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EVALUATION OF FACTORS ASSOCIATED WITH INCREASED
PLASMODIUM FALCIPARUM GAMETOCYTE-SPECIFIC TRANSCRIPTS IN
TWO POPULATIONS FROM SUB-SAHARAN AFRICA

by

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“The malaria parasite has been killing children and sapping the strength of whole populations for tens of thousands of years. It is impossible to calculate the harm malaria has done to the world.”

Bill Gates

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DEDICATION

To my family, especially my husband, for their unwavering support and unconditional love.

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May 15, 2020

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ABSTRACT

Evaluation of Factors Associated with Increased *Plasmodium falciparum* Gametocyte-Specific Transcripts in Two Populations from sub-Saharan Africa

Deborah M. Stiffler, Doctor of Philosophy, 2020

Thesis directed by: V. Ann Stewart, D.V.M., Ph.D., Professor, Department of Preventive Medicine and Biostatistics, Division of Tropical Public Health

Malaria is a major cause of morbidity and mortality in sub-Saharan Africa due to the high prevalence of *Plasmodium falciparum* in that region. Molecular methods for malaria detection can be useful in identifying individuals with sub-microscopic infections who may be transmitting malaria. To identify individuals with sub-microscopic gametocytemias, we developed a panel of qPCR assays that quantify gametocyte stage-specific RNA transcripts. These assays were validated using both highly synchronized cultured gametocytes and a small subset of field samples before being used to characterize gametocyte carriage in two larger studies.

In the first study, gametocyte transcripts were quantified in adults from the Kisumu region of Kenya who had asymptomatic malaria infections and were seeking voluntary testing for HIV. In this population, approximately 20% of individuals had detectable gametocyte-specific transcripts. Additionally, being HIV positive was associated with an increased risk of gametocyte positivity (relative risk = 1.820). Among

gametocyte positive individuals, those who were HIV-positive had higher copy numbers of gametocyte transcripts. These data suggest that HIV-positive individuals may be at a higher risk of transmitting malaria to mosquitoes, although standard membrane feeding assays are needed to confirm that the increase in transcripts correlates to an increase in infectivity.

Additionally, these assays were used to characterize gametocyte transcripts and asexual transcripts in children under 5 years of age who were treated with artemether-lumefantrine (AL) for uncomplicated malaria. In this cohort, we found that AL differentially affects gametocyte and ring-stage transcripts, and that a majority of children maintained ring-stage transcripts through the period of follow-up. Although the epidemiological significance of this is not yet known, these findings suggest that AL may not be capable of full clearance of *P. falciparum* infections. Several factors were also associated with gametocyte transcript positivity at one- or two-weeks post-treatment, including a higher gametocyte burden during the first 48 hours of follow-up, and a large drop in hemoglobin levels in the first week of follow-up.

Overall, these data show the utility of this qPCR panel in detecting stage-specific transcripts and provide important insight into potential risk factors for gametocyte carriage in regions with high malaria transmission.

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CHAPTER 1: General Introduction

THE COST OF INFECTIOUS DISEASES IN SUB-SAHARAN AFRICA

For many years, communicable diseases have been the leading cause of death in sub-Saharan Africa (173), (182). In the past 40 years, human immunodeficiency virus (HIV) has become a major cause of morbidity and mortality, joining malaria, lower respiratory infections, and diarrheal diseases as the top four causes of adult mortality. Generally, mortality due to infectious diseases is declining in sub-Sahara Africa, but morbidity due to infectious disease remains high and has a tremendous impact on quality of life. The disability created by these diseases contributes to the under-development of the region, reviewed in (26).

This underdevelopment, and the lack of public health infrastructure associated with it, functions as a substantial barrier to the reduction of infectious disease-related morbidity and mortality, but this barrier is not insurmountable. In recent years, great progress has been made to improve overall health in Africa. Much of this progress has been achieved through the combined efforts of targeted local programs and global outreach in support of improving the overall quality of life in developing nations. Non-governmental organizations and private foundations, such as the Bill and Melinda Gates Foundation, have been strong financial supporters of a push for the global eradication of malaria, along with many other initiatives to improve global development. Currently, one of the most comprehensive current partnerships is spearheaded by the United Nations, who in 2015, adopted a set of seventeen goals, called the Sustainable Development Goals (223). While not all of these goals are explicitly related to public

health, all of the targets, such as improved gender equality and universal access to quality education, will indirectly reduce the burden of infectious disease by raising the overall standards of living. Ultimately, it is hoped that the overall improvements made to health care infrastructure as a result of achieving these goals will reduce disease-related morbidity and mortality, and lead to the reduction, or even elimination, of some of the most debilitating infectious diseases.

In the interim, both the treatment and prevention of infectious diseases remain high priorities. To facilitate better clinical management, a better understanding of the transmission biology of these important pathogens is needed.

***PLASMODIUM SPP.* PARASITES AND HUMAN MALARIA**

Importance of Human Malaria

Malaria is a mosquito-borne disease caused by *Plasmodium spp.* parasites and is one of the oldest known diseases affecting humans. The name comes from the Italian “*mal aria*,” or “bad air,” as the disease had been associated with swampy environments capable of supporting the mosquito vector since long before the discovery of an arthropod vector in 1897 by Ronald Ross. Records of the symptoms of malaria date back to ancient Mesopotamia (105), and molecular techniques for the detection of malaria parasites have confirmed that ancient Egyptians suffered from malaria as far back as 3200 BC (142). Morbidity and mortality due to malaria have shaped many important events in history, including the American Civil War (13), World War II (96), and the Vietnam conflict (11). Malaria has sustained the presence of sickle cell trait and other

red blood cell defects that confer resistance to the parasite (42). Additionally, malaria has had a major effect on human settlement patterns because until recently, areas with large vector capacities, including regions of the southern United States, were made undesirable for habitation due to the high likelihood of contracting malaria during all or part of the year (105), (196).

Today, malaria continues to have a profound global impact, and there are long-term repercussions of this disease which extend beyond the immediate health care costs for treatment. Early exposure to malaria is associated with an increased risk of childhood mortality (59). For children who do survive, childhood bouts of malaria are associated with decreased academic performance, whereas the eradication of malaria has been shown to substantially increase educational attainment and family income, especially in individuals from lower socioeconomic groups (117), (193). Adults are also impacted by malaria; women in Tanzania reported having had to choose between seeking outside employment or maintaining their household's insecticide treated mosquito nets, which are vital for protecting their families while sleeping (60). More recently, outbreak of the Ebola virus resulted in such widespread fear of exposure that access to health care decreased substantially and an estimated 6,818 patients in Guinea, Liberia, and Sierra Leone died of malaria because they did not seek timely treatment (164).

While malaria was once ubiquitous in areas capable of hosting the mosquito vector, malaria control techniques such as the use of residual insecticide spraying, window screens, insecticide-treated bed nets (ITNs), and vector habitat removal have helped to eliminate or reduce malaria in many areas of the world. However, almost half

of the world remains at risk, and those living in resource-limited settings, especially in sub-Saharan Africa and Southeast Asia, are most likely to contract malaria.

Malaria Life Cycle and Vectors

Plasmodium spp. parasites are apicomplexan protozoan parasites. All members of the phylum Apicomplexa are obligate intracellular parasites and are named for the presence of an apical complex, which is important for invading host cells. Most member of the Apicomplexa phylum also possess a unique organelle called the apicoplast, which is believed to be derived from secondary endosymbiosis of algal organism and is the location of key metabolic functions (124). In addition to *Plasmodium*, several members of this phylum are important human pathogens, including *Babesia*, which causes the tick-borne illness babesiosis, and *Toxoplasma gondii*, which causes toxoplasmosis.

There are currently five species of *Plasmodium* parasites known to cause human malaria, described in Table 1. Although small enough to reside in red blood cells and hepatocytes, these organisms are eukaryotic with complex genomes comprised of 14 chromosomes and approximately 5,300 genes (82). The *P. falciparum* genome has been sequenced since 2002 and is notable for being composed of 80.6% adenine and thymine (AT) (82), making it one of the most AT rich genomes currently known and creating additional challenges for molecular genomic work, which often requires a particular guanine and cytosine (GC) content for successful primer annealing (82).

Plasmodium parasites are distributed throughout the world and can be found in North America, South America, Europe, Africa, and Asia, with different parasite species

predominating in different regions (Table 1) (236). Globally, *P. falciparum* and *P. vivax* are the two most prevalent species.

Table 1. Parasite characteristics for the causative agents of human malaria.
Modified from (181).

Species	<i>Plasmodium falciparum</i>	<i>Plasmodium vivax</i>	<i>Plasmodium malaria</i>	<i>Plasmodium ovale</i>	<i>Plasmodium knowlesi</i>
Geographic Distribution	Widespread	Widespread but rare in West Africa	Africa, Southeast Asia, Oceania, South America	West Africa, Western Pacific	South Asia
Length of Exo-Erythrocytic Cycle	5.5-7 days	6-8 days	9 days	12 days	16 days
Length of Intra-Erythrocytic Cycle	43-52 hours	48 hours	72 hours	48 hours	24 hours
Age of Red Blood Cell Infected by Parasites	All ages	Reticulocytes	Older red cells	Reticulocytes	All ages
Number of Merozoites from Erythrocytic Schizonts	16-20, rarely seen in peripheral blood due to sequestration	20-24 merozoites, generally >12	6-12 merozoites	6-14 merozoites	8-16 merozoites, usually more <12
Earliest Appearance of Gametocytes	10 days	3 days	Unknown	Unknown	Unknown

Plasmodium spp. parasites have a complex life cycle that includes a mammalian host and an arthropod vector (Figure 1). *Plasmodium* can be transmitted to humans when an infected *Anopheles* mosquito takes a blood meal. While probing, the mosquito injects *Plasmodium* sporozoites with saliva. In less than an hour, these sporozoites move to hepatocytes, where they replicate as schizonts unassociated with any clinical signs or

symptoms. During the hepatic life cycle stage, parasites replicate by schizogony, which is a form of asexual replication found in some protozoa that allows each maturing schizont to replicate into many merozoites simultaneously. *P. vivax* and *P. ovale* share the ability to become dormant in the liver. These dormant parasites are called hypnozoites and can persist for month to years.

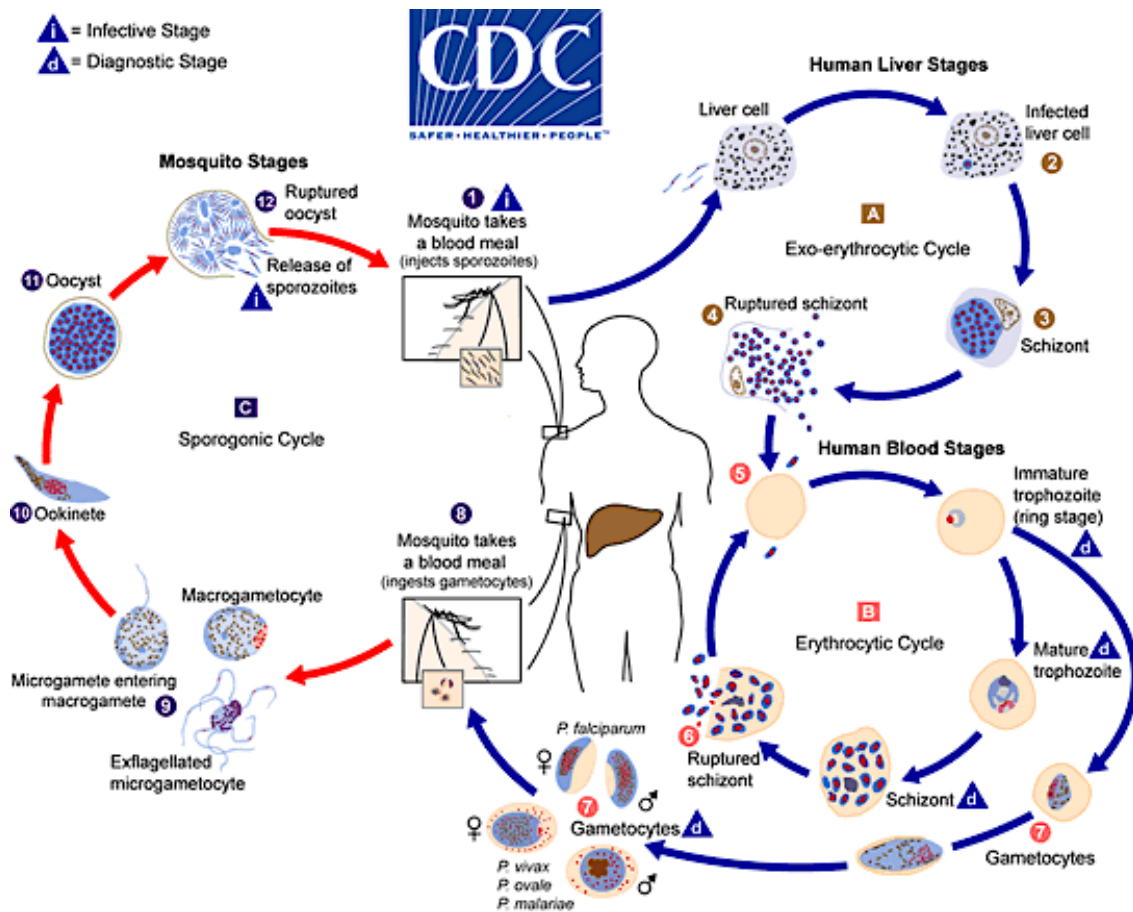


Figure 1. Life cycle of *Plasmodium* spp. that cause human malaria. Adapted from (43).

Clinical illness does not occur until merozoites emerge from the hepatocytes and begin replicating asexually in erythrocytes, first as trophozoites and then as erythrocytic schizonts that ultimately lyse and release more merozoites into the blood stream. The

number of resulting merozoites that a single schizont can divide into depends on the species of parasites (Table 1). During the erythrocytic stage of the life cycles, parasitemia increases with each round of invasion and schizogony. *P. falciparum* parasites sequester in the vasculature during much of their erythrocytic development; as a result, only ring stages are seen in peripheral blood smears. The attachment to the walls of the vasculature prevents clearance of the infected RBC in the spleen and is mediated by the erythrocyte membrane protein-1 (PfEMP-1), which is capable of extensive antigenic variation that simultaneously allows the parasite to develop in a protected compartment and evade the host's adaptive immune response (165).

During the erythrocytic life cycle, some parasites commit to forming gametocytes, which are the sexual and transmissible stages of the parasite in humans. In *P. falciparum* malaria, triggers for the formation of gametocytes are incompletely understood, but both the hosts increasing specific immunity and several different types of drug therapy appear to trigger increased gametocytogenesis. Gametocyte commitment occurs at a low frequency at all times. Gametocyte maturation occurs through five stages of development, referred to numerically as Stage I through Stage V, with the earlier stages being indistinguishable from trophozoites by microscopy. Gametocyte maturation takes place over the course of approximately ten days, at which time mature gametocytes circulate through the body and are capable of being picked up by a mosquito taking a blood meal. In *P. falciparum*, developing gametocytes are sequestered primarily in the bone marrow, but also in the spleen (108). Unlike developing erythrocytic stages, this sequestration is not mediated by PfEMP1 and may be related to morphological changes that occur in red blood cells containing gametocytes, which could physically restrict the

ability of the developing gametocyte to move freely through circulation until they become more deformable mature gametocytes (108), (56).

The sexual stage of the parasite life cycle occurs after a mosquito ingests both male and female stage V gametocytes during a blood meal. Immediately upon ingestion, environmental cues in the mosquito midgut, including a reduction in temperature, trigger the development of gametocytes into gametes. These gametes then form diploid zygotes, which mature into motile ookinetes, which are also diploid. These ookinetes are able to travel through the wall of the midgut into the salivary gland, where they form oocysts that become quadruploid and undergo two rounds of recombitorial meiosis before each of four haploid daughter cells undergoes schizogony. The oocysts release mature sporozoites that migrate to the salivary gland to continue the cycle of infection in another human host.

CLINICAL PRESENTATIONS OF MALARIA

In 2017, there were estimated to be between 203 and 262 million cases of clinical malaria worldwide, of which 92% occurred in Africa (236). Additionally, in 2017, approximately 435,000 deaths could be attributed to malaria, with 61% of malaria deaths occurring in children less than 5 years of age (236). The World Health Organization estimated that in 2016, malaria was responsible for the loss of over 34 million disability adjusted life years (DALYs) in Africa alone (235).

Only the erythrocytic schizont life stages of the parasites cause illness; the hepatic stage and sexual stage of the parasites are clinically silent. Clinical malaria can be divided into two presentations: uncomplicated malaria and severe malaria.

Uncomplicated Malaria

The most common clinical presentation of malaria is uncomplicated malaria, which is characterized by severe chills, fever, and hyperhidrosis, and if left untreated, occurs in episodes, referred to as malaria paroxysms. These symptoms are due to the immunopathology caused by the release of hemozoin, a byproduct of parasite metabolism, and other toxic byproducts of the parasite, which occurs during the rupture of RBCs and subsequent release of merozoites. Additionally, some individuals experience prodromal symptoms, including myalgia, headache, and mild fever, several days prior to a more acute episode of illness. The incidence of clinical malaria decreases with exposure to infected mosquito bites due to the development of a partial immune response.

In areas of endemic malaria transmission, clinical malaria is most common in young children who lack a sufficient immune response to control parasitemia. In areas with low or seasonal transmission, malaria can occur at any age since infrequent exposure to the parasite is insufficient to sustain broad and lasting immunity. Although uncomplicated malaria itself is not considered a threat to life, it can very quickly progress to severe malaria, as replication by schizogony rapidly increases parasitemia over a short period of time. Thus, uncomplicated malaria must be treated as a serious and potentially fatal condition, especially in groups which are at high risk of developing severe malaria, including naïve individuals, children, and pregnant women.

Severe Malaria

Severe malaria can occur when treatment is delayed, or in malaria-naïve individuals whose immune systems are unable to control parasite replication. Sub-Saharan Africa experiences the highest rates of severe malarial disease and malaria mortality due to the predominance of *P. falciparum*, which is the parasite species that causes the most severe clinical disease. Although often considered less severe when compared to *P. falciparum*, *P. vivax* can also cause severe symptoms and mortality, particularly in infants and pregnant women. The term severe malaria broadly describes any organ failure associated with symptomatic malaria, but the two most common and serious presentations are cerebral malaria (CM) and severe malarial anemia (SMA).

CM occurs more often in children than in adults and is characterized by the presence of *P. falciparum* parasitemia and decreased mental status (defined by a Blantyre Coma Score ≤ 2 in children or a Glasgow Coma Score ≤ 9 in adult) unattributable to any other pathology. Additional signs and symptoms of CM can include seizures, posturing, and abnormal respirations (ie: Cheyne-Stokes respirations). Ultimately, some children with CM suffer increased intracranial pressure resulting in herniation and death. In children who survive CM, improvement occurs rapidly after treatment; most return to normal mental status within 48 hours. The pathogenesis of CM is not completely understood but is thought to be due to a combination of parasite sequestration in the brain, endothelial cell dysfunction, and dysregulation of cytokines (reviewed in (102)).

SMA occurs secondarily to the hemolysis of both infected and uninfected RBCs and erythropoietic dysfunction. Lysis of RBCs during the release of merozoites destroys infected RBCs, but up to 90% of malaria-related hemolysis is from uninfected RBCs

(131), (170). Lysis of non-infected RBCs is not fully understood but is hypothesized to be caused by aberrant immune responses and complement (219), (77). SMA can be particularly devastating in children who were previously suffering from anemia due to poor nutrition or concurrent helminth infections prior to becoming infected with malaria, and pre-existing anemia has been associated with a slower recovery from their malaria-related anemia (170).

Asymptomatic Malaria

In areas with high levels of malarial transmission, a large portion of the population is likely asymptotically, and chronically, infected with malaria. These individuals maintain a parasite burden without exhibiting any signs or symptoms of malaria infection due to partial immunity to the parasite. There has never been a documented case of naturally acquired sterilizing immunity to malaria, but it is common for partial immunity to malaria to develop after repeated infection. This partial immunity is predominantly antibody-mediated and develops slowly due to the extensive antigen variation that is encoded in each parasite genome (reviewed in (136) and (129)). As immunity to the erythrocytic forms of the parasite develops, there is a reduction in clinical episodes over time; this explains why most malaria deaths in holoendemic areas occur in children under the age of 5 (Figure 2). In addition to maintaining a low, sometimes undetectable, asexual parasite burden, individuals with asymptomatic infections are capable of producing gametocytes and thus could be contributing to malaria transmission. Because these individuals do not feel sick and are therefore carrying out their normal day-to-day business, it is possible that they might contribute

more to the reservoir of transmission than an individual who is sick and resting in bed under a mosquito net.

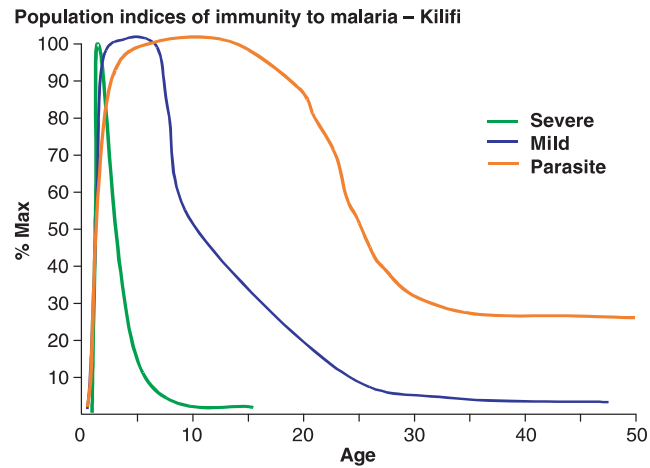


Figure 2. Population indices of immunity to malaria in Kilifi, Kenya.
Modified from (136)

The true prevalence of asymptomatic malaria is currently unknown and likely varies by transmission intensity, but our laboratory found that approximately 63% of asymptomatic adults seeking voluntary HIV testing in the Kisumu region of western Kenya were positive for *Plasmodium* by qPCR detection of *Plasmodium 18S* rRNA (Kifude, C, in prep.). The incidence of asymptomatic infection and the prevalence of gametocytes in asymptotically infected individuals are both poorly characterized; thus, it is unknown to what extent these chronically infected individuals contribute to malaria transmission. Our laboratory thus set out to perform a molecular characterization of both asexual transcripts and gametocyte-specific transcripts in order to better understand the transmission potential of asymptotically infected individuals.

TREATMENT

Clinical malaria is treatable with a variety of drugs, although drug resistance has been reported for many commonly used antimalarials, including sulfadoxine/pyrimethamine (55), mefloquine (19), (204), halofantrine (83), (32), and chloroquine (183), (27). Currently, most episodes of clinical malaria are treated by artemisinin combination therapies (ACT), which use two or more different drugs simultaneously to reduce the likelihood of treatment failure and the development of drug-resistant parasites. In reality, febrile illness is often treated empirically, leading to overuse of antimalarials and risking increased drug resistance.

Effects of Antimalarials on Gametocytes

Since the goal of antimalarial treatment is to reduce clinical disease, the effect of the drug on gametocytes is not always considered. The effect of antimalarial drugs on gametocytes is difficult to study *in vivo* due to a lack of control groups in human studies and the use of drug in combination, which can obscure the true effect of a single drug. Primaquine, methylene blue, and atovaquone are the only drugs currently known to be directly gametocidal (58). However, even those drugs which do not directly act on gametocytes are able to reduce gametocyte burden through elimination of the asexual stages that are precursors to gametocytes. Additionally, treatment with antimalarial drugs may impact the infectivity of gametocytes to mosquitos (161). Often, the sensitivity to antimalarial drugs varies throughout the course of gametocyte development, as seen with chloroquine; only very early gametocytes are sensitive to the drug (198), (200). Conversely, some drugs, including sulfadoxine-pyrimethamine (SP), have been shown to

increase gametocyte formation, especially in the context of drug resistance, although these gametocytes may be less infective to mosquitos than those found circulating prior to treatment (176), (90), (12). Overall, several factors complicate our understanding of the impact of antimalarials on gametocytes, including the use of drugs in combination, the differential impact of drugs on gametocytemia and infectivity to mosquitoes, and the dynamic nature of drug resistance.

METHODS OF MALARIA DETECTION

Clinical Diagnostics

The analysis of Giemsa-stained blood films by microscopy has long been considered the gold standard of malaria diagnosis in the clinic; in fact, the observation of pigmented bodies in the blood of patients with malaria preceded the actual discovery of the *Plasmodium* parasite as the causative agent of disease (53). Quantification of parasitemia can be done by microscopy, although for quantification and speciation to be accurate, slides must be read by an experienced microscopist. Another advantage of microscopy is the ability to directly visualize parasites for species identity and staging. Finally, the simplicity and low cost of sample collection and staining are well-suited to low resource settings.

Although used extensively in the clinic, microscopy has several significant limitations that make it poorly suited for epidemiological studies. First, microscopy has a lower limit of detection (LOD) of approximately 50 parasites per μl of blood (146), which makes it unsuitable for low parasitemia infections, which are commonly seen during asymptomatic malaria infection in areas of endemic transmission. Examination of

blood smears is a time-consuming process that is difficult to automate, making it impractical for high-throughput applications. Despite these limitations, microscopy remains a vital diagnostic tool for clinically ill patients.

For clinical diagnosis, rapid diagnostic tests (RDTs) are often used in place of microscopy. RDTs are simple to conduct and yield results in as little as 5 to 10 minutes. However, RDTs can be even less sensitive than microscopy, averaging a LOD of approximately 100 parasites per μl of blood (146), which can cause false negatives in patients with low parasitemia infections. RDTs are based on the detection of protein antigens, which remain in the blood after parasite clearance, leading to false positive results in individuals who have recently cleared their infections. Additionally, many RDTs detect the presence of histidine-rich protein 2 (PfHRP2), and an increase in the prevalence of *pfhrp2/3* gene deletions has led to false negative rates that exceed 50% (228). False negatives can also occur in individuals with exceedingly high parasitemias (when >5% of red blood cells are infected), a phenomenon called the prozone effect, which occurs because the amount of antigen present in the sample binds to all binding sites on both the detection and capture antibodies, preventing proper antibody complex formation (85).

Molecular Diagnostics

In light of the limitations of microscopy and RDTs, molecular techniques for malaria detection have become the preferred tools for epidemiological studies. These techniques range from loop-mediated isothermal amplification (LAMP), which can be conducted in the field, to quantitative nucleic acid sequence based amplification (QT-

NASBA) and quantitative real time PCR (qPCR), which are more suited to laboratory settings due to the requirement for stringent sample storage conditions. LAMP is particularly well-suited to low-resource setting because it is comprised of an entirely closed system; the amplification of DNA occurs in a single tube without the need of a thermocycler. LAMP is more rugged and portable than QT-NASBA and qPCR, but it is also less sensitive and still subject to issues of contamination and error. QT-NASBA and qPCR both use RNA as the nucleic acid template, which allows for the characterization of gene expression but also requires more careful handling due to the highly labile nature of RNA. QT-NASBA is similar to LAMP in that it is conducted in a closed-tube system without need of a thermocycler. qPCR requires the use of a thermocycler but is generally considered to be more sensitive than isothermal methods of amplification. qPCR chemistries can be based either on SYBR Green or TaqMan probes and can detect either genomic DNA or cDNA prepared from RNA. SYBR Green is an intercalating dye, which binds to double-stranded nucleic acid during amplification and fluoresces to generate a signal. TaqMan probes consist of a fluorophore and a quencher tagged on opposing ends of a DNA oligonucleotide that is complimentary to a section of the amplicon of interest. This fluorophore only becomes spectrophotometrically detectable once the probe is bound to the target DNA and the DNA polymerase reaches the probe, displacing the quencher molecule. TaqMan chemistries are more sensitive than SYBR Green because the probe is specific to the target sequence of interest, further reducing the likelihood of non-specific detection.

Molecular assays have been developed for numerous different malaria markers spanning all developmental stages of the *Plasmodium* life cycle. Examples of these

targets include *Plasmodium* 18S ribosomal RNA (112), which has both variable and highly conserved regions, *Pfsbp1*, which is a ring-stage marker (74), and *Pfs25*, which is commonly used as a *P. falciparum* gametocyte marker (16). Although these techniques are extremely sensitive and specific, the transcription of mRNA does not always directly correlate with immediate protein translation. Thus, gene expression data must be interpreted cautiously until verified by protein quantification or functional analyses, particularly when using ultra-sensitive methods, which may detect very low copy numbers that are below the threshold of being biologically relevant.

MALARIA TRANSMISSION

Gametocyte Commitment

The molecular mechanisms and processes of gametocytogenesis are still being investigated, but recent interest in transmission-blocking malaria vaccines has spurred additional research into the biology of gametocytes. Gametocyte commitment is believed to occur before schizogony, as all resulting merozoites from a given schizont will either develop into asexual parasites, male gametocytes, or female gametocytes, but never a combination (34), (194), (203). In the past ten years, several key players have been identified in the molecular pathways of gametocytogenesis, including *PfAP2-G*, which is the master transcriptional regulator of gametocyte formation (111). The activation of *PfAP2-G* has been linked to epigenetic changes mediated by several upstream proteins, including *P. falciparum* histone deacetylase 2 (PfHda2) (52), *P. falciparum* heterochromatin 1 (PfHP1) (30), and the gametocyte development 1 protein (PfGDV1)

(69), (78). PfHda1 and PfHP1 are involved in silencing *PfAP2-G* expression (52), (30), while PfGDV1 is thought to remove PfHP1 to facilitate *PfAP2-G* expression (78) (Figure 3). Although additional work is needed to fully understand that changes that occur during gametocyte commitment, epigenetic regulation is now thought to play a critical role in many of transcriptional changes that occur when the parasite switches from asexual to sexual reproduction (51), (reviewed in (110)).

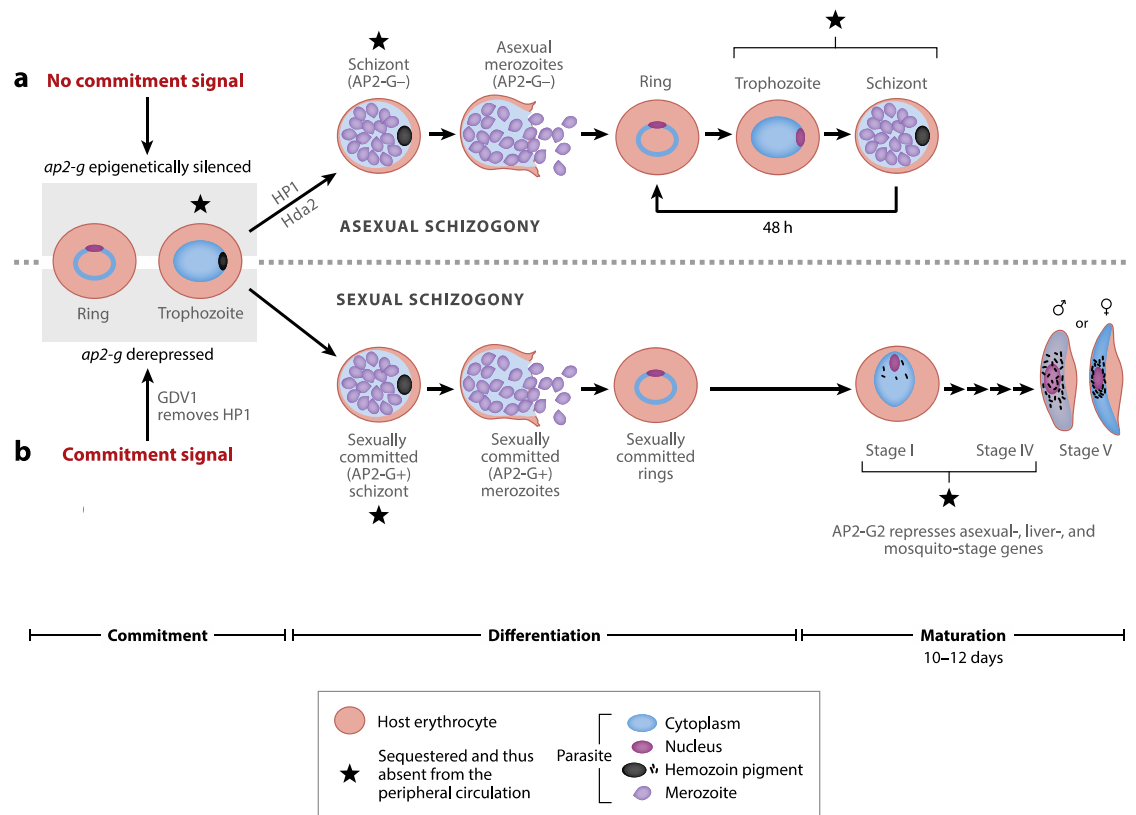


Figure 3. Current model for gametocytogenesis.
 Modified from (110).

Factors that Induce Gametocytogenesis

While gametocyte formation is known to occur nearly constantly at low levels, several environmental factors have been associated with increased gametocyte commitment, including treatment with antimalarial drugs. As previously discussed, treatment with sulfadoxine/pyrimethamine (SP) increases gametocyte burden (172), (176), and this also can occur after chloroquine treatment (36). Additionally, the detection of SP-resistant strains shows up first as increased gametocyte carriage after SP treatment (21).

Other environmental factors have been shown to increase gametocyte formation. In mice, mixed genotype infections caused increase gametocyte formation (216), suggesting that competition between strains and species of *Plasmodium* may be an important factor for gametocyte commitment. Host-specific factors such as host anemia (63), (169) and increased immune pressure (199) can contribute to gametocytogenesis. Recently, human lysophosphatidylcholine (LysoPC) has been identified as a regulator of gametocyte formation; this is the first time that a specific molecule has been identified as an environmental regulator of gametocytogenesis and provide proof-of-concept that parasites are capable of upregulating gametocyte commitment in response to an environmental change (31).

Importantly, increased gametocyte formation does not necessarily translate to increased infectivity to mosquitos. Decreased infectivity to mosquitos has been seen in spite a greater overall number of gametocytes; conversely, very low gametocytemias have been shown to be capable of infecting mosquitoes (149), (187). Additionally, the sex ratio of male to female gametocytes has long been suspected to play a role in ability

for sexual reproduction to occur in the mosquito midgut (7), and an increase in gametocytemia is not always evenly distributed between male and female gametocytes.

Transmission Blocking Immunity

Transmission blocking immunity (TBI) is able to develop slowly over many decades in response to repeated exposure (139), (88), (62). TBI primarily functions to block development of the parasites in the mosquito midgut and generally occurs through the ingestion of human serum, which contains antibodies and other host immune factors, during a mosquito bloodmeal. Many of these antibodies, including those specific to Pfs230, Pfs25, and Pfs48/45, have been or are currently being explored for use as transmission-blocking vaccine candidates (reviewed in (116)).

Although this vaccine strategy has the potential to be highly effective, the utility of a transmission-blocking vaccine may be limited because it is an “altruistic” vaccine; vaccination would not prevent the vaccinated individual from getting malaria but would help reduce the spread of the disease to other people. Preliminary surveys suggest that a vast majority of caregivers would give their children a transmission-blocking vaccine, but significant other challenges remain, including the logistical challenges of administering a new vaccine in a resource-limited-setting (50). Ultimately, the most effective use of a transmission-blocking vaccine may be in conjunction with chemoprophylaxis (40).

Importance of Transmission Control for Malaria Elimination

From 1955 until 2015, 27 countries and two territories have successfully eliminated malaria, including the United State of America, Australia, and several other countries scattered across Asia, Europe, South America, and Africa (233). Historically,

countries that have been able to eliminate or substantially reduce malaria prevalence have done so through the use of transmission-centric control strategies. These include a diverse range of methods, including the use of insecticides, removal of mosquito habitats, and use of window screens and bed nets, and these strategies remain effective tools for malaria control (33). Success also requires the implementation of substantial healthcare infrastructure and a firm commitment to eradication in order to be successful (10).

For example, the United States began their final elimination campaign during World War II by treating areas around important military bases in the South, and were able to expand that success after the war through cooperation between local and state health departments, the Centers for Disease Control, and the US Public Health Service (44). The use of DDT for indoor residual spraying was a primary means of malaria control and was used in combination with vector habitat removal and outdoor insecticide spraying. Previous attempts to eradicate malaria had failed, highlighting the need for coordinated and sustained commitment to treatment. The use of DDT was a primary means for elimination and eradication, but this insecticide is of very limited availability globally. Although once very effective, mosquitos have developed resistance to DDT, and resistance to other insecticides is a major emerging issue (79), (119). It is likely, then, that there is a limited window of opportunity for some vector control strategies to be used before the parasite is able to develop resistance to the insecticide or drug being used. Overall, it is expected that any successful control strategy will need to focus at least partially on intervening during the transmission stages of the malaria life cycle, and this will require sufficient infrastructure for coordinated and sustained initiatives.

MALARIA AND HIV CO-INFECTION

Human Immunodeficiency Virus (HIV)

The human immunodeficiency virus (HIV) has been widespread for less than 40 years, but in that time, it has caused an estimated 35 million deaths worldwide. HIV is a retrovirus that infects CD4+ T cells and causes acquired immune deficiency syndrome (AIDS), which is a fatal inability of the immune system to control opportunistic infections. Retroviruses are positive-stranded RNA viruses possess reverse transcription machinery to create DNA, which is then inserted into the host genome to form a provirus. The term “retro” refers to the incorporation of the viral genome into the host genome, as it is the opposite of how RNA is normally transcribed from genomic DNA. HIV is an example of a specific kind of retrovirus called a lentivirus, which is characterized by a particularly long incubation period.

There are two types of HIV, referred to as HIV-1 and HIV-2. In general, the term “HIV” refers to HIV-1, which is by far the most common and virulent type of HIV. HIV-1 is distributed throughout the world while HIV-2 is found predominantly in Africa. HIV-2 is less pathogenic and less transmissible than HIV-1 and is similar to certain subtypes of simian immunodeficiency virus (SIV). In addition to being more prevalent, HIV-1 is also more diverse than HIV-2. HIV-1 can be classified into four groups: M, N, O, and P. Group M is the predominant group and can be further divided into 11 subtypes. These subtypes generally follow geographical patterns and each have unique characteristics that may affect disease progression, severity, and transmission, along with drug susceptibility (215).

When a person is first infected with HIV, they experience acute HIV syndrome, which is characterized by flu-like symptoms, high HIV viral load, and a drop in CD4+ T cell count (Figure 4). During this initial infection, the virus disseminates widely throughout the body. After initial infection, HIV viral load drops and the virus can remain latent for several years before viral replication increases. During this latent period, viral load is low or even undetectable, and CD4+ T cell count can remain in the normal range while slowly declining. Reactivation of the virus leads to a rapid decline in CD4+ T cells that occurs concurrently with an increase in viral load, causing constitutional symptoms, including weight loss, fatigue, fever, malaise, dyspnea, night sweats, hyperhidrosis, decreased appetite, and pain. As CD4+ T cells decline, the patient begins to present with opportunistic infections or viral-induced cancers which eventually overwhelm the already-impaired immune system and lead to death. These infections and cancers are commonly referred to as “AIDS defining illnesses,” and examples of these illnesses include pneumocystis pneumonia, candidiasis, histoplasmosis, tuberculosis, Burkitt’s lymphoma and Kaposi’s sarcoma.

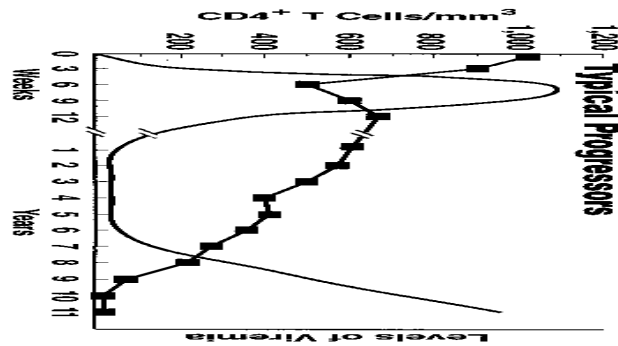


Figure 4. Natural History of HIV Infection.

Viremia peaks within the first 12 weeks of infection before it rapidly declines. After a long and variable latent period lasting several years, viremia begins to increase. CD4+ T cell count shows the inverse trend, with decrease T cells during periods of high viremia. Figure adapted from (163).

Clinical management of HIV infection is guided by the stage of the disease and aims to reduce viral load while preventing opportunistic infections. Both the World Health Organization (WHO) and the Center for Disease Control (CDC) have established guidelines for the classifying the stage of HIV infection (Table 3). In general, a patient is considered to have AIDS once their CD4+ T cell count has dropped below 200 cells/ul, even if their T cell count later increases above that threshold. In the United States, the CDC recommends antiretroviral drugs (ARTs) for all HIV positive patients upon diagnosis. The WHO has recently updated their recommendations to match those of the CDC (232). In 2016, the WHO estimated that approximately 46% of individuals who are known to be HIV positive are receiving treatment with ARTs (238), but this had increased to 64% by the end of 2018 (237), suggesting that ART scale-up programs are effectively improving ART coverage.

Current guidelines for antiretroviral drugs call for combination therapy to reduce drug resistance. For individuals who are unable to take the recommended first line drugs,

due to resistance, age, or pregnancy, there are alternative second-line drugs availability. Treatment failure rates vary based on a variety of factors, including specific drug usage, disease stage, and patient adherence, but overall, drug resistance is increasing and remains a major concern (reviewed in (49)).

Table 2. Summary of the WHO and CDC guidelines for classifying the stage of HIV infection.

World Health Organization Classification System for HIV (2007) (231)	
Primary HIV Infection	May be either asymptomatic or associated with acute retroviral syndrome
Stage I	HIV infection is asymptomatic with a CD4 ⁺ T cell count greater than 500 per µl of blood. May include generalized lymph node enlargement
Stage II	Mild symptoms which may include minor mucocutaneous manifestations and recurrent upper respiratory tract infections. A CD4 count of less than 500/µl.
Stage III	Advanced symptoms which may include unexplained chronic diarrhea for longer than a month, severe bacterial infections including tuberculosis of the lung, and a CD4 count of less than 350/µl
Stage IV (AIDS)	Severe symptoms which include toxoplasmosis of the brain, candidiasis of the esophagus, trachea, bronchi or lungs and Kaposi's sarcoma. A CD4 count of less than 200/µl.
United States Center for Disease Control and Prevention Classification System for HIV (2014) (190)	
Stage 0	The time between a negative/indeterminate test followed less than 180 days by a positive test
Stage 1	CD4 count $\geq$ 500 cells/µl and no AIDS defining conditions
Stage 2	CD4 count 200 to 500 cells/µl and no AIDS defining conditions
Stage 3	CD4 count $\leq$ 200 cells/µl or AIDS defining conditions

Epidemiology of HIV in sub-Saharan Africa

The human immunodeficiency virus was discovered in the early 1980s after clusters of young homosexual men presented with advanced immunocompromise and unusual opportunistic infections in several major US cities (45), (46). During the early

days of the American HIV epidemic, it was realized that the virus was well established in heterosexual individuals in central and east Africa as well and had likely been circulating on the African continent long before it appeared in the United States. To date, the origins of the virus have been traced to the 1920s in the Democratic Republic of the Congo, then known as the Belgian Congo (73). In the United States, an increased likelihood of infection has always been associated with high risk behaviors such as intravenous drug use, anal sex, and prostitution (166). In sub-Saharan Africa, the opposite is seen. HIV infection is not limited to those who participate in such high-risk behaviors; in fact, prevalence is higher in so-called “low risk” groups (168), (38), (37).

When this pattern was first noticed in Africa, HIV researchers and physicians feared that high HIV prevalence would similarly expand beyond high-risk populations and infect a large proportion of the general public elsewhere in the world (70). However, it soon became clear that this unique pattern of transmission is specific to sub-Saharan Africa, and likely is due to a complicated combination of socioeconomic and cultural factors.

African colonialism is hypothesized to be one of the leading factors that allowed HIV to slowly gain traction in the time between the 1920s and the 1980s, when it was widely recognized as a global pathogen. During this colonial period, there was an unprecedented increase in travel between villages and pathogens suddenly had new avenues for expansion, especially because this migration led to an increase in sexual intercourse between individuals from different villages or geographical locations (70). Additionally, the introduction of modern medical techniques, such as immunization, may have also inadvertently led to the spread of bloodborne diseases, as the same needle was

often reused for many patients without decontamination or sterilization between patient contacts (241). This could have potentially provided an opportunity for serial passaging of a virus over a short amount of time, which is known to increase virulence and enhance the ability of a pathogen to infect a new host species (70). Collectively, all of these factors may have provided an opportunity for the spread of a new and more potent virus, such as HIV-1, although the details of the development and spread of HIV prior to its discovery in the 1980s may never be known.

One current hypothesis for the more recent rapid spread and high prevalence of HIV is that long-term concurrent sexual relationships, rather than low-frequency high-risk encounters, can be an important aspect of African relationships (148), (91). In parts of sub-Saharan Africa, there are many adults who regularly have multiple concurrent long-term sexual partners, referred to as concurrency (41). This differs from the United States and other countries, where serial monogamy is more common and there is a slower spread of HIV in non-high risk populations (147). Serial monogamy can reduce the spread of HIV when compared to concurrency because of the low viral load that occurs during the latent period. When an individual first contracts HIV, their viral load is high (Figure 4). During concurrent relationships, there is a high risk of spreading HIV to another long-term partner during the initial acute infection; serial monogamy does not carry the same risk because the acute infection is relatively short and the chance of moving on to a new partner during that acute period is relatively lower (66). Despite the high prevalence of concurrent sexual relationships, condom use remains low in many regions of sub-Saharan Africa, which may facilitate high rates of HIV transmission (150).

Regardless of the cause, the HIV incidence remains high in sub-Saharan Africa. The major differences in HIV epidemiology complicates efforts to reduce the spread of HIV in Africa because some of the tried-and-true techniques for reducing HIV in places such as the United States, where healthcare and public health initiatives are well-funded, do not translate well to Africa, where funds are lacking to research and develop novel comprehensive public health campaigns. For example, needle exchange programs have been found to reduce the risk of HIV in intravenous drug users in the United States but would have limited efficacy in Africa.

There have been several substantial victories in the fight to reduce HIV incidence in sub-Saharan Africa. Educational campaigns have successfully increased knowledge about HIV transmissions and sexual health, increased condom usage, and perhaps most importantly, are de-stigmatizing conversations about HIV (75). Voluntary male circumcision programs are gaining traction and have been shown to reduce the incidence of HIV (138). Additionally, the number of individuals who know their HIV status has been steadily increasing due to the successes of community-based voluntary HIV testing and counseling (210), and several pilot programs are examining the efficacy of HIV self-testing to reduce the stigma of publicly attending a voluntary testing center (95), (39). As previously discussed, ART coverage has been steadily increasing in Africa (238). Collectively, these interventions have resulting in a global reduction of HIV incidence and mortality, although in Africa in 2017, over one million new cases of HIV were diagnosed and approximately 660,000 people died from AIDS-related illnesses (238). Reducing the incidence of new infections and improving access to ARTs remain high priorities in Africa.

Impact of HIV on Malaria

Generally, HIV-1 infection is associated with more frequent and severe bouts of clinical malaria (230), (80). Conversely, malaria infection may cause a transient increase in viral load and faster progression of HIV infection towards AIDS (100), (8). The impact of HIV on malaria gametocytogenesis remains unclear, although several studies, including both *in vivo* animal studies and human observational studies, have suggested that HIV may increase gametocytemia. Striking data in a rhesus macaque model of Simian Immunodeficiency Virus (SIV) and *Plasmodium fragile* showed a tremendous increase in gametocyte carriage in SIV-infected animals who were challenged with *P. fragile* (220). In humans, the increase in clinical malaria episodes in co-infected individuals is suspected to increase overall parasite biomass, leading to increase malaria transmission (1).

Areas of endemic *P. falciparum* transmission overlap with areas of high HIV-1 prevalence in sub-Saharan Africa. Due to the high frequency of HIV-1 and *P. falciparum* in these areas, it is likely that there is a significant portion of the population that is co-infected with both pathogens. Much of our current understanding of malaria and HIV co-infections focus on outcomes as they relate to HIV or clinical malaria (reviewed in (197), (195), (175), (6), (120)), as this information is important for establishing guidelines for patient care. However, it has been established that a large proportion of the adult population in a holoendemic area of malaria transmission are asymptotically infected with parasites, and the effect of HIV-1 on asymptomatic malaria transmission has not

been determined. Additionally, the prevalence of both diseases varies widely, so the results from one geographic area may not translate to a nearby area with a different vector capacity, or different HIV reduction strategies in place. This also complicates our understanding of co-infection. As treatment and prevention of both malaria and HIV improve, the proportion of individuals with well-controlled and asymptomatic disease will likely increase, requiring a better understanding of the malaria transmission potential of these individuals.

STUDY AREAS

Kisumu, Kenya

Kisumu, Kenya is a major port city located on the eastern side of Lake Victoria in Western Kenya. Over 1 million people live in the city and surrounding metropolitan region. The Kisumu area is a region of holoendemic malaria transmission. The climate is generally tropical, although there are periods with especially high rainfall, generally from March to June and November to December. A recent study estimated that in the Kombewa region of the Kisumu district, individuals receive 31.1 malaria-infective mosquito bites per person per year (113).

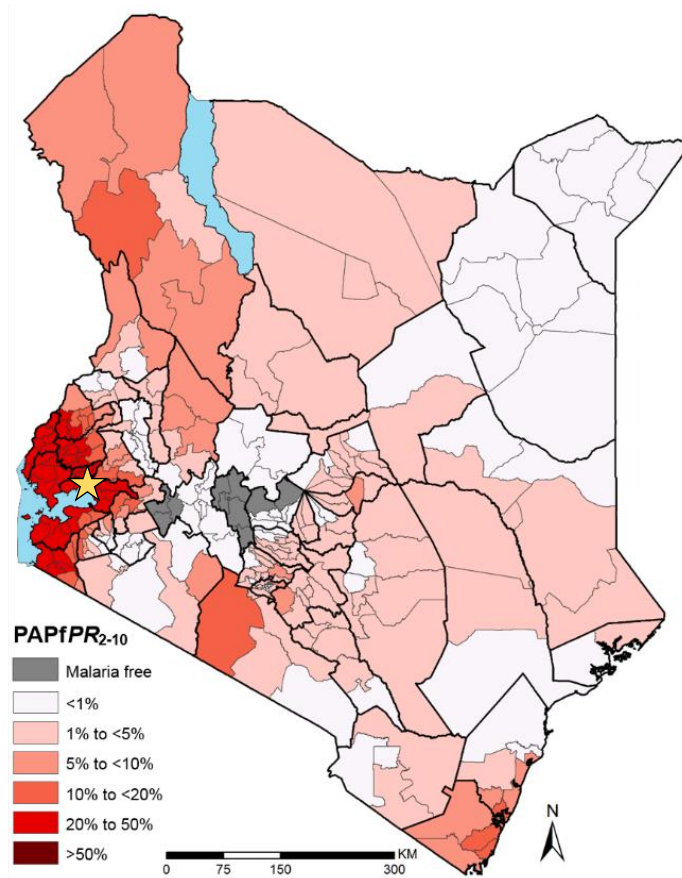


Figure 5. 2015 population adjusted prevalence of *P. falciparum* malaria in children between 2 and 10 years of age. The yellow star indicates the approximate location of Kisumu. Adapted from (143)

Additionally, Kenya is considered to be one of the four “high burden HIV” countries in Africa. Fortunately, recent campaigns to encourage voluntary HIV testing have helped reduce the incidence of new cases of HIV by 30% between 2010 and 2018 (222), but there remains a large proportion of the population that are HIV-positive. As of 2018, an estimated 1.6 million people living in Kenya were living with HIV (222).

The US Army Medical Research Unit-Kenya (USAMRU-K) maintains research stations in Kisumu and works closely with the Kenya Medical Research Institute

(KEMRI). Additionally, President's Emergency Plan for AIDS Relief (PEPFAR) maintains an active presence in this region as well. These projects contribute to the large number of epidemiological and clinical studies that are ongoing in the area.

Nchelenge, Zambia

The Republic of Zambia is located in southern Africa and has a population of over 17 million. The entire country experiences high malaria transmission year-round (Figure 6), although transmission peaks during the rainy season, which generally runs from November to April. Annually there are approximately 5 million cases of clinical malaria (234). In recent years, the incidence of malaria has fluctuated, with an overall decrease from 2001 until 2013, followed by a slight increase from 2013 to 2016 (240). Despite robust eradication efforts in the recent decade, malaria prevalence remains nearly constant for the past decade (240). Several factors may be contributing to these difficulties in reducing malaria. Zambia is bordered by 8 different countries, which creates a tremendous potential for human migration (128). Additionally, climate change may be supporting an increase in vector populations (14).

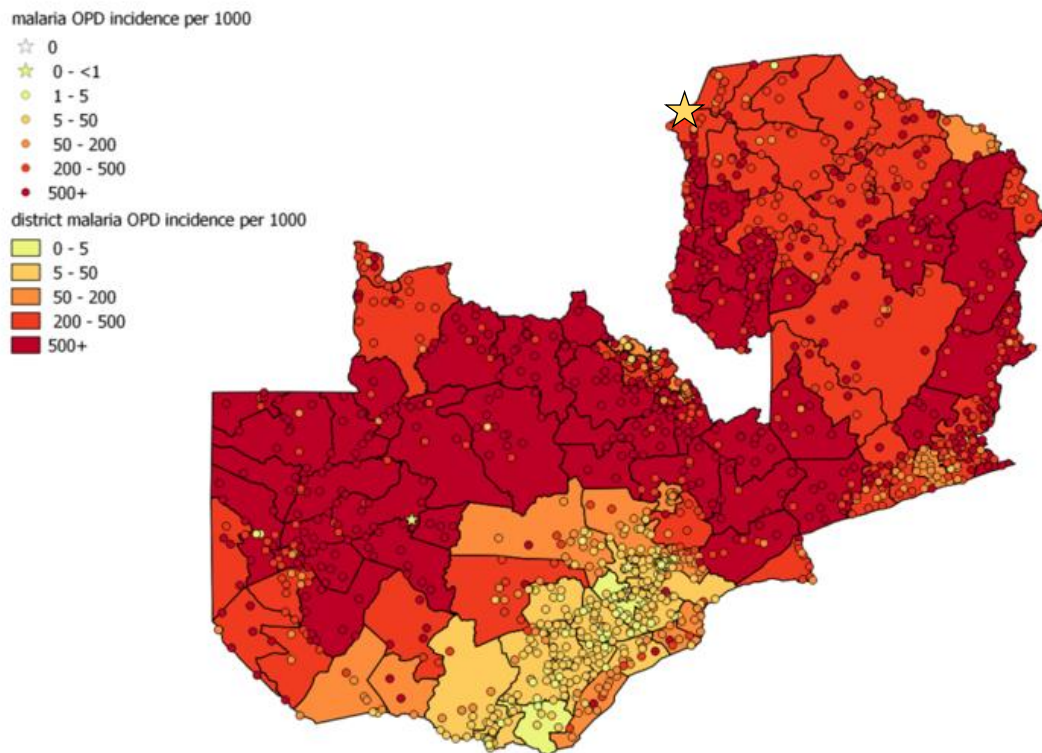


Figure 6. Prevalence of clinical malaria cases at outpatient departments (OPD) in Zambia, 2017. Circles represent healthcare facilities. The gold star indicates the approximate location of Nchelenge. Adapted from (240)

The Nchelenge District of Zambia is located in the Luapula Province and borders the Democratic Republic of the Congo in the northern part of the country. Many of the residents are fishermen or fishmongers, as the region borders Lake Mweru. While there is a paved road to the nearest hospital, the rest of the district is unpaved and difficult to travel by road during the rainy season, which runs from November to April. Electricity is very limited, but phone and Internet coverage is better and relatively reliable. 30% of the population does not receive schooling past primary school, and literacy is low in the region. As with Kisumu, there are several on-going research studies centered in the Nchelenge region.

RESEARCH GOALS, OBJECTIVES, AND RATIONALE

Research Goals

The overall goal of this research is to quantify the prevalence of *P. falciparum* gametocyte-specific transcripts in order to gain a better understanding of factors that are associated with increased gametocyte carriage.

Research Objectives

The objectives of this dissertation research are to develop a novel panel of highly sensitive qPCR assays, which will then be used to quantify the prevalence and abundance of falciparum gametocyte-specific RNA transcripts in order to evaluate factors important for falciparum gametocyte commitment and carriage in two different populations:

1. Asymptomatically falciparum malaria-infected adults from Western Kenya with and without HIV-1 who are not on antimalarial or antiretroviral drug therapy
2. Children from Zambia who are clinically ill with uncomplicated malaria and are being treated with artemether-lumefantrine (AL).

Research Rationale

Use of Molecular Methods to Quantify RNA Transcripts

For this research, gametocyte-specific transcripts are quantified as an indirect measure for the prevalence of falciparum gametocytes. While this is an indirect measure of gametocyte carriage, it is superior to traditional microscopy because it is more sensitive and is able to detect transcripts from gametocyte life stages that would be impossible to distinguish from early ring stages. Additionally, while most early

gametocytes are known to sequester in the bone marrow, molecular methods are still able to detect transcripts from these developing gametocytes (4). The targets of these assays have been validated using cultured parasites prior to use on clinical samples.

HIV and Malaria Co-Infection

Gametocyte carriage has not yet been quantified in adults who are asymptotically infected with both malaria and HIV and are naïve to antiretroviral treatments. As previously discussed in “The Impact of Malaria on HIV,” gametocyte carriage may be enhanced in these individuals. 1752 samples were collected in the form of dried blood spots (DBS) from adults seeking voluntary HIV testing who have no apparently clinical disease. Our novel panel of assays will characterize the prevalence of gametocyte-specific transcripts in this population to provide a snapshot of gametocyte carriage in asymptomatic individuals with and without HIV.

Longitudinal Study on the Effects of Artemether-Lumefantrine

The impact of antimalarial treatment on gametocyte carriage is not well characterized, especially over the course of time several weeks after treatment. Although known to be initially very effective at reducing the number of gametocytes, the lasting effects of artemether-lumefantrine (AL) on gametocytes is contentious. Some studies have shown either complete clearance of gametocytes (218) or reduced gametocytemia after treatment (22), (239), (86), (161), (179). A limited number of other studies have brought into question the long-term efficacy of AL against gametocytes, either by showing that gametocytemia may transiently increase after treatment (186), or by identifying individuals with persistent parasitemias and gametocytemias after treatment

(16). *In vitro* studies are of limited utility because they cannot accurately reflect the sequestration that occurs during gametocyte development. Our longitudinal study sample include multiple time points after treatment (0h, 6h, 12h, 18h, 24h, 30h, 36h, 42h, 48h, 72h, 1w, and 2w post-treatment), which allows us to evaluate the immediate effects of the drugs and the longer term efficacy against gametocytes, while also evaluating factors such as starting parasitemia.

AIM 1

Develop a panel of highly sensitive and specific *P. falciparum* gametocyte stage-specific qPCR assays for use with dried blood spots from clinical samples.

AIM 2

Quantify gametocyte-specific RNA transcripts from asymptotically infected adults with and without HIV co-infection to identify factors that are associated with increased gametocyte carriage in chronic malaria infection.

AIM 3

Quantify gametocyte-specific RNA transcripts using samples from a longitudinal study of clinically ill children to determine the immediate and long-term effects of artemether-lumefantrine on gametocyte carriage.

The results of these studies will be used to identify risk factors for enhanced malaria transmission with the ultimate goal of informing treatment guidelines in order to reduce the transmission of *P. falciparum*.

CHAPTER 2: Development of *P. falciparum* Gametocyte-Specific qPCR Assays

ABSTRACT

Molecular methods for gametocyte detection are superior to microscopy because they are able to quantify low gametocytemias and can be used to detect early gametocyte stages which are morphologically indistinguishable from asexual parasite forms. The most popular molecular target for detecting gametocytes is *Pfs25*, which is a Stage V female gametocyte marker. Because *Pfs25* is only expressed very late in the process of gametocyte development and is not highly expressed in Stage V male gametocytes, it is possible that gametocyte carriage is being underestimated when only this marker is being quantified. In this study, we have developed a panel of quantitative PCR (qPCR) assays to be used as a tool for detecting and quantifying stage-specific *P. falciparum* gametocyte transcripts. The panel includes amplicons from target genes: *Pfsbp1*, *PfAP2-G*, *Pfs16*, *Pf27/25*, *Pfs48/45*, *Pfs230p*, and *Pfs25*. Collectively, these targets span the entire process of gametocyte development, from commitment to gametocytogenesis to development of male and female Stage V gametocytes (5), (122), (158), (132), (111), (123). Stage-specific expression of these markers was verified by quantification of gene expression in highly synchronous cultured gametocytes. These assays are able to detect very low gametocytemias and can quantify copy numbers as low as 10 copies per µl of cDNA template. Additionally, use of this panel was validated for quantifying nucleic acid extracted from dried blood spots collected from individuals with who have uncomplicated falciparum malaria or who are asymptotically infected with falciparum

malaria. This highly sensitive and specific panel will be used to quantify gametocyte-specific RNA transcripts in clinical samples.

INTRODUCTION

Globally, morbidity and mortality due to malaria has been declining, but asymptomatic infections are still highly prevalent in areas of endemic malaria transmission (236). These asymptomatic infections are difficult to characterize because individuals likely do not realize that they are maintaining parasites and they may maintain lower parasitemias that can be difficult to detect (24). Additionally, asymptomatic individuals are capable of transmitting malaria (212), and may be more likely to do so because they are not ill and are carrying out their regular day-to-day activities, potentially incurring mosquito bites as they do so. In fact, individuals with low gametocyte burdens can still be infective to mosquitos (187). The transmission of gametocytes to mosquitos and subsequent sexual development is seen as an evolutionary bottleneck in the life cycle of the malaria parasite, making it a potential target for intervention strategies (201).

In order to continue making progress towards the goal of eliminating malaria, it is important to identify asymptomatic individuals who may be transmitting malaria (24). Although standard membrane feeding assays are the best way to measure infectivity to mosquitos, gametocyte burden can be used as an indirect estimate of potential transmissibility (107), (167). In many asymptomatic individuals, gametocyte burden may be too low to be quantified by microscopy. Additionally, developing gametocytes generally sequester in the bone marrow or spleen (108), and very early gametocytes are

indistinguishable from ring stage trophozoites. Although these developing gametocytes are not in circulation, some RNA transcripts expressed by those life stages have been detected in peripheral blood (4). For these reasons, sensitive molecular tools are needed to quantify gametocytes in these individuals (217).

Previously, several gametocyte-specific transcripts have been identified and used to quantify gametocyte burden (152), (133), (188), (92), (89). One of the most commonly used gametocyte targets is *Pfs25*, which is very highly expressed by Stage V female gametocytes and has been found to correlate well with infectivity to mosquitos (167). Because this transcript is so highly expressed, it is readily detected in low volume samples, even when the overall parasitemia is quite low (206), (208), (213). However, because *Pfs25* is only expressed by Stage V gametocytes, it is not expressed until about two weeks after gametocyte commitment. Using this marker as the sole determinant of gametocyte positivity may result in an underestimate of gametocyte positive individuals, especially in cross-sectional studies, as individuals may be harboring developing gametocytes that have not yet reached maturity at the time of sample collection. The use of *Pfs25* alone also does not allow for the quantification of male Stage V gametocytes, and the sex ratio of male to female gametocytes has been shown to impact transmissibility (144), (188). Additionally, when characterizing the effect of antimalarial drugs on gametocytes, additional markers for immature gametocytes would be needed to determine the immediate effects of the drug and to evaluate if there are differential effects on the different stages of developing gametocytes.

To better characterize gametocyte carriage, a panel of highly sensitive qPCR assays is needed that covers the full development of both male and female gametocytes,

from commitment to gametocytogenesis to maturity. This panel could then be used to identify potential reservoirs of transmission and to identify factors that increase gametocyte carriage, including antimalarial drug treatment.

OVERALL STUDY AIMS

The goal of this study is to develop a panel of stage-specific *P. falciparum* gametocyte assays that can be used to quantify RNA transcripts extracted from dried blood spots (DBS) using quantitative reverse transcription PCR (qPCR).

METHODS

Selection of Markers

A search of the existing literature was conducted to identify potential *P. falciparum* gametocyte genes. Stage specificity was confirmed using transcriptomics data available on PlasmoDB (<https://plasmodb.org>). mRNA sequences for each gene were obtained from PlasmoDB and analyzed using NCBI BLAST™ (<https://blast.ncbi.nlm.nih.gov/>) to ensure that targets sequences were unique to *P. falciparum* and that primer and probe sequences were free of identifiable single nucleotide polymorphisms.

Primer and Probe Design

Primers and probes were designed and checked for the absence of hairpins and primer-dimers using the Primer Express™ 3.0 software (Applied Biosystems, Foster City, CA, USA). All synthetic primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). Custom TaqMan probes in 1X TE buffer were

manufactured by Life Technologies (Carlsbad, CA, USA). Primer and probe sequences are listed in Table 3.

Table 3. Primer and probe sequences for gametocyte-specific qPCR assays. Sequences were obtained from PlasmoDB using GeneID listed below target name.

Target	Forward Primer	Reverse Primer	Probe	Amplicon Size (bp)
<i>PfAP2-G</i> PF3D7_1222600	5' TTC AAC CCA AAC ATT TAA ACT TAC TCA 3'	5' AAT CTC GAA GAT ACG ATT ATC AAC GA 3'	ABY 5' CGA ATG GGA AGA GAG CAT GCA ATG AAG 3' QSY	160
<i>Pfs16</i> PF3D7_0406200	5' GGA TCC CCT TCA ACT TTG CA 3'	5' CCT TGA GAT AGT CCA CCT TGA TTA GG 3'	JUN 5' TTC TTC AGG TGC CTC TCT TCA TGC TGT TG 3' QSY	89
<i>Pf27/25</i> PF3D7_1302100	5' AGC CCT TGG ATA AAT TTG GAA AT 3'	5' AAA GTT GGG GAT ATT GAG TTT CAT G 3'	JUN 5' AAA CAC ATG CCC CTC TCT CAC CTC GTA TT 3' QSY	105
<i>Pfs48/45</i> PF3D7_1346700	5' TGT AAG CCT AGC TCT TTG AAT AGT GAA 3'	5' TCA CGC ATA TCT GGC TTT AAA TTA TG 3'	VIC 5' TAT CTG GAT TCA TAG GAT ATA AG 3' MGBNFQ	101
<i>Pfs230p</i> PF3D7_0208900	5' CCC AAC TAA TCG AAG GGA TGA A 3'	5' TTA CCA AAA AAT GCT CCT AAA CGT T 3'	VIC 5' CAA AAC GAT CAA ACC ATC TC 3' MGB	196
<i>Pfs25</i> PF3D7_1031000	5' TCT GAA ATG TGA CGA AAA GAC TGT 3'	5' AGC GTA TGA AAC GGG ATT TCC 3'	FAM 5' ATA AAC CAT GTG GAG ATT T 3' MGBNFQ	88
<i>Pfsbp1</i> PF3D7_0501300	5' AAA GTA CTC CTT GTT GGC AAC GTA 3'	5' TTA ATG AAT ACG AAG TAG AAT CTC CAG C 3'	FAM 5' AAT GGC TCA AGA AGC 3' MGBNFQ	73

Creation of Plasmid Standards

A serial dilution of a plasmid standard was created for each amplicon of interest to be used for quantifying samples by qPCR. For each amplicon, primer sets were verified by amplification from *P. falciparum* genomic DNA by conventional PCR and gel electrophoresis. Then, each PCR product was cloned into the pCR 2.1-TOPO TA vector and transformed into competent *E. coli* using the TOPO TA Cloning Kit for Subcloning with OneShot Top10 Chemically Competent *E. coli* (Invitrogen, Carlsbad, CA, USA). Plasmid DNA was isolated from cultured bacteria using the QIAprep MiniPrep kit (Qiagen, Hilden, Germany), and was then quantified by Nanodrop. Verification of the successful insertion of each amplicon was conducted by Genewiz (Frederick, MD, USA) using Sanger sequencing (data not shown). Ten-fold dilutions were created over a range of 100,000 copies per μl to 0.1 copies per μl using dilution calculations based off of the mass of the amplicon of interest and the concentration of the plasmid DNA as measured by Nanodrop.

Quantitative PCR

All qPCR reactions were conducted using the Applied Biosystems 7500 Fast machine (Applied Biosystems, Foster City, CA, USA) using either TaqMan™ Fast Universal PCR Master Mix (2X), no AmpErase™ UNG (ThermoFisher Scientific, Waltham, MA, USA) for singleplex reactions or TaqMan™ Multiplex Master Mix (ThermoFisher Scientific, Waltham, MA, USA) for multiplex reactions. Each reaction was a total of 20 μl , including 10 μl of master mix, 1 μl of template (cDNA), and primers and probe to a final concentration of 250 nM. Each plate included a series of ten-fold

standard dilutions and all samples and standards were run in triplicate. Reactions were set up in 96 well optical plates and sealed with optical film. All runs were conducted for 40-45 cycles with the following cycling parameters: Initial denaturation: 50°C for 2 minutes, 95°C for 10 minutes; 40-45 cycles of denaturation at 95°C for 15 seconds and annealing, extension, and detection of fluorescence at 60°C for 1 minute.

Assay Validation and Optimization

A range of diluted plasmid standards were used to determine the limits of detection and quantification. Ten-fold standard dilutions ranging from 100,000 copies per μl to 0.1 copies per μl were used to broadly define the lower limits of the assay, followed by additional two-fold dilutions ranging from 10 copies per μl to 0.625 copies per μl to more accurately determine the lower limits of the assays. The specificity of each assay was verified by testing both cDNA from cultured *P. falciparum* parasites and uninfected human blood. For assays that were multiplexed (*Pfs16*, *Pfs48/45*, and *Pfs25*), 1 μl of each template was included for each plasmid standard replicate and the absence of cross-reactivity was verified by comparing curves and Ct values when amplified in the presence and absence of primers, probes, and standards for the other two targets.

Analysis of qPCR Data and Statistical Analysis

Ct values for standards were calculated by the 7500 Fast software, input into GraphPad Prism 6 (GraphPad, San Diego, CA, USA), and analyzed using a semi-logarithmic nonlinear regression analysis. Ct values for field samples were interpolated onto this semi-logarithmic line to calculate copy number per μl . Plates were excluded

from analysis if NTCs were positive or if the PCR threshold was below 90% or above 105%.

Culture of Highly Synchronous Gametocytes

To validate stage-specific gene expression, highly synchronous gametocytes were cultured using the following method. 3D7 and HB-2 parasite lines were obtained from MR4 (Manassas, Virginia, USA). Parasites were thawed from liquid nitrogen storage by the dropwise addition of NaCl solutions (145). Both 3D7 and HB-2 parasites were cultured using a protocol adapted from (141), briefly described as follows. Parasite cultures were established with a 4% hematocrit. Once reaching approximately 5% parasitemia and predominately ring stage, parasites were synchronized using 5% D-sorbitol, which was repeated after 48 hours. The volume of media in each flask was doubled and parasites were maintained in culture for 48 hours. At that time, heparin (obtained as heparin sodium salt from porcine intestinal mucosa, Sigma Aldrich, St. Louis, MO, USA) was added to culture media at a final concentration of 20 U/ml to inhibit invasion of RBCs by merozoites (28), (141). Media with added heparin was changed daily.

Thin smears were made daily to monitor growth of cultures and 50 µl dried blood spots were made on Whatman 903 Proteinsaver cards (GE Healthcare Life Sciences, Chicago, IL, USA) every 24 hours until the appearance of Stage V gametocytes by microscopy, or until no parasites could be observed on a thin film. To make each 50 µl DBS, 200 µl of parasite culture was spun down and rinsed twice in complete media to remove heparin before being resuspended at approximately 50% hematocrit in complete

medium. DBS were allowed to dry quickly (within 30 minutes) and completely on the air intake of a biological safety cabinet, and then were stored frozen at -80°C in individual bags with desiccant packets.

Nucleic Acid Extraction from Dried Blood Spots and cDNA Synthesis

Using a single use razor blade to reduce contamination, individual dried blood spots were cut out of the filter paper cards and minced into squares less than 3mm by 3mm. Filter paper pieces were then treated with Buffer AL, Proteinase K, and Buffer ATL (Qiagen, Hilden, Germany) to rehydrate the samples, lyse red blood cells and parasites, and degrade unwanted enzymes. Half of the resulting lysate was used for an RNA extraction using the RNeasy® Mini Kit (Qiagen, Hilden, Germany), while the other half was retained for genomic DNA extraction using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany). The RNA lysates were treated with a genomic DNA elimination step followed by cDNA synthesis using the QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany), using the included oligonucleotide primers, following the Qiagen protocol as written, and using 3 µl of RNA per 20 µl reaction. Due to both the very low volume of RNA samples and the low concentration of RNA in each sample, quality assessment and quantification of RNA was unable to be conducted on these samples prior to amplification by qPCR. No-template controls (NTCs), in which water replaces RNA, were regularly included during the reverse transcription step as a negative control.

***PfAQP* Genomic DNA Assay**

In order to control for variations in parasitemia between samples made from cultured parasites, a single copy genomic DNA target was quantified by qPCR. The assay was modified from (225) and uses the following primers and probes:

Forward Primer: 5' CCATCAAGAGATTTAGGATCCAGATT 3'

Reverse Primer: 5' GCTACAAGAGGTACCCAAAAATAAAAA 3'

Probe: FAM 5' TTGCATATGGAAAAGATACCT 3' MGB

This assay was conducted using TaqMan™ Fast Universal PCR Master Mix (2X), no AmpErase™ UNG (ThermoFisher Scientific, Waltham, MA, USA), and in optical plates with optical film, as described for other assays. Each reaction volume totaled 20 µl, including 1 µl of template, and each primer and probe had a final concentration of 312.5 nM.

Field Samples for Assay Validation

To test the utility of each assay on field samples, a subset of samples was selected from two different molecular epidemiology studies. RNA was extracted from these samples and cDNA was synthesized as previously described.

Asymptomatic Adults in Western Kenya

Samples for this study were collected under the supervision of the USUHS, WRAIR, and KEMRI Institutional Review Boards (KEMRI protocol #388266) as part of a cross-sectional molecular epidemiology study investigating the relationships between HIV and malaria in asymptomatic adults. These samples were obtained as dried blood spots from healthy adults seeking voluntary HIV testing in Kombewa, Kenya at either

Kombewa County Hospital or Manywanda Sub-County Hospital. From each patient, one to five 50 µl blood spots were blotted onto Whatman® 903 Protein Saver filter paper cards (GE Healthcare Life Sciences, Chicago, IL, USA) in the outpatient laboratory. Cards were allowed to air-dry rapidly on the air intake of a biological safety cabinet and once dry, were placed into individual sample storage bags with desiccant packets and stored frozen at -80C° to minimize RNA degradation. A subset of 130 samples was selected from the first 300 samples collected and tested for the presence of gametocyte transcripts by qPCR.

Children with Uncomplicated Falciparum Malaria in Zambia

Samples for this study were collected under the supervision of the Tropical Diseases Research Centre Ethics Review Committee and the Ministry of Health of the Government of the Republic of Zambia (protocol no. TDRC/C4/09/2014) and registered with the Pan African Clinical Trial Registry (PACTR201905783261453). These samples were obtained in the form of dried blood spots from children between 6 months and 5 years of age who were treated with a standard course of artemether-lumefantrine for uncomplicated falciparum malaria. Samples were collected every 6 hours for the first 48 hours, at 72 hours, and weekly for up to 5 weeks. Due to variations in sizes of the DBS, the diameter of each DBS was measured and used to calculate an area. These areas were used to normalize expression data to the size of the DBS from which it was obtained. A subset of samples was selected comprising 6 patients who were known to be gametocyte positive by microscopy at the initiation of treatment. A total of 71 samples from these 6 patients were tested for the presence of gametocyte transcripts by qPCR.

RESULTS

P. falciparum Stage-Specific Targets

A total of seven RNA-based qPCR assays were developed to quantify asexual ring stages, gametocyte-committed rings, and developing gametocytes. The functions of these proteins and predicted stages during which mRNA is expressed are summarized in Table 4.

Table 4. *P. falciparum* markers, protein function, and predicting timing of mRNA expression

Marker	Protein Function	Predicted Timing of mRNA Expression
<i>PfAP2-G</i>	Transcriptional master regulator of gametocytogenesis (111)	Prior to gametocyte development, in committed parasites (111)
<i>Pfs16</i>	Expressed on the membrane of the parasitophorous vacuole of gametocytes, non-essential for gametocyte development (118)	Highly expressed early in gametocyte development; expressed at a lower level during later stages, equally expressed by male and female (122), (132), (123)
<i>Pf27/25</i>	Important for formation of cellular membranes during gametocyte development (155)	Expressed early in gametocyte formation (5)
<i>Pfs48/45</i>	6-cystein protein, important for male gamete fertility (224)	Expressed from Stage II through Stage V (132)
<i>Pfs230p</i>	6-cysteine protein, important for ookinete formation and transmission to mosquitos (135)	Expressed by Stage V male gametocytes (132), (123)
<i>Pfs25</i>	Ookinete surface protein (114)	Highly expressed by Stage V female gametocytes (132), (123)
<i>Pfsbpl</i>	Maurer's cleft protein, traffics PfEMP-1 to the cell surface (18), (134)	Expressed by ring stage trophozoites (158)

Optimization and Validation of qPCR Targets

Primer pairs were validated by conventional PCR and gel electrophoresis during the creation of plasmid standards. Before quantifying transcripts in samples containing

parasite cDNA, each assay was validating by running dilutions of plasmid standards to verify appropriate amplification curves and PCR efficiency. Curves were considered acceptable if PCR efficiency was between 90% and 105%. Sample standard curves are shown in Figure 7, and the slopes, R^2 values, and PCR efficiencies are shown for those curves are shown in Table 5. Lower limits of detection (LOD) were determined by running two-fold dilutions at the lower end of the linear dynamic range (data not shown), and were determined to be a follows: 2.5 copies/ μ l for *Pfsbp1*, 1 copy/ μ l for *Pfs16*, *Pfs48/45*, and *Pfs25*, and between 1 and 10 copies/ μ l and for *PfAP2-G*, *Pf27/25*, and *Pfs230p*, which were not used as extensively as the other assays and therefore not as well-characterized.

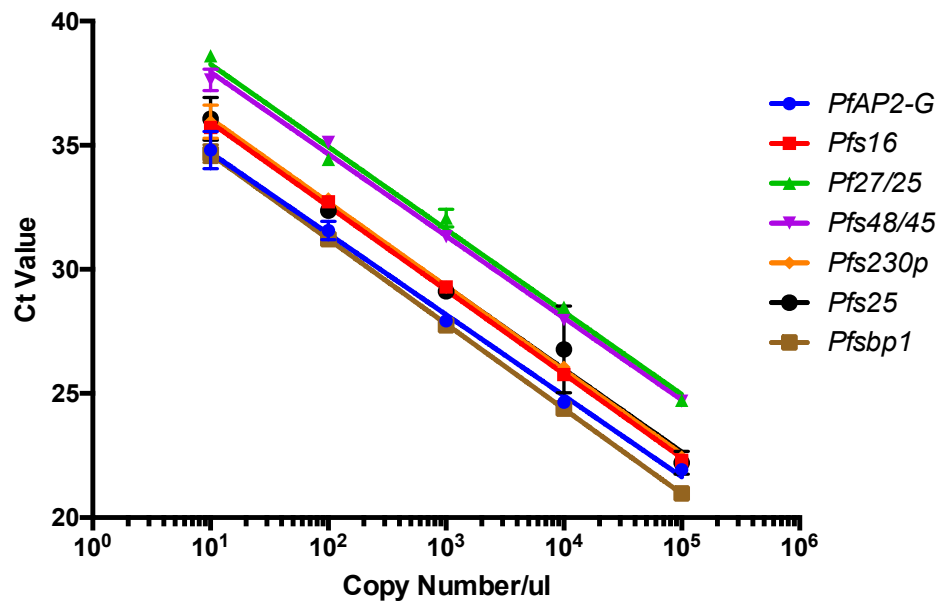


Figure 7. Representative standard curves for all seven *P. falciparum* stage-specific assays.

Table 5. Slope, R² values, and PCR efficiencies for representative standard curves.

Marker	Slope	R ²	PCR Efficiency
<i>PfAP2-G</i>	-3.264	0.9928	102.48%
<i>Pfs16</i>	-3.378	0.9984	97.71%
<i>Pf27/25</i>	-3.325	0.9908	99.87%
<i>Pfs48/45</i>	-3.302	0.9954	100.84%
<i>Pfs230p</i>	-3.380	0.9961	97.63%
<i>Pfs25</i>	-3.330	0.9678	99.66%
<i>Pfsbp1</i>	-3.420	0.9990	96.06%

Additionally, assays for *Pfs16*, *Pfs48/45*, and *Pfs25* were multiplexed to allow for simultaneous quantification of all three targets from a single ul of template, which is especially advantageous in low volume samples. To verify that cross-reactivity was not affecting amplification, assays were run first as a singleplex assay, then run with additional primer and probes added to the master mix, and finally were run with the primers, probes, and template for all three assays in a single reaction (Figure 8).

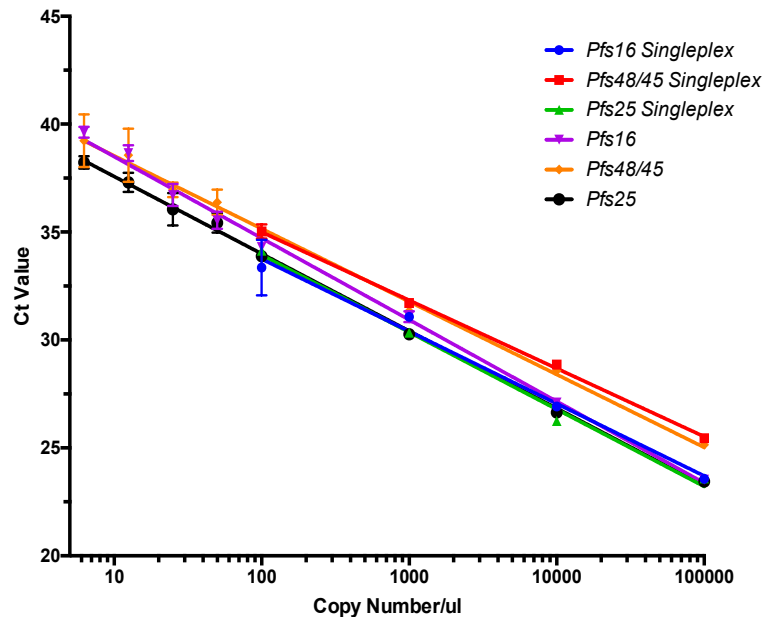


Figure 8. Comparison of standard curves when run as a singleplex or a multiplex reaction.

Amplification of RNA from Field Samples

All markers could be amplified from cultured parasites, but due to the sequestration of immature gametocytes and the lower parasitemias found in asymptomatic individuals, it was predicted that not all assays would be suitable for use in field samples. To determine which assays would be best for quantifying transcripts in dried blood spots, a subset of samples from two field studies were tested.

First, a subset of 126 samples from a study of asymptomatic adults in Kenya were tested using the assays for *PfAP2-G*, *Pfs16*, *Pfs48/45*, and *Pfs25* (Table 6). At the time of testing these samples, the assays for *Pfs27/25* and *Pfsbpl* had not been developed, so they were not included. Very few samples were positive for *PfAP2-G*, and only one sample was found to be positive for *Pfs230p* with a Ct value for that sample was below the limit of quantification for the assay. Based on this data, *Pfs16*, *Pfs48/45*, and *Pfs25* were selected as the markers used to analyze the full cohort (presented in Chapter 3).

Additionally, 71 samples from 6 patients were selected from a longitudinal study of clinically ill children from Nchelenge, Zambia, and these samples were testing using the assays for *Pfs16*, *Pfs48/45*, *Pfs27/25*, *Pfs25*, and *Pfsbpl* (Table 6). Samples were not tested for *PfAP2-G* or *Pfs230p* because the signal was previously determined to be too small to be detected in samples from the Kenya study. In this population, all assays successfully amplified transcripts in at least some samples. *Pfs27/25* and *Pfs48/45* had similar positivity rates, although *Pfs27/45* was expressed at a slightly higher level. Based on this data, *Pfs16*, *Pfs25*, and *Pfsbpl* were selected as the markers used to analyze the full cohort (presented in Chapter 4).

Table 6. Percentage of positive samples and average copy number for pilot samples from two different studies.

Marker	Asymptomatic Adults		Clinically Ill Children	
	% Positive Samples	Avg Copy Number	% Positive Samples	Avg Copy Number
<i>PfAP2-G</i>	0.8	29.47	--	--
<i>Pfs16</i>	20.0	24.28	78.9	141.88
<i>Pf27/25</i>	--	--	43.7	22.56
<i>Pfs48/45</i>	2.4	4.90	26.8	6.61
<i>Pfs25</i>	24.0	29.88	81.7	124.95
<i>Pfs230p</i>	0.0	--	--	--
<i>Pfsbpl</i>	--	--	73.2	104.6

Confirmation of Gametocyte Specificity

Synchronous cultured 3D7 gametocytes were used to confirm stage-specific gametocyte expression of the final markers selected for use with field samples (*Pfsbpl*, *Pfs16*, *Pfs48/45*, and *Pfs25*). To control for variation in parasitemia between DBS, *PfAQP*, a single copy gene, was quantified in each sample and used to normalize gene expression data for each sample to genomes per μ l. Gene expression was also measured in HB2 parasites, which are gametocyte deficient (17). HB2 culture was discontinued after day 6 because parasites could no longer be visualized by thin smear, while 3D7 culture was continued until day 12, at which point, stage V gametocytes were present by microscopy.

Pfsbpl expression decreased rapidly after the addition of heparin to the media on day 1 (Figure 9A), while *Pfs16* expression began increasing soon after the doubling of media on day -1 (Figure 9B). *Pfs48/45* was expressed during intermediate stages of

gametocyte development (Figure 9C), while *Pfs25* peaked during the late stages of gametocyte development (Figure 9D). Overall, *Pfs48/45* was expressed at a much lower level than the other markers.

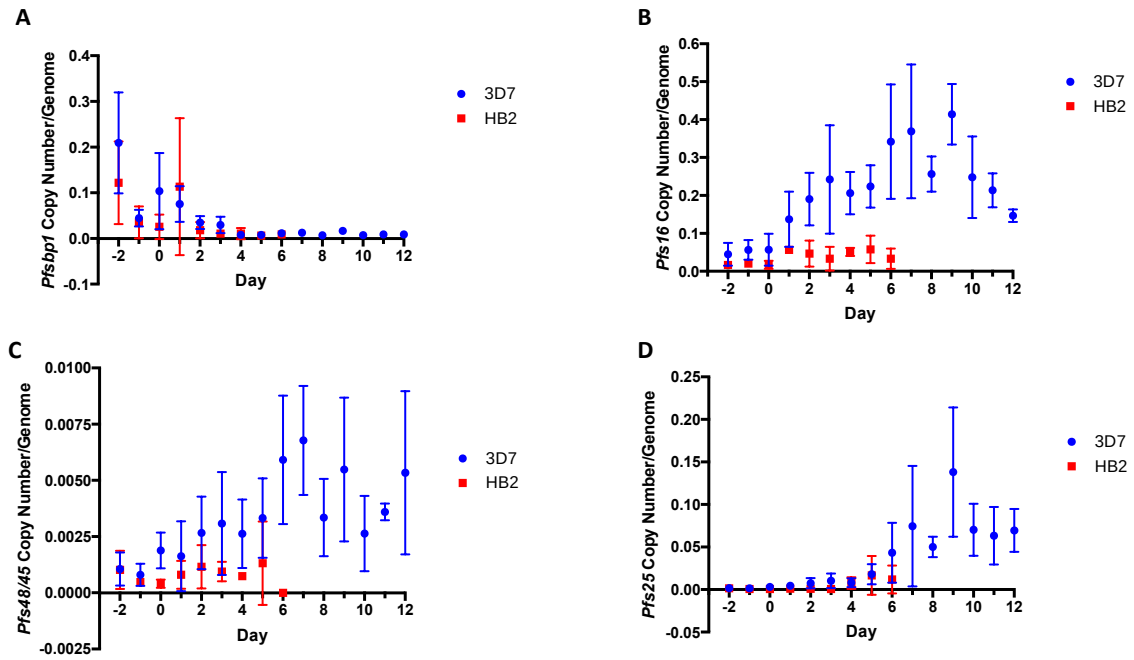


Figure 9. Expression of A) *Pfsbp1*, B) *Pfs16*, C) *Pfs48/45*, and D) *Pfs25* over time during gametocyte development for 3D7 and HB2 parasites. Media was doubled on day -1 and heparin was added to culture media from day 1 to day 12.

Although *Pf16* and *Pfs25* are concurrently expressed over much of the development of the gametocyte (Figure 9B, 9D), we found that the ratio of *Pfs16* to *Pfs25* expression changes as the gametocyte develops, with a higher proportion of *Pfs16* to *Pfs25* during the earliest stages of concurrent expression (Figure 10).

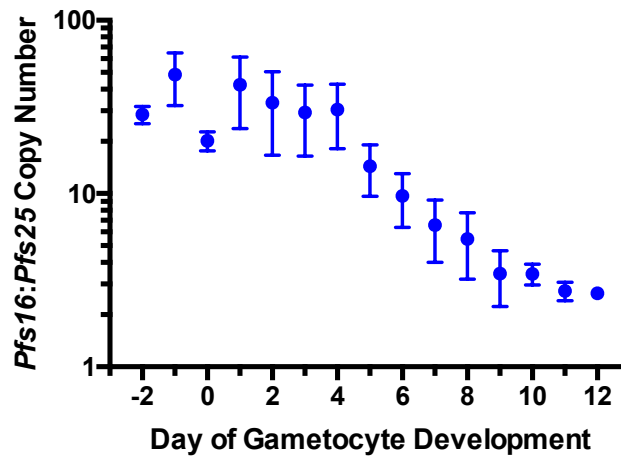


Figure 10. Ratio of *Pfs16* to *Pfs25* expression in 3D7 parasites during gametocyte development

DISCUSSION

This panel of gametocyte-specific marker is a comprehensive tool for quantifying gametocyte stage-specific transcripts at extremely low concentrations. All seven assays were capable of detecting transcripts in nucleic acid extracted from cultured parasites, and *Pfsbp1*, *Pfs16*, *Pf27/25*, *Pfs48/45*, and *Pfs25* were successfully used to quantify transcripts from dried blood spots. For the four assays (*Pfsbp1*, *Pfs16*, *Pfs48/45*, and *Pfs25*) that were used to characterize transcript abundance in larger molecular epidemiology studies, stage-specific expression was confirmed using highly synchronized cultured parasites.

The expression data from cultured parasites supports previously published data which shows that *Pfs16* expression begins early in the process of gametocyte development and remain present throughout all stages, while *Pfs48/45* is expressed during the intermediate stages of development, and *Pfs25* is expressed at the latest stages

of development (132). Additionally, our data shows that *Pfs48/45* is expressed at quite low levels in comparison to *Pfs16* and *Pfs25* (132).

By testing these assays in cultured parasites, clinically ill children, and asymptomatic adults, the utility of the panel was verified at three different levels of parasitemia and gametocytemia. In cultured parasites, the signal from immature parasites is likely much higher than it would be in human samples both because of the lack of sequestration in culture and because the gametocytemia can be quite high in culture. Additionally, certain markers, including *PfAP2-G* and *Pfs230p* are lowly expression and may not be detectable in human samples, particularly with the limited volume of blood obtained in a single dried blood spot. For these reasons, we evaluated the efficacy of the assays when used with human samples early in the process of optimization and validation.

This panel has several limitations that must be considered when interpreting the results, especially when analyzing transcripts in human blood samples. Negative data in human studies must be interpreted cautiously because it is impossible to determine whether a particular patient lacks a positive signal for a given marker because it is too low to detect, because it is sequestered in the bone marrow or vasculature, or because the individual is truly parasite negative. The ratio of *Pfs16* to *Pfs25* has the potential of being used to differentiate between early and late stage gametocytes, but in individuals with multiple gametocyte stages present, the distinction may become obscured. Ideally, a highly expressed marker that is restricted to early gametocyte stages would be identified for inclusion in this panel, potentially as a replacement for *Pfs16*. As more is learned

about gametocyte biology, there is the potential to replace markers with more suitable targets as they are identified.

Overall, this panel is highly sensitive and effective at amplifying transcripts from all stages of gametocyte development, making it an essential tool for characterizing gametocyte carriage on a molecular level.

CHAPTER 3: HIV-1 Infection Increases the Prevalence and Quantity of *Plasmodium falciparum* Gametocyte-Specific RNA Transcripts in Chronically Malaria Infected Adults in Western Kenya

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ABSTRACT

In an era of declining malaria morbidity and mortality, individuals with a high likelihood of transmitting malaria needed to be identified to further reduce the prevalence of malaria. In areas of holoendemic malaria transmission, asymptotically infected adults may be infected with transmissible gametocytes. It has been hypothesized that co-infection with HIV-1 may lead to an increase in gametocytemia (220), (229), (207). In this study, a panel of qPCR assays was used to quantify gametocyte stage-specific transcripts present in dried blood spots obtained from asymptotically adults seeking voluntary HIV testing in Kombewa, Kenya. A total of 1116 malaria-positive samples were tested, and 20.5% of individuals had detectable gametocyte-specific transcripts. Individuals also infected with HIV were 1.820 times more likely to be gametocyte positive ($P < 0.0001$) and had significantly higher gametocyte copy numbers when compared to HIV-negative individuals. Additionally, HIV positivity was associated with higher gametocyte prevalence in men and increased gametocyte carriage with age. Overall, this suggests that HIV-positive individuals may have an increased risk of transmitting malaria in regions with endemic malaria transmission and therefore should be at a higher priority for treatment with gametocidal antimalarial drugs.

INTRODUCTION

In the past decade, morbidity and mortality from *Plasmodium falciparum* malaria has substantially decreased in sub-Saharan Africa, but parasite prevalence remains high (236). In highly endemic areas of malaria transmission, many adults are able to asymptotically maintain *P. falciparum* parasites through partial immunity to the parasite (64), (202). These chronically-infected asymptomatic adults are suspected to encompass a large percentage of endemic populations and are likely contributing to the sustained presence of the parasite in regions with declining malaria morbidity and mortality (162), (24), (185). Generally, individuals with asymptomatic infections have lower parasite burdens than individuals with clinical malaria, although moderate to high parasite burdens can still occur without symptoms (104). The contribution of asymptomatic infection to malaria transmission is unknown, but since gametocytes are known to be intermittently present even in very low parasitemia infections, these asymptomatic individuals could be a major reservoir of transmission (187), (159), (127), (125), (74). Identifying individuals with an increased likelihood of transmitting parasites is a high priority for reducing the prevalence of malaria (24).

Gametocyte prevalence within a population is known to be dependent on several factors, including age and malaria endemicity (160), (205), (3), but gametocytemia has been difficult to accurately characterize because most of the early stages of the gametocyte are sequestered in the bone marrow (177), (108) and gametocytes are underreported by microscopy, especially at very low parasitemias. Molecular diagnostic techniques for gametocyte detection have been developed and are highly effective in quantifying gametocyte-specific RNA transcripts as an indirect measure for gametocyte

carriage (152), (227), (167). *Pfs25*, which is highly expressed by female Stage V gametocytes, is commonly used as a marker for gametocyte carriage, as it most accurately represents the number of mature gametocytes that are infective to mosquitos (152), (189). Additional gametocyte-specific markers have been identified, such as *Pfs16*, which is expressed very highly in the early stages of gametocyte development and remains expressed at a lower level throughout subsequent stages of development (35), (57), (189), and *Pfs48/45*, which is expressed by intermediate gametocytes, albeit at a lower level than either *Pfs16* or *Pfs25* (152), (4). Although the majority of gametocytes appear to sequester in the bone marrow during their development, low levels of transcripts for early and intermediated stages can still been found circulating through peripheral blood (4). The sensitivity of these molecular methods and the ability to detect multiple gametocyte life stages make molecular diagnostics promising techniques for measuring gametocyte carriage.

Despite the large geographical overlap between high HIV-1 prevalence and high malaria prevalence, the impact of HIV-1 on malaria is unknown for asymptomatic adults in areas of endemic malaria transmission. Immunosuppression due to HIV-1 infection is believed to contribute to the increase in episodes of clinical malaria and parasitemia in co-infected individuals (157), (214), (180). The effect of co-infection on malaria transmission, including in asymptomatic adults, is less defined, but several previous findings suggest that HIV-1 might cause an increase in gametocyte carriage. Gametocyte burden was found to be greatly increased in a *Rhesus macaque* model of simian immunodeficiency virus (SIV) and *P. fragile* (220). Additionally, gametocyte-specific antibodies develop slowly but are known to be important in controlling gametocyte

burden in older adults, and co-infection with HIV-1 has been found to be associated with a reduction in *P. falciparum*-specific antibody responses (229), (207). Some antiretroviral drugs (ARTs) have been shown to have gametocidal properties (99), (98), but despite recent efforts to improve HIV testing and education, there are still individuals who are unaware of their HIV status or who are not receiving treatment with ART after a diagnosis of HIV-1 infection (151). Much of the current research into HIV and malaria co-infection has focused on groups at high risk for clinical episodes of malaria, including children (103), (154), (137) and pregnant women (47), (29), (2), and the most common outcome of interest has been clinical severity (230), (174) or HIV disease progression (1). Much of the work pertaining to asymptotically infected adults uses less-sensitive methods for malaria detection, such as microscopy or RDTs (157), (106), (156), (153). Thus, there remains a notable gap in the literature concerning the effect of HIV on malaria transmission in ART-naïve asymptomatic adults.

OVERALL STUDY AIMS

The aim of this study is to evaluate the impact of HIV on *P. falciparum* gametocyte carriage in chronically malaria infected adults living in an area of endemic malaria transmission. To address this aim, a panel of gametocyte stage-specific qPCR assays was used to quantify gametocyte transcripts in dried blood spots from 1116 asymptotically infected adults seeking voluntary HIV testing in Kombewa, Kenya in order to evaluate the impact of HIV on gametocyte prevalence.

METHODS

Sample Collection in Kombewa, Kenya

Samples for this study were collected under the supervision of the USUHS, WRAIR, and KEMRI Institutional Review Boards (KEMRI protocol #388266) as part of a cross-sectional molecular epidemiology study investigating the relationships between HIV and malaria in asymptomatic adults. These samples were obtained as dried blood spots from healthy adults seeking voluntary HIV testing in Kombewa, Kenya at either Kombewa County Hospital or Manywanda Sub-County Hospital. From each patient, one to five 50 µl blood spots were blotted onto Whatman® 903 Protein Saver filter paper cards (GE Healthcare Life Sciences, Chicago, IL, USA) in the outpatient laboratory. Cards were allowed to air-dry rapidly on the air intake of a biological safety cabinet and once dry, were placed into individual sample storage bags with desiccant packets and stored frozen at -80C° to minimize RNA degradation. Comparison of this method with fresh RNA extractions and similarly prepared cultured parasite material had indicated <10% reduction in RNA signal (data not shown). A total of 1762 samples were collected as part of this study. At the time of sample collection, patients were also tested for HIV using the Alere Determine™ HIV 1/2 rapid diagnostic test (Abbott Laboratories, Chicago, IL, USA) and for malaria using Parascreen® rapid diagnostic test (Zephyr Biomedicals, Verna, Goa, India). Subsequently, samples were also tested for the presence of malaria-specific transcripts using a qPCR assay for plasmodial *18S* ribosomal RNA ((112), data not shown). Only the 1116 samples positive for plasmodial *18S* positive samples were tested for the presence of gametocytes.

Nucleic Acid Extraction from Dried Blood Spots and cDNA Synthesis

Individual entire dried blood spots were cut out of the filter paper cards and minced with a single use razor blade to eliminate the risk of cross-contamination. Filter paper pieces were then treated with Buffer AL, Proteinase K, and Buffer ATL (Qiagen, Hilden, Germany) to rehydrate the samples, lyse red blood cells and parasites, and degrade unwanted enzymes. Half of the resulting lysate was used for an RNA extraction using the RNeasy® Mini Kit (Qiagen, Hilden, Germany), while the other half was retained for additional studies. The RNA lysates were treated with a genomic DNA wipeout step followed by cDNA synthesis using the QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany), using the included oligonucleotide primers, following the Qiagen protocol as written, and using 3 µl of RNA per 20 µl reaction. Due to both the very low volume of RNA samples and the low concentration of RNA in each sample, quality assessment and quantification of RNA was unable to be conducted on these samples prior to amplification by qPCR. No-template controls (NTCs) were regularly included during the reverse transcription step as a negative control.

Selection of Markers and Primer and Probe Design

After identifying potential gametocyte markers by literature search, gametocyte transcriptomics data available on PlasmoDB (<https://plasmodb.org>) were used to select targets which are highly expressed and thus likely to be detectable in individuals with very low parasitemias. mRNA sequences were obtained for genes of interest from PlasmoDB and primers and probes were designed and checked for melting point and hairpin absence using the Primer Express™ 3.0 software (Applied Biosystems, Foster

City, CA, USA). Amplicon sequences were checked using NCBI BLAST™ (<https://blast.ncbi.nlm.nih.gov/>) to ensure that primer and probe sequences were free of single nucleotide polymorphisms. All synthetic primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). Custom TaqMan probes resuspended in 1X TE buffer were manufactured by Life Technologies (Carlsbad, CA, USA). Primer and probe sequences are listed in Table 7.

Table 7. Primer and probe sequences for gametocyte-specific qPCR assays. Sequences were obtained from PlasmoDB using GeneID listed below target name.

Target	Forward Primer	Reverse Primer	Probe	Amplicon Size (bp)
<i>Pfs16</i> PF3D7_0406200	5' GGA TCC CCT TCA ACT TTG CA 3'	5' CCT TGA GAT AGT CCA CCT TGA TTA GG 3'	JUN 5'TTC TTC AGG TGC CTC TCT TCA TGC TGT TG 3' QSY	89
<i>Pfs48/45</i> PF3D7_1346700	5' TGT AAG CCT AGC TCT TTG AAT AGT GAA 3'	5' TCA CGC ATA TCT GGC TTT AAA TTA TG 3'	VIC 5' TAT CTG GAT TCA TAG GAT ATA AG 3' MGBNFQ	101
<i>Pfs25</i> PF3D7_1031000	5' TCT GAA ATG TGA CGA AAA GAC TGT 3'	5' AGC GTA TGA AAC GGG ATT TCC 3'	FAM 5' ATA AAC CAT GTG GAG ATT T 3' MGBNFQ	88

Creation of Plasmid Standards

To quantify gene expression during qPCR, a serial dilution of a plasmid standard was created for each target. To create these standards, the amplicon of interest was amplified from cultured parasite material by conventional PCR and verified by gel electrophoresis. The PCR product was then cloned into the pCR 2.1-TOPO TA vector and transformed into competent *E. coli* using the TOPO® TA Cloning® Kit for

Subcloning with OneShot™ Top10 Chemically Competent *E. coli* (Invitrogen, Carlsbad, CA, USA). The QIAprep® MiniPrep kit (Qiagen, Hilden, Germany) was used to isolate plasmid DNA, which was then quantified by NanoDrop™2000C (ThermoFisher Scientific, Waltham, MA, USA). Insertion of the desired target amplicon into the plasmid was verified by Sanger sequencing conducted by Genewiz (Frederick, MD, USA) (data not shown). Dilution calculations were based off of the mass of the amplicon of interest and the concentration of the plasmid DNA as determined by NanoDrop™.

Quantitative Real Time PCR

All qPCR reactions were conducted using the Applied Biosystems 7500 Fast System (Applied Biosystems, Foster City, CA, USA) using TaqMan™ Multiplex Master Mix (ThermoFisher Scientific, Waltham, MA, USA). All three gametocyte markers were simultaneously quantified in a single triplex reaction in which the final concentration of each primer and probe was 250 nM. Each plate included a series of ten-fold standard dilutions of a mixture of all three amplicon-specific plasmids and a plain water no-template control. All samples and standards were run in triplicate. Each reaction was conducted using 1 µl of cDNA, 10 µl of appropriate master mix, 250 nM of each primer and probe, and RNase-free water to bring the total volume up to 20 µl. Reactions were set up in 96 well optical plates and sealed with optical film. All runs were conducted for 40-45 cycles with the following cycling parameters: Initial denaturation: 50°C for 2 minutes, 95°C for 10 minutes; 40-45 cycles of denaturation at 95°C for 15 seconds and annealing, extension, and detection of fluorescence at 60°C for 1 minute.

qPCR Assay Validation and Optimization

Ten-fold standard dilutions ranging from 100,000 copies per μl to 0.1 copies per μl were used to determine the limits of detection and quantification. Additional two-fold dilutions ranging from 10 copies per μl to 0.625 copies per μl were conducted at the lower end of the dilution series to more accurately determine the lower limits of the assays. For all three markers, the lower limit of quantification was 2.5 copies per μl of template and the lower limit of detection was 1 copy per μl of template. All concentrations were run in triplicate and run for 45 cycles. Specificity was verified by running each assay with cDNA from cultured *P. falciparum* parasites and uninfected human blood. To verify the absence of cross-reactivity when amplifying all markers in a single triplex assay, the amplification of each individual target was compared in the presence and absence of primers, probes, and standards for the other two targets (data not shown) and determined to be the same as the singleplex results (data not shown).

Analysis of qPCR Data and Statistical Analysis

To facilitate comparisons between runs, the Ct threshold was manually adjusted to 0.2. Ct values for standards were input into GraphPad Prism 6 (GraphPad, San Diego, CA, USA) and were analyzed using a semi-logarithmic nonlinear regression analysis. Ct values for field samples were interpolated onto this semi-logarithmic line to calculate copy number per μl . Plates were excluded from analysis if NTCs were positive, if the PCR threshold was below 90% or above 105%, or if the average Ct value for any of the plasmid standard replicates differed more than two standard deviations from the

aggregated average of Ct values for that particular dilution. Representative standard curves for all markers are shown in Figure 11.

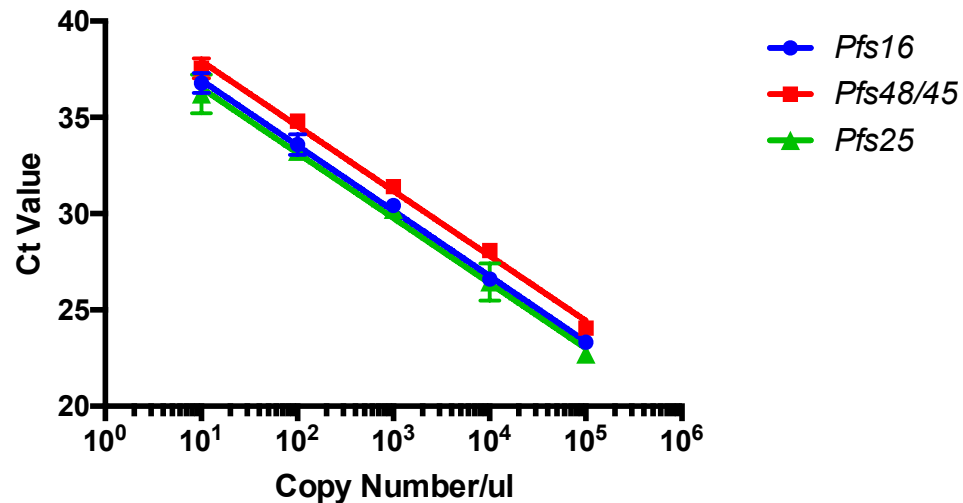


Figure 11. Representative standard curves for *Pfs16*, *Pfs48/45*, and *Pfs25*. For *Pfs16*, slope: -3.392, R^2 : 0.9953, and PCR efficiency: 97.16%. For *Pfs48/45*, slope: -3.372, R^2 : 0.9940, and PCR efficiency: 97.95%. For *Pfs25*, slope: -3.382, R^2 : 0.9846, and PCR efficiency: 97.55%.

Samples were considered positive if at least two of the three replicates were positive. If only one replicate was positive, the sample was re-run. After being re-run, the sample was considered to be positive if at least three wells were positive from both of the two runs. Samples were considered negative if only one or two out of six total replicates were positive.

Statistical Analyses

All statistical tests were conducted using GraphPad Prism 6 using non-parametric tests. Comparisons of prevalence between two groups were conducted using Fisher's exact test, while comparisons of more than two groups were conducted using Chi-square analyses. To evaluate differences in copy numbers, Mann-Whitney tests or Dunn's

multiple comparisons tests were used to compare two, or more than two groups, respectively.

RESULTS

Prevalence of Gametocyte-Specific Transcripts in Samples from Human Volunteers

Of the 1133 samples found to be positive for *Plasmodium* 18S ribosomal RNA (Kifude, C, in prep.), 1116 had adequate sample volumes remaining for gametocyte analysis by qPCR. This cohort was comprised primarily of young adults under the age of 30 years and included slightly more females than males (Table 2). Of these 1116 individuals, 229 (20.5%) were found to be positive for at least one gametocyte marker (*Pfs16*, *Pfs48/45*, and/or *Pfs25*). Although the overall percentage of individuals who were gametocyte positive was similar between males and females, the percentage of positive males differed between age brackets ($P=0.0186$, Chi-square analysis) and increased with age, while the percentage of positive females was not significantly different between different age brackets and tended to a slight, insignificant decrease with age (Table 8).

Table 8. Positivity for any gametocyte-specific marker (*Pfs16*, *Pfs48/45*, or *Pfs25*), divided by gender and age.

P values were calculated for each gender by Chi-square analysis.

Age Range	Gametocyte Positive	Gametocyte Negative	Total n (% Positive)	P Value
Male				
18-29	61	279	340 (17.9)	0.0178
30-39	34	78	112 (30.6)	
40-49	12	28	40 (30.0)	
50-56	5	11	16 (31.3)	
All Ages	112	396	508 (22.1)	
Female				
18-29	95	353	448 (21.2)	0.1905
30-39	13	78	91 (14.3)	
40-49	8	46	54 (14.8)	
50-56	1	14	15 (6.7)	
All Ages	117	491	608 (19.3)	

HIV-Positive Individuals are More Likely to Have Gametocyte-Specific Transcripts

129 individuals (11.6% of this cohort) were found to be HIV positive by rapid diagnostic test. Gametocyte positivity by any marker was significantly higher in the HIV positive individuals (44/129 or 34.1% of all HIV positive individuals in this cohort) than in HIV negative individuals (185/987 or 18.7% of all HIV negative individuals in this cohort) (Table 9). The relative risk of gametocyte positivity in the HIV positive group was 1.820 times higher than that of the HIV negative group ($P < 0.0001$, Fisher's exact test).

Table 9. Comparison of gametocyte positivity, as measured by detectable transcripts for one or more gametocyte marker, by HIV status.

Data displayed as number of samples and percentage gametocyte positive (right column) or HIV positive (bottom row). RR=1.820, P<0.0001.

	<i>Gametocyte Positive</i>	<i>Gametocyte Negative</i>	<i>Total</i>
<i>HIV Positive</i>	44	85	129 (34.1%)
<i>HIV Negative</i>	185	802	987 (18.7%)
<i>Total</i>	229 (19.2%)	887 (9.6%)	1116 (100.0%)

Prevalence of Gametocyte Transcripts Is Impacted by HIV Status, but Not Age or Gender

To evaluate if HIV status is driving the difference between males and females show in Table 8, the number of gametocyte positive samples was compared between HIV positive and HIV negative groups for each gender and age bracket (Table 10). The only significant difference (P=0.0082, Fisher's exact test) was seen between HIV negative males and females who were 30 to 39 years of age, indicating that there is little difference between males and females who are of similar age and the same HIV status.

Table 10. Comparison of the number of gametocyte positive samples (n) between age, and gender, by HIV status.

Percentage is the percent of the total cohort. Males and females for each age range were compared by Fisher's exact test and relative risk is given for groups which were significantly different.

HIV Positive					
Age	Male		Female		P Value RR
	n	%	n	%	
18-29	8	30.77%	15	36.59%	0.7925 ns
30-39	9	29.03%	2	20.00%	0.7004 ns
40-49	4	40.00%	3	60.00%	0.6084 ns
50-56	2	40.00%	1	100.00%	1.0000 ns
Total	24	33.33%	21	36.84%	0.7127 ns

HIV Negative					
Age	Male		Female		P Value RR
	n	%	n	%	
18-29	53	16.88%	80	19.66%	0.3837 ns
30-39	25	30.86%	11	13.58%	0.0133 2.273
40-49	8	26.67%	5	10.20%	0.0676 ns
50-56	3	27.27%	0	0.00%	0.0717 ns
Total	89	20.41%	96	17.42%	0.2505 ns

In this cohort, the number of samples in the 40-49 year old and 50-56 year old age brackets was quite small which may contribute to the lack of significant differences between males and females when comparing within age brackets. Since there was little difference between males and females, both genders were combined to increase the sample size in order to evaluate the impact of HIV status on the prevalence of gametocyte transcripts when separated by age (Table 11). In two of the four age groups (18-29, 40-49), there was a significant difference between HIV positive and HIV negative groups ($P=0.0035$ and $P=0.0154$, respectively, Fisher's exact test). Although this trend was not seen in all age groups, the two groups that were statistically significant include 79.2% of

the samples in the cohort because a large proportion of samples was from individuals ages 18-29. A Chi-square analysis was conducted to compare the number of positive samples between age ranges within either the HIV positive or the HIV negative groups and no significant differences were found (P= 0.3599 for HIV positive group, P=0.4879 for HIV negative group), but this may be due to the small sample size.

Table 11. Comparison of the number of gametocyte positive samples (n) between age by HIV status.

Percentage is the percent of all samples tested (n=1116). HIV positive and HIV negative groups for each age range were compared by Fisher's exact test and relative risk is given for groups which were significantly different. There was no difference in prevalence between age groups within HIV positive or HIV negative groups, evaluated by Chi-square test.

Age	HIV Positive		HIV Negative		P Value	RR
	n	%	n	%		
18-29	23	34.33%	133	18.45%	0.0035	1.861
30-39	11	26.19%	36	22.36%	0.5383	ns
40-49	7	46.67%	13	16.46%	0.0154	2.836
50-56	3	50.00%	3	12.00%	0.0688	ns
Total	44	34.11%	185	18.78%	0.0001	1.820

HIV Positive Individuals Have a Higher Burden of Gametocyte-Specific Transcripts

For each sample, a copy number/ μ l was calculated for *Pfs16*, *Pfs48/45*, and *Pfs25*, using Ct values calibrated against serial dilutions of a plasmid containing the amplicon of interest. To determine whether HIV infection is associated with higher numbers of gametocyte transcripts, the copy number per μ l was compared between HIV positive and HIV negative groups for each marker. Copy number per μ l was significantly higher in the HIV positive group compared to the HIV negative group for *Pfs25* (Figure 12B) and was trending towards significance for *Pfs16* (Figure 12A). There were few

samples that were positive for *Pfs48/45*, and there was no significant difference between HIV positive and HIV negative individuals for *Pfs48/45* (data not shown).

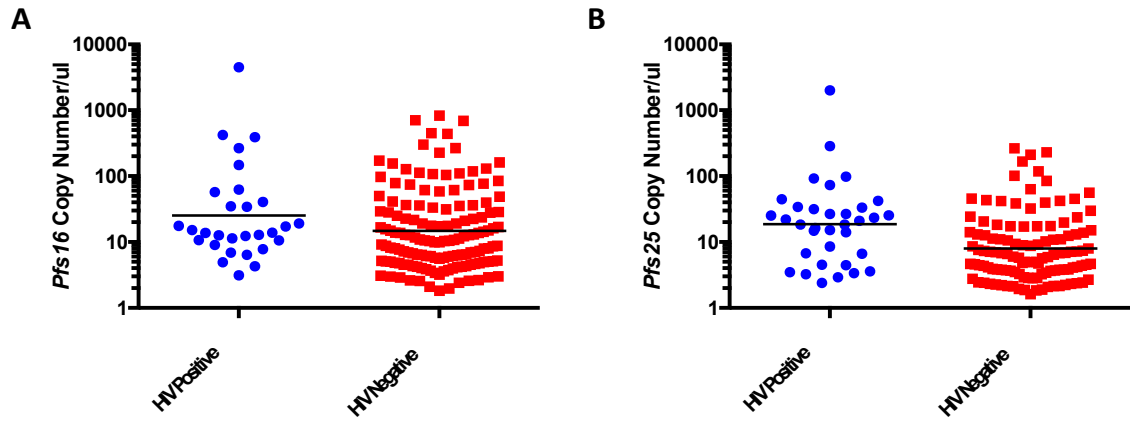


Figure 12. Comparison of copy number per µl by HIV status for *Pfs16* (A) and *Pfs25* (B). Black lines indicate geometric mean. Copy number per µl was significantly different between groups for *Pfs25* by Mann-Whitney test (*Pfs16*: $P=0.0581$, *Pfs25*: $P=0.0006$)

When comparing the prevalence of samples positive for 1, 2, or 3 markers, there were no significant differences between HIV positive and HIV negative groups (data not shown, Chi-square analysis); however, there were significant differences between HIV positive and negative groups when comparing median copy number by the number of concurrently positive markers (Figure 13). For *Pfs16*, the median copy number was highest in samples that were positive for all three markers, regardless of HIV status (Figure 13A). This trend was not seen for *Pfs25* copy numbers, which were much more consistent between groups (Figure 13B). When comparing 18S copy (data from Kifude, C., et al, in prep.), there was no difference in average 18S copy number between samples that were concurrently positive for two or three gametocyte markers in either HIV positive or HIV negative groups, suggesting that the difference in the quantity of

gametocyte transcripts shown in Figure 13A and 13C is only partially attributable to differences in overall parasitemia.

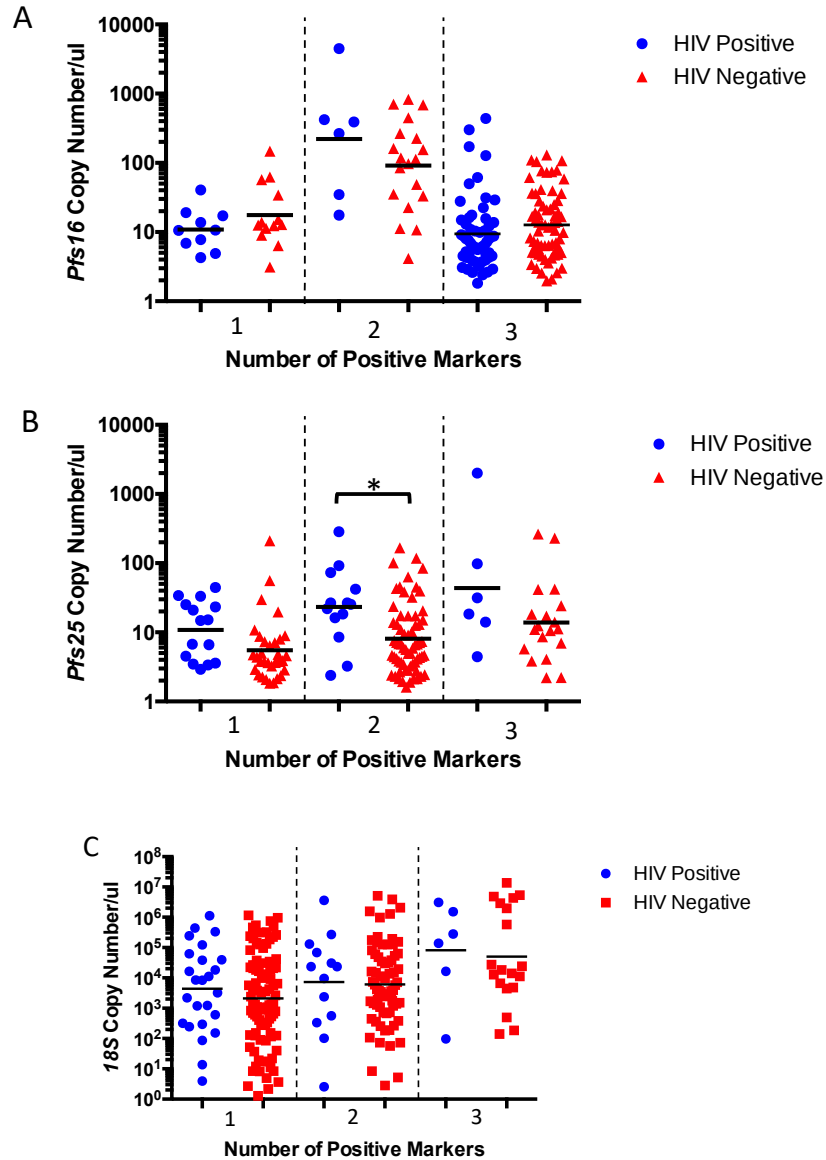


Figure 13. A) *Pfs16* and B) *Pfs25* copy number per µl for each combination of concurrently positive markers, by HIV status. Error bars show median and interquartile range. *Pfs48/45* positive samples were excluded from statistical analysis due to low n. Dunn's multiple comparisons analysis, * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, *** indicates $P \leq 0.001$, and **** indicates $P \leq 0.0001$. C) *18S* copy number by number of concurrently positive markers for HIV positive and HIV negative individuals. Dunn's multiple comparisons analysis, * indicates $P \leq 0.05$.

Positivity by Rapid Diagnostic Test Is Associated with an Increased Risk of Gametocyte Carriage

The malaria rapid diagnostic test (mRDT) used to test patients at the point of care was positive for only 45.4% those infections which were gametocyte positive by qPCR (Table 12). However, being mRDT positive at enrollment was associated with an increased risk of being gametocyte positive by any marker (RR=2.232, $P<0.0001$). There was no difference in gametocyte copy numbers between RDT positive and negative for *Pfs16* ($P=0.1157$, Mann-Whitney test) or *Pfs25* ($P=0.4844$, Mann-Whitney test,); however, *Pfs25* copy numbers were significantly different when comparing individuals who were positive for both malaria RDT and HIV RDTs with those who were not positive for either (Figure 14).

Table 12. Comparison of gametocyte positivity, as measured by detectable transcripts for one or more gametocyte marker, by positivity of malaria rapid diagnostic test. Data displayed as number of samples and percentage of total cohort. RR=2.232, $P<0.0001$.

	<i>Gametocyte Positive</i>	<i>Gametocyte Negative</i>	<i>Total</i>
<i>RDT Positive</i>	104 (9.32%)	199 (17.8%)	303 (27.2%)
<i>RDT Negative</i>	125 (11.2%)	688 (61.6%)	813 (72.8%)
<i>Total</i>	229 (20.5%)	887 (79.5%)	1116 (100.0%)

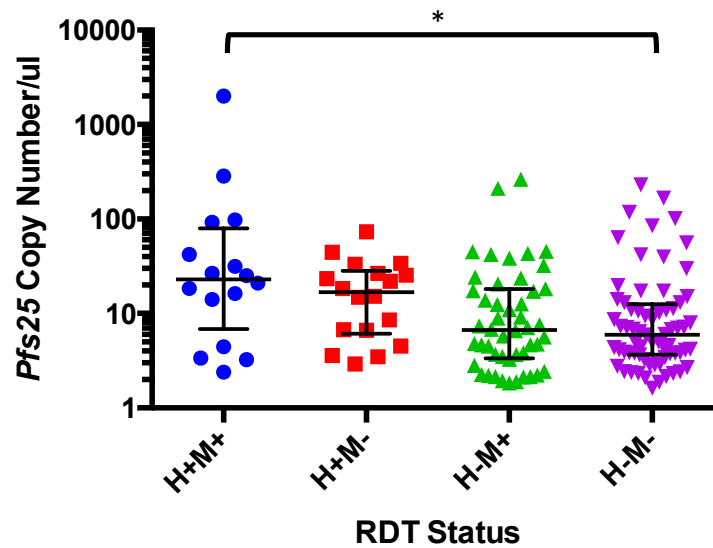


Figure 14. *Pfs25* copy number/ul by RDT status.

“H” indicates HIV RDT status and “M” indicates malaria RDT status. Error bars show median and interquartile range. Dunn’s multiple comparisons test, * indicates $P \leq 0.05$

DISCUSSION

This cohort provided a unique opportunity to quantify the gametocyte burden in an asymptomatic and antiretroviral therapy (ART)-naïve population in a region of holoendemic malaria transmission. In this population, 20.5% of asymptomatic adults had detectable gametocyte transcripts, and HIV positive individuals were 1.820 times more likely to have gametocyte transcripts ($P < 0.0001$). HIV positive individuals also had significantly higher gametocyte copy numbers, suggesting that HIV positive individuals may be both more likely to harbor gametocytes and maintain higher gametocytemias than HIV negative individuals. mRDTs are commonly used in the clinic to diagnose febrile illness, but they are unable to detect low parasitemia infections and were unsuitable for detecting gametocytes in this cohort. This could be because gametocyte carriage may occur in the absence of high parasitemias, as has been previously suggested (74).

Additionally, in this cohort, there were differences between gametocyte prevalence by both age and gender. Age has been shown to impact gametocyte carriage as transmission blocking immunity develops (3), but that trend was only seen in female participants. Male participants had a higher gametocyte prevalence, but interestingly, prevalence increased with age. This effect seems to be driven primarily by HIV status, as separating groups by HIV status resulted in almost identical gametocyte prevalence between males and females of each age group. Collectively, this suggests that HIV infection may interfere with gametocyte-specific immunity. In this study, however, a majority of participants were between 18 and 29 years of age; since previous research has shown that gametocyte prevalence is reduced in individuals 35 years of age or older (3), the number of individuals in this study who were old enough to have developed a broad range of gametocyte-specific antibodies is likely to be low.

In other molecular epidemiology studies, gametocyte carriage has been determined by the detection of *Pfs25*, which is highly abundant in Stage V female gametocytes and is considered an acceptable surrogate for the presence of mature gametocytes by microscopy. The exclusive use of *Pfs25* as a surrogate for gametocyte carriage likely leads to an underestimate of gametocyte positive individuals, especially in cross-sectional studies because it does not account for individuals who have immature gametocytes at the time of sampling. In our study, 79 samples, or 34.5% of gametocyte positive samples, were positive for only *Pfs16* and/or *Pfs48/45*. While molecular diagnostic methods have been shown to correlate well to mosquito infectivity (167), it has also been shown that gametocyte commitment can occur without maturity to stage V

(189) and that the number of gametocytes present is not the only predictor of mosquito infectivity (68).

Although the assays used in this study are extremely sensitive, it is likely that the panel is still underestimating the true prevalence of individuals harboring gametocytes, especially given the starting volume of each DBS was only 50 μ l. This technical challenge may become a larger issue as the prevalence of clinical malaria declines because the proportion of individuals with low parasitemia asymptomatic infections may increase. This issue is especially relevant for lower abundance targets, such as *Pfs48/45*, and targets which are expressed during stages which are primarily sequestered in the bone marrow, including *Pfs16* and *Pfs48/45*.

The specific mechanisms of increased gametocyte carriage in the context of co-infection remain to be elucidated but could be related to impaired T-cell dependent antibody responses impacting late-developing transmission blocking immunity. Co-infections that impact antibody development, including *Schistosoma haematobium*, have been found to be reduce gametocyte-specific antibody levels (9); other pathogens that impair B cell development, including HIV, are suspected to have a similar effect on gametocyte carriage. HIV has been shown to modulate B cell populations and impact *P. falciparum*-specific B cells (81), but the impact of these perturbations on gametocytes has yet to be determined. Additionally, angiogenic cytokines, including IL-1 β , IL-6, and IL-8, play a role in the release of mature gametocytes from the bone marrow (140), and these cytokines have been found to be dysregulated during HIV infection (115). This could lead to the premature release of developing gametocytes, resulting in an increase in circulating immature gametocytes. Future studies that include measurements of

immunological parameters, including CD4⁺ T cell counts, *P. falciparum*-specific antibodies, and cytokine levels, could provide important information regarding which, if any, aspect of HIV-related immune dysfunction have a role in gametocytemia. Currently, a follow-up longitudinal study is underway which follows both HIV positive and negative individuals over several weeks and includes a standard membrane feed assay component to directly measure infectivity to mosquitos. This study will provide an opportunity to evaluate the correlation between the presence of early gametocyte transcripts and the development of Stage V gametocytes and will also provide additional information pertaining to HIV infection, including viral load and CD4 T cell count.

The true measure of transmission potential can only be determined through direct quantification of infectivity to mosquitos. Thus, this data represents an indirect measure of an indirect measure inasmuch as RNA transcripts are used a proxy for gametocyte carriage, which is used as a proxy for mosquito infectivity. Ultimately, standard membrane feeding assays (SMFA) are the best tool for measuring malaria transmission, but the quantification of gametocyte-specific transcripts can serve as a more cost-effective and indirect means of measuring the potential for malaria transmission. In the future, additional studies should include SMFA and could focus on the sex ratio of male and female gametocytes, which is believed to impact infectivity to mosquitos and sexual reproduction in the mosquito midgut (97).

This study examined the impact of HIV co-infection on gametocyte development in asymptomatic adults, and highlights the potential importance of asymptomatic adults, particularly those co-infected with HIV-1, as potential reservoirs of transmission in areas that are holoendemic for malaria transmission. The identification of individuals at high

risk for transmitting malaria could be used to guide treatment with gametocidal drugs, further reducing the prevalence of the parasite and closing the gap between endemic malaria transmission and the global eradication of malaria.

CHAPTER 4: Measuring Gene Expression to Characterize Parasitemia and Gametocytemia after Artemether-Lumefantrine Treatment in Zambian Children with Uncomplicated *P. falciparum* Malaria

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ABSTRACT

Artemisinin combination therapy (ACT) is currently the treatment of choice for uncomplicated *P. falciparum* malaria. Although ACTs are known to be highly effective against asexual parasites, the *in vivo* effect of ACTs on various stages of gametocytes is incompletely characterized. Samples from 75 participants were selected from a therapeutic efficacy study of 100 children from Nchelenge, Zambia with uncomplicated *P. falciparum* malaria treated with artemether-lumefantrine (AL) and followed for five weeks. Dried blood spots (DBS) were collected from each participant every 6 hours for the first 48 hours, at 72 hours, and weekly; in this study, RNA was extracted from samples collected up to the second week of follow-up. This RNA was used for quantitative real-time PCR (qPCR) assays that quantify three stage specific transcripts: *Pfsbp1* (ring stage asexual parasites), *Pfs16* (highly expressed by early gametocytes, moderately expressed throughout gametocyte development), and *Pfs25* (stage V female gametocytes). We were able to successfully extract RNA and amplify transcripts present

in quantities as low as 2.5 copy/μl for *Pfsbpl*, and 1.25 copies/μl for *Pfs16* and *Pfs25*. In this cohort, it was found that treatment with AL was associated with a 30-fold reduction of mean *Pfsbpl* copy numbers within the first 36 hours. Despite this reduction in copy number, approximately 50% of the participants were positive for *Pfsbpl* by qPCR after 2 weeks. Many fewer samples were positive for *Pfs16* and/or *Pfs25* after 2 weeks, but mean *Pfs16* and *Pfs25* copy numbers were not reduced by treatment, consistent with stage-dependent variations in drug susceptibility to AL. Additional studies are needed to evaluate the epidemiological importance of such lowly expressed transcripts, to determine if the sustained prevalence of transcripts during the follow-up period is due to persistent parasitemia, reinfection, or recrudescence, and to determine the impact of drug treatment beyond two weeks.

INTRODUCTION

Malaria remains a major cause of morbidity and mortality in sub-Saharan Africa. In 2017, there were approximately 250 million cases of clinical malaria worldwide, predominately in Africa, and approximately 435,000 deaths could be attributed to malaria, with 61% of malaria deaths occurring in children less than 5 years of age (236). The transmission of the parasite from human host to mosquito vector represents an important bottleneck in the parasite's life cycle, making transmission an optimal target for the global reduction of malaria (201). In some instances, the treatment of malarial with antimalarial drugs may be facilitating the transition from human to mosquito by enhancing the infectivity of gametocytes to mosquitos (161) or increasing gametocyte development, especially in the context of drug resistance (176), (90).

Artemisinin combination therapies (ACTs), including artemether-lumefantrine (AL), are the current standard of care for most individuals with clinical malaria, and because these drugs are so widely used, it is important to fully understand the effects of ACTs on *P. falciparum* gametocytes and malaria transmission. Although artemisinin is not considered to be gametocidal, there is evidence that the drug is effective against early stage gametocytes by inhibiting gametocyte development (171). Early studies on the efficacy of AL showed clearance of gametocytes (226), (130), and reduced transmission to mosquitos was seen one week after treatment (211), suggesting that AL may be effective against both asexual and sexual life stages. However, other studies have reported incomplete gametocyte clearance after treatment with ACTs (22), (25), (86), or have actually shown enhanced infectivity of gametocytes to mosquitos after treatment (161). The selection of drug-resistance genes has been a concern since the scale-up of AL use, especially due to the potential of the survival of gametocytes with resistance alleles (93). Persistent submicroscopic parasitemia and gametocytemia, along with increased transmission to mosquitos after treatment with ACTs have been noted in several recent studies (16), (48).

A limitation of several of these studies was the use of microscopy to evaluate the impact of antimalarials on gametocytes, which may lead to the omission of submicroscopic gametocytemias that could be detected by molecular methods. The length of follow up may also lead to conflicting results, as the relatively slow development of gametocytes, combined with the tendency of immature gametocytes to sequester during development, could lead to a delayed treatment effect that may not be seen as a change in the number of mature gametocytes until several weeks after the

initiation of treatment. Finally, discrepancies in the effects of AL between studies conducted in the earlier years of AL usage and those conducted more recently may be because of changes in the drug susceptibility of the parasite.

Because of these limitations, additional studies are needed to understand the effects of AL on asexual parasites and gametocyte of currently circulating parasite strains. In this study, a panel of highly sensitive quantitative real-time PCR (qPCR) assays are used to evaluate the effects of AL on both asexual parasitemia and gametocytemia in children with uncomplicated malaria. These assays include two gametocyte stage-specific targets (*Pfs16* and *Pfs25*) that collectively cover the entire span of gametocyte development and individuals are followed up to two weeks after the initiation of treatment in order to understand the immediate and short-term effects of AL treatment.

OVERALL STUDY AIMS

The goal of this study is to evaluate the immediate and short-term impacts of artemether-lumefantrine treatment on asexual parasites and all stages of *P. falciparum* gametocytes in clinically ill children from Nchelenge Province, Zambia.

METHODS

Sample Collection in Nchelenge, Zambia

Samples for this study were collected under the supervision of the Tropical Diseases Research Centre Ethics Review Committee and the Ministry of Health of the Government of the Republic of Zambia (protocol no. TDRC/C4/09/2014) and registered

with the Pan African Clinical Trial Registry (PACTR201905783261453). These samples were collected in Nchelenge, Zambia from December 2014 through August 2015.

In this study, 100 children between the ages of 6 month and 5 years were treated with a standard 3 day course of artemether-lumefantrine for uncomplicated malaria. Once treatment was initiated, dried blood spots (DBS) were collected on Whatman® 903 Protein Saver filter paper cards (GE Healthcare Life Sciences, Chicago, IL, USA) every 6 hours for the first 48 hours, at 72 hours, and weekly for 5 weeks. DBS were allowed to rapidly air-dry and once dry, were placed into sample storage bags with desiccant and stored frozen at -80C° to minimize RNA degradation. Additionally, parasitemia and gametocytemia were monitored by microscopy at all time points. Hemoglobin levels were measured at the initiation of treatment (0h) and at one week.

For this study, a subset of 75 individuals were selected from the full cohort of 100 and only samples collected up to two weeks after the initiation of treatment were included.

Nucleic Acid Extraction from Dried Blood Spots and cDNA Synthesis

A 3mm round punch was removed from each dried blood spot and retained for later pharmacokinetic analysis. Due to variations in the size of the dried blood spots, the diameter of each sample was measured by caliper and was later used to normalize gene expression data to the size of the starting sample. Each spot was cut from the filter paper card and minced into pieces no larger than 3mm by 3 mm.

Filter paper pieces were then treated with Buffer AL, Proteinase K, and Buffer ATL (Qiagen, Hilden, Germany) to rehydrate the samples, lyse red blood cells and

parasites, and degrade unwanted enzymes. Half of the resulting lysate was used for an RNA extraction using the RNeasy® Mini Kit (Qiagen, Hilden, Germany), while the other half was retained for additional studies. The RNA lysates were treated with a genomic DNA elimination step followed by cDNA synthesis using the QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany), using the included oligonucleotide primers, following the Qiagen protocol as written, and using 3 µl of RNA per 20 µl reaction. Due to both the very low volume of RNA samples and the low concentration of RNA in each sample, quality assessment and quantification of RNA was unable to be conducted on these samples prior to amplification by RT-qPCR. No-template controls (NTCs) were regularly included during the reverse transcription step as a negative control.

Primer and Probe Design

mRNA sequences were obtained for genes of interest from PlasmoDB and primers and probes were designed and checked for melting point and hairpin absence using the Primer Express™ 3.0 software (Applied Biosystems, Foster City, CA, USA). Amplicon sequences were checked using NCBI BLAST™ (<https://blast.ncbi.nlm.nih.gov/>) to ensure that primer and probe sequences were free of single nucleotide polymorphisms. All synthetic primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). Custom TaqMan probes resuspended in 1X TE buffer were manufactured by Life Technologies (Carlsbad, CA, USA). Primer and probe sequences are listed in Table 13.

Table 13. Primer and probe sequences for gametocyte-specific qPCR assays.
Sequences were obtained from PlasmoDB using GeneID listed below target name.

Target	Forward Primer	Reverse Primer	Probe	Amplicon Size (bp)
<i>Pfsbp1</i> PF3D7_0501300	5' AAA GTA CTC CTT GTT GGC AAC GTA 3'	5' TTA ATG AAT ACG AAG TAG AAT CTC CAG C 3'	FAM 5' AAT GGC TCA AGA AGC 3' MGBNFQ	73
<i>Pfs16</i> PF3D7_0406200	5' GGA TCC CCT TCA ACT TTG CA 3'	5' CCT TGA GAT AGT CCA CCT TGA TTA GG 3'	JUN 5'TTC TTC AGG TGC CTC TCT TCA TGC TGT TG 3' QSY	89
<i>Pfs25</i> PF3D7_1031000	5' TCT GAA ATG TGA CGA AAA GAC TGT 3'	5' AGC GTA TGA AAC GGG ATT TCC 3'	FAM 5' ATA AAC CAT GTG GAG ATT T 3' MGBNFQ	88

Creation of Plasmid Standards

To quantify gene expression during qPCR, a serial dilution of a plasmid standard was created for each target. To create these standards, the amplicon of interest was amplified from cultured parasite material by conventional PCR and verified by gel electrophoresis. The PCR product was then cloned into the pCR 2.1-TOPO TA vector and transformed into competent *E. coli* using the TOPO® TA Cloning® Kit for Subcloning with OneShot™ Top10 Chemically Competent *E. coli* (Invitrogen, Carlsbad, CA, USA). The QIAprep® MiniPrep kit (Qiagen, Hilden, Germany) was used to isolate plasmid DNA, which was then quantified by NanoDrop™2000C (ThermoFisher Scientific, Waltham, MA, USA). Insertion of the desired target amplicon into the plasmid was verified by Sanger sequencing conducted by Genewiz (Frederick, MD,

USA) (data not shown). Dilution calculations were based off of the mass of the amplicon of interest and the concentration of the plasmid DNA as determined by NanoDrop™.

Quantitative Real Time PCR

All qPCR reactions were conducted using the Applied Biosystems 7500 Fast System (Applied Biosystems, Foster City, CA, USA) using TaqMan™ Fast Universal PCR Master Mix (2X), no AmpErase™ UNG (ThermoFisher Scientific, Waltham, MA, USA) for *Pfsbp1* or TaqMan™ Multiplex Master Mix (ThermoFisher Scientific, Waltham, MA, USA) for *Pfs16* and *Pfs25*. Both gametocyte markers were simultaneously quantified in a single duplex reaction, in which each plate included a series of ten-fold standard dilutions of the appropriate amplicon-specific plasmids and a plain water no-template control. All samples and standards were run in triplicate. Each reaction was conducted using 1 µl of cDNA, 10 µl of appropriate master mix, 250 nM of each primer and probe, and RNase-free water to bring the total volume up to 20 µl. Reactions were set up in 96 well optical plates and sealed with optical film. All runs were conducted for 40-45 cycles with the following cycling parameters: Initial denaturation: 50°C for 2 minutes, 95°C for 10 minutes; 40-45 cycles of denaturation at 95°C for 15 seconds and annealing, extension, and detection of fluorescence at 60°C for 1 minute.

qPCR Assay Validation and Optimization

Ten-fold standard dilutions ranging from 100,000 copies per µl to 0.1 copies per µl were used to determine the limits of detection and quantification. Additional two-fold dilutions ranging from 10 copies per µl to 0.625 copies per µl were conducted at the

lower end of the dilution series to more accurately determine the lower limits of the assays. For *Pfsbp1*, the lower limit of quantification was 2.5 copies per μl of template and for *Pfs16* and *Pfs25*, the lower limit of quantification was 1 copy per μl of template. All concentrations were run in triplicate and run for 40 cycles. Specificity was verified by running each assay with cDNA from cultured *P. falciparum* parasites and uninfected human blood. To verify the absence of cross-reactivity when amplifying all markers in a single duplex assay, the amplification of each individual target was compared in the presence and absence of primers, probes, and standards for the other targets and determined to be the same as the singleplex results (data not shown).

Analysis of qPCR Data and Statistical Analysis

To facilitate comparisons between runs, the Ct threshold was manually adjusted to a fixed analysis threshold of 0.2. Ct values for standards were input into GraphPad Prism 6 (GraphPad, San Diego, CA, USA) and were analyzed using a semi-logarithmic nonlinear regression analysis. Ct values for field samples were interpolated onto this semi-logarithmic line to calculate copy number per μl . Samples were considered positive if at least two of the three technical replicates were positive. Plates were excluded from analysis if NTCs were positive, if the PCR threshold was below 90% or above 105%, or if the average Ct value for any of the plasmid standard replicates differed more than two standard deviations from the aggregated average of Ct values for that particular dilution. Representative standard curves for all markers are shown in Figure 15.

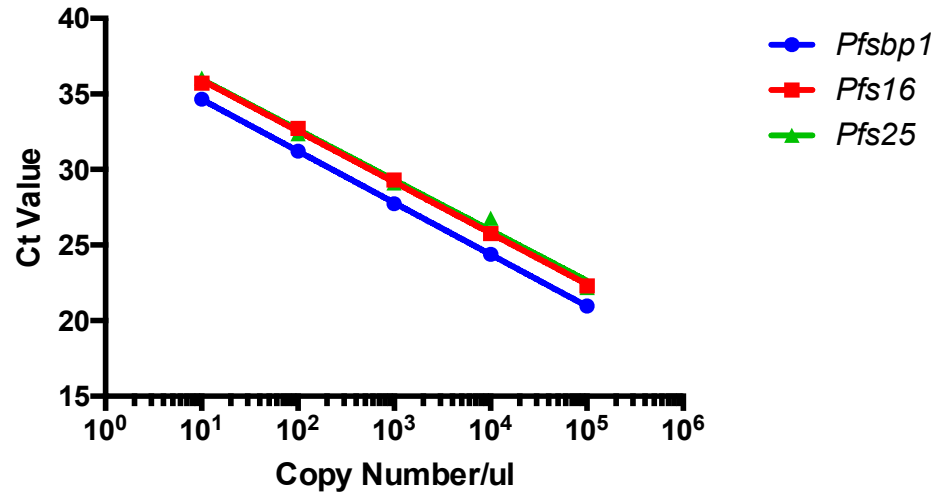


Figure 15. Representative standard curves for *Pfsbp1*, *Pfs16*, and *Pfs25*. For *Pfsbp1*, slope: -3.420, R^2 : 0.9990, and PCR efficiency: 96.06%. For *Pfs16*, slope: -3.378, R^2 : 0.9984, and PCR efficiency: 97.71%. For *Pfs25*, slope: -3.330, R^2 : 0.9878, and PCR efficiency: 99.66%.

Statistical Analyses

All statistical tests were conducted using GraphPad Prism 6 using non-parametric tests. Comparisons of prevalence between two groups were conducted using Fisher's exact test. To evaluate differences in copy numbers, Mann-Whitney tests or Dunn's multiple comparisons tests were used to compare two, or more than two groups, respectively. Area under the curve for the first 48 hours (AUC_{0-48}) was calculated for each marker in all samples using Prism 6.

RESULTS

***Pfsbp1* gene expression strongly correlates with asexual parasitemia determined by microscopy**

Pfsbp1 copy number per μl was found to be significantly higher in samples that were positive by microscopy for asexual parasites when compared to samples that were negative by microscopy ($P < 0.0001$, Mann-Whitney test) (Figure 16A). For samples that were positive by microscopy, the asexual parasitemia was found to correlate strongly with *Pfsbp1* copy number per μl (Figure 16B). Unsurprisingly, this suggests that asexual parasitemia correlates well with *Pfsbp1* copy number, but that molecular methods of detection are more sensitive than microscopy.

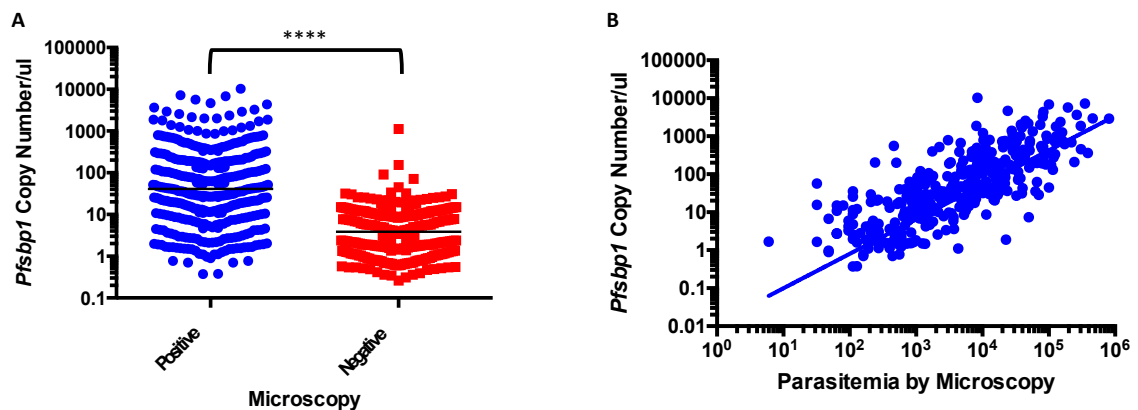


Figure 16. Comparison of *Pfsbp1* copy number and microscopy

A) *Pfsbp1* copy number/ μl for all *Pfsbp1* positive samples by positivity of microscopy. B) *Pfsbp1* copy number/ μl for all samples positive by microscopy and *Pfsbp1* qPCR, by parasitemia as determined by microscopy; Spearman Correlation: $r=0.7874$, $P < 0.0001$; Linear Regression: Slope=0.9076, $R^2=0.8595$. **** indicates $P < 0.0001$.

***Pfsbp1*, but not *Pfs16* or *Pfs25*, transcript levels declined on average 18 hours after initiation of treatment**

When compared to the mean *Pfsbp1* transcript level at the initiation of treatment (0h), mean *Pfsbp1* copy numbers were significantly different starting from 18 hours until

2 weeks after the initiation of treatment (Dunn's multiple comparison test; $P < 0.0001$, Kruskal-Wallis test) (Figure 17A). The mean *Pfsbp1* copy number remained relatively constant from 30 hours onward (Figure 17A, Table 14). A similar trend was not seen for either gametocyte marker. Although *Pfs16* copy number varied significantly across time points ($P = 0.02$, Kruskal-Wallis test), there were no significant differences between time 0 and subsequent time points (Figure 17B). For *Pfs25*, there were no significant differences between any of the time points ($P = 0.8200$, Kruskal-Wallis test) (Figure 17C).

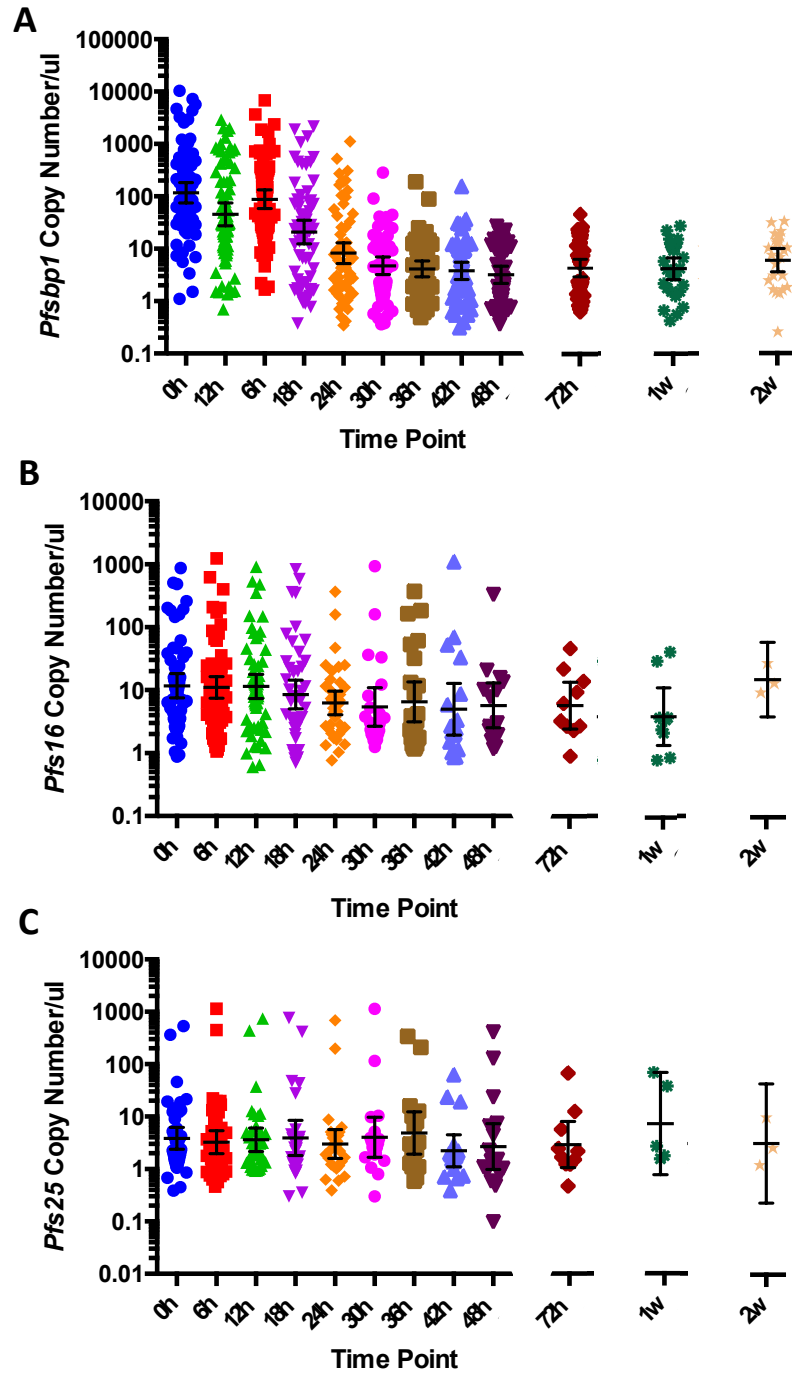


Figure 17. Copy number per μl for A) *Pfsbp1*, B) *Pfs16*, and C) *Pfs25*. Error bars show geometric mean and 95% confidence intervals.

Prevalence of *Pfsbp1*-positive samples remained high throughout follow up period

Although average copy number/ul did not decline over time, the proportion of samples that were positive for *Pfs16* or *Pfs25* declined over time, and only 6.1% of samples were positive for each marker at 2 weeks (Table 14). Both copy number and percentage of positive samples declined over time for *Pfsbp1*, but approximately half of the samples still had quantifiable transcripts at 2 weeks post-treatment (Table 14).

Table 14. Number of samples at each time point (n), and the percentage and geometric mean copy number of positive samples for each marker.

Time	n	<i>Pfsbp1</i>		<i>Pfs16</i>		<i>Pfs25</i>	
		# (% of n)	Mean Copy Number/ul	# (% of n)	Mean Copy Number/ul	# (% of n)	Mean Copy Number/ul
0h	75	75 (100.0)	116.90	59 (78.7)	11.76	42 (56.0)	3.84
6h	74	74 (100.0)	88.00	64 (86.5)	11.07	42 (56.8)	3.27
12h	75	73 (97.3)	45.33	59 (78.7)	11.47	36 (48.0)	3.60
18h	75	68 (90.7)	20.75	47 (62.3)	8.53	26 (34.7)	3.0
24h	75	68 (90.7)	8.23	38 (50.7)	6.30	27 (36.0)	2.99
30h	74	58 (78.4)	4.71	23 (31.1)	5.42	19 (25.7)	4.04
36h	74	53 (71.6)	4.12	25 (33.8)	6.52	17 (23.0)	4.85
42h	75	54 (72.0)	3.78	18 (24.0)	4.97	17 (22.7)	2.23
48h	75	47 (62.7)	3.18	15 (20.0)	5.73	18 (24.0)	2.69
72h	72	41 (56.9)	4.26	10 (13.8)	5.67	10 (13.9)	2.88
1w	55	27 (49.1)	4.13	9 (16.4)	3.77	5 (9.1)	7.16
2w	49	24 (48.9)	6.01	3 (6.1)	14.61	3 (6.1)	3.00

Pfsbp1 transcript levels changed more quickly than *Pfs16* or *Pfs25* during and after treatment with AL

As the prevalence of gametocyte-positive samples decreases, the proportion of samples that were concurrently positive for only one marker increases (Figure 18). During the first 24 hours, most samples were concurrently positive for multiple markers, but by 30 hours, most samples were positive for only *Pfsbp1*.

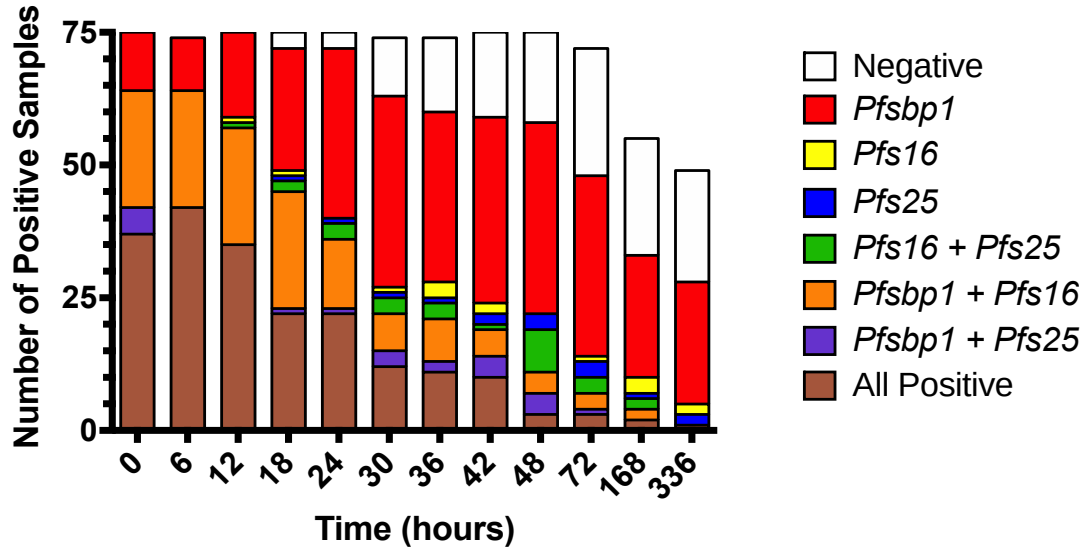


Figure 18. Number of samples positive for each combination of concurrent positivity.

To confirm this apparent difference in the rate of change of transcript levels between the three markers of interest, we compared fold change from the starting copy number per μl for each marker at all time points that had detectable transcripts for that marker (Figure 19). *Pfs16* fold change correlated more strongly than *Pfs25* with *Pfsbp1* fold change, although the fold change of both gametocyte markers correlated to *Pfsbp1* fold change (Figure 19). Additionally, the rate of change for *Pfs16* was less than that of *Pfsbp1* (shown by a slope <1 on Figure 19), and the rate of change of *Pfs25* was even less than *Pfs16* (Figure 19).

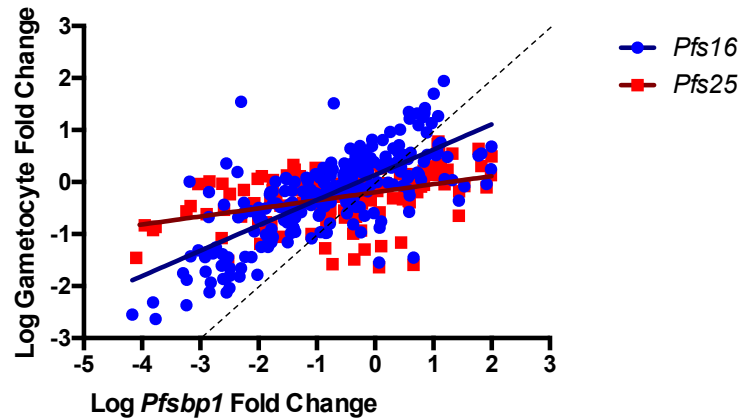


Figure 19. Log fold change from initial copy number for all positive time points during follow up.
Dashed line represents a slope of 1. For *Pfs16*: Spearman Correlation $r=0.7267$, $P<0.0001$; Linear regression slope= 0.4861 , $R^2=0.5353$. For *Pfs25*: Spearman Correlation $r=0.4260$, $P<0.0001$; Linear regression slope= 0.1550 , $R^2=0.1692$

High Asexual Parasitemia at start of treatment is not associated with higher gametocyte burden or positivity at 1 or 2 weeks

A higher starting asexual parasitemia, as measured by *Pfsbp1* transcripts, was inversely associated with being gametocyte positive at 1 or 2 weeks (Figure 20A). However, gametocyte positivity at 1 or 2 weeks was associated with a higher area under the curve (AUC) for both *Pfs16* and *Pfs25* (Figure 20B, 20C).

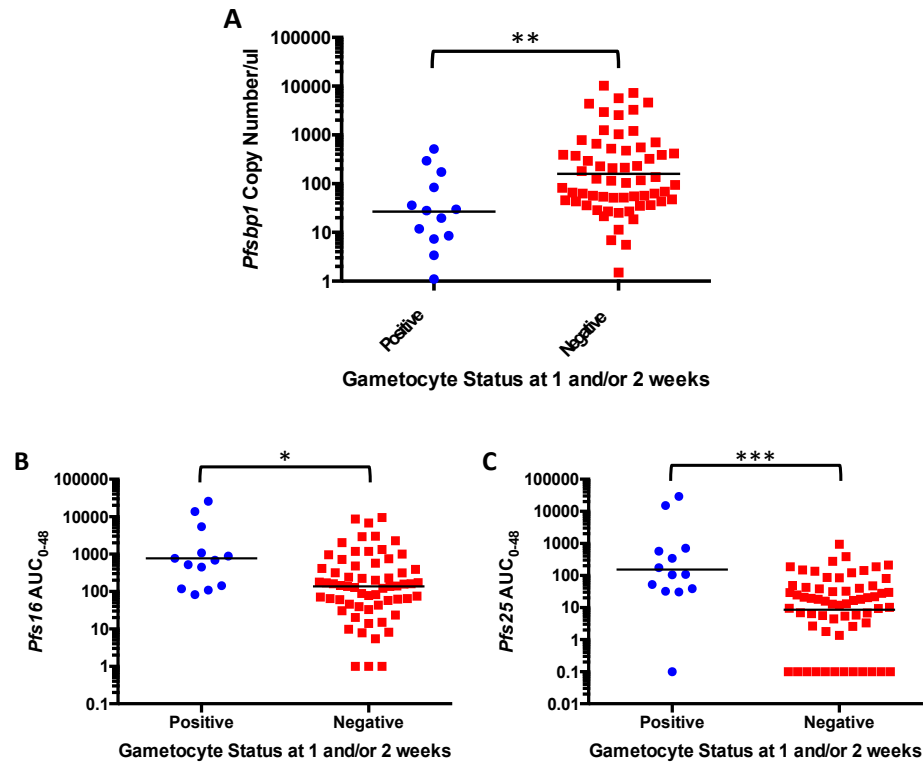


Figure 20. Impact of starting copy number and gametocyte AUC on gametocyte positivity after treatment

A) Starting asexual parasitemia sorted by gametocyte positivity at 1- or 2-weeks post-treatment; $P=0.0029$, Mann-Whitney test. AUC calculated from 0h to 48h for *Pfs16* (B) and *Pfs25* (C), sorted by gametocyte positivity at 1- or 2-weeks post-treatment. Negative samples were assigned an AUC value of 10% of the lowest positive value, which was 1 for *Pfs16* and 0.1 for *Pfs25*. Bars show geometric mean. Copy number/ μ l was significantly higher in gametocyte positive individuals ($P=0.0108$ for *Pfs16* and $P=0.0003$ for *Pfs25*, Mann-Whitney test). * indicates $P<0.05$, ** indicates $P<0.01$, and *** indicates $P<0.001$.

13 samples were negative for *Pfs25* during the entire time of this study (0h to 2w); of these, 3 were also negative for *Pfs16* at all time points. These geometric mean *Pfsbp1* copy number of those *Pfs25* negative samples was 49.77 copies/ μ l compared to 139.8 copies/ μ l for samples that were *Pfs25* positive at any point during follow up; this difference was statistically significant ($P=0.0437$, Mann-Whitney test). Thus, although

high *Pfsbp1* numbers were not predictive of long-term gametocyte positivity, there was an association between low parasitemia infections and an absence of mature gametocytes.

Gametocytemia at 1- or 2-weeks post-treatment is associated with a larger drop in hemoglobin levels

Previously, an association between low hemoglobin and the presence of gametocytes has been reported (23), (209). In this population, a similar trend was seen. The decrease in hemoglobin between day 0 and day 7 was significantly greater in individuals who were gametocyte positive by either marker at day 7 ($P=0.0084$, Mann-Whitney test) (Figure 21A). This trend was not seen when comparing individuals who were positive or negative for *Pfsbp1* ($P=0.8312$, Mann-Whitney test) (Figure 21B). Several other factors, including age, gender, and insecticide-treated bed net (ITN) usage were evaluated for an association between starting copy number, AUC_{0-48} , or gametocyte positivity at 1- or 2-weeks post-treatment. There was no association between age, gender, or ITN usage and any of our outcomes.

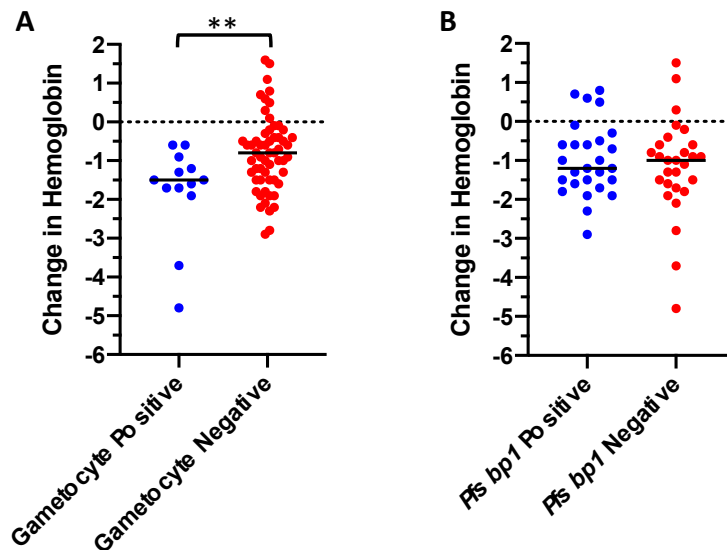


Figure 21. Hemoglobin and transcript positivity at 1 or 2 weeks.

A) Change in hemoglobin levels in g/dl from 0 hours to 1 week, by gametocyte positivity at 1 or 2 weeks. B) Change in hemoglobin levels in g/dl from 0 hours to 1 week, by *Pfsbp1* positivity at 1 or 2 weeks. Bars show median. * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$.

DISCUSSION

In this study, the prevalence of samples that were *P. falciparum*-positive by RT-qPCR dropped after treatment, but at 2 weeks, over half of the samples were still quantifiably positive for *Pfsbp1*, *Pfs16*, or *Pfs25*. Low copy numbers (below the limit of quantification) of *Pfsbp1* were detected in 75% of samples at 2 weeks, and the positivity of these samples was verified by the presence of *Plasmodium* 18S ribosomal RNA (data not shown). These results are similar to recent findings of submicroscopic parasitemia after treatment with ACTs, although the prevalence of an asexual marker at two weeks post-treatment was higher in this cohort (16), (48). This could be due the use of different

assays for detection of transcripts or different susceptibilities of the circulating parasite strains to treatment with ACTs.

Pfsbp1 copy numbers declined on average 18 hours after the initiation of treatment with AL, but gametocyte transcript copy numbers showed variable patterns. The average copy number of gametocyte-specific markers was not significantly reduced after AL treatment in individuals with detectable gametocyte transcripts, but the prevalence of individuals with detectable transcripts was greatly reduced. Pfsbp1 copy number changed more quickly than either gametocyte transcript levels, although the prevalence of gametocyte positivity dropped more quickly than the prevalence of Pfsbp1 positivity.

Additionally, several factors were identified that are associated with an increased risk of gametocyte carriage, as measured by these two markers, at 1- or 2-weeks post-treatment. Gametocyte carriage during the first 48 hours was found to be associated with long term gametocyte positivity. Pfsbp1 AUC during the first 48 hours was not associated with gametocyte positivity at 1 or 2 weeks, and the average starting Pfsbp1 copy numbers were lower in individuals who were gametocyte positive at 1 or 2 weeks. Children who were gametocyte positive at later time points also experienced a larger drop in hemoglobin levels between the initiation of treatment. These data also confirm previous reports that gametocyte carriage is not dependent on asexual parasite density (176).

One of the primary goals of this study was to verify the clearance of gametocytes by ACTs using a sensitive molecular assay. Several studies have previously reported complete clearance of gametocytes within two weeks of treatment with AL or other

ACTs, but many of these studies used microscopy as an endpoint (226), (130). While most individuals in this study did show clearance of gametocyte-specific transcripts after treatment, complete clearance by all individuals was not shown by either gametocyte marker (Pfs16 or Pfs25). Additionally, some individuals experienced a transient increase in gametocyte transcript copy numbers after treatment, which has been previously seen by microscopy (109). In a recent community sampling study of asymptomatic children under the age of 5, Pfs25 positivity was estimated to be about 22% and positivity to Pfs16 and/or Pfs25 was estimated to be 51% (71). The prevalence of Pfs16 and Pfs25 transcripts at the initiation of treatment was higher in this study, which was expected because the children in this study were experiencing a bout of clinical malaria at the time of sampling.

AL has previously been reported to be effective against developing gametocytes, but there was a sustained presence of Pfs16 in this cohort, even in the absence of concurrent Pfs25 expression, suggesting that the Pfs16 transcripts being detected are from early gametocytes. It is unclear if this is because the drug is not effectively clearing early gametocytes or if it is due to the sustained presence of asexual parasites. However, the decrease of Pfsbp1 copy numbers while Pfs16 copy numbers remain nearly constant raises the possibility that, in at least some individuals, early gametocyte development is still occurring.

Further planned analyses of the data presented in this study will assess for additional factors associated with gametocyte carriage and use computational modeling to discern patterns of gametocyte clearance after treatment in order to better understand the efficacy of AL as a gametocytocidal drug. Additional samples were collected as part of

this study that cover the time from 3 weeks to 5 weeks after the initiation of treatment, and the inclusion of these samples will allow for a better understanding of the long-term effects of AL treatment. Future studies are planned to include additional treatment arms, which will facilitate comparisons of the effects of different ACTs on both asexual parasites and gametocytes.

This study highlights the potential for persistent submicroscopic parasitemia and gametocytemia after AL treatment, but the epidemiological impact of these very lowly expressed transcripts is unknown. Because recent studies have shown a trend of increasing submicroscopic carriage (16), (48), additional studies are urgently needed to verify this finding and to determine if the sustained prevalence of transcripts during the follow-up period is due to persistent parasitemia, reinfection, or recrudescence. These transcripts may also represent low levels of contamination due to sample preparation and storage conditions or could be lingering nucleic acid from dying or phagocytosed parasites.

Overall, these results reinforce the critical importance of molecular tools for both the detection of submicroscopic infections and the early detection of potential drug resistance or treatment failure. The use of these sensitive tools allows for a better understanding of parasite dynamics after treatment and provides additional information on drug efficacy, which collectively can help improve the reduction of clinical malaria worldwide.

CHAPTER 5: Summary and General Conclusions

DISSERTATION SUMMARY

The research objectives for the dissertation were to characterize gametocyte carriage in two different African populations: 1) chronically infected adults from Kisumu, Kenya who may or may not be co-infected with HIV and 2) clinically ill children from the Nchelenge region of Zambia who have been treated with artemether-lumefantrine (AL). To characterize gametocyte carriage, a novel panel of qPCR assays was developed which targets gametocyte-specific mRNA transcripts that are only expressed during particular stages of gametocyte development. This gene expression was verified using synchronized cultured gametocytes that were grown in the lab. This work has shown the utility of molecular methods to quantitate gametocyte carriage, especially given the challenges in differentiating very early gametocytes from ring stages and the inability to observe a majority of developing gametocyte without examining bone marrow aspirates. Using these assays, we found both an increase in gametocyte prevalence and gametocyte copy numbers in HIV-positive individuals. Additionally, we identified several factors that were associated with sustained gametocyte transcript prevalence in children treated with AL, including a large drop in hemoglobin levels and the presence of gametocyte transcripts at the initiation of treatment. While standard membrane feeding assays are needed to verify that this increase in transcripts leads to higher levels of mosquito infections, this data suggests the need to prioritize certain individuals for treatment with gametocidal drugs in order to reduce transmission, especially in resource-limited environments.

THE UTILITY AND LIMITATIONS OF QPCR FOR EVALUATING PARASITEMIA AND GAMETOCYTE

The data presented in this dissertation shows the suitability of stage-specific assays for highly expressed markers such as Pfsbp1, Pfs16, and Pfs25. Importantly, this panel was able to detect transcripts in populations with submicroscopic parasitemias and infections that were undetectable by rapid diagnostic tests. This panel can also be used to monitor both the short- and long-term impacts of interventions, such as treatment with antimalarials.

Although all of the assays presented in Chapter 2 were able to amplify transcripts in cultured parasites, not all assays were suitable for use with field samples. Because developing gametocytes sequester, the amount of transcript specific to intermediate gametocyte stages that is detected in peripheral blood is a fraction of what would be detected if bone marrow aspirates were assayed (4). Many of the stage-specific transcripts used in this project are expressed at a fairly low level, which makes the detection of these transcripts even more challenging, especially in samples with low parasitemias. Additionally, almost all of the markers used in these assays are predicted to span multiple development stages. Thus, these assays were unable to definitively determine the stage of gametocytes being detected.

Despite that challenge, this panel can be used to make broad conclusions about the stage of gametocyte development. Because Pfs16 is most highly expressed in very early gametocytes and Pfs25 is only expressed in stage V gametocytes, gametocyte populations can be roughly divided into early gametocyte or late gametocyte groupings by the ratio of Pfs16 to Pfs25. This can be clearly seen in cultured parasites, which have

a very high ratio of Pfs16 to Pfs25 early in development but have a much lower ratio once Pfs25 expression can be detected (Figure 22A). This can be more difficult to understand in field samples because, unlike the synchronized parasites used to confirm expression in vitro, the samples obtained from human volunteers could have a mix of different stage gametocytes that obscure the interpretation of gametocyte staging. Still, some conclusions might be drawn from the changes in Pfs16:Pfs25 over time, as shown in the samples from the Zambia project. These samples have higher Pfs16 copy numbers compared to Pfs25 copy numbers at early time points, as shown by more points in the lower right quadrant of the graph displaying the first 18 hours after treatment initiation (Figure 22B, 22C).

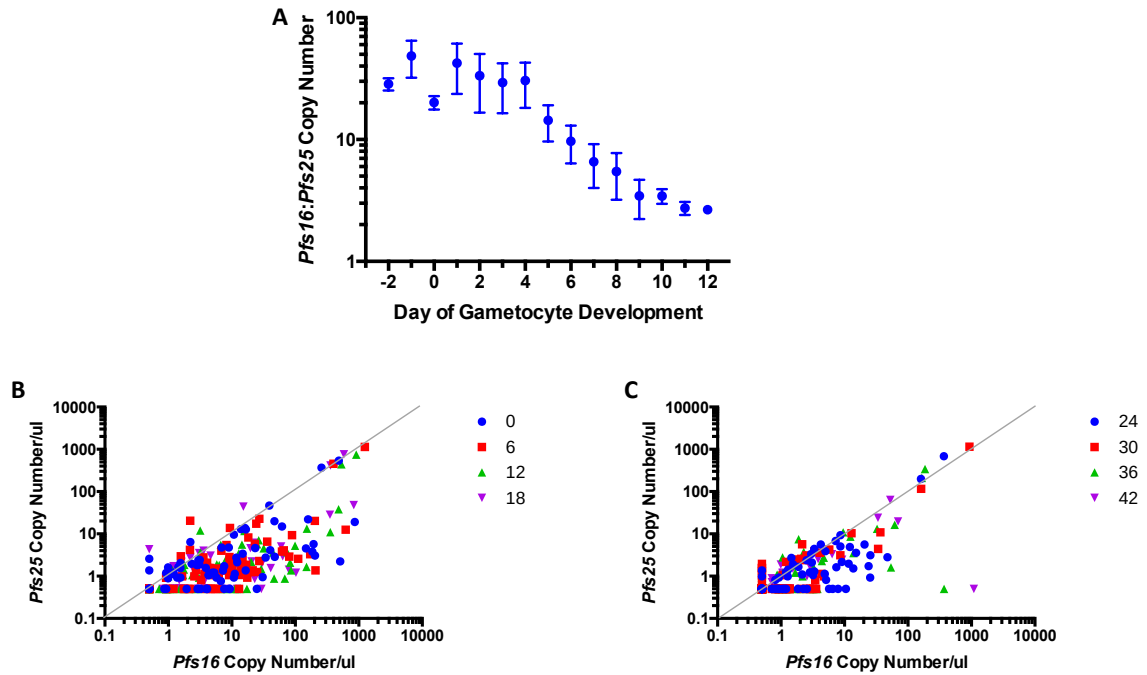


Figure 22. Ratio of *Pfs16* to *Pfs25*.

A) Ratio of *Pfs16* to *Pfs25* copy number/ul in 3D7 cultured parasites. Media was doubled on day -1 and heparin was added to culture media from day 1 to day 12. B and C) *Pfs16* copy number plotted against *Pfs25* copy number for all samples that were positive for both markers from the Zambia study at 0, 6, 12, and 18 hour time points (B), or 24, 30, 36, and 42 hour time points (C). Grey line indicates a slope of 1.

As more is learned about gametocyte biology, additional markers have been and continue to be identified. Since the development of this panel, several promising gametocyte markers have been reporting, including Pfg17, PfGEXP5, PfGK, and Pf13, (72), (74), (184). Ideally, markers with more restricted temporal expression will be discovered and adapted for use in the panel to allow a more conclusive determination of stage specificity. In the absence of such a marker, compartmental modeling may be a suitable tool for estimating gametocyte kinetics (25).

The most epidemiologically important limitation of this panel is the lack of a direct measurement of transmission to mosquitos. Gametocyte number can be a misleading indicator of infectivity, as submicroscopic gametocytemias can be very infective (187), (159), but in some circumstances, gametocytemia has been shown to be a good measure of transmission (149), (107), (167). The amount of correlation may vary based on prior interventions, as drug treatment in particular has been shown to create large numbers of gametocytes that are incapable of infecting mosquitos (12), (161). The use of standard membrane feeding assays in combination with gene expression data will likely be the most accurate measurement of transmission potential in a given population.

POTENTIAL MECHANISMS OF INCREASED PARASITEMIA IN HIV/MALARIA CO-INFECTION

To date, this is the first report of increased gametocyte carriage in asymptomatic adults with HIV co-infection. Although somewhat limited by the cross-sectional nature of the study design and sparse demographic information collected on study participants, this important finding shows both increased prevalence and increased levels of gametocyte transcripts in HIV-positive individuals when compared to HIV-negative individuals.

Since these individuals are newly diagnosed, but not necessarily newly infected, it is likely that the HIV positive group was comprised of individual in a range of disease stages. HIV status was determined by rapid diagnostic test, but viral load was not available. An attempt was made to quantify HIV-specific transcripts from the dried blood spots, but the volume of starting material was inadequate for testing (data not shown). Thus, it seems likely that the effects that are being seen may not be directly

related to viral load and instead could be due to other factors such as the general immune dysfunction seen throughout the course of HIV infection. Although the complexities of the immune responses to both HIV and malaria make it challenging to identify specific mechanisms involved in increase gametocyte carriage, there are several possible explanations.

The concept of an immunological balancing act is well established in HIV infection. In the presence of co-infection with HIV and several other pathogens, such as *Mycobacterium tuberculosis*, treatment with antiretroviral drugs (ARTs) results in improvement in immune function and concurrent decline in the patient's clinical status, a presentation referred to as IRIS, or immune reconstitution inflammatory syndrome (reviewed in (121)). In IRIS, the secondary pathogen elicits a strong immune response that facilitates a decrease viral load, but this overly robust immune response also causes more severe illness from the second pathogen, similar to an autoimmune disease. Interestingly, despite the ability of the malaria parasite to strongly activate the innate immune system, the treatment of HIV has not been documents to result in an IRIS-like worsening of malaria; however, the existence of IRIS highlights the potential for unusual immune responses in the context of HIV-infected individuals who are co-infected with an additional pathogen.

In malaria, partial immune response is considered permissive for the large number of asymptomatic infections in adults, but sterile immunity to any species of human malaria has never been observed during natural infection. Thus, it is hypothesized that partial immunity represents a balancing act between host immune response and parasite burden. In HIV infected individuals, impaired T cell function may be preventing

appropriate activation of antibodies, as T cells are known to be important for antibody activation in the context of malaria (221). In co-infected individuals, it is possible that perturbations in the immune system due to HIV may impact the ability of the immune system to maintain adequate control of parasitemia so as to prevent clinical illness, causing the increase in clinical malaria episodes that is a well established as a consequence of HIV and malaria co-infection (230). I propose that an increase in asexual parasitemia may also result in an increase in gametocyte-committed ring stages, leading to higher numbers of gametocytes.

Gametocyte-specific antibodies have been well documents to develop slowly over the course of multiple infections (87), (84), (54). HIV infection is associated with impairment of some *P. falciparum*-specific antibodies (94). In this study population, the significant increase in Pfs25 copy numbers in HIV positive individuals seen in Figure 12B may be explained be a decrease in antibody responses due to HIV infection, as these late-stage gametocytes are most likely to come into contact with antibodies due to their persistence in circulating blood. Future studies looking at the levels of gametocyte-specific antibodies in HIV infected individuals could shed additional light on the plausibility of this mechanism.

A recent study that has identified several pro-angiogenic cytokines as contributing factors for the release of mature gametocytes from the bone marrow may have some application to the understanding of increase gametocytemia in HIV positive individuals (140). HIV is associated with dysregulation of a variety of cytokine and chemokines, include the cytokines that have recently been implicated as major players in gametocyte sequestration in the bone marrow (115), (192), (15), (140). Thus, HIV may also be

impacting the ability of gametocytes to sequester in the bone marrow. This could explain the slight increase in the level of Pfs16 transcripts shown in Figure 12A.

A longitudinal study is currently underway to better understand how HIV affects asymptomatic malaria and gametocyte carriage. This study will allow a better understanding of the long-term effects of HIV and antiretroviral treatment, while also providing a more comprehensive characterization of how parasitemia and gametocytemia may change in chronically malaria-infected but HIV-negative individuals over time. Additionally, this study will include more comprehensive demographic and health care data, which will allow a broader range of factors to be evaluated for their effect on parasitemia. Critically, this study includes standard membrane feeding assays (SMFA) to determine how closely transcript abundance aligns with gametocyte infectivity to mosquitoes. Analysis of this longitudinal data may provide additional information about the mechanism of increased gametocytemia in HIV-positive individuals.

IMPLICATIONS FOR THE MANAGEMENT OF MALARIA IN THE ERA OF ERADICATION

For the past decade, the feasibility and timeline for the global eradication of malaria has been discussed intensively, and some estimate that malaria eradication will be achievable by 2050 (76). In order to effectively reduce the global burden of malaria, funding is needed for both the implementation and maintenance of large-scale interventions. Given the finite amount of financial support for global malaria initiatives, it is important to identify interventions that are able to reduce malaria while also being cost-effective and sustainable. To that end, evaluating the effect of interventions, such as

treatment with antimalarials, along with the identification of groups at a higher risk of transmitting malaria will allow for better public health decision making.

Despite the differences between the two study populations presented in this work, similarities were seen in the prevalence of asexual parasitemia in asexual adults and in children who were two weeks post-treatment. The sustained presence of asexual transcripts after treatment with AL highlights the concern of the emergence of increasing resistance to ACTs. While *kelch13* alleles associated with increased parasite clearance time are becoming more widespread in Southeast Asia, early reports of similar alleles have started to appear in Africa (178), (67). The efficacy of these drugs and the presence of drug-resistance alleles will both need to be monitored carefully. In the future, triple artemisinin combination therapies may be needed to prevent drug resistance (191), (61).

When comparing gametocyte carriage between the two study populations, the prevalence of gametocyte-specific transcripts was higher in the asymptomatic adults than in the children who were two weeks post-treatment, but the copy numbers of gametocyte transcripts were similar between both groups. In general, *Pfs25* levels were lower in the children with clinical malaria, both before and after treatment, when compared with the asymptomatic adults. Since these children were quite young and may not have a well-developed partial immunity to malaria, it is possible that they sought treatment so early in their infection that fewer stage V gametocytes had time to develop. This hypothesis is somewhat supported by the observation that *Pfs16* copy numbers were more similar between the two groups than *Pfs25* copy numbers, which could indicate that young gametocytes were developing at the time of treatment.

Overall, these data confirm that in areas of holoendemic malaria transmission, gametocyte carriage can be seen in asymptotically infected individuals as well as those experiencing episodes of clinical malaria (20), (65), (208). Additionally, incomplete gametocyte clearance is seen after treatment with AL, suggesting that an additional gametocidal drug should be included with ACTs, especially since the emergence of drug-resistant parasites can often be seen first in gametocytes that harbor these mutations (21). HIV-positive individuals showed by high prevalence of gametocyte transcripts and high copy numbers of transcripts in positive individuals, and these individuals may constitute a group at higher risk of transmitting malaria.

As previously mentioned, the level of gametocyte-specific transcripts may not correlate to increased transmission to mosquitos, and additional work will need to be conducted to determine how this gene expression data correlates to infectivity to the mosquito host. Because immunity to transmission stages develops very slowly after repeated exposure, the transmission intensity and age of the individual may impact the gametocyte carriage following drug treatment, especially when standard membrane feeding assays are used as the measure of transmission potential.

Although the presence of residual submicroscopic parasitemia after treatment with AL is of some concern, these low parasitemia infections may not be high enough to be of epidemiological significance. Interesting, mathematical modeling has already shown that treatment with ACTs does not reduce transmission in areas of high malaria transmission because the large number of asymptomatic individuals who do not receive antimalarial treatment outweighs the benefit of treatment of sick individuals with (101). Additionally, asymptomatic infections may not play a major role in preventing the

elimination or eradication of malaria. Asymptomatic infections are largely a problem in high-transmission settings where frequent exposure maintains the level of immunity that is permissive for asymptomatic infections. As the overall prevalence of malaria declines, the frequency of infective bites will also decline, leading to a reduction in the number of infections that remain asymptomatic, and a slight increase in clinical episodes (126). Thus, rather than focusing on asymptomatic individuals as potential transmitters of malaria to mosquitos, the combination of proven reduction strategies, such as vector habitat removal and insecticide use, with effective antimalarials that reduce the infectivity of gametocytes to mosquitos, may continue to be useful for attempting malaria eradication.

CONCLUDING REMARKS

In this dissertation, we have identified several circumstances in which gametocyte carriage may be elevated. While standard membrane feeding assays are needed to verify that this increase in transcripts leads to higher levels of mosquito infections, this data suggests the need to prioritize certain individuals for treatment with gametocidal drugs in order to reduce transmission, especially in resource-limited environments.

Malaria is a problem that has confounded mankind for thousands of years, and the human cost of this disease is truly incomprehensible. While malaria eradication is likely several decades away, the recent advancements in reducing the burden of malaria are heartening. The progress made in the past decade alone has saved hundreds of thousands of lives, and each new piece of information about the disease, the parasite, or the vector brings the malaria research community one step closer to solving this problem. Although

the work presented here is just a small part of the bigger malaria story, these findings may be of importance in shaping the guidelines for managing the care and treatment of individuals with malaria in order to reduce the future spread of the disease. For the individuals who have suffered from malaria and the families that have been diminished by it, it is my sincere hope that this research contributes to the achievement of a world that is free of malaria.

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