosquitoes (one group per human blood sample) tested positive for oocysts (at least one mosquito with at least one midgut oocyst). Among the associated human blood samples collected on the day of or within 2 days of mosquito feeding for oocyst-positive SMFA, 50% (17/34) of these blood samples were negative for *18S* and 35% (12/34) were negative for all gametocyte markers. Among the associated human blood samples collected on the day of or within 2 days of mosquito feeding for an oocyst-negative SMFA, 54% were positive for *18S* and 47.7% were positive for at least one gametocyte marker (Table 7). By Chi-square analysis, there were no differences in the proportion of *18S* or gametocyte positive samples by study group or oocyst positivity.

Among the oocyst positive samples, a range of 12-39 mosquitoes per human blood sample were fed and dissected for oocysts. The mean number of total oocysts per mosquito grouping for *18S* positive samples was 1.94 oocysts (range 1-11 oocysts) while the mean for *18S* negative was 1.71 oocysts (range 1-7 oocysts) (Table 8). The mean number of total oocysts per mosquito grouping for gametocyte positive by ddPCR was 2.14 oocysts (range 1-11 oocysts) while the mean for gametocyte negative by ddPCR was 1.25 oocysts (1-3 oocysts). There was one mosquito grouping with a total oocyst count of 31 oocysts. This outlier sample was excluded from the mean and ranges calculated and was from a group of mosquitoes

fed on a HIV-1 negative sample with detectable parasites by *18S* and gametocytes by ddPCR. There was no statistical difference between oocyst counts by *18S* or gametocyte positivity or study group (unpaired t-test).

In an effort to determine whether any study variables predicted oocyst positivity (successful transmission), selected clinical and diagnostic predictors were analyzed using GEE analysis (Table 9). Overall, neither gametocyte positivity nor any individual gametocyte-specific marker were significant predictors of oocyst positivity. Additionally, none of the selected clinical markers were a significant predictor of oocyst positivity.

Due to lack of data at certain time points (Figure 20B), we were unable to compare oocyst positivity over time. However, for HIV-1 positive newly diagnosed volunteers, week 1 and week 2 account for 44.4% of all oocyst positivity. There were no oocyst positive samples in the subsequent month 1 and month 2.

Table 7. Oocyst Positivity by Study Group and Molecular Diagnostic Positivity. Number of samples of dissected mosquitoes (~25 per patient blood sample) that had at least one mosquito with at least one midgut oocyst (positive sample) or no mosquitoes with oocysts (negative sample). Each category includes the distribution by study group as well as the respective number of positive samples by molecular marker and their accompanying percentage of the total in each subgroup. *Volunteers can be positive for more than one gametocyte transcript marker. HIV (+) ND is HIV-1 positive newly diagnosed volunteers. HIV (-) is HIV-1 negative volunteers.

	Group: # Analyzed	***18S* Positive (% of total)**	***Pfs25* Positive (% of total)**	***PfMGET* Positive (% of total)**	***Pfs16* Positive (% of total)**	**Total**
Oocyst Positive	HIV (+) ND: 99	7 (38.9%)	9 (50%)	10 (55.6%)	1 (6%)	18
	HIV (-): 85	10 (62.5%)	6 (37.5%)	7 (43.8%)	0 (0%)	16
	Total	17 (50%)	15 (44.1%)	17 (50%)	1 (2.9%)	
Oocyst Negative	HIV (+) ND: 99	38 (46.9%)	27 (33.3%)	32 (39.5%)	7 (8.6%)	81
	HIV (-): 85	43 (62.3%)	22 (31.9%)	17 (24.6%)	3 (4.3%)	69
	Total	81 (54%)	49 (32.7%)	49 (32.7%)	10 (6.7%)	

Table 8. Oocyst Enumeration per Oocyst Positive Samples.

The total number of oocysts calculated per grouping of mosquito dissections in oocyst positive samples.

Subset sample	HIV-1 group	Gametocyte Positive vs Negative (ddPCR)	Parasite Positive vs Negative (*18S* qPCR)	Number of Mosquitoes Dissected	Oocysts per Midgut for Each Dissected Mosquito	Total Oocyst Count
1	HIV-1 Neg.	Negative	Negative	25	[0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,2,0,0,0,0,0,0,0,0,0]	2
2	HIV-1 Pos. ND	Positive	Negative	25	[0,0,3,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	3
3	HIV-1 Pos. ND	Negative	Positive	23	[0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,1]	1
4	HIV-1 Pos. ND	Positive	Negative	25	[0,6,0,0,0,1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	7
5	HIV-1 Neg.	Positive	Negative	25	[1,0,0,0,0,0,0,0,0,0,3,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	4
6	HIV-1 Neg.	Positive	Negative	26	[0,1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	1
7	HIV-1 Pos. ND	Positive	Negative	26	[0,0,0,1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	1
8	HIV-1 Pos. ND	Positive	Positive	25	[0,0,0,0,0,1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	1
9	HIV-1 Pos. ND	Positive	Negative	25	[0,0,1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	1
10	HIV-1 Pos. ND	Positive	Negative	26	[0,0,0,0,1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	1
11	HIV-1 Pos. ND	Positive	Positive	26	[0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,1,0,0,0,0,0]	1
12	HIV-1 Pos. ND	Negative	Positive	39	[0,0,0,0,1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	1
13	HIV-1 Pos. ND	Negative	Negative	39	[0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,1,0]	1
14	HIV-1 Pos. ND	Negative	Negative	12	[0,0,0,0,0,0,1,0,0,0,0,0]	1
15	HIV-1 Neg.	Negative	Positive	25	[1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	1
16	HIV-1 Pos. ND	Negative	Negative	26	[0,0,0,0,0,1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	1
17	HIV-1 Pos. ND	Positive	Positive	22	[0,0,0,8,0,0,0,0,0,0,0,3,0,0,0,0,0,0,0,0,0,0]	11
18	HIV-1 Neg.	Negative	Negative	25	[0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,1,0,0,0,0,0,0,0]	1
19	HIV-1 Neg.	Positive	Positive	25	[0,4,0,0,0,0,26,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,1,0,0,0,0]	31
20	HIV-1 Pos. ND	Positive	Negative	24	[0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,1,0,0,0,0]	1
21	HIV-1 Neg.	Positive	Negative	25	[0,0,1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	1
22	HIV-1 Neg.	Negative	Positive	27	[0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,1,0,0,0,0,0,0,0,0,0,0,0,0]	1
23	HIV-1 Neg.	Negative	Positive	26	[0,3,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	3
24	HIV-1 Pos. ND	Positive	Positive	25	[0,0,0,0,0,0,2,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	2

25	HIV-1 Neg.	Positive	Positive	26	[0,0,0,0,0,0,0,0,0,1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	1
26	HIV-1 Neg.	Negative	Negative	24	[0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,1,0,0]	1
27	HIV-1 Neg.	Negative	Positive	25	[0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,1,0,0,0,0]	1
28	HIV-1 Pos. ND	Positive	Negative	24	[0,1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	1
29	HIV-1 Neg.	Positive	Positive	25	[0,1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	1
30	HIV-1 Neg.	Positive	Positive	25	[0,0,0,0,0,1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	1
31	HIV-1 Pos. ND	Positive	Positive	25	[0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,3,0,0,0,0,0]	3
32	HIV-1 Pos. ND	Positive	Negative	24	[0,0,0,0,0,0,0,0,0,0,1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	1
33	HIV-1 Neg.	Positive	Positive	25	[0,0,1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	1
34	HIV-1 Neg.	Positive	Positive	25	[0,0,0,0,0,1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0]	1

Table 9. GEE Transmission Omnibus Results.

Predicting overall oocyst positivity using gender, study group, age, CD4+ T cell levels, hemoglobin (HGB) levels (g/L), red blood cell (RBC) counts (x 10^{12}/L), binary results for positivity of gametocyte-specific transcripts (positive vs negative), and log *18S* copy numbers/µL). When log transforming *18S* copy numbers/µL undetermined results were replaced with "0.01" for the transformation. HIV+ ND is HIV-1 positive newly diagnosed volunteers. HIV- is HIV-1 negative volunteers. *Specific gametocyte transcript positivity was analyzed using a separate GEE model than the accompanying predictors.

Predictor of Oocyst positivity	**Exponentiated aOR**	**95% Wald Confidence Interval**	**Adjusted p-value (Holm's)**
Gender (Male:Female)	0.613	0.184 to 2.045	1.000
Study Group (HIV+ ND:HIV-)	1.133	0.409 to 3.140	1.000
Age	0.951	0.906 to 0.998	0.336
CD4+ T cells	1.001	0.999 to 1.002	1.000
HGB	0.975	0.718 to 1.325	1.000
RBC	0.652	0.211 to 2.014	1.000
Gametocyte Positivity (Binary)	1.895	0.842 to 4.264	0.854
Pfs25 Positivity (Binary)*	1.056	0.403 to 2.766	1.000
PfMGET Positivity (Binary)*	1.790	0.626 to 5.119	1.000
Pfs16 Positivity (Binary)*	0.578	0.090 to 3.697	1.000
Log *18S* Copy numbers/µL	0.984	0.840 to 1.153	1.000

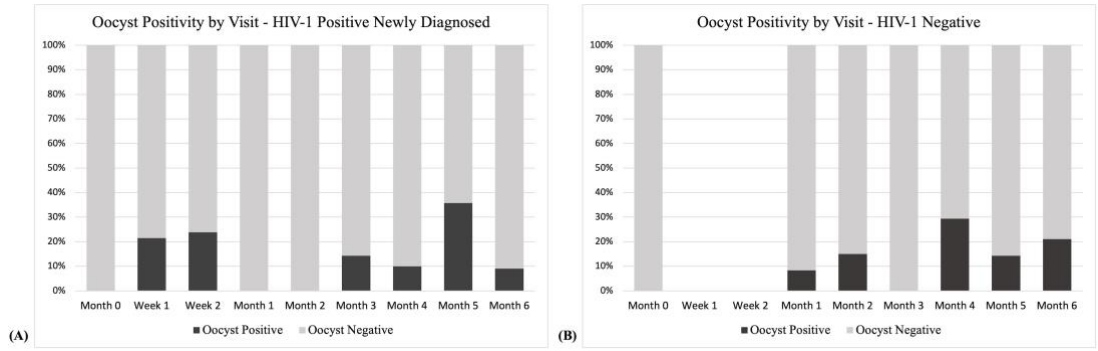

Figure 20. Oocyst Positivity by Study Group and Time.
A distribution of the total number of oocyst positive samples as percentage of the total analyzed at each time point for **(A)** HIV-1 positive newly diagnosed volunteers and **(B)** HIV-1 negative volunteers. As part of the study design, HIV-1 negative volunteers did not have a scheduled visit at week 1 or week 2, so no samples were analyzed at these time points.

RDT Positivity at Baseline Was Positivity Associated with Gametocyte Prevalence at Baseline for HIV-1 Positive Newly Diagnosed Volunteers

If a participant was HIV-1 positive newly diagnosed and RDT positive at baseline (month 0), they had a significantly higher probably of also being positive for at least one gametocyte specific transcript when compared to HIV-1 negative volunteers and HIV-1 positive volunteers on treatment (Chi-square test *p*-values 0.005 and 0.001, respectively) (Table 10).

Table 10. Comparison of Gametocyte Positivity at Month 0 by RDT positivity and Study Group
A comparison by study group of the interaction between gametocyte positivity and RDT positivity at baseline (month 0). Numbers of positive or negative samples and the percentages (in paratheses) of the total number analyzed are provided. HIV (-) is HIV-1 negative volunteers. HIV (+) ND is HIV-1 positive newly diagnosed volunteers. HIV (+) OT is HIV-1 positive volunteers on treatment for ART and TS.

Study Group		RDT Negative (% of Total)	RDT Positive (% of Total)	Total
HIV (-)	Negative for Gametocytes	26 (68.4%)	12 (31.6%)	38
	Positive for Gametocytes	20 (64.5%)	11 (35.5%)	31
HIV (+) ND	Negative for Gametocytes	19 (70.4%)	8 (29.6%)	27
	Positive for Gametocytes	4 (20.0%)	16 (80.0 %)	20
HIV (+) OT	Negative for Gametocytes	13 (100%)	0 (0.0%)	13
	Positive for Gametocytes	11 (84.6%)	2 (15.4%)	13

Impact of ART and Antimalarials on Gametocyte Positivity After Treatment

If a volunteer was positive for malaria by RDT at any time, they were treated with AL in accordance with Kenyan MoH guidelines. Among the 782 samples that were subsequently tested for gametocytes (*18S* positive), HIV-1 positive newly diagnosed volunteers had a significantly lower probability of being gametocyte positive in the subsequent month (month 1) after initiation of ART, TS, and AL at month 0 (RDT positive) compared to HIV-1 negative volunteers after initiation of just AL at month 0 (Table 11 and Figure 21A-B). The difference was significant using Pearson's Chi-square (*p*-value: 0.003) despite there being no statistical difference in asexual parasite clearance (*18S* positivity) (*p*-value: 0.1892) (Table 11 and Figure 21C). Comparisons between HIV-1 volunteers on treatment were not conducted because only two samples were positive by RDT at month 0.

This trend was only observed from month 0 to month 1 when comparing gametocyte positivity in the subsequent month after testing positive for malaria by RDT. There were no other time points that showed a significant difference in gametocyte positivity after prescribed antimalarial treatment.

In an effort to determine which intervention (ART and TS or AL) was associated with the change in gametocyte prevalence, we compared the same groups that were malaria RDT positive at month 0 to those that were malaria RDT negative at month 0. HIV-1 newly diagnosed volunteers no longer had a significantly lower or higher probability of being gametocyte positive in the subsequent month (month 1) after initiation of HIV-1 treatment compared to HIV-1 negative volunteers who were RDT negative (*p*-value 0.231) (Table 11 and Figure 21A). HIV-1 negative volunteers who did not receive AL (RDT negative) exhibited a decrease in the percentage of individuals who were positive for gametocytes at month 1 (Table 11 and Figure

21B). Neither group received AL treatment; however, HIV-1 positive newly diagnosed volunteers still received ART and TS. Using a proportions test to compare the two different RDT groups (AL vs no AL), there was a significant difference in the ratio of gametocyte positivity at month 1 for the HIV-1 negative volunteers (*p*-value 0.023) while HIV-1 positive newly diagnosed volunteers maintained a similar difference in the ratio of gametocyte positivity between the two categories (*p*-value 1.000).

Table 11. Comparison of Gametocyte and *18S* Positivity at Month 0.
Gametocyte prevalence by ddPCR after malaria treatment at month 1 if the volunteer was positive by RDT at month 0. Gametocyte prevalence by ddPCR in the absence of malaria treatment at month 1 if the volunteer was negative by RDT at month 0. *18S* prevalence by qPCR after malaria treatment at month 1 if the volunteer was positive by RDT at month 0.

	Study Group	**Positive for Gametocytes** (% within group)	**Negative for Gametocytes** (% within group)	**Total**
RDT Positive Month 0	HIV (-)	**11** (73.3%)	**4** (26.7 %)	15
	HIV (+) ND	**2** (16.7%)	**10** (83.3%)	12
	Total	13	14	27
RDT Negative Month 0	**Study Group**	**Positive for Gametocytes** (% within group)	**Negative for Gametocytes** (% within group)	**Total**
	HIV (-)	**12** (42.9%)	**16** (57.1 %)	28
	HIV (+) ND	**1** (16.7%)	**5** (83.3%)	6
	Total	13	21	34
RDT Positive Month 0	**Study Group**	**Positive for *18S*** (% within group)	**Negative for *18S*** (% within group)	**Total**
	HIV (-)	**15** (78.9%)	**4** (21.1 %)	19
	HIV (+) ND	**12** (54.6%)	**10** (45.4%)	22
	Total	27	14	41

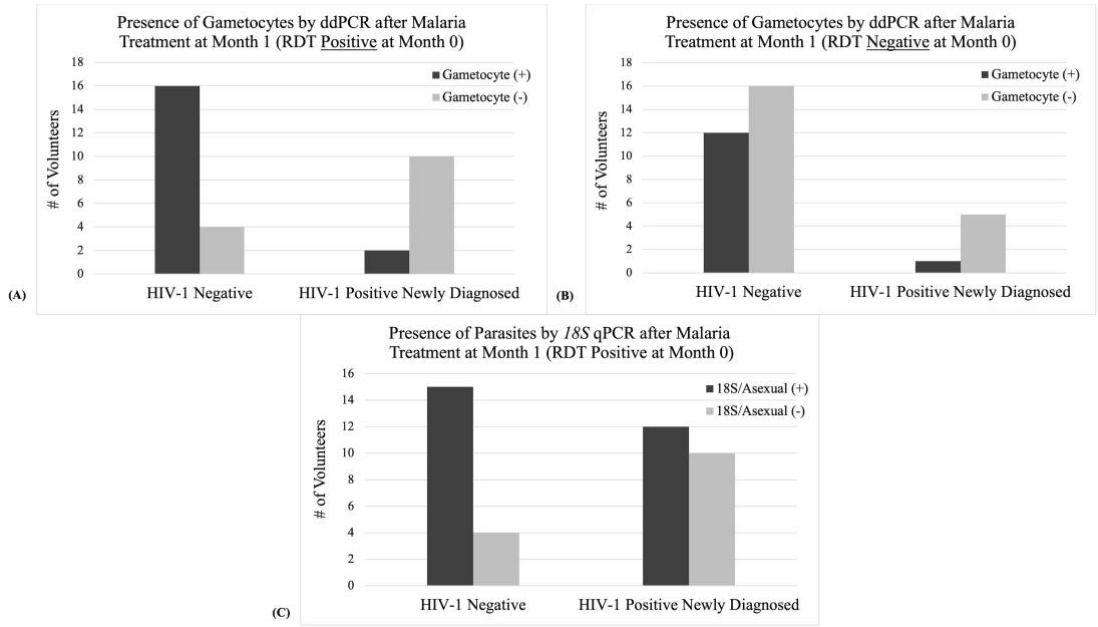

Figure 21. Presence of Gametocytes and Asexual Parasites at Month 1 by Study Group

The column graph on the top left (A) shows the presence of gametocytes by ddPCR after AL treatment at month 1 if the volunteer was positive for malaria by RDT at month 0. The column graph on the top right (B) shows the presence of gametocytes by ddPCR without AL treatment at month 1 if the volunteer was negative for malaria by RDT at month 0. The column graph on the bottom center (C) shows the presence of asexual parasites by 18S qPCR after AL treatment at month 1 if the volunteer was positive for malaria by RDT at month 0.

Discussion

This study investigated the longitudinal impact of HIV-1 co-infection and drug treatment on gametocyte transcript prevalence and parasite transmission to *A. gambiae* from asymptomatic volunteers in a region of holoendemic malaria transmission. The overall prevalence for at least one gametocyte specific transcript in parasite carriers (*18S* positive) across all groups and at all time points was 51.1% (400/782) using ddPCR assays. The GEE models showed that there was no significant effect on, or interaction between, the HIV-1 status and/or the visit number (time) and gametocyte positivity. However, as expected, there was a significant relationship between the log transformed *18S* copy numbers and gametocyte transcript prevalence (aOR: 1.202, adjusted *p*-value 0.018). The odds of being

gametocyte positive varied by HIV status and time point, but there was no significant predictive interaction. There was a tendency for increased *Pfs25* and *PfMGET* transcript prevalence after month 0 and/or month 1 (Figures 14,15,17, and 18). For HIV-1 positive newly diagnosed volunteers, the initiation of ART and TS at month 0 (despite receiving antimalarials if RDT positive) was associated with a significant impact on the reduction of gametocyte transcript prevalence in the subsequent month (Figure 21A). Interestingly, 50% of the blood samples that were associated with mosquito infection by oocysts were negative for malaria by *18S* qPCR and 35% were negative for all gametocyte specific transcript markers by ddPCR. There were no significant differences in HIV-1 status, time, or gametocyte prevalence and oocyst positivity.

In areas of holoendemic malaria transmission, such as the study area near Kisumu, Kenya, clinical immunity to malaria develops during childhood and continues into adulthood due to the frequency of new infections (76; 206). The high density of semi-immune asymptomatic individuals likely serves as a primary reservoir for malaria parasite transmission. Among the 300 malaria asymptomatic volunteers enrolled in the study, 263 (87.7%) were positive for malaria parasites by *18S* qPCR at least once throughout the 6-month course of the study and 73% of those malaria positive volunteers were positive for gametocyte specific transcripts at least once by ddPCR. With this longitudinal study design, we could include patients that shifted from detectable to undetectable parasitemias and gametocytemias, a cyclic commonality well described for malaria parasites (54). When compared to the baseline point prevalence (47.7% parasite prevalence and 45.5% gametocyte prevalence of those parasite positive) (Figure 6), the inclusion of multiple time points increased the ability to detect parasites in individuals by 84 percent and to detect

gametocytes in individuals by 60 percent. The high positivity seen throughout the study reflect the endemicity of asymptomatic parasitemias and the fact that semi-immune individuals, regardless of HIV-1 status, cycle between detectable and undetectable parasitemias and gametocytemias. Eight HIV-1 negative volunteers were positive for malaria parasites by *18S* qPCR every month over the 6-month study period; however, they were not positive for gametocytes at every time point. From this study, we were unable to identify any unique predictors about these individuals and why they maintained chronic parasitemias despite prescribed AL treatment.

HIV-1 and malaria co-infection has been associated with poor clinical outcomes (87; 107; 129; 134), but little is known about the impact of asymptomatic co-infection on malaria parasite transmission potential. In our previous point-prevalence study of parasites and gametocytes in asymptomatic individuals at the time of HIV-1 testing (n=1,116), the relative risk for gametocyte positivity was 1.82 higher for HIV-1 positive samples than HIV-1 negative samples (231). However, when evaluating the point prevalence at baseline (n=143) of our current study, we did not see the same trend. In fact, gametocyte positivity was nearly equal between HIV-1 negative and HIV-1 positive (32/70 and 33/73 respectively) (Figure 6). It is likely that the sample size in this longitudinal study was not large enough to replicate the same results.

An important aim of our longitudinal study was to evaluate the impact of ART and TS treatment on asymptomatic gametocytemias over an extended period of time. The antifolate drugs TS and sulfadoxine-pyrimethamine (SP) share pharmacological similarities and SP resistance is well described in the geographical area of our study (20; 168). TS has been described to have an inhibitory effect on

gametocyte burden and parasite transmission in *in vitro* models (108; 109). Another study demonstrated that antifolate therapy enhances gametocytemia almost immediately after drug initiation and peaks at about two weeks into therapy (40), but there was only one month of follow-up in that study. Our longitudinal study design included visits for HIV-1 newly diagnosed volunteers at one week and two weeks post initiation of ART and TS. Within those two weeks, there was an observed increase in *Pfs25* and *PfMGET* prevalence with a decrease in *Pfs16* prevalence (Figure11A). Unfortunately, due to the study design, week 1 and week 2 data were not collected on HIV-1 negative volunteers to use as a reference for statistical analysis and, therefore, the HIV-1 positive newly diagnosed data for these time points was not included. However, throughout the course of our longitudinal study we did not observe any significant reduction in gametocyte transcript prevalence or ddPCR concentration that could be associated with ART or TS treatment.

Based on GEE analysis to compare results from month 0 to month 6, HIV-1 positive newly diagnosed volunteers exhibited a tendency for increased gametocyte positivity after month 1 and a drop in positivity at month 4. HIV-1 negative volunteers also exhibited a moderate tendency for increased gametocyte positivity over time with an inexplicable drop in positivity at month 5. A tendency for early increased gametocyte positivity after antifolate treatment was reported by Bousema et al. (40), but the increases we observed were independent of HIV status and treatment. The unexplained drops were not a result of seasonality as the drops fell on different calendar months (Figure 9), suggesting a biological pattern that deserves further study. There were significant differences among groups, but there were no differences between groups over time. Given that only 42% of the 300 enrolled volunteers presented at every visit/time point which limited our analyses, the lack of

significance could have resulted from high malaria prevalence and sample sizes that were too small to detect differences in positivity.

Within the HIV-1 positive newly diagnosed group, we observed a steep increase in *PfMGET* positivity between enrollment (month 0) to week 2 (8% to 31%) (Figure 11A). While positivity by the gametocyte specific transcript marker *PfMGET* is not a direct indicator of the quantity of male gametocytes present, it does indicate that at least one male gametocyte was present in the dried blood spot. Changes in quantity of male gametocytes have been associated with anemia, parasitic competition, and changes in immunity [reviewed in (203)]. Anemia (RBC and/or hemoglobin levels) was analyzed in this study, but there were no significant associations between *PfMGET* positivity or any other gametocyte specific marker with RBC or hemoglobin levels. Parasites are believed to alter their sex ratios with an increase in male gametocytes as a form of fertility insurance (197; 201). This prediction also suggests that transmission blocking immunity (TBI) might drive an increase in male gametocyte production. While further study is required to test mechanism, it is possible the first two weeks of ART and changes to host immunity contribute to increased positivity of male specific transcripts to a level that was equal to that of female specific transcript positivity.

We also examined associations with antimalarial treatment in the subsequent month after treatment. Compared to HIV-1 negative volunteers, there was a significant difference in gametocyte positivity at month 1 for the HIV-1 newly diagnosed volunteers who were RDT positive at month 0 and prescribed AL (*p*-value 0.003) (Table 10 and Figure 21A). Interestingly, a significantly lower proportion of samples from HIV-1 positive newly diagnosed volunteers were gametocyte positive Comparing the groups at the same time point but without antimalarial treatment,

gametocyte prevalence outcome for HIV-1 positive newly diagnosed volunteers stayed the same while the outcome for HIV-1 negative volunteers changed. In samples without AL treatment at month 0, there was a significantly higher likelihood for lower gametocyte positivity than if they were treated (*p*-value 0.023). These observations suggest that the combination of ART and TS with antimalarials could be associated with increased clearance of gametocytes compared to AL treatment alone, which was associated with increased gametocyte prevalence.

Gametocyte prevalence is often described to have an association with transmission potential, simply because gametocyte maturation is the precursor required for parasite fertilization and oocyst development in the mosquito. However, transmission potential and success should not be defined without the context of infectivity to the mosquito through oocyst or sporozoite enumeration (159). This concept remains true regardless of improved methods for detection of gametocytes. There were no significant predictors of oocyst positivity or oocyst enumeration, including HIV-1 status, log transformed *18S* copy numbers/µL or presence or concentration of common gametocyte specific markers. In fact, only 65% of mosquito groups considered to be oocyst positive were associated with gametocyte-positive volunteer blood samples collected on the day of or within 2 days of SMFA. Further, half of all blood samples associated with oocyst-positive mosquito groups failed to test positive for parasites by *18S* qPCR (Table 7). It is well known that submicroscopic infections can successfully transmit to mosquitoes [reviewed in (16; 166)], but our data show that transmission can also occur at densities that are below detection of highly sensitive molecular assays. This underscores recent findings from a controlled human malaria infection that show infectivity occurred at rates as low as

1.6 gametocytes/μL of blood (5 million RBC) (67), and confirms that actual mosquito infectivity remains the only accurate measurement of transmission.

Increased *PfMGET* positivity from enrollment (month 0) to week 2 for HIV-1 newly diagnosed volunteers was associated with 44% of all oocyst-positive mosquito groups fed on volunteer blood in weeks 1 and 2 from this group (Figure 20A). Despite a lack of data from the HIV-1 negative control group for comparison, these observations suggest that analyses of the effects of ART and TS on parasite sex ratio and potential for transmission success to mosquitoes might be warranted. Altered sex ratio could drive diversity of infecting drug resistant genotypes. Competition and inbreeding can increase male gametocyte production (199), so drug resistant parasite genotypes might infect mosquitoes more efficiently (149; 152).

The use of ddPCR, which is more sensitive than microscopy or qPCR (244), requires multiple calculations and assumptions to determine absolutely copy number concentration that can be a limitation of this technology (131; 244). The use of plasmid standards to compare concentrations versus copies/μL yielded agreement, but the concentrations were always smaller than the equivalent copies/μL determined by standards in qPCR. Many of the samples analyzed were positive for gametocyte specific transcripts but at very low ddPCR concentrations that made it difficult to compare concentrations between samples. Additionally, ddPCR assays require a user to set a threshold to determine the cutoff amplitude at which a droplet is either positive or negative. The separation between positive and negative droplets specifically within the *PfMGET* marker included "rain" droplets that were difficult to classify as positive or negative. Rain droplets can be due to false positive readings, an inhibited or "lagging" PCR reaction, coagulation of multiple droplets, or primer-target mismatch (237; 253). To design a non-biased approach for setting the

threshold for the *Pfs25* and *PfMGET* duplex assay, we used a conservative method to determine concentrations (ddpcRquant) (253). In optimizing the *Pfs16* marker for ddPCR, we observed more distinct separation between positive and negative droplets (rain droplets were not common) which allowed a manual threshold to be set with minimal bias.

While we did not observe any significant associations with predicted clinical factors and HIV-1 status in this longitudinal study, our data highlight some of the complexities surrounding HIV-1 malaria co-infection and transmission potential to mosquito vectors. We suggest that future studies should focus on the impact that ART, antimalarials, and antifolate drugs have on gametocyte burden and subsequent mosquito infectivity using more frequent visits over time. Additionally, we recommend that future studies should closely monitor changes in parasite genotypes and chronicity of infection over a longer study duration to better track changes in CD4+ T-cell counts. From a global health perspective, our findings highlight the ongoing need to focus on areas with high asymptomatic infection rates and complex transmission patterns and to support malaria elimination strategies.

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CHAPTER 4: *Plasmodium vivax* Gametocyte Kinetics Using Orthologous qPCR Assays in *Aotus nancymaae* Monkeys and Febrile Patients in Peru

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Abstract

Despite the widespread geographical distribution of *Plasmodium vivax*, this species of malaria is often understudied. Certain biological aspects of the species make it difficult to grow in culture and subsequently challenge our research and development efforts in the global fight for malaria control and elimination. A critical component to malaria elimination involves reduction and prevention of transmission which includes understanding the biology and kinetics of the sexual stage parasite, the gametocyte. In this study, we used orthologous genetic markers from known *P. falciparum* gametocyte genes to evaluate *P. vivax* gametocyte-specific transcript expression in *Aotus nancymaae* sporozoite induced infections as well as symptomatic human infections in Peru. Our assay validation experiments resulted in highly sensitive and specific qPCR assays targeting the orthologs *Pvs25, PvMGET, Pvs16,* and *PvAP2-G.* In both human and monkey infections, *Pvs25* transcript abundance and prevalence was significantly higher than the other molecular markers and often expressed before initial parasite detection by microscopy in monkey infections. Additionally, in the monkey infections, we observed an increase in gametocyte transcript sex-ratio (female to male) for two to five days after mefloquine treatment. The combination of qPCR assays proved their utility in the field by detecting 32.3% more gametocyte positive samples than by microscopy. Overall, these assays and data contribute to our understanding of *P. vivax* gametocyte kinetics and help guide future research towards reducing malaria transmission.

Introduction

Plasmodium vivax is geographically the most widely distributed cause of human malaria, with 2.5 billion people at risk every year. Because the parasite is extremely

difficult to grow in culture, *P. vivax* malaria is not as well studied as the more readily cultured *P. falciparum* malaria. However, understanding the unique biology of *P. vivax* may help advance efforts to control and eliminate all *Plasmodium* species. Gametocytes, the sexual stage responsible for onward parasite transmission to mosquitoes, are present in the absence of clinical symptoms (42; 157), and due to a shorter gametocytogenesis (as compared to *P. falciparum*), *P. vivax* infections may be able to successfully transmit the disease to mosquitoes before any clinical presentation and prescribed treatment regimen. The successful transmission of any *Plasmodium* parasite depends on the presence of both male and female gametocytes in the circulating blood at the time of a mosquito blood-meal. The male to female ratio and densities are important elements in the malaria transmission cycle, yet these ratios are not well understood in *P. vivax*.

Advanced gametocyte-specific genomic research on *P. falciparum*, to include RNA-sequencing, has described certain gametocyte molecular markers (138; 148; 216; 233; 262) which have become valuable tools in understanding *P. falciparum* gametocytes. Additional data from advanced genomic studies between *P. vivax* and *P. falciparum* genomes have been described and highlight important *Plasmodium* genetic similarities and differences (57); however, their genomes are similar enough to identify and potentially utilize genetic orthologs in *P. vivax* molecular assays to understand overall gametocytemia, gametocyte sex-ratio, and gametocytogenesis.

In the absence of a sufficient *ex vivo P. vivax* laboratory model, leaders in the field of *P. vivax* research developed *P. vivax* - animal model using *Aotus nancymaae* owl monkeys. In this model, the *Ao. nancymaae* demonstrates similar biological and immunological responses to human malaria infections, making the model ideal for *P.*

vivax gametocyte studies (68). In order for *P. vivax* merozoites to invade the host erythrocyte, they must bind with the Duffy antigen (Fy) receptor on the erythrocyte surface (146). Like humans, *Ao. nancymaae* species have duffy-positive erythrocytes, making the species permissive to induced pre-erythrocytic infections.

In South America, four countries accounted for 82% of all malaria cases in 2020: The Bolivarian Republic of Venezuela, Brazil, Colombia, and Peru (176). All four countries share a unique border — the Amazon Rainforest (Figure 22). While many South American countries have seen a decrease in malaria incidence and mortality since 2000, the country of Peru has seen a greater than 48% increase in incidence from their lowest incidence year, 2011, until 2019 (176). Data from 2020 and 2021 require a unique interpretation due to the restrictions in movement and subsequent changes in occupational exposure risk and access to care from the COVID-19 pandemic. In 2020, the Peruvian Amazonian region of Loreto accounted for approximately 84.5% of all Peruvian cases and had an approximate distribution of *P. vivax* and *P. falciparum* of 78% and 22% respectively (62). In addition to the increase in incidence, the region has seen a change in *P. vivax* disease severity. *P. vivax* has historically been considered a more benign malaria infection; however, recent evidence has shown an increase in disease severity (17; 88; 196).

In this study, we developed novel real-time PCR (qPCR) assays to detect *P. vivax* gametocytes using orthologous genetic markers *PvMGET*, *Pvs25*, *Pvs16*, and *PvAP2-G*. These qPCR assays were validated to determine the sensitivity, specificity, limit of detection, and limit of quantification/linearity. The assays were then applied to controlled monkey infections to determine the kinetics of these markers to include the timeline of

detection during an infection, the changing sex ratios, and gametocyte prevalence after treatment. Continued characterization of these molecular markers was further assessed in the field by identifying and quantifying gametocyte expression in febrile human patients in Peru, mainly in the Loreto region. These gametocyte specific *P. vivax* assays can be utilized to better understand *P. vivax* gametocyte kinetics and the epidemiology of infection in support of global elimination efforts.

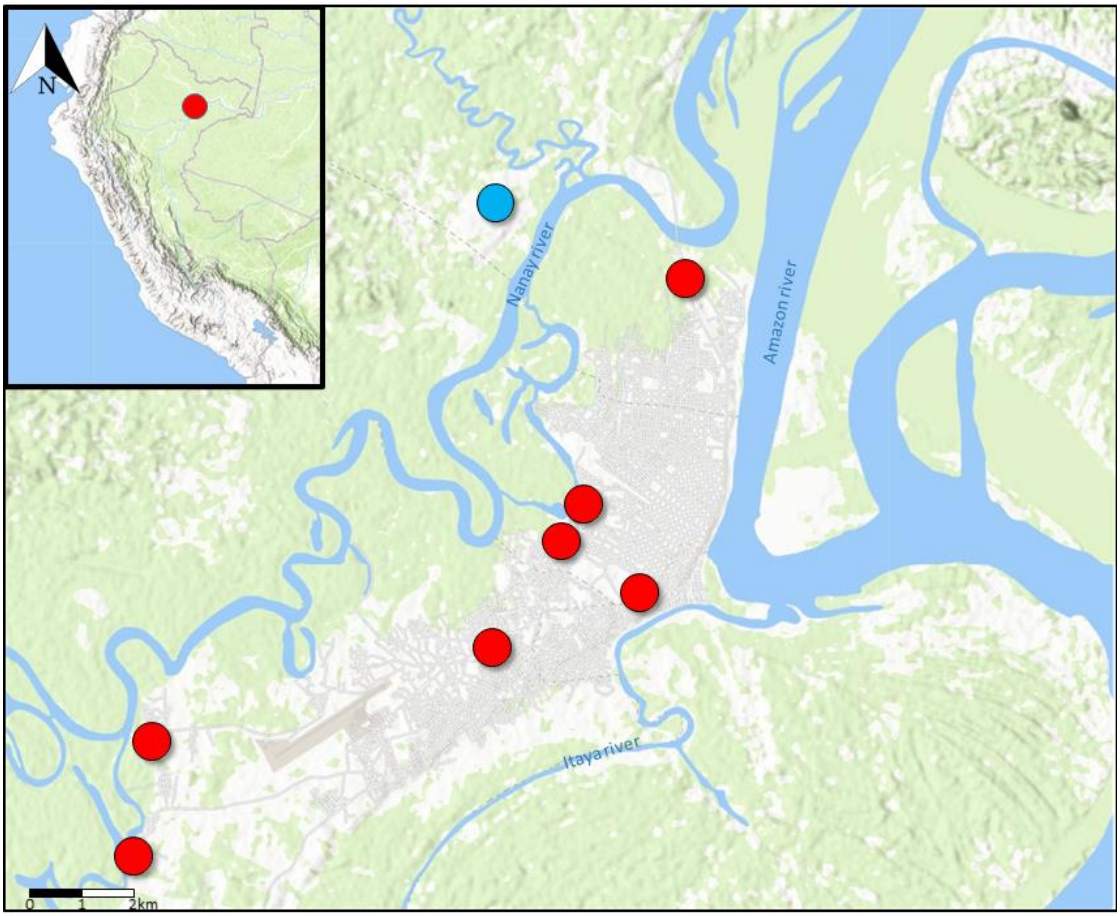

Figure 22. Locations of Study Sites in the Loreto Region of Peru.
Red circles are Peruvian health center study sites. The blue circle is the Iquitos Apoyo Hospital study site.

Methods

Selection of Marker Transcripts

Four genes were selected to detect overall gametocytemia, gametocyte sex ratio, and gametocytogenesis. *P. vivax* orthologs were selected from PlasmoDB (https://plasmoDB.org) or OrthoMCL DB (https://orthomcl.org) relative to known *P. falciparum* molecular markers (Table 11). In *P. falciparum* infections, *Pfs16* is an early molecular marker of gametocytes (31) which has shown high transcript levels with a relatively equal female to male ratio of 1.28:1.00 (138); therefore, the ortholog *Pvs16* was selected to putatively detect overall gametocytemia. The male marker *PvMGET* was identified as potential ortholog to the male maker *PfMGET*, which has been shown to have more abundant gene transcripts than other previously used male markers (138). The most well described *P. vivax* gametocyte marker, *Pvs25*, is highly expressed in mature female gametocytes (4; 138; 262). Transcriptional factor *PfAP2-G*, a master regulator for gametocyte commitment, was identified early in *P. vivax* patients with the ortholog *PvAP2-G* (4; 119) and was used as a potential marker for gametocytogenesis.

Primer and Probe Design

Apart from *Pvs25*, primer pairs and probes (Table 1) were designed using the OligoArchitect™ Online (Sigma-Aldrich, St. Louis, MO, USA). *Pvs25* primer pairs and probes were adapted as previously described (33; 263). Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA), and custom TaqMan QSY probes by Applied Biosystems (Applied Biosystems, Foster City, CA, USA).

Table 12. qPCR Primers and Probe Sequences.

Pv Gene ID	Pf Ortholog	Relevance and Product	Primers and Probes

PVX_116970	PF3D7_146990	**Male** (Male Gametocyte-Enriched Transcript: MGET)	**Forward**: 5'- CCCCGCCAGTTTACTTCTCA - 3' **Reverse**: 5'- ATGCGCATTCGGACAATTTT - 3' **Probe**: FAM – CTGTGAGCTCTCCACGACGGCGTAA -QSY
PVP01_0616100	PF3D7_1031000	**Female (**Ookinete surface protein P25)	**Forward**: 5' - ACACTTGTGTGCTTGATGTATGTC - 3' **Reverse**: 5' - ACTTTGCCAATAGCACATGAGCAA - 3' **Probe**: FAM – TGCATTGTTGAGTACCTCTCGGAA - QSY
PVP01_0305600	PF3D7_0406200	**Overall/Early** (Sexual stage-specific protein precursor: s16)	**Forward**: 5' TCAGCACGACTGTGATTAACAT - 3' **Reverse**: 5' - CCTCCTTTACGCTGCCCATT - 3' **Probe**: FAM – AGCCCAGATAACAATACCAAC AGAACC - QSY
PVX_123760	PF3D7_1222600	**Gametocyte commitment** (AP2 domain transcription factor AP2-G)	**Forward**: 5' - TCTCCAACAACTGCCCATAC - 3' **Reverse**: 5' - TCACCCTTCATACTCCCATTTG - 3' **Probe**: FAM – ATATGCGGACGGAGGCGAATCATT - QSY

Initial Assay Development

PCR reactions consisted of 12.5 μL DreamTaq™ Hot Start PCR Master Mix (ThermoFisher Scientific, Waltham, MA, USA), 350 nm of each primer, one μL of template, and nuclease free water as needed for a final reaction volume of 25 μL. *P. vivax* specific gDNA (BEI Resources, NIAID, NIH, Bethesda, MD, USA) from the Sal I strain was used as template for initial assay development. Reactions were performed on the MJ Research PTC-200 Thermal Cycler (Marshall Scientific, Hampton, NH, US) under the following program: initiation denaturation at 95°C for 10 minutes, 40 cycles of 95°C for 30 seconds, 60°C for 45 seconds, 72°C for 1 minute, and final extension at 98°C for 10 minutes. PCR products were visualized on 3% agarose gel stained with ethidium bromide.

The amplified fragment was cloned into the pCR 2.1 TOPO TA Vector (Life Technologies, Carlsbad, CA, USA) in accordance with manufacturers' protocols (Invitrogen TOPO TA Cloning Kit with pCR 2.1-TOPO, One Shot TOP10 Chemically Competent *E. coli*). Plasmid DNA was purified using the QIAprep Spin MiniPrep kit (Qiagen, Hilden, Germany), quantified using a spectrophotometer (NanoDrop 2000C, ThermoFisher Scientific, Waltham, MA, USA), and verified by Sanger sequencing by GENEWIZ from Azenta Life Sciences (South Plainfield, NJ, USA).

Real-time Quantitative PCR to Detect P. vivax Gametocytes

PCR reactions consisted of 10 μL TaqMan™ Fast Advanced Master Mix (ThermoFisher Scientific, Waltham, MA, USA), 250 nm of each primer and probe, one μL of template, and nuclease free water as needed for a final reaction volume of 20 μL. Reactions were performed on the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following fast thermal cycling program: initial denaturation at 95°C for 20 seconds followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. qPCR results were analyzed using Applied Biosystems 7500 Real-Time PCR System Software (Applied Biosystems, Foster City, CA, USA) to generate Ct values and corresponding copy number per μL, and GraphPad Prism 9 (GraphPad, San Diego, CA, USA) was used to calculate slope of the standard curve, qPCR efficiency, and coefficient of correlation (R^2). Samples were considered positive if at least two of three replicates were positive. If only one replicate was positive, samples were rerun using two μL of template to differentiate between noise and low transcript prevalence. Plates were excluded if the NTC was positive or if the qPCR efficiency was below 90% or greater than 110%.

Blood Collection and Extraction of Nucleic Acids from Ao. nancymaae – P. vivax Infections

65 blood samples were collected from five different New World *Ao. nancymaae* monkeys infected with *P. vivax* parasites through either sporozoite induced infections or blood stage induced infections. Specific details of the study design are being published elsewhere but are approved in accordance with Institutional Animal Care and Use Committee (IACUC) and Institutional Review Board (IRB) guidelines (NAMRU-6 18-07; NMRCD.2007.0004). In brief, one splenectomized monkey (Monkey A) was injected with infected red blood cells (iRBCs) from roughly equal amounts of three *P. vivax* strains (Brazil-I from Brazil, ONG from Vietnam, and Sal-I from El Salvador). Laboratory-reared *Anopheles darlingi* mosquitoes were fed on Monkey A and infectious sporozoites were extracted. Another splenectomized monkey (Monkey B) was infected intravenously with approximately 24,000 extracted sporozoites (establishing a new strain of *P. vivax*, BOS – first generation). Once the infection was established, *Anopheles darlingi* mosquitoes were fed on Monkey B and infectious sporozoites were extracted. Monkeys C, D, and E remained spleen intact and were infected intravenously with an infectious dose of approximately 5,000 sporozoites each (strain BOS – second generation).

Blood was drawn from the saphenous vein using a fine tuberculin needle every other day or daily starting three – eight days post infection until day 64 or cure. Blood samples were collected in EDTA anticoagulant and mixed with the same volume of RNA*later*™ Stabilization Solution (Invitrogen™, Waltham MA, USA). RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the

manufacturer's standard protocol for RNA extraction. Genomic DNA was digested as part of this procedure prior to the reverse transcription of RNA. cDNA synthesis was conducted using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) and following the manufacturers standard protocol. Parasitemias were determined by expert microscopy or a multiplex 18S rRNA qPCR for low parasitemia infections (assay published elsewhere).

Blood Collection and Extraction of Nucleic Acids from Human Volunteers in Peru

105 blood samples were collected from individuals who were referred to one of eight different study sites in Peru (Figure 1) (19) after presenting with suspected or confirmed malaria as part of a surveillance study by the U.S. Naval Medical Research Unit No. 6. (NAMRU-6, Lima, Peru) (protocol # NMRCD.2007.0004). Enrollment occurred from September 2018 to March 2020. Whole blood samples were collected in accordance with IRB protocols and with signed informed consent. Inclusion criteria consisted of volunteers aged five or older with documented fever (axillary temperature ≥ 37.5°C or oral temperature > 38°C) or history of fever during the previous 72 hours in the absence of another obvious cause of fever. Two thick and two thin blood smears were prepared for each volunteer. Positive cases determined by microscopy were referred to the Peruvian MoH facility for treatment. Blood was blotted onto Whatman® 903 Protein saver filter paper cards (GE Healthcare Life Sciences, Chicago, IL USA) and processed for RNA and DNA extraction. Single air-dried blood spots (DBS) were minced, and nucleic acids extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's standard protocol for RNA extraction. Genomic DNA was digested as part of this procedure prior to the reverse transcription of RNA. cDNA synthesis was

conducted using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) and following the manufacturers standard protocol.

Plasmodium genus photo-induced electron transfer-PCR (PET-PCR) as previously described was used to quantify parasite density for available samples (19) (144) as well as to determine *Plasmodium* species (135).

Results

qPCR Assay Validation and Optimization

Ten-fold serial dilutions of plasmids containing each target gene, ranging from 100,000 copies per µL to 1.0 copy per µL, were generated by diluting in water. Dilutions were calculated using the mass of the amplicon of interest and the spectrophotometer measured concentration. Additional two-fold dilutions were used to determine lower limits of detection. The limits of detection for *Pvs25*, *PvMGET*, *Pvs16*, and *PvAP2-G* were 1.0, 2.5, 1.0, and 2.5 copies/µL respectively. The limits of quantification or linearity for *Pvs25*, *PvMGET*, *Pvs16*, and *PvAP2-G* were 10 copies/µL. Annealing temperatures and final primer and probe concentrations were optimized as necessary. The ten-fold serial dilution series was utilized as a standard curve during optimization, validation, and subsequent sample analysis.

The specificity of each marker was analyzed using cDNA templates from non-*P.vivax* species in samples for human blood and monkey blood. cDNAs from *P. falciparum* strain 3D7 (MR4, Manassas, Virginia, USA), laboratory purified stage V *P. falciparum* strain NF54 gametocytes (gift from Dr. Surendra Prajapati, Uniformed Services University of Health Sciences, Bethesda, MD, USA) and human samples with

known *P. falciparum* asexual and sexual parasitemias by qPCR from Kenya were used as templates. cDNAs from field positive *P. ovale* and *P. malariae* (determined by expert microscopy and verified by species specific qPCR assays) human samples from Kenya were also used as templates. Additionally, cDNA from infected blood obtained from a laboratory induced Rhesus macaque - *P. cynomologi* (M/B strain) were utilized as template (gift from Dr. Brandon Wilder, Oregon Health & Science University, Portland, OR, USA). cDNA from two different uninfected human blood samples were used also used. All non-*P.vivax* DNA samples tested for specificity were negative by qPCR for each *P. vivax* gametocyte marker of interest.

Field validation and assay utility was evaluated using known (by expert microscopy) high gametocytemia samples from three human infections in Peru and high gametocytemia samples from three different strains of *P. vivax* induced infections in *Ao. nancymaae* monkeys (Brasil-I, ONG, and Sal-I). All assays were successful in detecting gametocyte transcripts from these samples. Quantitative parasitemia and gametocytemia by expert microscopy was compared to copy number per µL for each *P. vivax* gametocyte specific transcript marker based on standard curves. In the human infections, correlation between parasites/µL by microscopy and *Pvs25*, *PvMGET*, and *Pvs16* copy number/µL by qPCR were $R^2 = 0.9474$, 0.9137, 0.7227 respectively (Figure 23A) and correlation between gametocytes/µL by microscopy and *Pvs25*, *PvMGET*, and *Pvs16* copy number/µL by qPCR were $R^2 = 0.9926$, 0.9985, and 0.9798 respectively (Figure 23B). In the single stain monkey infections, correlation between parasites/µL by microscopy and *Pvs25*, *PvMGET*, *Pvs16*, *and PvAP2-G* copy number/µL by qPCR were $R^2 = 0.2558$,

0.9629, 0.7716, and 0.8921 respectively (Figure 23C). Microscopy data was not available for gametocytes/µL in the monkey infections.

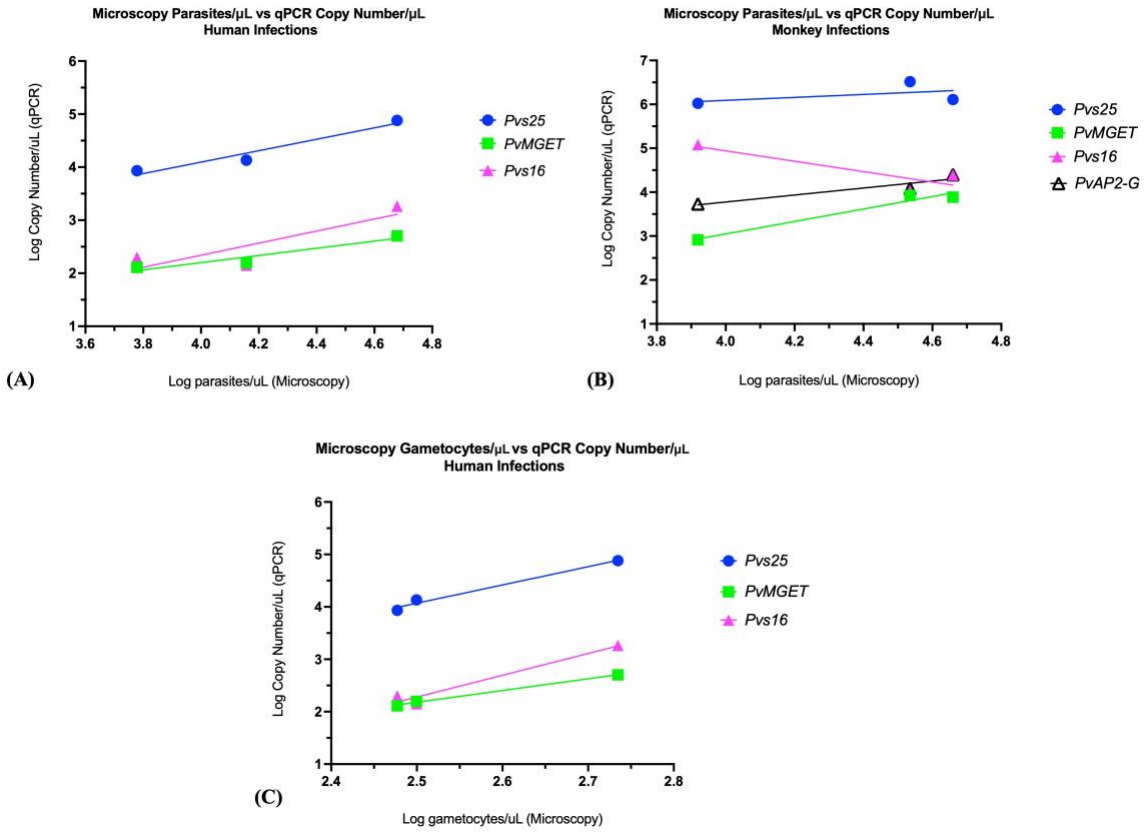

Figure 23. Correlation Between Microcopy Log Parasites or Gametocytes/µL and qPCR Log Gametocyte-Specific Transcripts Copy Numbers/µL in Monkey and Human Infections.
(A). Microscopy Parasites/µL vs qPCR Log Gametocyte-Specific Transcripts Copy Numbers/µL in Human Infections **(B).** Microscopy Log Parasites/µL vs qPCR Log Gametocyte-Specific Transcripts Copy Numbers/µL in Monkey Infections **(C).** Microscopy Log Gametocytes/µL vs qPCR Log Gametocyte-Specific Transcripts Copy Numbers/µL in Human Infections.

Gametocyte-Specific Transcripts in Monkey A

15 samples of blood were collected for gametocyte analysis beginning on day three post-infection until 30 days post-infection from Monkey A (splenectomized – induced blood stage infection with mixed strains). Asexual parasitemia was determined

by expert microscopy due to high parasitemias observed when the spleen is unable to clear parasites. By qPCR, *Pvs25* was first detected at low quantities five days post-infection (~ seven days prior to asexual detection by microscopy), *Pvs16* was first detected at low quantities seven days post-infection (~ five days prior to asexual detection by microscopy), and *PvMGET* and *PvAP2-G* were first detected at low quantities 10 days post-infection (~ two days prior to asexual detection by microscopy) (Table 13) (Figure 24). *Pvs25* transcript abundance increased after treatment with Mefloquine (MQ) on day 18; all other markers had a decrease in transcript abundance. Asexual parasites were no longer detected by microscopy after day 21, while all gametocyte-specific transcripts were no longer detected by qPCR after day 24. The gametocyte sex ratio (female:male) increased after day 19 by 1054% until after day 24.

Table 13. Results from qPCR Copy Numbers/µL of Each Gametocyte-Specific Transcript Marker on Respective Days Post Infection in Monkey A. "Und." are the undetermined samples that did not show any positive amplification by qPCR.

Day Post Infection	***Pvs25* Copy Number/µL**	***PvMGET* Copy Number/µL**	***Pvs16* Copy Number/µL**	***PvAP2-G* Copy Number/µL**	**Sex Ratio (F:M)**	**Asexual Parasites/µL (Microscopy)**
3	Und.	Und.	Und.	Und.	N/A	0
5	187	Und.	Und.	Und.	N/A	0
7	1956	Und.	16	Und.	N/A	0
10	7878	31	153	167	254	0
12	41697	497	609	1201	84	2738
14	56070	98	400	950	571	3494
15	338378	1024	2664	9521	331	8846
17	2056359	2629	25959	39063	782	9605
18*	1544313	2432	27754	29811	635	22103
19	1029446	3123	6270	15214	330	10038
21	1859904	488	4821	402	3809	3150
24	18518	15	40	7	1238	0
30	Und.	Und.	Und.	Und.	N/A	0

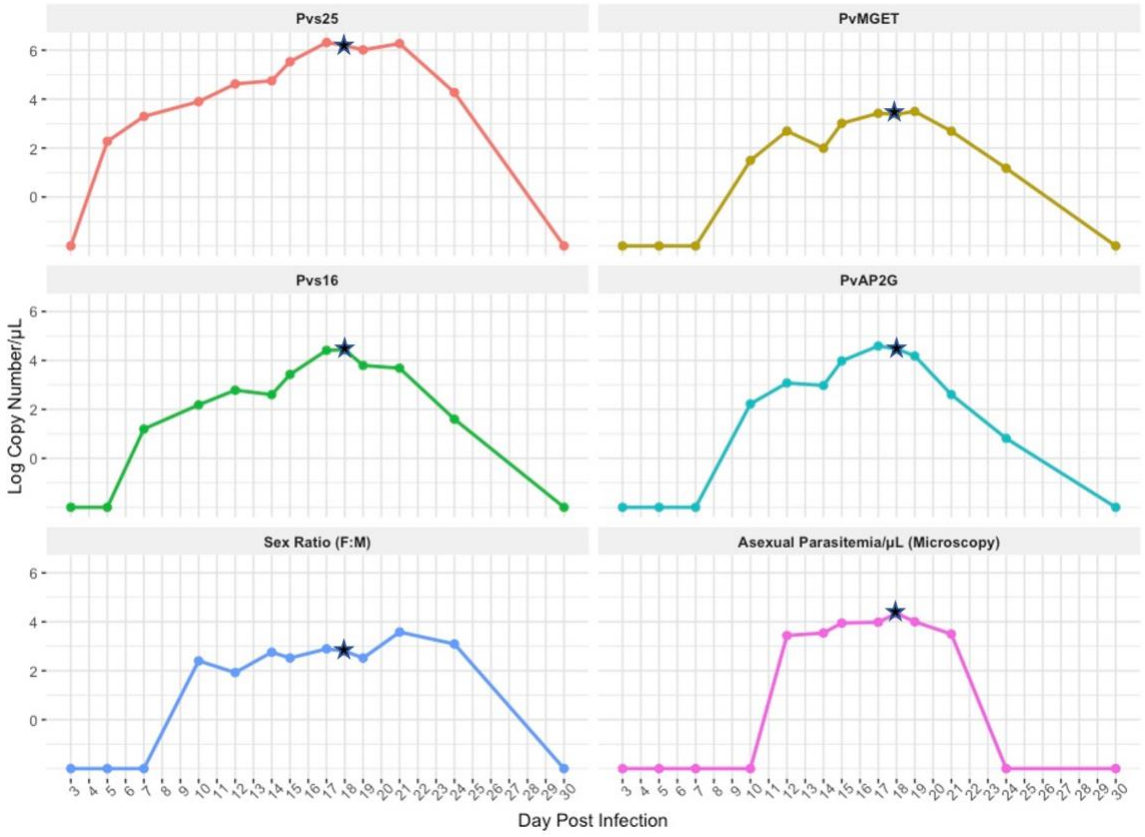

Figure 24. Log Copy Numbers/µL of Each Gametocyte-Specific Transcript Marker on Respective Days Post Infection in Monkey A. The star marks the day that the monkey was treated with MQ.

Gametocyte-Specific Transcripts in Monkey B

20 samples of blood were collected for gametocyte analysis beginning on day seven post-infection until 64 days post-infection from Monkey B (splenectomized – induced sporozoite stage infection with new BOS first generation strain). Asexual parasitemia was determined by expert microscopy. By qPCR, *Pvs25* was first detected at low quantities 19 days post-infection (~ five days after asexual detection by microscopy), *Pvs16* was first detected at low quantities 23 days post-infection (~ nine days after asexual detection by microscopy), and *PvMGET* and *PvAP2-G* were first detected at low quantities 26 days post-infection (~ 12 days after asexual detection by microscopy)

(Table 14) (Figure 25). However, asexual parasite detection by microscopy was intermittently negative on days 19, 21, and 26. *Pvs25* transcript abundance increased after treatment with MQ on Day 35, all other markers had a decrease in transcript abundance. Asexual parasites were no longer detected by microscopy after day 35, while all gametocyte-specific transcripts were no longer detected by qPCR after day 37 except for *Pvs25* which was still detected at day 40 post-infection. The gametocyte sex ratio (female:male) increased after day 35 by 243% until after day 37.

Table 14. Results from qPCR Copy Numbers/µL of Each Gametocyte-Specific Transcript Marker on Respective Days Post Infection in Monkey B. "Und." are the undetermined samples that did not show any positive amplification by qPCR.

Day Post Infection	***Pvs25* Copy Number/µL**	***PvMGET* Copy Number/µL**	***Pvs16* Copy Number/µL**	***PvAP2-G* Copy Number/µL**	**Sex Ratio (F:M)**	**Asexual Parasites/µL (Microscopy)**
7	Und.	Und.	Und.	Und.	N/A	0
9	Und.	Und.	Und.	Und.	N/A	0
12	Und.	Und.	Und.	Und.	N/A	0
14	Und.	Und.	Und.	Und.	N/A	131
16	Und.	Und.	Und.	Und.	N/A	222
19	31	Und.	Und.	Und.	N/A	0
21	80	Und.	Und.	Und.	N/A	0
23	494	Und.	8	Und.	N/A	436
26	15273	27	562	572	556	0
28	16912	28	313	553	613	565
30	6325	170	178	302	37	531
33	29236	19	263	399	1554	1815
34	275	Und.	320	141	N/A	1078
35*	3189	70	596	469	46	3030
37	7419	47	292	215	158	0
40	51	Und.	Und.	Und.	N/A	0
43	Und.	Und.	Und.	Und.	N/A	0
46	Und.	Und.	Und.	Und.	N/A	0
49	Und.	Und.	Und.	Und.	N/A	0
64	Und.	Und.	Und.	Und.	N/A	0

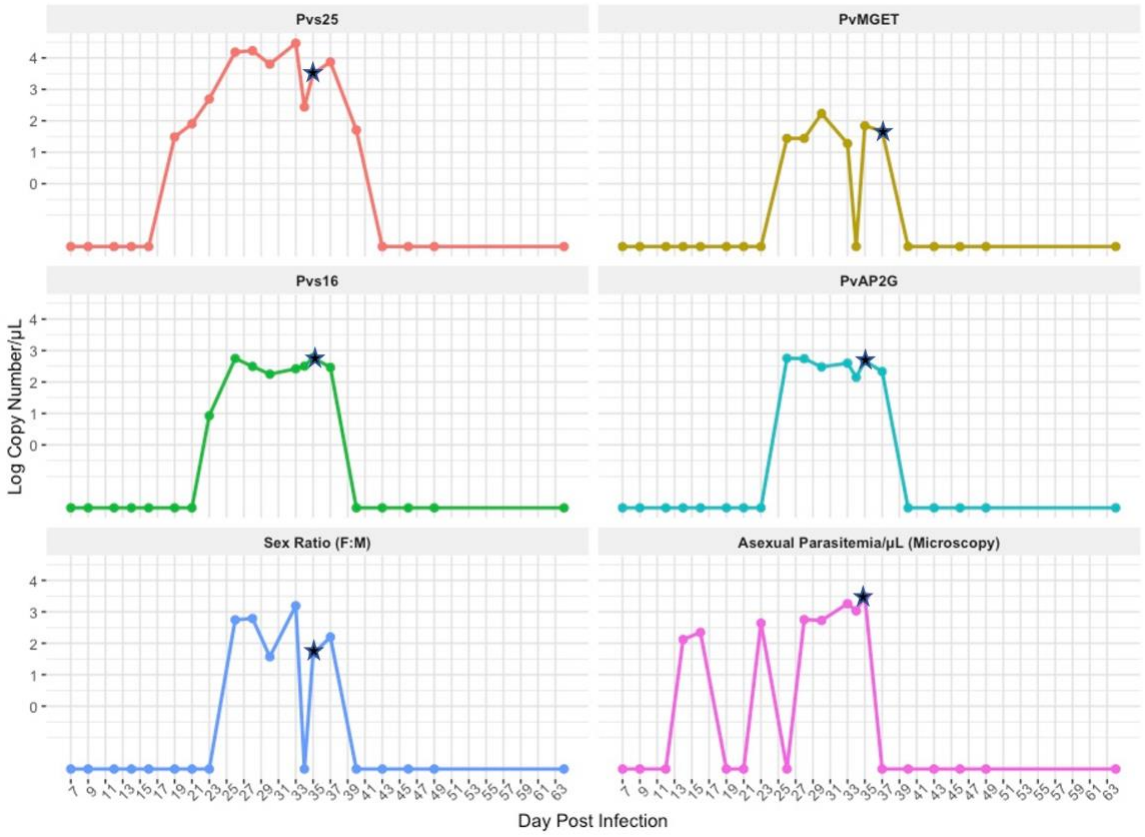

Figure 25. Log Copy Numbers/µL of Each Gametocyte-Specific Transcript Marker on Respective Days Post Infection in Monkey B. The star marks the day that the monkey was treated with MQ.

No Parasites Detected in Monkey C and D

Six samples of blood were collected for gametocyte analysis beginning on day eight post-infection until 60 days post-infection from Monkey 3 and 4 (spleen intact – induced sporozoite stage infection with second generation BOS strain). Asexual parasitemia was determined by expert microscopy and was negative throughout the entire course of infection. By qPCR, all gametocyte-specific transcript markers were also negative.

Gametocyte-Specific Transcripts in Monkey E

17 samples of blood were collected for gametocyte analysis beginning on day eight post-infection until 60 days post-infection from Monkey E (spleen intact – induced sporozoite stage infection with second generation BOS strain). Asexual parasitemia was determined by qPCR due to low observed parasitemia by microscopy. By qPCR, *Pvs25* was first detected at low quantities 28 days post-infection (~13 days after asexual detection by qPCR and on the same day as asexual detection by microscopy), *Pvs16* was first detected at low quantities 25 days post-infection (~10 days after asexual detection by qPCR and ~ three days before asexual detection by microscopy), *PvMGET* was only detected in low quantities on days 41 and 51, and *PvAP2-G* was never detected throughout the course of the infection (Table 15) (Figure 26). However, asexual parasite detection by microscopy was intermittently negative on days 31, 34, 36, 39, and 48. Monkey E was treated with MQ on day 54, however, there was no available RNA for gametocyte analysis on this day or until six days after treatment in which there was no detection of asexual and sexual parasites. Asexual parasites/μL by qPCR were in extremely low abundance from day 15 until day 31 (range: 1-19 parasites/μL). There were no detectable gametocytes by qPCR or asexual parasites by microscopy on day 48 despite there be detectable asexual parasites by qPCR. Due to low prevalence of *PvMGET*, there were only two days with quantifiable sex ratio.

Table 15. Results from qPCR Copy Numbers/μL of Each Gametocyte-Specific Transcript Marker on Respective Days Post Infection in Monkey E. "Und." are the undetermined samples that did not show any positive amplification by qPCR.

Day Post Infection	***Pvs25* Copy Number/ μL**	***PvMGET* Copy Number/ μL**	***Pvs16* Copy Number/ μL**	***PvAP2-G* Copy Number/ μL**	**Sex Ratio (F:M)**	**Asexual Parasites /μL (qPCR)**	**Asexual Parasites/μL (Microscopy)**
8	Und.	Und.	Und.	Und.	N/A	0	0
11	Und.	Und.	Und.	Und.	N/A	0	0

13	Und.	Und.	Und.	Und.	N/A	0	0
15	Und.	Und.	Und.	Und.	N/A	1	0
18	Und.	Und.	Und.	Und.	N/A	2	0
20	Und.	Und.	Und.	Und.	N/A	1	0
22	Und.	Und.	Und.	Und.	N/A	7	0
25	Und.	Und.	25	Und.	N/A	19	0
28	220	Und.	Und.	Und.	N/A	8	151
31	10	Und.	Und.	Und.	N/A	49	0
34	5	Und.	Und.	Und.	N/A	33	0
36	406	Und.	16	Und.	N/A	42	0
39	10	Und.	14	Und.	N/A	125	0
41	1296	47	119	Und.	28	242	169
48	Und.	Und.	Und.	Und.	N/A	336	0
51	2983	8	152	Und.	355	565	514
60	Und.	Und.	Und.	Und.	N/A	0	0

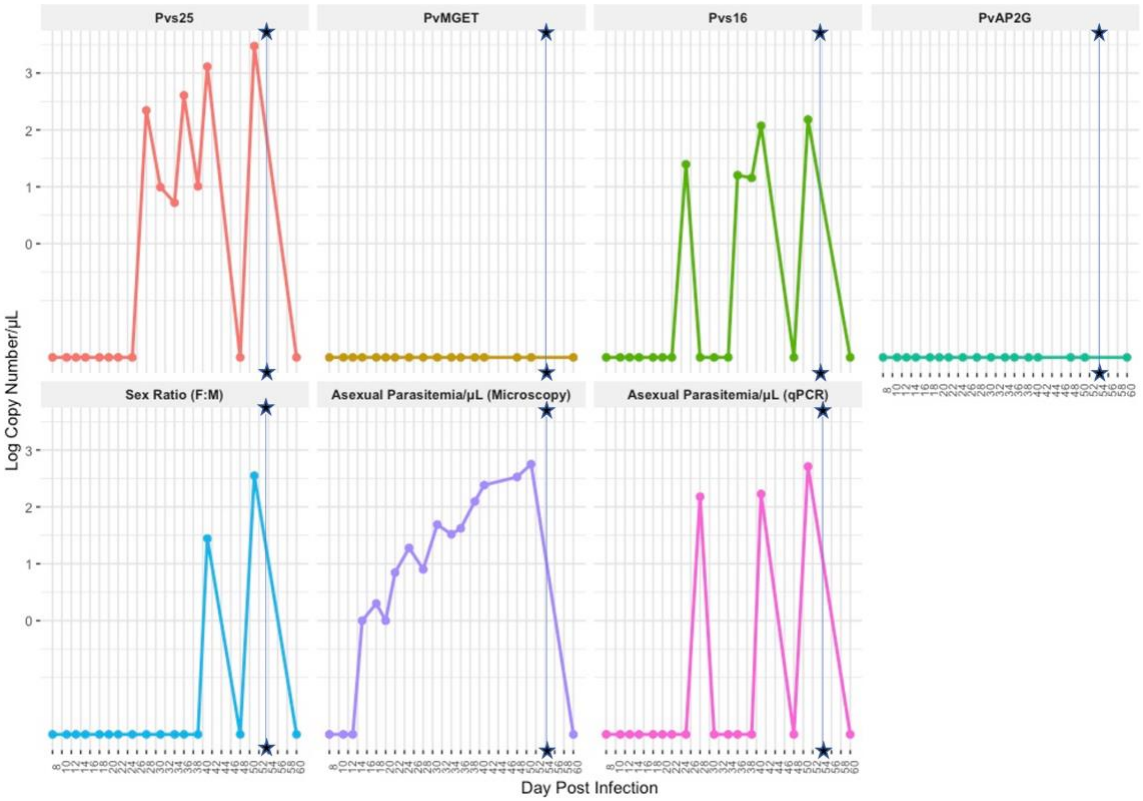

Figure 26. Log Copy Numbers/µL of Each Gametocyte-Specific Transcript Marker on Respective Days Post Infection in Monkey E. The star and line mark the day that the monkey was treated with MQ.

Gametocyte-Specific Transcript Abundance in All Monkeys

Pvs25

Copy numbers/µL were consistently higher in the *Pvs25* gametocyte-specific transcript marker than the other markers. *Pvs25* regularly was detected prior to any other gametocyte-specific transcript marker. Abundance of *Pvs25* transcripts ranged from 187 – 2,056,359 copy numbers/µL in Monkey A, 31 – 29,236 copy numbers/µL in Monkey B, and 5 – 2,983 copy numbers/µL in Monkey E. In the reference single strain infections (blood induced), *Pvs25* abundance was 1,285,141 copy numbers/µL for Brasil-I, 1,054,190 copy numbers/µL for ONG-I, and 3,274,917 for Sal-I.

PvMGET

Copy numbers/µL were consistently lower in the *PvMGET* gametocyte-specific transcript marker than the other markers. Abundance of *PvMGET* transcripts ranged from 15 – 3,123 copy numbers/µL in Monkey A, 19 - 170 copy numbers/µL in Monkey B, and 8 - 47 copy numbers/µL in Monkey E with only two time points with detectable levels. In the reference single strain infections (blood induced), *Pvs25* abundance was 7,675 copy numbers/µL for Brasil-I, 819 copy numbers/µL for ONG-I, and 8,442 for Sal-I.

Pvs16

Copy numbers/µL were consistently lower in the *Pvs16* gametocyte-specific transcript marker compared to *Pvs25* but higher when compared to *PvMGET*. *Pvs16* was regularly detected after *Pvs25* but prior to *PvMGET and PvAP2-G.* Abundance of *Pvs16* transcripts ranged from 16 – 27,754 copy numbers/µL in Monkey A, 8 - 596 copy

numbers/µL in Monkey B, and 14 – 152 copy numbers/µL in Monkey E. In the reference single strain infections (blood induced), *Pvs16* abundance was 24,507 copy numbers/µL for Brasil-I, 120,905 copy numbers/µL for ONG-I, and 10,783 for Sal-I.

PvAP2-G

Copy numbers/µL were consistently lower in the *PvAP2-G* gametocyte-specific transcript marker compared to *Pvs25* but higher when compared to *PvMGET*. Abundance of *PvAP2-G* transcripts ranged from 7 – 39,063 copy numbers/µL in Monkey A, 141 - 572 copy numbers/µL in Monkey B and was never detected in Monkey E (spleen intact, induced sporozoite infection). In the reference single strain infections (blood induced), *PvAP2-G* abundance was 25,059 copy numbers/µL for Brasil-I, 5,386 copy numbers/µL for ONG-I, and 12,110 for Sal-I.

Sex-Ratio

Sex-ratio of female to male log copy numbers/µL ranged from 1.44 to 3.58 across all monkey infections. In the reference single strain infections (blood induced), the sex-ratio of female to male log copy numbers/µL was 2.22 for Brasil-I, 3.11 for ONG-I, and 2.59 for Sal-I. Using simple linear regression, the correlation between log *Pvs25* and log *PvMGET* was significant (*p*-value: <0.0001, R^2: 0.8317).

Distribution of Samples from Human Volunteers in Peru

105 samples were included for gametocyte analysis from microscopy confirmed malaria infections from 8 different study sites in Peru. Of the 105 volunteers, 39% were female (41/105) and 61% were male (64/105) with an age distribution of 10 – 79 years old (mean: 37 years old). The majority (29.5%) of samples came from Moronacocha

Health Center (Table 16) and the majority (39%) classified themselves in the "other" category for their occupation followed by 25.7% as agriculture workers (Table 6). By expert microscopy, asexual parasites/µL ranged from 12 to 61,773 (mean: 6,890 parasites/µL) and sexual parasites were detected in 75 of 104 samples with a range of 12 – 761 parasites/µL (mean: 139 parasites/µL). One sample did not have microscopy enumeration. By PET qPCR, 95/105 (90.5%) were determined to be single *P. vivax* infections, 9/105 (8.6%) were single *P. falciparum* infections, and 1/105 (0.1%) was a mixed *P. vivax* and *P. falciparum* infection.

Table 16. Distribution of Samples by Study Site in Peru.

Site	Frequency	Percent of Total
Moronacocha Health Center	31	29.5%
Bellavista Nanay Health Center	10	9.5%
San Juan Health Center	11	10.5%
Santa Clara de Nanay Health Center	24	22.9%
Santo Tomas Health Center	5	4.8%
Tupac Health Center	8	7.6%
Padrecocha Health Post	12	11.4%
Hospital Apoyo Iquitos	4	3.8%
Total	**105**	**100%**

Table 17. Distribution of Samples by Occupation.

Occupation	Frequency	Percent of Total
Agriculture	27	25.7%
Health Worker	1	1%
Housewife	9	8.6%
Mining	15	14.3%
Other	41	39%
Student	12	11.4%

Total	**105**	**100%**

Gametocyte-Specific Transcripts in Samples from Human Volunteers in Peru

Of the 105 samples included for gametocyte analysis, 98 (93.3%) were positive for at least one gametocyte-specific molecular marker. 95/98 (96.9%) were positive for *Pvs25*, 67/98 (68.3%) were positive for *PvMGET*, and 77/98 (78.6%) were positive for *Pvs16*. There were 62 samples (63.3%) that were positive for all three gametocyte-specific molecular markers. Copy numbers/µL ranged from 6 to 76,122 copy numbers/µL for *Pvs25* (mean: 4,698 copy number/µL), 3 to 506 copy numbers/µL for *PvMGET*, (mean: 66 copy numbers/µL) and 2 – 1,844 copy numbers/µL for *Pvs16* (mean: 104 copy numbers/µL). Copy numbers/µL for all molecular markers had a significant positive association with microscopy confirmed asexual parasites/µL and sexual parasites/µL (Pearson's correlation analysis) (Table 18). Sex-ratio of female to male log copy numbers/µL ranged from -0.397 to 3.360 across all volunteer samples with a mean of 1.604 log copy numbers/µL. Of the 65 samples that were positive for both *Pvs25* and *PvMGET*, only 4 samples had higher *PvMGET* copy numbers/µL than *Pvs25*.

Table 18. Pearson's Correlation Between Diagnostic Tests and Gametocyte-Specific qPCR Results

		PET-PCR (parasites /µL)	Microscopy Asexual (parasites/µL)	Microscopy Sexual (parasites/µL)	qPCR *PvMGET* (copy numbers/µL)	qPCR *Pvs25* (copy numbers/µL)	Sex Ratio F:M	qPCR *Pvs16* (copy numbers/µL)
PET-PCR (parasites/ µL)	Pearson Correlation	1	.890**	.607**	.315*	.416**	-0.020	.302*
	p-value		0.000	0.000	0.014	0.000	0.881	0.014
Microscopy Asexual (parasites/µL)	Pearson Correlation	.890**	1	.600**	.511**	.574**	-0.056	.509**
	p-value	0.000		<0.001	<0.001	<0.001	0.658	<0.001

Microscopy Sexual (parasites/μL)	Pearson Correlation	.607**	.600**	1	.367**	.409**	-0.076	.322*
	p-value	0.000	<0.001		0.006	<0.001	0.587	0.011
qPCR *PvMGET* (copy numbers/μL)	Pearson Correlation	.315*	.511**	.367**	1	.692**	-0.089	.863**
	p-value	0.014	<0.001	0.006		<0.001	0.481	<0.001
qPCR *Pvs25* (copy numbers/μL)	Pearson Correlation	.416**	.574**	.409**	.692**	1	.391**	.812**
	p-value	0.000	<0.001	<0.001	<0.001		0.001	<0.001
Sex Ratio F:M	Pearson Correlation	-0.020	-0.056	-0.076	-0.089	.391**	1	0.021
	p-value	0.881	0.658	0.587	0.481	0.001		0.872
qPCR *Pvs16* (copy numbers/μL)	Pearson Correlation	.302*	.509**	.322*	.863**	.812**	0.021	1
	p-value	0.014	<0.001	0.011	<0.001	<0.001	0.872	

**Correlation is significant at the 0.01 level
*Correlation is significant at the 0.05 level

Gametocyte Prevalence by qPCR vs Microscopy

Overall, the qPCR gametocyte assays were able to detect 24 gametocyte positive samples that were negative for gametocytes by microscopy. There were 2 samples that were negative for any gametocyte-specific molecular marker but were positive for gametocytes by microscopy. The overall difference between gametocyte prevalence by qPCR and gametocyte prevalence by microscopy was significant (McNemar's chi-square test, p-value <0.0001). Gametocyte prevalence by microscopy was 70.5%, while gametocyte prevalence by qPCR was 93.3%.

Detection in P. falciparum Infections

Of the nine samples that were determined to be *P. falciparum* infections by qPCR, eight of them were positive for *P. vivax* gametocytes. All eight were positive for *Pvs25*, four of eight were positive for *Pvs16*, and two of eight were positive for *PvMGET* (Table 19). There were two samples (sample 6468 and 6693) that were positive for all three *P.*

vivax gametocyte-specific molecular markers. Two of the eight samples were originally determined to be *P. vivax* (sample 6468*) *or P. vivax/P. falciparum* mixed (sample 6661*) infections by microscopy but only *P. falciparum* by PET-PCR.

Table 19. Parasites/µL by PET-PCR and Microscopy and Copy Numbers/µL Of Gametocyte-Specific Transcript Markers by qPCR

Sample	PET-PCR (parasites/ µL)	Microscopy Asexual (parasites/ µL)	Microscopy Sexual (parasites/ µL)	qPCR *PvMGET* (copy numbers/ µL)	qPCR *Pvs25* (copy numbers/ µL)	Sex Ratio F:M	qPCR *Pvs16* (copy numbers/ µL)
6467	32,542	28,429	0	Und.	Und.	N/A	Und.
6468*	2,478	3,765	88	59.6	4115.7	69.1	44.1
6485	1,568	1,389	148	Und.	5.7	N/A	17.7
6493	Unavailable	2,660	0	Und.	7.3	N/A	Und.
6661*	794	Unavailable	Unavailable	Und.	124.9	N/A	Und.
6676	398	971	0	Und.	452.6	N/A	Und.
6693	62	120	0	12.6	253.9	20.2	6.5
6733	8,598	7,864	0	Und.	49.5	N/A	Und.
6757	Unavailable	7,681	116	Und.	34.6	N/A	15.4

Associations Between Gametocyte-Specific Transcript Abundance

There was no significant difference between qPCR log gametocyte copy numbers/µL and gender (unpaired t-test; *Pvs25* p-value: 0.983, *PvMGET* p-value: 0.951, *Pvs16* p-value: 0.747). Using interquartile range for age groups, there was a significant difference between *Pvs25* copy numbers/µL and age, where ages 51-79 had significantly higher *Pvs25* log copy numbers/µL than ages 37-50 (one-way ANOVA, adjusted p-value: 0.033), there were no other differences between ages groups for any other molecular marker. Additionally, there was no difference between the study site location and log copy numbers/µL for any molecular marker (one-way ANOVA). While there was no significant difference between occupations and log copy numbers/µL for *Pvs16* and

Pvs25, volunteers that worked in the mining sector had significantly higher *PvMGET* log copy numbers/µL than those in the Agriculture and Other sectors (one-way ANOVA, adjusted p-values: 0.020 and 0.008 respectively). Those that worked in the mining sector also had overall higher *PvMGET* log copy numbers/µL than all other sectors (albeit not significant likely due to small sample size).

Discussion

Using four *P. vivax* gametocyte-specific transcript markers that are orthologs of known *P. falciparum* markers, we were able to detect gametocyte prevalence and abundance (copy numbers/µL) in both induced *P. vivax* specific monkey infections and natural human infections in Peru. *Pvs25* was consistently a more robust molecular marker as it presented with higher prevalence and higher abundance than the other three markers in both the monkey and human infections. Additionally, *Pvs25* was often the first molecular marker to show up and last marker to remain detectable over the course of a monkey infection. qPCR gametocyte-specific assay sensitivity was significantly higher than microscopy gametocyte sensitivity in both monkey and human infections. qPCR gametocyte-specific assay specificity was also high while analyzing available reference controls from different *Plasmodium* species and strains; however, there were eight human samples that had *P. vivax* gametocyte prevalence by qPCR but were classified as *P. falciparum* by PET-PCR. During specificity optimization, all samples containing *P. falciparum* DNA were negative for each *P. vivax* gametocyte marker.

Using the combination of qPCR assays to further understand gametocyte kinetics in controlled monkey infections, there was a difference in prevalence and abundance between iRBC induced infections and sporozoite induced infections as expected. The

iRBC induced infections had gametocyte detection approximately seven days prior to any microscopy confirmed parasite detection (Table 13). *P. vivax* gametocytes take two to three days to mature after exoerythrocytic schizont rupture (10), therefore, it is likely that gametocytes were present during inoculation but the infection was not established for detection by microscopy until later in the infection (day 12). Gametocyte positivity remained for at least three days after MQ treatment despite being negative for parasites by microscopy. These observations are consistent with various studies that show gametocyte clearance may be delayed after MQ treatment in *P. falciparum* infections (229). The effects of MQ on *P. vivax* gametocyte clearance specifically in *Ao. nancymaae* models are relatively inconclusive despite MQ's chemical relation to the *P. vivax* gametocide quinine (63; 102; 186; 192).

In the first generation sporozoite infection, gametocytes (*Pvs25*) were not detected by qPCR until five days after asexual parasite detection by microscopy (Table 14). As reviewed by Bousema and Drakeley (42), *P. vivax* infectious (mature) gametocytes often appear within two to three days after asexual parasite detection by microscopy. However, throughout the course of infection for Monkey B, the parasitemias were so low that there was a change in microscopic detection; over the course of seven days only one of four sample collection days (days 19, 21, 23, and 26 post infection) were positive for parasites by microscopy despite being gametocyte positive by qPCR for all four sample collection days (Table 14). Sexual positivity remained for at least five days after MQ treatment for *Pvs25* and two days after MQ treatment for all other markers despite being negative for parasites by microscopy.

Gametocyte prevalence and abundance was much lower in the spleen intact monkey which supports prior evidence for parasite immune clearance in the spleen. For this infection, parasitemia was calculated using qPCR and microscopy; parasites were observed in very low abundance by qPCR approximately 13 days prior to any gametocyte detection by qPCR. While it should not take 13 days for *P. vivax* gametocytes to appear or reach maturity, the difference in time to detection may be attributed to the enhanced optimization and utilization of the *18S* rRNA qPCR assay as gametocytes also express *18S* rRNAs. When compared to microscopic detection of asexual parasites, there were intermittent negative asexual observations despite prevalence of gametocytes by qPCR; *Pvs16* was detected three days prior to microscopic detection of asexual parasites. Overall gametocyte prevalence by qPCR was so low that *PvAP2-G* failed to test positive throughout the entire course of infection. Recent evidence suggests that *PvAP2-G* is present in *P. vivax* infection; transcripts of *PvAP2-G* were identified in early blood stage infections in 5 of 12 infected samples using RNAseq methodologies (4). While the observed lack of *PvAP2-G* prevalence by qPCR may be explained by limitations in the qPCR assay sensitivity, it equally highlights an area for future studies to investigate the role that the immune system and spleen have in gametocyte commitment in *P. vivax* infections. *PvMGET* was also remarkably low in Monkey E with only three samples testing positive over the course infection while 13 samples were positive for parasites by *18S* rRNA qPCR. It is described that *P. vivax* gametocytes circulate for a maximum of three days (42; 59), therefore, it is reasonable to observe that transcript abundance or prevalence at low levels may fluctuate throughout the course of infection. Unfortunately, due to the discordant timelines between blood sampling and MQ treatment, any

conclusions into gametocyte prevalence or abundance after MQ treatment in Monkey E were inconclusive. For the other spleen intact monkeys infected with sporozoites (Monkey 3 and 4), all qPCR gametocyte-specific markers were negative which agreed with asexual and sexual microscopy results and confirmed that there were not established *P. vivax* infections in either monkey.

Sex-ratio was difficult to interpret as the difference in transcript abundance was much higher with the *Pvs25* marker than *PvMGET*. In *P. falciparum* infections, *PfMGET* presented with more abundant gene transcripts than other potential male markers (138), however, this may not apply to *PvMGET* in *P. vivax* infections. In a recent study showing *P. vivax* gene expression in male and female gametocytes by scRNA-seq, the marker PvP01_1412100 was observed to show high transcript abundance in male gametocytes (207) and may be another candidate for male gametocyte detection. Additionally, in *P. vivax* infections, gametocyte sex ratios are typically female biased by at least 2:1 (59). If one were to assume equal transcript expression of *Pvs25* in females and *PvMGET* in males, then this study would suggest an average female to male ratio of 129:1 as copy numbers/ µL or 2.2:1 as log copy numbers/ µL. This data suggests that transcript expression is not equal between *Pvs25* and *PvMGET. P. falciparum* parasites are believed to alter their sex ratio with an increase in male gametocyte production as a form of fertility insurance (201) especially in an environment of increase host immune response such as immediately after pharmaceutical treatment (185; 197). However, the opposite was observed in our monkey *P. vivax* infections after MQ treatment where *Pvs25* increased after treatment and *PvMGET* decreased. Additionally, in *P. falciparum* infections, an increase in male gametocyte production is associated with host anemia,

parasitic competition, or host immune response (review in (203)). Unfortunately, none of the above host or parasitic factors were investigated in this study but provide opportunities for future studies to examine how the factors affect both male and female *P. vivax* gametocyte production and subsequent transmission to the mosquito vector. Transmission success is often associated with fluctuating sex ratios, particularly in *P. vivax* infections where it has been documented that transmission success was dependent only on male gametocyte densities (147).

In *P. falciparum*, *Pfs16* is expressed at low levels throughout each stage of gametocyte development infection but is most associated with stage II *P. falciparum* gametocytes (84; 217). Additionally, recent observations from a cross sectional cohort in Kenya detected *Pfs16* at higher levels than *Pfs25* (231). Given what we know about *Pfs16*, we expected *Pvs16* to be detected prior to *Pvs25* as an early indicator of gametocyte development and possibly at higher levels throughout the infection; however, *Pvs16* was rarely detected before *Pvs25* and was always in lower abundance. These observations are supported by conflicting *P. vivax* specific results where *Pvs16* was identified as an early marker by RNAseq in one study (4), but was mostly detectable in asexual parasites rather than male or female gametocytes in another scRNA-seq study (207). Further investigation is required to better understand the utility and kinetics of *Pvs16* as an ortholog to *Pfs16* in *P. vivax* infections.

The combination of *Pvs25, PvMGET,* and *Pvs16* qPCR assays proved to be a reliable assay for field detection of gametocytes in natural human infections. All three markers statistically agreed with microscopy asexual parasites/μL and microscopy sexual parasites/μL (Table 18). As seen in the monkey infections, transcript abundance was

highest in *Pvs25* and lowest in *PvMGET*. The qPCR assays detected 24 more gametocyte positive samples when compared to gametocyte detection by microscopy, highlighting the increased sensitivity of molecular assays for sexual parasite detection. Gametocyte prevalence by microscopy was 70.5%, while gametocyte prevalence by qPCR was 93.3%. There were only two *P. vivax* samples that were positive for gametocytes by microscopy but negative for any gametocyte-specific marker by qPCR.

Specificity studies showed no cross reactivity with multiple *P. falciparum* strains and infections, *P. ovale, P. malariae, P. cynomologi*, or uninfected blood. However, when utilized in the field, eight human volunteer samples that were positive for *P. vivax* gametocytes by qPCR were classified as *P. falciparum* infections by PET-PCR. One sample was originally determined to be a *P. vivax* infection by microscopy but perhaps was a mixed species infection with low level *P. vivax* transcripts. The discordant results demonstrate the limitations of any assay, specifically the utilization in low density infections. Speciation by a more sensitive assay such a highly sensitive and highly species-specific *18S* sRNA assay may be beneficial. Another explanation for the discordant results may be that during specificity optimization of the qPCR gametocyte assays we did not have access to confirmed *P. falciparum* DNA from parasites isolates circulating in South America and therefore, we cannot completely exclude the possibility that cross-reactivity did exist. Specificity was limited by testing samples from known *P. falciparum* infections from Kenya and would be further supported by testing both *P. falciparum* and *P. vivax* samples from other endemic areas of the world, to include South America and Southeast Asia.

While there was no difference in gametocyte abundance by age or gender, there was an interesting observation of increased *PvMGET* copy numbers/μL in individuals that worked in the mining sector. While little can be interpreted from this specific observation, it is worth considering which parasite isolates are circulating in those work (or living) conditions and if there is parasitic competition from multiple infections which can lead to increased male gametocyte production (reviewed in (203)).

The linear dynamic range was determined to be between 10 copies copies/μL to over 100,000 copies per μL with a lower, non-linear limit of detection between 1.0 and 2.5 copies per μL. The lower limit of detection confirms the assay's utility in identifying gametocyte prevalence at low-level parasitemias, specifically because low-density infections are capable of transmitting to the mosquito vector and low-density infections often are present in Amazonian populations (6; 7). One limitation of the qPCR gametocyte-specific assays was that reproducibility was not evaluated in this study and is an important component of assay validation. Additionally, because each assay was run independently, DNA sample volume was not able to be conserved for continued analyses. Future studies could consider optimizing the molecular markers for duplex or multiplex assays in order to conserve DNA template, time, and costs.

While the exact kinetics of these molecular markers may not be fully described from this study, we were able to prove that orthologous markers are viable candidates for continued investigation into the kinetics of the different stages of *P. vivax* gametocytes, gametocyte sex ratio, and gametocyte commitment. Because *P. vivax* gametocytes are difficult to detect by microscopy, molecular methods such as the ones presented will aid

in future research efforts supporting campaign efforts to eliminate gametocytes and prevent malaria transmission.

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Chapter 5: Summary and General Conclusions

DISSERTATION SUMMARY

Plasmodium gametocytes are the specialized sexual precursor to gametes which are required to continue the life cycle of malaria transmission to the mosquito. Because they are not associated with any clinical manifestations, they often are not the priority in global funding and research campaigns. There is still much to be learned about gametocyte biology and its complex relationship with malaria transmission. The overarching goal of this dissertation was to contribute to the understanding of malaria transmission by assessing the relationship between gametocyte sex ratio/density and mosquito infectivity relative to various host dynamics. To address our main research objectives, we investigated orthologous *P. falciparum* and *P. vivax* gametocyte-specific mRNA transcripts to be used in novel molecular assays and assessed their utility in asymptomatic infections from a *P. falciparum* holoendemic region, symptomatic human *P. vivax* infections, and sporozoite induced *P. vivax* monkey infections. Using sensitive ddPCR and qPCR technologies to detect and quantify gametocyte gene expression, we were able to evaluate male and female gametocyte biology in study groups that are often underrepresented in malaria research.

Aim 1: **Develop, apply, and evaluate a *Plasmodium falciparum* gametocyte molecular panel for sensitive detection in a longitudinal prevalence study of asymptomatic malaria patients with varying HIV-1 status.** This aim was accomplished by designing a highly sensitive ddPCR assay using three independent gametocyte-specific molecular transcripts. The assay utility proved useful in detecting gametocytes in over half of all asymptomatic *18S* positive volunteers. To our knowledge, this was the first utilization of ddPCR to detect gametocytes in the field specifically in a

low-density high sample size study. During analysis, we confirmed that transmission potential should not be determined by only parasite or gametocyte presence but should include actual data from mosquito infections. Additionally, we found an interesting relationship between HIV-1 ART and TS and gametocyte burden. The complex logistics of such a study yielded a sample size that proved too small to answer some aspects of our hypothesis discussed below.

Aim 2: **Determine and evaluate molecular markers for sensitive detection of *Plasmodium vivax* gametocytes in monkey and human infections.** This aim was accomplished by designing four sensitive gametocyte-specific qPCR assays that detected transcript expression in multiple monkey infections as well as in natural infections from volunteers in Peru. Thankfully, we were able to collaborate with multiple partners to obtain access to sample sets, however, we were limited by the fact that we did not have control over the entire study design with access to data that would have augmented our ability to answer our hypothesis. Many of these limitations are discussed below.

THE COMPLEXITY OF MALARIA/HIV-1 CO-INFECTION AND MALARIA TRANSMISSION

As reviewed in chapter 2, the biological complexities of both HIV-1 and malaria lead to significant gaps in understanding how the two pathogens interact in the human host and further how they impact malaria parasite transmission. Individually, the disease mechanisms are well understood, however, when presented as a co-infection, interpretations are often conflicting. Many studies that looked at the interaction between the two diseases focused on clinical outcomes. The work detailed in chapter three focused on evaluating the interaction between the diseases with an emphasis on critical components of malaria transmission. Using gametocyte-specific transcript markers of

mature female and male gametocytes (*Pfs25* and *PfMGET)* as well as a marker hypothesized to be expressed at earlier developmental stages (*Pfs16)*, we were able to detect the presence of gametocytes in asymptomatic individuals from the endemic region of Kisumu, Kenya. Furthermore, we were able to observe gametocyte expression over time relative to HIV-1 status, CD4+ T-cell levels, and other components of laboratory performed CBCs.

Contrary to results from a preliminary cross-sectional study (231), HIV-1 positive individuals were not more likely to harbor gametocytes than HIV-1 negative in our longitudinal study. These discordant results could be attributed to statistical challenges to include a large difference in study sample sizes and the requirement to correct for multiple comparisons. Additionally, different molecular detection techniques and markers were used. Biologically, we were limited in our access to immuno- and hematological confounders that are often associated with either disease. We were able to conclude that neither CD4+ T-cell levels nor RBC were predictors of gametocyte prevalence or abundance. The study would have been further enhanced if we were able to better evaluate each volunteer's stage of HIV-1 with viral load numbers.

Independently, we did not observe any significant fluctuations of gametocyte expression levels or positivity over time for any of the study groups. However, due to the nature of the study design, we were able to assess the longitudinal impact of the initiation of ART and TS and/or AL on gametocyte expression levels. Volunteers who were prescribed ART, TS, and AL (all HIV-1 positive) saw a significant reduction in gametocyte transcript prevalence the following month when compared to volunteers who were only prescribed AL (HIV-1 negative). These observations suggest that the current

health guidelines and use of ART and TS may contribute to a reduction in asymptomatic malaria reservoirs.

As discussed in chapter 2, to make conclusions on transmission potential it is important to look at the entirety of the malaria life cycle and study the relationship between overall parasite prevalence, gametocyte prevalence, and mosquito infectivity. In this present work, we were able to conclude that neither the presence of male or female gametocytes nor their transcript abundance can accurately predict successful transmission. Using even the most sensitive molecular techniques, parasite transmission to the mosquito occurred in the absence of detectable parasites and gametocytes (ddPCR LOD: 1.2 copy numbers per μL). Unfortunately, due to assay and study limitations, no concluding observations were made in reference to gametocyte sex-ratio in this population. The *PfMGET* molecular marker was detected less often than the *Pfs25* marker. *Pfs25* was detected in 314 samples while *PfMGET* was detected in 208 samples (approximate ratio 1.5:1.0) with average concentrations of 17.5 and 20.8 respectively. These results somewhat agree with the typical female bias observed in natural infections (3 to 5:1.0), however, our original selection of *PfMGET* as a representative for male gametocyte-enriched transcripts stemmed from a study that showed *PfMGET* transcripts were expressed at comparable levels to *Pfs25* (138). The challenges of using transcripts to quantify sex ratio may also have been impacted by the low sample volume of blood used in DBS as well as the higher limit of detection for *PfMGET* compared to *Pfs25*. While we were unable to fully assess the utility of *PfMGET*, this is one of the first studies to investigate a male specific molecular marker in low-density field samples. Additionally, the molecular marker *Pfs16* was observed significantly less often than in

the cross-sectional qPCR study (231). The difference in prevalence of the *Pfs16* transcripts is worth investigating further. It is important to consider the temporal separation between the two studies; overall parasite and gametocyte prevalence may be changing in our study area due to a multitude of environmental or social factors as well as enhancements in clinical diagnostics and treatment/preventions.

This research study was limited by several factors. The most significant limitation was in the development and utility of the ddPCR assay. While the ddPCR assay was able to detect gametocyte transcripts in 51.1% of all parasite positive samples (compared to the cross-sectional study with 20.5% positive transcripts using a similar qPCR assay in a similar parasite positive population (231)), the calculated absolute concentration of each gametocyte specific transcript marker was significantly lower than calculated copy numbers/uL of plasmid standards by qPCR. These low absolute concentrations were also observed in the field samples. With such low concentrations, any analysis of transcript abundance was difficult and often very left skewed (limited range). The difference between ddPCR concentrations and qPCR plasmid copy numbers/uL were also observed in the first published use of ddPCR to detect human malaria parasites by Koepfli et al. (131) These combined results also agree with recent studies that plasmids can result in an overestimation of template concentrations using qPCR and spectrophotometric measurements when compared to ddPCR (25) (112) (69; 78) (208) (221) .

ddPCR is a newer molecular technique and its application in detecting pathogens has only been reported in recent years [reviewed in (141)]. To our knowledge, this study is the first of its kind to assess the utility of ddPCR in gametocyte detection, specifically in high sample size (n) and low-density parasite field samples. Unfortunately, because

ddPCR has rarely been used outside of controlled malaria transcript studies, it was difficult to follow any specific regulations or recommendations for determining thresholds for positive and negative droplets. Even after recommended optimizations, both the *Pfs25* and *PfMGET* marker presented with what is classified as "rain" in a ddPCR reaction (237; 253). The presence of "rain" droplets promoted an investigation in how to determine the threshold limit without introducing researcher bias. When the same method was applied to determine the threshold of the *Pfs16* molecular marker, the method was not sufficient and inaccurately set the threshold to include negative droplets.

Another limitation of the study was in the study design itself. We used two different molecular techniques to determine parasite prevalence and gametocyte prevalence: an *18S* rRNA qPCR assay for parasite prevalence and ddPCR assay for gametocyte prevalence. If ddPCR is hypothesized to be more sensitive than qPCR, we may have unknowingly excluded potential gametocyte positive samples by only analyzing samples for gametocytes that were *18S* positive. Additionally, study time points at week 1 and week 2 were not included for all study groups and therefore were not included in statistical analyses despite observations worth investigating (i.e. increase in *PfMGET* positivity within the first two weeks of ART/TS initiation).

Continued investigation into the complexities of this sample set is recommended. Future studies should determine parasite genotypes to assess the chronicity of infection as well as determine if there is a significant difference in *P. falciparum dhfr* and *dhps* haplotypes amongst HIV-1 positive individuals as previously observed (248). These mutations are associated with antifolate (TS) resistance and could result in higher gametocyte prevalence and potentially enhance transmission. Additionally, the ddPCR

assay would benefit from investigations into the reproducibility of the assay and/or include newer molecular markers that have the potential to improve detection across all gametocyte stages and specifically the male sex (85; 202; 210; 262).

USE OF ORTHOLOGOUS GAMETOCYTE MARKERS IN *P. VIVAX* INFECTIONS

In support of the objectives for Aim 2, the work detailed in chapter four focused on the development of qPCR assays to quantify expression of *P. vivax* gametocyte molecular markers in monkey and human infections. Due to advanced genomic technologies, we were able to identify orthologous genes between *P. falciparum* and *P. vivax* and develop multiple qPCR assays to detect *P. vivax* gametocyte-specific transcript prevalence and abundance. The combination of four unique markers allowed us to further characterize gametocyte prevalence, gametocyte sex-ratio, and gametocytogenesis. All four markers were successful in detecting gametocytes with high agreement with gametocytes/μL determined by microscopy (the current gold-standard). Compared to microscopy, the qPCR assays were able to detect significantly more gametocytes in both monkey and human infections, improving overall *P. vivax* gametocyte detection. Additionally, these assays were evaluated based on several parameters to include limits of detection and quantification and specificity. Overall, these assays were highly sensitive and specific.

Our sex specific assays were based on known expression levels of mature *P. falciparum* female (*Pfs25)* and male (*PfMGET)* gametocytes. In a *P. vivax* sporozoite induced infection of *Aotus nancymae* monkeys, ortholog *Pvs25* was expressed earlier than *PvMGET* and in higher abundance. When applied to field samples from Peru, *Pvs25* was also expressed in higher abundance than *PvMGET*. *Pvs25* was detected early in

monkey infections, leading to a significant finding that gametocytes are present and therefore possibly transmissible before diagnostic detection in most clinical settings. Additionally, *Pvs25* was detected after other gametocyte markers were no longer detected and often after parasites were no longer detectable by microscopy. Another significant finding was the change in sex-ratio after MQ treatment in the monkeys. There was an observed increase in *Pvs25* abundance two to five days after treatment while *PvMGET* abundance decreased. These results are contrary to general hypotheses that parasites increase their male gametocyte commitment when their existence is threatened (201).

The two other orthologs used were *Pvs16,* as a possible early-stage marker, and *PvAP2-G* as a marker for gametocytogenesis. While both markers were observed during assay optimization, *PvAP2-G* was not detected in the spleen intact monkey infection nor during natural human *P. vivax* infections. *Pvs16* was expressed at moderate and intermittent levels throughout both monkey and human infections but was not expressed any earlier than *Pvs25* as hypothesized.

There were several limitations in this study. First, despite specificity optimization to include various strains of *P. falciparum*, *P. vivax* gametocytes were detected in eight of nine human samples that were determined to be *P. falciparum* infections by PET-PCR. The discordant results may be due to gametocyte or species assay limitations and should be investigated further, perhaps by genomic sequencing methods. Another limitation is the utility of gametocyte-specific transcripts to accurately quantify sex-ratio. Especially in low-density infections, transcript abundance may not accurately reflect gametocyte quantity due to the distribution of parasites in a single DBS, the quality and use of plasmids in qPCR assays, or the overall limits of quantification in each assay. *PvAP2-G*

failed to test positive in spleen intact and human infections. From the little that we know about *PfAP2-G,* it is possible that the transcripts were expressed at levels too low to detect by our designed assay. Additionally, while we know that *PfAP2-G* is expressed in low levels of *in vitro* sexually committed parasites, we do not know if *PvAP2-G* follows the same kinetics or where in the human body that these parasites may be developing if not in circulating blood. Continued investigation into the mechanisms of the immune system and the role of the spleen in gametocyte commitment is worth considering.

To further enhance these assays, we would recommend continuing to investigate the specificity of these assays by incorporating samples of *P. vivax* and *P. falciparum* from multiple global regions, to include areas in and around the Amazonian basin. Additionally, the qPCR assays would benefit from investigations into the reproducibility of the assays and/or include alternative molecular markers that have the potential to distinguish between stages of gametocyte development or alternate markers for male gametocyte detection (207). Particular attention should be placed on the utility of the *Pvs16* assay for detection of gametocytes due to conflicting results surrounding the kinetics of the ortholog in *P. vivax* infections from recent transcriptomic studies (4; 207). Future studies should continue to evaluate the kinetics of gametocytes by incorporating full genome sequencing of parasites circulating in both the human infections of Peru and in the newly adapted strain that was used to infect monkeys.

FINAL CONCLUSIONS

P. falciparum and *P. vivax* are two of the most harmful vector-borne parasitic pathogens in the world. Despite centuries and decades of research into the mechanisms of the malarial disease they cause, almost half of the world is still at risk for infection. The

overall goal of this research was to contribute to our current understanding of malaria parasite transmission by utilizing novel techniques and methodologies in understudied populations. We presented two different molecular approaches to help achieve this goal and highlighted unique populations and areas of concern that should be considered when developing global and public health strategies and campaigns. In an era focused on the elimination and eradication of vector-borne diseases such as malaria, the final challenges will be controlling the influence of sub-clinical reservoirs and their understudied contribution to disease transmission. A shared vision with continuous and incremental experimental contributions will open doors to a world where malaria no longer threatens the lives of today and tomorrow. As the Dalai Lama once said, “If you think you are too small to make a difference, try sleeping with a mosquito.”

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