UNIVERSITAT DE BARCELONA

Targeting Malaria Transmission: A Transdisciplinary Approach

Harvie P. Portugaliza

"I have been teaching the population about malaria...I met along the way two people going to the healers with a child who was convulsed with a swollen belly."

Doctoral Thesis

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Dit proefschrift is tot stand gekomen in het kader van het ‘International Doctorate in Transdisciplinary Global Health Solutions’ - Erasmus Mundus Joint Doctorate Trans Global Health Programme, met als doel het behalen van een gezamenlijk doctoraat. Het proefschrift is voorbereid in de Institut de Salut Global de Barcelona (ISGlobal) van de Universitat de Barcelona en in het Faculteit der Geneeskunde van de Universiteit van Amsterdam.

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Abbreviations

ACT	Artemisinin-based Combination Therapy
AL	Artemether-Lumefantrine
AMA-1	Apical Membrane Antigen 1
AMETRAMO	Associação de Médicos Tradicionais de Moçambique
AP2/ERF	Apetala2/Ethylene Response Factor
APE	Agentes Polivalentes Elementares
apiAP2	Apicomplexan Apetala 2
ART	Artemisinin
ASAQ	Artesunate-Amodiaquine
ASMQ	Artesunate-Mefloquine
ASSP	Artesunate-Sulfadoxine-Pyrimethamine
BSA/PBS	Bovine Serum Albumin/Phosphate-Buffered Saline
cAMP	Cyclic Adenosine Monophosphate
Cas9	CRISPR Associated Protein 9
CD36	Cluster of Differentiation 36
cDNA	Complementary Deoxyribonucleic Acid
CDP	Cytidine Diphosphate
ChIP-seq	Chromatin Immunoprecipitation Sequencing
CQ	Chloroquine
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSA	Chondroitin Sulfate A
DALY	Disability-adjusted Life Year
DAPI	4′,6-diamidino-2-phenylindole
DHA-PPQ	Dihydroartemisinin-Piperaquine
EPCR	Endothelial Protein C Receptor
ER	Endoplasmic Reticulum
ETRAMP	Early Transcribed Membrane Protein
EV	Extracellular Vesicle
FACS	Fluorescence-activated Cell Sorting
FGD	Focus Group Discussion
FSC/SSC	Forward Scatter/Side Scatter Plot
G6PD	Glucose-6-phosphate Dehydrogenase
gDNA	Genomic Deoxyribonucleic Acid
GDV1	Gametocyte Development 1
GlucNAC	N-acetyl-d-glucosamine
H3K9Me3	Histone 3 Lysine 9 Trimethylation
Hb	Hemoglobin
hDHFR	Human Dihydrofolate Reductase
HS	Heat Shock
IC50	Inhibitory Concentration 50
ICAM1	Intercellular Adhesion Molecule 1
IFA	Immunofluorescence Assay
IPTi	Intermittent Preventive Treatment In Infants
IPTp	Intermittent Preventive Treatment In Pregnancy
iRBC	Infected Red Blood Cell
IRS	Indoor Residual Spraying
ISWI	Imitation Switch Gene
ITN	Insecticide-treated Nets
KAHRP	Knob-associated Histidine-rich Protein
KO	Knockout
LISP1	Liver-specific Protein 1
LLINS	Long-Lasting Insecticidal Nets
LMICs	Low- and Middle-income Countries

LOD	Limit of Detection
LSD2	Lysine-specific Histone Demethylase 2
LysoPC	Lysophosphatidylcholine
MAPK	Mitogen-activated Protein Kinase
MDA	Mass Drug Administration
MDV1	Male Development Gene-1
mRNA	Messenger Ribonucleic Acid
MSP	Merozoite Surface Protein
MSRP	Merozoite Surface 7-Related Proteins
NCC	Next Cycle Conversion
PA	Artesunate-Pyronaridine
PbAP2-FG	*Plasmodium berghei* Apetala2-Female Gametocyte
PEG-3	Protein of Early Gametocyte 3
PfAP2-G	*Plasmodium falciparum* Apetala2-Gametocyte
PfAP2-I	*Plasmodium falciparum* Apetala2-Invasion
PfAP2-O	*Plasmodium falciparum* Apetala2-Oocyst
PfCRT	*Plasmodium falciparum* Chloroquine Resistance Transporter
PfEBA	*Plasmodium falciparum* Erythrocyte-binding Antigen
PfEK	*Plasmodium falciparum* Ethanolamine Kinase
PfEMP1	*Plasmodium falciparum* Erythrocyte Membrane Protein 1
PfGECO	*Plasmodium falciparum* Gametocyte Erythrocyte Cytosolic Protein
PfGEXP	*Plasmodium falciparum* Gametocyte Exported Protein
PfGK	*Plasmodium falciparum* Glycerol Kinase
PfHDAC2	*Plasmodium falciparum* Histone Deacetylase 2
PfHP1	*Plasmodium falciparum* Heterochromatin Protein 1
PfMDR1	*Plasmodium falciparum* Multidrug Resistance 1
PfMGET	*Plasmodium falciparum* Male Gametocyte–enriched Transcript
PfPUF2	*Plasmodium falciparum* Pumilio and Fem-3 Binding Factor 2
PfRH	*Plasmodium falciparum* Reticulocyte Binding Protein-like Homolog
PMT	Phosphoethanolamine Methyltransferase
PQ	Primaquine
PVM	Parasitophorous Vacuole
QT-NASBA	Quantitative Nucleic Acid Sequence-Based Amplification Assay
RBC	Red Blood Cell
RDT	Rapid Diagnostic Test
RIFINs	Repetitive Interspersed Families of Polypeptides
RON	Rhoptry Neck Protein
RT-LAMP	Reverse Transcriptase Loop-mediated Isothermal Amplification Assay
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction Assay
SAMS	S-Adenosylmethionine Synthetase
SCC	Same Cycle Conversion
SRB	Sexual Ring Biomarker
SERRS	Serine-tRNA Ligase
SMC	Seasonal Malaria Chemoprevention
SNF2L	Sucrose Non-Fermentable 2l
SP	Sulfadoxine-Pyrimethamine
SPR	Slide Or Rapid Diagnostic Test Positive Rate
STEVOR	Subtelomeric Variable Open Reading Frame
UCE	Ubiquitin-conjugating Enzyme
VAR2CSA	Variant Surface Antigen 2-Chondroitin Sulfate A
WHO	World Health Association
WT	Wild-Type
yFCU	Yeast Enzyme Cytosine Deaminase And Uridyl Phosphoribosyl Transferase

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Chapter 1

General Introduction

1. Malaria as a global health concern

1.1. Brief history of malaria

According to ancient writings and artifacts, malaria existed long before the Common Era: the symptoms that killed Neolithic dwellers and ancient folks resembled those that we now associate with severe clinical malaria. As early as 850 BC, Greek philosophers chronicled the association between the common malaria symptoms characterized by periodic fever and splenomegaly and those individuals who lived near swamplands. For 2500 years, natural philosophers took literally what was obvious – the unpleasant smell emanating from swamps called the 'miasmas' – and attributed this smell to the disease termed 'mala aria', a medieval Italian phrase for 'bad air'. With a primitive understanding of medicine, ancient researchers most likely never suspected a protozoan parasite as a malaria-causing agent, although they probably considered the role of mosquitoes in transmitting the disease because anecdotal writings described insect control as a measure to avoid malaria (1,2).

In 1676, the discovery of microorganisms termed 'animalcules' by Antonie Philips van Leeuwenhoek marked the beginning of a revolution in human understanding of infection. This discovery incriminated an array of microbes to any type of sickness and kick-started the race to discover the causative agent of malaria. In the 1800s, Corrado Tommasi-Crudeli and Theodor Albrecht Edwin Klebs were the lead scientists that proposed the role of microbial inhalation from miasma and microbial ingestion from swamps as the causes of malaria. However, their works were proven erroneous after Charles Louis Alphonse Laveran meticulously demonstrated in the blood of malaria patients the unusual appearance of

pigmented crescent cells and flagellated round cells. These are now identified as the mature sexual forms of the malaria parasite. The later works of Ronald Ross and several Italian scientists proved the crucial role of mosquitoes in transmitting malaria parasites, thereby linking swamplands (a notorious mosquito breeding ground) as a risk factor for malaria epidemics (1,3).

In the 1900s, the malaria research community started to unravel the complex biology of the parasite. The liver stage of *Plasmodium* species was discovered in monkeys and humans, revealing one of the earliest phases in the life cycle of the mammalian malaria parasites (4). The invention of the first *in vitro* culture system for *Plasmodium falciparum* catalyzed the current progress on the biological understanding of the most dangerous human malaria species (5). The isolation of artemisinin from the plant *Artemisia annua* marked the birth of what is currently the most vital antimalarial drug (6). Finally, the omics era permitted the malaria research community to dive deeper into the invisible world of *Plasmodium* molecules. Among the numerous exciting discoveries were the mapping of the whole genome of malaria (7) and gene manipulation using the CRISPR-Cas9 system (8). In this Ph.D. work, we applied some of these technologies to understand one of the less explored research areas in malaria – the early stages of sexual development.

1.2. Global burden of malaria

The disease burden of malaria has significantly declined over the past two decades but remains a major threat to human life (9,10), especially to those living in low- and middle-income countries (LMICs) (**Figure 1**). In 2017, malaria was among the leading five communicable diseases with the highest disability-adjusted life years (DALYs), which is an indicator of disease burden based on the years lost due to death, illness, and disability (11). In 2018, there were 228 million (95% CI: 206 to 258 million) malaria cases recorded worldwide and 93% (213 million) of these cases were from the World Health Organization (WHO) African region, mainly in sub-Saharan Africa where *P. falciparum* continued to be

the most prevalent human malaria parasite. In terms of mortality, the overall malaria cases resulted in 405 thousand deaths with the majority (94%) of the mortality found in the African region. Children and pregnant women are most vulnerable to severe malaria whereby deaths among children below the age of 5 years accounted for 67% (272 thousand) of malaria-related mortality in 2018 (10).

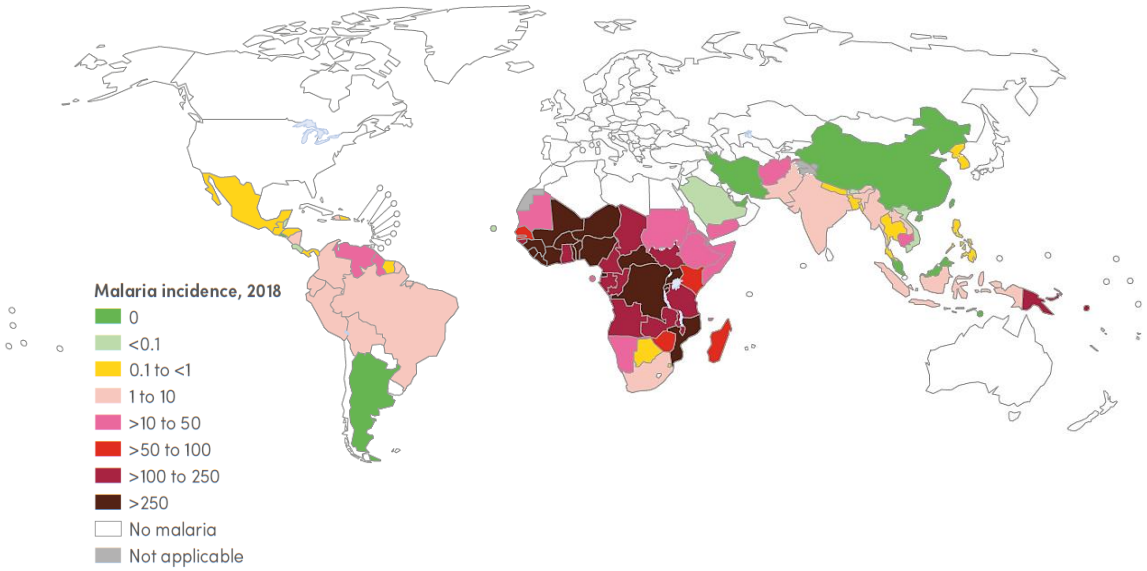

Figure 1. The global map of malaria incidence rate (cases per 1000 population at risk) in the year 2018. The global incidence rate of malaria in 2018 was around 57 cases per 1000 population at risk. As of 2018, there were 31 countries endemic to malaria. Figure was taken from the World Health Organization (WHO) World Malaria Report (2019) (10).

What makes malaria common across vulnerable groups and prevalent in LMICs can be broadly linked to two factors: ecological conditions and poverty. Malaria transmission is a dynamic event where several interconnected factors, including the natural environment and man-made alteration of the natural landscape, contribute to the success of the parasite's life cycle in endemic areas (12). A conducive ecological condition for malaria transmission requires a suitable temperature, rainfall, humidity, and vegetation, which are all provided by forest canopies (12,13). Moreover, population migration and the shift to modern

agricultural practices, including the construction of dams, irrigation systems, and deforestation, have contributed to the intensity of malaria disease burden as these activities enhance the risk of malaria reintroduction and spread through vector mosquitoes (13,14).

Although the relationship between malaria and poverty has long been identified, the connections remain complex (14). This makes malaria a 'wicked problem' that can only be solved through close collaborations between disciplines using a transdisciplinary approach. The interlocking complexity between malaria and poverty is exemplified by a scenario where poverty sustains the ideal environment for malaria transmission, which in turn impedes the economic growth of the affected community (15). The striking influence of malaria on sustained poverty can be explained by multiple networks that the malaria burden undermines. These networks include population growth and fertility, economic saving and investment, workforce productivity, school and work absenteeism, medical expenses, and early deaths (14). Hence, malaria and poverty have dual causality where poor households are trapped in a reinforcing cycle between the two (15).

1.3. Malaria treatment

In the absence of treatment, malaria is often fatal. It is therefore essential to ensure a very early diagnosis protocol to provide treatment within 24 to 48 h from the onset of symptoms. Under WHO guidelines, only malaria-diagnosed patients by microscopy or rapid diagnostic test (RDT) are allowed to receive antimalarial drug therapy, which must be taken as a full treatment course. To prevent parasite resistance, the WHO recommends administering at least two effective antimalarial drugs as a combination therapy, with dosages calculated based on the bodyweight (BW) of the patient. Currently, the frontline drugs for malaria are generally artemisinin-based combination therapies (ACTs), which consist of the fast-acting drug component artemisinin (ART or its derivatives) and one of the long-acting partner drugs (**Table 1**). ART demonstrates a rapid killing

effect against the replicating parasites but displays a short elimination half-life in human serum (see also Section 7.3). All partner drugs may not have a rapid efficacy against the parasites but their longer elimination half-lives complement the activity of ART (16).

Table 1. Artemisinin-based combination therapies (ACTs) for clinical use

Combination*	Abbreviation	Geographic use
Artemether + lumefantrine	AL	Mostly in African countries
Artesunate + amodiaquine	ASAQ	Mostly in western African countries
Dihydroartemisinin + piperaquine	DHA-PPQ	Southeast Asian countries; also used for MDA and IPTp in Africa
Artesunate + mefloquine	ASMQ	Preceded DHA-PPQ in Southeast Asia
Artesunate + sulfadoxine-pyrimethamine	ASSP	India, Middle East countries, and eastern African countries
Artesunate + pyronaridine	PA	Registered in Africa and Asia

*Therapeutically indicated combinations for the treatment of uncomplicated *P. falciparum* or *P. vivax* malaria.
Note: Data adapted from Blasco et al. (2017)(17).

In uncomplicated malaria cases, ACT is given to children and adults for three days. For first-trimester pregnant women, ACT is not recommended so that their treatment course includes a combination of 7-day quinine and clindamycin. For severe malaria cases, patients are given intravenous or intramuscular artesunate injection within 24 h and a complete treatment course of ACT once they can tolerate the oral route. In the absence of parenteral artesunate, the next drug in line would be artemether (18). To reduce transmission in low endemicity settings, concomitant treatment with single-dose primaquine (PQ) is employed along with ACT. PQ is a potent gametocytocidal drug with a mode of action linked to mitochondrial metabolic activity disruption. Although WHO recommends a low treatment dose (0.25 mg/kg BW) without prior screening for glucose-6-phosphate dehydrogenase (G6PD) deficiency, it is still needed to test the patient for G6PD deficiency when giving relatively high PQ doses to avoid any life-threatening adverse reactions, specifically severe hemolysis (16,18).

Unfortunately, ART resistance has emerged in Southeast Asia, characterized by delayed parasite clearance (>5 h) or microscopic parasitemia on day 3 after the first treatment dose (19–21). Past research

has suggested that the emergence of ART resistance is related to indiscriminate monotherapy and poor drug quality, whereas its persistence is linked to parasite genetic diversity, transmission settings, and host immunity (17,22). More recently, treatment failures have been documented due to the simultaneous resistance to ART and its partner drugs (23–25). The non-adherence to the ACT regimen complicates more the situation as patients' compliance with a 3-day treatment course is affected by various factors, including the level of education and the supervision of healthcare providers (26).

1.4. Malaria prevention and control

In the absence of an effective vaccine, malaria control and prevention hold a vital role in stopping the spread of malaria. Chemoprevention and vector control play a role in this.

1.4.1. Chemoprevention

The intermittent preventive treatment in pregnancy (IPTp) using the antimalarial drug sulfadoxine-pyrimethamine (SP) is recommended in African countries to combat maternal anemia and low neonatal birth weight associated with *P. falciparum* infection during pregnancy. Pregnant women in their second trimester are provided with at least 3 doses of SP, one month apart. However, compliance among pregnant women is affected by varied social, economic, and cultural complexities of antenatal care (27). Infants less than 1 year of age also receive SP as an intermittent preventive treatment in infancy (IPTi) in areas with moderate to high endemicity and where SP remains effective. However, the implementation of IPTi still depends on the decision of the country and oftentimes is not adopted as a national policy. Seasonal malaria chemoprevention (SMC) is also recommended in the sub-Sahel region of Africa (south of the Sahara Desert), where intense transmission occurs during the 3 to 4 months rainy season. SMC entails giving amodiaquine and SP monthly during each transmission period to children below the age of 6 years (18).

The major concern about SP as chemoprevention is its low efficacy (~30%) due to high levels of drug-resistant parasites (28), thereby prompting the recommendation to use ACT as a substitute for SP. This has been the case in Tanzania in 2006 after experiencing some cases of treatment and/or chemoprevention failure due to SP-resistant parasites (29). A multiple-site clinical trial in western Kenya demonstrated better efficacy of ACT (DHA-PPQ) when compared to SP in abrogating the incidence of clinical malaria in pregnant women by substantially reducing the risk of malaria and anemia during delivery (30). The main issue, however, is that ACT poses safety concerns during the early trimester of pregnancy, raising doubts about whether its benefits outweigh its risks (18). Nonetheless, a recent multinational clinical trial on different types of ACT showed that all ACTs did not cause any undesirable effects when given during the second and third trimester of pregnancy, and also no adverse reaction was reported among infants (31).

Meanwhile, for travelers and returning residents to malaria-endemic countries, chemoprophylaxis is recommended by providing either of the following antimalarial drugs: atovaquone-proguanil, chloroquine, doxycycline, mefloquine, primaquine, and tafenoquine. Three of these antimalarial drugs are considered the 'gold-standard'. These include (i) a weekly intake of mefloquine, (ii) a daily intake of atovaquone–proguanil, and (iii) a daily intake of doxycycline (32).

1.4.2. Vector control

Core interventions against mosquito vectors are high coverage of insecticide-treated nets (ITN) and indoor residual spraying (IRS). High coverage means a 100% ITN distribution in all affected households and at least 85% of yearly IRS application (33). Bhatt and colleagues (2015) highlighted the advantage of using ITN and IRS, revealing that these interventions can avert 68% and 10% of malaria cases in endemic settings, respectively (34).

ITNs are used as a protective barrier against mosquito bites during sleeping and have been reported to decrease malaria cases in children by

20%. Typically, the recommended bed nets have a lifespan of 3 years and are washable up to 20 times without losing the efficacy of the impregnated insecticide (32). In all malaria-endemic settings, only pyrethroid-treated long-lasting insecticidal nets (LLINS) are recommended by WHO as a core vector intervention. In areas with proven pyrethroid resistance, the next in line would be the pyrethroid-piperonyl butoxide bed nets. Unfortunately, as of 2014, resistant mosquitoes to at least one of the insecticides used in vector control have been reported in 60 countries, posing a threat to ongoing malaria control programs and to the prospects of malaria elimination (35). Meanwhile, IRS targets the mosquito's resting sites such as the surfaces of the house and domestic animal shelter, or where vectors might come into contact. Depending on the type of houses, different products can be used. The insecticide dichlorodiphenyl-trichloroethane is used in houses with mud walls while the insecticide pirimiphos-methyl is applied in houses with concrete walls (36). Unfortunately, both ITN and IRS do not protect individuals from other forms of mosquito biting episodes (also known as residual transmission), as in the case of forest malaria and diurnal biting (33,36). There are also issues with the misuse of bednets (e.g., used as fishing nets) and poor community acceptance of IRS (e.g., perceived bad smelling) (32,36).

1.5. Malaria elimination goal

Malaria eradication is an ambitious goal of completely eradicating malaria around the globe through a series of region-specific malaria elimination campaigns (e.g., targeting the Greater Mekong Subregion in Southeast Asia and the Magude District in Mozambique). The drive for local, national, or regional elimination is prompted by the impacts of malaria control efforts, which have drastically shrunk the burden of malaria worldwide (37). In general, the malaria elimination strategy emphasizes aggressive malaria control by scaling up intervention tools to make them accessible to the entire at-risk population (38).

1.5.1. Phases of the malaria elimination program

According to WHO, malaria elimination requires the absence of local transmission of all human malaria parasites for at least three consecutive years. To obtain a malaria-free status, four distinct programmatic phases are set by the Roll Back Malaria Initiative-Global Malaria Action Plan: these are the control phase, pre-elimination phase, elimination phase, and prevention of reintroduction phase (**Figure 2**).

In targeted areas undergoing a malaria control phase, substantial reductions in malaria incidence, prevalence, morbidity, and death that meet a locally acceptable level become the new trend as a result of deliberate efforts to intensify malaria control strategies. One of the bases to move from the control phase to the pre-elimination phase is when less than 5% of fever cases are attributable to malaria infection, as assessed by microscopy and/or RDT (39–41).

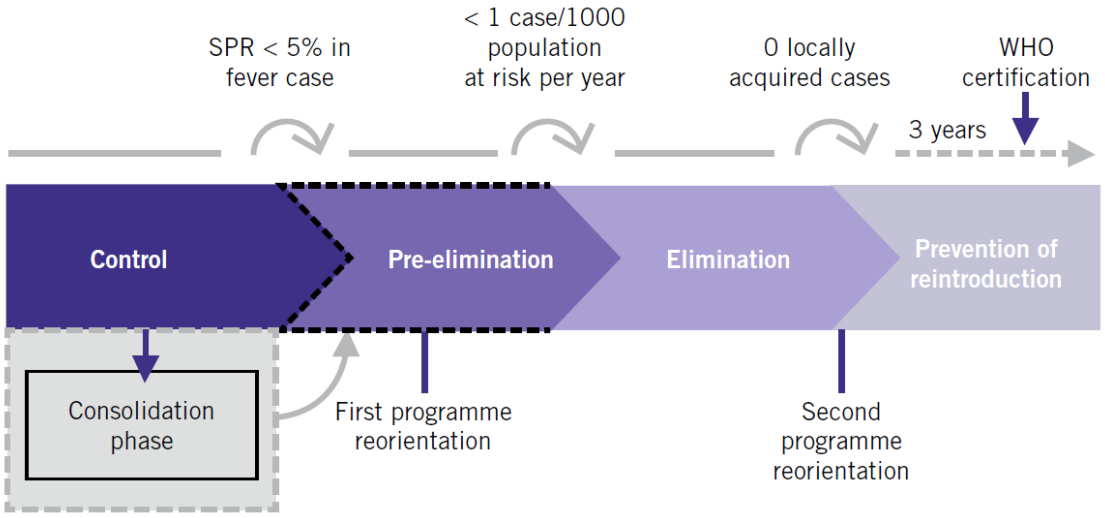

Figure 2. Phases of the malaria elimination program. The path to malaria elimination consists of four steps: the control, pre-elimination, elimination, and prevention-of-reintroduction phases. Implementation of each phase may vary depending on the targeted site, malaria caseload, and the extent to which the program can realistically manage malaria cases. SPR = slide or rapid diagnostic test positive rate. Figure was taken from WHO (2016) (41).

In the pre-elimination phase, malaria control programs are reoriented to transition from sustained malaria control to the elimination phase. Here,

high coverage in terms of diagnostic facilities and clinical services is expected along with reinforced reporting and surveillance. This is then followed by other programmatic modifications intended to stop malaria transmission across the country or in the targeted area (39–41).

When the population at risk per year reaches less than 1 malaria case per 1000 population, the country is qualified to begin the elimination phase. In the elimination phase, four programmatic approaches that require huge local expertise and resources are employed. The approach includes (i) effective management of all malaria cases, (ii) prevention of onward transmission from indigenous cases, (iii) prevention and detection of imported malaria cases, and (iv) management of malaria foci (39–41).

Lastly, the prevention-of-reintroduction phase takes place as an essential measure to sustain the absence of malaria cases. This is achieved by putting strict surveillance and quick response programs accompanied by vector control strategies and other interventions that target the high-risk population (39–41).

1.5.2. High-risk population in a malaria elimination setting

In elimination settings, as malaria transmission decreases, the infection dynamics shifts from previously vulnerable groups (infants and pregnant women) to all-age groups. As a result, the clustering of parasite reservoirs becomes apparent in defined geographical foci and in a new high-risk population. In Southeast Asia and South America, the hard-to-reach ethnic minorities and mobile tribes are among the high-risk populations that might challenge malaria elimination efforts. These groups rarely seek medical attention, and if they want to, their remote location hinders access to healthcare providers. For a similar reason, delivering programmatic healthcare services and disease monitoring linked to malaria elimination campaigns have proven challenging (42,43). Moreover, in many Southeast Asian countries, individuals engaging in forest works contribute to persistent malaria transmission as they are often exposed to malaria vectors (44,45). The majority of the forest goers are adult males with submicroscopic parasitemia, forming a cluster of asymptomatic

infections that when left untreated could contribute to seasonal malaria outbreaks (46). The role of asymptomatic individuals during malaria elimination is crucial as they cover at least 75% of malaria infections in the community (see Section 2.3.2) (47).

1.5.3. Main challenges in malaria elimination initiatives

While malaria elimination efforts were successful in some countries, a few countries that initially experienced substantial reductions in malaria cases are now struggling to maintain their gains. Previous reasons for malaria resurgence after intensive elimination efforts were attributed to a lack of sustained funding (e.g., India), political instability and insurgency (e.g., Azerbaijan and Tajikistan), weak malaria program vigilance (e.g., Mauritius), resistance to antimalarial drugs (e.g., countries in the Greater Mekong subregion), resistance to insecticides (e.g., countries in sub-Saharan Africa), malaria importation (e.g., countries in southern Africa), and weak health system with limited program capacity (e.g., Solomon Islands and Vanuatu) (35). Hence, in the absence of new effective interventions, malaria eradication is expected to be delayed as current tools are confronted with diverse challenges, in addition to unforeseen circumstances in targeted sites (48).

As new prospects for intervention, malaria vaccine and targeted mass drug administrations (MDAs) are likely to accelerate the impact of currently in-place strategies for malaria elimination. However, the most advanced malaria vaccine today (RTS,S/AS01) provides very limited protection in infants (26% efficacy) and young children (36% efficacy), needing additional developments to improve its efficacy (49). Meanwhile, the MDA strategy relies on a single type of antimalarial drug (i.e., ACT) that has been under threat for losing its efficacy due to parasite resistance (16). Moreover, community perception of MDA is not always positive and thus requires intensive community engagement to cover all the target population, which is a resource-draining and time-consuming process (50,51). A more recent malaria elimination project in southern Mozambique revealed that low coverage rate and/or treatment

abandonment were among the identified reasons why even multiple rounds of MDA along with extensive vector control failed to eliminate malaria in the Magude District (36). The outcome of that project suggests that the current tools are not sufficient to completely interrupt the transmission of malaria in a moderately endemic setting in Africa.

2. The causative agent of the deadliest form of malaria

2.1. The evolution of *P. falciparum*

The relationship between water and malaria is a never-ending affair: recent research suggests that the origin of *P. falciparum* can be traced back from ancient species inhabiting the sea (52). Analyses of the parasite's plastid or apicoplast, which is a non-photosynthetic relic of plant and algal chloroplasts, pointed to the potential ancestral origin of the current malaria parasites. A phylogenetic analysis of the apicoplast genome revealed that the closest ancestors of *Plasmodium* are the photosynthetic coral algae (*Chromera* and *Vitrella*), predatory marine flagellates (*Colpodella, Voromonas,* and *Alphamonas*), and an unknown lineage linked to the coral environment called the Apicomplexan Related Lineage-5 (53–55). Although the analysis of the apicoplast exposes the existence of free-living ancestors thriving in the coral reefs, the transition of an ancient species into an obligate intracellular parasite remains unresolved. Primitive insects probably of mosquito descents thriving both in the sea and land might have played an important part in the 'host jump' of *Plasmodium* (52).

Alterations in host preference appear to be a common phenomenon in the evolution of many *Plasmodium* species (56). Previous phylogenetic analysis showed that *P. falciparum* is more related to the bird parasite *P. gallinaceum* than the other four human malaria parasites, namely *P. vivax, P. ovale, P. malariae,* and *P. knowlesi* (57). Further genome studies however revealed an uncanny genetic similarity between *P. falciparum* and the parasite found in chimpanzees *P. reichenowi*, implying that the

former directly evolved from the latter (58,59). This chimpanzee origin theory was however debunked after finding that all current *P. falciparum* isolates formed a monophyletic clade within several malaria species from wild Western African gorillas (60). The *P. praefalciparum* species under the sub-genus *Laverania* is now considered to be the direct ancestor of *P. falciparum*, indicating that the epidemic of human malaria occurred from a single event of gorilla-to-human transmission (61). Recent developments suggest that all species from the sub-genus *Laverania* might only infect apes because of their unique genes involved in erythrocyte invasion (62,63).

2.2. The life cycle of *P. falciparum*

Like any other *Plasmodium* species, *P. falciparum* requires two separate hosts to complete its life cycle – a female *Anopheles* mosquito (definitive host) and a human being (intermediate host). The definitive host is where sexual reproduction takes place (56). When a malaria-infected mosquito bites a human host, it inoculates an average of 15 motile sporozoites into the human dermal capillaries during the course of a blood meal (**Figure 3**) (64). To reduce the odds of getting blocked by the host immune cells, the sporozoites avoid the lymphatic system by traveling via the bloodstream to reach the liver within 30-60 minutes (min) (65).

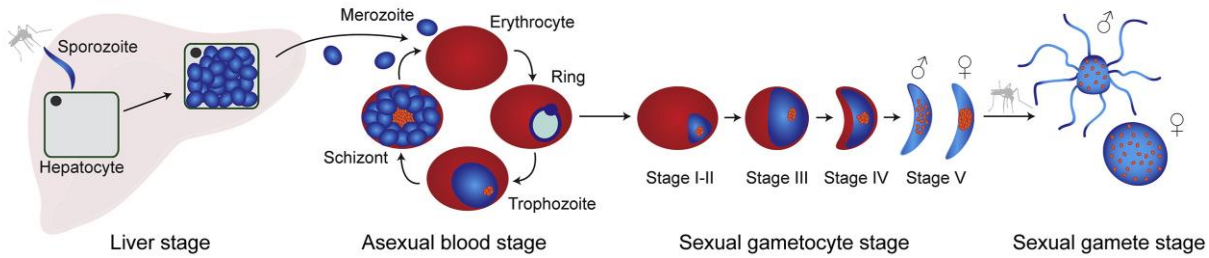

Figure 3. The life cycle of *P. falciparum*. Sporozoites are inoculated into a human host by the female Anopheline mosquito and travel through the bloodstream to reach the liver. The initial hepatic schizogony releases thousands of merozoites, which invade the red blood cells (RBCs) to undergo repeated cycles of erythrocytic schizogony. In every erythrocytic cycle, a small proportion of the parasites convert into the sexual form called gametocytes that develop in the bone marrow. Mature Stage V male and female gametocytes are released into the circulation and taken by the mosquito during a blood meal. Gametocytes

are activated inside the mosquito midgut to become sexual gametes. Fertilization takes place and a zygote is formed. The zygote develops into a motile ookinete and then oocyst. The oocyst undergoes sporogony to produce thousands of sporozoites, which travel into the salivary gland of the mosquito, waiting for a new host to infect. Figure illustrated by and modified from Vanaerschot et al. (2020) (66).

After the hepatic invasion, sporozoites form a parasitophorous vacuole and initiate the first round of replication called the exoerythrocytic or hepatic schizogony, which produces thousands of merozoites. Unlike *P. vivax* and *P. ovale*, the dormant liver stage hypnozoite has never been observed in *P. falciparum* infection (32). Once hepatic schizogony is completed, merozoites are released back into the circulation through a membrane-bound cluster called merosome, a structure designed to ensure a smooth migration and evasion from the host immune system (67). Next, merozoites invade the RBCs in rapid but multi-level events that comprise of pre-invasion, active invasion, and echinocytosis processes, which all take place in a short span of two minutes (**Figure 4**) (68).

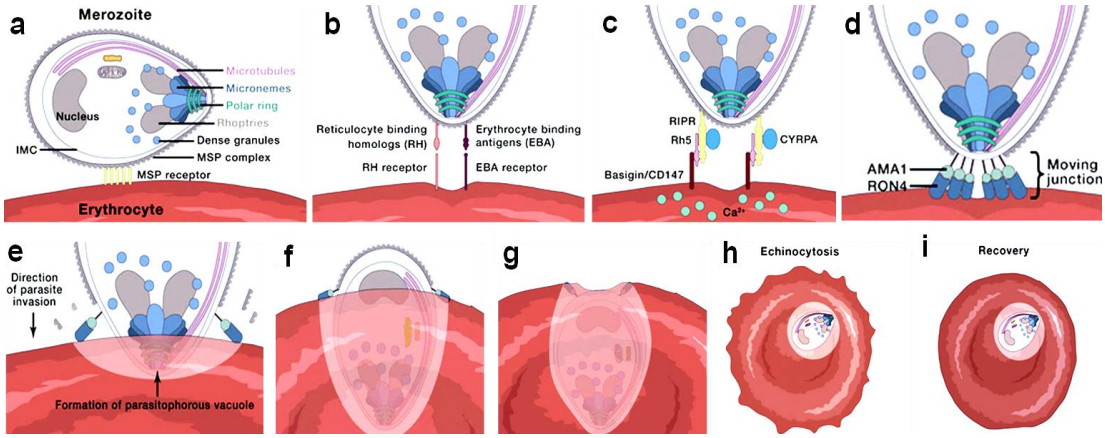

Figure 4. The process of merozoite invasion in *P. falciparum*. (a) Initial contact occurs between the merozoite surface proteins (MSP) and RBC, followed by a reorientation process that (b) directs the apical end of the parasite to the RBC where specific ligand-receptor interactions are mediated by the parasite proteins PfEBA and PfRH. (c) This is followed by the binding of the PfRh5 complex to the Basigin receptor found on the RBC surface. This interaction stimulates Ca^{2+} influx into the RBC that allows (d) deposition of the RON complex and binding of AMA-1, making the attachment irreversible. (e-g) The merozoite propels itself using its internal actomyosin complex while its rhoptry generates a lipid-rich parasitophorous vacuole membrane covering the merozoite. (h-i) The RBC membrane seals off and becomes echinocytic as it losses water during

merozoite invasion but later on will fully recover its normal morphology. Figure illustrated by and modified from Cowman et al. (2016) (68).

After merozoite invasion, a series of morphologically distinct stages develop inside the RBC, from ring stage within 0 to 22-hour post-invasion (hpi) to trophozoites (23 to 38 hpi) and schizonts (39 to 48 hpi). The ring stage is relatively non-feeding and dormant with little hemoglobin uptake (69) until it reaches the trophozoite stage where active feeding based on hemoglobin and nutrients from human serum takes place to support the process of multiple nuclear divisions called schizogony. Schizogony is a unique process of asynchronous nuclear division in the absence of the usual cytokinesis, creating a multinucleated syncytium called the schizont (70). Erythrocytic schizogony produces 8 to 32 daughter merozoites that egress and invade new RBCs, such that the whole repetitive process creates an indefinite number of rounds of the asexual cycle (**Figure 5**) (65).

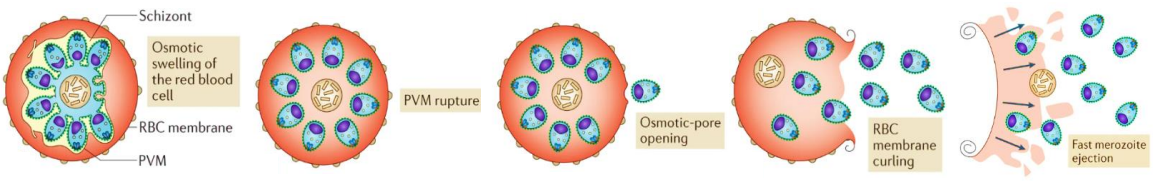

Figure 5. The process of merozoites egress in *P. falciparum*. The parasitophorous vacuole (PVM) ruptures after a buildup of pressure in mature schizonts. Merozoites become mobile inside an intact red blood cell (RBC), followed by the formation of an osmotic pore in the RBC membrane where one or two merozoites undergo a very rapid osmotic release. Next, the RBC membrane undergoes curling and buckling that allow the dispersion of the remaining merozoites into various directions with projectiles up to 10 µm away towards the adjacent RBCs. Figure illustrated by and modified from De Niz et al. (2017) (71).

Sexual development starts when a subpopulation of the parasites in every cycle commits to become gametocytes, which are a non-replicative sexual stage and the only form of the parasite that is infectious to the mosquito vector. Gametocytes develop in the bone marrow for an average of 10 days, which ranges from 4 to 12 days (72). Mature Stage V male and

female gametocytes that are released into the circulation will remain infectious for several days (3.4 to 6.4 days) (73) or even weeks (1.8 to 216.3 weeks) (74). Typically, the gametocyte sex ratio is female-biased, with 3 to 5 females per 1 male. This ratio is optimal for successful malaria transmission (75). The number of gametocytes in the blood capillaries needs to be at the threshold limit of 1000 per milliliter of blood to establish a patent mosquito infection (76). Although there is no robust correlation between gametocyte density and mosquito infection under laboratory conditions, the rate of mosquito infection is positively correlated to the number of gametocytes in the blood (73,77).

Once a mosquito takes a blood meal, it picks up the circulating mature Stage V male and female gametocytes. In the mosquito midgut, gametocytes exit from the erythrocytes using proteases and immediately after 8-15 min they develop into eight male microgametes or one female macrogamete (**Figure 6**). The process of gamete activation inside the mosquito, termed gametogenesis, is a result of environmental alterations when reaching the insect's midgut, specifically the sudden drop in temperature of ~5 ºC, the increase in pH to ~8, and the presence of xanthurenic acid (65). When the microgamete fertilizes the macrogamete, which happens within 30-60 min, a zygote is formed after ~2 h that later transforms into a motile ookinete after ~20 h. The ookinete penetrates the midgut epithelium and becomes an oocyst that undergoes asexual sporogony for 7 to 12 days. Sporogony produces thousands of motile sporozoites that travel through the mosquito hemolymph and into the salivary glands. The infected mosquito finds a new human host to feed and inoculates the sporozoites and so the life cycle of malaria continues (78,79).

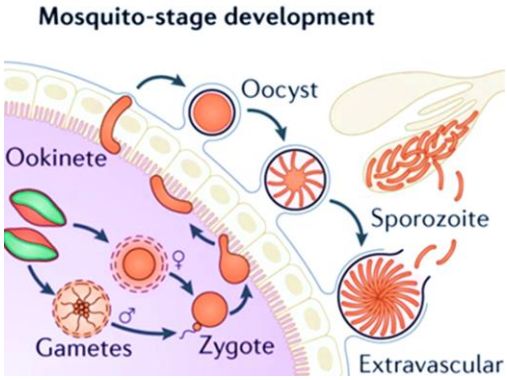

Figure 6. The development of *P. falciparum* inside the mosquito vector. Fertilization of a female gamete with a male gamete forms a zygote that develops into an ookinete and oocyst. The oocyst undergoes multiple divisions to form the sporozoites. Figure illustrated by Venugopal et al. (2020) (65).

2.3. Symptomatology of *P. falciparum* infection

Malaria symptomatology can be classified into three forms: uncomplicated malaria, severe malaria, and asymptomatic malaria. In general, the initial symptoms of malaria are non-specific and resemble influenza, although fever is considered to be the most consistent characteristic of malaria symptom.

2.3.1. Uncomplicated and severe malaria

In uncomplicated malaria cases, an infected person experiences the typical malaria symptoms, such as fever, chills, headache, profuse sweating, nausea, vomiting, diarrhea, and anemia without any signs of severe organ damage (32). By contrast, the hallmarks of severe malaria are multiple organ dysfunctions that involve the brain (cerebral malaria), lungs (respiratory failure), kidneys (acute renal failure), and in combination with (or without) severe anemia (i.e., hemoglobin <5 g/100mL or hematocrit <15%) (80,81). According to WHO, a patient is also considered to have severe malaria when *P. falciparum* parasitemia is beyond 10% despite the apparent absence of vital organ dysfunctions (18). Whether a patient will experience uncomplicated or severe malaria might be associated with the immune system's ability to manage the level of parasitemia. Previous research in hyperendemic settings demonstrated that severe forms were prevalent in children less than five years of age but not in older patients, whereas age dependency appeared inconspicuous in

low endemicity areas. This indicates that clinical immunity or tolerance to suppress clinical symptoms of malaria may develop over time, depending on the degree and frequency of parasite exposure (3,82).

2.3.2. Asymptomatic malaria

Asymptomatic malaria indicates that a patient has malaria parasites but experiences no malaria symptoms at all. In the natural course of the parasite life cycle, a typical asymptomatic period would occur during the prepatent period from 6 to 9 days, which is the duration of hepatic schizogony until the release of hepatic merozoites into circulation. Asymptomatic infection happens even on the initial days of intraerythrocytic blood-stage infection (i.e., around 5 days) when the erythrocytic cycles are merely starting. An individual with only sequestered and circulating gametocytes, which cause no significant pathology but are essential for transmission, can also be tagged as an asymptomatic carrier (32,80).

On the population level, at least 75% of malaria infections in the community are asymptomatic individuals, who are characterized by the absence of any malaria symptoms and typically carry malaria parasites at the submicroscopic level. Submicroscopic infection can persist for several months and is more widespread in low transmission settings (with community malaria prevalence of ≤10% by microscopy) than in high transmission settings (with a prevalence of ≥75% by microscopy) (47,83). The shift from high to low transmission intensity brought by effective malaria control and case management has been shown to increase the prevalence of submicroscopic infection (84). This can be explained by the residual immunity at the population level (47), which is involved in suppressing the multiplication of parasites below a critical level associated with malaria symptoms (85,86).

In areas where low transmission intensity is sustained for many years, it is unclear what other factors affect the high prevalence of asymptomatic infection despite the presumed loss of protective immunity at the population level. However, it is possible that protective immunity is

retained in a subpopulation with frequent exposure to parasites (e.g., forest goers in Vietnam) and possibly in the form of prolonged acquired immunity (47).

2.4. Pathophysiology of *P. falciparum* infection

Once the parasite gains RBC entry, it hijacks the cell in a progressive manner by consuming the hemoglobin and exporting parasite proteins to facilitate the nutrient acquisition, cytoadherence, and sequestration. Hemoglobin digestion through a specialized food vacuole is necessary to acquire all amino acids (except for isoleucine, which is taken extracellularly) to sustain the parasite's rapid and nutrient-demanding growth, which is needed to generate new daughter merozoites. *P. falciparum* has also the capacity to invade RBC of all ages because of its multiple and redundant invasion pathways. This explains a much higher parasite density in *P. falciparum* infection when compared to other human malaria species (87). In the case of hyperparasitemia, the egress of merozoites will result in a massive RBC hemolysis that leads to severe anemia, although dyserythropoiesis of an unclear mechanism (probably an immune-mediated red cell precursor apoptosis) also contributes to anemia during the course of malaria infection (88). Merozoites egress is also accompanied by the release of toxins and parasite DNAs, which induce fever through their interaction with the toll-like receptor 9 and subsequent production of tumor necrosis factor (32).

One of the unique features of *P. falciparum* is the ability of the late stages (trophozoites and schizonts) to cytoadhere on the surface of the vascular endothelium. The parasite surface proteins encoded by the *var* gene family, the *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1), are implicated in the process of cytoadherence. PfEMP1 protrudes through a knob-like structure of the infected RBCs (iRBCs) and interacts with host endothelial receptors CD36, Endothelial Protein C receptor (EPCR), Intercellular Adhesion Molecule 1 (ICAM1) and Chondroitin Sulfate A (CSA), among others. In cerebral malaria, a variant of PfEMP1 interacts

with ICAM1 and EPCR, whereas in placental malaria the PfEMP1 variant VAR2CSA interacts with the CSA receptor (32).

In general, for successful parasite sequestration, the process shall consecutively involve adherence, rolling, and firm attachment of the rigid iRBC into the endothelium of capillaries. Once sequestration is established, it disrupts the blood flow, which results in tissue hypoxia and lactic acidosis. In severe malaria, lactic acidosis is the major contributor to metabolic acidosis, which is an important determinant of the patient's survival (89). Sequestration also causes localized dissemination of toxins that trigger excessive and harmful inflammatory reactions in the affected tissues. PfEMP1, together with RIFINS, also mediates another form of cytoadherence called the 'rosetting' (90), in which iRBCs attach to RBCs and platelets, thereby forming a big clump that can block vasculature circulation. The Complement Receptor 1, Heparan Sulfate, and the ABO blood group are the erythrocytic factors associated with 'rosetting' (81).

Overall, the pathology of malaria is a result of cellular, metabolic, and immune-mediated damages as a consequence of the parasite erythrocytic asexual cycles. The principal causes of deaths in malaria include severe anemia, cerebral malaria, and respiratory arrest (17).

3. *P. falciparum* sexual development

3.1. Terminologies in early sexual development

The terminologies 'gametocytogenesis', 'sexual differentiation', 'sexual commitment', and 'sexual conversion' are used interchangeably in many articles to describe the initial phase of the transition from asexual stage parasite into gametocyte. This broad usage of the term, irrespective of the temporal and developmental phase, oftentimes generates potential semantic confusion, as in the case of 'sexual differentiation'. On one hand, sexual differentiation refers to the transition from asexual to the sexual stage (91), on the other hand, it refers to the differentiation of male and female gametocytes (92,93), which also denotes 'sex determination' (93).

The term ‘gametocytogenesis’ is similar to the former definition of sexual differentiation except that it describes the whole process of gametocyte development from sexual ring to mature Stage V gametocyte (94). Certainly, some of the events require semantic clarity to define a specific step occurring within sexual development.

Bancells and colleagues (2019) proposed a new use of the nomenclature that clarifies the semantics of ‘sexual commitment’ and ‘sexual conversion’, which both occur in the earliest phase of sexual development (**Figure 7**). In their definition, ‘sexual commitment’ and ‘sexual conversion’ are two distinct processes that occur sequentially within the same parasite cycle or until the next cycle. They proposed that sexual commitment underpins the very first episode of switching from the asexual cycle to sexual development, in which cells deterministically commit to sexual development at a later time point. Asexual parasite stages can sexually commit by activating the expression of the master regulator *pfap2-g*[1] (PF3D7_1222600). By turning on *pfap2-g*, the resulting sexually committed parasites are locked in a sexual pathway but remain morphologically similar to the asexual stages. Meanwhile, sexual conversion kicks in when a sexually committed parasite develops into a sexual ring, which is technically the first gametocyte stage (also known as gametoring). By definition, “*sexual conversion is marked by the onset of gametocyte-specific expression of proteins absent from any replicating blood stages*” (95).

In this thesis, the term ‘sexual conversion’ is used to describe a process of gametocyte formation when the readouts rely on relative gametocyte levels. Although ‘sexual commitment’ overlaps across the whole process of gametocyte formation, it may be more appropriate to use this terminology for experiments in which readouts focus on quantifying sexually committed cells (e.g., committed schizonts), which are yet difficult to identify due to limited markers and tools availability.

[1] Italized and lowercase name refers to the gene name, while non-italized and uppercase refers to the protein.

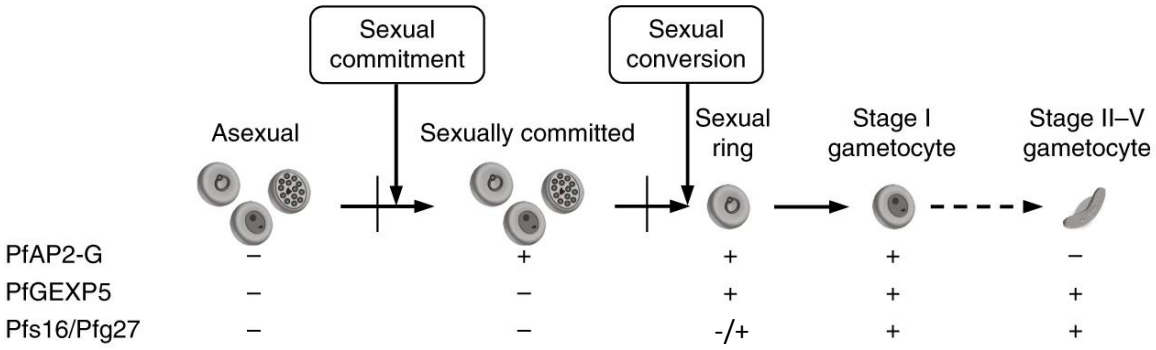

Figure 7. The nomenclature of *P. falciparum* early sexual development. In sexual commitment, the asexual stage parasite develops into a sexually committed parasite after activating *pfap2-g*. The sexually committed parasite later becomes the sexual ring that expresses some of the early gametocyte proteins (e.g., PfGEXP5, Pfs16, and Pfg27) – the expression of gametocyte-specific proteins absent from any replicating blood stages marks sexual conversion. All sexually converted parasites are fated to become mature gametocytes, which is the only parasite stage transmissible to the mosquito vector. Figure modified from Bancells et al. (2019) (95).

3.2. Sexual commitment

The current understanding of how asexual parasites transition to the sexual stage revolves around the transcription factor *pfap2-g* (*P. falciparum Apetala2 – Gametocyte*) (**Figure 8**). PfAP2-G belongs to the Apicomplexan AP2 (apiAP2) family of DNA-binding proteins that is homologous to a group of transcription factors called the Apetala2/Ethylene Response Factor (AP2/ERF) protein family, which functions during plant development and stress regulation (96).

In *P. falciparum*, the apiAP2 family has 27 gene members that are mainly conserved across *Plasmodium* species, with functions linked to parasite stage switching and developmental transitions in humans and mosquitoes (99). Previous research on *ap2-g* revealed that nonsense genetic mutations, as well as gene knockout and conditional knockdown, resulted in the absence of gametocytes in *P. falciparum* (100) and the murine malaria parasite *P. berghei* (101). Concomitant growth advantage over the parental wild-type was also observed among parasites with defective *ap2-g* (100–102), demonstrating that its loss of function favors asexual multiplication and therefore *ap2-g* operates at the time of

commitment rather than at later steps of sexual development. Furthermore, the expression of *ap2-g* under the control of strong constitutive promoters was shown to generate an almost 100% sexual conversion rate in *P. berghei* (103) and *P. falciparum* (104), reinforcing the idea that gametocyte production is dependent on *ap2-g* activation. All these findings strongly pinpoint *ap2-g* as the master switch of sexual commitment.

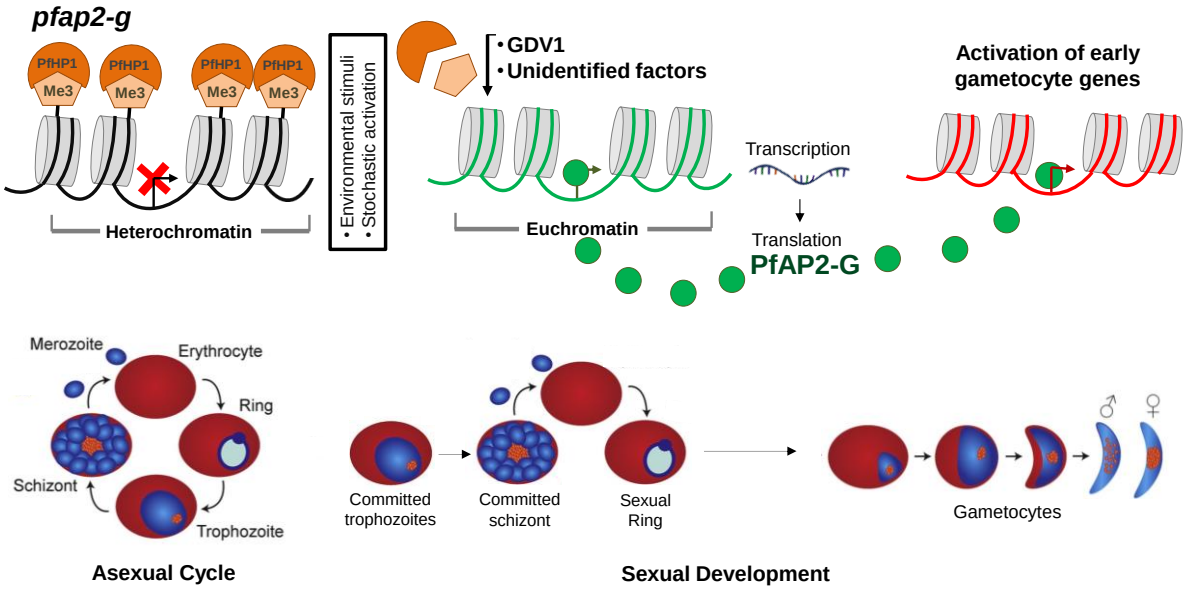

Figure 8. The initial steps of *P. falciparum* sexual development. The *pfap2-g* locus is silenced in all asexual parasites (typically >90% of the population) by epigenetic marks H3K9Me3 and PfHP1 – these are the signatures of heterochromatin silencing. GDV1 (97) and possibly other unidentified factors assist in evicting some factors involved in *pfap2-g* silencing, making the gene euchromatic or active. Once activated, *pfap2-g* undergoes a positive-feedback mechanism producing a sufficient amount of PfAP2-G protein that drives the transcription of early gametocyte genes. Early gametocyte gene activation is one of the essential events to attain gametocyte development (98). Figure adapted from Josling and Llinás 2015 (98); parasite cycle illustrated by Vanaerschot et al. (2020) (66).

Although PfAP2-G is essential in executing sexual commitment, new evidence suggests that its activation requires the cooperation of other factors. In all asexual stages, the gene itself is silenced by the heterochromatic mark histone H3 lysine 9 Trimethylation (H3K9Me3), which is bound by Heterochromatin Protein 1 (PfHP1; PF3D7_1220900)

(105). To some extent, its silencing is also controlled by Histone Deacetylase 2 (PfHDA2; PF3D7_1008000) (106). Essentially, loosening the default heterochromatic state of *pfap2-g* needs the cooperation of other molecules to start its transcription. Filarsky et al. (2018) demonstrated that Gametocyte Development 1 protein (GDV1; PF3D7_0935400) evicts the epigenetic repressor PfHP1 during the trophozoite stage, thereby derepressing *pfap2-g* from its silenced state (97). GDV1 was previously described as a candidate protein implicated in sexual commitment after a genome-wide analysis revealed a correlation between gametocyte-deficient parasite lines and *gdv1* deletion in chromosome 9. Functional restoration of *gdv1* rescued the normal gametocytogenesis of the defective lines and its overexpression led to an enhanced gametocytemia (107). Further research on GDV1 was directed on the role of a multiexon-long noncoding *gdv1* antisense RNA, which is located downstream and overlapping with the *gdv1* locus (97,108). This antisense performs as a potential *gdv1* negative regulator that is responsive to choline level alteration, a known environmental stimulus of sexual commitment, suggesting a possible molecular mechanistic link between external stimuli and parasite's ability to adjust its rate of sexual conversion (97).

Other regulators that are putatively involved in chromatin-remodeling, transcriptional control, and post-transcriptional regulation appear to interact indirectly with PfAP2-G and play a role in sexual commitment. However, many of these genes have not undergone functional characterization or targeted gene studies to define their role during sexual commitment (96,98,109,110). For instance, a single-cell RNA-sequencing in a parasite line with conditional PfAP2-G degradation revealed a concomitant upregulation of the epigenetic factors *lysine-specific histone demethylase 2* (*lsd2*; PF3D7_0801900), *histone deacetylase 1* (*hda1*; PF3D7_1472200), *iswi* (PF3D7_0624600), and *snf2l* (PF3D7_1104200) in PfAP2-G-positive cells (111). The latter two may function as putative chromatin-modifiers associated with nucleosomal rearrangement in the

pfap2-g locus. The ISWI protein was previously reported to localize predominantly in the euchromatic area of the nucleus and was implicated in the transcriptional activation process (111,112).

The ApiAP2 transcription factor AP2-G3 (PF3D7_1317200) was also predicted to cooperate with AP2-G in initiating sexual commitment as its loss of function reduced the level of gametocyte production both in *P. yoelii* (gene name: *pyap2-g3*) and *P. falciparum* (113,114). The detailed role of AP2-G3 in *P. falciparum* is unclear, but in *P. berghei*, it associates with female gametocyte development, rather than playing a role at the commitment phase (115). The orthologue of another apiAp2 transcription factor, PF3D7_1408200, in the murine malaria parasite *P. berghei* (gene name: *pbap2-g2*) was also suggested to cooperate in the initiation of sexual commitment by repressing the expression of genes necessary for asexual replication, thereby promoting a smooth transition to sexual development. However, a knockout of *pbap2-g2* neither eliminated the production of gametocytes nor conferred a growth advantage over the parental line, suggesting that this transcription factor operates downstream of *ap2-g* (101,116).

Another factor that may be involved in the regulation of sexual commitment is the RNA-binding protein PfPUF2 (PF3D7_0417100). This is a member of the translation repressor Puf family (Pumilio and fem-3 binding factor). PfPUF2 was shown to block sexual development in *P. falciparum* since its loss of function enhanced the production of gametocytes (117).

In summary, current data on sexual commitment suggest that the whole commitment process may cover a complex chain of events. This may involve the activation of different stimulating factors and the downregulation of operations related to asexual replication.

3.3. Sexual conversion

The activation of *pfap2-g* follows a positive-feedback loop as its protein binds to a region of its own promoter with GNGTAC motifs, thereby

amplifying its own protein production. Once PfAP2-G levels reach a certain threshold, it binds to the early gametocyte gene promoters (again with GNGTAC motifs) to commence the sexual conversion process (100). More recently, a chromatin immunoprecipitation followed by sequencing (ChIP-seq) analysis confirmed the direct binding of PfAP2-G to hundreds of genes involved in gametocytogenesis, including the well-established early gametocyte genes *gexp5* (PF3D7_0936600), *pfs16* (PF3D7_0406200), *etramp 10.3* (PF3D7_1016900), *pfg14-744* (PF3D7_1477300), and *pfg14-748* (PF3D7_1477700) (118).

In the canonical process of sexual conversion, called the Next Cycle Conversion (NCC), *pfap2-g* is activated at the trophozoite stage or before the first nuclear division so that the resulting PfAP2-G-positive parasites go through an additional round of replication after commitment. Committed schizonts already contain a homogenous progeny of sexual merozoites (92,119,120). By contrast, the vast majority of schizonts are PfAP2-G-negative and contain all asexual merozoites destined for the asexual replication cycle (120). However, multiple lines of evidence recently showed that activation of PfAP2-G can occur at the early ring stage, and then parasites convert directly into gametocytes without going through an additional round of multiplication (95,103) (**Figure 9**). This pinpoints a new sexual pathway called the Same Cycle Conversion (SCC) (95), which resembles how gametocytes are produced in other haemosporidian species (121). The timing of *pfap2-g* activation may signal the decision to convert, such that activation at the early ring stage promotes SCC while activation at the late stage promotes NCC (95).

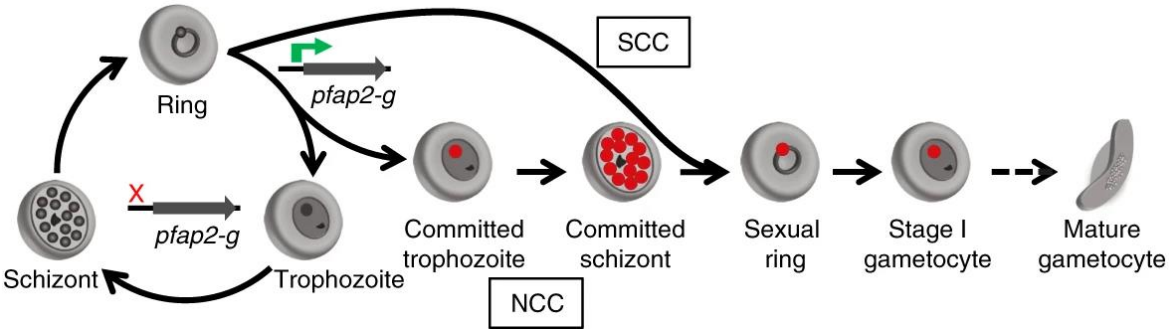

Figure 9. The two sexual conversion pathways of *P. falciparum*. The Same Cycle Conversion (SCC) bypasses a nuclear dividing-cell stage called the committed schizont, so that *pfap2-g* activation in asexual ring results in its direct conversion to the sexual ring that will later develop into gametocyte. The Next Cycle Conversion (NCC) follows the relatively longer canonical process in which *pfap2-g* activation leads to the production of sexually committed schizonts that release around 8-32 offspring that are fated to become gametocytes. Figure illustrated by Bancells et al., 2019 (95).

3.4. Sex determination

Male and female gametocytes are indistinguishable from one another until they reach Stage IV, when sexual dimorphism starts to show. However, other methods of sex determination that target sex-specific antigens can distinguish male and female gametocytes as early as Stage III (see Section 4.3). The system of sex determination in *P. falciparum* is not governed by sex chromosomes because one haploid parasite can produce both female and male fertile gametocytes (122). Although the mechanism behind sex determination remains unclear, the current model proposes that the initial decision to become male or female takes place before the start of schizogony. The timing itself implies that sex determination could occur when *pfap2-g* is activated, or immediately before or after its activation (122,123). The model of sex determination is based on observations that a committed schizont can have either all male or all female progeny but never a mixture of both sexes, implying that a certain sex differentiating factor strikes before cell division (92,119).

To date, no single molecular factor explains what drives the decision to become a male or female gametocyte nor confirmed when the divergence happens within the course of the erythrocytic cycle. However, it

is likely that sex determination is orchestrated by one or a few transcription factors, epigenetic regulators, and/or post-transcriptional modifiers, of which timing shall fall within the commitment phase (123).

As for transcription factor, PbAP2-FG (PyAP2-G3; PF3D7_1317200) was shown to regulate many essential genes for normal female gametocyte development in the murine malaria parasite *P. berghei*. However, PbAP2-FG cannot qualify as the master switch for sex determination because its disruption only stopped the normal development of the female gametocyte but did not abolish its production (115). Recently, a single-cell transcriptomics approach performed from early Stage III to late Stage V gametocytes in *P. falciparum* identified clusters of genes distinctly expressed for each gametocyte sex. The female cluster included two ApiAp2 transcription factors, namely *pfap2-o* (PF3D7_1143100) and the uncharacterized ApiAP2 gene PF3D7_1107800, as potential candidates involved in sex determination. However, both genes may also play a role in the latter sexual stage development, specifically during oocyst formation (124).

Genes involved in kinase-mediated signaling were also identified to associate with different parasite sex development. In particular, the mitogen-activated protein kinase *mapk1* (PF3D7_1431500) and *mapk2* (PF3D7_1113900) were linked to female and male formation, respectively (124,125). However, more research is needed to validate their role in sex determination.

One of the members of the translation repressor Puf family, *pfpuf1* (PF3D7_0518700), was also shown to regulate female gametocyte development since its disruption decreased the level of female gametocytes along with reduced total gametocyte production (126). Disrupting the other Puf member, *pfpuf2* (PF3D7_0417100), which is also involved in repressing sexual development, was shown to increase the level of total gametocytes with an abnormally male-biased sex ratio, indicating its potential role in repressing male gametocyte development (117).

Overall, while all these studies indicate a single or group of genes that might be involved in sex determination, most of the approaches appear limited to analyzing the later gametocyte stages. The main barrier in dissecting the initial steps of sex determination is the absence of a reliable and early biomarker for each parasite sex (127).

3.5. Sexual merozoites

The mechanism of sexual merozoite invasion of new RBC is most likely similar to the asexual merozoite. During sexual commitment, the most consistently upregulated invasion gene is *msrp1* (PF3D7_1335000), which is a target of PfAP2-G and PfAP2-I (PF3D7_1007700) (118). In many datasets, *msrp1* has been a consistent marker found in mature sexually committed schizonts expressing *gdv1* and *pfap2-g* (97,104,118,128). The upregulation of *msrp1* suggests that sexual merozoites may have altered their invasion pathway for a more precise cell-binding mechanism that could be related to immature erythrocyte preference (118). To gain bone marrow entry and infect immature erythrocytes, sexual merozoites are reported to penetrate the endothelial cell junctions or attach to human P-selectin receptors. Of note, the human P-selectin receptors were found to interact with merozoite surface 7-related proteins (MSRPs), including the MSRP1 (65,129).

Observations of immature gametocyte enrichment in the haematopoietic niche (65,130,131) presumed that sexual merozoites and/or sexual rings have tropism to the erythroblastic island of the bone marrow. This hypothesis is known as ‘homing’, a paradigm that explains why Stage I to IV gametocytes accumulate in the bone marrow (65). In *P. berghei*, the preferential homing of sexual stages was demonstrated using intravital microscopy. Here, the ‘homing’ process was associated with a vascular leakage in the hematopoietic niche as a result of inflammation (129).

The concept of sexual merozoite traveling directly to the hematopoietic niche is not supported by the detection of sexual ring

biomarkers in the peripheral blood samples (91,132,133). Moreover, a more recent study demonstrated the absence of reticulocyte preference for both sexual and asexual merozoites (104). These observations may suggest that the 'homing' mechanism may occur within or after the sexual ring development (0-22 hpi) rather than during the sexual merozoite stage. Of note, sexual merozoites need to find new RBCs within 2 minutes in order to survive (68).

As an alternative to the 'homing' hypothesis, the presence of sequestered gametocytes can be explained by a cryptic asexual cycle occurring within the bone marrow, thereby producing a pool of sexual forms upon environmental triggers (65,134) (**Figure 10**). Therefore, it is possible that asexual rings or merozoites can also travel to the bone marrow and undergo sexual conversion. The bone marrow is a perfect environment for sexual commitment because it has limiting levels of lysophosphatidylcholine, the absence of which promotes sexual conversion in *P. falciparum* (135).

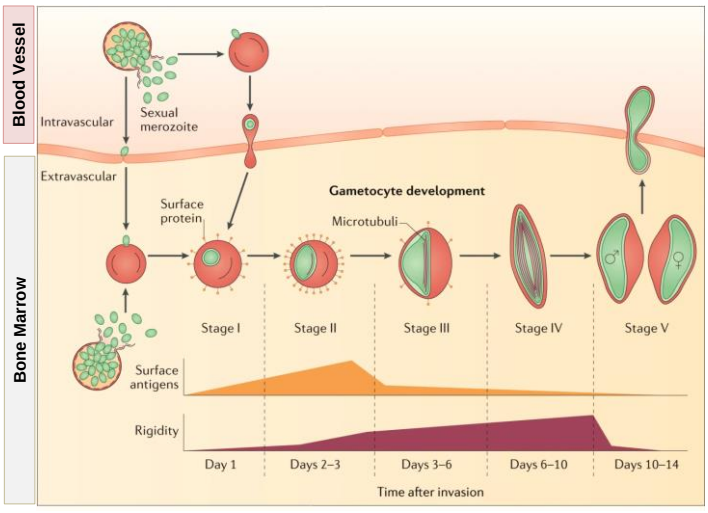

Figure 10. Sexual stages homing and development in the bone marrow. Sexual merozoites may 'home' to the bone marrow or invade new erythrocytes and become sexual rings, which may also undergo homing. Additionally, inside the bone marrow, committed schizonts release the sexual merozoites, which invade the RBCs and its precursors to develop into gametocyte stages. Stage V gametocytes are release from the parenchyma to the intravascular circulation. Figure adapted from Venugopal et al. (2020) (65).

3.6. Sexual rings

Sexual rings are found in the hematopoietic environment and the blood circulation, indicating that sexual commitment and invasion by sexual merozoites are not exclusively occurring in the hematopoietic niche

(132,133). Furthermore, the detection of sexual ring biomarkers and the presence of gametocytes that directly develop from freshly collected ring stages in malaria patients support the idea that sexual rings circulate in the bloodstream (91). Hence, the peripheral blood of a malaria-infected individual may contain Stage V gametocytes, asexual rings, sexual rings, asexual merozoites, and sexual merozoites, although the latter two stages have a very short circulation half-life (68). The circulation of sexual rings has significant implications in the early clinical diagnoses of a transmissible person and when performing epidemiological studies on malaria transmission, of which very little has been reported (**Chapter 4**).

3.7. Stage I to V gametocytes

3.7.1. Morphology

When a sexual ring progresses to Stage I gametocyte after ~24 h post-invasion (hpi), it becomes morphologically round until ~40 hpi, resembling the morphology of asexual trophozoites (136). Stage I gametocytes transform into Stage II gametocytes after day 3 or 4 through an initial elongation process (**Figure 11**), which is attributed to the developing cytoskeleton of the longitudinal microtubule and facilitated by an inner membrane complex. Stage II gametocytes are tear-drop shaped in the beginning and D-shaped later on, occupying half of the hemoglobin-depleted but non-deformed RBC (136,137).

Once Stage III gametocyte is reached after day 5 to 7, it marks the end of hemoglobin digestion where the feeding process halts as gametocytes undergo a relatively dormant state (94). Stage III gametocytes have bluntly rounded ends and their minor elongation distorts the RBC morphology. They continue to transform into Stage IV after day 7 to 9, which are full-sized gametocytes with pointed ends (136,137). Sexual dimorphism also starts to show in Stage IV gametocytes, particularly in terms of nuclear size, which is bigger in males than in females (138).

Mature Stage V gametocytes on day 9 to 14 have rounded ends and the sexual dimorphism becomes more obvious. In Giemsa-stained blood

smear, the female gametocyte has a more elongated shape, centrally aggregated pigment granules and chromatin, as well as blue-staining cytoplasm in comparison to the male. In human infection, the morphology of mature Stage V gametocyte is the basis for identification and detection by routine microscopy, although the inherently low gametocytemia impedes both accurate gametocyte quantification and sex ratio determination (73,139).

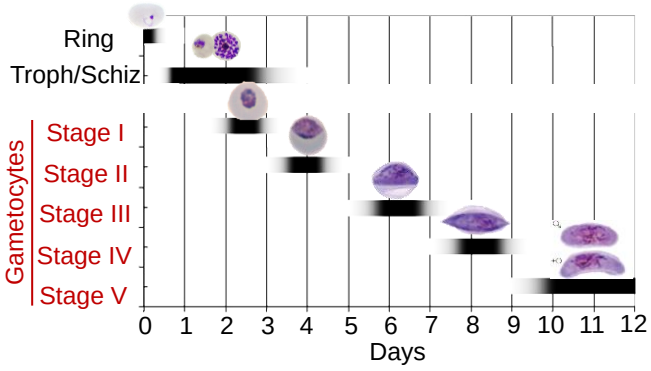

Figure 11. Different stages of gametocytes. *P. falciparum* culture treated with N-Acetyl-glucosamine (GlcNAc) monitored for different parasite stages using Giemsa-stained blood smear. The peak for the individual stage is represented by dark shades. Data taken from Fivelman et al. (2007) (136); Gametocyte photos were taken from Josling and Llinás (2015) (98).

3.7.2. Gametocyte distribution within the human host

In human infection, Stage I to Stage IV gametocytes are enriched in the bone marrow, while mature Stage V gametocytes are released in the peripheral circulation (73). The mechanism of Stage I to Stage IV gametocyte sequestration is not fully understood but previously it was assumed that their adhesion to the bone marrow parenchyma is associated with the structure called knobs. Knobs are mainly formed by knob-associated histidine-rich protein (KAHRP) and are present mainly on the surface of Stage I gametocytes and partly on early Stage II gametocytes, indicating that sequestration in the hematopoietic niche occurs as early as Stage I gametocytes as they are absent in the circulation. Knobs assist the variant surface antigen PfEMP1 in facilitating the adherence of trophozoites and schizonts in the endothelial cells of different organs by binding to receptors such as CD36 and Intercellular Adhesion Molecule 1 receptors. These receptors are found in the bone

marrow and thus presumed to mediate binding to PfEMP1 during the adherence of Stage I gametocytes (73,140). However, Tibúrcio and colleagues (2013) demonstrated the absence of knobs and KAHRP, as well as the downregulation of PfEMP1, on the surface of Stage I gametocyte-infected RBC (141), proposing that other mechanisms of sequestration are involved.

The binding of immature gametocytes to the bone marrow may also be facilitated by the gametocyte-exported proteins GEXP07 (PF3D7_1301700) and GEXP10 (PF3D7_0113900). These two parasite proteins can bind to the human chemokine fractalkine (CX3CL1) receptors in the endothelial cells of several organs and mesenchymal stem cells of the bone marrow (65). Both GEXP07 and GEXP10 are also present on the surface of trophozoites, suggesting that the mechanisms of binding between parasites and human cells are shared between sexual and asexual stages (65,142).

Immature gametocyte sequestration is also attributed to gametocyte-facilitated erythrocyte membrane rigidity, leading to the mechanical retention of the immature gametocytes in the bone marrow parenchyma. The Subtelomeric Variable Open Reading Frame (STEVOR) protein is incriminated for the mechanism of membrane rigidity as it interacts with the Ankyrin complex, which is a component of the RBC cytoskeleton. The reversion of the rigid RBC membrane occurs in Stage V gametocytes when a cyclic adenosine monophosphate (cAMP)-dependent protein kinase phosphorylates the STEVOR. Overall, the dephosphorylation of STEVOR along with the disassembly of the microtubular cytoskeleton contributes to the deformability of Stage V gametocytes, which translates into a bone marrow exit mechanism (65,140).

4. Biomarkers of sexual stages

4.1. Biomarkers of sexual rings

Transcriptomic data revealed a list of sexual ring biomarkers but only a few of them were validated in *in vitro* or field studies (110,111,118,133). These include the sexual commitment gene *pfap2-g* (PF3D7_1222600) (95,143), the early gametocyte gene *pfs16* (PF3D7_0406200) (144,145), and the sexual ring gene *gexp5* (PF3D7_0936600) (91,132,146). In **Chapter 2**, we characterize a new biomarker called *gexp02* (PF3D7_1102500), thus adding to the limited collection of genes that are expressed in sexual rings.

Capturing the mRNA of sexual rings in the circulation has been performed using the biomarker *pfs16* (144,145), which is also a marker of all gametocyte stages. However, the transcript levels of *pfs16* in ring stages are relatively abundant in parasites with disrupted *pfap2-g* function or in non-gametocyte producer parasite line (**Figure 12**), thus requiring caution when interpreting its mRNA levels (95). Nonetheless, the specificity of *pfs16* at the protein level from Stage I to V gametocytes has been confirmed by multiple *in vitro* immunofluorescence assays, indicating that only its mRNAs are non-specific (95,143).

The mRNAs of *gexp5* also show similar promiscuity in asexual stages (95), which led some authors to propose that it works independently from PfAP2-G function (146). However, new ChIP-seq data revealed that *gexp5* is one of the targets of PfAP2-G, with further transcriptomic analysis showing an increase of its transcription levels upon PfPAP2-G activation (118). In any case, *gexp5* and *pfs16* transcript levels may increase when sexual rings are abundant but are not sufficiently specific to exclude that they arise from circulating asexual rings.

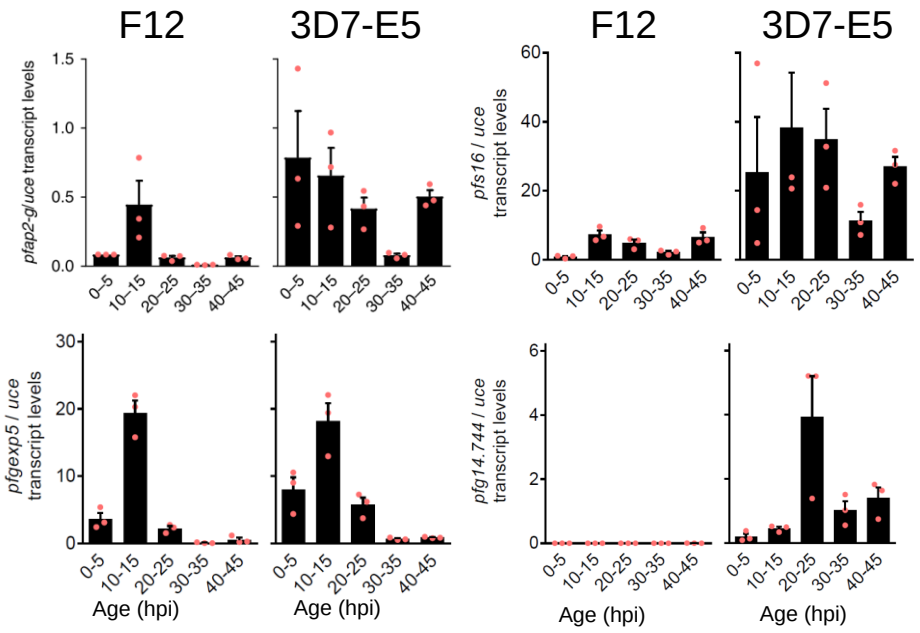

Figure 12. The specificity of early sexual genes transcript levels. Two parasite lines were tested to quantify the mRNAs of selected sexual rings and early gametocytes biomarkers: F12 has a nonsense mutation in *pfap2-g* and is a known non-gametocyte producer (*pfap2-g* transcripts are still detected but do not produce functional protein that can activate its own expression or that of other early gametocyte genes), while 3D7-E5 is a high gametocyte producer subclone of 3D7. Note: sexual biomarkers are normalized to the *ubiquitin-conjugating enzyme* (*uce*) (PF3D7_0812600). Data were taken from Bancells et al. (2019) (95).

4.2. Biomarkers of early stage gametocytes

In Stage I gametocytes, the following proteins are functionally validated to be sexual stage-specific: Pfs16 (PF3D7_0406200) (147), Pfg27 (PF3D7_1302100) (148), PfGECO (PF3D7_1253000) (149), Pfg14-744 (PF3D7_1477300), Pfg14-748 (PF3D7_1477700) (150,151), and GEXP02 (PF3D7_1102500) (143,152). The most common feature among them is their exportation outside the parasites, within the RBC cytoplasm and membrane. Their putative functions may therefore involve host-cell remodeling, protein trafficking (153,154), RNA binding (e.g., Pfg27), and gametocyte maturation (155). In particular, Pfs16 (~24 hpi) and Pfg27 (~30 hpi) are commonly used as a tool to differentiate Stage I gametocytes from the asexual trophozoites by immunofluorescence assay and flow cytometry (95,156) (**Figure 13**).

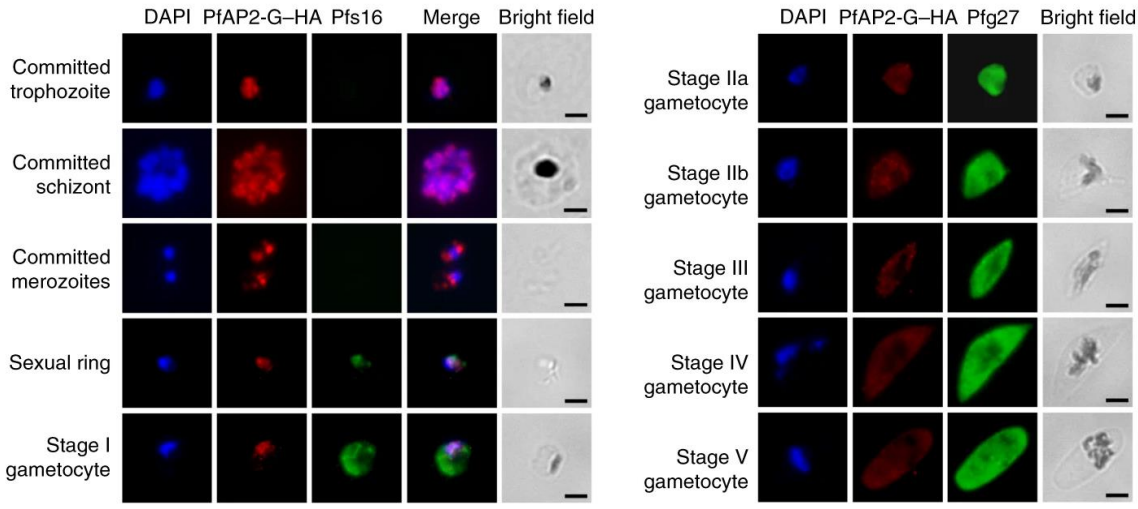

Figure 13. Selected biomarkers used for sexually committed parasites and gametocyte stages. Immunofluorescence assay detecting PfAP2-G, Pfs16, and Pfg27 in sexually committed stages, early sexual stages, and late gametocyte stages, respectively. Anti-Hemagglutinin (HA) antibodies were used to detect HA-tagged PfAP2-G (95). Figure was taken from Bancells et al. (2019) (95).

The genes *mdv1* (*peg3*; PF3D7_1216500) and *etramp10.3* (*peg4*; PF3D7_1016900) (139) are two of the highly expressed biomarkers in Stage II gametocytes, although both can start expressing at Stage I gametocyte. MDV1 and ETRAMP10.3 proteins are present in the parasitophorous vacuole of the parasite and are also exported to the host RBC (157,158). The *etramp10.3*, including its promoter and coding sequence, is widely used as a biomarker to quantify gametocytes as early as Stage II and during the mosquito stages (150,159–161).

4.3. Biomarkers of gametocyte sexes commonly used for *in vitro* studies

Differentiating gametocyte sexes can be performed by detecting a sex-specific gametocyte protein and mRNA levels, with the former usually applied *in vitro* while the latter is applied in field studies (see Section 4.4). To differentiate gametocyte sexes *in vitro*, the Pfg377 (PF3D7_1250100) and α-tubulin II (PF3D7_0422300) are typically used in an

immunofluorescence assay (75,119,162) (**Figure 14**). Pfg377 is expressed in Stage III to Stage V female gametocytes and is involved in the formation of the osmiophilic body, a structure presumably needed during the release of the (female) macrogamete from the RBC in the mosquito midgut (156,162). By contrast, the α-tubulin II (PF3D7_0422300) is considered a male-specific biomarker only at Stage V gametocytes because its expression from Stage I to IV is promiscuous between sexes (163,164). The α-tubulin II is a component of the axonemal microtubules that are needed for the exflagellation of (male) microgamete (164).

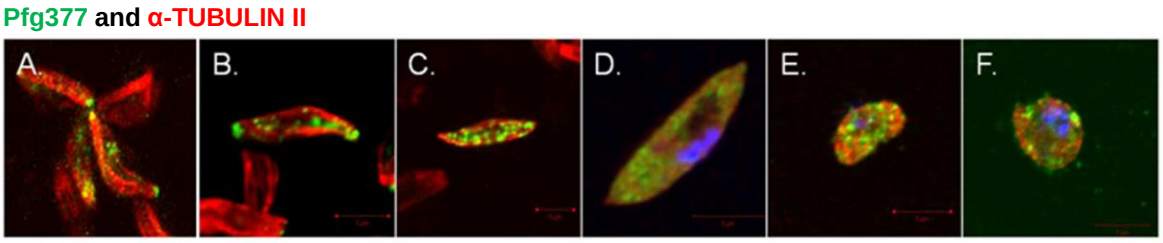

Figure 14. Gametocyte sex biomarkers using an immunofluorescence assay. Pfg377 (green) and α-tubulin II (red) are commonly used biomarkers for female and male gametocytes, respectively. Pfg377 is expressed in females at Stage III (A-C), Stage IV (D), mature Stage V (E), and activated Stage V or macrogamete (F). The α-tubulin II is expressed in all gametocyte stages but specifically abundant in Stage V male gametocytes. DAPI was used to stain the nucleus. Figure was taken from Schwank et al. (2010) (164).

4.4. Biomarkers of mature gametocytes commonly used in studies with human samples

The detection of Stage V gametocytes in field studies is gaining more attention in malaria research because it serves as a surrogate test for active malaria transmission. The molecular techniques used to detect the degree of gametocytemia are intended for RNA transcript quantification of a given gametocyte gene (**Table 2**). The commonly used techniques to quantify the gametocyte RNA in field samples include a reverse transcriptase PCR assay (RT-PCR), quantitative nucleic acid sequence-based amplification assay (QT-NASBA), and reverse transcriptase loop-mediated isothermal amplification assay (RT-LAMP). The rationale of

these assays is to capture genes expressed in Stage V gametocytes while excluding the detection of parasite's genomic DNA (gDNA) (73).

Table 2. List of mature gametocyte-specific markers currently employed for molecular diagnosis of *P. falciparum*

Gene Identification	Gene Name	Description	LOD	F:M*	Remarks
Female-biased biomarkers					
PF3D7_1031000	*pfs25*	Zygote and ookinete surface protein	0.01 F/µL	35.58	Necessary for mosquito infection
PF3D7_0630000	*None*	CPW-WPC family protein	0.3 F/µL	47.83	Zygote and ookinete protein; contains 9 introns Refs
PF3D7_1351600	*pfgk*	Glycerol kinase	0.3 F/µL	41.41	
PF3D7_0903800	*ccp4*	LCCL domain-containing protein	0.1 F/µL	38.49	Sexual-stage adhesion; contains 1 intron
Male-biased biomarkers					
PF3D7_0208900	*pfs230p*	6-cysteine protein	1.8 M/µL	0.02	
PF3D7_1469900	*pfmget*	Gamete surface protein	0.01 M/µL	0.02	KO has no effect on fertilization; contains two introns
PF3D7_1311100	*pf13*	Meiosis-specific nuclear structural protein	22 M/µL	0.005	
All gametocyte sexes biomarker					
PF3D7_1319800	*pfg17*	Conserved Plasmodium protein, unknown function	0.1 G/µL	0.60	Detect all gametocyte sexes

LOD = Limit of Detection; F = Female Gametocyte; M = Male Gametocyte; G= Gametocyte; KO = Knockout. *F:M is the ratio of female to male gametocyte mRNA transcripts as measured by RNA-seq in sorted female and male gametocytes. Data taken from Tadesse et al. (2019) (75).

Of the known mature gametocyte biomarkers, the mRNA of *pfs25* (PF3D7_1031000) is widely used because of its high sensitivity and specificity for mature gametocytes (73,165,166). QT-NASBA analysis of *pfs25* using the *NF54* strain showed a limit of detection (LOD) of one gametocyte per microliter (µL), which is 16-fold better compared to thick blood smear microscopy (145). The use of a quantitative (q) RT-PCR assay targeting *pfs25* mRNA can permit a much higher sensitivity depending on the volume of blood samples used for RNA extraction, with LOD of 0.02-0.05 gametocyte per µL of blood when extracted from 50 µL

culture. In this assay, one gametocyte equates to 87 (95% CI: 65.55-115.60) *pfs25* mRNA copies (167). Moreover, the qRT-PCR assay showed better precision in quantifying *pfs25* transcript levels compared to QT-NASBA. Although *pfs25* is more abundant in female gametocytes than in males, its transcript levels positively correlate to both total gametocytemia and mosquito infection rate (168).

The female gametocyte biomarker *pfg377*, which has been used *in vitro*, is comparable to *pfs25*, except that it contains many genetic polymorphisms among field parasites, thereby causing potential variations across sample collections and inaccuracies in detecting transmission burden (73,169). The most recent additions to the list of female biomarkers are PF3D7_0630000, *pfgk* (PF3D7_1351600), and *ccp4* (PF3D7_0903800). The PF3D7_0630000 gene belongs to the uncharacterized group of CPW-WPC protein family and is specifically expressed in mature female gametocytes. The PF3D7_0630000 gene has nine introns that allow the design of intron-spanning primers for RT-PCR, thus avoiding an additional step of DNase treatment that is usually necessary to eliminate parasite's gDNA contamination (170). The transcript levels of *pfgk* and *ccp4* are also considered female-biased as both showed high expression levels in female gametocytes with negligible transcript levels in male gametocytes (165,166).

Quantifying male gametocytes along with female gametocytes is vital to determine the gametocyte sex ratio, which along with mature gametocyte density, may determine the infectiousness to mosquitoes. Several studies revealed that male gametocytes may dominate over females in areas where total gametocyte density is low, reflecting a strategic investment to maximize the likelihood of transmission (171,172). The identification of male biomarkers is relatively recent because male gametocytes are inherently scanty in numbers compared to their female counterparts. To date, the male biomarkers used to analyze field samples are *pfs230p* (PF3D7_0208900), *pf13* (PF3D7_1311100), and *pfmget* (PF3D7_1469900). The *pfs230p* is the first widely used male-biased

biomarker with a limit of detection of two male gametocytes per µL of blood using the qRT-PCR method (145). However, the *pfs230p* is inferior in terms of clinical sensitivity compared to the *pf13* in a qRT-PCR assay (165). Meanwhile, the *pfmget* is used alongside *pfs25* for the determination of the gametocyte sex ratio because both biomarkers have comparable specificity and sensitivity (75,166,171).

So far, *pfg17* (PF3D7_1319800) is the only biomarker that can equally detect all gametocyte sexes and therefore useful in providing an accurate molecular estimate of the total gametocyte density (173,174). As a general biomarker of male and female gametocytes, *pfg17* can be considered superior to *pfs25* in determining total gametocyte density (173). However, the gametocyte stage specificity of *pfg17* has yet to be proven in a qRT-PCR assay using different parasite stages and gametocyte dilutions (174).

5. Basal sexual conversion in *P. falciparum*

The human malaria parasite *P. falciparum* has diverse sexual conversion rates that range from 0 to 20%, depending on parasite strains[2], clones[3], and subclones[4] (100,175,176). In human infections, the estimated sexual conversion rate ranges from 0.3 to 1% with an average of 0.54% (74). Despite these variations, the rate of spontaneous sexual conversion appears stable across time, such that one subclone can maintain a relatively consistent sexual conversion rate in many consecutive cycles. The underlying mechanism is unclear but implies that sexual conversion occurs as an intrinsic property of the parasite that is most likely operated at the genetic and epigenetic levels. A constitutive sexual conversion contributes to the low-frequency but consistent baseline production of gametocytes (100,104).

In evolutionary biology, the strategy of the parasite to minimize the rate of sexual conversion while investing in growth expansion of asexual

[2] A parasite strain refers to a recognized culture-adapted field isolate (e.g., NF54).
[3] A parasite clone is a progeny of the parasite strain derived from a single parasite (e.g., 3D7 is a clone of NF54).
[4] A subclone is a progeny from the parasite clone derived from a single parasite (e.g., E5 is a subclone of 3D7).

stages within the human host is referred to as 'reproductive restraint'. Through reproductive restraint, a certain malaria parasite subclone can outperform other competing subclones and even other malaria parasite species, since investing in asexual replication means dominance over host resources and better survival (177–179). Reproductive restraint is also a strategy of the transmissible stage to evade the host immune system as it lowers the odds of gametocyte antigens exposure, thus resulting in a non-neutralizing antibody response against the sexual forms (180–182). Although such a strategy is not advantageous for parasite spread because it reduces total gametocyte production, the contrary would also come with a cost as dramatic gametocyte investment might erode the parasite strategy to survive within the host (183).

The reason why reproductive restraint appears to be a default strategy in *P. falciparum* is unclear but evolutionary biologists suspect it is connected to competition between parasite species and strains (177–179). However, this hypothesis remains controversial because, in some human studies, mixed infection between *P. malariae* and *P. falciparum* promotes a much higher gametocytemia of the latter (184,185), and multiclonal *P. falciparum* infection may enhance high gametocyte carriage (186). Under *in vitro* conditions, each *P. falciparum* subclone demonstrates unique levels of gametocyte investment, with either low (~1%) or high (~15%) sexual conversion rates even in the absence of competition (100). These findings suggest that coinfection is not only the driving force of reproductive restraint and that other factors are at play.

6. External triggers of sexual conversion in *P. falciparum*

It is widely thought that sexual conversion is a stress-related adaptation because gametocytes are relatively resistant to harmful conditions commonly encountered by the parasite. This means that gametocyte investment is a strategy for future species preservation when asexual stages sense an extreme microenvironment that might compromise parasite's survival within the human host (183). In *P.*

falciparum, the rate of sexual conversion is low compared to asexual multiplication. What triggers the holdback from increasing the sexual conversion rate beyond the baseline level is still a continuous debate. Mounting evidence shows that microenvironmental factors can modulate malaria transmission by changing the rate of parasite's sexual conversion. Yet, key information on how microenvironmental factors are being translated into a signal and received by epigenetic regulators that control the sexual commitment gene *pfap2-g* remains a long-standing question (109,187,188).

6.1. Phospholipids biosynthesis factors

Thus far, the most consistent and reproducible among sexual conversion stimuli is a low level of the human serum factor lysophosphatidylcholine (LysoPC) and its derived metabolite choline (135). LysoPC is a normal component of the human serum and is taken by the parasites as a source of choline and fatty acids to build its cell membrane during the erythrocytic cycle. When the parasite reaches the trophozoite and schizont stages, the nutrient requirements become demanding, including for the *de novo* biosynthesis of phospholipids. This cell demand may reach up to 6-fold relative to the demand of the ring stage. In infected erythrocytes, the most abundant phospholipid is phosphatidylcholine (PC), which is primarily formed via a stepwise enzymatic process called the cytidine diphosphate (CDP)-choline-dependent Kennedy Pathway (**Figure 15**) (189–191).

In the absence of choline, the parasite can use ethanolamine for PC biosynthesis via the phosphoethanolamine methyltransferase (PMT) pathway. The parasite nucleus has its own stock of ethanolamine, which is derived from the decarboxylation of serine – a byproduct of hemoglobin degradation. In brief, the PMT pathway involves triple sequential methylation of phosphoethanolamine to generate phosphocholine via S-adenosylmethionine donors (189–191).

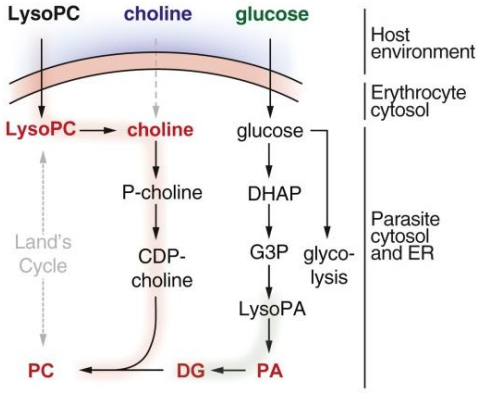

Figure 15. Phosphatidylcholine synthesis in *P. falciparum*. PC synthesis requires LysoPC or choline and glucose. When LysoPC enters the parasite, it is hydrolyzed to form choline, which is phosphorylated by choline kinase to form phosphocholine (P-choline). P-choline is used as a precursor to creating cytidine diphosphate choline (CDP-choline), which is transformed into phosphatidylcholine (PC) by 1,2-diacylglycerol choline phosphotransferase (190). Glucose also participates via glycolysis, which forms the PC precursors – the phosphatidic acid (PA) and diglyceride (DG). Moreover, LysoPC can be acylated to form a PC (Land's Cycle) (135). Figure illustrated by Brancucci et al. (2017) (135).

The level of LysoPC or choline is inversely correlated to the level of sexual conversion rate in different *P. falciparum* strains (**Table 3**). The depletion of LysoPC and choline will dramatically enhance the rate of sexual conversion but will also negatively impact the rate of asexual stage multiplication (97,135). LysoPC may be depleted during the course of severe malaria infection and most likely in many specialized tissues (e.g., bone marrow) under normal conditions (91,135). Previous *in vitro* studies describing a method where parasites were allowed to reach a level of hyperparasitemia (150,192) and where a spent culture medium was applied to induce gametocytemia (159,193) are now considered an effect associated with LysoPC depletion (136,159). The relationship between LysoPC depletion and the parasite sexual conversion will also depend on the parasite factors and nutrients present in the serum. For instance, the bioavailability of LysoPC in the serum will depend on its binding to the protein albumin (135).

Table 3. The sexual conversion rate of *P. falciparum* under inducing and non-inducing levels of lysophosphatidylcholine and choline

External factors	Parasite line/strain	Approximate SC rate (%)		Method	Reference
		CTL	Induce		
Lysophosphatidyl choline (serum depletion)	Pf2004/164tdTom	1	30	FACS	(135)
	HB3/748tdTom	2	20	FACS	
	NF54	7	30	Giemsa	
Choline depletion	3D7/3×HA-GDV1	2	30	Giemsa	(97)
	NF54-gexp02-Tom	7	60	FACS	(143)
	NF54-10.3-Tom	20	40	FACS	
	3D7/E5-gexp02-Tom	2	17	FACS	
	3D7/E5-10.3-Tom	3	14	FACS	

SC= Sexual conversion; CTL= Non-induced condition; FACS= Flow cytometry
Note: Choline depletion data is part of Chapter 2 of this thesis.

When LysoPC is depleted, it follows a stepwise activation of genes that are involved in epigenetic regulation, phospholipid biosynthesis, and kinase signaling. In particular, a limiting level of LysoPC will stimulate two of the most important sexual commitment biomarkers, *pfap2-g* and *gdv1,* in addition to the upregulation of genes involved in the parasite metabolism, including the *ethanolamine kinase* (*pfek*; PF3D7_1332500), *phosphoethanolamine N-methyltransferase* (*pfpmt*; PF3D7_1343000), and *S-adenosylmethionine synthetase* (*sams*; PF3D7_0922200), among others (135). The latter set of genes has something to do with the upregulation of the PMT pathway to compensate for the Kennedy Pathway due to choline deprivation. However, how sexual commitment gets turned on after LysoPC depletion is still an enigma. One possible explanation is that when parasites divert into the PMT pathway, the pathway snatches certain resources (e.g., SAMS enzyme) that are needed for the silencing of the *pfap2-g* locus (e.g., histone methylation), thus making the gene active (194,195).

6.2. Erythrocytic factors

RBC conditions have been associated with an increase in *P. falciparum* gametocytemia, as described in several cross-sectional studies and *in vitro* experiments. Increased prevalence of gametocyte carriage has been linked to anemia, a pathological condition in patients characterized

by decreased hemoglobin and low hematocrit levels. Two independent studies in Thailand showed that patients with anemia are likely to be gametocyte carriers, but several other factors, including drug treatment and the severity of malaria infection, have also been found to associate with the increase in prevalence of gametocyte carriage (196,197). In The Gambia and Ghana, low hematocrit levels have been associated with an increase in gametocyte carriage among children (198,199). However, several studies also described that a high gametocyte carriage in children commonly associates with parasite density, immune response, and the severity of infection (200,201).

Under controlled conditions, immature types of RBCs, specifically reticulocytes and orthochromatic normoblasts, have been shown to favor gametocyte production (202–204). Furthermore, stimulation with erythropoietin *in vitro* did not increase the rate of sexual conversion, thereby excluding the possibility that the erythropoietic hormone directly triggers gametocyte production (203) (**Table 4**). The increase in gametocyte numbers in the presence of immature RBCs was previously explained as a form of stage-switching when available resources are favorable to the parasite (205). Because reticulocytes are more metabolically enriched than mature RBCs and may have an advantage in terms of longer circulation period, it is speculated that these factors drive the conversion of asexual parasites to gametocytes (206). However, one may argue that instead of resource availability, the stimulating factor might be the lack of resources in reticulocyte, such as the deficiency of hemoglobin (207).

To connect between the observations of cross-sectional studies on anemia and *in vitro* experiments on reticulocytemia, it is hypothesized that the putative increase in gametocyte levels under field settings is attributed to the massive production of reticulocytes in response to anemia. In human malaria physiopathology, widespread parasite-induced hemolysis usually leads to severe anemia, which then activates an adaptive response in the erythropoietic system by accelerating the production and

release of hematopoietic cells, including the early circulation of reticulocytes (198,208,209). However, recent studies demonstrated that reticulocyte occupancy of the parasite did not affect the levels of sexual conversion in *P. falciparum* (104,135), arguing that if a true increase in sexual conversion occurs during parasite-induced anemia, it likely arises from indirect host changes. These findings imply an indirect relationship between erythrocyte factors and sexual conversion (210,211).

Table 4. Erythrocytic factors and *in vitro* gametocyte production in *P. falciparum*

External factors	Source	Parasite line/strain	Approximate Gametocyte level		Method	Reference
			CTL	Expose		
Reticulocytes	HbS	HB-3	0.5%	1.92%	Giemsa	(204)
	HbS	3D7	3.5%	5.5%		
	HbS	HB-3	3.35%	10%		(202)
	PNH	HB3	2.4%	7.3%		
	NUT	HB3	2.4%	10.4%		
	AI	HB3	1.3%	13%		
	HbC	HB3	0.9%	10.8%		
Reticulocytes and orthochromatic normoblasts	Umbilical cord blood	3D7-Pfs16	200 counts	400 counts	FACS	(203)
Erythropoietin	Commercial	3D7-Pfs16	200 counts	190 counts	FACS	(203)
Lysed uninfected erythrocytes	Normal human blood	NF54	0.53%	0.75%	Giemsa	(212)
		NF54	17 counts	26 counts	Giemsa	(211)
Lysed infected erythrocyte	Normal human blood	NF54	17 counts	25 counts	Giemsa	(211)
Hemoglobin depletion	Hypotonically shocked RBC	GC03	0.25%	2.5%	IFA	(207)
		Dd2	0.4%	1.5%	IFA	
		HB3	0.25%	0.25%	IFA	

CTL = Non-inducing control condition (e.g., normal RBC); Expose = exposure to erythrocytic conditions. HbS = Patients with sickle cell anemia; PNH = Paroxysmal nocturnal hemoglobinuria; NUT = Nutritional anemia under treatment; AI: Autoimmune hemolytic anemia; HbC = Homozygous hemoglobin C disease; RBC = Red blood cells

6.3. Immunological factors

The role of host immunity on gametocyte production has been documented under *in vitro* conditions by exposing *P. falciparum* to immune cells and antibodies. Combined malaria-immune sera and lymphocytes from naturally infected Gambian children were shown to enhance gametocyte numbers up to 5-fold relative to the control group (sera from malaria-naïve individuals) in culture-adapted *P. falciparum* (213). Moreover, the anti-*Plasmodium falciparum* antibodies derived from

hybridoma cells demonstrated a similar pattern of gametocyte increase (214–216), again indicating the role of immunity as a trigger of sexual conversion. Interestingly, one of the studies showed that exposure to anti-*Plasmodium falciparum* antibodies may display a stage-dependent stimulation of sexual conversion, such that exposure at the trophozoite and ring stages may increase and decrease the number of gametocytes, respectively (215).

In human infection, the impact of immunological factors on sexual conversion is not well explored, although there are studies associating immunity to the degree of malaria transmission. The main effect of immunity however is linked to selective pressure that may result in a population-wide adaptation of gametocyte production levels, such that under low transmission settings, parasites tend to be selected for transmission investment by producing more sexual forms when compared to those parasites from high transmission settings (217). This observation may be of paradox to *in vitro* studies (214–216), where the presence of malaria immune response (a relatively comparable situation to high transmission intensity) was shown to increase gametocyte production.

Acquired immunity against the asexual stages may also affect the level of gametocytes since most field studies found that semi-immune adults are less likely to be gametocyte carriers than the less-immune children (200,201). Interestingly, despite the anti-correlation between age and gametocyte prevalence, quantification of gametocyte numbers relative to asexual stages across age groups revealed that semi-immune adults showed a higher relative gametocyte density than those less-immune children (218). Although these observations do not imply a direct impact on sexual conversion, it supports the previous notion that immunity may play a role in determining the levels of gametocyte production (73). Hence, future works should address how gametocyte dynamics is affected by an immune-induced sexual conversion and an immune-mediated damage to the sexual forms.

6.4. Hormonal factors

Previous reports examined whether different human hormones can influence the production of gametocytes on the basis that parasites always encounter human hormones in natural infections. *In vitro* studies in *P. falciparum* that tested the effect of hydrocortisone, progesterone, 17-beta-estradiol, testosterone, and insulin, showed an induced gametocyte production up to 3 folds by steroid hormones, while exposure to insulin only increased the level of parasitemia but not gametocytemia (219,220). However, the effect of corticosteroid *in vitro* was also shown to be inconsistent or probably dependent on parasite strain (221).

6.5. Parasite factors

Parasite extracellular vesicles (EVs) may serve as a vehicle to encapsulate parasite RNA, DNA, proteins, organelles, or even acquired drugs, and secrete them outside for parasite-to-parasite and parasite-to-host cell communication. Malaria parasites can generate exosomes (40 to 120 nm) and microvesicles (50 to 1000 nm) that are formed by membrane invagination and membrane outward budding, respectively (222,223). Two studies demonstrated that microvesicles and exosomes from asexual parasites can be internalized by other parasites and then stimulate both gametocyte production and sexual conversion rate (224,225). These findings suggest that parasites communicate among themselves to modulate sexual commitment. However, it is unclear what part of the EV cargo is involved in signal transduction, since it needs to be at a much higher than physiological concentrations to induce an increase in the sexual conversion rate. In line with this, recent research demonstrated that EVs only play a minor role in stimulating sexual conversion (135).

Parasite growth dynamics was also shown to affect sexual conversion in *P. falciparum*. Dyer and Day (2003) postulated two models of how asexual growth governs sexual commitment: (i) the presence of internal metabolic factors that are sensitive to growth dynamics and (ii) the presence of diffusible parasite growth factors that influence each parasite's

decision. By co-culturing different pairs of *P. falciparum* strains across semi-permeable membranes, they observed a 3-fold reduction in the sexual conversion in one group when the other group is undergoing a log-phase asexual replication. They concluded that the diffusible molecules, which stimulate asexual replication, negatively affect sexual commitment. However, they were unsuccessful in isolating any type of diffusible molecules that can trigger growth enhancement or repress sexual commitment (226). By contrast, new research development clearly shows that the modulation of the parasite's sexual conversion is strongly connected to the level of human LysoPC in the serum rather than the involvement of diffusible parasite factors (135).

6.6. Signaling pathway modulators

Pharmacological agents interacting with the parasite's signaling pathways have been documented to modulate sexual conversion rates. The cyclic adenosine monophosphate (cAMP), which is implicated in intracellular signal transduction by activating protein kinase A, has been reported to operate in the process of sexual commitment. The addition of caffeine, theophylline, and Na_2EDTA, which are all antagonists of phosphodiesterase that is involved in cAMP regulation, was shown to enhance gametocyte production at a modest level (1.5- to 2-fold increase) (227). However, the direct addition of cAMP into the parasite cultures yielded divergent results, raising doubts about its role in sexual commitment (228,229).

The phorbol ester and G protein pathways, which also operate in signal transduction, have been reported to play a role in sexual conversion. Phorbol ester is involved in the activation of protein kinase C or the serine/threonine protein kinases and has been shown to enhance gametocyte production by ~3-fold in *P. falciparum* cultures (220). The heterotrimeric G protein-dependent system was also linked to gametocyte formation, as shown by the effect of cholera toxin and Mas 7 (Mastoparan analogue) compounds, which both interact with the classical components

of G protein signaling. In a few experiments, these compounds increased the gametocyte production by 2 to 3 folds in *P. falciparum* cultures (230,231). Further validations however are still needed to confirm that signal transduction truly takes part in sexual commitment (94).

6.7. Endoplasmic reticulum stressors

Bioinformatics analysis revealed that *P. falciparum* lacks a certain component of the canonical endoplasmic reticulum (ER) stress response, whereby the ER response machinery is presumed to be dependent mainly on the eIF2-α-mediated protein translational attenuation. Hence, it was postulated that sexual conversion may underpin an escape mechanism against the massive load of misfolded proteins during ER stress, as it overwhelms the less-functional ER stress response. This hypothesis is supported by a 2- to 3-fold increase in gametocyte production and *pfap2-g* levels upon parasite exposures to drugs that cause ER stress (232,233).

6.8. Pharmacological agents

Treatment with diminazene aceturate (234) and ammonium compounds (ammonium carbonate and ammonium bicarbonate) (235) were shown to enhance *P. falciparum* gametocyte production *in vitro*. Diminazene aceturate is a known inhibitor of nucleic acid synthesis and S-adenosyl-l-methionine decarboxylase, an enzyme involved in polyamine biosynthesis. It is also used for the treatment of other protozoan parasites, such as *Babesia* and *Trypanosoma* (234). The role of ammonium compounds, specifically ammonium bicarbonate, in parasite cultures is limited to its use as a buffer agent (236), and it's unclear whether it affects the parasite's sexual commitment. Like any other stimuli, the mechanisms of action of how these drugs may induce sexual conversion remain unexplored along with a thorough validation of the sexual conversion rate (139). The impact of clinically-relevant drugs on sexual conversion is discussed in the next section.

7. Impact of antimalarial drugs on sexual conversion

Antimalarial drugs are designed to cure malaria patients by eliminating parasite stages in the blood and tissues. Although all groups of antimalarial drugs are effective against the replicating asexual stages, their efficacies are limited against gametocytes, which are clinically silent and resistant to most antimalarial drugs. There are several groups of antimalarial drugs with various modes of action that are clinically used for chemoprevention and treatment (**Figure 16**). To date, antimalarials have been (at least initially) highly efficacious in curing infections; prolonged use however leads to the selection of drug-resistant parasite strains (17). On top of that, reports of increasing gametocyte production after treatment triggers additional concern for the sustainability of current antimalarials. The effect of antimalarial drugs on sexual conversion is explored using various parasite models, experimental designs, and tools to measure gametocyte levels. With previously limited tools and understanding of how gametocytes are formed and on best approaches to testing drug induction hypotheses, many past studies lack the conclusive data to understand fully the impact of treatment on sexual conversion (73,237).

In the following sections, I discuss in detail the different studies performed to determine whether clinically relevant antimalarial drugs, such as chloroquine, sulfadoxine-pyrimethamine, and artemisinin, can promote gametocyte production *in vitro* and *in vivo*. In **Chapters 3 and 4**, we interrogate the sexual conversion of *P. falciparum* upon exposure to some of the currently available antimalarial drugs.

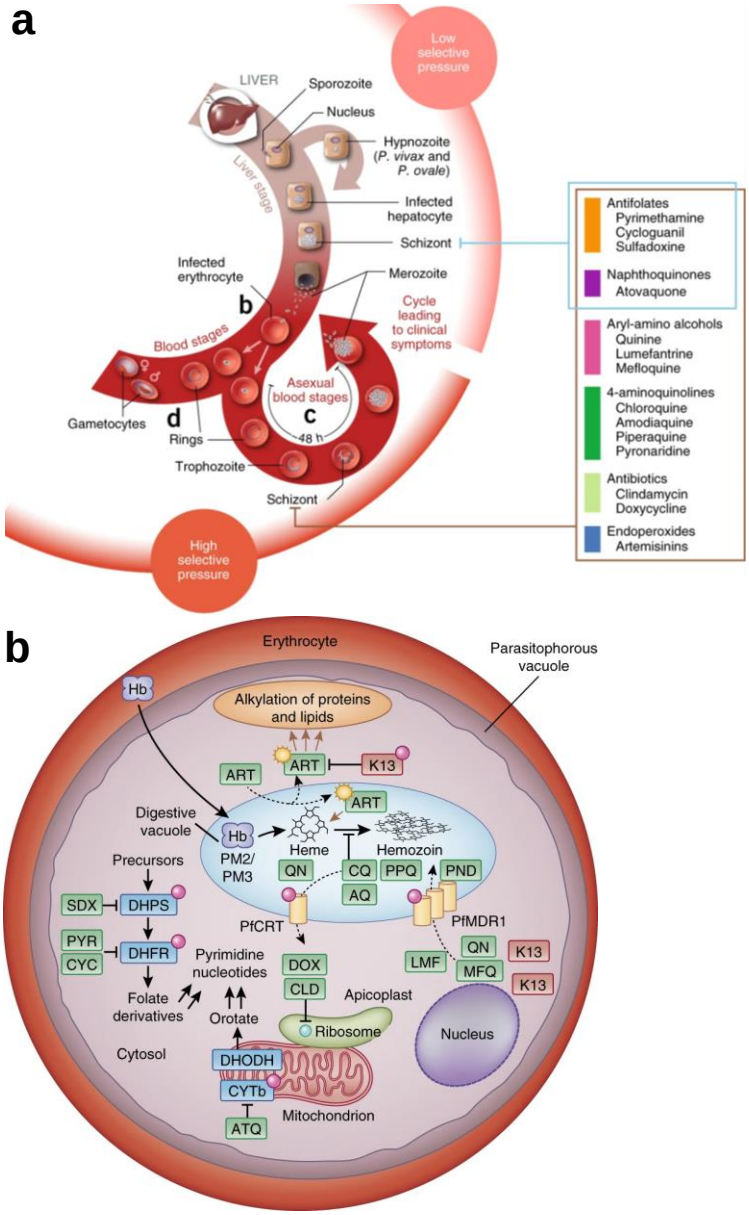

Figure 16. Antimalarial drugs. (a) Six different groups of antimalarial drugs and the targeted asexual stages. (b) Mode of actions and mechanisms of resistance of common antimalarial drugs. The targeted biological pathways where antimalarial drugs (green boxes) take effect include heme detoxification in the digestive vacuole, folate and pyrimidine biosynthesis (blue boxes) in the cytosol, electron transport (blue boxes) in the mitochondrion, and protein translation inside the apicoplast. The modes of resistance include mutations (pink circles) in the transporters PfCRT and PfMDR1 for 4-aminoquinolines group, plasmepsin 2 and 3 for piperaquine (PPQ), PfK13 for artemisinin (ART), key enzymes DHPS and DHFR for sulfadoxine (SDX) and both pyrimethamine (PYR) and cycloguanil (CYC), and CYTb for atovaquone (ATQ). Other abbreviations: chloroquine (CQ); amodiaquine (AQ); aryl-amino alcohol quinine (QN); pyronaridine (PND); lumefantrine (LMF); mefloquine (MFQ); clindamycin (CLD); doxycycline (DOX). Figure Illustrated by Blasco et al. (2017)(17).

7.1. Chloroquine (CQ)

7.1.1. CQ characteristics and mechanism of action

Chloroquine belongs to the group of 4-aminoquinolines and has been used to treat malaria since the 1940s. CQ has the advantage of being highly effective against malaria parasites with few human side effects and being affordable as a result of its relatively cost-effective production (238). As with most commonly used antimalarial drugs, CQ targets the mature

forms of the parasites in which host hemoglobin (Hb) degradation takes place. However, CQ is ineffective against gametocytes (17).

In the parasite food vacuoles, Hb degradation produces a large amount of noxious hematins that are normally deactivated into a neutral product called hemozoin, a chemically inert crystal lattice of heme dimers. CQ molecules enter into the food vacuole by simple diffusion and start protonating due to the acidic pH (pH ~5) environment, making the molecules trapped inside the vacuole. The diprotic CQ interferes with the parasite detoxification process by inhibiting heme transformation into hemozoin, resulting in oxidative stress and thereby severe parasite damage (239). Over the years of CQ application, parasites overcome this effect by developing several point mutations in the genes coding for digestive vacuole membrane proteins, specifically the *P. falciparum chloroquine resistance transporter* (*pfcrt*) and *multidrug resistance 1* (*pfmdr1*). These mutations mediate the H^+-dependent efflux of CQ outside the digestive vacuole, rendering CQ molecules unavailable inside the vacuole (17).

7.1.2. Impact of CQ on sexual conversion

Subcurative CQ has been shown to increase sexual conversion rates by up to 5-fold in *P. falciparum* under laboratory conditions (240,241). Buckling and colleagues (1999) exposed the parasites to CQ for 17 h starting from the late ring stages and observed an increasing level of sexual conversion rates along with a decreasing asexual growth, all relative to the untreated control. The induction varied depending on the parasite strains, with 3D7 and SUD124/8 showing a 2-fold greater effect compared to HB3 and 7G8, supporting the idea that natural genetic variation may influence sexual conversion. Moreover, sexual conversion rates and net gametocyte production did not significantly vary between CQ-resistant strains (SUD124/8 and 7G8) and CQ-sensitive strains (3D7 and HB3), suggesting that previous drug pressure selection did not modify the inherent sexual conversion response (240). In another study, following CQ exposure at different inhibitory concentrations (IC10 to IC50) in a 3D7

parasite background, a 4-fold increase of net gametocytemia was observed at IC10 but not at other doses, supporting the previous finding that sublethal concentrations may enhance the production of sexual forms. Antimalarial drugs with an almost identical mode of action with CQ (e.g., aryl-amino alcohol and 4-aminoquinoline groups) showed a net gametocyte increase upon IC10 and IC50 exposures (241). In contrast to these findings, Reece and colleagues (2010) showed that *P. falciparum* field isolates underwent reproductive restraint upon subcurative CQ exposure, explaining that decreasing gametocyte production upon sublethal stimuli is advantageous for their survival by producing more progeny through asexual replication (242).

In a rodent malaria model, subcurative CQ treatment of *P. chabaudi* was shown to increase the rates of sexual conversion up to 2.5 fold, although the net gametocytemia was much lower than in the untreated control (243,244). The low net gametocytemia was due to the killing effect of CQ that affected the growth rate of the parasite. This suggests a correlation between enhancing sexual conversion and the destruction of asexual replication (243).

In several human infections, CQ treatment has been implicated for increased risk of gametocytemia on days 7 and 14 post-initial drug exposures. However, detailed analyses revealed that the effect was more directly associated with CQ resistance. Therapeutic failure linked to CQ-resistant parasites significantly enhanced the odds of having circulating gametocytes on days 7 to 14 compared to those patients harboring CQ-sensitive parasites (245–247). Hence, one of the explanations on days 7 and 14 gametocytemia is the inability of CQ to substantially reduce the parasite burden throughout the treatment course.

7.2. Sulfadoxine-pyrimethamine (SP)

7.2.1. SP characteristics and mechanism of action

In the 1990s, SP replaced CQ as malaria treatment and chemoprevention during pregnancy. To date, SP is used as IPTp with

proven benefits of improving neonatal birth weight (248). Sulfadoxine and pyrimethamine inhibit dihydropteroate synthase and dihydrofolate reductase enzymes, respectively, which are involved in folic acid synthesis. For a rapidly growing malaria parasite, folate derivatives are essential cofactors for nucleotides and amino acid synthesis. Therefore, asexual parasites treated with SP do not progress to the end of the cycle. Gametocytes are partially to totally resistant to SP owing to their relatively dormant metabolic state (17,249).

In terms of drug resistance, pyrimethamine resistance due to mutations in *pfdhfr* and sulfadoxine resistance due to point mutations in *pfdhps* are spreading across malaria-endemic areas. With cases of multiple mutations, the drug efficacy of SP is severely affected, thereby limiting its use as a chemopreventive and curative drug combination (17).

1.1.1. Impact of SP on sexual conversion

The correlation between SP treatment and enhanced sexual conversion is presumed in many clinical reports that observed an increased prevalence of gametocyte carriage and density after treatment (73). However, molecular evidence suggests that the increase is rather immediate and does not correspond to the timing expected if there was induction of sexual conversion followed by normal gametocyte development and release from the bone marrow (237,250). Therefore, the efflux of immature gametocytes from the bone marrow, rather than induction of sexual conversion, has been proposed as the reason for increased gametocyte density after SP treatment. Supporting the idea of premature gametocyte expulsion, mosquito infection assays revealed that gametocytes from SP-treated patients are less infectious despite the relatively higher density in the blood (251). One alternative explanation for the reduced infectiousness to mosquito was the sterilizing effect of SP: SP-exposed gametocytes are likely to fail to develop further inside the mosquito midgut because of selective killing of male gametocytes by SP (252).

Only a few studies on SP-induced sexual conversion have been reported *in vitro* because performing antimalarial assay that involve sulfadoxine and pyrimethamine is not straightforward. The culture medium RPMI 1640 used for *in vitro* culturing of *P. falciparum* contains para-aminobenzoic acid and folic acid, which are both antagonists of antifolate drugs. While adjusting the culture conditions successfully allows the *in vitro* tests with pyrimethamine and to a lesser extent with sulfadoxine, the use of *in vivo* tests remains the preferred option (253). Nevertheless, Reece and colleagues examined the *in vitro* sexual conversion rates of *P. falciparum* upon pyrimethamine exposure and found a decreasing trend, corroborating their working hypothesis on reproductive restraint (242). The same group performed a sexual conversion experiment in a rodent malaria model by exposing *P. chabaudi* to varying pyrimethamine dosages. They concluded that a subcurative level of the drug repressed gametocyte investments while a curative dose resulted in the total allocation of all resources to sexual conversion (a.k.a. terminal investment). In contrast to their findings, a previous study on subcurative pyrimethamine in *P. chabaudi* revealed an enhanced gametocytemia after treatment, suggesting that, like many other stressful stimuli, this drug also triggers sexual conversion (244).

7.3. Artemisinin (ART) and its derivatives

7.3.1. ART characteristics

Artemisinin (ART) is a phytochemical extract derived from the wormwood plant *Artemisia annua*, which is used in traditional Chinese medicine for the treatment of fever. Artesunate, artemether, artemotil, and dihydroartemisinin (DHA) are among the derivatives of ART with improved potency, bioavailability, and outstanding human safety profile (16,254). All forms of ART are converted into the active metabolite DHA *in vivo* through the liver cytochrome P450 CYP2A6 and plasma esterases. DHA is 1.4 times more active than artesunate and likely the most potent form among the ART derivatives. The main drawback of artemisinin is its very short

elimination half-life (~1 to 2 h) in human patients. For ART monotherapy, it may need 7 consecutive days to completely eliminate a 2% initial parasitemia in a human adult. Nevertheless, ART administration can drastically decrease the parasitemia after 4 to 6 h resulting in a 10,000-fold reduction in parasite numbers after 48 h. Affected parasites become pyknotic and are rapidly cleared from the bloodstream through a splenic pitting mechanism (255,256).

To compensate for its short half-life, ART is co-administered with a partner drug that is long-acting, such as piperaquine with ~33 days half-life, hence creating the so-called artemisinin-based combination therapies (ACTs). In ACTs, the ART component is responsible for a rapid bulk reduction of parasite loads in the first two days while the long-acting partner drug takes over the killing of the remaining parasites in the succeeding days. This strategy optimizes the therapeutic effects of both components while minimizing the odds of acquiring resistance to both antimalarial drugs (16,254,257).

7.3.2. ART mechanism of action

ART has a sesquiterpene lactone with a unique endoperoxide bridge that is responsible for antimalarial activity. The interaction between ART's distinct endoperoxide bridge and the parasite's free heme or ferrous ions in the food vacuole results in the reductive scission of the drug. The process of ART reductive scission generates reactive oxygen species and carbon-centered free radicals that cause widespread protein and lipid damage in parasites. Recent research has pinpointed a DHA-mediated killing mechanism that damages the proteostasis network of the parasite by causing protein misfolding and inhibiting newly synthesized proteins to undergo proper folding (258–260). The DHA-killing mechanism also underpins a prolonged ER stress that leads to an increased eIF2-α phosphorylation and protein synthesis attenuation (259).

7.3.3. ART resistance

Previous studies in Southeast Asia identified a correlation between delayed parasite clearance after ART treatment and specific mutations in the propeller domain of a Kelch protein gene (*pfk13,* PF3D7_1343700), specifically 580Y, 539T, 543T, 493H, and 446I (261,262). It was unclear how these mutations decrease the efficacy of ART until recently when Birnbaum and colleagues (2020) demonstrated that PfK13 regulates hemoglobin (Hb) endocytosis, such that mutations in this domain would diminish the intake of Hb in rings (**Figure 17**). Depletion of Hb means lesser Hb degradation and so the levels of free heme and ferrous ions in the parasite food vacuole would reduce, which in turn halts the activation of DHA (263). Phenotypically, resistant parasites are characterized by ring stages that undergo delayed development upon ART exposure, meaning ring-stage parasites would delay its development until ART is eliminated from the blood (264).

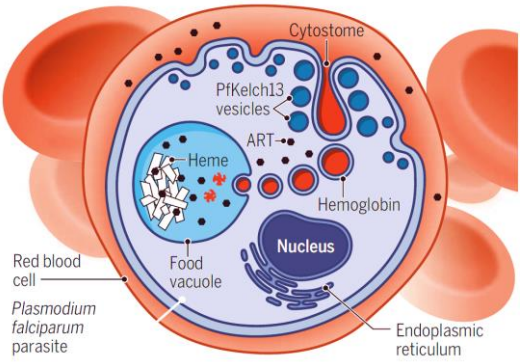

Figure 17. The process of ART activation inside the malaria parasite. The parasite engulfs the host hemoglobin (Hb) using its cytostome. Hb traffic in the parasite cytoplasm is regulated by PfK13-containing vesicles, of which mutation results in a lesser amount of Hb entering the parasite food vacuole in ring stages. Once Hb is degraded in the food vacuole, it releases heme and ferrous ions as by-products that bind to ART. This binding activates the ART, generating reactive oxygen species and free radicals that are extremely toxic to the parasite (263). Figure was taken from Marapana and Cowman (2020) (332).

7.3.4. Geographical distribution of ART resistance

ART resistance in the form of delayed parasite clearance has initially been reported in Western Cambodia in 2008 (265) and soon after in the borders of Thailand-Myanmar, Thailand-Cambodia, and Cambodia-Vietnam (266). Now, artemisinin resistance in the Greater Mekong subregion is becoming more evident and is characterized not only by

delayed parasite clearance (suspected resistance) but also by treatment failure (**Figure 18**). However, in many scenarios, treatment failure of the ACT regimen is more likely associated with parasites harboring multiple mutations against partner drugs such as mefloquine and piperaquine, in addition to the ART itself (25,266,267).

In other countries, the prevalence of suspected ART resistance was relatively low, although mutations in the K13 domain may appear common. In several African countries, K13 mutations were found at a low prevalence rate without being associated with treatment failure and delayed parasite clearance phenotypes (266,268–270). The low frequency of K13 mutants in African settings suggests that they reflect the richness of locally different parasite genetic background rather than a result of drug selection or transfer of K13 mutant parasites from Southeast Asia (266,271).

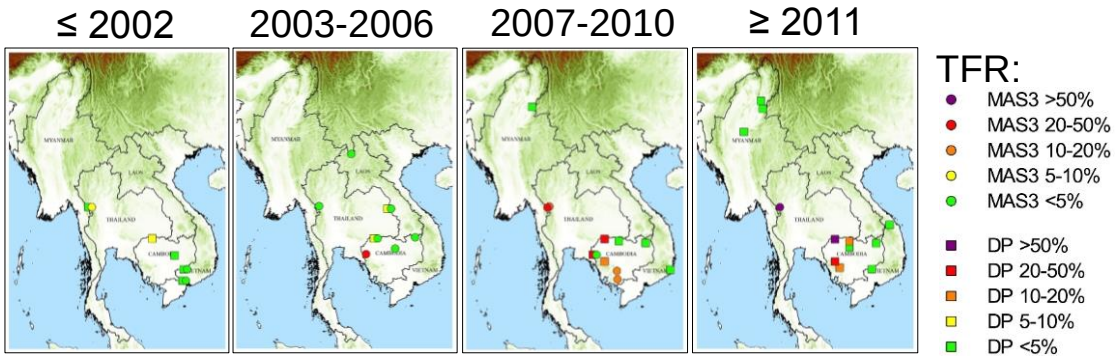

Figure 18. Reports of ACT failure in the Greater Mekong subregion. Treatment failure (TFR) is characterized by the following WHO criteria: persistence of parasite until day 7 or presence of parasite on day 3 and recrudescence on day 28 or 42 after treatment, given that ART reaches adequate blood concentration. Studies with ≥42-day treatment follow-up were included in the map. MAS3 = artesunate + mefloquine; DP = Dihydroartemisinin + Piperaquine.

7.3.5. Impact of ART on sexual conversion

In field settings, all forms of ACT almost always diminish the transmission potential of a malaria infection by significantly reducing gametocyte density and carriage, as well as by rapidly decreasing asexual

replication, which provides a continuous source of gametocytes. Because ART is highly effective against immature gametocytes, it can also deplete the sequestered gametocyte reservoir in the bone marrow that in turn lessens the number of circulating mature gametocytes (272).

Under *in vitro* conditions, Peatey and colleagues (2009) examined the levels of net gametocyte production in a 3D7 strain (chimeric Pfs16-GFP reporter line) after a 48-h treatment with ART (starting at the ring stage) and observed a significant increase of raw gametocyte numbers at the IC50 (10 nM) level compared to the untreated control. However, this potential increase of sexual conversion was not observed by another group after treating with DHA an *etramp10.3*-tagged Pf2004 strain (159). Meanwhile, anecdotal observation (not well-controlled study) in K13 mutant strains revealed that parasites exposed to curative ART dose (700 nM) that underwent dormancy may show a relatively higher gametocytemia than the ART-sensitive NF54 strain (273).

8. Gaps and challenges in sexual conversion studies

8.1. Sexual conversion rate *in vitro*

To measure whether a certain factor enhances the rate of sexual conversion, one must accurately quantify the level of gametocytes and the total parasitemia in a synchronous population at a given cycle. This approach would allow the interrogation of stimuli to determine whether prior exposures lead to changes in sexual commitment and subsequent sexual conversion. The net gametocyte number is an informative measure of malaria transmission as it correlates with mosquito infection (77,168,274). However, as a sole readout in sexual conversion assay, as described in many previous studies, the net gametocyte number may not be an accurate method to determine the impact of external stimuli on the sexual conversion rate.

The potential biases of relying solely on gametocyte numbers are exemplified by the following conditions: (i) the starting parasitemias

between treatment groups and its comparator are different from the time of stimulus or the stimulus itself affects the multiplication rate of the parasite, (ii) the multiplication rates of two parasite lines being compared are not similar, (iii) and the method of gametocyte culture did not involve the addition of compounds to stop further asexual replication. On top of that, sexual forms are inherently low in numbers and are difficult to distinguish from the asexual parasites at the early stages, resulting in significant variability when counted by microscopy (159,237). In **Chapter 2**, we describe the common methods used to examine sexual conversion and present a robust technique to accurately measure both gametocytemia and the rate of sexual conversion.

8.2. Sexual conversion rate in human studies

In a human study, the impact of external factors on sexual conversion has never been clearly demonstrated because of difficulties in disentangling other factors, including host immunity (see Section 6.3) and parasite stage dynamics, that may interact with the stimulus in question (73). As a result, almost all studies are limited to the statistical association of certain factors with different gametocyte readouts but not directly on sexual conversion rates. Most of these factors involve clinical features, type of interventions or human and parasite factors, among others (see Section 6). Also, most of the readouts are based on gametocyte density and/or gametocyte carriage in a cross-sectional study. Gametocyte density indicates how many Stage V gametocytes are there in each patient while gametocyte carriage tells how many individuals are positive and negative for gametocytes. Neither one of the two parameters can directly measure sexual conversion when assessed in a cross-sectional study. When measuring gametocyte levels, a one-time snapshot gives little information on the effect of factors on sexual conversion as the presence of circulating gametocytes today indicates an event that happened ~1-2 weeks before. Therefore, a cohort study with a multiple sampling design is the most

appropriate approach to assess whether certain factors could enhance sexual conversion (274).

To address whether there is an increase or decrease in gametocyte production, molecular tools using female gametocyte biomarkers are used as a surrogate test because of its high mRNA abundance, with well-established gametocyte sensitivity and specificity. In particular, the *pfs25* biomarker has been widely employed as a well-suited tool as it correlates with gametocyte density and mosquito infection rates (77,168,274). More recently, sexual ring biomarkers are employed to assess human patients for early signs of malaria transmission potential (91,275). Therefore, a time-course analysis of mature gametocytes to examine sexual conversion can be improved by initial analysis of sexual rings, which are relatively numerous and immediately present in the circulation before they are sequestered in the tissues as young gametocytes.

9. Hypothesis

Understanding what drives the production of more gametocytes is a crucial facet for the improvement of current tools and strategies used to implement malaria elimination initiatives. We hypothesize that artemisinin, the main drug component of the frontline treatment against clinical malaria, may stimulate sexual conversion by upregulating the sexual commitment gene *pfap2-g* in *P. falciparum*, which may result in the subsequent production of more mature gametocytes. This hypothesis underpins the adaptable nature of the parasites to perpetuate under adverse conditions and previous findings that suggest that sexual commitment can be modulated by environmental factors. Furthermore, because interrupting malaria transmission is a complex event, we postulate that such an endeavor requires an amalgamation of societal and scientific knowledge. In this way, we can tailor the right approach to introduce a new intervention, such as MDA, for a successful malaria elimination program in the targeted region.

10. Research objectives

In this Ph.D. project, we combined molecular biology, epidemiology, and qualitative social science to investigate how drug treatment affects the transmission potential of malaria parasites and to examine how community understanding of malaria influences the deployment of new interventions. Specifically, we addressed the following objectives:

1) To develop a robust assay for accurate quantification of sexual conversion rates in culture-adapted parasites.
2) To determine if the clinically relevant antimalarial drugs DHA and CQ affect *pfap2-g* activation and sexual conversion rates in culture-adapted parasites.
3) To examine if the frontline treatment of malaria (ACT) affects sexual conversion in naturally infected patients from different malaria-endemic countries.
4) To understand how community perceptions of malaria and its prevention and control affect the deployment of interventions intended for malaria elimination efforts.

We further linked the results generated from different disciplines to achieve meaningful findings that could contribute to the current goal of malaria elimination.

Outline of the Thesis

In **Article 1**, we optimized a robust sexual conversion assay to investigate the impact of external stimuli on the sexual conversion rate of *P. falciparum*. To specifically identify sexual stages, we generated new reporter lines using the CRISPR-Cas9 technology. Our new *gexp02* promoter-based lines showed the ability to distinguish sexual rings at a single-cell level. With our new reporter parasite lines, we were able to shorten the sexual conversion assay and dispense the need for anti-asexual multiplication compounds (e.g., GlucNAC).

In **Article 2**, we demonstrated *in vitro* that the impact of artemisinin on the sexual conversion of *P. falciparum* is affected by drug concentrations, the parasite stage, and the parasite metabolic state. Our results revealed that exposure to subcurative doses of DHA at the trophozoite stage, but not at the ring stage, upregulated *pfap2-g* expression levels and increased the rate of sexual conversion and the total gametocyte numbers. Gametocytes from artemisinin-induced cultures were infectious to the mosquito vector.

In **Article 3**, we evaluated the impact of artemisinin-based treatment on transcript levels of sexual ring markers in naturally infected patients from Vietnam, Burkina Faso, and Mozambique. We observed a clear upregulation of transcript levels for *pfap2-g* and other sexual ring biomarkers after treatment, suggesting an induced sexual conversion. Artemisinin resistant parasites (PfK13 mutants) from Vietnam showed significantly lower levels of *pfap2-g* induction, whereas sensitive parasites from African cohorts displayed higher *pfap2-g* induction immediately after treatment.

In **Article 4**, we examined the malaria perception of the community in the Magude district of Southern Mozambique. We identified potential constraints and opportunities that might affect the deployment of interventions for the malaria elimination initiative. Malaria awareness, trust in health institutions and openness for new chemoprophylaxis may positively influence the elimination effort. A lack of awareness of asymptomatic carriers, inadequate understanding of residual transmission and barriers to care-seeking might jeopardize the uptake of malaria interventions.

Results Chapters

Article 1

Portugaliza, H.P., Llorà-Batlle, O., Rosanas-Urgell, A. and Cortés, A., 2019. Reporter lines based on the *gexp02* promoter enable early quantification of sexual conversion rates in the malaria parasite *Plasmodium falciparum*.
*__Scientific Reports, 9:1-12. PMID: 31601834__

Article 2

Portugaliza, H.P., Miyazaki, S., Geurten, F.J., Pell, C., Rosanas-Urgell, A., Janse, C.J. and Cortés, A., 2020. Artemisinin exposure at the ring or trophozoite stage impacts *Plasmodium falciparum* sexual conversion differently.
__eLife, 9:60058. PMID: 33084568__

Article 3

Portugaliza, H.P., Guetens P., Magloire, N.H., van Hong, N., Rovira-Vallbona, E., Sitoe, A., Varo, R., Basat, Q., Cortés, A. and Rosanas-Urgell, A. Expression dynamics of early markers of *Plasmodium falciparum* sexual conversion in naturally infected patients treated with artemisinin.
Manuscript in Preparation.

Article 4

Portugaliza, H.P., Galatas, B., Nhantumbo, H., Djive, H., Murato, I., Saúte, F., Aide, P., Pell, C. and Munguambe, K., 2019. Examining community perceptions of malaria to inform elimination efforts in Southern Mozambique: a qualitative study.
***__Malaria Journal, 18:232. PMID: 31296238__

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**Quartile 1 (Microbiology; Medicine); 2019 Impact Factor: 7.080
***Quartile 1 (Infectious Diseases); 2019 Impact Factor: 2.631

Chapter 2

Reporter lines based on the *gexp02* promoter enable early quantification of sexual conversion rates in the malaria parasite *Plasmodium falciparum*

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Author Contributions

H.P.P. and A.C. conceived the project, designed and interpreted the experiments, and wrote the manuscript, with input from all authors. H.P.P. performed the experiments. O.L.-B contributed unpublished plasmids, the design of the CRISPR-Cas9 strategy, and scientific discussion. A.R.-U. contributed to supervision and scientific discussion.

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Reporter lines based on the *gexp02* promoter enable early quantification of sexual conversion rates in the malaria parasite *Plasmodium falciparum*

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Transmission of malaria parasites from humans to mosquito vectors requires that some asexual parasites differentiate into sexual forms termed gametocytes. The balance between proliferation in the same host and conversion into transmission forms can be altered by the conditions of the environment. The ability to accurately measure the rate of sexual conversion under different conditions is essential for research addressing the mechanisms underlying sexual conversion, and to assess the impact of environmental factors. Here we describe new *Plasmodium falciparum* transgenic lines with genome-integrated constructs in which a fluorescent reporter is expressed under the control of the promoter of the *gexp02* gene. Using these parasite lines, we developed a sexual conversion assay that shortens considerably the time needed for an accurate determination of sexual conversion rates, and dispenses the need to add chemicals to inhibit parasite replication. Furthermore, we demonstrate that *gexp02* is expressed specifically in sexual parasites, with expression starting as early as the sexual ring stage, which makes it a candidate marker for circulating sexual rings in epidemiological studies.

Malaria disease is produced by repeated cycles of asexual parasite replication in the human blood. During the ~48 h intraerythrocytic asexual replication cycle, parasites develop through the ring, trophozoite and schizont stages. This is followed by infected erythrocyte rupture and egress of merozoites, which invade erythrocytes to start a new round of multiplication.

The spreading of malaria between humans is mediated by *Anopheles* mosquitoes. Human to vector transmission requires that a subpopulation of the parasites abandons the asexual cycle and differentiates into non-replicative male or female sexual forms termed gametocytes. In the case of *P. falciparum*, which is responsible for the more severe forms of human malaria, parasites that undergo sexual development mature through the morphologically distinct gametocyte stages I to V over ~10 days. Only mature stage V male and female gametocytes are infectious to mosquitoes, where they are activated to form gametes[1,2]. Given their essential role for malaria transmission, gametocytes are considered a priority target for strategies aimed at eliminating malaria.

The initial steps of *P. falciparum* sexual differentiation are regulated by PfAP2-G, a transcription factor of the ApiAP2 family that drives the expression of early gametocyte genes[3–7]. In asexual parasites, the gene encoding this transcription factor, *pfap2-g*, adopts a heterochromatic conformation that results in epigenetic silencing, whereas activation to initiate sexual development requires a transition to a euchromatic state[3,5,8,9]. This transition is facilitated by the gametocyte development 1 (PfGDV1) protein, a recently identified upstream regulator of *pfap2-g*[10]. Expression of PfAP2-G marks the sexually committed stage, which according to recently proposed definitions[11] is a cell state that deterministically results in sexual development at a later point. Commitment is followed by sexual conversion, marked by the expression of gametocyte-specific proteins absent from any replicating blood stages.

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While previously it was thought that an additional round of replication after commitment was an obligate step[12], recent research in *P. falciparum* and the murine malaria parasite *P. berghei* demonstrated that sexual conversion can also occur within the same cycle of commitment[11,13]. For both sexual conversion pathways, termed same cycle conversion (SCC) and next cycle conversion (NCC)[11], conversion results in the formation of sexual ring stages that then develop into stage I to V gametocytes. The only sexual stages present in the circulation are sexual rings and mature stage V gametocytes[14,15], whereas stage I-IV gametocytes are sequestered in tissues such as the bone marrow[1,6,16,17].

While sexual stages mediate transmission, the asexual cycle results in within-host parasite expansion, providing the opportunity to generate more sexual forms. The relative investment in multiplication and sexual differentiation is tightly adjusted to ensure long-term survival, and in the case of *P. falciparum* only a small fraction of the parasites (typically <10%) differentiate into sexual forms at each cycle of multiplication[18,19]. The sexual conversion rate, defined as the proportion of parasites that become gametocytes at each replication cycle, underlies the trade-off between growth in the same host and transmission. Non-induced (baseline) sexual conversion rates vary between different parasite lines[3], and conversion can be induced by external cues. Several conditions, including drug treatment, have been proposed to stimulate sexual conversion[2,5,6,20], but depletion of the serum component lysophosphatidylcholine (LysoPC) stands out as a highly reproducible induction method that is likely relevant during human infection[21]. Addition or removal of choline, involved in the same metabolic pathway as LysoPC, can be used as a convenient alternative to repress or induce sexual conversion under culture conditions[10,21].

A common approach to measure sexual conversion rates consists of determining the gametocytemia of a culture relative to the initial rings parasitemia of the synchronized culture from which the gametocytes originated[3,11,14,22]. This reflects the proportion of sexual vs total rings in the initial culture. Gametocytemia is typically measured by light microscopy analysis of Giemsa-stained smears, which is laborious and has limited accuracy because gametocytemia is typically much lower than the asexual parasitemia. An additional limitation of this assay is that gametocytemia is typically measured >3 days after seeding the assay, as unambiguous morphological identification is not possible until gametocytes reach stage II[5,23]. Since the asexual parasites present in the culture continue multiplying every 48 h, to prevent culture collapse and to identify gametocytes more easily, cultures are usually treated with chemicals such as N-acetyl-D-glucosamine (GlcNAc) or heparin[24,25] that do not kill non-replicating gametocytes but inhibit asexual parasite multiplication. Altogether, this standard assay to determine sexual conversion rates is time-consuming, has limited accuracy, and is not suitable for high-throughput approaches. As an alternative, sexual conversion rates have been measured using immunofluorescence assay (IFA) analysis with antibodies against early gametocyte markers such as Pfs16, but this method still required quantification of the proportion of sexual parasites by fluorescence microscopy[10,26,27].

Assays that use flow cytometry to quantify gametocytes at an early stage of sexual development are ideally suited to accurately determine sexual conversion rates. Transgenic parasite lines expressing fluorescent proteins under the control of promoters from genes expressed in early gametocytes such as *pfg14-744*, *pfg14-748*, *etramp10.3*, *pfg27* and *pfs16* have been described[28–35]. However, in all cases the reporter constructs were maintained episomally, implying that continuous drug pressure was required to maintain the episome. Even in the presence of selective pressure, some parasites lose the episome at each division[30,35], and some drugs may affect sexual conversion, resulting in confounding effects[2,5,6,20]. Furthermore, the promoters used do not have high activity until stage I or II of gametocyte development, and are inactive or expressed at low levels at the sexual ring stage.

Beyond the early gametocyte markers that have been known for years such as Pfs16, Pfg27 or Pfg14.744[34,36,37], several new early gametocyte markers have been recently identified using genome-wide approaches[3,4,7,10,21,26,32,38]. New gametocyte markers with an earlier onset of expression during sexual development, already present in sexual rings, would be valuable not only to study sexual conversion *in vitro*, but also for epidemiological studies. Currently, the only well-validated specific transcript marker for sexually committed stages and sexual rings is *pfap2-g*. The expression of the protein GEXP05 starts as early as the sexual ring stage[39,40], but the high rate of *gexp05* expression in asexual parasites[11,40] suggests that assays based on these transcripts may not be sufficiently specific for sexual parasites. Among the new early gametocyte markers that have not been characterized in detail, *gexp02*[32] was consistently identified as one of the earliest responders to PfAP2-G activation[10,11,15,26,41]. Additionally, using a transgenic parasite line with inducible *pfap2-g* expression we also found that *gexp02* is one of the very few genes that is expressed at high levels in response to PfAP2-G activation as early as the sexual ring stage (Llorà-Batlle *et al.*, manuscript in preparation).

To develop a robust and simple sexual conversion assay, here we generated stable *P. falciparum* lines with a genome-integrated tandem Tomato (tdTom) fluorescent marker gene controlled by early gametocyte promoters. We show that transgenic lines using the *gexp02* promoter enable specific identification of sexual stages by flow cytometry or immunofluorescence assay (IFA) as early as the sexual ring stage, thereby shortening considerably the duration of the sexual conversion assay. We also show that *gexp02* transcripts provide a new marker to specifically detect sexual parasites as early as the sexual ring stage.

Results and Discussion

Generation of early gametocyte-reporter transgenic lines. The reporter lines were generated from two high gametocyte producer *P. falciparum* culture-adapted lines, NF54 and E5. The NF54 line shows high sexual conversion rates and is commonly used to infect mosquitoes, as it retains the ability to produce infective male and female gametocytes[42]. The E5 line is a subclone of 3D7 (itself a clone of NF54[43]) that has been used to study the early steps of sexual differentiation[3,4,11]. Cultures were maintained in 0.5% Albumax II supplemented with 2 mM choline for non-inducing conditions, and choline was removed to induce conversion[10,21].

Using CRISPR-Cas9 technology, we generated reporter lines with the *gexp02* (PF3D7_1102500) promoter controlling the expression of the fluorescent reporter *tdTomato* (tdTom) integrated in the *lisp1* (PF3D7_1418100)

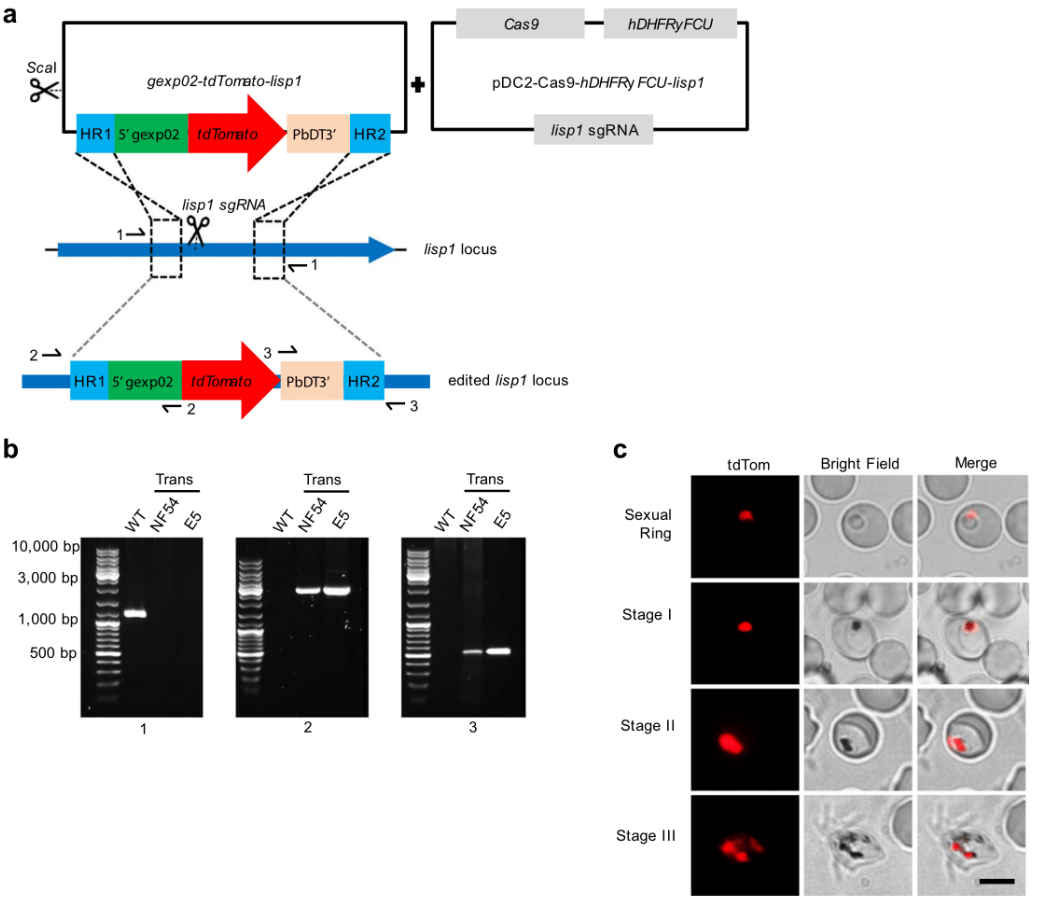

Figure 1. Generation and validation of gametocyte-reporter lines based on the *gexp02* promoter. **(a)** Schematic of the strategy to generate the transgenic lines using the CRISPR/Cas9 system. Half-arrows indicate the position of primers used for diagnostic PCR. **(b)** Diagnostic PCR confirmation of the integration of the *gexp02-tdTomato-lisp1* plasmid at the *lisp1* locus. Numbers at the bottom indicate the primer pair used for each PCR reaction. Genomic DNA from the wild type E5 line (WT) or the NF54 and E5 transgenic lines (Trans) was used. The size of the bands was as expected for the wild-type (1,184 bp for PCR 1) or correctly-edited locus (2,875 bp for PCR 2 and 550 bp for PCR 3). The size of the most intense and top bands of the size marker is indicated. A PCR product was not amplified with primer pair 1 in the transgenic lines, likely because of the too large size of the expected amplification product. **(c)** Live cell fluorescence microscopy analysis of induced (choline-depleted) *NF54-gexp02-Tom* cultures, revealing a subset of tdTom-positive cells consistent with the sexual ring stage (no detectable hemozoin pigment) (top panel). At days 1 to 3 after N-acetyl-D-Glucosamine (GlcNAc) treatment, tdTom signal was observed in parasites morphologically resembling gametocytes at different stages of development (other panels). Scale bar: 5 µm.

locus (*NF54-gexp02-Tom* and *E5-gexp02-Tom* lines) (Fig. 1a). The *lisp1* gene plays a role in liver stages and is not expressed in blood stage parasites[44]. Diagnostic PCR confirmed correct integration of the construct in both NF54 and E5. In all cases, bands corresponding to the wild type *lisp1* locus were not detected in the transfected parasites, indicating that editing occurred in virtually all parasites (Fig. 1b). Preliminary characterization by live cell fluorescence microscopy revealed that only a subset of the parasites expressed tdTom, consistent with the expected gametocyte-specific expression. A strong signal was observed for parasites that, based on morphology and time of collection, appeared to be at the sexual ring stage or stages I-III of gametocyte development (Fig. 1c and Supplementary Fig. 1a).

We also generated analogous transgenic lines in which the fluorescent marker was under the control of the *etramp10.3* (PF3D7_1016900) promoter, which was previously shown to drive strong expression of reporter genes in sexual parasites from stage I/II onwards[28,29,33]. While in these previous studies the plasmids were maintained episomally, we integrated the *etramp10.3*-tdTom expression cassette in the NF54 and E5 genomes to generate the *NF54-10.3-Tom* and *E5-10.3-Tom* lines (Supplementary Fig. 1b). We also generated a reporter line in which tdTom was under the control of the promoter of the early gametocyte marker Pfs16 (*E5-pfs16-Tom*), but preliminary analysis of this line revealed a general fluorescent signal in many multinucleated parasites (i.e. schizonts) that did not demarcate a distinct subset of cells (Supplementary Fig. 1c), so this parasite line was not further characterized. Reporter lines with an episomal *pfs16* promoter driving the expression of a fluorescent marker had

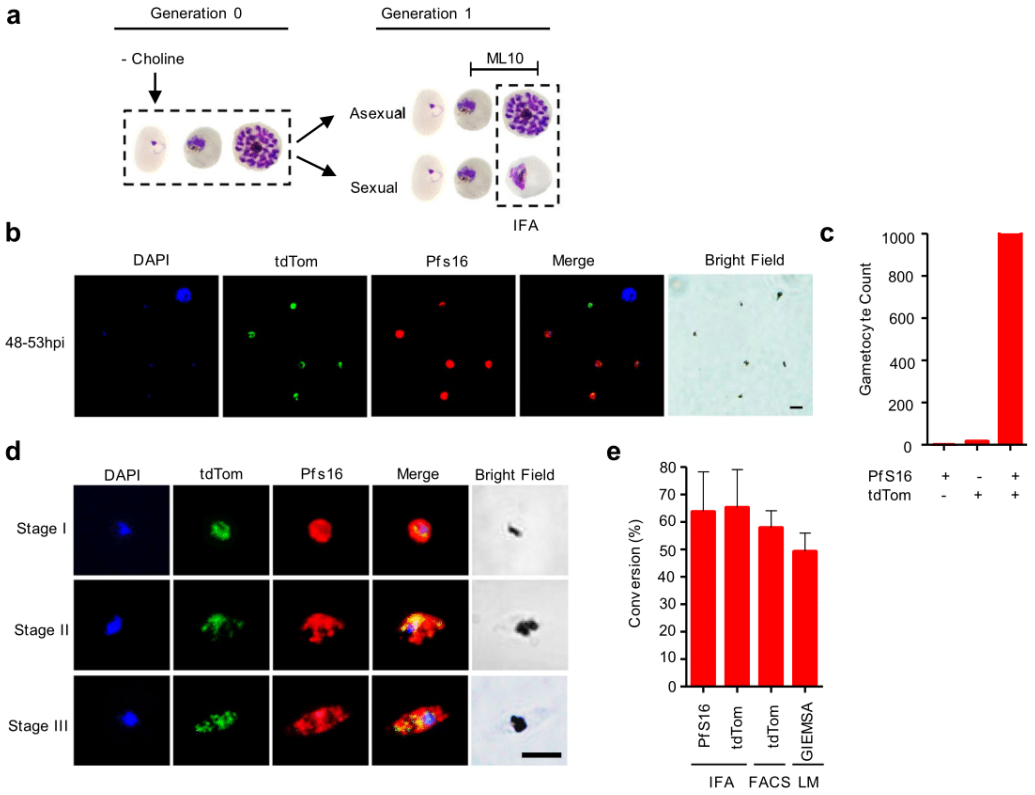

Figure 2. Expression of *gexp02-Tom* in sexual stage parasites. (**a**) Schematic overview of the experiment design with tightly synchronized *NF54-gexp02-Tom* cultures. Sexual conversion was induced by choline-depletion at the ring stage. ML10 was added at ~30–35 h post-invasion (hpi) of the next cycle to prevent schizont rupture. (**b**) Representative immunofluorescence assay (IFA) image of *NF54-gexp02-Tom* cultures at ~48–53 hpi. Mononucleated parasites (stage I gametocytes) express tdTom and Pfs16. Scale bar: 5 µm. (**c**) Distribution of parasites positive for the different markers by IFA (1,176 single-nucleated parasites scored). (**d**) Representative IFA images of gametocytes (stages I-III) showing expression of tdTom and Pfs16 in the same parasites. Scale bar: 5 µm. (**e**) Sexual conversion rates of the *NF54-gexp02-Tom* line as determined by IFA, flow cytometry (FACS) (gametocytemia determined at ~48–53 hpi), and Giemsa-stained blood smears (gametocytemia determined on day 4). Data are presented as the average and s.e.m. of three independent biological replicates.

been previously reported[30,31]. In one of the studies, fluorescence was observed in a subset of schizonts, which were hypothesized to be sexually committed[31]. However, using specific antibodies the Pfs16 protein was found to be an absolutely specific gametocyte marker expressed from stage I of gametocyte development or even earlier, but not in sexually committed schizonts[11,36]. Altogether, these observations suggest that the *pfs16* promoter may not be fully specific for sexual stages; instead, the absolutely sexual parasite-specific expression of the Pfs16 protein may be conferred by post-transcriptional mechanisms.

Sexual stage expression of the *gexp02* promoter. To confirm the ability of the *gexp02* promoter to identify sexual stages, we analyzed the *NF54-gexp02-Tom* line by IFA, co-staining parasites with antibodies against tdTom and Pfs16. To obtain high levels of gametocytes, we induced sexual conversion by removing choline[10,21] at the ring stage. At the following cycle, the cGMP-dependent protein kinase (PfPKG) inhibitor ML10 was added to the cultures ~30–35 h post-invasion (hpi) to prevent schizont rupture and reinvasion[45]. IFA analysis was performed when sexual parasites were at stage I of gametocyte development and replicating parasites were mainly at the schizont stage (~48–53 hpi) (Fig. 2a). Essentially all mononucleated parasites expressing endogenous Pfs16 (gametocytes) also expressed tdTom, and vice versa (Fig. 2b,c). However, a distinct small subset of schizonts that may correspond to sexually committed schizonts also expressed tdTom, but not Pfs16 (discussed below in following sections). After treating cultures with GlcNAc, we observed the two proteins co-expressed in developing gametocytes (Fig. 2d). Cross-reactivity of the secondary antibodies was excluded by experiments in which one of the primary antibodies was not added (Supplementary Fig. 2a). As expected from these results, the conversion rate determined by IFA with antibodies against Pfs16 or TdTom was almost identical, and roughly coincided with determinations based on flow cytometry analysis of tdTom or by microscopy analysis of Giemsa-stained smears (Fig. 2e). Altogether, these experiments demonstrate the specificity of our system based on the *gexp02* promoter to quantify gametocytemia. Analysis of sexual conversion rates in the *NF54-10.3-Tom* line also revealed consistent results between experiments based on IFA, flow cytometry or Giemsa-stained smears (Supplementary Fig. 2b).

Flow cytometry-based time-course analysis of *gexp*02-tdTom expression. To identify the earliest time for accurate gametocytemia measurements, we determined the temporal expression dynamics of tdTom driven by the *gexp02* or the *etramp10.3* promoters using flow cytometry. After defining the gates of tdTom-specific signal (Supplementary Fig. 3), cultures of the four transgenic lines were Percoll/sorbitol synchronized to a defined 5 h age window (lines based on the *gexp02* promoter) or sorbitol synchronized (lines based on the *etramp10.3* promoter) and grown under inducing (- choline) and non-inducing (+choline) conditions[10,21] (Fig. 3a). In both the *NF54-gexp02-Tom* and *E5-gexp02-Tom* lines, a population of tdTom-positive parasites was evident as early as 10–15 hpi (generation 1), both under inducing and non-inducing conditions, with a relatively stable level of expression until 96–101 hpi (Fig. 3b,c). In contrast, in the *NF54-10.3-Tom* and *E5-10.3-Tom* lines full expression of the marker was not observed until 72–94 hpi (stage II gametocytes), although weaker signal was already observed in many parasites at 48–70 hpi (Fig. 3b,d). Altogether, these results demonstrate that using the *gexp02* promoter lines, gametocytemia and sexual conversion rates can be accurately measured as early as 10–15 hpi. Since reinvasion does not occur until much later, the use of the *gexp02* promoter dispenses the need to add chemicals to inhibit the growth of asexual parasites.

Of note, the new transgenic lines showed similar sexual conversion rates to their parental lines and were inducible by choline depletion (6 to 7-fold higher conversion under inducing conditions in *gexp02* promoter-based lines) (Fig. 3), demonstrating that their sexual conversion phenotypes were not substantially altered during genome editing. Unexpectedly, the NF54 line used in this study shows an extremely high sexual conversion rate upon induction (~60%), comparable to the conversion rates observed upon depletion of HP1 or the *gdv1* antisense RNA[10,26]. We also observed that the multiplication rate was reduced in choline-depleted cultures, especially in NF54-derived lines (growth rate ~4 in the absence of choline vs 7–8 in its presence) (Supplementary Fig. 4). Since reduced growth was already observed during the initial cycle of multiplication upon choline removal, it cannot be explained by increased sexual conversion, suggesting that the reduced growth reflects the metabolic consequences of the lack of choline[21].

The *gexp02* promoter is active in a subset of schizonts. Unexpectedly, in the parasite lines *NF54-gexp02-Tom* and *E5-gexp02-Tom* we observed parasites at the schizont stage (according to their DNA content) that were positive for tdTom (Fig. 3b, top row, e.g. 48–53 hpi, and Fig. 4a). This raises the possibility that the *gexp02* promoter may be active not only in sexual rings and subsequent gametocyte stages, but also in some committed schizonts. Indeed, live cell fluorescent microscopy and IFA confirmed the occurrence of some schizonts that expressed tdTom (Fig. 4a,b). tdTom-positive schizonts were observed both in cultures with or without ML10, but they occurred infrequently in the cycle of choline removal (Generation 0) and were abundant only at the following cycle in choline-depleted cultures (Generation 1) (Fig. 4c). While some of the tdTom-positive schizonts may reflect new conversion events occurring during Generation 1, the much lower levels at Generation 0 in spite of abundant sexual commitment raise the intriguing possibility that a subset of tdTom-positive sexual rings may not develop directly into gametocytes, but rather multiply for an additional cycle as *gexp02*-expressing sexually committed forms. The higher parasitemia at Generation 1 may also influence the proportion of tdTom-positive schizonts, which unexpectedly also increased between Generations 0 and 1 in non-induced cultures. This may also reflect the inherent variability of sexual conversion rates even between consecutive generations. Of note, activation of the *gexp02* promoter doesn't imply that the endogenous GEXP02 protein is expressed, because post-transcriptional regulation is commonly observed during sexual development[5]. Expression in a subset of schizonts of fluorescent proteins under the control of gametocyte-specific promoters has been previously observed by others[28,31,34], and in some cases doesn't match the expression of the endogenous protein. Future research should establish the significance of the activity of early gametocyte promoters in a subset of schizonts. However, it is important to highlight that the occurrence of tdTom-positive schizonts doesn't affect the ability of our new transgenic lines to accurately measure sexual conversion rates, because gametocytemia can be measured long before tdTom-positive schizonts are observed (e.g. at ~20 hpi).

***gexp02* transcripts as a marker for sexual rings.** Given the very early expression observed for *gexp02*-driven reporters, we evaluated the potential use of *gexp02* transcripts as a marker for sexual rings. The ability to identify sexual rings is particularly relevant in human infections, because they are the only immature sexual stages that are present in the circulation[1,6,14,15]. To establish the specificity of *gexp02* transcripts for sexual forms, we first analyzed relative *gexp02* transcript levels in an already available collection of cDNA samples[11] from a transgenic parasite line in which a destabilization domain is appended to PfAP2-G[3]. The protein can be stabilized by addition of the Shield 1 ligand, which results in production of gametocytes, whereas in the absence of Shield 1 no gametocytes are formed. After adding Shield 1 at 0–5 hpi, abundant *gexp02* transcripts are detected, whereas they are almost absent from untreated cultures (Fig. 5a). This result confirms that *gexp02* is expressed specifically in parasites undergoing sexual development.

Next we analyzed *gexp02* transcripts in choline-depleted and choline-supplemented cultures at a time when the majority of parasites are at the mature schizont stage but some new rings have already formed (40–45 hpi), at the ring stage of the next cycle (~0–10 hpi), and in stage V gametocytes (Fig. 5b). The latter stage was included to determine if transcripts derived from mature gametocytes can potentially have a major contribution to the total level of *gexp02* transcripts in human blood samples. In parallel, we examined the transcript levels of the sexual commitment marker *pfap2-g*. In ring stage cultures, both *pfap2-g* and *gexp02* displayed higher transcript levels in induced than in uninduced cultures, reflecting the increase in the abundance of sexual parasites. Furthermore, *gexp02* transcript levels were substantially lower in stage V gametocyte cultures than in ring stage cultures (Fig. 5c), in spite of the former being a pure sexual population whereas the latter is a mixture of sexual and asexual parasites. Based on these results, the relative contribution of mature gametocytes to the total *gexp02* transcripts in an infected human blood sample is expected to be very low. The use of different genes to normalize expression

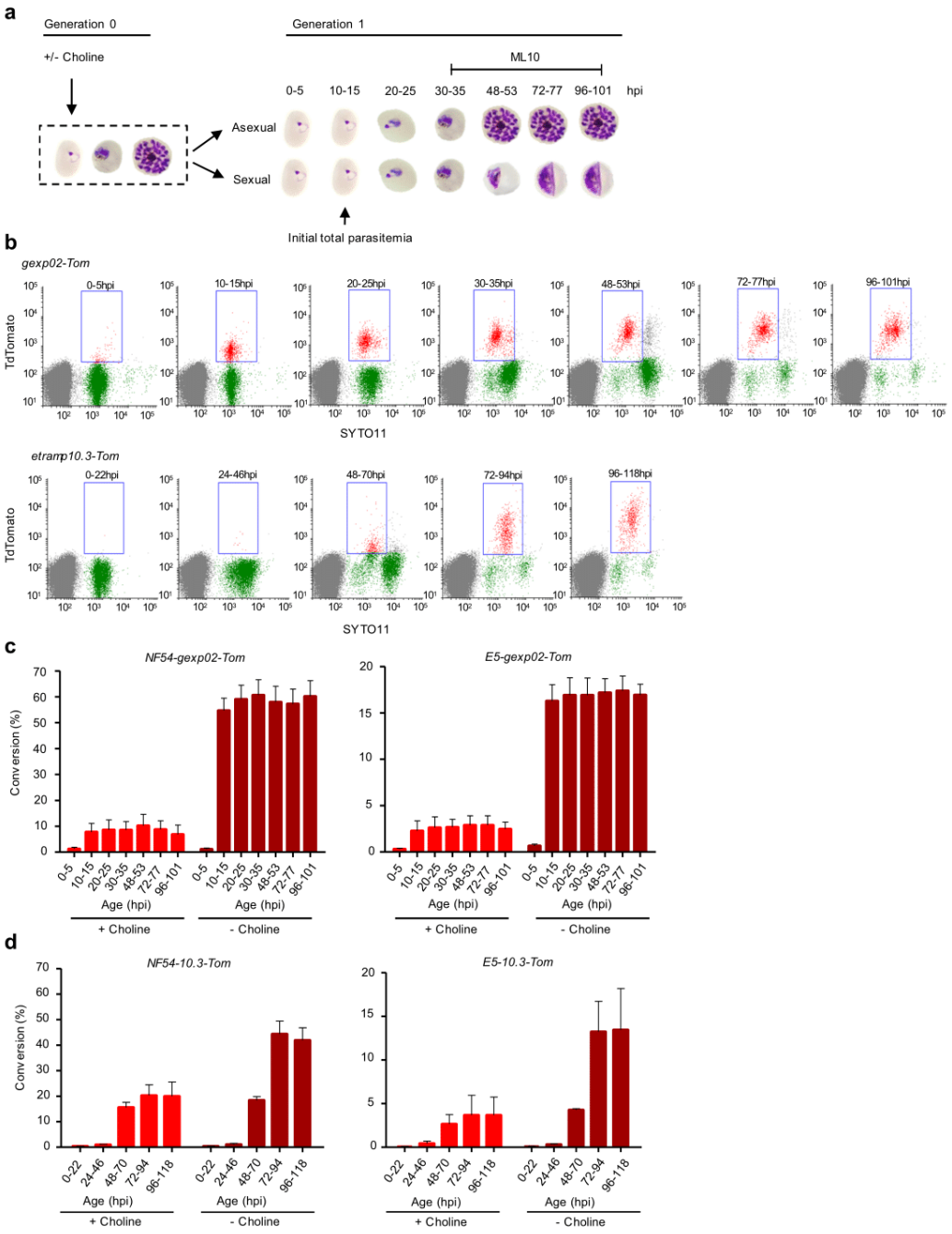

Figure 3. Flow cytometry-based time-course analysis of *gexp02*-tdTom and *etramp10.3*-tdTom expression during sexual development. (**a**) Schematic overview of the experiment design. Cultures were either maintained in the presence of choline, or with choline removed at the ring stage to induce sexual conversion. After reinvasion, the proportion of tdTom-positive parasites was determined by flow cytometry at several times (the times shown correspond to experiments with the *gexp02-Tom* lines, which were synchronized to a 5 h age window). ML10 was added at the trophozoite stage (generation 1, as indicated) to prevent schizont rupture and reinvasion. **(b)** Representative flow cytometry SYTO11 (stains DNA) – tdTomato plots for the *NF54-gexp02-Tom* and *NF54-etramp10.3-Tom* transgenic lines (- choline condition) at different times after invasion. Gates (rectangles) indicate SYTO11 and tdTomato double-positive parasites. **(c)** Quantification of sexual conversion rates at different times in the *NF54-gexp02-Tom* and *E5-gexp02-Tom* lines, based on detection of tdTomato fluorescence. Conversion rates (%) were determined as the gametocytemia relative to the initial rings parasitemia. Data are presented as the average and s.e.m. of three independent biological replicates. **(d)** Same as panel c, but for the *NF54-etramp10.3-Tom* and *E5-etramp10.3-Tom* lines. Data are presented as the average and s.e.m. of two independent biological replicates.

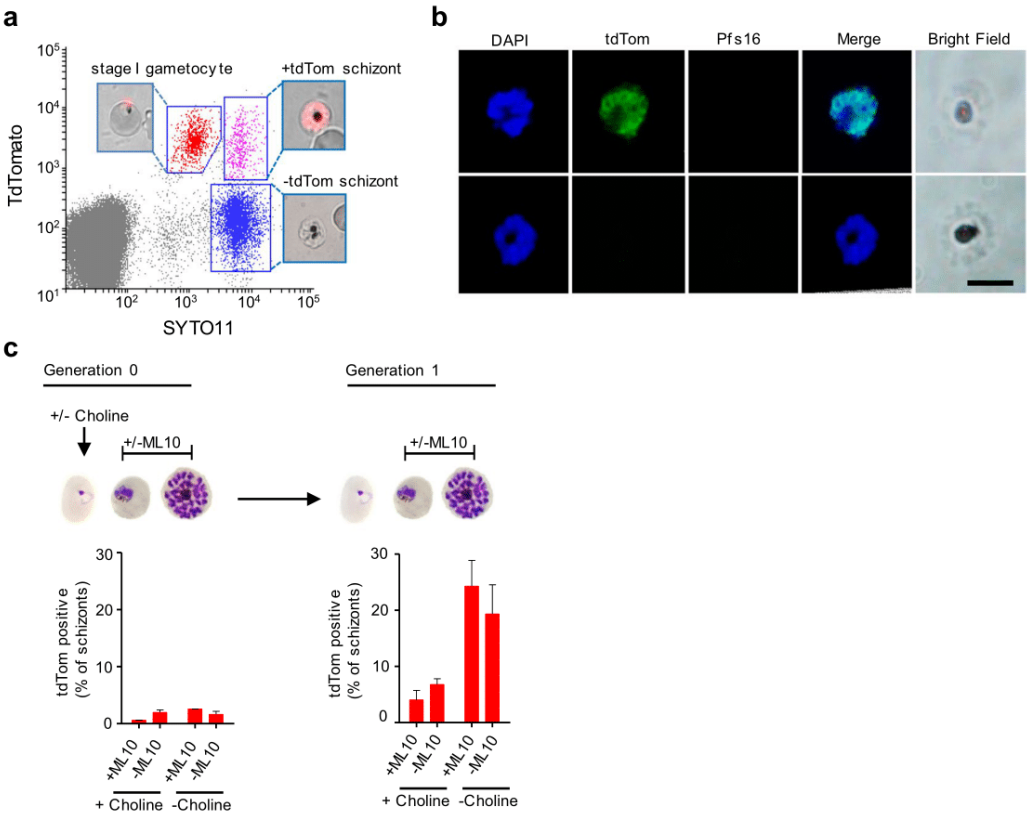

Figure 4. Expression of tdTom in schizonts of *gexp02-Tom* lines. (**a**) Representative flow cytometry analysis of the *NF54-gexp02-Tom* line at ~48–53 hpi (induced by choline depletion at the previous cycle and treated with ML10), showing presence of tdTom-positive schizonts (upper right gate), in addition to stage I gametocytes (upper left gate), and tdTom negative schizonts (lower right gate). Insets are live cell fluorescence microscopy images of the different subpopulations. **(b)** Representative IFA images of schizonts that are either tdTom positive or negative. All schizonts were Pfs16-negative. Scale bar: 5 µm. **(c)** Flow cytometry-based quantification of tdTom positive schizonts at the generation of induction and the following generation. Induced (choline-depleted) and uninduced (choline-supplemented) cultures, with or without addition of ML10, were tested. Data are presented as the average and s.e.m. of three independent biological replicates.

didn't affect any of the conclusions (Supplementary Fig. 5). Altogether, these results indicate that *gexp02* transcripts provide an appropriate marker for immature sexual parasites. The higher transcript levels of *gexp02* compared to *pfap2-g* in ring stage samples suggest that *gexp02* may be a more sensitive marker for sexual rings, but a detailed analysis of *gexp02* transcripts in field samples is clearly needed before this gene can be considered a useful marker for epidemiological studies.

Concluding remarks. The quantification of sexual stages is one of the limiting steps for an accurate determination of sexual conversion rates, which is key for studies on malaria transmission biology. Here we describe new transgenic parasite lines based on the *gexp02* promoter to quantify sexual stages. These new lines show two clear advantages over previously available tools: first, sexual parasite-specific reporter protein expression is detected as early as 10–15 hpi. This detection time enables a substantially shorter sexual conversion assay, dispensing the need to use compounds that inhibit parasite replication. Second, our genome-integrated constructs are stable and do not require drug selection pressure, which prevents potential confounding effects. Furthermore, the new transgenic lines retain the ability to respond to external conditions, which makes them suitable to study both baseline spontaneous sexual conversion, and induced conversion. Since the sequences of the *lisp1* locus targeted by the guide RNA and the homology regions in the constructs described here are highly conserved among commonly used culture-adapted parasite lines and field isolates (PlasmoDB.org), it would be straightforward to integrate the same reporter constructs in the genome of parasites of different genetic background.

We propose a sexual conversion assay that consists on culturing the *NF54-gexp02-Tom* or *E5-gexp02-Tom* lines in the presence and absence of choline (to measure basal and induced conversion, respectively), and at the next cycle measure total parasitemia and gametocytemia by flow cytometry when parasites are at the late ring or early trophozoite stage. Using this assay, the effect of different conditions (e.g. drugs) on sexual conversion rates can be assessed. The assay is robust, simple and accurate. In addition to facilitating the determination of sexual conversion rates, the new parasite lines that we have developed will enable the purification (by fluorescence-activated

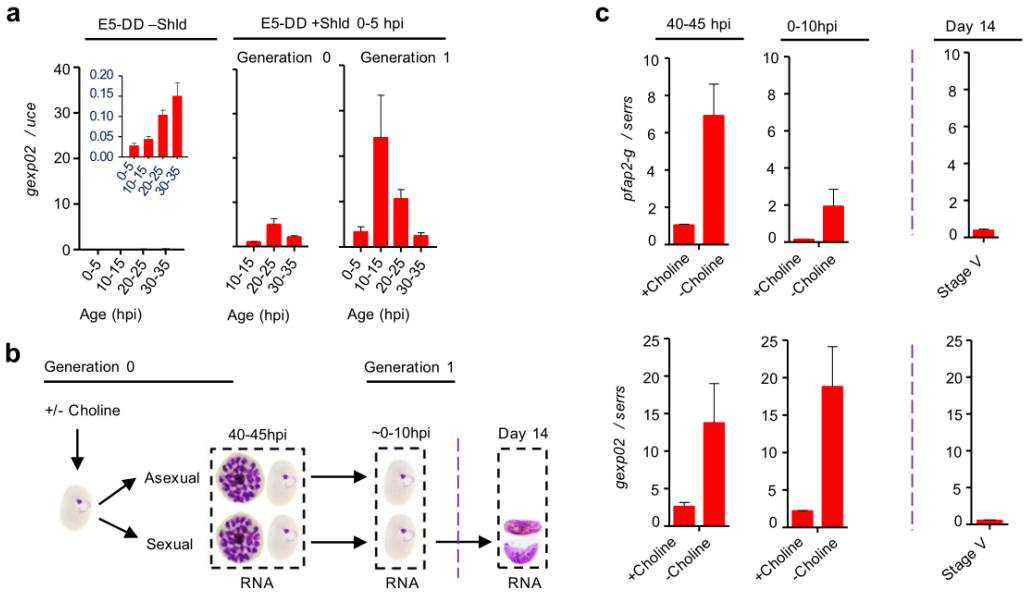

Figure 5. Transcriptional dynamics of the *gexp02* gene. (**a**) Transcriptional analysis of *gexp02* in the E5-HA-DD line, in which PfAP2-G is fused to the FKBP destabilization domain, cultured either without Shield 1 (Shld) (no gametocytes are formed) or with Shld added at 0–5 hpi to stabilize PfAP2-G. The inset graph in the –Shld panel shows the same data with a different y-axis range, because under these conditions *gexp02* transcripts levels were very low. Transcript levels are normalized against *ubiquitin-conjugating enzyme (uce)* (PF3D7_0812600). **(b)** Schematic overview of the design of experiments aimed at comparing the transcriptional dynamics of *pfap2-g* and *gexp02* in induced (choline-depleted) and uninduced (choline-supplemented) *NF54-gexp02-Tom* cultures. Analysis was performed at the schizont stage of the generation of induction (40–45 hpi, some new rings already present) and at the ring stage of the next generation (~0–10 hpi). Additionally, *gexp02* transcript levels were determined in mature stage V gametocytes (Day 14). **(c)** Transcript levels of *gexp02* and *pfap2-g* relative to the *serine-tRNA ligase* (*serrs*) gene (PF3D7_0717700). Data are presented as the average and s.e.m. of two independent biological replicates.

cell sorting) and characterization of sexual rings and early gametocytes. These transgenic lines can also be used to assess the susceptibility of early sexual forms to different drugs.

We also report that *gexp02* transcripts are highly specific for sexual stages and can be detected in sexually-developing parasites as early as the sexual ring stage. While several proteins are specifically expressed in sexual parasites, transcripts for some of these proteins appear to be more promiscuous. So far, only *pfap2-g* transcripts had been described as a mRNA marker that is specific for parasites undergoing sexual development and can be detected before stage I of gametocyte development. Further research using samples from infected humans is needed to determine the full potential of *gexp02* transcripts as a second marker for circulating immature sexual stages in epidemiological studies.

Methods

Parasites. The culture-adapted NF54[43,46] and E5[3] (a 3D7-B[47] subclone) lines were used to generate the transgenic lines. The NF54 stock used here was obtained from Sanaria[48]. Parasites were cultured in B+ erythrocytes (3% hematocrit) in RPMI 1640-based standard medium supplemented with 10% human plasma and incubated at 37 °C under hypoxia (2% O_2, 5.5% CO_2, balance N_2) and shaking (100 rpm) or static conditions. Regular culture synchronization was performed by standard sorbitol lysis, whereas for selected assays we used tight synchronization to a well-defined age window by combining Percoll purification of mature forms (followed by addition of fresh erythrocytes to establish new cultures) with sorbitol lysis 5 h later, as previously described[11]. Flow cytometry determination of parasitemia was performed essentially as described[49].

In our laboratory, NF54 is regularly cultured in media with human serum or plasma, which contains high levels of the metabolite LysoPC that represses sexual conversion[21], whereas E5 is regularly maintained in media with 0.5% Albumax II (with low levels of LysoPC and choline), and no human serum. Here, for most experiments we maintained both lines in Albumax II media supplemented with 2 mM choline chloride (Sigma-Aldrich no. C7527), which is analogous to supplementing with LysoPC[10,21]. To induce sexual conversion, we replaced the media by media without choline. This was always performed in combination with synchronization by sorbitol lysis, which in total involves two washing steps. Induced cultures were then maintained without choline until the end of the experiment.

For selected experiments, cultures were treated with ML10, a highly specific inhibitor of PfPKG. The compound was added at a 80 nM concentration at the trophozoite stage (typically 30–35 hpi) to inhibit merozoite

egress[45], thus preventing multiplication of asexual parasites. ML10-arrested schizonts appeared to decrease in number over time (Fig. 3b), suggesting that they progressively disintegrated.

Cultures at the ring stage (5 to 8% parasitemia) were used for transfection by electroporation with 60 µg of circular Cas9 plasmid and 30 µg of linearized donor plasmid. Starting the day after transfection, cultures were selected with 10 nM WR99210 for 4 consecutive days[50]. Diagnostic PCR analysis of genomic DNAs to validate integration of the plasmids was performed with the TaKaRa LA Taq DNA Polymerase (Takara Bio USA, Inc.). It is well established that different subclones of the same genetic background often show different phenotypes in terms of sexual conversion and ability to infect mosquitoes[3,42]. Thus, to retain the sexual conversion phenotype of the parental E5 and NF54 parasite lines, the transgenic lines obtained were not subcloned. It is likely that a subset of the parasites in the transgenic lines retained the *pDC2-Cas9-hDHFRyFCU-lisp1* plasmid and hence is resistant to WR99210. However, this plasmid contains the negative selection marker yFCU, which enables eliminating parasites containing this plasmid by selection with 5-fluorocytosine. We recommend performing this treatment before transfection of the transgenic lines with other plasmids for sequential editing, and to periodically monitor the absence of wild type parasites by PCR analysis of genomic DNA.

We defined the sexual conversion rate as the proportion of parasites that convert into sexual stages in a given parasite population[3,11]. Sexual conversion by flow cytometry or Giemsa-stained smears was calculated by dividing the gametocytemia over the initial parasitemia of a culture at the ring stage, determined when no additional replication was allowed. On the other hand, sexual conversion by IFA was measured by directly determining the proportion of Pfs16 or tdTom positive cells in >200 DAPI positive cells.

Plasmids. Plasmid *pDC2-Cas9-hDHFRyFCU-lisp1* was based on the pDC2-Cas9-U6-hDHFRyFCU plasmid[50,51]. The single guide RNA (sgRNA) specific for the *liver-specific protein-1* gene (*lisp1*, ID: PF3D7_1418100; previous ID: PF14_0179) was generated by cloning annealed oligonucleotides (spanning positions 5,526-5,545 from the *lisp1* start codon) into a *Bbs*I site using the In-Fusion system (Clontech).

The *etramp10.3-tdTomato-lisp1* donor plasmid used to insert the *etramp10.3-tdTomato* cassette into the *lisp1* locus was based on the plasmid *164-tdTomato*[29], which has the tdTom coding sequence under the control of the *etramp10.3* (ID: PF3D7_1016900; previous ID: PF10_0164) promoter and the *P. berghei dhfr 3′*terminator (PbDT-3′). We noticed that this plasmid contains an unexpected 162 bp fragment that includes positions 1–102 of the spliced coding sequence of a *rifin* gene (ID: PF3D7_0115300; previous ID: PFA0745W) between the *Not*I site (separates the *etramp10.3* promoter from tdTom) and the tdTom sequence. This fragment does not appear to interfere with the expression of the reporter gene, as correct expression was previously described[29]. This sequence is present in all plasmids generated here derived from the *164-tdTomato* plasmid. We first cloned PCR-amplified *lisp1* homology regions (HR1 and HR2) into *Kpn*I/*Sal*I and *SnaB*I/*Eco*RI sites flanking the *etramp10.3-tdTomato-PbDT3'* cassette. HR1 and HR2 consisted of positions 5,168-5,523 and 5,891-6,236 of the *lisp1* coding sequence (relative to the start codon), respectively.

To generate plasmid *gexp02-tdTomato-lisp1*, we replaced the *etramp10.3* promoter in the *etramp10.3-tdTomato-lisp1* plasmid with the *gexp02* promoter. The *gexp02* (ID: PF3D7_1102500; previous ID: PF11_0037) upstream region (position −2,457 to −25 bp relative to the start codon) was PCR-amplified from *P. falciparum* genomic DNA and cloned into plasmid *etramp10.3-tdTomato-lisp1* digested with *Sal*I and *Not*I using the In-Fusion system (Takara Bio USA, Inc.). An analogous procedure was followed with the *pfs16* promoter (position −863 to −1 relative to the start codon) to generate the *pfs16-tdTomato-lisp1* plasmid. All primers used to generate the plasmids are described in Supplementary Table 1.

Immunofluorescence assay (IFA). Blood smears for IFA were fixed with 1% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100/PBS for 5 min, and blocked with 3% BSA in PBS for 30 min, essentially as described[11]. Two washes with PBS were performed between steps (5–10 min, shaking at 120 rpm). Primary antibodies were incubated at room temperature for 3 h or at 4 °C overnight while shaking (60 rpm). After 3 PBS washes, secondary antibodies in a solution also containing DAPI (5 µg/ml) were incubated at room temperature for 3 h while shaking (60 rpm). All antibodies were prepared in 3% BSA/PBS. The primary antibodies used were rabbit-anti-RFP (1:500; Rockland Inc. no. 600-401-379) and mouse-anti-PfS16[52] (1:400), whereas the corresponding secondary antibodies were goat-anti-rabbit IgG-Alexa Fluor 488 (1:1000; ThermoFisher no. A11034) and donkey-anti-mouse IgG Alexa Fluor 546 (1:1000; ThermoFisher no. A10036). IFA slides were mounted with Vectashield medium (Palex Medical) and viewed under an Olympus IX51 epifluorescent microscope. Images were taken using an Olympus DP72 camera connected to CellSens Standard 1.11 software and were further processed using ImageJ software.

Flow cytometry determination of gametocytemia. Flow cytometry was performed at several time points to capture the expression of fluorescent reporters driven by gametocyte promoters in synchronized cultures at different stages of development. Samples for flow cytometry analysis (using a BD LSRFortessa™ apparatus) were prepared by mixing the nucleic acid stain Syto11 (0.016 µM) (Life Technologies no. S7573) and 10 µl of parasite culture at 3% hematocrit in 800 µl of PBS essentially as described[49]. Flow cytometry analysis (typically 100,000 cells per sample) was set to simultaneously detect tdTom (laser: 561 nm; filter: 582/15; power: 50 mW) and Syto11 (laser: 488 nM; Filter: 525/50-505LP; power: 50 mW). Initial gating was performed using the side scatter and forward scatter areas (SSC-A versus FSC-A plot) to define the RBC population and to exclude cells or debris outside the granularity and size of RBCs. Next, singlets were gated using the forward scatter height (FSC-H) versus FSC-A plot. Sexual stages were detected on the double positive gate as parasites positive for both tdTom signal and Syto11-stained nucleic acids (Supplementary Fig. 3). Downstream analysis was performed using Flowing Software version 2.5.1 (Perttu Terho).

Transcriptional analysis. RNA preparation and reverse-transcription quantitative PCR (RT-qPCR) were performed essentially as previously described, using a method suitable for low amounts of RNA[53]. In brief, RNA was collected in TRIzol (Invitrogen), purified with the RNeasy® Mini Kit (Qiagen no. 74104), DNAse treated (Qiagen no. 79254), and cDNA synthesized using the AMV Reverse Transcription System (Promega) with a mixture of oligo (dT) and random primers. The qPCR analysis of cDNAs was performed using the standard curve method, with a standard curve for each primer pair contained in each plate. Transcript levels were normalized against transcripts of *serine-tRNA ligase* (*serrs*, ID:PF3D7_0717700), *ubiquitin-conjugating enzyme* (*uce*, ID: PF3D7_0812600), and *18S ribosomal RNA* (*18S rRNA*, IDs: PF3D7_0112300, PF3D7_1148600 and PF3D7_1371000), which show relatively stable levels across asexual and sexual blood stages (PlasmoDB.org).The primers used are described in Supplementary Table 1. Primers for *pfap2-g*, *serrs*, *uce* and *18S rRNA* are based on previously described primers[11,54].

Data Availability

Materials and protocols are available from the corresponding author on reasonable request.

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Author Contributions

H.P.P. and A.C. conceived the project, designed and interpreted the experiments, and wrote the manuscript, with input from all authors. H.P.P. performed the experiments. O.L.-B. contributed unpublished plasmids, the design of the CRISPR-Cas9 strategy, and scientific discussion. A.R.-U. contributed to supervision and scientific discussion.

Additional Information

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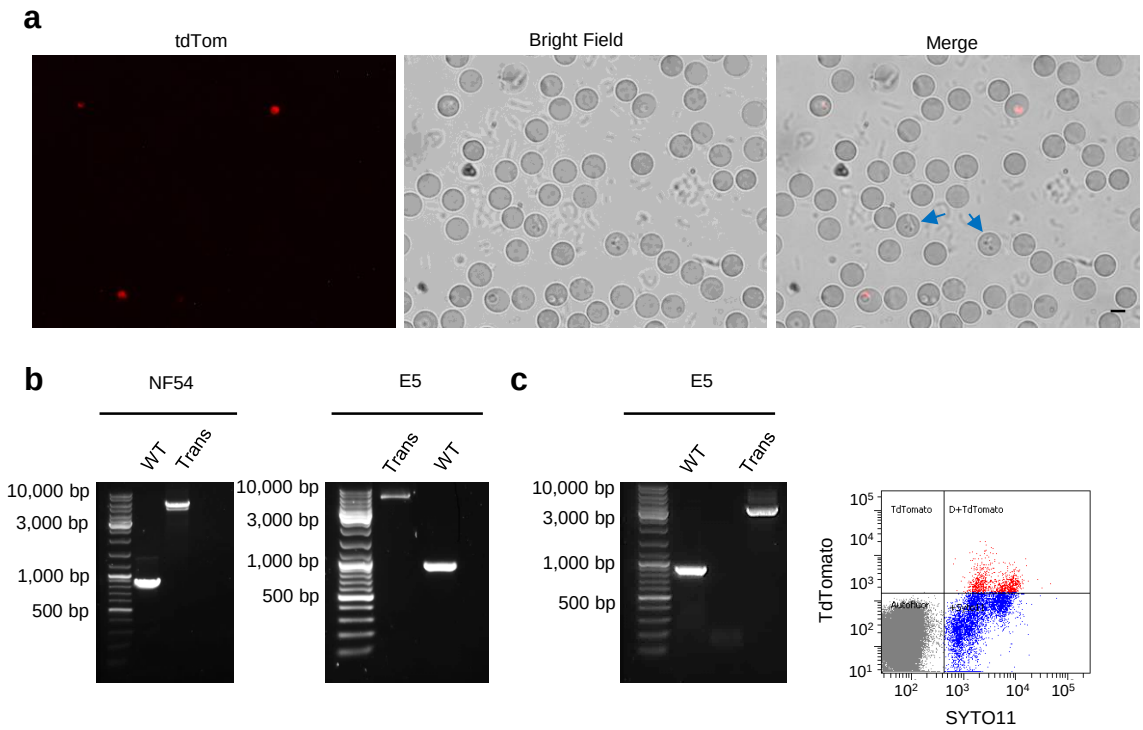
Supplementary information

Reporter lines based on the *gexp02* promoter enable early quantification of sexual conversion rates in the malaria parasite *Plasmodium falciparum*

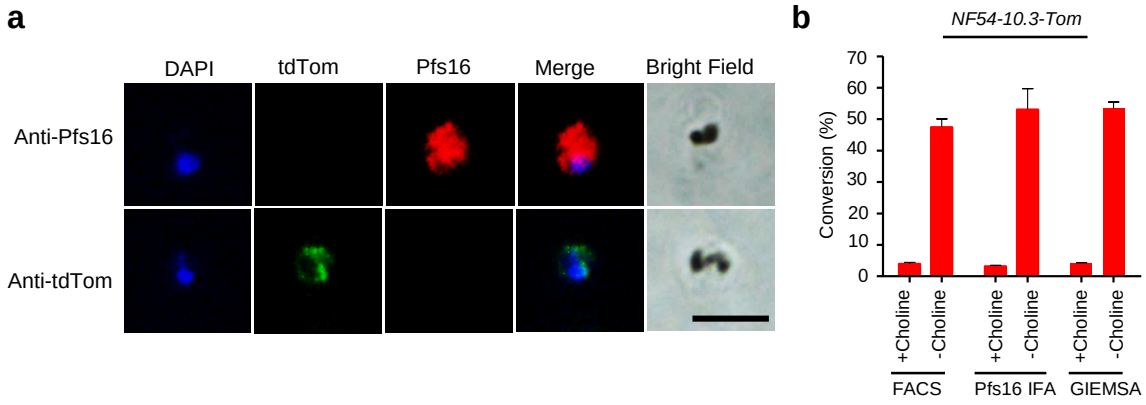
Harvie P. Portugaliza, Oriol Llorà-Batlle, Anna Rosanas-Urgell & Alfred Cortés

Supplementary Table 1. List of oligonucleotide used in this study. Restriction sites introduced in the primers are underlined

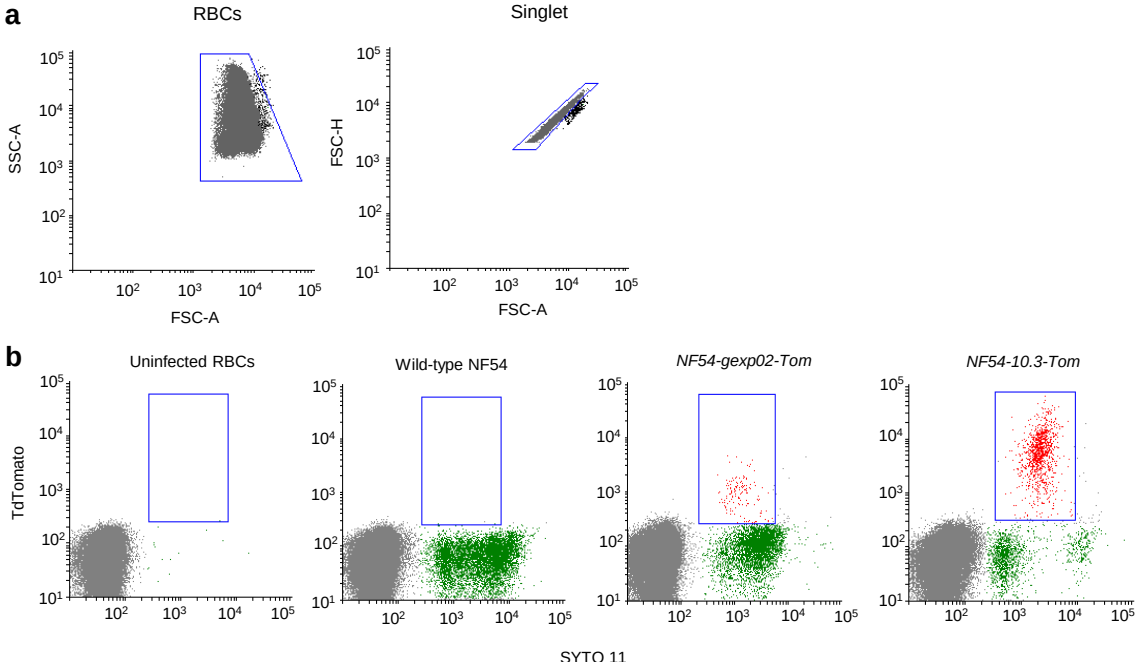
In-Fusion® primers for cloning *lisp1* homology regions		
Name	Sequence	Enzyme
lisp1_HR1_+5168_Fwd	aatactcgcggcccgGGTACC AAGACAATGGGAAATGGTGTTA	*Kpn*I
lisp1_HR1_+5523_Rev	acgttcatgcGTCGAC TTCTGGGACGACATTTATTGTT	*Sal*I
lisp1_HR2_+5891_Fwd	gcggccctgcagTACGTA TACCTATAGAGGATAAGGAGAA	*Sna*BI
lisp1_HR2_+6236_Rev	actcactataGAATTC GTAAGTGTTGTGGGTATGCTT	*Eco*RI
In-Fusion® primers for cloning *gexp02 and pfs16* promoters		
Name	Sequence	Enzyme
gexp02_-2457_Fwd	cgtcccagaaGTCGAC GGATTGGCACTTATACCTTTA	*Sal*I
gexp02_-46_Rev	gctcaccatGCGGCCGC TCAAACTCTAAATGATTATCC	*Not*I
pfs16_-863_Fwd	cgtcccagaaGTCGAC CCCCTCATTTCATAGTTTGTCTT	*Sal*I
Pfs16_-1_Rev	cgtcccagaaGTCGAC GTTGAAGAAAGTATAAATAGAAAAATGGC	*Not*I
In-Fusion® primers for *lisp1* guide in *pDC2-Cas9-hDHFRyFCU-lisp1* plasmid		
Name	Sequence	
lisp1_guide_+5526_Fwd	taagtatataatattGAGGAACTGGGAACATGTAGgttttagagctagaa	
lisp1_guide_+5545_Rev	ttctagctctaaaacCTACATGTTCCCAGTTCCTCaatattatatactta	
Primers for PCR confirmation of construct integration		
Name	Sequence	Remarks
lisp1_+5088_Fwd	TATGAAGAATATATTGAACGAATC	Upstream of HR1
lisp1_+6249_Rev	GATATTCATTAAACCTCTCATTG	Downstream of HR2
lisp1_+6006_Rev	AGTATACCCAGGAGTGGATAA	Within HR2
PbDT 3'_+694 _Fwd	TGTCGAAACAAAACTGGCATA	
gexp02_-46_Rev	TCAAACTCTAAATGATTATCC	
Primers for RT-qPCR		
Name	Sequence	Assay
gexp02 _+813_Fwd	GGAAATTAGGAGTAAGAAGAGG	SYBR Green
gexp02_+972_Rev	TCGCTATTTATTTCACTTGGCT	
pfap2-g_+3874_Fwd	AACAACGTTCATTCAATAAATAAGG	SYBR Green
pfap2-g_+3979_Rev	ATGTTAATGTTCCCAAACAACCG	
serRS_+590_Fwd	AAGTAGCAGGTCATCGTGGTT	SYBR Green
serRS_+747_Rev	TTCGGCACATTCTTCCATAA	
uce_+67_Fwd	GGTGTTAGTGGCTCACCAATAGGA	SYBR Green
uce_+155_Rev	GTACCACCTTCCCATGGAGTA	
18srRNA_+792_Fwd	GCTTTTGAGAGGTTTTGTTACTTTG	SYBR Green
18srRNA_+1006_Rev	CTCTGACATCTGAATACGAATGC	



Supplementary Figure 1. Characterization of the new reporter lines. (a) Representative live cell fluorescence microscopy image of an induced (- choline) *NF54-gexp02-Tom* culture at the ring stage, as in Fig. 1c. The low magnification images show the presence of tdTom-positive rings (red fluorescence inside the RBC) and tdTom-negative rings (blue arrows). Scale bar: 5 µm. **(b)** Diagnostic PCR confirmation of integration of the *etramp10.3-tdTomato* plasmid (analogous to the *gexp02-tdTomato-lisp1* plasmid shown in Fig. 1a, but with the *etramp10.3* promoter instead of the *gexp02* promoter) at the *lisp1* locus in the NF54 and E5 lines. The forward primer from primer pair 1 (see Fig. 1a) and a reverse primer recognizing the homology region 2 (HR2) (Supplementary Table 1) were used for this PCR analysis. The amplification band in the wild-type lines (WT) is of the expected size (919 bp), and in the transgenic lines (Trans) the band is of the expected size after correct editing (5,308 bp). **(c)** Analogous analysis for integration of the *pfs16-tdTomato-lisp1* plasmid in the *lisp1* locus in the E5 line (expected size of the PCR product after correct editing: 3,964 bp), and flow cytometry-based analysis of ~24-46 h post-invasion parasites of this line showing tdTom fluorescence in late-stage asexual parasites.

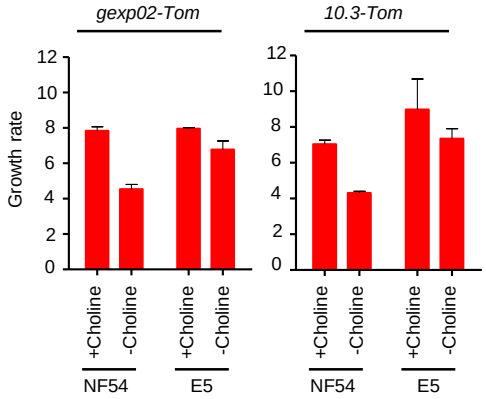


Supplementary Figure 2. Validation of the IFA assay and characterization of the *NF54-10.3-Tom* line. (a) Representative images of immunofluorescence assay (IFA) analysis of *NF54-gexp02-Tom* stage I gametocytes to exclude cross-reactivity of secondary antibodies. Only mouse-anti-Pfs16 or only rabbit-anti-RFP primary antibodies were used in the top and bottom panels, respectively. In both cases, two secondary antibodies (goat-anti-rabbit IgG-Alexa Fluor 488 and donkey-anti-mouse IgG Alexa Fluor 546) and DAPI were used. Images are representative of 1,001 and 1,017 infected erythrocytes analyzed for anti-Pfs16 and anti-tdTom conditions, respectively. Scale bar: 5 µm. **(b)** Sexual conversion rate under +/- choline conditions of the *NF54-10.3-Tom* line as determined by flow cytometry (FACS), Pfs16 IFA, and Giemsa-stained blood smears. To determine the conversion rate by FACS, gametocytemia and initial parasitemia were measured by flow cytometry using tdTom + Syto11 and Syto11 fluorescence, respectively. Results are presented as the average and s.e.m. of two independent biological replicates.

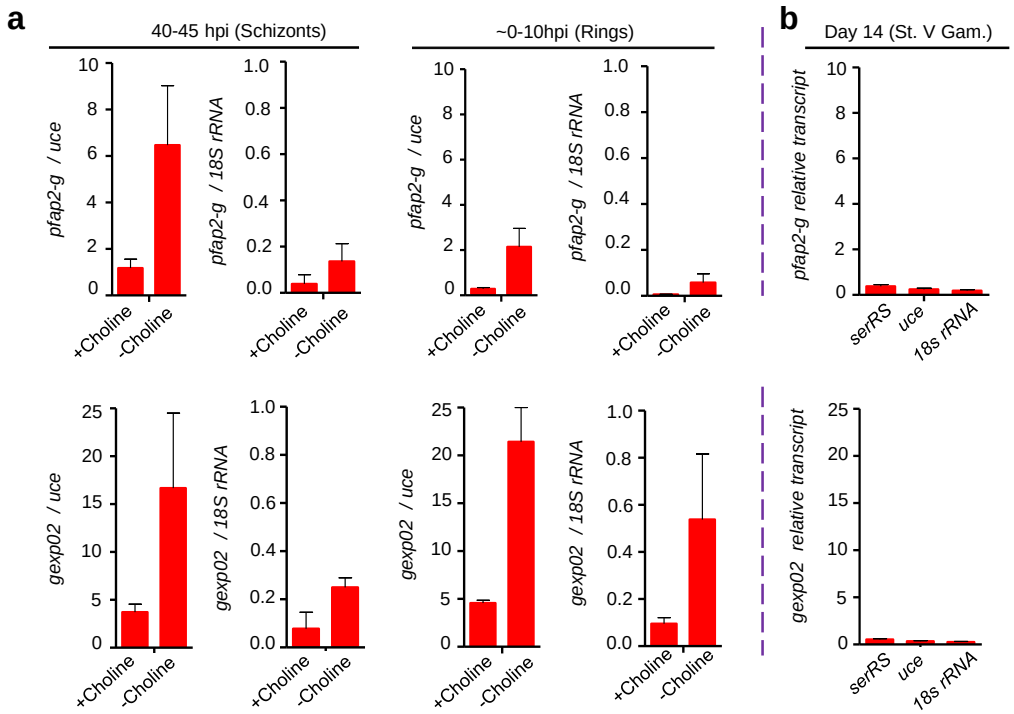


Supplementary Figure 3. Set-up of the flow cytometry assay. (a) Representative images of the initial gating strategy. The red blood cell (RBC) population was initially gated according to granularity and size (SSC-A versus FSC-A plot) and then gated to

define singlets (FSC-H versus FSC-A plot). **(b)** To identify the signal attributable to parasite DNA and to tdTomato expression, we used uninfected RBCs and wild-type NF54 controls (asynchronous culture), and the transgenic lines *NF54-gexp02-Tom* (30-35hpi, + choline) and *NF54-etramp10.3-Tom* (72-94hpi, - choline) showing gametocytes in the tdTom + SYTO11 double positive gate (rectangle).

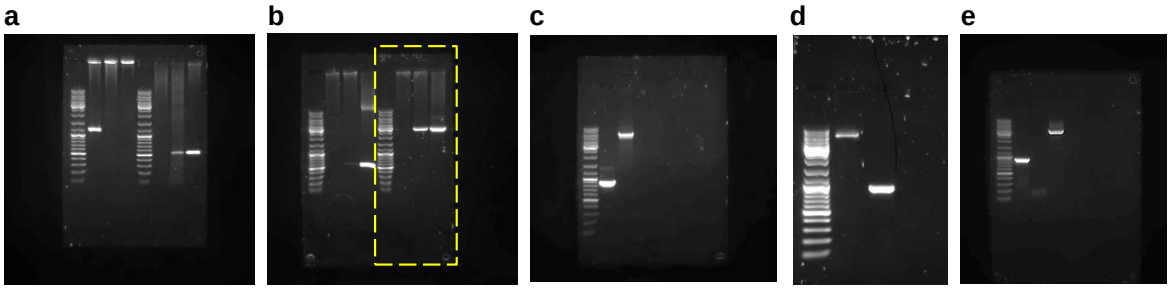


Supplementary Figure 4. Comparison of growth rates of *gexp02-Tom* and *10.3-Tom* transgenic lines under +/-choline conditions. Growth rates were calculated as the increase in parasitemia between two consecutive generations (starting with a ~1.2% parasitemia), determined by flow cytometry. Results are presented as the average and s.e.m. of two independent biological replicates.



Supplementary Figure 5. Transcript levels of *gexp02* and *pfap2-g* normalized against different reference genes. (a) Relative transcript levels of *pfap2-g* and *gexp02* in induced (- choline) and uninduced (+ choline) *NF54-gexp02-Tom* cultures analyzed at the schizont (40-45 hpi) and next generation ring (~0-10 hpi) stages. Samples are the same as in Fig. 5c in the main text, but normalized against transcript

levels of *ubiquitin-conjugating enzyme* (*uce*, ID: PF3D7_0812600), *serine-tRNA ligase* (*serrs*, ID:PF3D7_0717700) or *18S ribosomal RNA* (*18s rRNA*, ID: PF3D7_1148600). **(b)** Transcript levels of *pfap2-g* and *gexp02* in stage V gametocytes, as in Fig. 5c in the main text, but normalized against the different genes indicated. Results are presented as the average and s.e.m. of two independent biological replicates.



Supplementary Figure 6. Full length gels. (a) Full length gel for Fig. 1b (PCRs 1 and 3). **(b)** Full length gel for Fig. 1b (PCR 2). The dashed line indicates the part of the gel containing the relevant samples. **(c)** Full length gel for Supplementary Fig. 1a (left). **(d)** Full length gel for Supplementary Fig. 1a (right). **(e)** Full length gel for Supplementary Figure 1b.

Chapter 3

Artemisinin exposure at the ring or trophozoite stage impacts *Plasmodium falciparum* sexual conversion differently

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RESEARCH ARTICLE

Artemisinin exposure at the ring or trophozoite stage impacts *Plasmodium falciparum* sexual conversion differently

Harvie P Portugaliza[1,2,3], Shinya Miyazaki[4], Fiona JA Geurten[4], Christopher Pell[3,5], Anna Rosanas-Urgell[2], Chris J Janse[4], Alfred Cortés[1,6]*

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Abstract Malaria transmission is dependent on the formation of gametocytes in the human blood. The sexual conversion rate, the proportion of asexual parasites that convert into gametocytes at each multiplication cycle, is variable and reflects the relative parasite investment between transmission and maintaining the infection. The impact of environmental factors such as drugs on sexual conversion rates is not well understood. We developed a robust assay using gametocyte-reporter parasite lines to accurately measure the impact of drugs on sexual conversion rates, independently from their gametocytocidal activity. We found that exposure to subcurative doses of the frontline antimalarial drug dihydroartemisinin (DHA) at the trophozoite stage resulted in a ~ fourfold increase in sexual conversion. In contrast, no increase was observed when ring stages were exposed or in cultures in which sexual conversion was stimulated by choline depletion. Our results reveal a complex relationship between antimalarial drugs and sexual conversion, with potential public health implications.

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Introduction

Plasmodium falciparum is responsible for the most severe forms of human malaria. Repeated rounds of its ~48 hr intraerythrocytic asexual replication cycle result in an exponential increase in parasite numbers and are responsible for all clinical symptoms of malaria. At each round of replication, a small subset of parasites commits to differentiation into non-replicative sexual forms termed gametocytes, which are the only form transmissible to a mosquito vector. Sexual commitment is marked by epigenetic activation of the expression of the master regulator PfAP2-G, a transcription factor of the ApiAP2 family (***Josling et al., 2020***; ***Kafsack et al., 2014***; ***Llorà-Batlle et al., 2020***; ***Poran et al., 2017***). This is followed by sexual conversion, which according to our recently proposed definitions (***Bancells et al., 2019***) is marked by the expression of gametocyte-specific proteins absent from any replicating blood stages. After sexual conversion, parasites at the sexual ring stage develop through gametocyte stages I to V in a maturation process that lasts for ~10 days (***Josling et al., 2018***; ***Ngotho et al., 2019***). While immature gametocytes are sequestered in organs such as the bone marrow (***Venugopal et al., 2020***), mature gametocytes (stage V) are released into the circulation, where they are infectious to mosquitoes for several days or even weeks (***Cao et al., 2019***). To eliminate malaria, which the World Health Organization has adopted as a global goal, it is necessary to

block transmission, as well as killing asexual parasites to cure patients (**World Health Organization, 2017**).

To secure within-host survival and between-host transmission, the proportion of parasites that convert into sexual forms at each replicative cycle, termed sexual conversion rate, is variable and tightly regulated. In human infections, gametocyte densities are always much lower than asexual parasite densities, and basal *P. falciparum* sexual conversion rates in vivo are estimated to be ~1% (**Cao et al., 2019**; **Eichner et al., 2001**). This reveals a reproductive restraint for which multiple alternative hypotheses have been proposed (**McKenzie and Bossert, 1998**; **Mideo and Day, 2008**; **Taylor and Read, 1997**). Whatever the reason for the low levels of sexual conversion, multiple observations suggest that malaria parasites can respond to changes in the conditions of their environment by adjusting the trade-off between transmission and within-host survival. From an evolutionary perspective, the ability to adjust sexual conversion rates depending on the host conditions would be clearly advantageous for the parasite (**Carter et al., 2013**; **Schneider et al., 2018**). In *P. falciparum*, several conditions have been shown to increase sexual conversion rates, and sexual conversion is viewed by some as a response to stress, although this remains controversial (**Baker, 2010**; **Bousema and Drakeley, 2011**; **Dyer and Day, 2000**; **Josling et al., 2018**). The most commonly used method to enhance sexual conversion and obtain large numbers of gametocytes in vitro relies on overgrowing blood-stage cultures (the 'crash method') (**Delves et al., 2016**) and/or maintaining the cultures with parasite-conditioned (spent) medium (**Brancucci et al., 2015**; **Fivelman et al., 2007**). Recent research has established that depletion of the human serum lipid lysophosphatidylcholine (LysoPC) underlies the stimulation of sexual conversion by high asexual parasitemia or spent medium, providing the first mechanistic insight into how environmental conditions can influence the rate of sexual conversion (**Brancucci et al., 2018**; **Brancucci et al., 2017**). Low plasma LysoPC levels were also associated with increased sexual conversion rates in human infections (**Usui et al., 2019**). Depletion of LysoPC or choline, a downstream metabolite in the same metabolic pathway, has now been used by several groups to stimulate sexual conversion under culture conditions (**Brancucci et al., 2017**; **Filarsky et al., 2018**; **Portugaliza et al., 2019**).

Artemisinin and its derivatives (collectively referred to as ARTs) are potent antimalarial drugs that rapidly kill asexual parasites. After activation by cleavage of their endoperoxide bond by hemoglobin degradation products, ARTs produce reactive oxygen species and free radicals that result in widespread damage in parasite proteins and lipids. However, because ARTs have a very short elimination half-life in the human circulation (~1–3 hr), their application as monotherapy was discontinued to avoid infection recrudescence and development of drug resistance. Artemisinin-based combination therapies (ACTs), consisting of ART and a long-acting partner drug, are the current frontline treatment for uncomplicated as well as severe malaria cases (**Blasco et al., 2017**; **de Vries and Dien, 1996**; **Haldar et al., 2018**; **Talman et al., 2019**). Resistance to ARTs has emerged in South-East Asia in the form of delayed parasite clearance (**Dondorp et al., 2009**). ART resistance is associated with mutations in the PfKelch13 protein (**Ariey et al., 2014**) that prevent hemoglobin degradation in early ring-stage parasites. This in turn prevents ART activation, resulting in resistance of early rings to the drug (**Birnbaum et al., 2020**; **Yang et al., 2019**). Nowadays, ART resistance is frequently accompanied by simultaneous resistance to partner drugs such as mefloquine, piperaquine, or amodiaquine, resulting in high rates of treatment failure and limiting treatment options (**Mairet-Khedim et al., 2020**; **Phyo et al., 2016**; **van der Pluijm et al., 2019**).

Treatment with antimalarial drugs such as chloroquine (CQ) or sulfadoxine-pyrimethamine is usually associated with increased gametocytemia (density of gametocytes in the blood) on the days following drug administration, whereas treatment with ACTs results in reduced gametocytemia and transmission to mosquitoes (**Ippolito et al., 2017**; **Okell et al., 2008**; **Price et al., 1996**; **Sawa et al., 2013**; **von Seidlein et al., 2001**; **WWARN Gametocyte Study Group, 2016**). Despite the efficacy of ACTs in reducing gametocytemia, successfully treated patients can remain infectious for several days and contribute to transmission (**Bousema et al., 2006**; **Bousema et al., 2010**; **Karl et al., 2015**; **Targett et al., 2001**). The higher capacity of ACTs to reduce gametocytemia compared to other drugs is attributable to several factors: (i) faster killing of asexual parasites, which prevents the formation of new gametocytes; (ii) more efficient killing of immature gametocytes; (iii) partial clearance of mature gametocytes, which are insensitive to most other clinically relevant drugs (**Adjalley et al., 2011**; **Chotivanich et al., 2006**; **Plouffe et al., 2016**).

Although it has been proposed that the increase of gametocytemia observed after treatment with some drugs may reflect stimulation of sexual conversion, there is no direct linear relationship between conversion rates and the prevalence and density of circulating gametocytes (**Carter et al., 2013**; **Koepfli and Yan, 2018**; **Reece and Schneider, 2018**). The dynamics of circulating gametocyte densities after treatment can be explained without invoking an adjustment of sexual conversion rates: first, gametocytes are sequestered away from the circulation until ~10 days after sexual conversion, implying that the peaks of gametocytemia observed after treatment with some drugs (within less than 10 days) may reflect the dynamics of asexual parasite growth before treatment, rather than post-treatment changes in sexual conversion. Second, the effects of the drugs on sexual conversion rates in human infections cannot be disentangled from other drug-mediated actions such as the release of sequestered parasites or gametocyte clearance (**Babiker et al., 2008**; **Bousema and Drakeley, 2011**; **Butcher, 1997**; **Koepfli and Yan, 2018**).

To directly address the effect of drug treatment on sexual conversion, a small number of studies have used *P. falciparum* in vitro cultures, yielding inconsistent results. While some studies reported increased sexual conversion upon exposure to specific doses of drugs such as CQ or ART (**Buckling et al., 1999b**; **Peatey et al., 2009**; **Rajapandi, 2019**), others did not observe this effect with ART (**Brancucci et al., 2015**), or reported reduced sexual conversion upon exposure to low doses of CQ or pyrimethamine (**Reece et al., 2010**). Although the discrepancies may reflect methodological differences between these studies and limited accuracy in determining sexual conversion rates, the divergent conclusions also suggest a complex scenario in which conditions such as the specific drug used, the parasite stage at the time of exposure, and drug concentration may determine the effect of treatment on sexual conversion.

Given the widespread use of ACTs for malaria treatment and in mass drug administration campaigns aimed at malaria elimination, understanding the impact of ARTs on sexual conversion is an urgent research priority. Here we developed a robust assay based on recently described gametocyte-reporter parasite lines (**Portugaliza et al., 2019**) to accurately measure the impact of drugs on sexual conversion rates, independently from their gametocytocidal activity. Using this assay, we tested the effect of exposing parasites to dihydroartemisinin (DHA, the active metabolite of all ARTs) at different stages and under different metabolic conditions, to provide an accurate and comprehensive description of the direct effect of this drug on sexual conversion rates. We also tested the effect of another drug, CQ, and a different type of stress, heat shock, on sexual conversion rates.

Results

Exposure to DHA at the trophozoite stage enhances sexual conversion

To examine the effect of ARTs on *P. falciparum* sexual conversion, we administered a 3 hr pulse of DHA to synchronous cultures of the *NF54-gexp02-Tom* reporter line. This parasite line expresses the fluorescent reporter tdTomato under the control of the promoter of the sexual stage-specific gene *gexp02* (PF3D7_1102500), which allows accurate flow cytometry-based detection of very early gametocytes within a few hours after sexual conversion (**Portugaliza et al., 2019**). The short drug pulse mimics the short plasma half-life of ARTs (**de Vries and Dien, 1996**). Cultures were regularly maintained in choline-containing culture medium (Albumax-based medium with a supplement of choline) to mimic the repression of sexual conversion by healthy human serum, and choline was either maintained or removed during the experiment to repress or stimulate sexual conversion (**Brancucci et al., 2017**; **Filarsky et al., 2018**). The DHA pulse was administered at the trophozoite (**Figure 1A**) or the ring (see below) stage, using subcurative DHA concentrations (5 and 10 nM) that in trophozoites resulted in a reduction of growth of <40% (**Figure 1B**). The sexual conversion rate was calculated as the proportion of parasites that developed into gametocytes at the cycle after exposure (i.e., after reinvasion) (**Figure 1—figure supplement 1A**).

In cultures supplemented with choline, the sexual conversion rate increased from <10% in control cultures to ~40% in cultures exposed to a 5 or 10 nM DHA pulse at the trophozoite stage (**Figure 1C–D**). Importantly, total gametocytemia (determined at the next multiplication cycle after drug exposure, i.e., early gametocytes) was also clearly higher in DHA-exposed cultures than in control cultures (**Figure 1E**). This result indicates that the increase in the sexual conversion rate is not

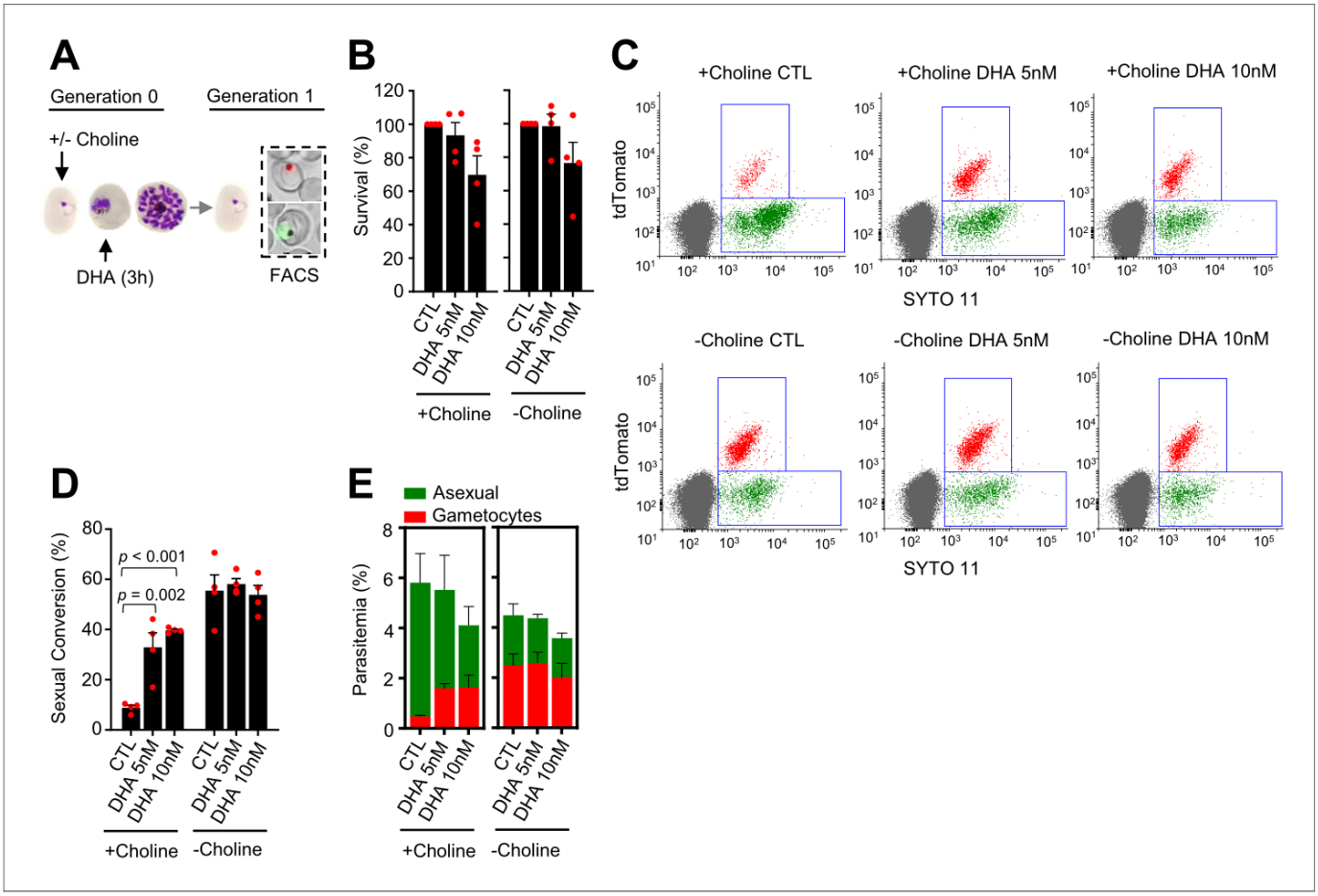

Figure 1. Effect of a dihydroartemisinin (DHA) pulse at the trophozoite stage on sexual conversion. (**A**) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-gexp02-Tom* line under non-inducing (+ choline) or inducing (– choline) conditions were exposed to a 3 hr DHA pulse at subcurative doses at the trophozoite stage (25–30 hpi). Sexual conversion was measured by flow cytometry (FACS) after reinvasion (~30–35 hpi of the next multiplication cycle). (**B**) Survival rate of cultures exposed to the different drug doses, using total parasitemia values (asexual + sexual parasites). For each choline condition, values are presented relative to the parasitemia in the control cultures (no drug). (**C**) Representative SYTO 11 (stains parasite DNA) versus TdTomato (marks gametocytes) flow cytometry plots. (**D**) Sexual conversion rate determined by flow cytometry. The p-value is indicated only for treatment versus control (no drug) significant differences ($p<0.05$). (**E**) Distribution of absolute parasitemia of asexual and sexual parasites (from the same flow cytometry measurements as in panel D). In all panels, data are presented as the average and s.e.m. of four independent biological replicates.

The online version of this article includes the following figure supplement(s) for figure 1:

Figure supplement 1. Schematic representation of the assays to determine sexual conversion rates.

Figure supplement 2. Effect of a dihydroartemisinin (DHA) pulse at the trophozoite stage on sexual conversion, determined using MitoTracker to identify viable parasites.

Figure supplement 3. Effect of a dihydroartemisinin (DHA) pulse at the trophozoite stage on sexual conversion in the *E5-gexp02-Tom* line.

Figure supplement 4. Effect of a dihydroartemisinin (DHA) pulse at the trophozoite stage in the NF54-10.3-Tom line on sexual conversion, determined by three different methods.

Figure supplement 5. Effect of a low concentration dihydroartemisinin (DHA) pulse at the trophozoite stage on sexual conversion.

Figure supplement 6. Effect on sexual conversion of a dihydroartemisinin (DHA) pulse at 5–30 nM concentrations during the trophozoite stage.

Figure supplement 7. Flow cytometry set-up for the identification of viable parasites in the 3D7-A parasite line using MitoTracker.

Figure supplement 8. Flow cytometry set-up for the identification of viable parasites in the *NF54-gexp02-Tom* line using MitoTracker.

only attributable to the lower number of asexual parasites after drug treatment, but also to a net increase in the number of gametocytes produced. By contrast, in cultures in which sexual conversion was already stimulated by choline depletion, DHA treatment did not result in a further increase in the sexual conversion rate or in the absolute number of gametocytes (***Figure 1C–E***). Similar results

were obtained in experiments in which sexual conversion rates were calculated based only on viable parasites as identified by a marker of active mitochondria (***Figure 1—figure supplement 2***), using an analogous reporter line generated in the 3D7-E5 genetic background that has lower levels of basal sexual conversion than NF54 (*E5-gexp02-Tom* line, ***Figure 1—figure supplement 3***; ***Portugaliza et al., 2019***), and using a transgenic line with the fluorescent reporter under the control of the *etramp10.3* (PF3D7_1016900) gametocyte-specific promoter (*NF54-10.3-Tom* line) (***Portugaliza et al., 2019***). Using this latter parasite line, we measured sexual conversion rates by flow cytometry, by immunofluorescence assay (IFA) detecting the Pfs16 (PF3D7_0406200) early gametocyte marker and by light microscopy analysis of Giemsa-stained blood smears (***Figure 1—figure supplement 1B***). All approaches yielded similar results and confirmed enhanced sexual conversion after exposure of trophozoites to subcurative doses of DHA (***Figure 1—figure supplement 4***).

In an additional set of experiments, we tested the impact of a DHA pulse at lower concentrations (≤5 nM) on sexual conversion (***Figure 1—figure supplement 5***). A 2 nM DHA pulse did not have a measurable effect on parasite growth (***Figure 1—figure supplement 5B***), but in choline-supplemented cultures it consistently resulted in a > twofold increase in the sexual conversion rate and the total number of gametocytes (although not statistically significant; ***Figure 1—figure supplement 5C–D***). Drug doses < 2 nM did not have a detectable effect on sexual conversion.

We also tested the impact on sexual conversion of higher DHA doses up to 30 nM, a concentration that kills ~90% of the parasites (***Figure 1—figure supplement 6A–B***). In choline-supplemented cultures, both sexual conversion rates and total gametocytemia were clearly enhanced upon exposure to DHA concentrations up to 15 nM, but the increase was lower upon exposure to higher concentrations. In choline-depleted cultures, increasing DHA doses resulted in a progressive reduction of sexual conversion (***Figure 1—figure supplement 6C–D***). However, it is important to note that the determination of sexual conversion rates is less accurate when the majority of the parasites are killed by the drug. Thus, given that maximum induction was observed at 10 nM and the difficulties to estimate sexual conversion accurately in experiments with higher drug doses, we used 5 and 10 nM DHA pulses for the majority of experiments described in the next sections.

Gametocytes of the *NF54-gexp02-Tom* line produced in cultures treated with 5 nM DHA at the trophozoite stage (in the presence of choline) matured through stages I to V without any apparent morphological alteration. Furthermore, mature, stage V gametocytes were able to exflagellate after activation and to infect mosquitoes productively (***Figure 2***). As expected from the increase in the number of early gametocytes in DHA-treated cultures, the gametocytemia 10–13 d later (mature gametocytes) and the number of exflagellation centers were also higher in DHA-treated cultures compared to control cultures (no DHA). Likewise, when using these cultures to infect mosquitoes, the number of oocysts and sporozoites per mosquito was higher in mosquitoes fed with DHA-treated cultures than in mosquitoes fed with control cultures. The magnitude of the increase was similar to that observed in mosquitoes fed with cultures in which sexual conversion was stimulated by choline depletion (***Figure 2B***). While only differences in the number of exflagellation centers and oocysts/mosquito were statistically significant ($p<0.05$) between DHA-treated and control cultures (which is likely attributable to the intrinsic variability of mosquito feeding experiments), a clear increase was observed in all independent biological replicates for all parameters tested.

DHA exposure at the ring stage does not enhance sexual conversion

A DHA pulse (5 or 10 nM) at the early ring stage that reduced growth by <25% (***Figure 3A–B***) did not enhance sexual conversion. Instead, it resulted in a reduction of sexual conversion and gametocytemia, both in choline-supplemented and choline-depleted *NF54-gexp02-Tom* cultures (***Figure 3C–E***; ***Figure 3—figure supplement 1***). This unexpected result was confirmed using the *NF54-10.3-Tom* reporter line and the different methods described above to assess sexual conversion (***Figure 3—figure supplement 2***). In an additional set of experiments using *NF54-gexp02-Tom* cultures, we tested higher concentrations of the drug, and again observed a decrease in sexual conversion rates that in choline-depleted cultures was more marked with higher concentrations of the drug (***Figure 3—figure supplement 3***). In these experiments, we determined sexual conversion by measuring expression of the gametocyte reporter at 24 and 48 hr post invasion (hpi), and observed no difference between sexual conversion determined at the two different time points. This result excludes the possibility that the lower conversion rates observed in cultures exposed to DHA at the

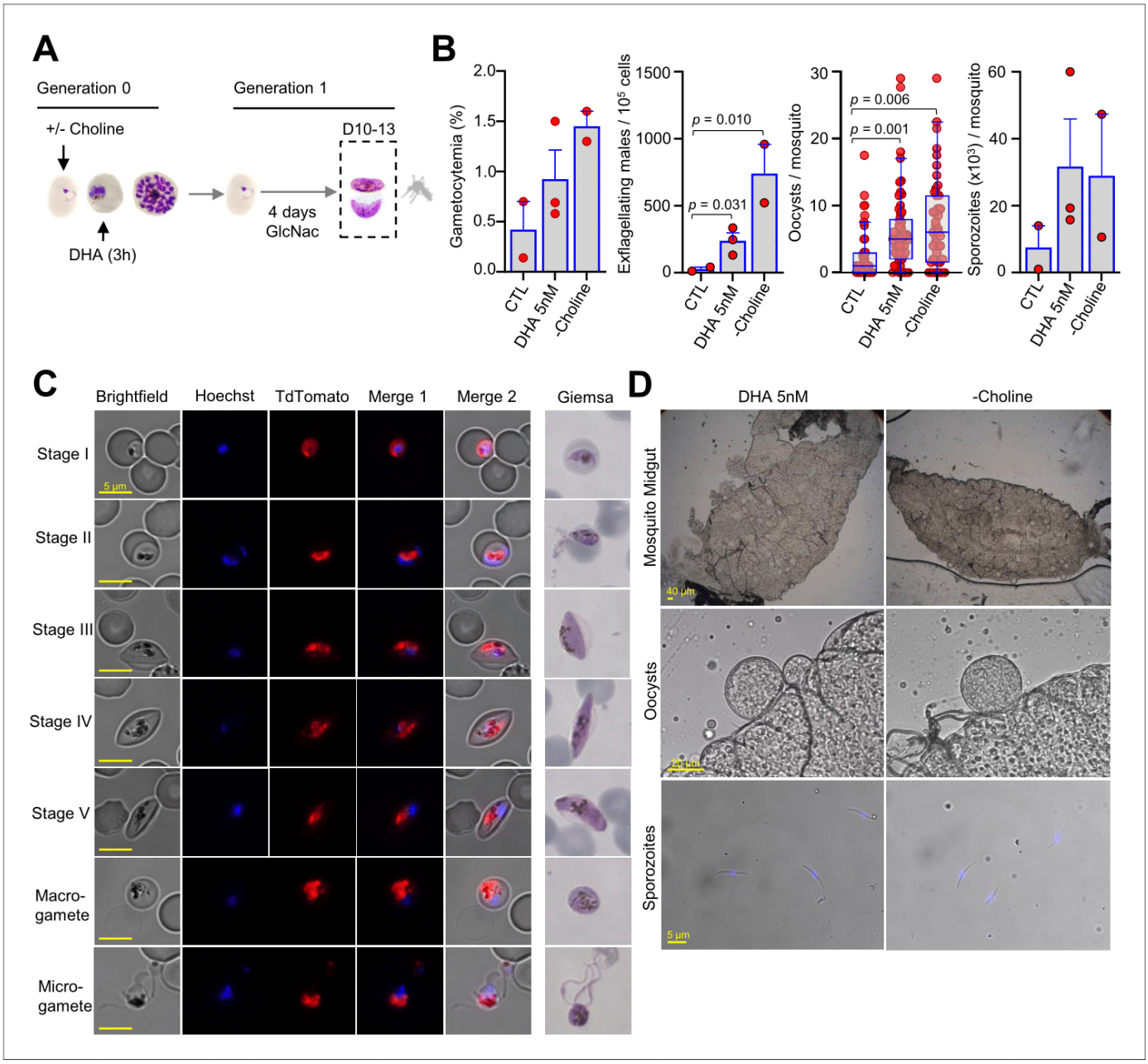

Figure 2. Mosquito infection by gametocytes from cultures exposed to dihydroartemisinin (DHA). (**A**) Schematic representation of the assay. Sorbitol-synchronized cultures of the *NF54-gexp02-Tom* line were maintained under control non-inducing conditions (+ choline, CTL), exposed to a 3 hr 5 nM DHA pulse (in the presence of choline) at the trophozoite stage, or maintained in the absence of choline (– choline, used as a positive control for a gametocyte-inducing condition). On the first day of Generation 1, N-acetylglucosamine (GlcNAc) was added and maintained for 4 d to eliminate asexual parasites and obtain pure gametocyte cultures. The different cultures were used to infect *Anopheles* mosquitoes by standard membrane feeding. (**B**) Gametocytemia at the time of mosquito infection (10–13 d after DHA treatment), exflagellation levels (after 10 min of activation with fetal calf serum), number of oocysts/mosquito (n = 53 for CTL, n = 103 for DHA 5 nM, and n = 79 for – choline, data for all individually dissected mosquitoes from all replicates is shown) and average number of sporozoites/mosquito in each independent biological replicate (obtained from pooled dissections; in total, n = 65 for CTL; n = 111 for DHA; n = 123 for – choline). Results are from three independent biological replicates, but in one experiment the CTL culture was lost and in another one the – choline control was not included. Data are presented as the average and s.e.m. of the independent biological replicates, except for oocysts/mosquito results that are presented as standard box and whisker plots. The p-value is indicated only for significant differences ($p<0.05$) between conditions. (**C**) Representative images of gametocytes at different stages and activated gametes from DHA-treated cultures, showing no apparent abnormality. Images from live cell fluorescence analysis (Hoechst stains nuclei; TdTomato is expressed under the

Figure 2 continued on next page

Figure 2 continued

control of the *gexp02* promoter) and Giemsa-stained smears are shown. (**D**) Representative images of mosquito midguts (transparent, circular structures are oocysts), oocysts, and sporozoites from DHA-treated and – choline cultures, showing no apparent abnormality.

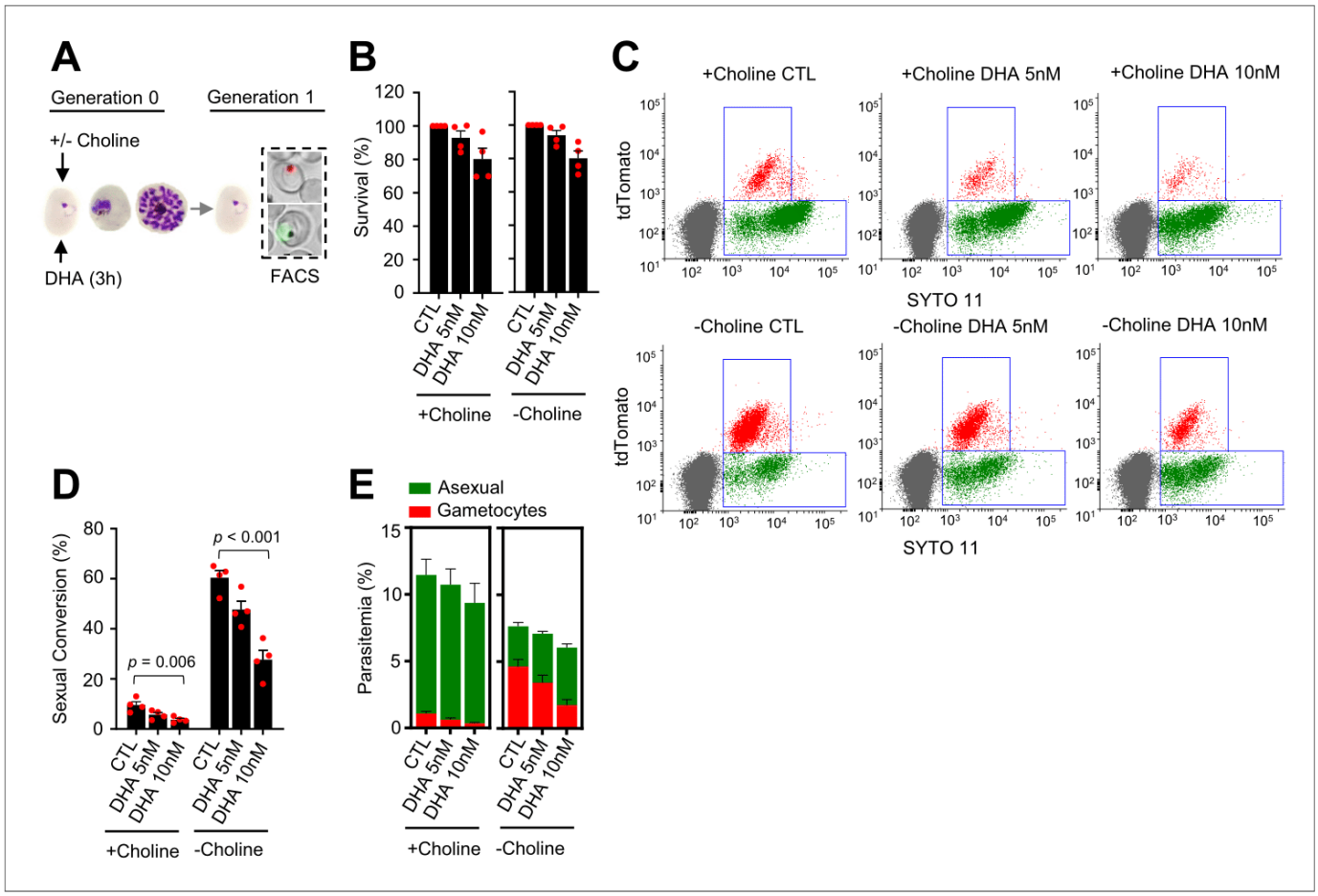

Figure 3. Effect of a dihydroartemisinin (DHA) pulse at the ring stage on sexual conversion. (**A**) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-gexp02-Tom* line under non-inducing (+ choline) or inducing (– choline) conditions were exposed to a 3 hr DHA pulse at subcurative doses at the early ring stage (0–10 hpi). Sexual conversion was measured by flow cytometry (FACS) after reinvasion (~30–40 hpi of the next multiplication cycle). (**B**) Survival rate of cultures exposed to the different drug doses, using total parasitemia values (asexual + sexual parasites). For each choline condition, values are presented relative to the parasitemia in the control cultures (no drug). (**C**) Representative SYTO 11 (stains parasite DNA) versus TdTomato (marks gametocytes) flow cytometry plots. (**D**) Sexual conversion rate determined by flow cytometry. The p-value is indicated only for treatment versus control (no drug) significant differences ($p<0.05$). (**E**) Distribution of absolute parasitemia of asexual and sexual parasites (from the same flow cytometry measurements as in panel D). In all panels, data are presented as the average and s.e.m. of four independent biological replicates.

The online version of this article includes the following figure supplement(s) for figure 3:

Figure supplement 1. Effect of a dihydroartemisinin (DHA) pulse at the ring stage on sexual conversion, determined using MitoTracker to identify viable parasites.

Figure supplement 2. Effect on sexual conversion of a dihydroartemisinin (DHA) pulse at the ring stage in the *NF54-10.3-Tom* line, determined by three different methods.

Figure supplement 3. Effect on sexual conversion of a dihydroartemisinin (DHA) pulse at 10–40 nM concentrations at the ring stage.

ring stage are attributable to a DHA-induced delay in sexual conversion or gametocyte development (***Figure 3—figure supplement 3***).

To explore the possibility that DHA exposure at the early ring stage may stimulate immediate sexual conversion via the same cycle conversion (SCC) pathway (***Bancells et al., 2019***), rather than by the canonical next cycle conversion (NCC) pathway, we assessed the effect of DHA exposure at the ring stage on the level of gametocytes produced within the same cycle of exposure (***Figure 4A***). We observed no apparent differences in sexual conversion rates via the SCC route between DHA-exposed cultures and their controls (***Figure 4B–C***; ***Figure 4—figure supplement 1***). Similar results were obtained using the *NF54-10.3-Tom* reporter line and flow cytometry or light microscopy analysis of Giemsa-stained smears to measure sexual conversion by the SCC pathway. However, IFA analysis of this parasite line using anti-Pfs16 antibodies revealed an increase in the proportion of parasites expressing this endogenous protein upon DHA exposure (***Figure 4—figure supplement 2***). The significance of this observation remains unclear but it may indicate a rapid effect of DHA on the expression of some gametocyte specific genes without further sexual development.

In these experiments, choline depletion did not increase sexual conversion via the SCC route (***Figure 4B***). This result may be explained by two alternative scenarios: (i) conversion via the SCC route is insensitive to choline depletion; (ii) ring stages are insensitive to stimulation of sexual conversion by choline depletion. To distinguish between these two possibilities, we assessed sexual conversion via the NCC pathway in cultures in which choline was depleted at different stages of the life cycle (***Figure 5A***). We found that choline depletion at the ring stage does not induce sexual conversion, in contrast to depletion at the trophozoite stage (***Figure 5B–C***). Altogether, these results show that in parasites at the ring stage neither a DHA pulse nor choline depletion induces sexual

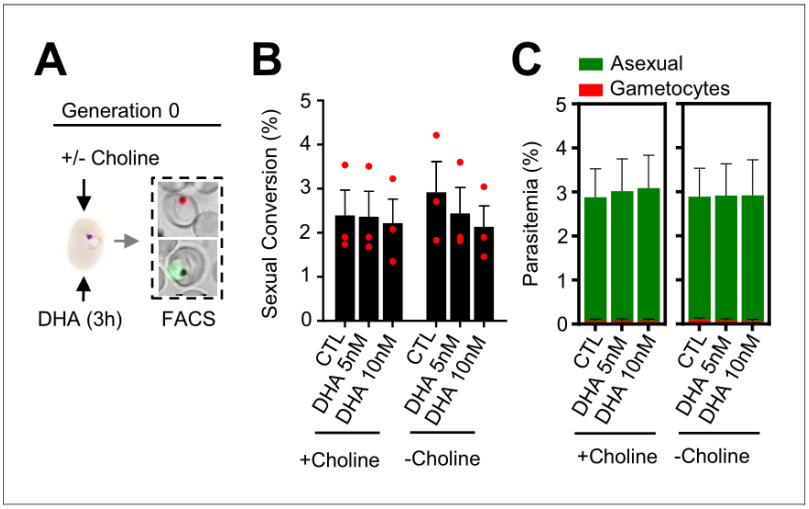

Figure 4. Effect of a dihydroartemisinin (DHA) pulse at the ring stage on sexual conversion by the same cycle conversion (SCC) route. (**A**) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-gexp02-Tom* line under non-inducing (+ choline) or inducing (– choline) conditions were exposed to a 3 hr DHA pulse at subcurative doses at the early ring stage (0–10 hpi). Sexual conversion was measured by flow cytometry (FACS) within the same multiplication cycle (~30–40 hpi) to determine the effect of the drug pulse only on production of new gametocytes by the SSC route. (**B**) Sexual conversion rate determined by flow cytometry. No significant difference ($p<0.05$) with the control (no drug) was observed for any treatment condition. (**C**) Distribution of absolute parasitemia of asexual and sexual parasites (from the same flow cytometry measurements as in panel B). In all panels, data are presented as the average and s.e.m. of three independent biological replicates.
The online version of this article includes the following figure supplement(s) for figure 4:

Figure supplement 1. Effect of a dihydroartemisinin (DHA) pulse at the ring stage on sexual conversion by the same cycle conversion (SCC) route, determined using MitoTracker to identify viable parasites.

Figure supplement 2. Effect of a dihydroartemisinin (DHA) pulse at the ring stage on sexual conversion by the same cycle conversion (SCC) route in the *NF54-10.3-Tom* line, determined by three different methods.

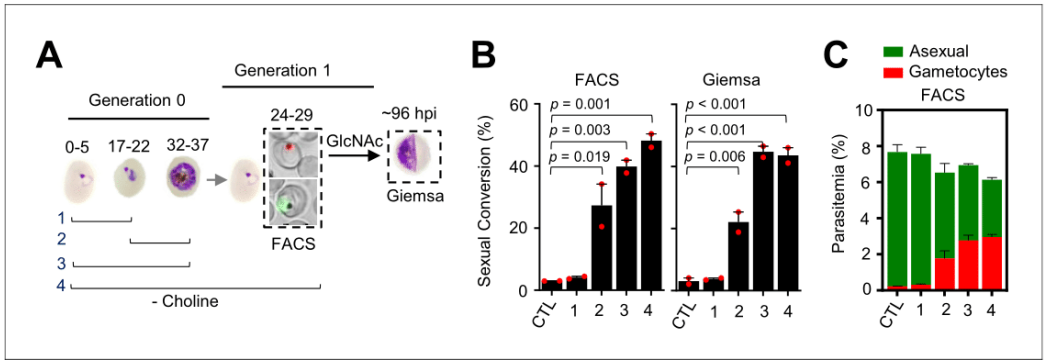

Figure 5. Changes in sexual conversion rates after choline depletion at different parasite stages. (**A**) Schematic representation of the assay. Choline was removed from tightly synchronized cultures of the *NF54-gexp02-Tom* line for the periods indicated, and sexual conversion rates measured after reinvasion by flow cytometry (FACS;~24–29 hpi of the following multiplication cycle) or by light microscopy analysis of Giemsa-stained smears (Giemsa;~96 hpi) in cultures treated with GlcNac. Control (CTL) cultures were maintained with choline all the time. (**B**) Sexual conversion rate for cultures under different conditions. The p-value is indicated only for choline depletion versus control significant differences ($p<0.05$). (**C**) Distribution of absolute parasitemia of asexual and sexual parasites, determined by flow cytometry (from the same flow cytometry measurements as in panel B). In all panels, data are presented as the average and s.e.m. of two independent biological replicates.

conversion, suggesting that this developmental stage is largely insensitive to environmental stimulation of sexual conversion.

Exposure to CQ or heat shock at the trophozoite stage can also enhance sexual conversion

Using the same drug pulse approach, we assessed whether CQ, a drug with a different mode of action than DHA (***Haldar et al., 2018***), also stimulates sexual conversion (***Figure 6A***). Exposure to 80 nM CQ at the trophozoite stage, a dose that induces ~40% lethality (***Figure 6B***), resulted in enhanced sexual conversion rates in choline-supplemented cultures (***Figure 6C–D***; ***Figure 6—figure supplement 1***). However, the level of induction was only ~ twofold, much lower than induction by DHA, and there was no consistent induction at higher or lower drug doses. Similar to DHA, CQ exposure at the trophozoite stage did not increase sexual conversion in choline-depleted cultures (***Figure 6C–D***), and exposure to CQ at the ring stage did not enhance sexual conversion by either the NCC or the SCC (***Figure 6—figure supplements 2–3***) routes. Reduced sexual conversion was observed in choline-depleted cultures treated with CQ doses that kill the vast majority of parasites, but this needs to be interpreted with caution because of the intrinsic limitations of sexual conversion assays when the majority of parasites are killed (***Figure 6***; ***Figure 6—figure supplements 1–2***).

We also tested the effect of an unrelated type of stress, a 3 hr heat shock at 41.5˚C mimicking a malarial febrile episode, on sexual conversion. Exposure of choline-supplemented cultures at the trophozoite stage to heat shock, which reduced survival by ~40%, resulted in a ~ fourfold increase in sexual conversion and gametocytemia (***Figure 7***).

Enhancement of sexual conversion by DHA operates via *pfap2-g*

To determine whether stimulation of sexual conversion by DHA involves the activation of the master regulator *pfap2-g* (PF3D7_1222600), we analyzed the transcript levels for this gene after a DHA pulse, and also for one of its earliest known targets, *gexp02* (***Filarsky et al., 2018***; ***Josling et al., 2020***; ***Llorà-Batlle et al., 2020***; ***Portugaliza et al., 2019***; ***Silvestrini et al., 2010***). Transcript levels for the two genes were determined at the schizont stage of the cycle of exposure and at the ring stage of the next cycle. A subcurative DHA pulse at the trophozoite stage resulted in upregulation of both *pfap2-g* and *gexp02* relative to the *serine-tRNA ligase* (PF3D7_0717700) reference gene in choline-supplemented cultures, but not in choline-depleted cultures (***Figure 8A–C***). By contrast, exposure to DHA at the ring stage resulted in reduced expression of both genes (***Figure 8D–F***). Analysis of transcripts only 2 hr after DHA exposure at the ring stage did not reveal induction of *pfap2-g* or *gexp02* (***Figure 8G–I***), ruling out activation of the genes at a time consistent with conversion via the SCC route. Identical results were obtained when normalizing *pfap2-g* or *gexp02*

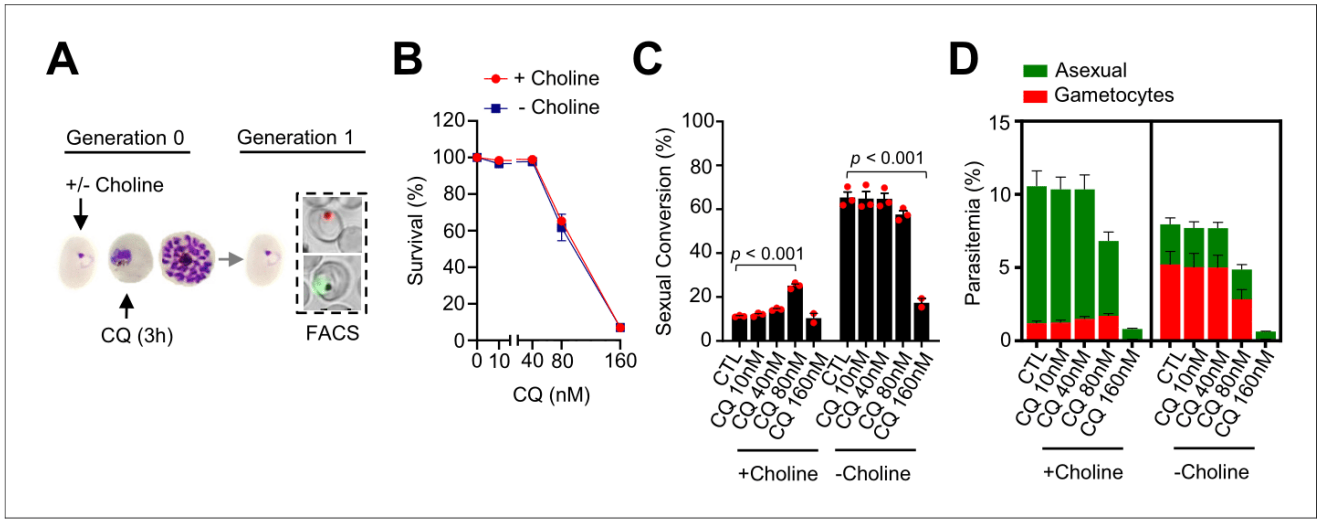

Figure 6. Effect of a chloroquine (CQ) pulse at the trophozoite stage on sexual conversion. (**A**) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-gexp02-Tom* line under non-inducing (+ choline) or inducing (– choline) conditions were exposed to a 3 hr CQ pulse at subcurative doses at the trophozoite stage (25–30 hpi). Sexual conversion was measured by flow cytometry (FACS) after reinvasion (~30–35 hpi of the next multiplication cycle). (**B**) Survival rate of cultures exposed to the different drug doses, using total parasitemia values (asexual + sexual parasites). For each choline condition, values are presented relative to the parasitemia in the control cultures (no drug). (**C**) Sexual conversion rate determined by flow cytometry. The p-value is indicated only for treatment versus control (no drug) significant differences ($p<0.05$). (**D**) Distribution of absolute parasitemia of asexual and sexual parasites (from the same flow cytometry measurements as in panel C). In all panels, data are presented as the average and s.e.m. of three independent biological replicates.

The online version of this article includes the following figure supplement(s) for figure 6:

Figure supplement 1. Effect of a chloroquine (CQ) pulse at the trophozoite stage on sexual conversion, determined using MitoTracker to identify viable parasites.

Figure supplement 2. Effect of a chloroquine (CQ) pulse at the ring stage on sexual conversion.

Figure supplement 3. Effect of a chloroquine (CQ) pulse at the ring stage on sexual conversion by the same cycle conversion (SCC) route.

transcript levels against *ubiquitin-conjugating enzyme* (PF3D7_0812600) as a reference gene (***Figure 8—figure supplement 1***). Overall, the findings of these transcriptional analyses clearly mirror the effect of the drug on sexual conversion rates, indicating that induction of sexual conversion by DHA is associated with *pfap2-g* activation.

Discussion

ARTs are the key component of ACTs, the most widely used treatment for clinical malaria. Additionally, ACTs may be widely administered in mass drug administration campaigns aimed at malaria elimination. Given that the success of malaria control and elimination efforts largely depends on preventing disease transmission, understanding the impact of ARTs on the production of transmission forms is of paramount importance. Our results show a complex effect of DHA on the trade-off between asexual proliferation and the formation of transmission forms. Exposure of parasites at the trophozoite stage to subcurative doses of DHA resulted in a large increase in sexual conversion rates and total number of gametocytes, which were viable and infectious to mosquitoes. However, this was not observed when parasites were exposed to the same drug doses at the ring stage. Furthermore, in cultures in which sexual conversion was already stimulated at the metabolic level (i.e., by depletion of choline), DHA did not further stimulate sexual conversion at either stage. The accurate determination of the impact of DHA on sexual conversion rates at different stages was possible thanks to the development of an assay that uses a short drug pulse and reporter parasite lines that enable very early detection of gametocytes by flow cytometry. A limitation of our study is that we only used wild type parasite lines that do not carry mutations in the PfKelch13 protein associated with artemisinin resistance (***Ariey et al., 2014***; ***Birnbaum et al., 2020***; ***Yang et al., 2019***). Future

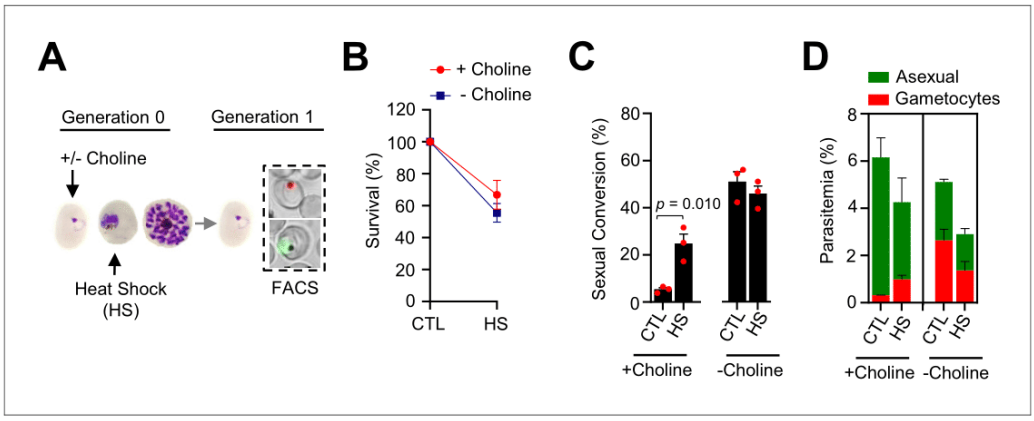

Figure 7. Effect of heat shock at the trophozoite stage on sexual conversion. (**A**) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-gexp02-Tom* line under non-inducing (+ choline) or inducing (– choline) conditions were exposed to a 3 hr heat shock (41.5˚C) at the trophozoite stage (25–30 hpi). Sexual conversion was measured by flow cytometry (FACS) after reinvasion (~30–35 hpi of the next multiplication cycle). (**B**) Survival rate of cultures exposed to heat shock (HS) or maintained at 37˚C (CTL), using total parasitemia values (asexual + sexual parasites). For each choline condition, values are presented relative to the parasitemia in the control cultures. (**C**) Sexual conversion rate determined by flow cytometry. The p-value is indicated only for heat shock versus control significant differences ($p<0.05$). (**D**) Distribution of absolute parasitemia of asexual and sexual parasites (from the same flow cytometry measurements as in panel C). In all panels, data are presented as the average and s.e.m. of three independent biological replicates.

studies should assess the effect of DHA on sexual conversion in parasite lines carrying such mutations, to determine whether or not the effect of the drug on sexual conversion is linked to its effect on parasite survival.

The overall impact of a drug on the transmission potential of an infection depends on its effect on the sexual conversion rate, and on several other factors. In the case of ARTs, the stimulation of sexual commitment at the trophozoite stage may not result in an overall increase in transmission due to rapid clearance of asexual parasites, which prevents new rounds of gametocyte production, and to the activity of the drug against developing and mature gametocytes. Indeed, several studies have observed that treatment with drug combinations containing ARTs reduce gametocyte density and the duration of gametocyte carriage (***Bousema et al., 2006***; ***Bousema et al., 2010***; ***Ippolito et al., 2017***; ***Karl et al., 2015***; ***Okell et al., 2008***; ***Price et al., 1996***; ***Sawa et al., 2013***; ***Targett et al., 2001***; ***von Seidlein et al., 2001***; ***WWARN Gametocyte Study Group, 2016***). Notwithstanding the net reduction of transmission potential commonly observed after ART treatment, it is possible that patients in which many of the parasites are at the trophozoite stage at the time of ART administration may experience a peak of circulating gametocytes ~ 10 days after treatment (the time required for gametocyte maturation), if the drug does not kill all parasites. In this regard, it is noteworthy that the largest stimulation of sexual conversion was observed at subcurative doses of the drug. Such low drug concentrations may occur during treatment with substandard or underdosed drugs, through poor compliance with the prescribed regimen, as a consequence of drug malabsorption, or as the drug is eliminated following its natural pharmacokinetics profile. Treatment associated with low ARTs concentration may enable survival of some parasites, and at the same time enhance the probability of sexual conversion. Thus, our findings have potential public health implications for the use of ARTs in treatment and elimination strategies. While the benefits of ARTs for malaria treatment clearly outweigh the potential risks, the possibility that ARTs increase the transmission potential of some patients should be taken into account when considering their massive use in preventive treatment or elimination campaigns.

There is ongoing debate regarding whether human malaria parasites can modulate their level of investment in producing transmission forms as a response to 'stress' (i.e., a condition that reduces the asexual multiplication rate). Whether the impact of stress on sexual conversion rates is positive (enhancement) or negative (reduction) also remains controversial (***Buckling et al., 1999a***; ***Buckling et al., 1999b***; ***Buckling et al., 1997***; ***Koepfli and Yan, 2018***; ***Peatey et al., 2009***; ***Schneider et al., 2018***). Evolutionary theory for life histories predicts that treatment with low doses

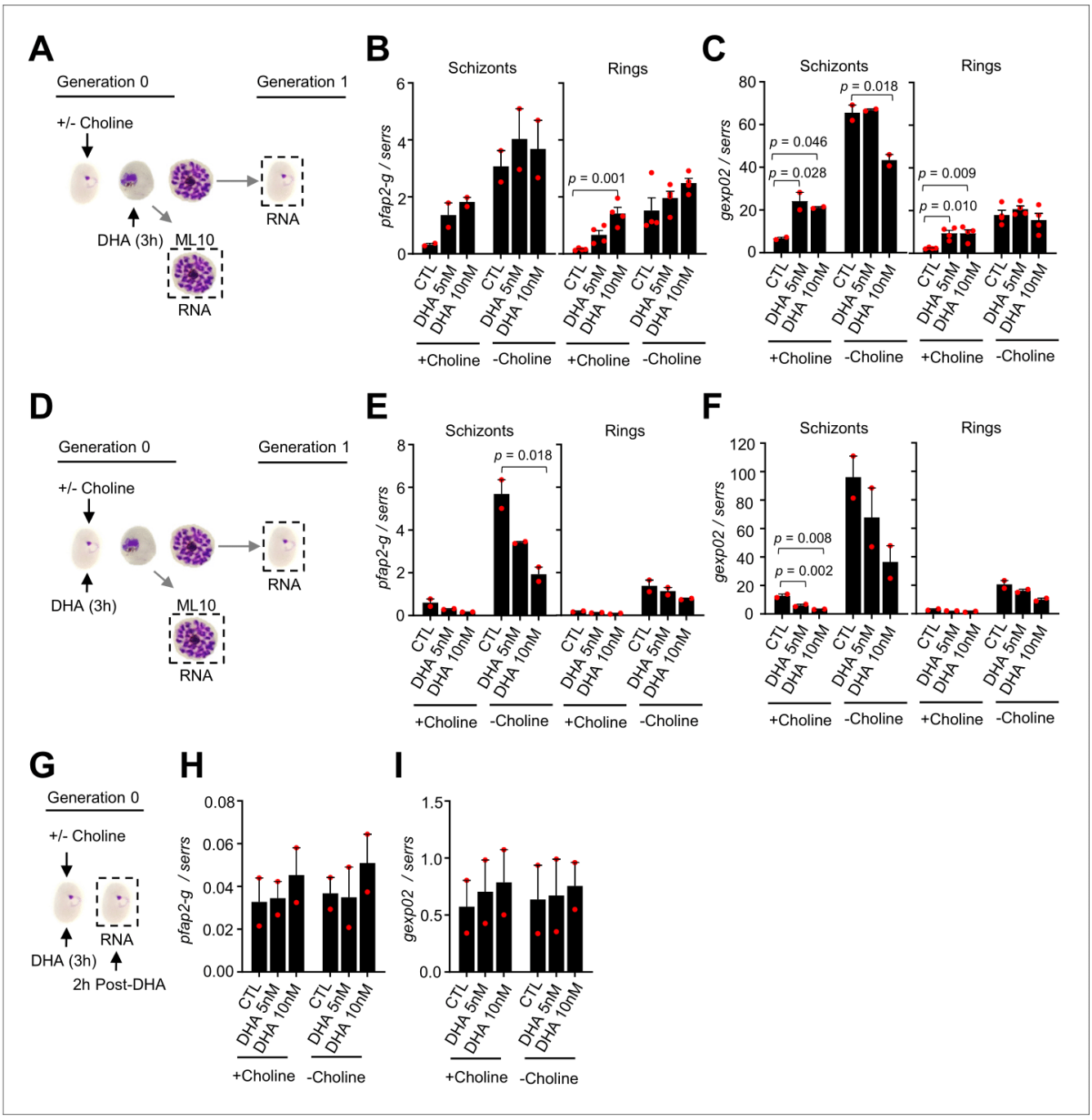

Figure 8. Changes in the expression of *pfap2-g* and *gexp02* after a dihydroartemisinin (DHA) pulse. (**A**) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-gexp02-Tom* line under non-inducing (+ choline) or inducing (– choline) conditions were exposed to a 3 hr DHA pulse at subcurative doses at the trophozoite stage (25–30 hpi). RNA for transcriptional analysis was collected from ML10-treated cultures at the mature schizont stage (48–53 hpi) and, after reinvasion, from cultures at the early ring stage (cultures not treated with ML10,~5 hpi). (**B–C**) Transcript levels of *pfap2-g* (**B**) or *gexp02* (**C**) normalised against the *serine-tRNA ligase* (*serrs*) gene. (**D–F**) Same as panels **A-C**, but cultures were exposed to DHA at the ring stage (0–10 hpi). (**G–I**) Same as panels **D-F**, but RNA for transcriptional analysis was collected only 2 hr after completing the drug pulse. Data are presented as the average and s.e.m. of four (panels **B-C**, rings) or two (other panels) independent biological replicates. The p-value is indicated only for treatment versus control (no drug) significant differences ($p<0.05$).

The online version of this article includes the following figure supplement(s) for figure 8:

Figure supplement 1. Changes in the transcript levels of *pfap2-g* and *gexp02* after a dihydroartemisinin (DHA) pulse, normalized against the *uce* gene.

of antimalarial drugs results in reproductive restraint (reduced sexual conversion) to facilitate within-host survival, whereas treatment with high doses that kill the majority of the parasites elicits terminal investment (increased sexual conversion). The results of a recent study using a murine model of malaria were consistent with this prediction (**Schneider et al., 2018**). Our experiments with in vitro cultured *P. falciparum* exposed to low doses of DHA at the ring stage were also consistent with these prediction, as this resulted in moderately reduced sexual conversion rates. In contrast, experiments in which a pulse of DHA or CQ at a low dose was administered at the trophozoite stage showed the opposite trend, such that the subcurative treatment stimulated sexual conversion. This latter result is in line with some previous studies using *P. falciparum* (**Buckling et al., 1999b**; **Peatey et al., 2009**) or a murine malaria model (**Buckling et al., 1999a**; **Buckling et al., 1997**). A possible explanation for the discrepancy with the predictions of evolutionary theory (when exposure occurs at the trophozoite stage) is that in the absence of stress, sexual conversion in *P. falciparum* is already restrained, with estimated conversion rates of ~1% in human infections (**Cao et al., 2019**; **Eichner et al., 2001**). Thus, a further reduction of the investment in transmission upon exposure to low drug doses would not have a substantial impact on within-host survival, implying that this response would not provide a selective advantage, whereas the opposite response can enhance the chances of transmission. Of note, the absence of LysoPC and choline (**Brancucci et al., 2017**), or heat shock, all of which reduce the multiplication rate of *P. falciparum* cultures and therefore can also be considered as sublethal stress signals, also stimulate sexual conversion. Together, the results of experiments with *P. falciparum* cultures exposed to low level of stress at the trophozoite stage do not support the predictions of evolutionary theory, whereas for murine malaria parasites different studies reported conflicting results. In this regard, it is possible that different *Plasmodium* species use different strategies to adjust sexual conversion rates upon stress: although the role of AP2-G as the master regulator of sexual conversion appears to be widely conserved in all malaria parasite species, upstream events involved in the regulation of sexual conversion are remarkably different between human and murine parasites. The latter show higher conversion rates, do not alter sexual conversion in response to LysoPC restriction, and their genomes lack a *gdv1* ortholog (**Ngotho et al., 2019**).

Our experiments establish that sexual conversion can be stimulated by exposure to DHA at the trophozoite stage, but not at the early ring stage. Of note, stimulation of sexual conversion by depletion of choline (as a proxy for LysoPC depletion) or by exposure to CQ shows a similar stage dependency, suggesting that the ring stage is largely insensitive to stimulation of sexual conversion. At the ring stage, some types of stress, such as exposure to DHA, may induce latency of a rather small fraction of the parasites as a means of population survival (**Barrett et al., 2019**; **Talman et al., 2019**), rather than enhancing sexual conversion. Furthermore, we found that in cultures in which sexual conversion is stimulated by choline depletion, it cannot be further stimulated by drugs, such that there are no additive or synergistic effects between drugs and choline depletion. Together, these observations suggest that the different stimuli converge into the same mechanism of *pfap2-g* activation, which likely involves cellular components that are absent during the ring stage. Because stimulation of sexual conversion by choline depletion has been shown to involve GDV1 (**Filarsky et al., 2018**), which is only expressed in the second half of the intraerythrocytic development cycle and is absent from ring stage parasites, we hypothesize that stimulation by DHA may also depend on GDV1. A possible explanation for the similar effects of DHA and choline depletion on sexual conversion is that treatment with DHA may result in choline depletion: DHA induces damage on membrane lipids (**Hartwig et al., 2009**), which may increase the use of LysoPC or choline, resulting in a reduction of their levels. However, heat shock, a completely different type of stress that is not known or predicted to affect LysoPC or choline levels, also enhanced sexual conversion. This result suggests that stimulation of sexual conversion can occur without the involvement of choline metabolism. As an alternative model, parasites may be able to sense a state of mild to moderate 'stress' or growth restriction (**Schneider et al., 2018**): the drug doses that result in increased sexual conversion, as well as LysoPC or choline restriction (**Brancucci et al., 2017**; **Portugaliza et al., 2019**) and heat shock, were all associated with a < 50% reduction of growth rates. The observation that a 2 nM pulse of DHA, which does not have any measurable effect on parasite survival, appears to stimulate sexual conversion may indicate that sexual conversion is triggered by the response associated with moderate stress, rather than by growth restriction per se. In this regard, activation of the cellular stress response has been proposed to be associated with enhanced gametocyte production

(**Chaubey et al., 2014**), and DHA triggers such a stress response (**Bridgford et al., 2018**; **Zhang et al., 2017**). Further research is needed to establish the molecular mechanisms underlying the modulation of sexual conversion rates by different environmental conditions.

Altogether, here we provide a detailed characterization of the changes in *P. falciparum* sexual conversion rates that occur in response to a pulse of DHA. We demonstrate remarkable plasticity in sexual conversion rates, and a complex response that depends on the stage of the parasites at the time when they are exposed to the drug, the drug dose, and the metabolic state (presence or absence of choline). This complex scenario may explain the discrepant results obtained by previous studies. The assay that we have developed to test the impact of DHA on sexual conversion rates can be used to test the impact of any other drug or condition, as shown here for CQ and heat shock. Of note, the success of malaria elimination efforts largely depends on the ability to reduce or interrupt transmission. Although our results are not of immediate public health concern because the overall impact of treatment with ACTs is a reduction of the transmission potential, at least when compared with other drugs, the capacity of ARTs to induce sexual conversion must be taken into account. Otherwise, under certain conditions, treatment may result in an increase in transmission that could jeopardize efforts to eliminate malaria.

Materials and methods

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Gene (*Plasmodium falciparum*)	*pfap2-g*	PlasmoDB	PF3D7_1222600	
Gene (*Plasmodium falciparum*)	*gexp02*	PlasmoDB	PF3D7_1102500	
Cell line (*Plasmodium falciparum*)	*NF54-gexp02-Tom*	PMID:31601834		Maintained in culture with 2 mM choline
Cell line (*Plasmodium falciparum*)	*E5-gexp02-Tom*	PMID:31601834		Maintained in culture with 2 mM choline
Cell line (*Plasmodium falciparum*)	*NF54-10.3-Tom*	PMID:31601834		Maintained in culture with 2 mM choline
Commercial assay or kit	RNeasy Mini Kit	Qiagen	Cat. No. 74104	
Chemical compound, drug	ML10	PMID:28874661; S. Osborne (LifeArc) and D. Baker (LSHTM)		cGMP-dependent protein kinase inhibitor
Chemical compound, drug	Dihydroartemisinin (DHA)	Sigma-Aldrich	Cat. No. D7439	
Chemical compound, drug	Chloroquine	Sigma-Aldrich	Cat. No. C6628	
Chemical compound, drug	Choline chloride	Sigma-Aldrich	Cat. No. C7527	
Chemical compound, drug	N-acetyl-d-glucosamine	Sigma-Aldrich	Cat. No. A8625	

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Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Software, algorithm	BD FACSDiva Software	BD Biosciences	RRID:SCR_001456	Flow cytometry acquisition and analysis using BD LSRFortessa machine
Software, algorithm	Flowing Software version 2.5.1	Perttu Terho	RRID:SCR_015781	Flow cytometry data analysis
Software, algorithm	Prism 8	GraphPad	RRID:SCR_002798	
Antibody	Pfs16 (mouse, monoclonal)	R.Sauerwein, Radboud University	32F717:B02	IFA (1:400)
Antibody	Goat-anti-mouse IgG–Alexa Fluor 488	Thermo Fisher	Cat. No. A11029	IFA (1:1000)
Other	SYTO 11	Life Technologies	Cat. No. S7573	Flow cytometry (0.016 µM)
Other	MitoTracker Deep Red FM	Invitrogen	Cat. No. M22426	Flow cytometry (0.6 µM)
Other	DAPI	Applichem lifescience	Cat. No. A4099.0005	IFA (5 µg/mL)
Other	Hoechst 33258	Thermo Fisher	Cat. No. H3569	Live cell fluorescence microscopy (2 µM)

Parasite cultures

The transgenic reporter lines *NF54-gexp02-Tom*, *E5-gexp02-Tom*, and *NF54-10.3-Tom* were previously described and characterized (**Portugaliza et al., 2019**). These parasite lines carry a *tdTomato* reporter gene under the control of either the *gexp02* or the *etramp10.3* promoters. Since these *P. falciparum* lines were generated and validated in our laboratory, and the expression pattern of the fluorescent markers confirms their identities, additional authentication was considered unnecessary. They were not tested for *Mycoplasma*, but *Mycoplasma* contamination is not known to affect any of the parameters analyzed in this study.

Cultures were regularly maintained at 37˚C under shaking (100 rpm) or static conditions in a hypoxic atmosphere (2% O_2, 5.5% CO_2, balance N_2), with B+ erythrocytes (3% hematocrit) and standard RPMI-HEPES parasite culture medium containing 0.5% Albumax and supplemented with 2 mM choline (**Filarsky et al., 2018**; **Portugaliza et al., 2019**). Erythrocytes were obtained from the Catalan official blood bank (Banc de Sang i Teixits). To obtain cultures of a well-defined age window, we used Percoll/sorbitol synchronization. In brief, Percoll-purified schizonts were used to establish a fresh culture that 5 or 10 hr later was subjected to 5% D-sorbitol lysis to obtain cultures of a defined 0–5 or 0–10 hpi age window. For heat shock experiments, synchronized cultures at the trophozoite stage were transferred for 3 hr to an incubator at 41.5˚C and then placed back at 37˚C (**Rovira-Graells et al., 2012**).

Cultures for the production of mature gametocytes for mosquito infection were maintained in a semi-automated shaker incubator system as described (**Mogollon et al., 2016**). Fresh human serum and erythrocytes for these experiments were obtained from the Dutch National Blood Bank (Sanquin Amsterdam, the Netherlands; permission granted from donors for the use of blood products for malaria research and microbiology; tested for safety). Erythrocytes and human serum from different donors were pooled.

Drug treatment and determination of drug survival rates

To test the impact of drugs on sexual conversion in the presence or absence of choline, after tight synchronization (0–5 or 0–10 hpi) cultures at ~1.5% parasitemia were split in two and one culture was maintained with a 2 mM choline supplement whereas the other had no choline added. Drug pulses with DHA (Sigma-Aldrich no. D7439) or CQ (Sigma-Aldrich no. C6628) were performed at 1–6 hpi (*NF54-10.3-Tom*) or 0–10 hpi (*NF54-gexp02-Tom*) for exposure at the ring stage, or at 25–30 hpi for exposure at the trophozoite stage. After 3 hr, the drug was removed and fresh, pre-warmed culture medium was added. In some experiments, 200 nM DHA was maintained for 48 hr as a 'kill' control (***Xie et al., 2014***).

The survival rate was calculated as the total parasitemia (asexual + sexual parasites) at the next cycle after drug exposure (measured at ~30–35 hpi or ~30–40 hpi) in treated cultures relative to control cultures, and expressed as percentage. Parasitemia was measured by flow cytometry (see below).

Determination of sexual conversion rates

A schematic of our approach to determine sexual conversion rates is provided in ***Figure 1—figure supplement 1***. We define day 0 (D0) as the first day of the next cycle after drug exposure, which corresponds to the first day of Generation one in the schematics in the figures. D1 corresponds to the day when new sexual parasites become stage I gametocytes. When using the *NF54-gexp02-Tom* and *E5-gexp02-Tom* lines, the sexual conversion rate was calculated as the sexual stage parasitemia divided by the total (sexual + asexual) parasitemia, and expressed as percentage. Asexual and sexual parasites were quantified by flow cytometry at ~30–35 hpi or ~30–40 hpi (D1) of the cycle after drug treatment, in the absence of chemicals that inhibit asexual replication. When using the *NF54-10.3-Tom* line, the sexual conversion rate was measured by dividing the gametocytemia on day 3 (D3) by the initial ring stage parasitemia on D0. In this case, cultures were treated with 50 mM N-acetyl-D-glucosamine (GlcNAc; Sigma-Aldrich no. A8625) from D1 onwards to inhibit asexual replication. In experiments with the *NF54-10.3-Tom* line, gametocytemia was also measured on D0 to identify gametocytes already present in the culture at the beginning of the experiment, but it was found to be negligible. In any case, it was subtracted from D3 gametocytemia, such that only gametocytes newly formed during the assay were considered. Unless otherwise indicated, statistical analysis of differences in sexual conversion was performed using one-way ANOVA with Tukey HSD as the post hoc test. Variance was assumed to be homogenous because the sample size was the same for all groups, and they contain the same type of data.

Flow cytometry

Flow cytometry analysis to measure parasitemia at the cycle of drug exposure was performed using the nucleic acid stain SYTO 11 (0.016 µM) (Life Technologies no. S7573) and a BD FACSCalibur machine as previously described (***Rovira-Graells et al., 2016***). To measure asexual and tdTomato-positive sexual parasites, we used a BD LSRFortessa machine as previously described (***Portugaliza et al., 2019***), with small modifications after the addition of the mitochondrial membrane potential MitoTracker Deep Red FM fluorescent dye (Invitrogen no.M22426) at 0.6 µM to identify live parasites (***Figure 1—figure supplements 7–8***; ***Amaratunga et al., 2014***). Briefly, the erythrocyte population was defined using the side scatter area (SCC-A) versus forward scatter area (FSC-A) plot, followed by singlet gating using the forward scatter height (FSC-H) versus FSC-A plot. From the singlet population, the parasites were simultaneously analyzed for tdTomato fluorescence (laser: 561 nm; filter: 582/15; power: 50 mW), SYTO 11 fluorescence (laser: 488 nm; Filter: 525/50-505LP; power: 50 mW), and MitoTracker fluorescence (laser: 640 nm; Filter: 670/14-A; power: 40 mW). Total gametocytes were quantified on the double-positive gate of the tdTomato versus SYTO 11 plot. Total asexual stages were quantified on the tdTomato-negative but SYTO 11-positive gate, whereas viable asexual stages were measured on the tdTomato-negative but MitoTracker-positive gate. Flowing Software version 2.5.1 (Perttu Terho) was used for downstream analysis.

Immunofluorescence assay

Immunofluorescence assays (IFA) were performed as previously described (***Bancells et al., 2019***; ***Portugaliza et al., 2019***). Briefly, an aliquot of culture was treated with 80 nM ML10 (cGMP-

dependent protein kinase inhibitor) (**Baker et al., 2017**), starting at ~30–35 hpi until ~48–53 hpi, to inhibit schizont rupture and allow maturation of gametocytes to the stage when all of them express Pfs16. Air-dried blood smears containing schizonts and stage I gametocytes (~48–53 hpi) were fixed with 1% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, and blocked with 3% BSA in PBS. The gametocyte-specific primary antibody mouse-anti-PfS16 (1:400; 32F717:B02, a gift from R. Sauerwein, Radboud University) and the goat-anti-mouse IgG–Alexa Fluor 488 secondary antibody (1:1,000, Thermo Fisher no.A11029) were used to identify stage I gametocytes, whereas DAPI (5 µg/mL) was added to stain parasite DNA. IFA slides were mounted using Vectashield (Palex Medical) and viewed under an Olympus IX51 epifluorescence microscope for determination of sexual conversion rates. A minimum of 200 DAPI-positive cells was counted for each sample.

Transcriptional analysis

Trizol reagent (Invitrogen no. 15596026) was used to collect and preserve total RNA, followed by extraction using a protocol designed for samples with low RNA concentration (**Mira-Martínez et al., 2017**). Briefly, RNA from Trizol samples was purified using a commercial kit (RNeasy Mini Kit, Qiagen no. 74104) with additional on-column DNAse treatment (Qiagen no. 79254). Next, cDNA synthesis was performed using the AMV Reverse Transcription System (Promega), with a combination of oligo (dT) and random primers. Quantitative PCR (qPCR) analysis of the cDNAs was performed as previously described (**Bancells et al., 2019**) using triplicate wells (technical replicates) for each biological replicate of each sample. Transcript levels of *pfap2-g* and *gexp02* were normalized against the housekeeping genes *serine-tRNA ligase* and *ubiquitin-conjugating enzyme*. All qPCR primers used have been previously described (**Bancells et al., 2019**; **Portugaliza et al., 2019**). Statistical analysis of transcript levels was performed using one-way ANOVA with Tukey HSD as the post hoc test, as for the analysis of sexual conversion rates.

Production of mature gametocytes and mosquito feeding

Cultures maintained in a medium containing 0.5% Albumax and supplemented with 2 mM choline were synchronized for ring stages by D-Sorbitol treatment and diluted to a final parasitemia of 1.5%. At 22 hr after synchronization, DHA (5 nM) was added to the cultures for 3 hr, and 24 hr later (i.e., after reinvasion) culture conditions were changed to medium with 10% human serum instead of Albumax and choline, and GlcNac (50 mM) was added to kill asexual stages. GlcNac was maintained for 4 d. Gametocyte cultures were followed during 9–13 d after DHA-treatment with medium changes twice a day, but without replenishing with fresh erythrocytes. At days 9–13, gametocyte development was analyzed in Giemsa stained blood smears and exflagellation was monitored after activation as described (**Marin-Mogollon et al., 2018**). Gametocytes (day 10–13) were fed to *Anopheles stephensi* mosquitoes using the standard membrane feeding assay (SMFA) (**Marin-Mogollon et al., 2018**; **Ponnudurai et al., 1989**). Oocysts (day 7 and 14) and salivary gland sporozoites (day 14) were counted as described (**Marin-Mogollon et al., 2018**). Statistical analysis of differences in the parameters measured (**Figure 2**) was performed using one-way ANOVA with Tukey HSD as the post hoc test, except for oocyst/mosquito values. For the oocyst/mosquito analysis, we used the Kruskal-Wallis test with post hoc Dunn's test (this is used because the data is not normally distributed, Shapiro-Wilk test $p<0.001$).

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Additional files

Supplementary files

- Transparent reporting form

Data availability

All data generated or analysed during this study are included in the manuscript and supporting files.

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Supplementary information

Artemisinin exposure at the ring or trophozoite stage impacts *Plasmodium falciparum* sexual conversion differently

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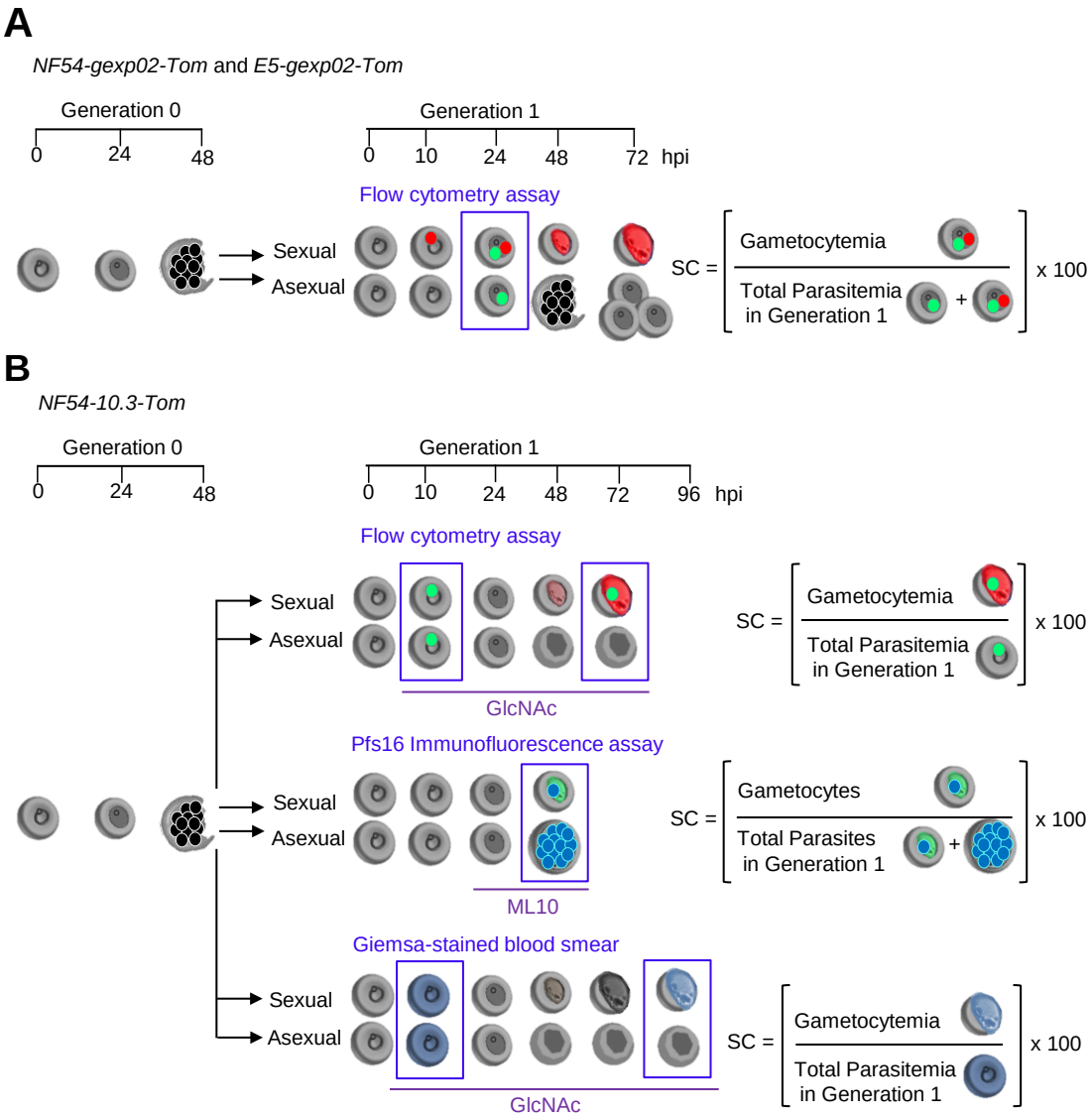

Figure 1-figure supplement 1. Schematic representation of the assays to determine sexual conversion rates. We used parasite lines expressing the fluorescent reporter tdTomato under the control of the *gexp02* **(A)** or *etramp10.3* **(B)** promoters. Generation 0 is the growth cycle at which the culture is exposed to different conditions (e.g., choline removal, or drugs), with parasite age indicated in h post-invasion (hpi). In all cases, the sexual conversion rate (SC) is calculated as the percentage of Generation 1 parasites that develop into non-replicative sexual forms (gametocytes). The total parasitemia (sexual + asexual parasites) at Generation 1 is measured by flow cytometry using fluorescent markers that stain DNA in all parasites (green dots) or active mitochondria in viable parasites (not represented here), by immunofluorescence assay using a fluorescent DNA stain (blue dots) or by light microscopy using Giemsa-stained smears (pale blue marks). Gametocytemia is measured by flow cytometry based on tdTomato signal (red marks), by immunofluorescence based on Pfs16 signal (pale green marks) or by light microscopy using Giemsa-stained smears (gametocyte-shaped pale blue marks). In immunofluorescence assays, the number of parasites and gametocytes in a given number of fields are determined instead of parasitemia and gametocytemia, but since both total parasites and gametocytes are determined simultaneously from the same samples (i.e., the total erythrocytes denominator is the same for both parameters), this does not affect the sexual conversion rate calculation. Detection of gametocytes based

on tdTomato expression in the *NF54-gexp02-Tom* and *E5-gexp02-Tom* lines is performed as early as 24 hpi in Generation 1. Detection based on Pfs16 expression is performed at 48 hpi, and requires addition of ML10 to prevent schizont bursting and reinvasion, which would increase the number of asexual parasites observed. Detection of gametocytes based on tdTomato expression or light microscopy analysis of Giemsa-stained smears in the *NF54-10.3-Tom* line is performed at 72 or 96 hpi, respectively, and requires addition of N-acetyl-D-glucosamine (GlcNAc) to prevent multiplication of asexual parasites, which would collapse the culture.

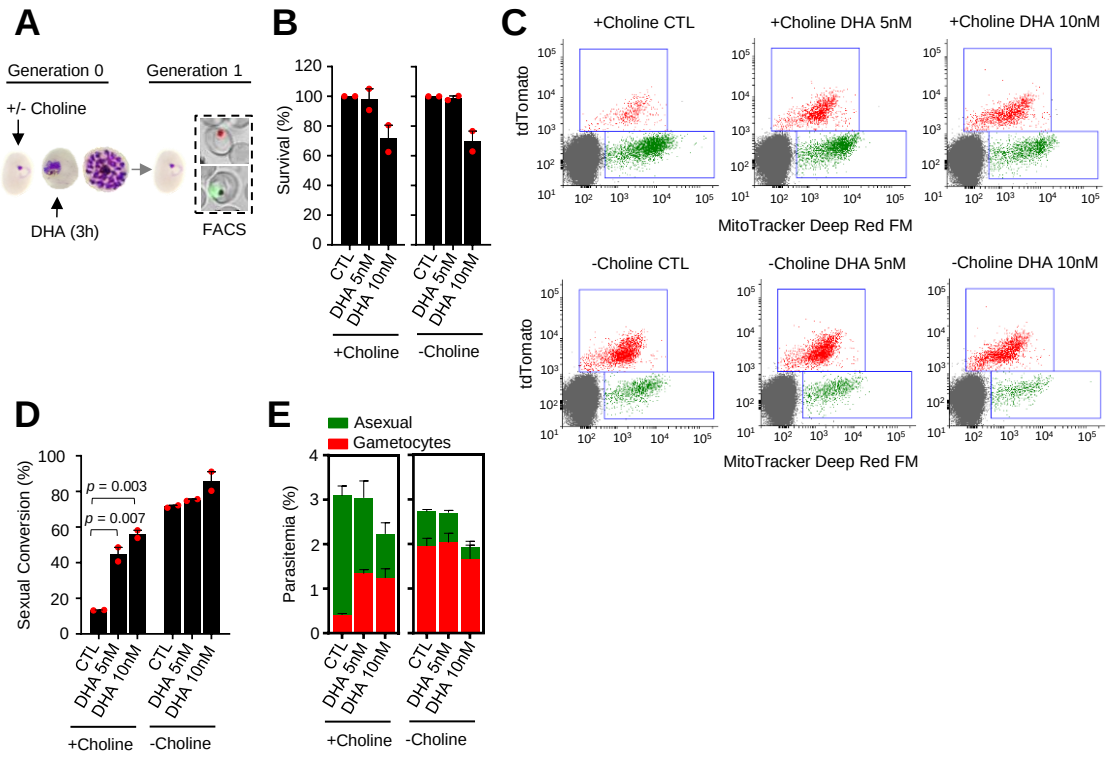

Figure 1-figure supplement 2. Effect of a dihydroartemisinin (DHA) pulse at the trophozoite stage on sexual conversion, determined using MitoTracker to identify viable parasites. (A) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-gexp02-Tom* line under non-inducing (+ choline) or inducing (- choline) conditions were exposed to a 3 h DHA pulse at subcurative doses at the trophozoite stage (25-30 hpi). Sexual conversion was measured by flow cytometry (FACS) after reinvasion (~30-35 hpi of the next multiplication cycle)**. (B)** Survival rate of cultures exposed to the different drug doses, using total parasitemia of live parasites (asexual + sexual parasites) determined with a mitochondrial membrane potential stain (MitoTracker Deep Red FM). For each choline condition, values are presented relative to the parasitemia in the control cultures (no drug). **(C)** Representative MitoTracker Deep Red FM vs TdTomato (marks gametocytes) flow cytometry dot plots. **(D)** Sexual conversion rates determined by flow cytometry, calculated using MitoTracker-positive cells only. The *p* value is indicated only for treatment vs control (no drug) significant differences ($p<0.05$). **(E)** Distribution of absolute parasitemia of asexual and sexual parasites (from the same flow cytometry measurements as in panel D). In all panels, data are presented as the average and s.e.m. of 2 independent biological replicates.

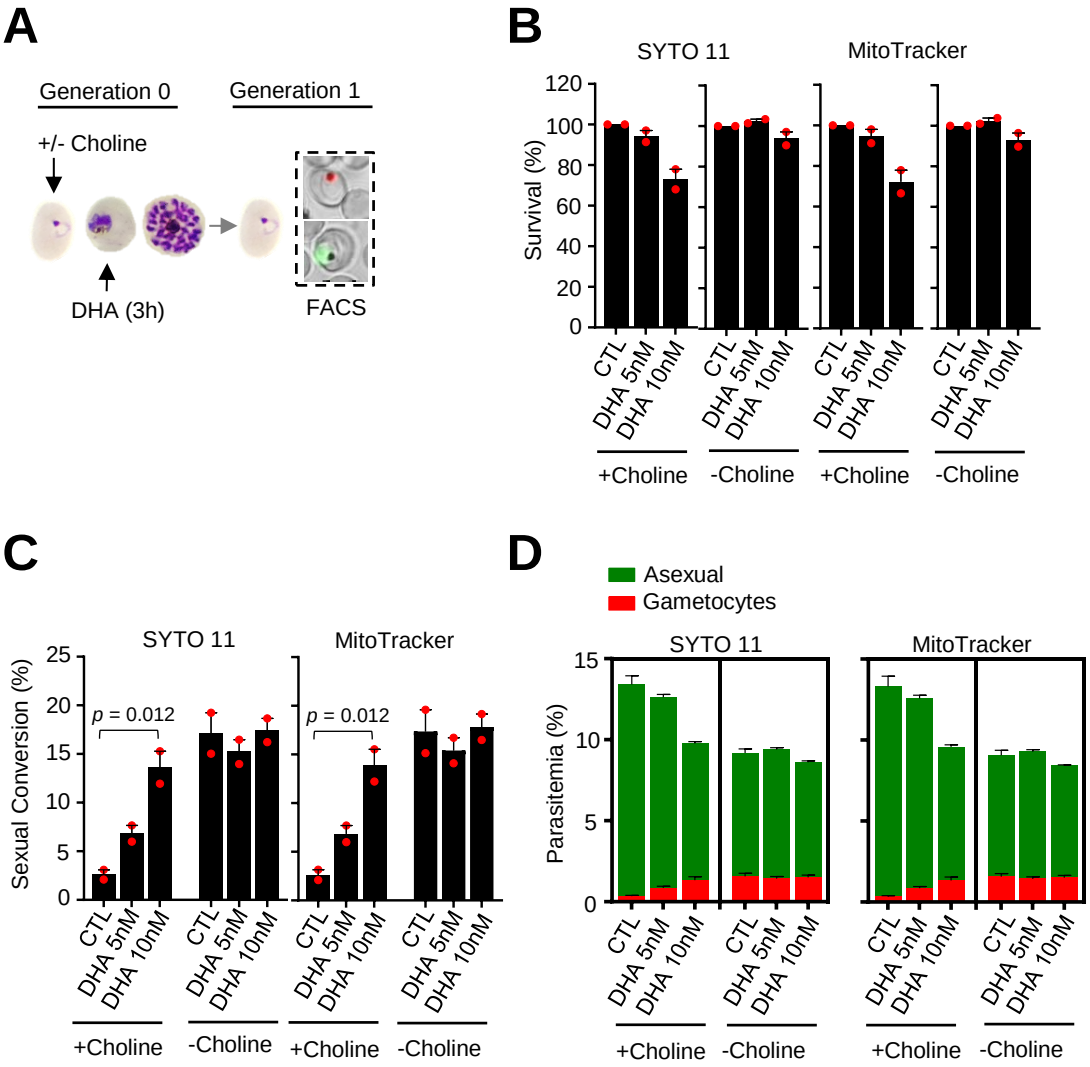

Figure 1-figure supplement 3. Effect of a dihydroartemisinin (DHA) pulse at the trophozoite stage on sexual conversion in the *E5-gexp02-Tom* line. (A) Schematic representation of the assay**.** Tightly synchronized cultures of the *E5-gexp02-Tom* line under non-inducing (+ choline) or inducing (- choline) conditions were exposed to a 3 h DHA pulse at subcurative doses at the trophozoite stage (25-30 hpi). Sexual conversion was measured by flow cytometry (FACS) after reinvasion (~30-35 hpi of the next multiplication cycle). **(B)** Survival rate of cultures exposed to the different drug doses, using total parasitemia values (asexual + sexual parasites) based on identification of all parasites with SYTO 11 or viable parasites only with MitoTracker Deep Red FM. For each choline condition, values are presented relative to the parasitemia in the control cultures (no drug). **(C)** Sexual conversion rates determined by flow cytometry. The *p* value is indicated only for treatment vs control (no drug) significant differences ($p<0.05$). **(D)** Distribution of absolute parasitemia of asexual and sexual parasites (from the same flow cytometry measurements as in panel C). In all panels, data are presented as the average and s.e.m. of 2 independent biological replicates.

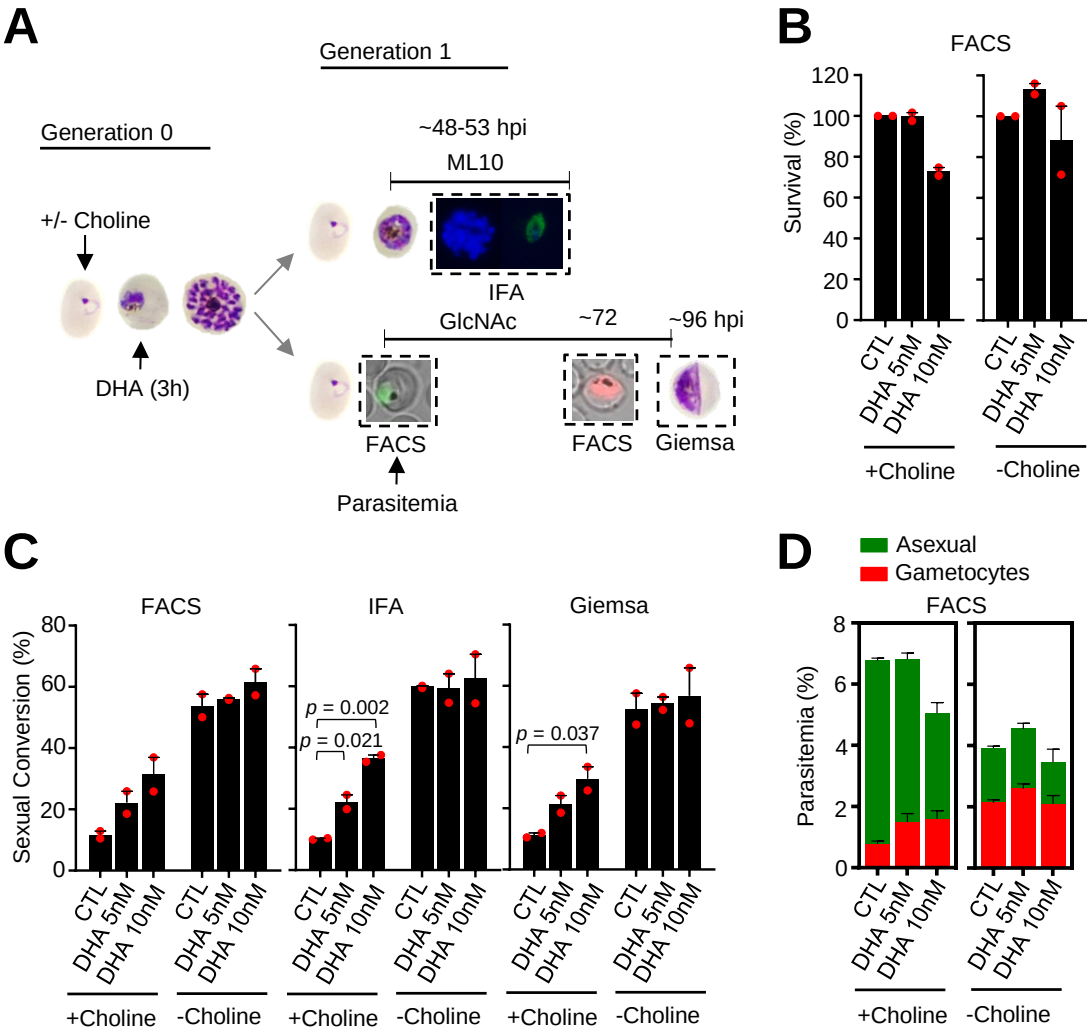

Figure 1-figure supplement 4. Effect on sexual conversion of a dihydroartemisinin (DHA) pulse at the trophozoite stage in the *NF54-10.3-Tom* line, determined by three different methods. (A) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-10.3-Tom* line (expression of the fluorescence reporter starts later during gametocyte development than in the *NF54-gexp02-Tom* line) under non-inducing (+ choline) or inducing (- choline) conditions were exposed to a 3 h DHA pulse at subcurative doses at the trophozoite stage (25-30 hpi). Sexual conversion was measured by: (i) flow cytometry analysis (FACS) on D1 (D0 is the first day of Generation 1) to determine the initial parasitemia (using the SYTO 11 stain), and on D3 to determine the gametocytemia (SYTO 11 and TdTomato signal) in cultures treated with N-acetylglucosamine (GlcNAc); (ii) immunofluorescence assay (IFA) analysis of cultures treated with ML10 using the Pfs16 marker; (iii) flow cytometry analysis on D1 to determine the initial parasitemia, and on D4 light microscopy analysis of Giemsa-stained blood smears (Giemsa) to determine the gametocytemia in cultures treated with GlcNAc. **(B)** Survival rate of cultures exposed to the different drug doses, using total D1 parasitemia values (asexual + sexual parasites) determined by flow cytometry (FACS). For each choline condition, values are presented relative to the parasitemia in the control cultures (no drug). **(C)** Sexual conversion rates determined by FACS, IFA, and Giemsa-stained blood smears. The *p* value is indicated only for treatment vs control (no drug) significant differences ($p<0.05$). **(D)** Distribution of absolute parasitemia of asexual and sexual parasites, determined by flow cytometry (from the same flow cytometry measurements as in

panel C). In all panels, data are presented as the average and s.e.m. of 2 independent biological replicates.

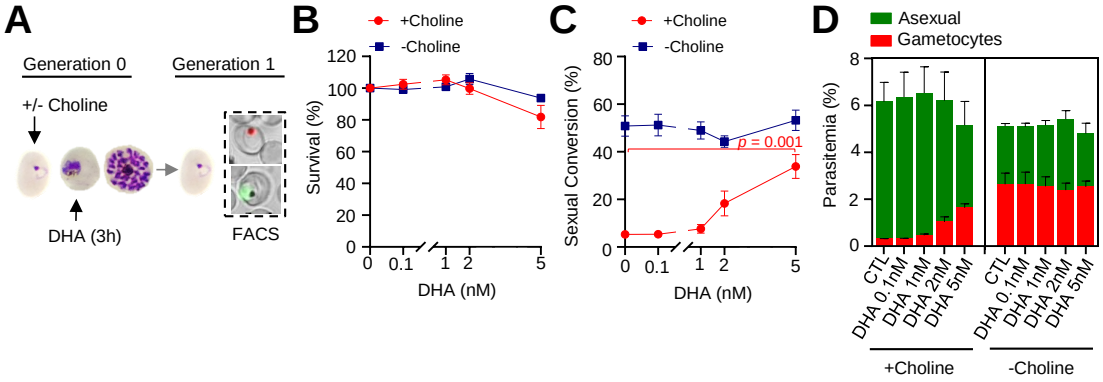

Figure 1-figure supplement 5. Effect on sexual conversion of a low concentration dihydroartemisinin (DHA) pulse at the trophozoite stage. (A) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-gexp02-Tom* line under non-inducing (+ choline) or inducing (- choline) conditions were exposed to a 3 h DHA pulse at various low doses at the trophozoite stage (25-30 hpi). Sexual conversion was measured by flow cytometry (FACS) after reinvasion (~30-35 hpi of the next multiplication cycle). **(B)** Survival rate of cultures exposed to the different drug doses, using total parasitemia values (asexual + sexual parasites) based on identification of all parasites using SYTO 11. For each choline condition, values are presented relative to the parasitemia in the control cultures (no drug). **(C)** Sexual conversion rates determined by flow cytometry. The *p* value is indicated only for treatment vs control (no drug) significant differences ($p<0.05$). **(D)** Distribution of absolute parasitemia of asexual and sexual parasites (from the same flow cytometry measurements as in panel C). In all panels, data are presented as the average and s.e.m. of 3 independent biological replicates.

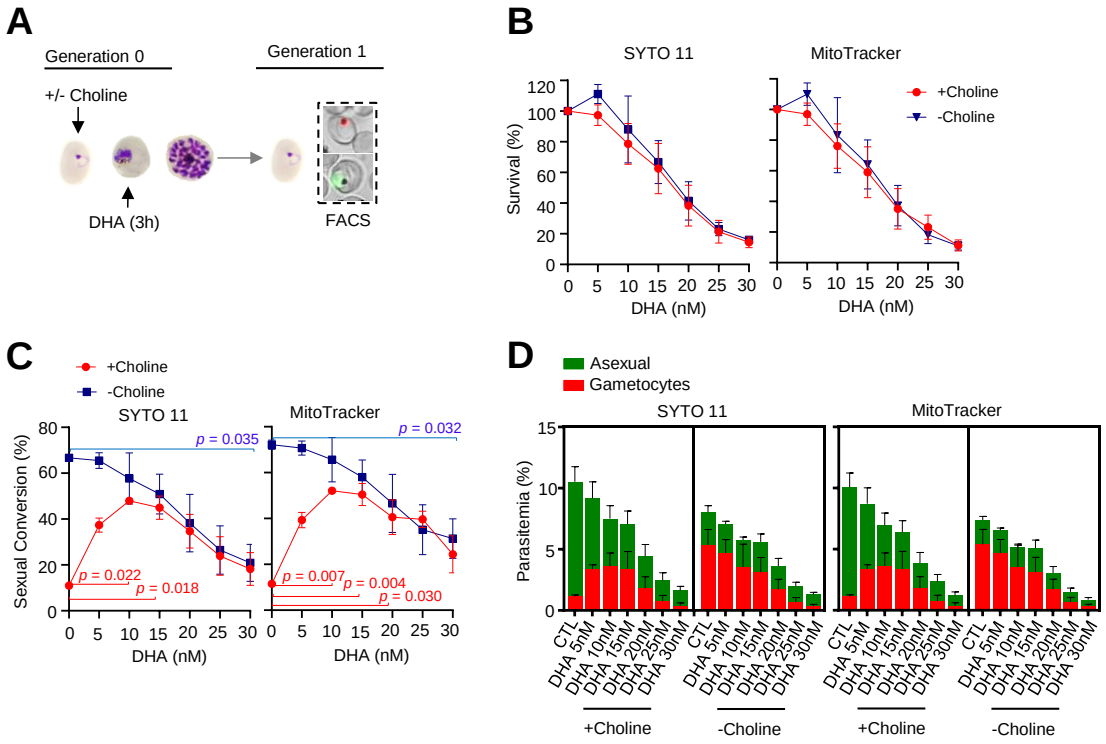

Figure 1-figure supplement 6. Effect on sexual conversion of a dihydroartemisinin (DHA) pulse at 5-30 nM concentrations during the trophozoite stage. (A) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-gexp02-Tom* line under non-inducing (+ choline) or inducing (- choline) conditions were exposed to a 3 h DHA pulse at various doses at the trophozoite stage (25-30 hpi). Sexual conversion was measured by flow cytometry (FACS) after reinvasion (~30-35 hpi of the next multiplication cycle). **(B)** Survival rate of cultures exposed to the different drug doses, using total parasitemia values (asexual + sexual parasites) based on identification of all parasites or viable parasites only, with SYTO 11 or MitoTracker Deep Red FM, respectively. For each choline condition, values are presented relative to the parasitemia in the control cultures (no drug). **(C)** Sexual conversion rates determined by flow cytometry. The *p* value is indicated only for treatment vs control (no drug) significant differences ($p<0.05$). **(D)** Distribution of absolute parasitemia of asexual and sexual parasites (from the same flow cytometry measurements as in panel C). In all panels, data are presented as the average and s.e.m. of 3 independent biological replicates.

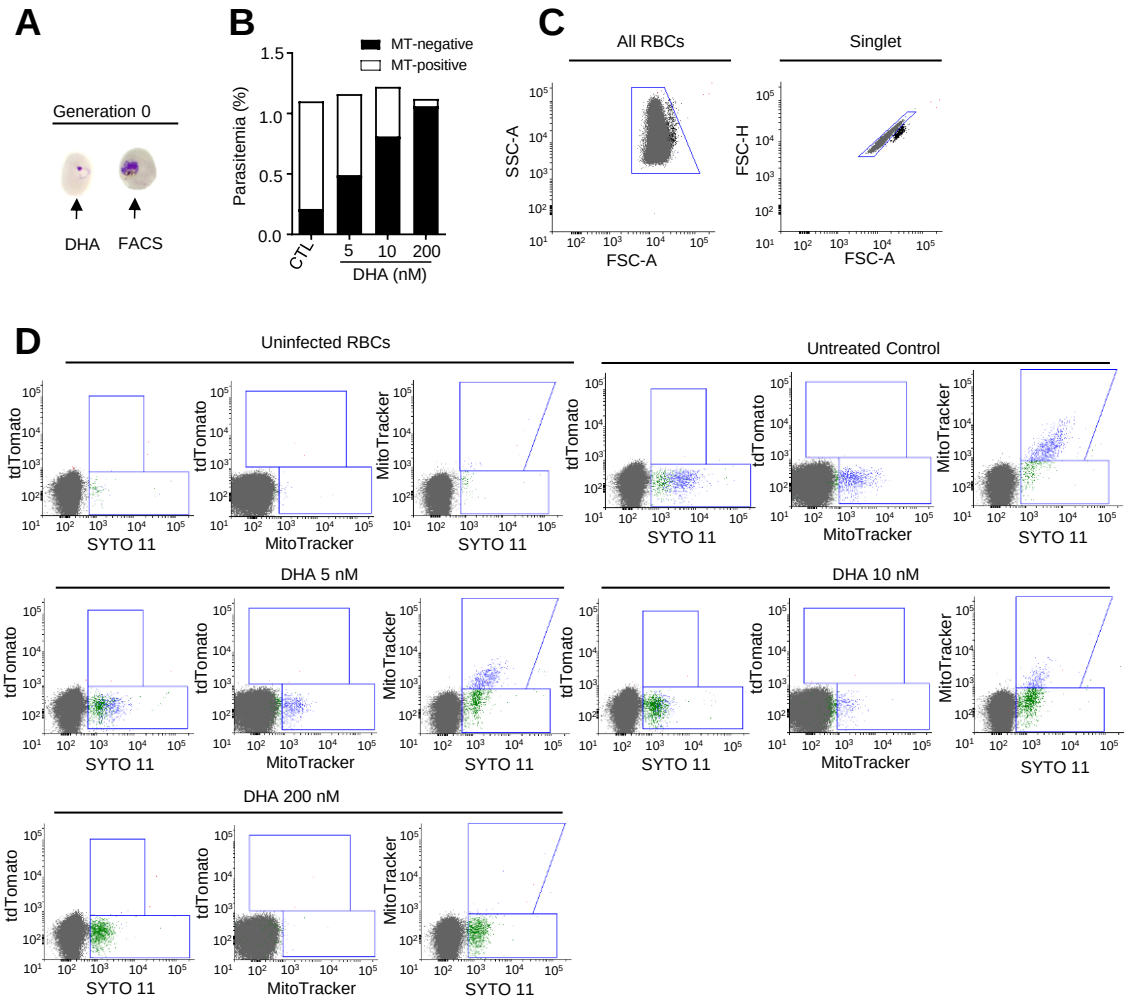

Figure 1-figure supplement 7. Flow cytometry set-up for the identification of viable parasites in the 3D7-A parasite line using MitoTracker. (A) Schematic representation of the assay. Tightly synchronized cultures of the 3D7-A wild-type parasite line (does not have the *TdTomato* gene, does not produce gametocytes) were exposed to a 3 h dihydroartemisinin (DHA) pulse at subcurative doses at the ring stage (0-20 hpi) or maintained under a lethal dose (200 nM, 'kill' control) for ~24 h. Flow cytometry measurements were performed the next day, within the same asexual cycle. **(B)** Total parasitemia as determined using SYTO-11, and distribution of MitoTracker (MT) Deep Red FM-positive (viable) and –negative (non-viable and non-stained) parasites after DHA exposure. **(C)** Dot plots of the initial gating strategy. The red blood cell (RBC) population was gated first for cell granularity and size (SSC-A versus FSC-A plot) and then for a defined singlet population (FSC-H versus FSC-A plot). **(D)** Flow cytometry dot plots for MitoTracker Deep Red FM, SYTO 11, and TdTomato (marks gametocytes in the gametocyte-reporter lines). Some presumably healthy parasites were not stained with MitoTracker, as revealed by the presence of MitoTracker-negative/SYTO 11-positive parasites in the no-drug control.

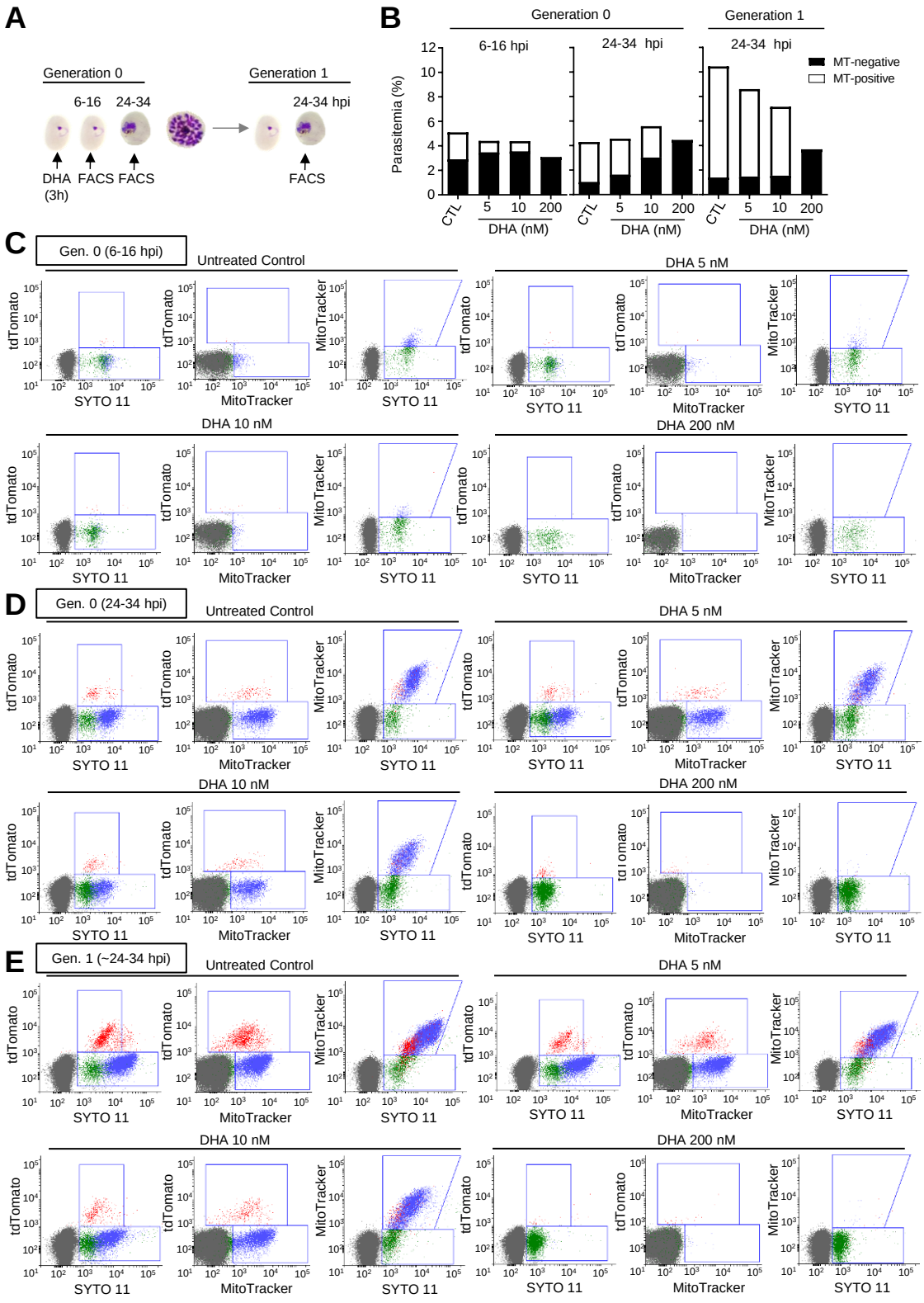

Figure 1-figure supplement 8. Flow cytometry set-up for the identification of viable parasites in the *NF54-gexp02-Tom* line using MitoTracker. (A) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-gexp02-Tom* line were exposed to a 3 h dihydroartemisinin (DHA) pulse at subcurative doses at the ring stage (0-10 hpi) or maintained under a lethal dose (200 nM, 'kill' control) for up to 48 h. Flow cytometry measurements were performed at different times within the same cycle, and after reinvasion, as indicated. **(B)** Total parasitemia as determined using

SYTO-11, and distribution of MitoTracker (MT) Deep Red FM-positive (viable) and –negative (non-viable and non-stained) parasites after DHA exposure. **(C-E)** Flow cytometry dot plots for MitoTracker Deep Red FM, SYTO 11, and TdTomato (marks gametocytes) at 6-16 hpi (C) and 24-34 hpi (D) of the same cycle of DHA treatment, and ~24-34 hpi of the next cycle (E). Some presumably healthy parasites were not stained with MitoTracker, as revealed by the presence of MitoTracker-negative/SYTO 11-positive parasites in the no-drug control (especially in ring-stage cultures).

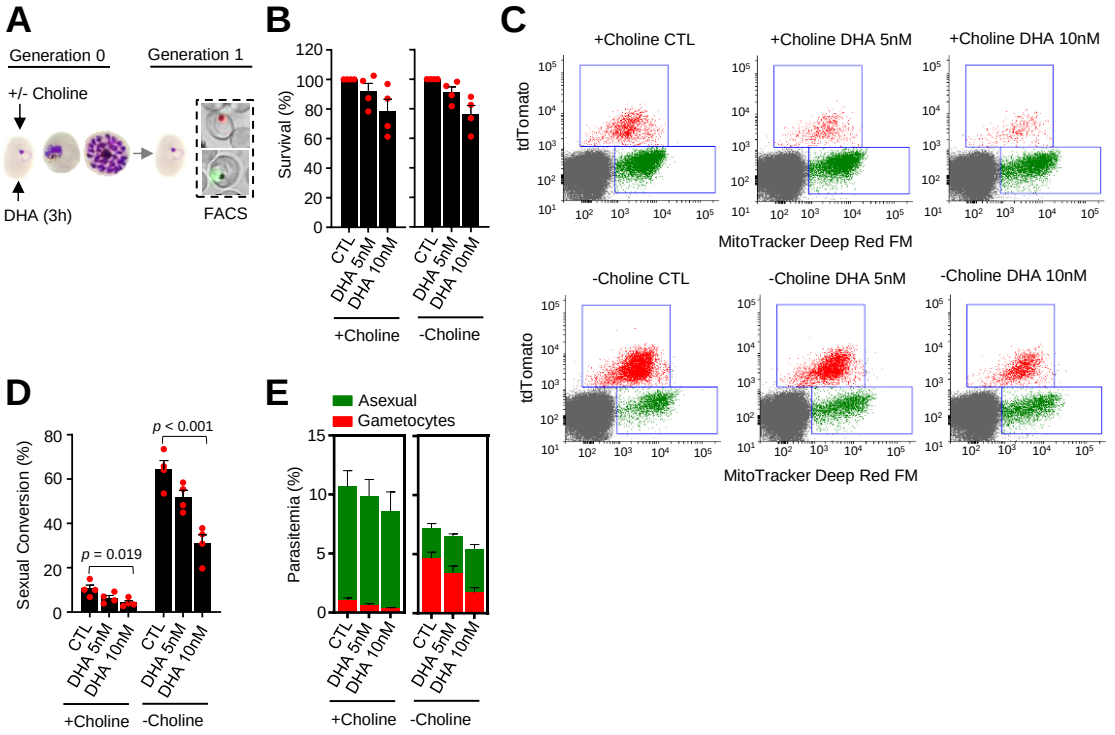

Figure 3-figure supplement 1. Effect of a dihydroartemisinin (DHA) pulse at the ring stage on sexual conversion, determined using MitoTracker to identify viable parasites. (A) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-gexp02-Tom* line under non-inducing (+ choline) or inducing (- choline) conditions were exposed to a 3 h DHA pulse at subcurative doses at the early ring stage (0-10 hpi). Sexual conversion was measured by flow cytometry (FACS) after reinvasion (~30-40 hpi of the next multiplication cycle). **(B)** Survival rate of cultures exposed to the different drug doses, using total parasitemia of live parasites (asexual + sexual parasites) determined with a mitochondrial membrane potential stain (MitoTracker Deep Red FM). For each choline condition, values are presented relative to the parasitemia in the control cultures (no drug). **(C)** Representative MitoTracker Deep Red FM vs TdTomato (marks gametocytes) flow cytometry dot plots. **(D)** Sexual conversion rates determined by flow cytometry, calculated using MitoTracker-positive cells only. The *p* value is indicated only for treatment vs control (no drug) significant differences ($p<0.05$). **(E)** Distribution of absolute parasitemia of asexual and sexual parasites (from the same flow cytometry measurements as in panel D). In all panels, data are presented as the average and s.e.m. of 4 independent biological replicates.

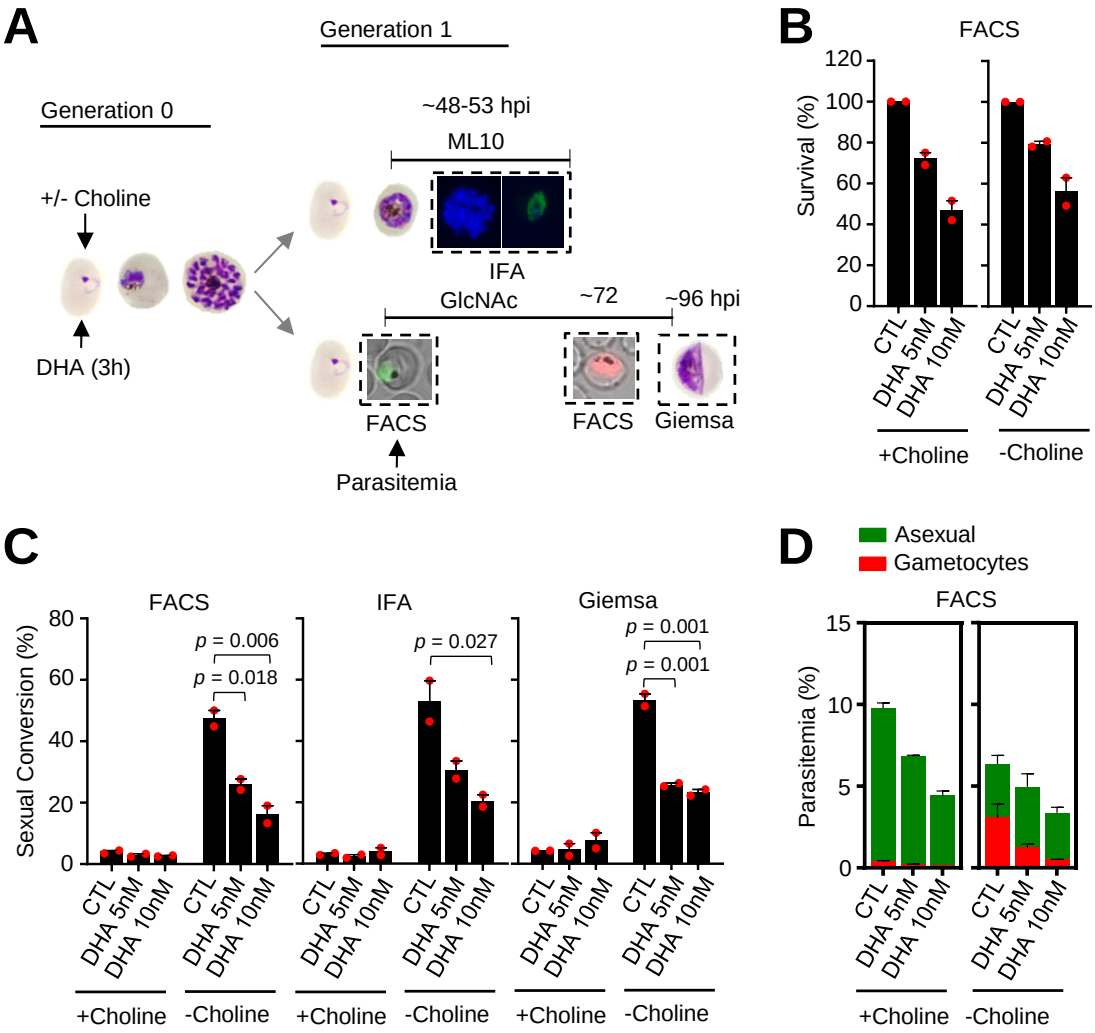

Figure 3-figure supplement 2. Effect on sexual conversion of a dihydroartemisinin (DHA) pulse at the ring stage in the *NF54-10.3-Tom* line, determined by three different methods. (A) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-10.3-Tom* line (expression of the fluorescence reporter starts later during gametocyte development than in the *NF54-gexp02-Tom* line) under non-inducing (+ choline) or inducing (- choline) conditions were exposed to a 3 h DHA pulse at subcurative doses at the ring stage (1-6 hpi). Sexual conversion was measured by: (i) flow cytometry analysis (FACS) on D1 (D0 is the first day of Generation 1) to determine the initial parasitemia (using the SYTO 11 stain), and on D3 to determine the gametocytemia (SYTO 11 and TdTomato signal) in cultures treated with N-acetylglucosamine (GlcNAc); (ii) immunofluorescence assay (IFA) analysis of cultures treated with ML10 using the Pfs16 marker; (iii) flow cytometry analysis on D1 to determine the initial parasitemia, and on D4 light microscopy analysis of Giemsa-stained blood smears (Giemsa) to determine the gametocytemia in cultures treated with GlcNAc. **(B)** Survival rate of cultures exposed to different drug doses, using total D1 parasitemia values (asexual + sexual parasites) determined by flow cytometry (FACS). For each choline condition, values are presented relative to the parasitemia in the control cultures (no drug). **(C)** Sexual conversion rates as determined by FACS, IFA, and Giemsa-stained blood smears. The *p* value is indicated only for treatment vs control (no drug) significant differences ($p<0.05$). **(D)** Distribution of absolute parasitemia of asexual and sexual parasites, determined by flow cytometry

(from the same flow cytometry measurements as in panel C). In all panels, data are presented as the average and s.e.m. of 2 independent biological replicates.

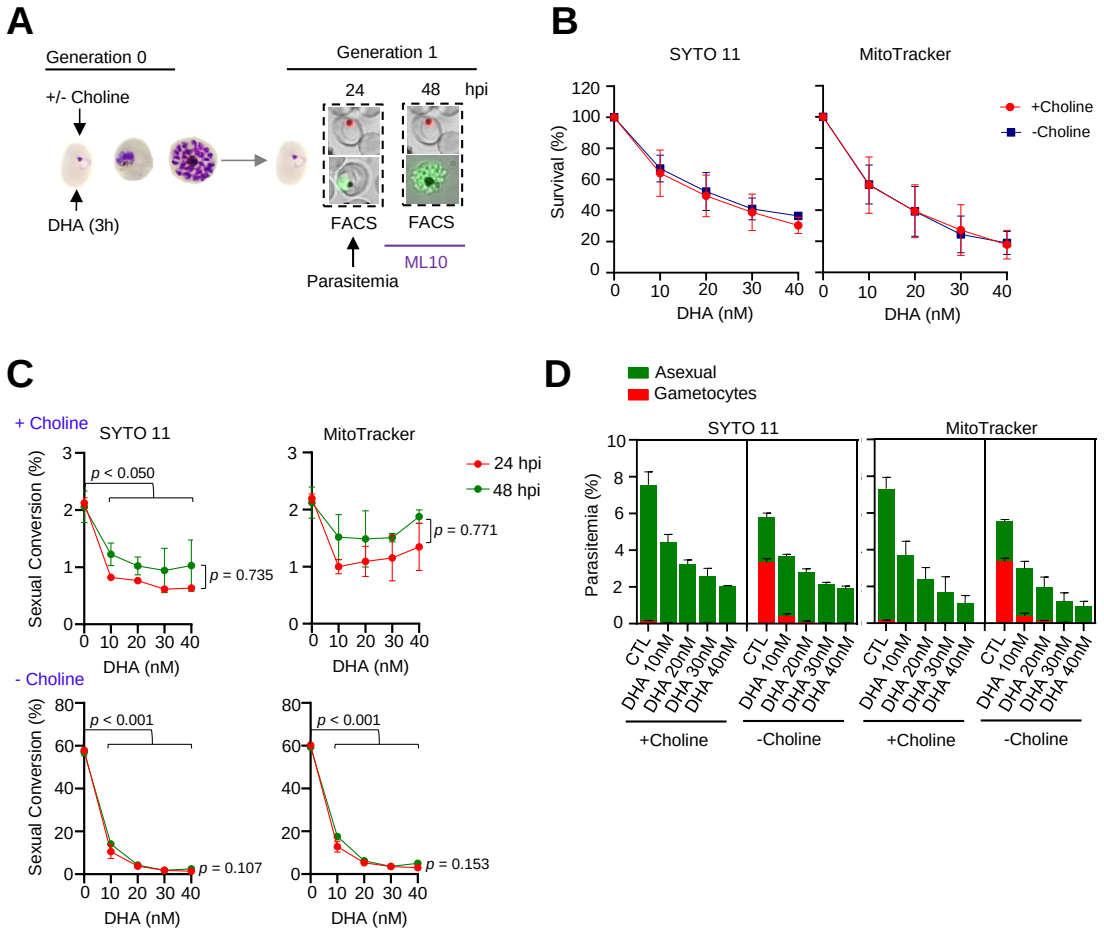

Figure 3-figure supplement 3. Effect on sexual conversion of a dihydroartemisinin (DHA) pulse at 10-40 nM concentrations at the ring stage. (A) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-gexp02-Tom* line under non-inducing (+ choline) or inducing (- choline) conditions were exposed to a 3 h DHA pulse at the early ring stage (0-10 hpi). Sexual conversion was measured by flow cytometry (FACS) after reinvasion, at 24 or 48 hpi of the next multiplication cycle. To measure sexual conversion at 48 hpi, cultures were treated with ML10 to prevent an additional round of reinvasion that would increase the asexual parasitemia. **(B)** Survival rate of cultures exposed to the different drug doses, using 24 hpi total parasitemia values (asexual + sexual parasites) based on identification of all parasites or viable parasites only, with SYTO 11 or MitoTracker Deep Red FM, respectively. For each choline condition, values are presented relative to the parasitemia in the control cultures (no drug). **(C)** Sexual conversion rates determined by flow cytometry. The *p* value is indicated only for comparison between 24 and 48 hpi readouts of sexual conversion and for treatment vs control (no drug) significant differences ($p<0.05$); a single value is shown when all drug concentrations tested yielded a similar *p* value. A two-way ANOVA test was used (time of readout was included as a variable). **(D)** Distribution of absolute parasitemia of asexual and sexual parasites (from the same flow cytometry measurements as in panel C). In all panels, data are presented as the average and s.e.m. of 2 independent biological replicates.

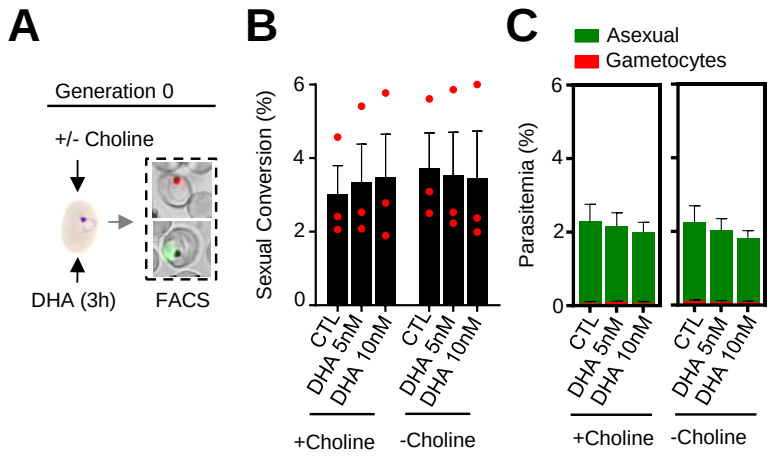

Figure 4-figure supplement 1. Effect of a dihydroartemisinin (DHA) pulse at the ring stage on sexual conversion by the same cycle conversion (SCC) route, determined using MitoTracker to identify viable parasites. (A) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-gexp02-Tom* line under non-inducing (+ choline) or inducing (- choline) conditions were exposed to a 3 h DHA pulse at subcurative doses at the early ring stage (0-10 hpi). Sexual conversion was measured by flow cytometry (FACS) within the same cycle (~30-40 hpi) to determine the effect of the drug pulse only on production of new gametocytes by the SSC route. **(B)** Sexual conversion rates determined by flow cytometry, calculated using MitoTracker-positive cells only. No significant difference (p<0.05) with the control (no drug) was observed for any treatment condition. **(C)** Distribution of absolute parasitemia of asexual and sexual parasites (from the same flow cytometry measurements as in panel B). In all panels, data are presented as the average and s.e.m. of 3 independent biological replicates.

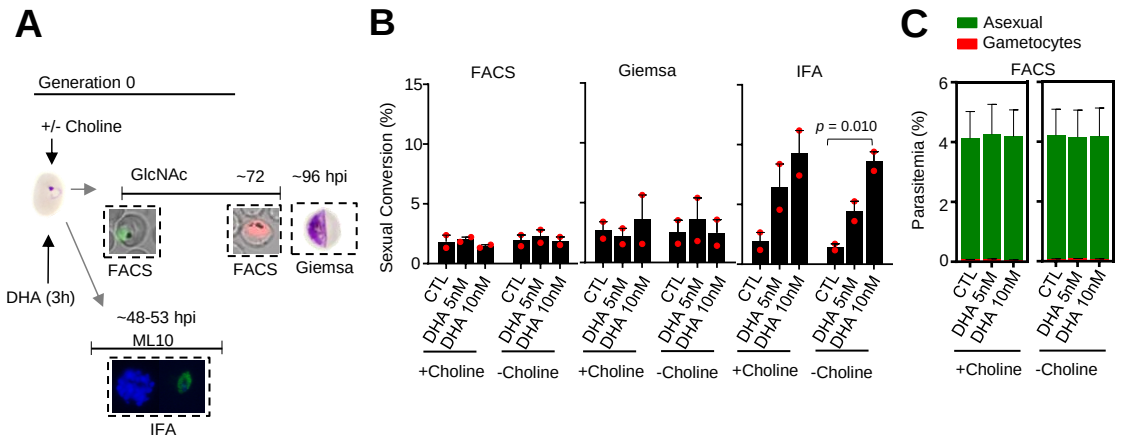

Figure 4-figure supplement 2. Effect of a dihydroartemisinin (DHA) pulse at the ring stage on sexual conversion by the same cycle conversion (SCC) route in the *NF54-10.3-Tom* line, determined by three different methods. (A) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-10.3-Tom* line under non-inducing (+ choline) or inducing (- choline) conditions were exposed to a 3 h DHA pulse at subcurative doses at the early ring stage (1-6 hpi). Sexual conversion was measured by: (i) flow cytometry analysis (FACS) on D1 (D0 is the first day of Generation 0, i.e., the day of drug treatment) to determine the initial parasitemia (using the SYTO 11 stain), and on D3 to determine the gametocytemia (SYTO 11 and

TdTomato signal) in cultures treated with N-acetylglucosamine (GlcNAc); (ii) immunofluorescence assay (IFA) analysis of cultures treated with ML10 using the Pfs16 marker; (iii) flow cytometry analysis on D1 to determine the initial parasitemia, and on D4 light microscopy analysis of Giemsa-stained blood smears (Giemsa) to determine the gametocytemia in cultures treated with GlcNAc. **(B)** Sexual conversion rates as determined by FACS, IFA, and Giemsa-stained blood smears. The *p* value is indicated only for treatment vs control (no drug) significant differences ($p<0.05$). **(C)** Distribution of absolute parasitemia of asexual and sexual parasites, determined by flow cytometry (from the same flow cytometry measurements as in panel B). In all panels, data are presented as the average and s.e.m. of 2 independent biological replicates.

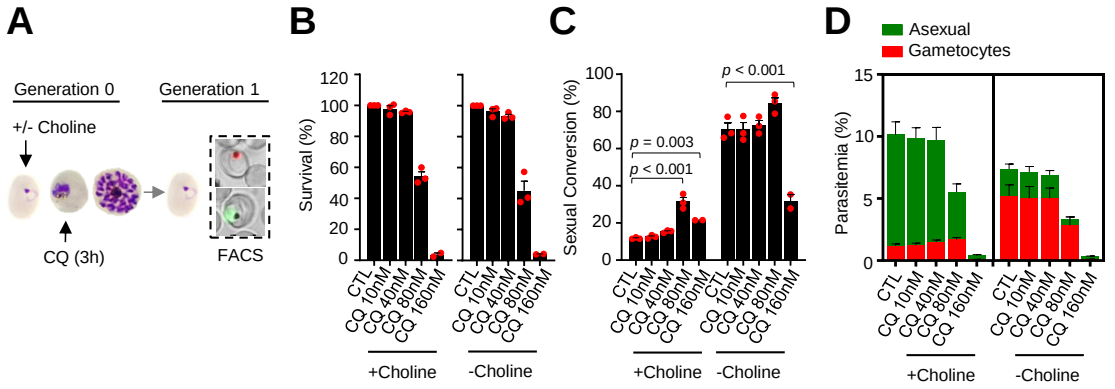

Figure 6-figure supplement 1. Effect of a chloroquine (CQ) pulse at the trophozoite stage on sexual conversion, determined using MitoTracker to identify viable parasites. (A) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-gexp02-Tom* line under non-inducing (+ choline) or inducing (- choline) conditions were exposed to a 3 h CQ pulse at subcurative doses at the trophozoite stage (25-30 hpi). Sexual conversion was measured by flow cytometry (FACS) after reinvasion (~30-35 hpi of the next multiplication cycle). **(B)** Survival rate of cultures exposed to the different drug doses, using total parasitemia of live parasites (asexual + sexual parasites) determined with a mitochondrial membrane potential stain (MitoTracker Deep Red FM). For each choline condition, values are presented relative to the parasitemia in the control cultures (no drug). **(C)** Sexual conversion rates determined by flow cytometry, calculated using MitoTracker-positive cells only. The *p* value is indicated only for treatment vs control (no drug) significant differences ($p<0.05$). **(D)** Distribution of absolute parasitemia of asexual and sexual parasites (from the same flow cytometry measurements as in panel C). In all panels, data are presented as the average and s.e.m. of 3 independent biological replicates.

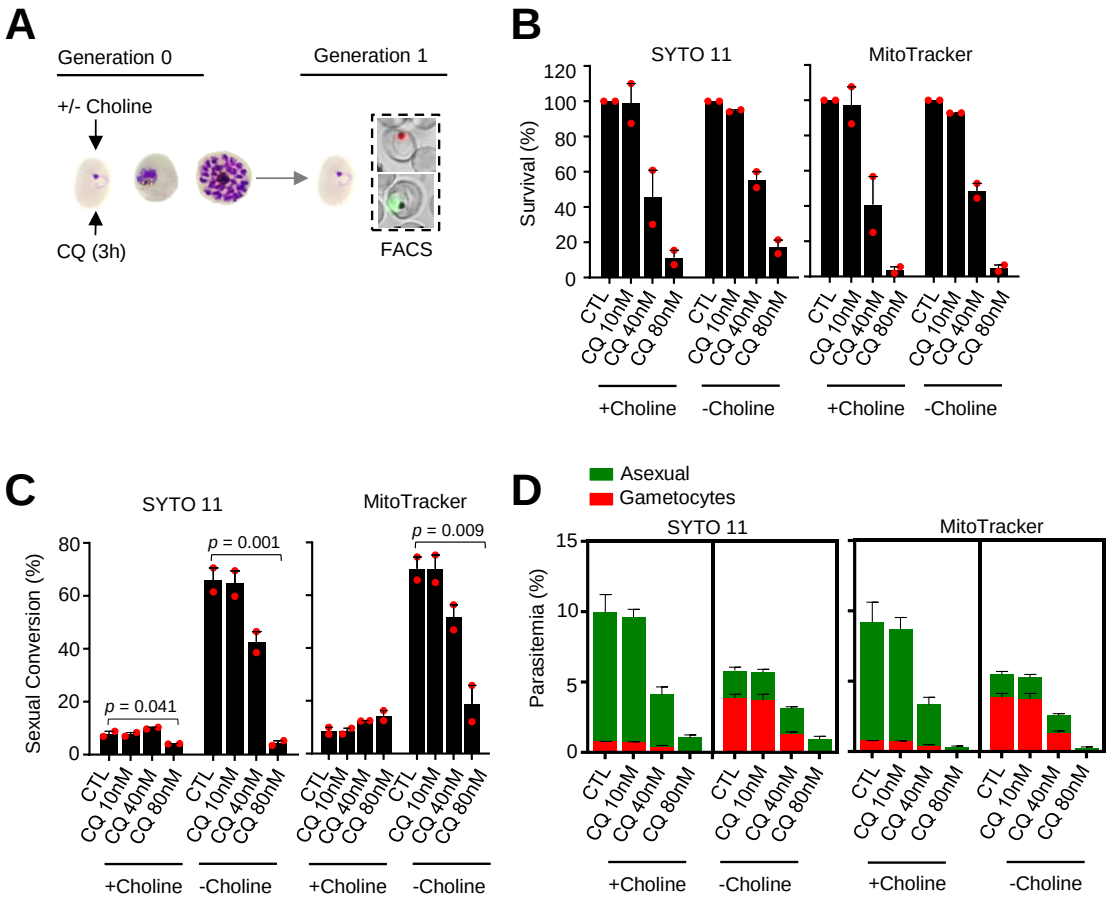

Figure 6-figure supplement 2. Effect of a chloroquine (CQ) pulse at the ring stage on sexual conversion. (A) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-gexp02-Tom* line under non-inducing (+ choline) or inducing (- choline) conditions were exposed to a 3 h CQ pulse at subcurative doses at the ring stage (0-10 hpi). Sexual conversion was measured by flow cytometry (FACS) after reinvasion (~30-40 hpi of the next multiplication cycle). **(B)** Survival rate of cultures exposed to the different drug doses, using total parasitemia values (asexual + sexual parasites) based on identification of all parasites with SYTO 11 or viable parasites only with MitoTracker Deep Red FM. For each choline condition, values are presented relative to the parasitemia in the control cultures (no drug). **(C)** Sexual conversion rates determined by flow cytometry. The *p* value is indicated only for treatment vs control (no drug) significant differences ($p<0.05$). **(D)** Distribution of absolute parasitemia of asexual and sexual parasites (from the same flow cytometry measurements as in panel C). In all panels, data are presented as the average and s.e.m. of 2 independent biological replicates.

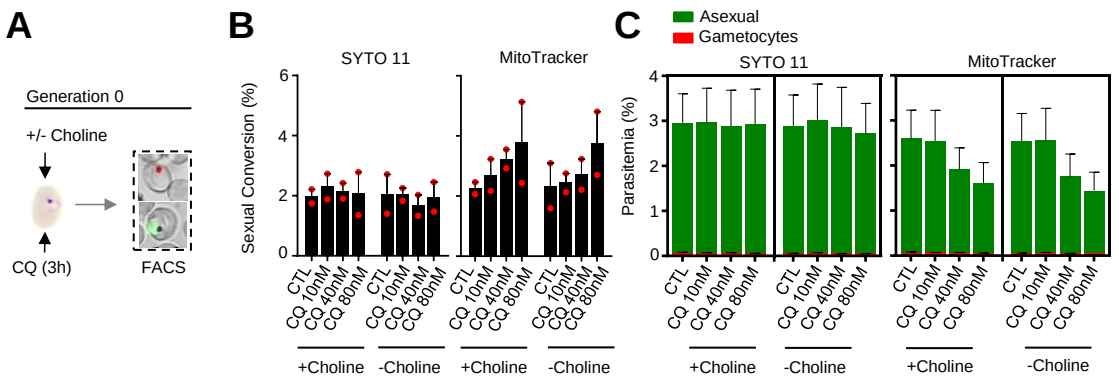

Figure 6-figure supplement 3. Effect of a chloroquine (CQ) pulse at the ring stage on sexual conversion by the same cycle conversion (SCC) route. (A) Schematic representation of the assay. Tightly synchronized cultures of the *NF54-gexp02-Tom* line under non-inducing (+ choline) or inducing (- choline) conditions were exposed to a 3 h CQ pulse at subcurative doses at the early ring stage (0-10 hpi). Sexual conversion was measured by flow cytometry (FACS) within the same cycle (~30-40 hpi) to determine the effect of the drug pulse only on production of new gametocytes by the SSC route. **(B)** Sexual conversion rates as determined by flow cytometry using SYTO 11 or MitoTracker Deep Red FM to identify all parasites or viable parasites only, respectively, in addition to TdTomato to identify gametocytes. No significant difference (p<0.05) with the control (no drug) was observed for any treatment condition. **(C)** Distribution of absolute parasitemia of asexual and sexual parasites (from the same flow cytometry measurements as in panel B). In all panels, data are presented as the average and s.e.m. of 2 independent biological replicates.

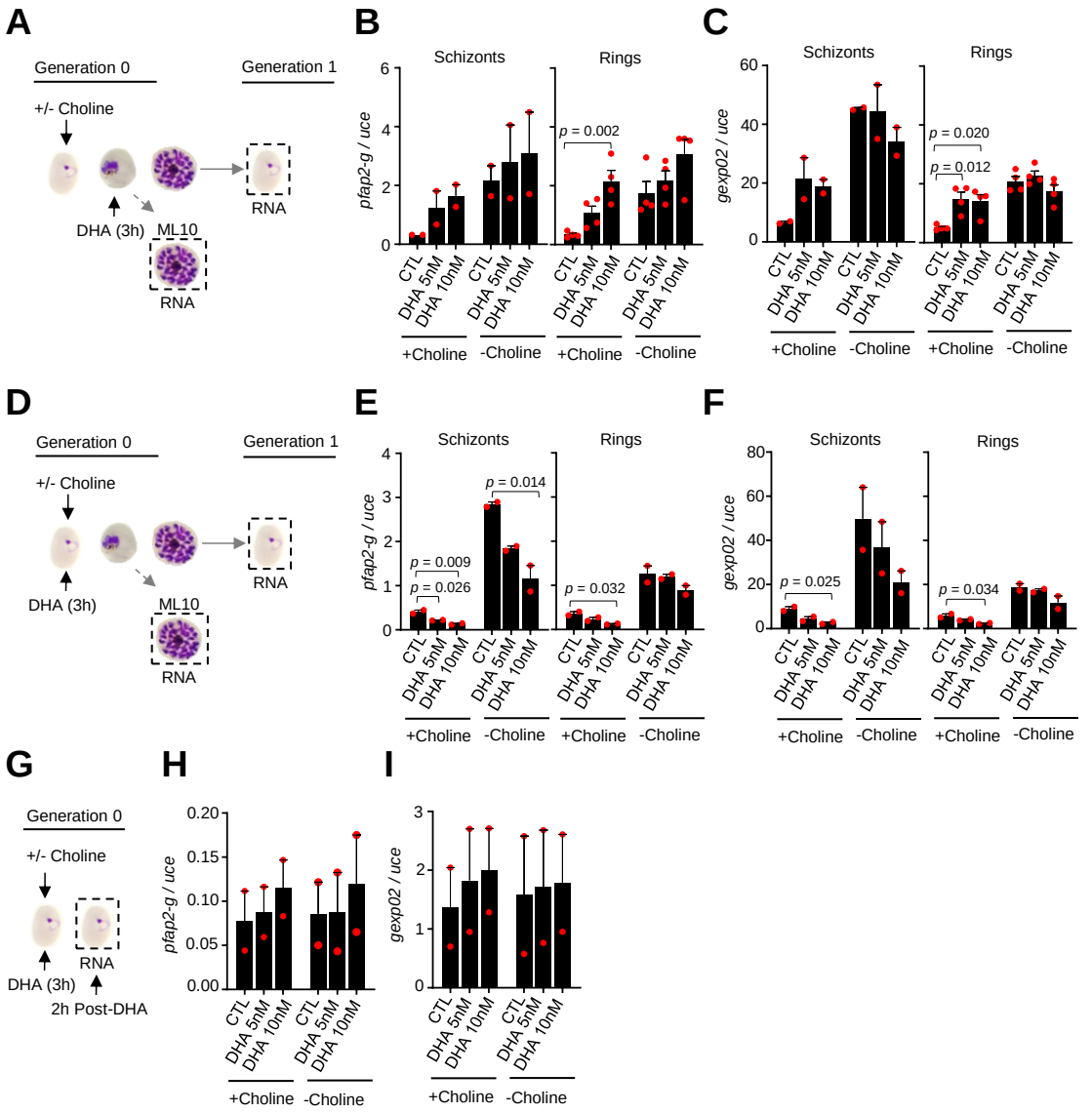

Figure 8-figure supplement 1. Changes in the transcript levels of *pfap2-g* and *gexp02* after a dihydroartemisinin (DHA) pulse, normalized against the *uce* gene. **(A)** Schematic representation of the assay. Tightly synchronized cultures of the *NF54-gexp02-Tom* line under non-inducing (+ choline) or inducing (- choline) conditions were exposed to a 3 h DHA pulse at subcurative doses at the trophozoite stage (25-30 hpi). RNA for transcriptional analysis was collected from ML10-treated cultures at the mature schizont stage (48-53 hpi) and, after reinvasion, from cultures at the early ring stage (cultures not treated with ML10, ~5 hpi). **(B-C)** Transcript levels of *pfap2-g* (B) or *gexp02* (C) normalized against the *ubiquitin-conjugating enzyme* (*uce*) gene. **(D-F)** Same as panels A-C, but cultures were exposed to DHA at the ring stage (0-10 hpi). **(G-I)** Same as panels D-F, but RNA for transcriptional analysis was collected only 2 h after completing the drug pulse. Data are presented as the average and s.e.m. of 4 (panels B-C, rings) or 2 (other panels) independent biological replicates. The *p* value is indicated only for treatment vs control (no drug) significant differences (p<0.05).

Chapter 4

Expression dynamics of early markers of *Plasmodium falciparum* sexual conversion in naturally infected patients treated with artemisinin

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Author Contributions

HPP: Conceptualization; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review and editing. PG: Analysis; Writing - review and editing. NHM: Sample collection; Resources; Writing - review and editing. AMS, MA, TH, NvH, and AS: Sample collection; Resources; Writing - review and editing. ERV: Analysis; Writing - review and editing. RV: Resources; Writing - review and editing. QB: Supervision; Resources; Writing - review and editing; Funding acquisition. AS: Conceptualization; Resources; Supervision; Funding acquisition; Writing - original draft; Project administration; Writing - review and editing. ARU: Conceptualization; Resources; Supervision; Funding acquisition; Writing - original draft; Project administration; Writing - review and editing.

Publication Information

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Abstract

Artemisinins are the key component of the frontline treatment against malaria. The impact of artemisinin-based treatment on *Plasmodium falciparum* sexual conversion levels in natural malaria infections is not known. This is an important gap of knowledge because sexual conversion is the first essential step for human-to-mosquito transmission, and is a key determinant of the number of transmissible forms (gametocytes) that are produced. To assess the direct impact of artemisinin on sexual conversion, we analyzed the expression of sexual ring markers (*pfap2-g*, *gexp02*, *gexp5*, *pfg14-744,* and *pfs16*) in longitudinally-collected (12 h intervals) patient samples from three independent cohorts in Vietnam, Burkina Faso, and Mozambique (n=109). In the majority of infections from Burkina Faso and Mozambique, we observed upregulation of the master regulator of sexual commitment, *pfap2-g*, and the other markers immediately after artemisinin treatment, indicating a treatment-induced sexual conversion. However, this was not observed in parasites from Vietnam with delayed clearance upon treatment and carrying artemisinin-protective mutations in the *pfk13* gene. In the Vietnam cohort, enhanced transcript levels of early sexual markers were associated with a peak of circulating mature gametocytes one week later. Our results underscore the importance of correct antimalarial drug use to avoid potential induction of malaria transmission.

Introduction

The malaria parasite *Plasmodium falciparum* causes half a million deaths each year, mainly in children under 5 years of age from low- and middle-income countries (1). The disease burden of malaria is a result of continuous erythrocytic replication of asexual parasite stages, which involves cycles of circulating young asexual rings progressing to tissue-sequestered mature trophozoites and schizonts. To initiate malaria transmission, in each asexual replicative cycle a variable proportion of the parasites (typically ~1%) develop into non-replicating sexual forms (gametocytes), which are the only parasite stage able to infect anopheline mosquito vectors (2).

Parasite commitment to sexual conversion and subsequent sexual differentiation depends on the transcription factor PfAP2-G, the master regulator

of the process (3,4). Activation of the expression of *pfap2-g* is a stochastic process influenced by the environment and regulated at the epigenetic level (5,6). In asexual stages the *pfap2-g* locus is silenced by epigenetic mechanisms, thus sexual commitment occurs when the chromatin at the *pfap2-g* locus transitions from a transcriptionally repressive to a permissive state. This transition is triggered by the parasite protein gametocyte development 1 (GDV1) (7). The first sexual stage found in the circulation after activation of *pfap2g* expression is the sexual ring stage. The only known molecular marker for very young sexual rings is *pfap2-g* itself. Apart from *pfap2-g*, older sexual rings also express early gametocyte markers such as *gexp02*, *gexp05*, *pfg14-744*, and *pfs16* (5,8–10). Recent work has shown that sexual rings are indeed found circulating in the bloodstream (11–14) and therefore markers for this stage can be used to predict future transmission potential. Sexual rings develop to gametocyte stages I to V in a process that lasts ~10 days and occurs away from the blood circulation, mainly in the bone marrow, where maturing gametocytes are sequestered. Mature male and female stage V gametocytes, the only stages that are infective to mosquitos, are released back into the peripheral blood.

Artemisinin-based combination therapies (ACTs) are the frontline treatment against *P. falciparum* infection (15). In addition to their use to treat malaria disease, ACTs are being employed in mass drug administration campaigns aiming to interrupt transmission and eliminate malaria (16), because of their high efficacy against asexual stages and partial efficacy against immature gametocytes (17–22). While treatment with drugs such as chloroquine or sulfadoxine-pyrimethamine results in an increase in gametocyte carriage (23–26), treatment with ACTs typically reduces gametocyte density and mosquito infectiousness. However, circulating gametocytes are not fully eliminated from the blood circulation after treatment, indicating that some patients treated with ACTs can remain infectious to mosquitos for several days (27–30). The efficacy of artemisinin and derivatives (collectively referred to as ART) is seriously threatened by the emergence of parasite resistance. *P. falciparum* ART resistance is defined as delayed parasite clearance with microscopy detectable asexual parasites on day 3 and is associated with non-

synonymous mutations in the *P. falciparum* gene *pfk13* (encoding the PfK13 propeller domain protein) (31). *P. falciparum* resistance to ART first emerged in Cambodia and has rapidly spread throughout Southeast Asia (31–34). Currently, it also poses a threat for malaria control in high-transmission areas in Africa (34–37).

Plasmodium spp. are able to modify their basal sexual conversion levels in response to multiple external factors (38,39), possibly as an adaptive response to stressful conditions (40). Several antimalarial drugs have been proposed to alter sexual conversion rates on the basis of their effect on gametocyte levels after treatment in natural infections. However, changes in gametocyte carriage upon treatment do not necessarily reflect changes in sexual conversion, because gametocyte levels also depend on the ability of the drug to kill immature and mature gametocytes and to induce the release of gametocytes from the bone marrow (41,42).

Several studies have also addressed the role of antimalarial drugs on sexual conversion using *in vitro* parasite cultures (43–46) or rodent malaria models (47–49), but the results were often discordant between studies. This may be explained by a complex response that depends on multiple factors that may be different between specific studies. We recently measured the impact of chloroquine and dihydroartemisinin (DHA), the active metabolite of all ARTs, on *P. falciparum* sexual conversion in *in vitro* cultures, and indeed found that it depends on multiple factors including parasite stage, drug dose, and metabolic state (50). For this study, we used improved gametocyte-reporter lines that enable very early quantification of sexual forms (8) and a new assay with a short drug pulse to measure the effect of the drug on sexual conversion independently from its gametocytocidal effect. We showed that exposure to subcurative doses of the drug at the trophozoite stage significantly increased sexual conversion rates, whereas exposure at the ring stage had the opposite effect. Furthermore, the enhanced conversion was not observed in parasites in which sexual conversion was previously stimulated by depletion of choline (50).

To elucidate whether exposure to drugs results in changes in gametocyte conversion rates in natural infections, it is necessary to disentangle the effect of the drugs on sexual conversion from their effects on gametocyte viability and

release from the bone marrow. This requires the use of very early markers of sexual conversion (41) to detect sexual rings, the only circulating sexual form apart from mature gametocytes. It also requires a study design with frequent sampling after treatment. Therefore, here we investigated the impact of ART treatment on sexual conversion by analyzing transcript levels of the markers *pfap2-g*, *gexp02*, *gexp5*, *pfg14-744*, and *pfs16* (all expressed in circulating sexual rings) in longitudinal patient samples collected before and every 12 h after treatment in three independent cohort studies in Vietnam, Mozambique and Burkina Faso. We also measured mature gametocyte markers between 1 and 2 weeks after treatment.

Results

Characteristics of study participants

We analyzed the impact of ART on the sexual conversion of *P. falciparum* in naturally infected malaria patients from three independent longitudinal studies conducted in Vietnam (n = 34), Burkina Faso (n = 30), and Mozambique (n = 45). The common key characteristics between study sites were ART-based treatment, multiple sample collections for gene expression analysis from before treatment (0h) to 48 h after treatment at 12 h intervals , and an additional sample between days 7 and 14 for mature gametocyte analysis (**Figure 1A; Supplementary Figure 1**). The type of antimalarial drug treatment in the first 3 days and clinical presentation of the patients varied between cohorts, with parenteral artesunate (AS) and severe malaria cases in Mozambique, and oral ACT and uncomplicated malaria cases in Vietnam (DHA-piperaquine, PPQ) and Burkina Faso (artemether-lumefantrine, AL). In addition, in Vietnam, one of the two treatment arms received a 3-day treatment of oral AS monotherapy followed by 3 days of DHA-PPQ (see Methods). Most study participants from Vietnam were 15 to 30-year-old males, which in this area are a high-risk group of malaria infection due to forest-related activities (51). In Burkina Faso and Mozambique, all participants were children 1-11 years old. In all cohorts, the majority of patients showed submicroscopic gametocytemia on day 0. The delayed parasite clearance phenotype, defined as light microscopy (LM)-

detectable asexual parasitemia on day 3 after treatment, was only observed in the Vietnam cohort (**Supplementary Figure 2**).

pfap2-g expression levels after artemisinin treatment and its association with PfK13 mutations

We observed a clear upregulation of *pfap2-g* relative transcript levels (normalized to *uce*) immediately after the first dose of ART treatment in the majority of isolates from Burkina Faso and Mozambique (African cohorts), whereas in the Vietnam cohort we observed mainly downregulation (**Figure 1 B-C**). These changes in *pfap2-g* expression levels suggest an overall induction of sexual conversion after ART-based treatment in African but not in Vietnam parasites. We hypothesized that differences in *pfap2-g* changes between African and Vietnam cohorts may be due to the presence of *pfk13* mutations and associated decrease in susceptibility to ART in Vietnam isolates (52). Indeed, the majority (87.51%) of isolates from Vietnam carried mutations in the *pfk13* gene, with 62.5% of PfK13 mutant isolates carrying the C580Y mutation (**Figure 1D**), dominant in Vietnam and the Greater Mekong sub-region (**36**). Next, we compared *pfap2-g* relative transcript levels between wild-type and *pfk13* mutant isolates and found that *pfap2-g* expression levels significantly decreased over time in PfK13 mutant isolates after ART treatment, whereas the opposite trend was observed in the few isolates from Vietnam carrying wild type PfK13 (**Figure 1E**). Similarly, a comparison based on median fold-change (Median $FC^{12\text{-}48h}$), defined as the median of the changes in normalized transcript levels across four time-points after treatment relative to before treatment levels, revealed a significant induction of *pfap2-g* in wild-type isolates compared to *pfk13* mutants (**Figure 1F**). This suggests that PfK13 mutant isolates may be refractory to the inducing effect of ART on sexual conversion.

Next, we compared changes in *pfap2-g* expression upon treatment (based on median $FC^{12\text{-}48h}$) in patients with delayed parasite clearance phenotype (indicative of ART resistance) compared to patients with fast parasite clearance (indicative of ART sensitivity) (**Figure 1G**). Isolates from patients with delayed clearance had significantly lower *pfap2-g* Median $FC^{12\text{-}48h}$ than patients that cleared infections adequately. Of note, 67% (8/12) of infections with a fast

clearance phenotype carried PfK13 mutant alleles, but 75% of them (6/8) showed non-induced *pfap2-g* levels (i.e., fold-change below 1).

All patients enrolled in the African cohorts showed fast parasite clearance after treatment, similar to wild-type parasites from Vietnam. Likewise, the majority of African samples displayed a significant *pfap2-g* induction after ART treatment, with a higher *pfap2-g* expression increase than Vietnam samples in both fast and delayed clearance groups (**Figure 1G**). A negative association between *pfap2-g* induction upon treatment and parasite clearance time was observed in the African cohorts and in Vietnam, but it was statistically significant only when the Vietnam samples were included (**Figure 1H**). Overall, these results suggest that ART treatment results in an increase in sexual conversion in wild type but not in *pfk13* mutant parasites. Results were consistent when *serrs* and *18s rRNA* genes were used for normalization, indicating that the choice of the normalizing gene did not affect the conclusions (**Supplementary Figure 3A-D**).

Changes on expression levels of sexual ring biomarkers (SRBs) after artemisinin treatment

We also analyzed the relative transcript levels of *gexp02*, *gexp5*, *pfg14-744*, and *pfs16* in the three cohorts. The activation of these biomarkers is PfAP2-G-dependent but the onset of expression occurs at different times during the sexual ring stage for the different markers, and there are also differences in the level of specificity for sexual versus asexual rings. While *pfap2-g* is expressed in sexual rings of all ages, as the gene is already active in the preceding sexually committed schizonts, expression of *gexp02* and *gexp05* starts at the middle of the sexual ring stage (~10 hpi), and expression of *pfg14-744* and *pfs16* starts even later during the sexual ring stage (~20 hpi). Only *pfap2-g*, *gexp02*, and *pfg14-744* transcripts appear to be completely absent from asexual parasites. Transcripts for all markers are detected in circulating mature gametocytes, but in general at very low levels compared with sexual rings (**Figure 2A**) (5,8,10,53,54).

Correlation analysis for the changes in expression levels after treatment (Median $FC^{12\text{-}48h}$) detected with different SRBs revealed important differences

between them. While the correlation of *pfap2-g* expression levels and the other four SRBs was low in the three cohorts, correlations between *gexp02*, *gexp5*, *pfg14-744*, and *pfs16* were low to moderately positive (r = 0.3–0.5; $p < 0.05$) (**Figure 2B**), likely reflecting differences in SRB transcription dynamics across the sexual ring stage.

Despite the differences in the expression dynamics of each SRB, time-course analyses of *gexp02*, *gexp5*, *pfg14-744*, and *pfs16* after ART treatment showed a trend relatively similar to *pfap2-g* in all the cohorts (**Figure 2C; Supplementary Figure 4A-E**). SRBs showed induction in the majority of patients from the African cohorts, and in a lower proportion of patients from Vietnam (i.e., the prevalence of infections showing induction from highest to lowest was Mozambique > Burkina Faso > Vietnam) (**Figure 2D**). In spite of the similar general trend, the prevalence of infections in which induction was observed and the magnitude of induction varied between SRBs; for instance, the proportion of patients showing upregulation of *gexp02* (based on >2 Median FC^{12-48}) was three-fold higher than that for *pfap2-g* (i.e., 34.5% vs. 11.8%) in the Vietnam cohort (**Figure 1C; Figure 2D**).

We also measured basal transcript levels of SRBs in the three cohorts using samples collected before treatment (0h) (**Supplementary Figure 5**). First, we observed that each SRB had different transcripts abundance, with *pfg14-744* consistently showing the lowest abundance. In contrast, *gexp05* and *pfs16* transcripts showed the highest abundance in the three cohorts. Second, isolates from Vietnam showed the highest basal expression levels of *pfap2-g* and all other SRBs compared to African samples, which might reflect a higher parasite investment in gametocyte production in this low endemicity setting (**Supplementary Figure 6A-D**).

The increase in SRB expression after ART treatment results in mature gametocyte carriage only in the Vietnam cohort

We measured the levels of mature gametocytes from before treatment until 7 to 14 days after treatment, when new gametocytes formed immediately after treatment are expected to be mature and present in the circulation. For this, we used the *pfs25* biomarker, which detects female mature gametocytes

and correlates with overall mature gametocyte density and mosquito infection rates (41,55,56). In the three cohorts, gametocytemia decreased gradually after treatment but ACT failed to completely clear all gametocytes by days 7 and 14, such that between 25 and 50% of the study participants remained a potential source of transmission (**Figure 3A-B**). In samples from Mozambique, we measured the gametocyte sex ratio based on transcript levels of *pfs25* and *pfmget* (male gametocyte marker); we observed a pronounced decrease with time of *pfs25* compared to pfmget transcripts, indicating that female gametocytes are likely more vulnerable to ART than male gametocytes, resulting in a progressive reduction in the female to male ratio (**Supplementary 7A-C**).

Next, we investigated whether the peak of mature gametocytes observed on days 7 to 14 in several patients was associated with ART induction of sexual conversion. In Vietnam, the *pfap2-g* Median $FC^{12\text{-}48h}$ was significantly higher in samples with detected gametocytes on day 7 (i.e., detection of *pfs25* biomarker) than in those without, but the difference was not significant in the Burkina and Mozambique cohorts (**Figure 3C**). There was also a significant positive correlation between induced *pfap2-g* levels and gametocyte carriage 1 week later in Vietnam but not in Burkina and Mozambique (**Figure 3D**).

In Vietnam, a distinct mature gametocytes peak was apparent on day 7 only in patients with an induced *pfap2-g* expression upon treatment (defined as *pfap2-g* Median $FC^{12\text{-}48h} > 1$, **Figure 3E-G**, purple), but gametocyte density (*pfs25* copy numbers) on day 7 was not significantly different between samples showing or not showing *pfap2-g* induction (**Figure 3E**). In African cohorts, we did not observe an increase in day 10-14 gametocyte carriage associated with *pfap2*-g induction (**Figure 3E-F**).

The expression fold-change after treatment for *gexp02*, *gexp5*, *pfg14-744*, and *pfs16* did not correlate with mature gametocyte levels on days 7 to 14 in any of the three cohorts (**Supplementary Figure 8A-F**). We also assessed if the levels of sexual rings before treatment contribute to mature gametocytes on days 7 to 14. Basal transcript levels for *gexp02*, *gexp5*, and *pfs16* showed a weak correlation with gametocyte carriage on days 7 to 14 in one of the African cohorts but not in the Vietnam cohort (**Supplementary Figure 9A-G**). Together,

our findings suggest that mature gametocytes found in the circulation 1-2 weeks after treatment may be the result of ART-induced sexual conversion, but a significant association was only observed in one of the cohorts. Moreover, mature gametocyte levels may also depend on multiple other factors, possibly including a limited contribution from sexual rings present before treatment.

Other factors that may contribute to determining the effect of ART on *pfap2-g* induction

We observed variability in the expression changes in *pfap2-g* and other SRBs after ART treatment both between and within the three cohorts. Hence, we examined whether these differences could be explained by differences in parasite and patient/clinical factors, using univariate and multivariable linear regression analyses (**Supplementary Figures 10 and 11**).

The presence of mature gametocytes on day 7 is associated with increased *pfap2-g* Median $FC^{12\text{-}48h}$ in the Vietnam cohort (**Supplementary Figure 10A**, univariate analysis). Therefore, to investigate whether additional factors may have an effect on *pfap2-g* induction, we constructed two different multivariate models for the Vietnam cohort: one including the variable 'day 7 gametocyte' (Model 1) and one excluding it (Model 2).

Model 1 confirmed the positive association between increased *pfap2-g* levels and mature gametocytes on day 7 (reg. coef. 1.3, 95% CI 0.59 to 2.13, $p = 0.001$). In addition, younger patients were more likely to have induced *pfap2-g* levels after treatment than older ones (reg. coef. -0.04, 95% CI -0.07 to -0.01, $p = 0.019$). In Model 2, PfK13 mutations were significantly associated with *pfap2-g* levels, when adjusted for other variables and excluding the 'day 7 gametocyte' variable. Carrying a *pfk13* wild-type allele was significantly associated with higher *pfap2-g* induction levels after treatment (reg. coef. -1.69, 95% CI -2.89 to -0.65, $p = 0.006$), consistent with the results presented in **Figure 1F**.

In Burkina Faso, we did not observe significant associations between explanatory variables and changes in *pfap2-g* levels (**Supplementary Figure 10B**). In Mozambique, blood glucose concentrations and treatment with rosiglitazone (insulin sensitizer) were significantly associated with *pfap2-g* levels, when adjusted for other variables (**Supplementary Figure 10C**). A

higher blood glucose concentration on day 0 was associated with higher *pfap2-g* levels increase after parenteral artesunate treatment (reg. coef. 1.22, 95% CI 0.16 to 2.30, p = 0.025). Rosiglitazone as an adjunct therapy was associated with lower *pfap2-g* Median $FC^{12\text{-}48h}$ after parenteral artesunate treatment (reg. coef. -3.99, 95% CI -7.95 to -0.32, p = 0.048).

In the final model combining the three studies, the type of treatment and parasite clearance (day 3 positivity) were significantly associated with *pfap2-g* induction (**Supplementary Figure 11**). Artesunate monotherapy was associated with higher *pfap2-g* levels (reg. coef. 2.54, 95% CI 0.53 to 4.56, p = 0.014), whereas delayed parasite clearance was associated with lower *pfap2-g* levels (reg. coef. -0.07, 95% CI -0.11 to -0.03, p < 0.001). The Mozambique cohort had several unique characteristics, such as using artesunate monotherapy and including severe malaria patients. It is unclear which of the conditions could explain the increased levels of *pfap2-g* in this cohort.

Overall, the results of the multivariate analysis confirm our previous observations that the presence of mature gametocytes on day 7 and carrying wild-type *pfk13* alleles were associated with higher *pfap2-g* induction in the Vietnam cohort. It also identified that blood glucose levels before treatment and rosiglitazone treatment of severe malaria patients may have an effect on *pfap2-g* induction levels.

Discussion

While the dynamics of *P. falciparum* gametocyte clearance after ACT treatment has been described in detail (17–22,57), studies addressing the direct impact of treatment on parasite sexual conversion in natural infections are lacking. The extent to which *P. falciparum* parasites modify sexual conversion rates in response to environmental factors has been difficult to investigate in natural infections, mainly due to the lack of specific early markers of sexual conversion and the need for demanding study designs with frequent patient sampling (40,41). Previous studies reporting changes in mature gametocyte carriage after treatment cannot be interpreted as treatment affecting sexual conversion, because the presence of gametocytes in the blood circulation after treatment depends on many other factors (41). Here we characterized the

impact of ART treatment on parasite sexual conversion in naturally infected patients recruited in three different cohorts, using time-course analyses of biomarkers for both sexual rings and mature gametocyte.

Our results reveal upregulation of the master regulator of sexual commitment *pfap2-g*, which in patients from the Vietnam cohort often translated into subsequent mature gametocyte carriage. We observed a relatively similar trend for *gexp02*, *gexp5*, *pfg14-744*, and *pfs16* in all three cohorts, suggesting that parasites can induce sexual conversion to increase their investment in transmissible forms in response to treatment. By comparing the three cohorts, we observed that *pfap2-g* induction is associated with ART sensitivity. Parasites carrying PfK13 wild-type alleles and showing a fast clearance phenotype show higher induction of *pfap2-g* expression than parasites with PfK13 mutations or exhibiting delayed clearance.

A possible explanation for the difference observed between wild type parasites and parasites from Vietnam carrying mutant *pfk13* alleles is that the level of ART exposure was insufficient to activate *pfap2-g* expression in the mutants. Parasites carrying PfK13 mutations tend to be less sensitive to the damaging effect of ART as a result of delayed drug activation (33). In a previous analysis of the Vietnam samples, patient isolates with delayed parasite clearance after treatment were associated with PfK13 mutations and higher ring survival rates in *ex vivo* drug susceptibility assays (52). However, the insufficient activity of the drug to activate *pfap2-g* expression in PfK13 mutants cannot explain why we observe a decline of *pfap2-g* levels, rather than unaltered expression, even after multiple ART doses. The reduction in *pfap2-g* transcript levels might be the result of induced parasite dormancy (58) or reflect a parasite response involving higher investment in asexual replication (48).

Differences in the *pfap2-g* response between individuals and between cohorts might also be explained by the predominant stage of the parasites at the time of ART treatment. Recently, we demonstrated *in vitro* a stage-dependent impact of ART on sexual conversion, with increased sexual conversion rate upon drug exposure at the trophozoite stage, but reduced sexual conversion upon exposure at the ring stage (50). Altered developmental progression, with a prolongation of the ring stage even in the absence of ART

exposure, is a well-documented phenomenon in PfK13 mutant parasites (58–60). The dominant downregulation of *pfap2-g* levels observed in the Vietnam cohort may be a consequence of parasites from Vietnam extending the ring-stage development and shortening the trophozoite-stage development, such that the time window at which parasites are susceptible to ART-mediated enhancement of sexual conversion is shorter. According to the model described by Khoury et al. (2020) (61), in patients with PfK13 mutant parasites, an ACT dose may overlap with the highly vulnerable trophozoite stage only in a lower proportion (~30%) of patients than in wild type parasites (>50%). Even for a complete 3-day treatment course, the drug may coincide only with ring stages in some patients infected with parasites carrying *pfk13* mutations. This model also explains the prevalent upregulation of *pfap2-g* levels in African cohorts, where all parasites are ART sensitive and thus do not have an extended ring stage. In these patients, ART is more likely to coincide with the trophozoite stage (61), which is susceptible to the enhancement of sexual conversion. In our datasets, upregulation of sexual ring biomarkers is prominent at 12 to 24 h time-points, indicating that it mainly reflects induction after the first ART dose, when parasite density is still relatively abundant.

Parasites in which sexual conversion is induced by one external factor (e.g., choline depletion) cannot be additionally induced by another external factor (e.g., ART), suggesting that there is a level of saturation for the rate of sexual conversion (50). Isolates from Vietnam showed a higher level of expression of *pfap2-g* and other SRBs before treatment than isolates from the African cohorts, suggesting that, even under basal conditions, Vietnam parasites may be in a constitutively partially-induced state. The difference in SRB basal levels (before treatment) between Vietnam and African cohorts may be explained by differences in the intensity of transmission and prevalence of ART resistance between the study settings. Low transmission and PfK13 mutations were both previously associated with higher gametocyte densities in field studies (62,63). Lysophosphatidylcholine (LysoPC) plasma levels, which have been shown to correlate with sexual ring density in Ghanaian patients (11), are not available in our study. Thus, we could not assess whether LysoPC

levels play a role in determining the different basal expression levels of SRBs observed between the cohorts.

Our results show that while gametocyte density drops after ART treatment, several patients remain gametocyte carriers, as previously observed in other field settings (17,27–30). Past studies demonstrated that gametocytes exposed to ART treatment (27,64) as well as ART-induced gametocytes (50) were capable of infecting *Anopheles* mosquitos. This suggests that gametocytes observed in our study cohorts after treatment potentially contribute to malaria transmission. In the Vietnam cohort, *pfap2-g* induction was associated with gametocyte carriage on day 7 after treatment. However, we did not observe this association in the African cohorts. One possible explanation is that in African parasites, which have a higher sensitivity to the drug, the gametocytes formed after treatment are killed by the 3-day treatment course during the sexual ring or immature gametocyte stages (17). By contrast, in parasites from Vietnam carrying *pfk13* mutant alleles, developing gametocytes may be partially resistant to the drug. In this scenario, enhanced sexual conversion can lead to increased numbers of mature gametocytes about a week after treatment. Of note, all participants in these cohorts received complete treatment, but patients receiving incomplete treatment or suboptimal drugs in non-controlled settings may have peaks of infective gametocytes some days after treatment even if they carry parasites with wild type *pfk13*.

To the best of our knowledge, our study is the first to compare the expression of multiple SRBs in epidemiological samples. These markers enable the quantification of parasites at the sexual ring stage, the only sexual stage found in the circulation in addition to mature gametocytes. We observed that *gexp05* and *pfs16* biomarkers consistently have the highest transcript abundance in all study cohorts, which is an advantage because in field samples parasite density is typically low, especially after drug treatment, and often only a low volume of blood is available. However, other factors need to be considered for the selection of SRBs for epidemiological studies: i) the stage specificity of the markers. Some of the SRBs are also expressed in other circulating stages such as asexual rings (*gexp05* and *pfs16*) or mature gametocytes, albeit typically at lower levels than in sexual parasites (53); ii) the fine temporal

expression dynamics of the markers within the sexual ring stage, with *pfap2-g* being the only marker expressed in sexual rings of all ages. Taking into account all of these factors and the results of our study, *pfap2-g* and *gexp02* may be the most informative markers. However, it is important to keep in mind that different SRBs have different features and a combination of them can be a powerful tool to clearly define the dynamics of early malaria transmission in field settings.

In conclusion, our field data show that induction of sexual conversion after ART treatment occurs in patients infected with ART-sensitive parasites, which is relevant for countries using ACTs to treat malaria or even for malaria elimination efforts. Our study not only highlights the importance of correct antimalarial drug use to avoid potential induction of malaria transmission in the field, but also defines a framework (study design and markers of sexual conversion) that can be used to investigate the impact of other environmental and host factors on sexual conversion.

Materials and Methods

Study design

This study examined the changes in relative transcript levels of sexual ring biomarkers (SRBs) upon treatment in three independent longitudinal studies conducted in Vietnam (Krong Pa District), Burkina Faso (Nanoro Department), and Mozambique (Manhiça District) (**Supplementary Figure 1**). All three cohorts had involved drug efficacy studies in malaria patients, with ACT and/or AS monotherapy as the primary treatment in the first three days. Multiple collections of blood samples within the first 1 to 2 weeks were key characteristics of these cohort studies.

Patients with uncomplicated *P. falciparum* mono-infections in the Vietnam cohort were recruited for a two-arm randomized open-label ART efficacy study between April 2015 and September 2017, as previously described (52). Patients in arm 1 were treated with oral dihydroartemisinin-piperaquine (DHA-PPQ) (Eurartesim®, 40 mg / 320 mg) once a day for 3 days while those enrolled in arm 2 were treated with oral AS (Co-Artesun®, 50 mg) once a day for 3 days, followed by a standard DHA-PPQ treatment for another 3 days.

In Burkina Faso, a longitudinal sub-cohort study intended to assess the effect of ACT on sexual conversion was performed in patients with uncomplicated falciparum malaria between January 2019 and February 2020. Patients were confined in the hospital for 3 days for clinical and diagnostic assessment, and treated with the standard oral artemether-lumefantrine (AL) (Coartem®, 20 mg / 120 mg) twice a day for 3 days. They were advised to return after 8 to 10 days for additional clinical examination and blood collection.

In Mozambique, patients with severe falciparum malaria were recruited from June 2018 to May 2019 in hospital-based rosiglitazone (insulin sensitizer drug) Phase IIb clinical trial. Previous information on the Phase IIa clinical trial has been reported elsewhere (65). In Phase IIb, with severe malaria cases involved, the standard treatment regimen was a parenteral AS (Artesun®, 2.4 mg/kg) given via intravenous route during hospital admission, 12h, and 24h later; after which, intravenous AS was administered for at least 24h. A complete AL (Coartem®, 20 mg / 120 mg) regimen was given to patients that can tolerate oral administration. In addition, a group of patients received parenteral rosiglitazone as an adjunct therapy.

Inclusion criteria and sample size

For Vietnam and Burkina Faso cohort, the main inclusion criteria to recruit the participants were the following: at least 1 year of age, single infection with *P. falciparum* assessed by microscopy, parasite density greater than 1000 p/µL (Vietnam) or 10,000 p/µL (Burkina Faso), absence of severe malaria symptoms, and absence of underlying diseases (e.g., cardiopulmonary conditions). For the Mozambique study, the main inclusion criteria were the following: 1 to 12 years of age, *P. falciparum* positive using rapid diagnostic test and confirmed by microscopy with parasite density greater than 2500 p/µL, at least 1 sign of severe malaria (i.e., at least 2 episodes of generalized seizure within 24h, prostration, impaired consciousness, and respiratory distress), and requiring hospitalization.

The estimated sample size was calculated based on the initial data in Krong Pa district, Gia Lai province. With estimated inhabitants of 70,000 and a

malaria prevalence of 3% in 2015 (52), the number of individuals infected with *P. falciparum* would be roughly 2,100 patients. Assuming a 20% expected frequency of *pfap2-g* induction upon treatment, 5% margin of error, 80% confidence levels, and a design effect of 1 in three clusters, the estimated sample size would be ≥ 34 patients per cluster or a total of ≥ 102 patients in 3 clusters (Epi Info™, CDC). The actual number of patients included in this study was 34 in the Vietnam cohort, 30 in Burkina Faso, and 45 in Mozambique, for a total of 109.

Blood sample, RNA, and DNA preparations

For Vietnam and Burkina Faso cohorts, a total of 300 µL of the venous blood sample was collected on day 0, from which 100 µL was aliquoted for RNA extraction in 500 µL of RNAprotect Cell Reagent (Qiagen, cat. no. 76526) and 200 µL of blood was preserved in EDTA tube for DNA extraction using the QIAamp® 96 DNA blood Kit (Qiagen, cat. no. 51161). After every 12h intervals, finger pricked blood samples were collected using the same volumes that on day 0. For the Mozambique cohort, a 500 µL whole blood was immediately processed for low RNA extraction protocol, as previously described (66). Briefly, the erythrocyte pellet was lysed and homogenized with 4.5 mL Trizol reagent (Invitrogen, cat. no. 15596026) and froze directly at -80ºC until RNA extraction.

The total RNA from Vietnam samples was processed following the protocol of RNeasy Plus 96 Kit (Qiagen, cat. no. 74192). For Burkina Faso and Mozambique samples, a modified protocol that combines both Trizol reagent and RNeasy® Mini Kit (Qiagen no. 74104) was performed for the total RNA extraction (66). All RNA samples were subjected to on-column DNase I treatment (Qiagen no. 79254). In all RNA samples, the first-strand cDNA was synthesized by using the AMV Reverse Transcription System (Promega, cat. no. A3500), with both oligo (dT) and random primers included in the reaction.

Quantitative PCR (qPCR) analysis

To quantify the transcript levels, a qPCR analysis of the cDNA was performed in triplicate wells using a standard curve method, with each set of oligonucleotide primers included for each target gene (8,53). Transcript levels of

pfap2-g (PF3D7_1222600), *gexp02* (PF3D7_1102500), *gexp5* (PF3D7_0936600), *pfg14-744* (PF3D7_1477300), and *pfs16* (PF3D7_0406200), were normalized with transcript levels of housekeeping genes *ubiquitin-conjugating enzyme* (*uce*) (PF3D7_0812600), *serine-tRNA ligase* (*serrs*) (PF3D7_0717700), and *18S rRNA* (PF3D7_1148600; PF3D7_0112300; PF3D7_1371000) to obtain their relative expression levels (8). The female gametocyte biomarker *pfs25* (PF3D7_1031000) was analyzed by qPCR in all samples. In Mozambique samples, we included the analysis of the male gametocyte biomarker *pfmget* (PF3D7_1469900). Transcript levels of mature female and male gametocytes were expressed as copy numbers with respect to their standard curves, which were based on constructed plasmids that contain either *pfs25* or *pfmget* mRNA sequence (52,67). The total parasite density was also estimated by qPCR using the *18S rRNA* transcript levels (PF3D7_1148600; PF3D7_0112300; PF3D7_1371000) for Vietnam and Mozambique samples (52) and *varATS* genomic DNA for Burkina Faso samples (68). All qPCR primers are listed in **Supplementary Table 1**.

Data analysis

The main outcome variables used to evaluate the effect of treatment on sexual conversion were the changes in expression levels of SRBs from the first treatment dose of ACT or AS until the end of follow up. These changes were expressed as fold-change (FC) unit, which is the ratio of relative expression levels in the post-treatment time point (12h to 48h) to the relative expression levels in the pre-treatment time point (0h). In all analyses, we used the median FC from 12h to 48h (Median $FC^{12\text{-}48h}$) to represent the central fold-change value observed after treatment. In selected analyses, we grouped the patient sample using cut-off values of 1 or 2 Median $FC^{12\text{-}48h}$. Time points beyond 48h were excluded from the final analysis because of very low parasite density or absence of parasites as a result of ACT treatment.

Relative transcript levels and fold changes were $\log_2$ transformed to meet the assumption of data normality, as assessed using the Shapiro–Wilk test. One-way ANOVA with Tukey HSD as a post hoc test was used to compare the median FC of more than two variables while Student's t-test was used to

compare two variables. A paired t-test was performed to determine the average difference in SRBs relative expression levels before and after treatment, with effect size (Cohen's *d*) included in the computation. Pearson's correlation coefficient (*r*) was calculated to assess the linear correlations between SRBs. Spearman's correlation coefficient (r_s) was also used to evaluate the correlation between SRBs and mature gametocyte carriage (presence or absence of *pfs25*) on days 7 to 14. Linear regression analysis was performed with the median *pfap2-g* FC as the dependent (outcome) variable while parasite and patient/clinical factors as independent variables, which are described in detail in **Supplementary Figures 11**.

Ethical approval

The Vietnam cohort study was approved by the ethical review board of the National Institute of Malariology, Parasitology and Entomology (351/QD-VSR) and Ministry of Health (QD2211/QD-BYT) in Vietnam, as well as of the Institute of Tropical Medicine (936/14) and Antwerp University Hospital (14/15/182) in Belgium. It was also registered at ClinicalTrials.gov (Identifier: NCT02604966). The Burkina Faso cohort study was approved by the ethical review board of the Comité d'Ethique pour la Recherche en Santé (CERS: 2018/10/131)) in Burkina Faso, as well as of the Institute of Tropical Medicine (1261/18) and Antwerp University Hospital (19/06/064) in Belgium. Ethical approvals of the Mozambique study were given by the ethical review board of the Comite Institucional de Bioética em Saude de Manhiça (230/CNBS/15) and Departamento farmacéutico, Ministry of health, Maputo (374/380/DF2016) in Mozambique, as well as of the Comité de Bioética del Hospital Clínic (HCB/2015/0981) in Spain. The Mozambique study was registered at ClinicalTrials.gov (Identifier: NCT02694874).

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FIGURE LEGENDS

Figure 1. *pfap2-g* expression levels and their association with PfK13 mutations. (A) Study design of the three cohorts. Red lines indicate the time of blood sample collection and RNA analysis. Blue and violet arrows indicate the time of treatment. Abbreviations: dihydroartemisinin-piperaquine (DHA-PPQ); artesunate (AS); artemether-lumefantrine (AL). **(B)** Time-course analysis of *pfap2-g* transcript levels before (0h) and after treatment (12-48h). *p* values were calculated using paired t-test (i.e., 0h vs 12h or 24h or 36h or 48h). Only significant *p* values ($p < 0.05$) are shown, with their effect size (Cohen's d). **(C)** Prevalence rate and 95% confidence interval (CI) of patients with greater than 1 and 2 median *pfap2-g* fold-change (relative to before treatment levels) between 12-48h (Median $FC^{12\text{-}48h}$). **(D)** Prevalence rate and 95% CI of PfK13 wild-type (WT) and mutant isolates from Vietnam. The colored bar indicates the proportion of different PfK13 mutations. **(E)** Time-course analysis of *pfap2-g* transcript levels before and after treatment in PfK13 WT or mutant isolates. Only significant *p* values ($p < 0.05$) are shown, with their effect size (Cohen's d). **(F)** Comparison of the *pfap2-g* Median $FC^{12\text{-}48h}$ between PfK13 WT and mutant isolates. The *p* value was calculated using a t-test. **(G)** Comparison of the *pfap2-g* Median $FC^{12\text{-}48h}$ between patients with fast and delayed parasite clearance time (defined as the absence or presence of ring stages by microscopy on day 3, respectively). Blue dots indicate PfK13 WT isolates. *p* values were calculated using One-way ANOVA with Tukey HSD as post hoc test. **(H)** Linear association of *pfap2-g* Median $FC^{12\text{-}48h}$ with parasite clearance. The blue line represents the linear prediction with 95% CI (gray shade). Red dots are the actual values of *pfap2-g* FC. *p* values from linear regression analysis are shown.

Figure 2. Comparison of transcript levels after treatment for different sexual ring biomarkers (SRBs). (A) Specificity of sexual ring biomarkers transcript levels among parasite stages found in the blood circulation, based on published studies (Bancells et al., 2019; Portugaliza et al., 2019; Van Biljon et al., 2019; Tibúrcio et al., 2015). Abbreviations: asexual rings (AR); sexual rings (SR); mature gametocytes (G); young sexual rings (Y), e.g., 0-5 hour post-invasion (hpi); mid sexual rings (M), e.g., 10-15 hpi; and late sexual rings (L), e.g., 20-25 hpi. **(B)** Linear correlation matrix between the Median $FC^{12\text{-}48h}$ for different SRBs. SRBs transcript levels were normalized to *uce*. Pearson correlation coefficients (*r*) are shown, with dark blue (1.00) being the highest correlation and white (0) the lowest. Absence of *r* value means insufficient sample size for the calculation. *r* values ≥ 0.3 showed statistical significance at an alpha level of 0.05. **(C)** Time-course analysis of SRBs relative transcript levels before (0h) and after treatment (12-48h). *p* values are calculated using a paired t-test (i.e., 0h vs 12h or 24h or 36h or 48h). Only significant *p* values ($p <$

0.05) are shown, with their effect size (Cohen's d). **(D)** Prevalence rate and 95% confidence interval (CI) of patients with greater than 2 value for the *gexp02, gexp5, pfg14-744,* and *pfs16* Median $FC^{12\text{-}48h}$.

Figure 3. Association of *pfap2-g* fold-change after treatment with mature gametocyte circulation 1 to 2 weeks later. (A) Prevalence rate and 95% confidence interval (CI) of gametocyte carriage (*pfs25* biomarker) before treatment and 1 to 2 weeks after treatment. **(B)** Temporal dynamics of mature gametocyte biomarker *pfs25*, showing gametocyte carriage 1-2 weeks later. The average (red dot) with 95% CI, median (blue dot) with 95% CI, and individual data in grey dots are shown. **(C)** Differences on *pfap2-g* median fold-change (Median $FC^{12\text{-}48h}$) after treatment in patients with detected (yes) or undetected (no) mature gametocyte biomarker *pfs25* on days 7 to 14. **(D)** Spearman correlation coefficient (r_s) with 95% CI of Median $FC^{12\text{-}48h}$ after treatment and *pfs25* positivity on day 7 to 14. Only *p* value <0.05 is shown. **(E)** Comparison of mature gametocyte (*pfs25*) density on days 7-14 between patients with greater than or less than 1 value of Median $FC^{12\text{-}48h}$ after treatment. Bar graphs with 95% CI and individual dots as actual values are shown. Differences between the two groups were not significant using a t-test. **(F)** Patients grouped according to Median $FC^{12\text{-}48h}$ of 1. **(G)** Median $FC^{12\text{-}48h}$ greater than 1 corresponds to *pfs25* levels in bar graph with purple bar on days 7 to 14 and less than 1 corresponds to *pfs25* levels in bar graph with blue bar on days 7 to 14. Bar graphs are the average of *pfs25* with 95% CI, with dots representing the actual values.

FIGURES

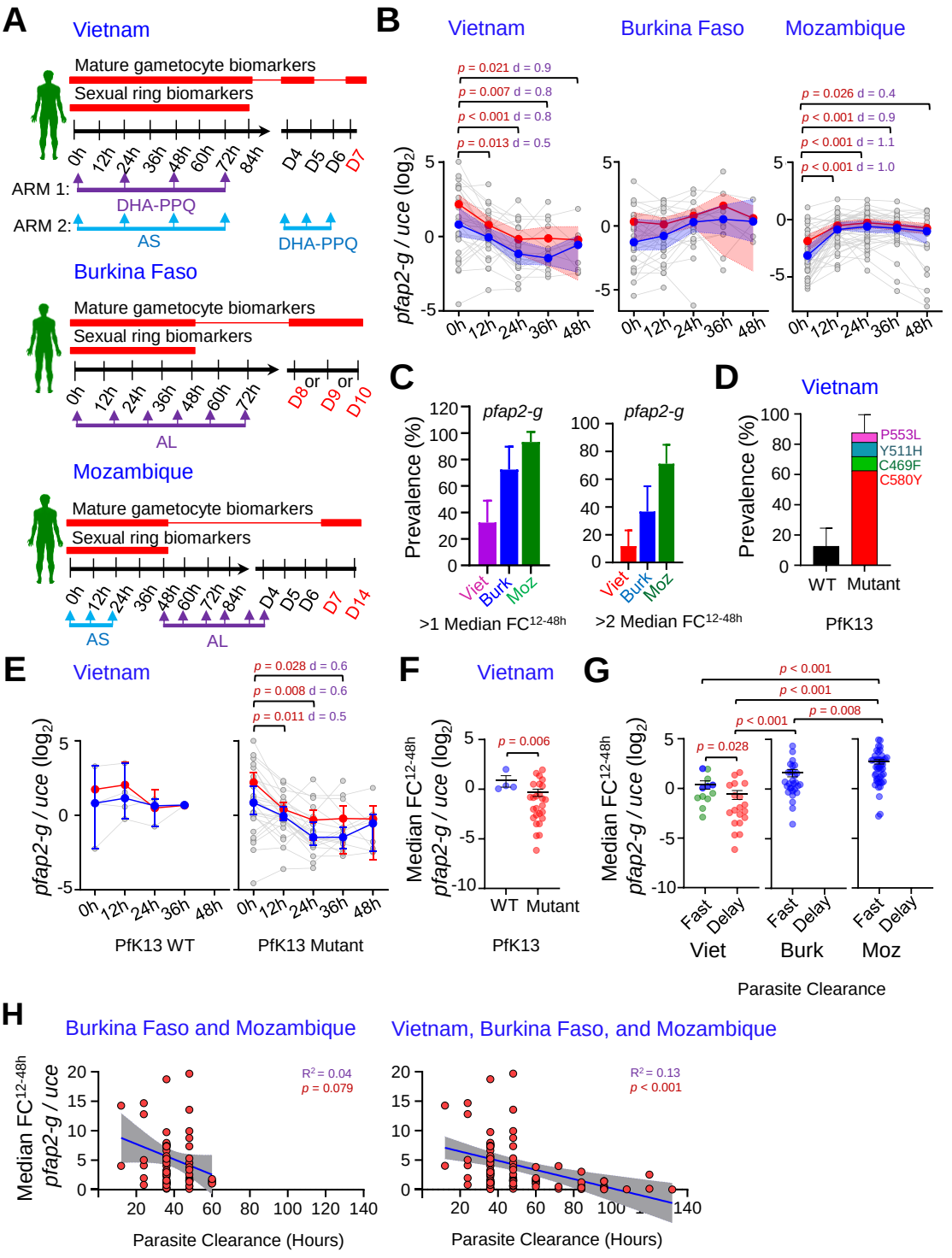

Figure 1. *pfap2-g* expression levels and their association with PfK13 mutations

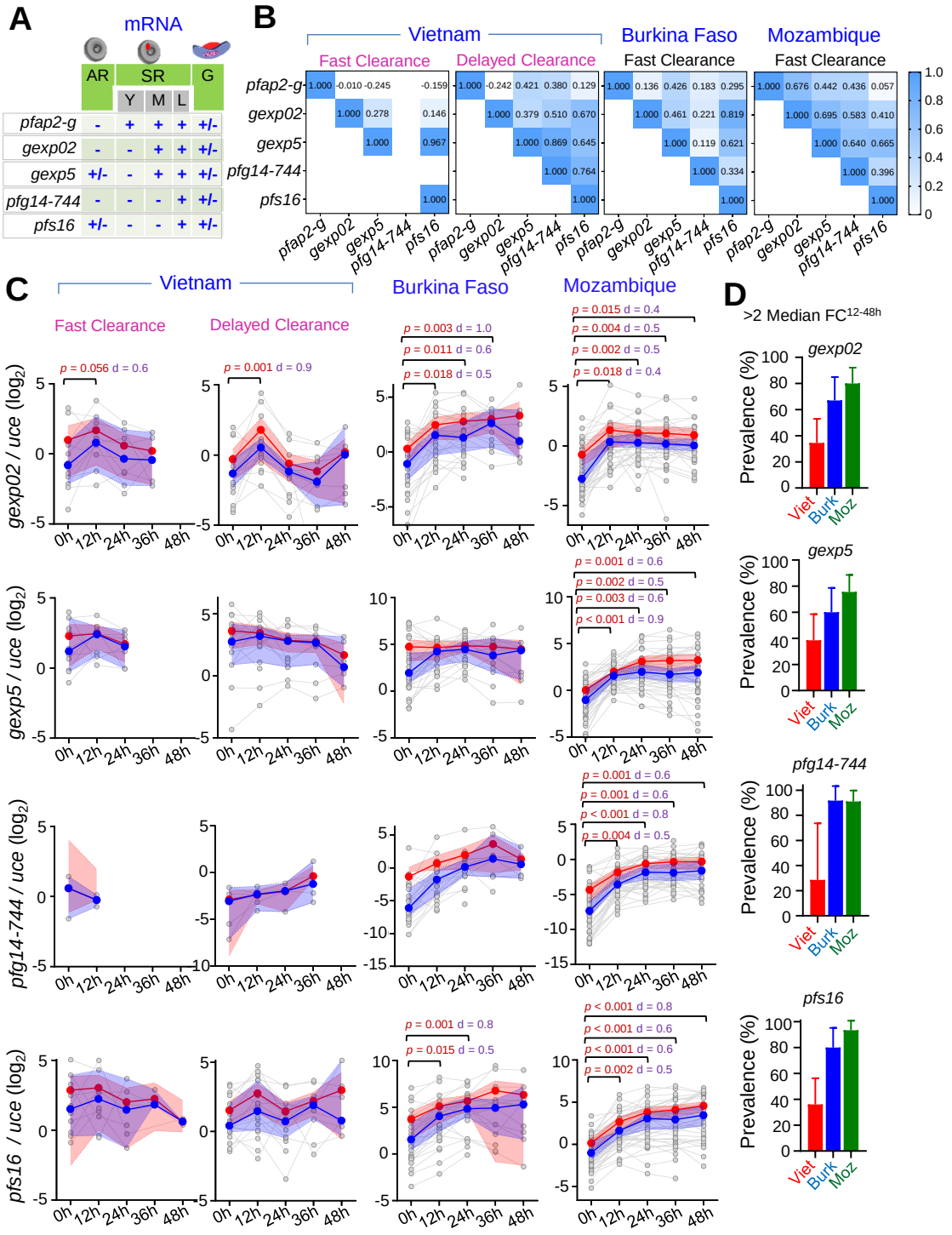

Figure 2. Comparison of transcript levels after treatment for different sexual ring biomarkers (SRBs).

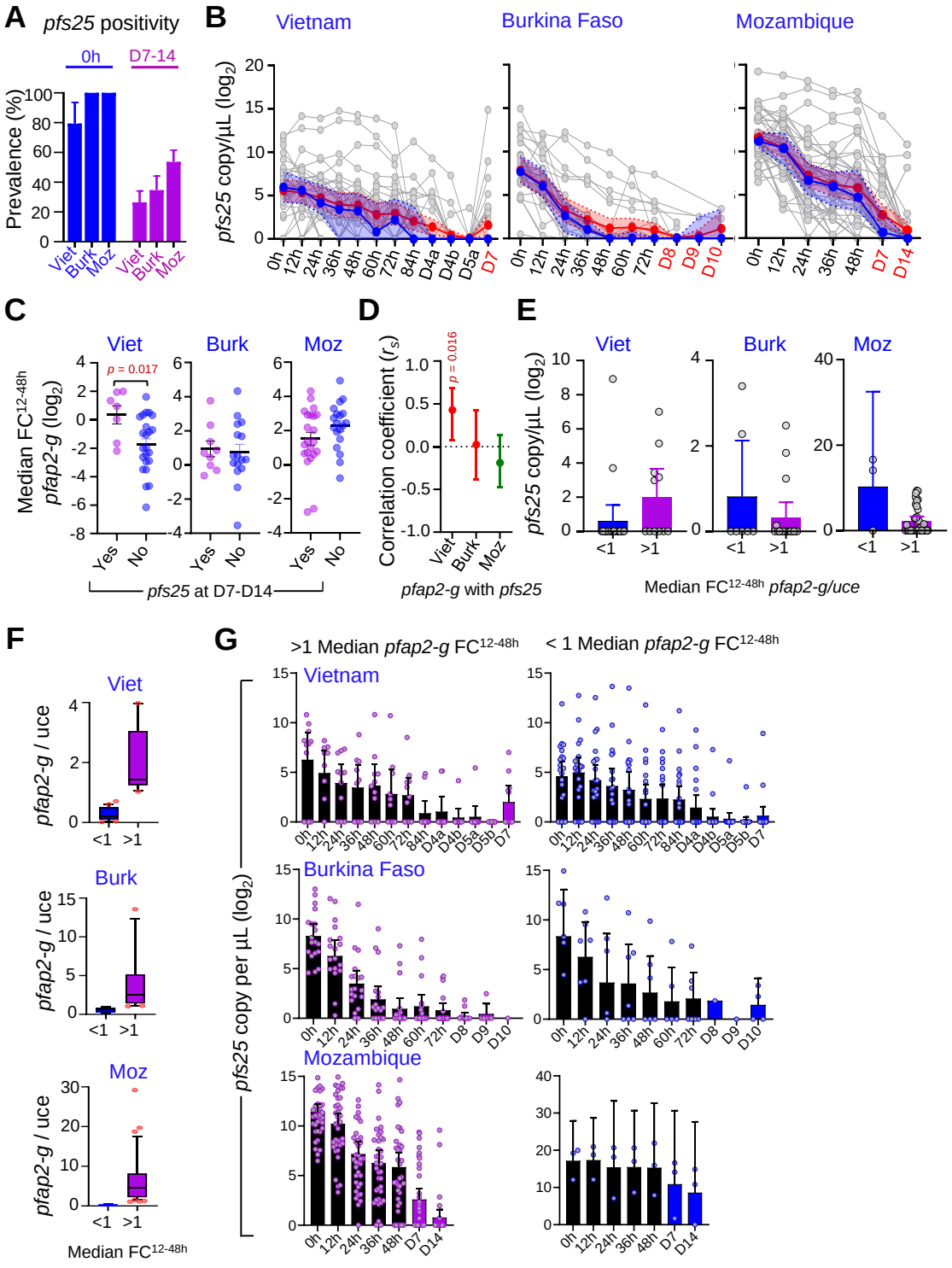

Figure 3. Association of *pfap2-g* fold-change after treatment with mature gametocyte circulation 1 to 2 weeks later.

Supplementary Figures

Expression dynamics of early markers of *Plasmodium falciparum* sexual conversion in naturally infected patients treated with artemisinin

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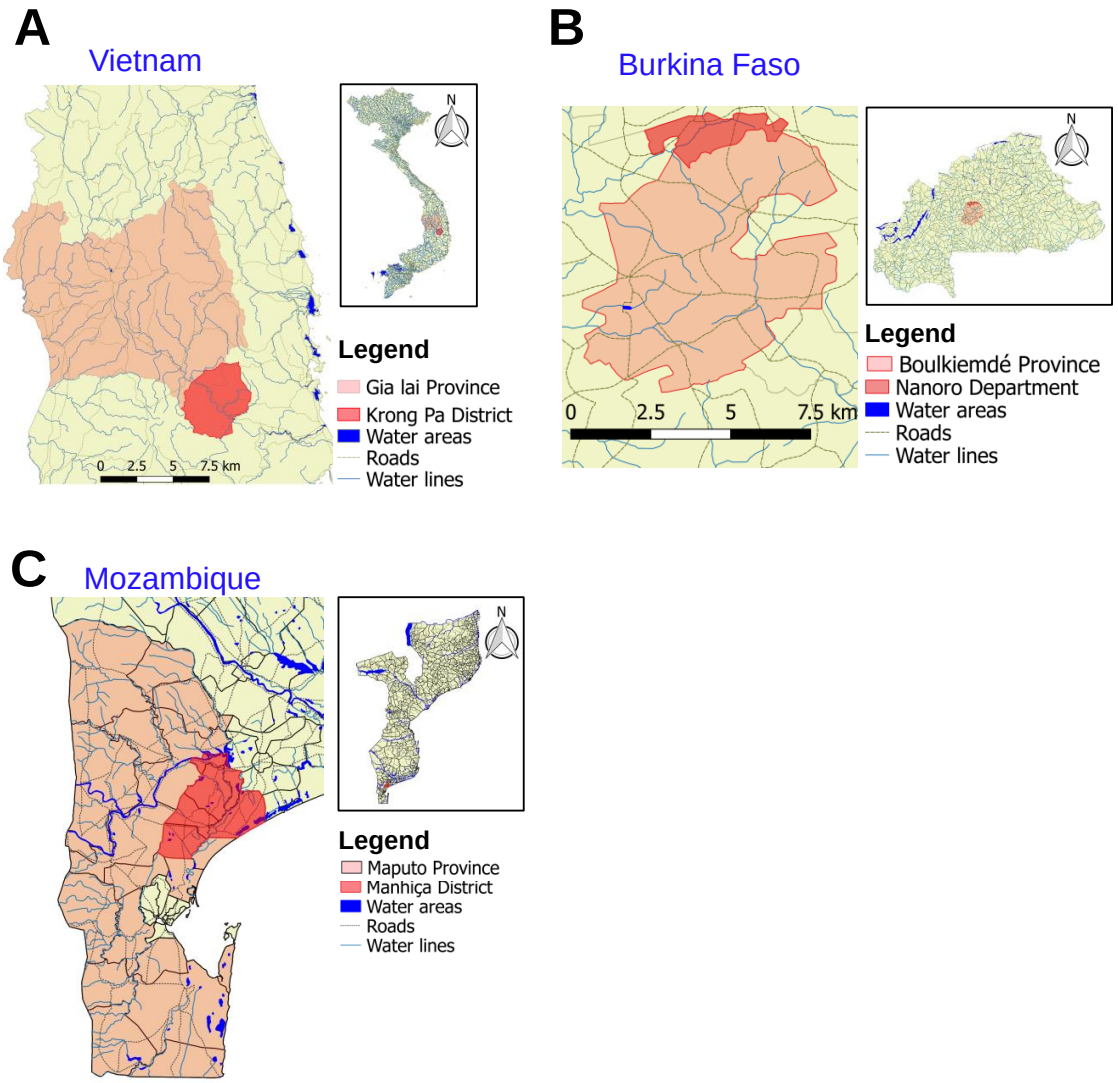
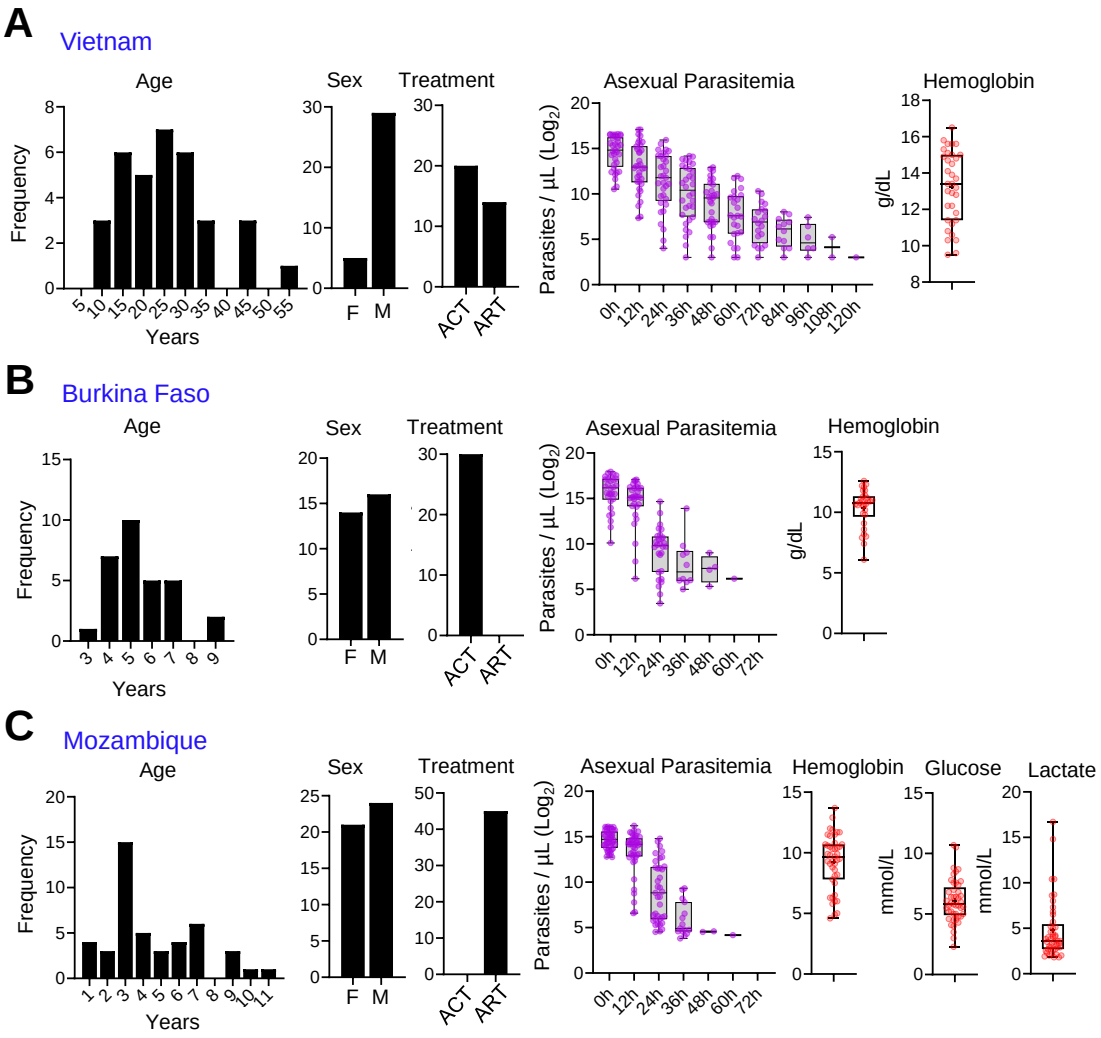
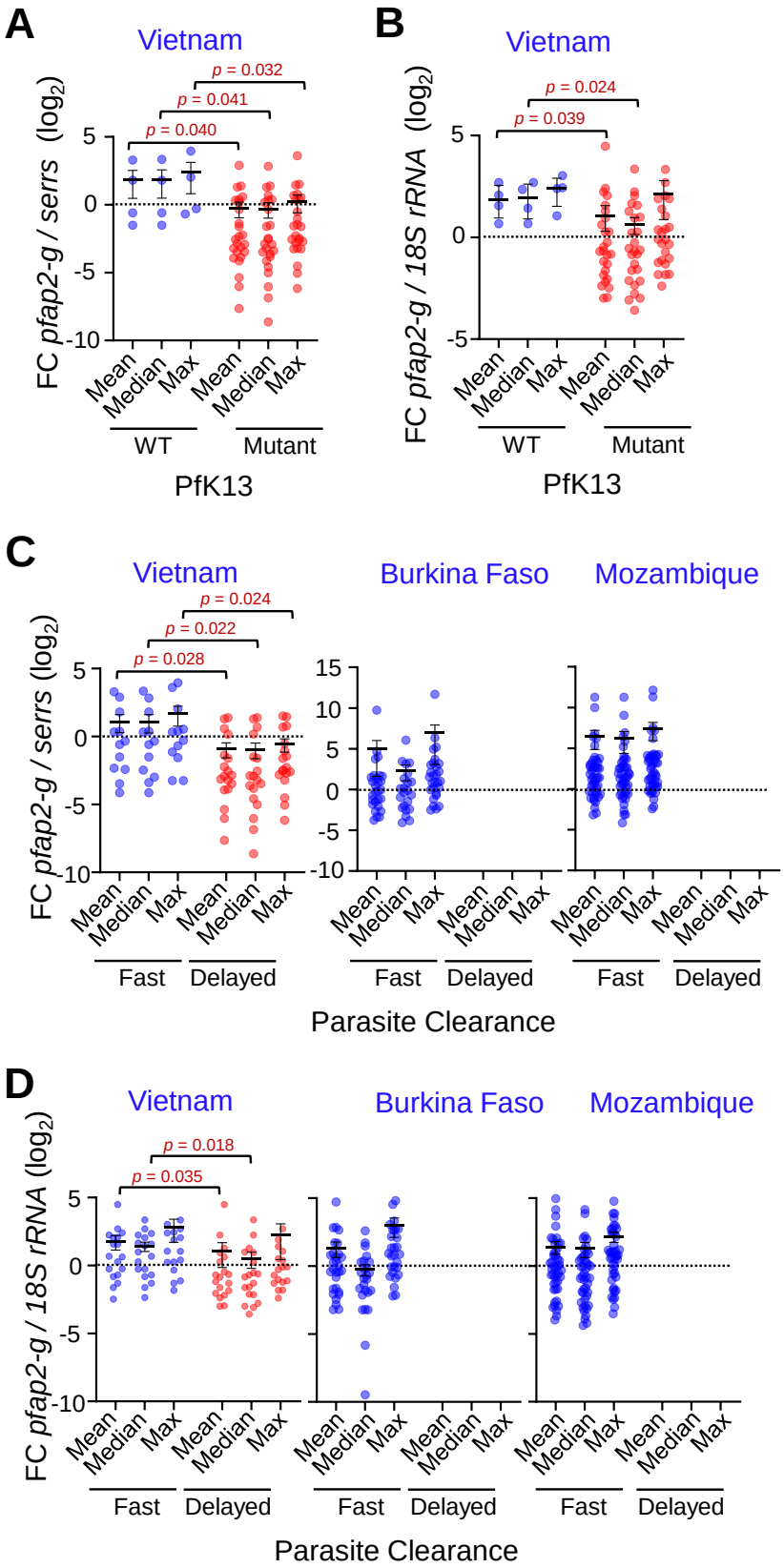


Supplementary Figure 1. Map of the study site. (A) The Vietnam cohort was conducted in Krong Pa District of Gia Lai Province, Central Vietnam. **(B)** The Burkina Faso cohort was conducted in Nanoro department of Boulkiemdé Province, Central Burkina Faso. **(C)** The Mozambique cohort was conducted in Manhiça District Hospital in Maputo Province, Southern Mozambique.

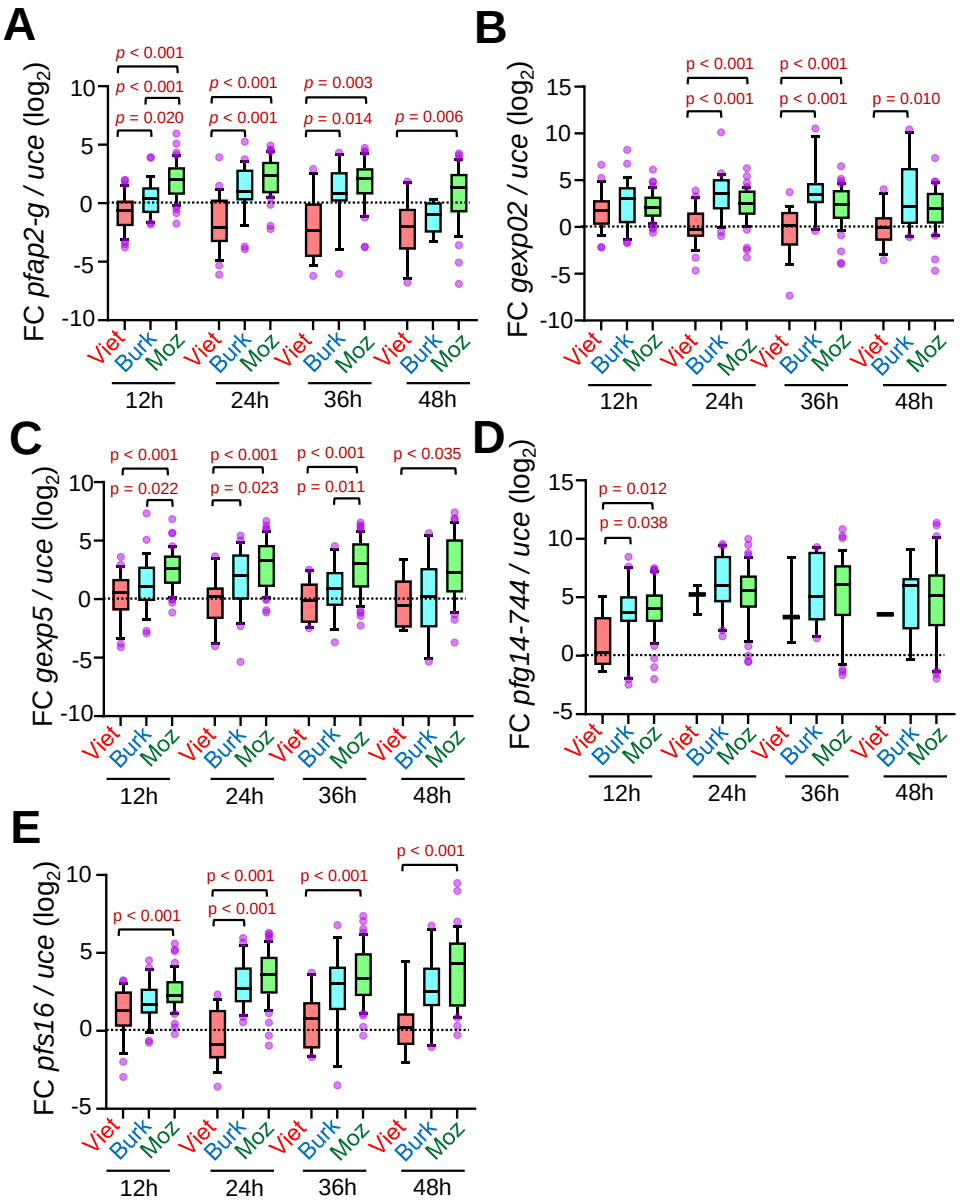


Supplementary Figure 2. Selected patients' information. (A) Age histogram, sex, treatment type, parasite density (microscopy), and hemoglobin data in the Vietnam cohort. **(B)** Similar to panel A, but in the Burkina Faso cohort. **(C)** Similar to panel A, but in Mozambique study. Abbreviations: Female (F); Male (M); Artemisinin-based combination therapy (ACT); artemisinin (ART) monotherapy in the first 2-3 days.

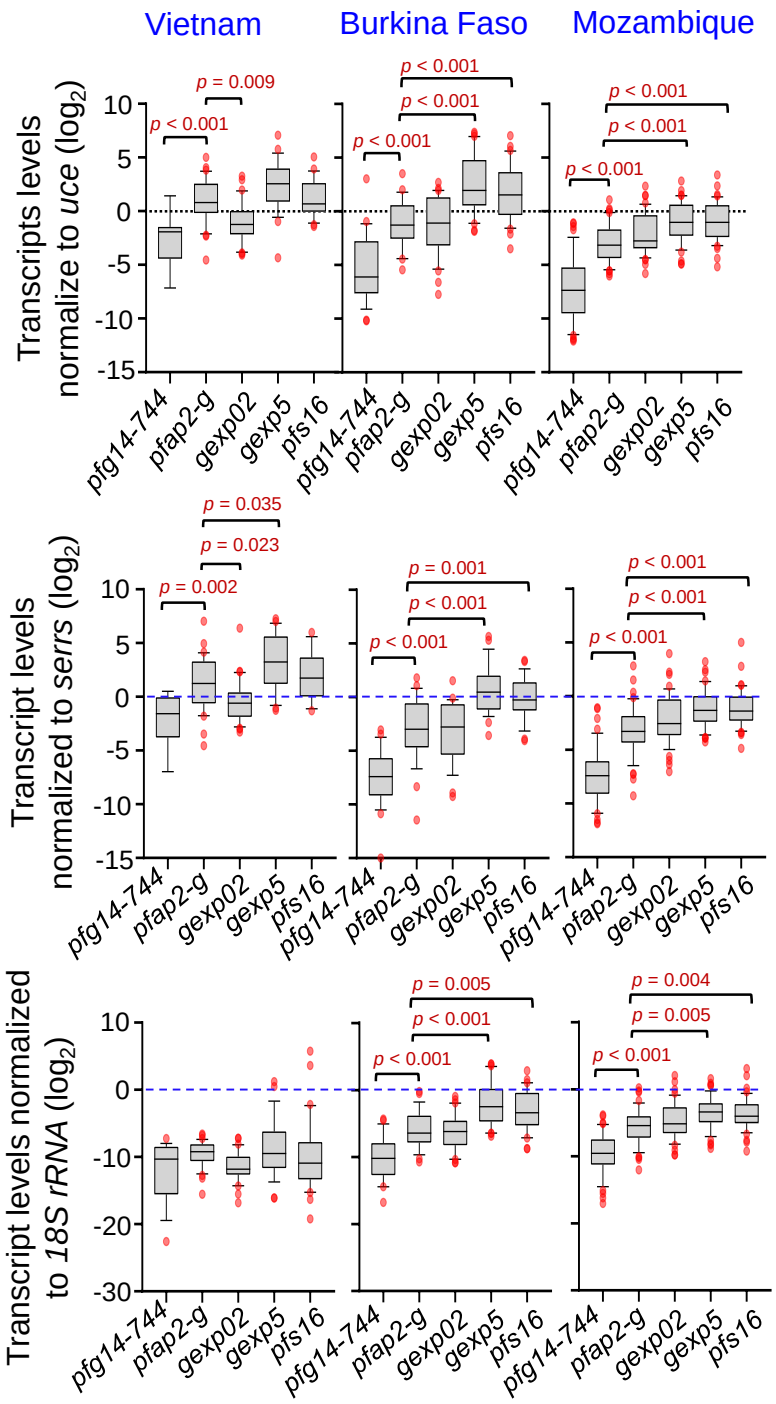


Supplementary Figure 3. *pfap2-g* expression levels and its association with PfK13 mutations. (A) Comparison of *pfap2-g* fold change (FC) relative to 0h in PfK13 wild-type and mutant parasites in Vietnam. FC is shown as the mean, median, and average values from 12-48h. *p* values are calculated using t-test, with less than 0.05 are shown. *pfap2-g* transcript levels normalized to *serine t-RNA ligase* (*serrs*). **(B)** Similar to panel A but *pfap2-g* is normalized to

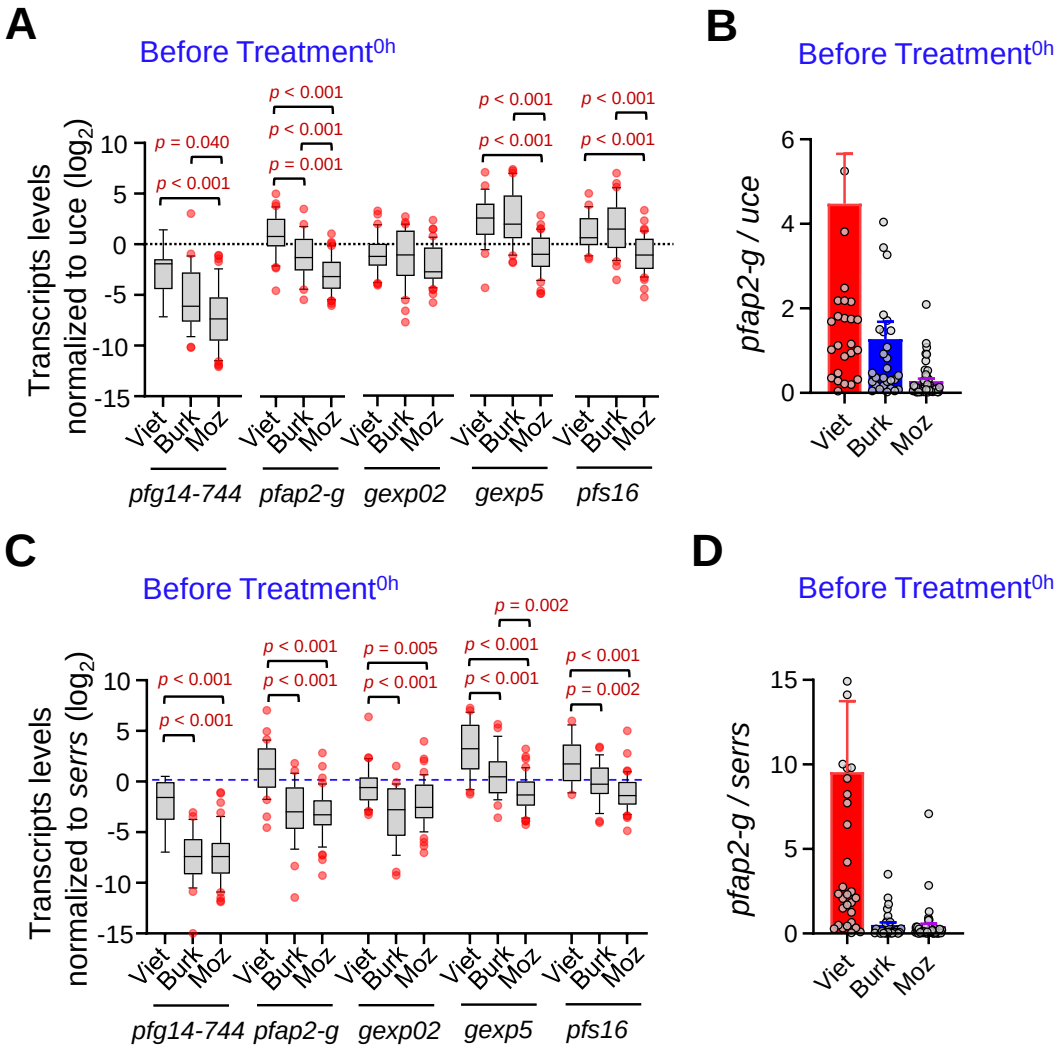
18S rRNA. **(C)** Comparison of *pfap2-g* fold change (FC) relative to 0h between parasite clearance phenotype**.** Fast clearance indicates the absence of ring stages by microscopy on day 3, whereas delayed clearance indicates the presence of ring stages on or beyond day 3. *pfap2-g* normalized to *serrs*. **(D)** Similar to panel C but *pfap2-g* is normalized to *18S rRNA*. In Vietnam cohort, *18s rRNA* was measured by a one-step qPCR using RNA samples while in Burkina Faso *18s rRNA* was measured by a two-step qPCR using cDNA samples.



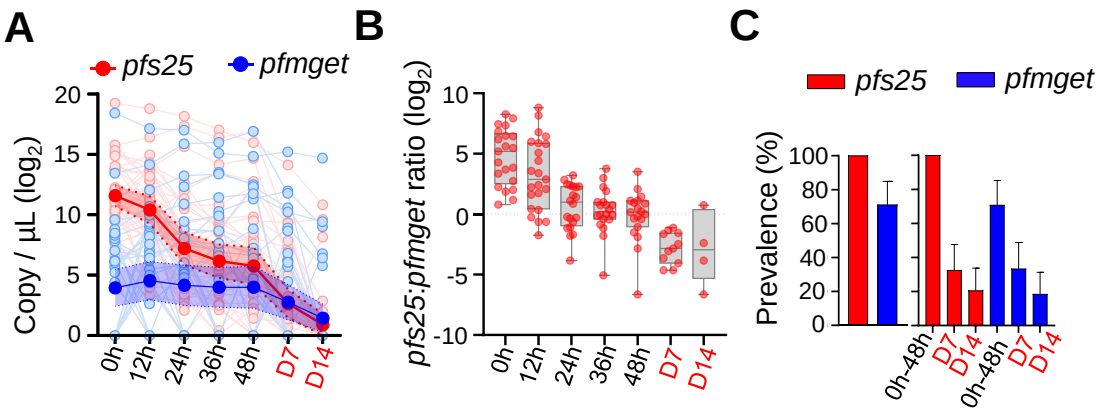
Supplementary Figure 4. Sexual ring biomarkers expression levels after treatment based on fold-change. (A-E) Sexual ring biomarkers fold-change (FC) 12-48h after treatment, all relative to 0h time-point (before treatment). *p* values between study cohorts were calculated using One-way ANOVA with Tukey HSD as post hoc test. *p* values less than 0.05 are shown.



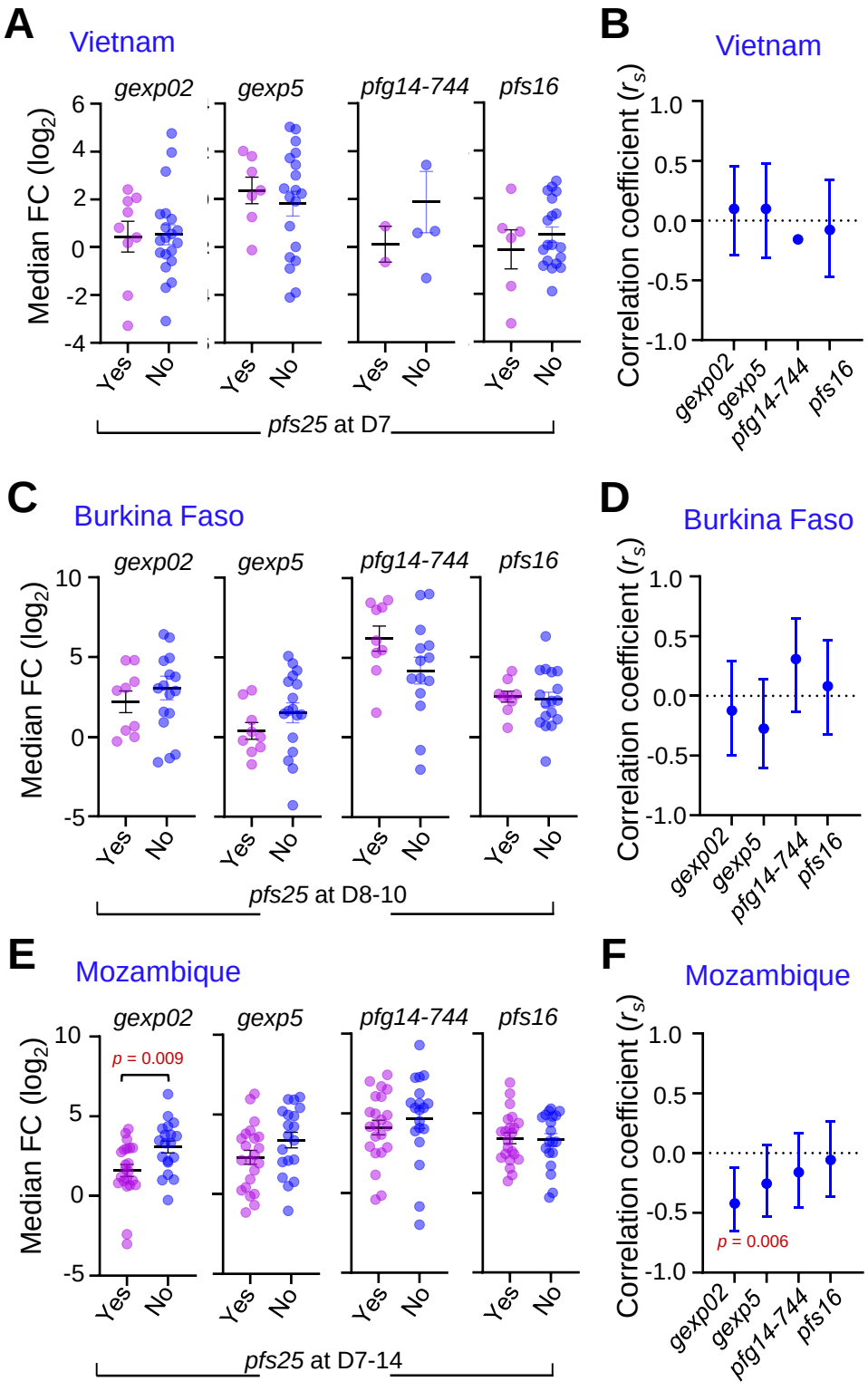
Supplementary Figure 5. Comparison of sexual ring biomarkers expression levels before treatment. Comparison of 5 sexual ring biomarkers transcript levels normalized to *uce*, *serrs,* and *18s rRNA* before treatment. *p* values were calculated using One-way ANOVA with Tukey HSD as post-hoc test, with *p* values less than 0.05 between *pfap2-g* (comparator) and other sexual ring biomarkers are shown.



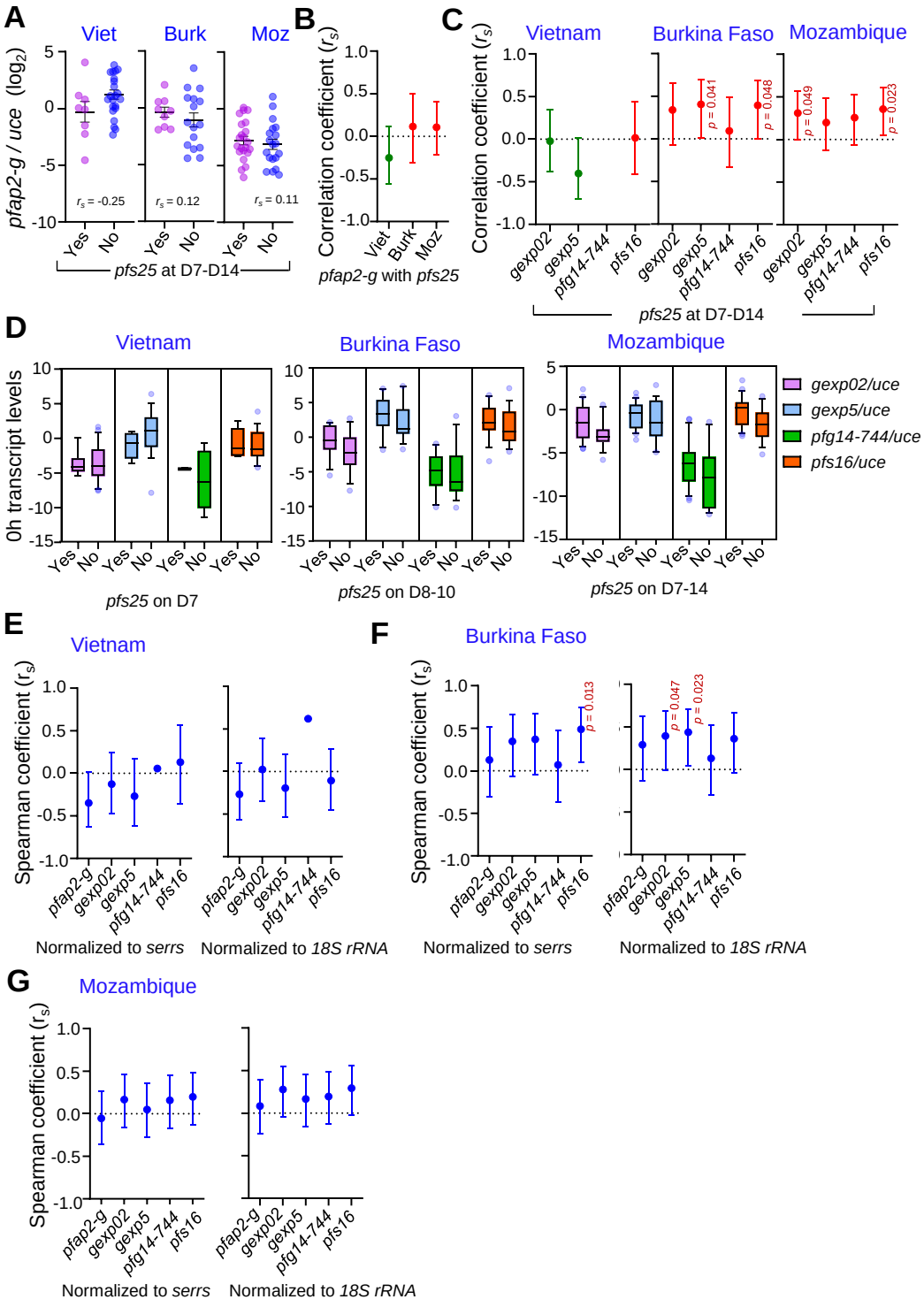
Supplementary Figure 6. Sexual ring biomarkers expression levels before artemisinin treatment between study cohorts. (A) Comparison of 5 sexual ring biomarkers expression levels in three study sites before treatment. *p* values were calculated using One-way ANOVA with Tukey HSD as post-hoc test. *p* values less than 0.05 are shown. **(B)** Average *pfap2-g* relative transcript levels before treatment. Error bar: s.e.m. **(C-D)** Similar to Panel A and B, but normalized to *serrs*.



Supplementary Figure 7. Male and female mature gametocytes information in Mozambique cohort. (A) Average copy numbers with 95% CI of *pfs25* (red dots and lines) and *pfmget* (blue dots and lines) mRNA, with individual data in the background. **(B)** Ratio between *pfs25* and *pfmget* biomarkers. **(C)** Prevalence rate and 95% confidence interval (CI) of gametocyte carriage using *pfs25* and *pfmget* biomarkers.

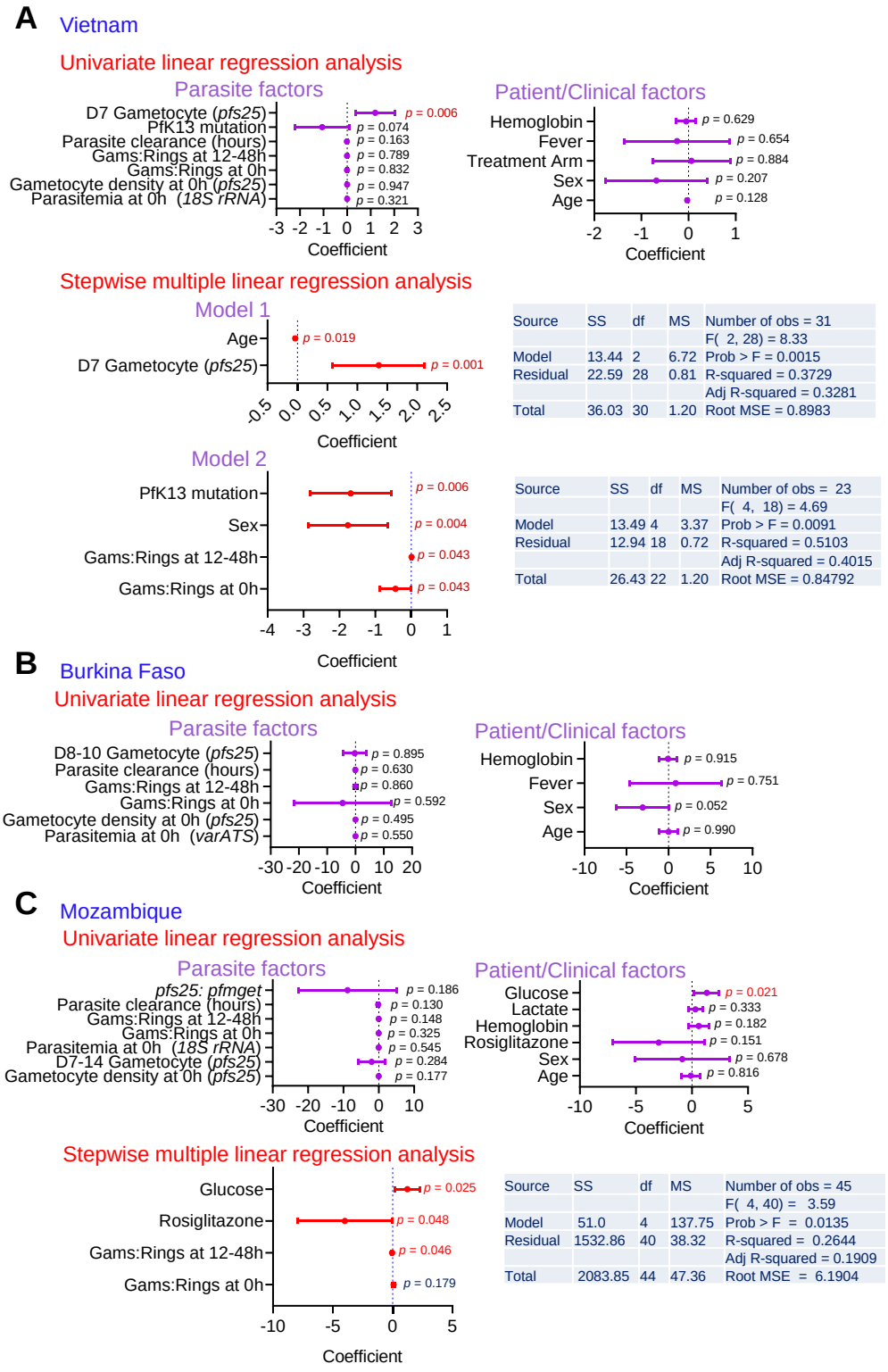


Supplementary Figure 8. Correlation of four sexual ring biomarkers fold-changes after treatment with mature gametocyte circulation 1 to 2 weeks later. (A) Differences on SRBs median fold-change (Median $FC^{12\text{-}48h}$) after treatment in patients with detected (yes) or undetected (no) mature gametocyte biomarker pfs25 on days 7 to 14 in Vietnam cohort. **(B)** Spearman correlation coefficient (r_s) with 95% CI of SRBs FC after treatment and *pfs*25 positivity on day 7 to 14. **(C-D)** Similar to panel A-B, but in Burkina Faso cohort. **(E-F)** Similar to panel A-B, but in Mozambique cohort. Only *p* value < 0.05 is shown.



Supplementary Figure 9. Correlation between sexual ring biomarkers (SRBs) expression levels before treatment and gametocyte circulation 1-2 weeks later. (A) Differences on *pfap2-g* transcript levels before treatment at 0h time-point in patients with detected (yes) or undetected (no) mature gametocyte biomarker *pfs25* on days 7 to 14. **(B)** Spearman correlation coefficient (r_s) with 95% CI of *pfap2-g* transcript levels before treatment and *pfs25* positivity on day 7 to 14. **(C)** Similar to panel B, but with other four SRBs. Only *p* value < 0.05 is shown. **(D)** Differences on *gexp02, gexp5, pfg14-744,* and *pfs16* transcript levels before treatment at 0h time-point in patients with detected (yes) or undetected (no) mature gametocyte biomarker

pfs25 on days 7 to 14. **(E-G)** Spearman correlation coefficient (r_S) with 95% CI of five SRBs transcript levels before treatment and *pfs25* positivity on day 7 to 14 in three study cohorts. SRBs were normalized to either *serrs* or *18s rRNA*. Only *p* value < 0.05 is shown.

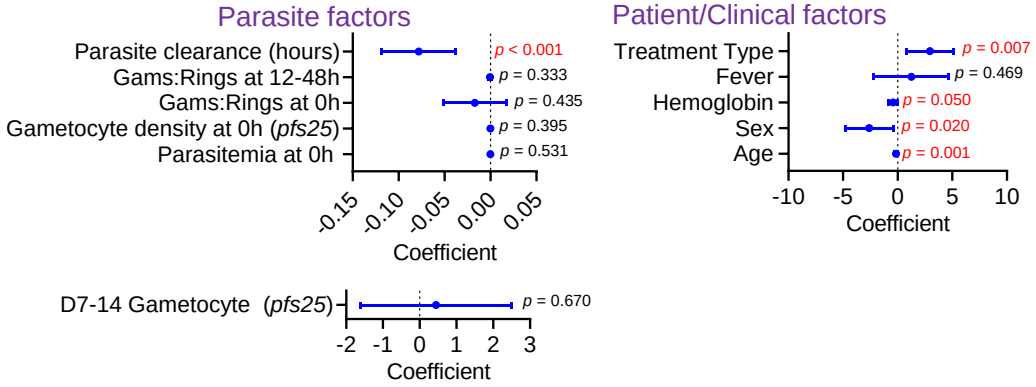


Supplementary Figure 10. Linear regression analysis of *pfap2-g* expression levels (median fold change from 12-48h) with parasite, patient, and clinical factors at individual cohort level. (A) Analysis of factors associated with *pfap2-g* expression levels in Vietnam cohort. Two models are presented after forward and backward stepwise multiple linear

regression analysis. Model 1 included the 'Gametocyte on day 7' variable while Model 2 excluded the 'Gametocyte on day 7' variable. **(B)** Analysis of factors associated with *pfap2-g* expression levels in Burkina Faso cohort. In the multiple linear regression analysis, no variables showed significant association (p >0.05). **(C)** Analysis of factors associated with *pfap2-g* expression levels in Mozambique cohort. Result of stepwise multiple linear regression analysis is shown. Variable descriptions: Gametocyte at D7-14 (0 = absent of *pfs25*, 1 = present of *pfs25*); PfK13 mutation (0 = wild-type; 1 = mutant); parasite clearance (hours where no parasite is observed under the microscope); Gams:Rings at 12-48h (average ratio of *pfs25* to *18S rRNA* or *varATS* from 12-48h after treatment); Gams:Rings at 0h (ratio of *pfs25* to *18S rRNA* or *varATS* at 0h time-point); Gametocyte density at 0h (*pfs25* copies/µL at 0h time-point); parasitemia at 0h (PCR parasitemia based on *18S rRNA* or *varATS* at 0h time-point); Hemoglobin (g/dL) levels at 0h time-point; Fever (0 = 37ºC, 1 = > 37ºC) at 0h time-point; Treatment arm (0 = artemisinin-combination therapy in the first 2-3 days, 1 = artesunate monotherapy on the first 2-3 days); Sex (0 = female, 1 = male); Age (years); *pfs25:pfmget* (ratio of female to male gametocyte transcript levels at days 7-14); Glucose (mmol/L) levels at 0h time-point; Lactate (mmol/L) levels at 0-h time-point; Rosiglitazone (0 = not administered, 1 = administered).

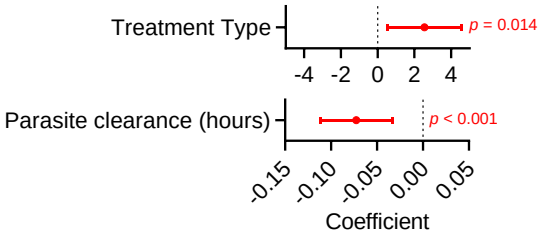
A

Univariate linear regression analysis



B

Stepwise multiple linear regression analysis



Source	SS	df	MS	Number of obs = 105
				F(2, 102) = 10.94
Model	574.81	2	287.41	Prob > F = <0.001
Residual	2678.97	102	26.26	R-squared = 0.1767
				Adj R-squared = 0.1605
Total	3253.78	104	31.29	Root MSE = 5.1249

Supplementary Figure 11. Linear regression analysis of *pfap2-g* expression levels (median fold change from 12-48h) with parasite, patient, and clinical information in three cohorts, a combination of Vietnam, Burkina Faso, and Mozambique datasets. (A) Results of univariate linear regression analysis of factors associated with *pfap2*-g expression levels. **(B)** Result of stepwise multiple linear regression analysis of factors associated with *pfap2-g* expression levels in three cohorts. Variable descriptions can be found in Supplementary Figure 10.

Supplementary Table 1. List of oligonucleotide sequences for qPCR analysis

Gene	Gene ID	Sequence	Conc.	Assay	Reference
pfap2-g	PF3D7_1222600	Fwd+3874:AACAACGTTCATTCAATAAATAAGG Rev+3979: ATGTTAATGTTCCCAAACAACCG	0.5 µM	SYBR Green	Bancells et al., 2019
gexp02	PF3D7_1102500	Fwd+813: GGAAATTAGGAGTAAGAAGAGG Rev+972: TCGCTATTTATTTCACTTGGCT	0.5 µM	SYBR Green	Portugaliza et al., 2019
gexp5	PF3D7_0936600	FW+373: GTCCGAGGTAGTGAGAAATTG Rev+480: TGTGTACATGATTCCATTGGT	0.5 µM	SYBR Green	This paper
pfg14-744	PF3D7_1477300	Fwd+445: GATGTACCGAAGTATGAGAATGATT Rev+547: TGGATAACGGCAAGGATATTTCTT	0.2 µM	SYBR Green	Bancells et al., 2019
pfs16	PF3D7_0406200	Fwd+148:TCAGGTGCCTCTCTTCATGCT Rev+247: GCTGAGTTTCTAAAGGCATTTTGTC	0.2 µM	SYBR Green	Bancells et al., 2019
serrs	PF3D7_0717700	Fwd+590: AAGTAGCAGGTCATCGTGGTT Rev+747: TTCGGCACATTCTTCCATAA	0.2 µM	SYBR Green	Bancells et al., 2019
uce	PF3D7_0812600	Fwd+67: GGTGTTAGTGGCTCACCAATAGGA Rev+155: GTACCACCTTCCCATGGAGTA	0.25 µM	SYBR Green	Bancells et al., 2019
18S rRNA	PF3D7_1148600; PF3D7_0112300; PF3D7_1371000	Fwd: GCTTTTGAGAGGTTTTGTTACTTTG Rev: CTCTGACATCTGAATACGAATGC	0.2 µM	SYBR Green	Portugaliza et al., 2019
pfs25	PF3D7_1031000	Fwd+254: GAAATCCCGTTTCATACGCTT Fwd+390: AGTTTTAACAGGATTGCTTGTATC Probe: HEX-TGT AAG AAT GTA ACT TGT GGT AAC GGT-BHQ1	0.4 µM (primer) 0.2 µM (probe)	SYBR Green; Taq-Man (probe)	This paper (SYBR Green); Rovira-Vallbona et al., 2017 (TaqMan)
pfmget	PF3D7_1469900	Fwd+52: GGTCCAAATATAAAATCCTGTTC Rev+251: TGTGTAACGTATGATTCATTTTC	0.4 µM	SYBR Green	This paper; Gruenberg et al., 2020
varATS domain		Fwd: CCCATACACAACCAAYTGGA Rev: TTCGCACATATCTCTATGTCTATCT Probe: 6-FAM-TRTTCCATAAATGGT-NFQ-MGB	0.8 µM (primer) 0.4 µM (probe)	TaqMan	Hofmann et al., 2015

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Chapter 5

Examining community perceptions of malaria to inform elimination efforts in Southern Mozambique: a qualitative study

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Author Contributions

HPP organized the data, performed data coding/analysis and interpretation, and drafted the manuscript. BG contributed in study design, data analysis, interpretation, and writing of the manuscript. HN, HD, IM, participated in the study design, data collection and transcription. FS, PA participated in the conception of study design, supported in study implementation, and contributed to the writing of the manuscript. CP directly supervised HPP throughout the study and contributed to data analysis, interpretation, and manuscript writing. KM conceptualized the study design, trained field researchers, and supervised data collection and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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RESEARCH

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Examining community perceptions of malaria to inform elimination efforts in Southern Mozambique: a qualitative study

Harvie P. Portugaliza[1,2,3*†], Beatriz Galatas[1,4†], Hoticha Nhantumbo[4], Helder Djive[4], Ilda Murato[4], Francisco Saúte[4], Pedro Aide[4,5], Christopher Pell[2,6,7] and Khátia Munguambe[4,8]

Abstract

Background: In a background of renewed calls for malaria eradication, several endemic countries in sub-Saharan Africa are contemplating malaria elimination nationally or sub-nationally. In Mozambique, a strategy to eliminate malaria in the south is underway in the context of low endemicity levels and cross-border initiatives to eliminate malaria in South Africa and Eswatini. In this context, a demonstration project aiming to interrupt malaria transmission through mass antimalarial drug administrations and intensified vector control programmes accompanied by community engagement and standard case management was implemented in the Magude District. To ensure the necessary uptake of these interventions, formative qualitative research explored the perceptions, beliefs, attitudes, and practices related to malaria, its prevention and control. The current article describes the results of this study.

Methods: Seventeen focus group discussions were conducted between September and October of 2015 with the community leaders (6), adult men (5), women of reproductive age (5), and traditional healers (1) in Magude prior to the implementation of the project interventions. Respondents discussed perceptions around malaria symptoms, causes, preventions, and treatments.

Results: Knowledge of malaria was linked to awareness of its clinical presentation, and on-going vector control programmes. Perceptions of malaria aetiology were fragmented but related mainly to mosquito-mediated transmission. Reported preventive measures mostly involved mosquito control although participants were aware of the protective limitations of vector control tools. Awareness of asymptomatic carriers and the risk of outdoor malaria transmission were varied. Fever and malaria-like symptoms triggered immediate care-seeking community at health facilities. The identified barriers to malaria treatment included fear/mistrust in Western medicine, distance to health facilities, and lack of transportation.

Conclusions: Several constraints and opportunities will potentially influence malaria elimination in Magude. Malaria awareness, trust in health institutions, and the demand for chemoprophylaxis could facilitate new interventions, such as mass drug administration. A lack of awareness of asymptomatic carriers, inadequate understanding of residual transmission, and barriers to care seeking could jeopardize uptake. Hence, elimination campaigns require strong community engagement and grassroots mobilization.

Keywords: Community, Elimination, Magude, Malaria, Perceptions, Qualitative

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Background

In the past decade, the renewed vision of malaria eradication has triggered substantial political attention and financial support in many endemic countries. Malaria eradication is the long-term goal of relieving the world from the burden of malaria through the short-term elimination of disease and transmission in specific regions. To achieve elimination, new malaria intervention packages are needed, as well as technical and economic support to the areas targeting elimination [1, 2]. Although the malaria-related burden of disease remains highest in sub-Saharan Africa (contributing 92% of global cases in 2017) the prospect of elimination has been raised for some previously endemic areas, such as Botswana, South Africa, and Swaziland [3].

According to the 2018 World Malaria Report (WMR) [3], Mozambique ranked 3rd and 8th in terms of number of estimated malaria cases and deaths, respectively. This translates to around 14 thousand deaths and 10 million cases in 2017, with the majority amongst children below the age of five [3]. The high malaria burden in Mozambique results from continuous year-round transmission with a seasonal peak during the rainy period from December to April. Transmission intensity is heterogeneously distributed throughout the country, with higher prevalence estimates in the centre and north (29–68%) compared to the south (2–23%) [4, 5].

Current efforts to prevent and control malaria in Mozambique primarily consist of the nationwide distribution of long-lasting insecticidal bednets (LLINs) through mass campaigns (every 3 years) and routine antenatal care and yearly indoor residual spraying (IRS) in selected districts. Rapid diagnostic tests and artemisinin-based combination therapy (ACT) are implemented as frontline case management measures to avert malaria deaths. Additionally, Intermittent Preventive Treatment of Pregnant Women (IPTp) is offered to address substantial malaria risk in mothers and unborn children [4, 5]. However, concerns have been expressed about the limited coverage and uptake of these programs, i.e., only 16%, 34%, and 88% for bed net, IPTp, and IRS, respectively in 2015 [4, 6, 7]. Meanwhile, malaria cases are increasing whereas funding for national malaria prevention and control has stagnated over the last 5 years [3].

Despite the concerns of increasing malaria incidence, the National Malaria Control Programme (NMCP) has identified southern Mozambique as a potential target area for elimination [4, 5]. To support the design of the NMCP's malaria elimination plan for southern Mozambique, a malaria elimination project was implemented in the district of Magude [8]. This project aimed to interrupt malaria transmission through mass antimalarial drug administration (MDA) and vector control (IRS and LLINs) combined with a campaign of strong community engagement and standard case management.

The effectiveness of these interventions depends significantly on achieving high coverage levels (at least 80% for MDA, 85% for IRS, and 100% for LLINs [9–11]) in target communities. This can be a challenge, particularly in the case of MDA, which requires high uptake among all members of a community regardless of infections status [12]. Achieving high MDA coverage requires the design of tailored interventions combined with intense community engagement activities according to local contexts and understandings of malaria [13, 14].

To inform the design of the elimination strategy, and the accompanying community engagement in Magude, formative qualitative research was conducted to examine local understandings of malaria and its prevention and control prior to the deployment of interventions. This article describes community members' reported malaria-related perceptions, beliefs, attitudes, and practices, and examines their relevance for the design of malaria elimination strategies.

Methods

Study area

Magude District, in northwestern Maputo Province, southern Mozambique, borders South Africa's Kruger National Park. Magude covers 6961 km^2 with five administrative posts, and is home to approximately 50,000 residents spread across 11,000 family compounds. Agriculture and fishing are the main livelihood activities. Magude has open forests, savannahs, and one permanent river (*Incomati*). The majority of houses are traditional round or rectangular huts with walls constructed mainly using cane, cement, and mud bricks. More than 80% of the households count on either a traditional latrine or no form of sanitation facility. In 2015, Magude counted with seven rural health centres (but no district hospital), which are all located near main roads in its respective administrative posts. The median distance from households to the nearest health facility is 2.7 km (interquartile range [IQR] 1.4–7.9 km), although households can be as close as 15 m or as far as 38.8 km from the health facility. The far-flung areas within this district are covered by 27 community health workers or *Agentes Polivalentes Elementares* (APEs) who provide basic healthcare services and referrals to the closest health centre. The average distance between households and the assigned APE is around 6.3 km (median of 4.4 km, IQR 2.8–5.4 km). According to the guidelines of Mozambique's Community Health Programme, one APE is expected to cover 500 to 1200 inhabitants [4, 5]. However, to our knowledge, there has been no assessment on the actual APE

coverage in Magude district. Malaria has historically been the main cause of morbidity in the district, with a yearly incidence higher than 200 cases per 1000 (Galatas et al. pers. commun.).

Study design

A qualitative study examined community perceptions of malaria in the district of Magude prior to the implementation of an intervention package aiming to interrupt malaria transmission in the area. This study used a grounded theory approach to generate and understand emergent theories and patterns about the local perceptions of malaria and leaned towards post-positivism by considering participants' perspectives around malaria-related concepts while maintaining objectivity (guided by the biomedical explanatory models) and awareness of potential biases [15].

Sampling strategy

Participants were selected to elicit the opinions and capture the lay perspectives of MDA recipients as well as household and community-level decision-makers. Purposive sampling was performed to achieve uniform representation of community groups and administrative locations through consultations with community key informants. Key informants acted as an intermediary between the research team and their communities, and supported the identification and mobilization of participants.

Data collection

A semi-structured focus group discussion (FGD) guide was designed to capture perceptions of malaria and its related concepts (i.e., symptoms, causes, controls, and treatments). The guide questions were prepared in Portuguese, and pilot tested in the local language Changana. After subsequent refinements, all FGDs were conducted in Changana. The interviewers, who are all fluent in Portuguese and Changana, were trained to facilitate the FGDs. FGDs were carried out between September and October 2015, with community leaders, traditional healers, adult men ($\geq$ 18 years old), and women of reproductive age (15–49 years old) in all administrative posts until saturation was reached, i.e., no new additional information emerged.

Data analysis

FGDs were audio-recorded, transcribed, and translated from Changana into Portuguese and then into English by certified local translators. Transcripts were initially read and examined for themes relevant to the research question. Transcripts were then imported into NVivo 12 software (QSR International) for in-depth content analysis. The analysis approach was mainly inductive, with flexibility in identifying relevant themes. Topics that showed within- and between-group consensus, divergence, and uniqueness were further examined. Finally, the themes were critically discussed until researchers reached agreement on their relevance and the patterns observed across the different respondent groups.

Results

In total, 17 FGDs were conducted with community leaders comprising village chiefs, traditional leaders, and heads of large families (6); men older than 18 years-old (5); women of reproductive age (15–49 years-old) (5); and, traditional healers from the Association of Traditional Healers of Mozambique (AMETRAMO) (1). The number of participants per FGD ranged from three to 12 (Additional file 1).

Demographic characteristics

Table 1 presents the demographics of participants of each community group represented in the FGDs. Community leaders comprised mainly of male participants aged 30–83 years (median 63). Traditional healers were mainly female aged 21–51 years (median 30). The age of men and women groups ranged between 18 and 76 years (median 26) and 15–60 years (median 30), respectively. Most participants were married or living in marital union, had a low level of education (mostly primary level), and worked as farmer or charcoal producers. Participants were largely Christian, but a few identified themselves as Atheists, Muslims, Animists, and Zionists.

Perceptions of malaria and its symptoms in Magude

In the local dialect, Changana, the collective symptoms of malaria were referred to as the traditional illness *Mututumelo* (syn. *Muzototo, Mututumela*) or *Dzedzedze* (syn. *Madzedzedze*). 'Malaria' connoted a diagnosis made by the healthcare provider after presenting with signs traditionally linked to *Mututumelo* or *Dzedzedze*. Participants believed that these terms represented a single disease on the basis of symptoms or after a diagnosis. Hence, in the following quotation (and all others), 'malaria' is used to describe syndromes that may or may not overlap with the biomedical definition.

> *It's the same thing… when you go to the hospital they say you have malaria. Traditionally they say that you have Muzototo.*
> FGD4_HA10 adult men

> *Dzedzede is the same as malaria…because Dzedzedze is when you do not feel the body being good and feeling cold.*
> FGD5_MIR13 women of reproductive age

Table 1 Demographics of participants per community group

Variables	Community leaders	Men	Women	Healer
Gender				
Male	94.2% (49/52)	100% (39/39)	0 (0/53)	10% (1/10)
Female	5.7% (3/52)	0 (0/39)	100% (53/53)	90% (9/10)
Age (years)				
Range	30–83	18–76	15–60	21–51
Median	63	28	30	30
Mean	59	31	31	32
Marital status				
Single	1.9% (1/52)	30.8% (12/39)	35.8% (19/53)	40% (4/10)
Married	11.5% (6/52)	2.6% (1/39)	0 (0/53)	10% (1/10)
União (Marital Union)[b]	81% (42/52)	66.7% (26/39)	51.8% (28/53)	50% (5/10)
Widowhood	5.8% (3/52)	0 (0/39)	11.3% (6/53)	0 (0/10)
Education				
None	28.9% (13/45)	5.1% (2/39)	20.8% (11/53)	10% (1/10)
Primary	57.8% (26/45)	46.2% (18/39)	37.7% (20/53)	80% (8/10)
Secondary	11.1% (5/45)	43.6% (17/39)	41.5% (22/53)	10% (1/10)
Tertiary	2.2% (1/45)	5.1% (2/39)	0 (0/53)	0 (0/10)
Religion				
Christianity	59.6% (31/52)	74.4% (29/39)	71.2% (38/53)	0 (0/10)
Atheism	17.3% (9/52)	23.1 (9/39)	15.1% (8/53)	100% (10/10)
Animism	19.2 (10/52)	2.6 (1/39)	5.7% (3/53)	0 (0/10)
Islam	3.8% (2/52)	0 (0/39)	0 (0/53)	0 (0/10)
Zionism	0 (0/52)	0 (0/39)	7.5% (4/53)	0 (0/10)
Occupation				
Farmer	63.5% (33/52)	20.5.7% (8/39)	54.7% (29/53)	0 (0/10)
Coal producer	0 (0/52)	23.1% (9/39)	0 (0/53)	0 (0/10)
Trad. Healer	0 (0/52)	0 (0/39)	0 (0/53)	100% (10/10)
Home-based[a]	1.9% (1/52)	15.4% (6/39)	32.1% (17/53)	0 (0/10)
Salesperson	7.7% (4/52)	7.7% (3/39)	1.9% (1/53)	0 (0/10)
Service/laborer	3.8% (2/52)	7.7% (3/39)	5.6% (3/53)	0 (0/10)
Driver	1.9% (1/52)	5.1% (2/39)	0 (0/53)	0 (0/10)
Teacher	1.9% (1/52)	2.6% (1/39)	0 (0/53)	0 (0/10)
Student	0 (0/52)	2.6% (1/39)	3.8% (2/53)	0 (0/10)
Others	19.2 (10/52)	15.4% (6/39)	1.9% (1/53)	0 (0/10)
Total	52	39	53	10

[a] *Housewives/househusbands*

[b] União: Marital union without marriage certificate

For respondents, 'malaria' was a disease that causes suffering among children and adults. Awareness of malaria and the perceived symptoms was noticeably high in all groups. Participants identified fever, cold, joint pain, headache, and vomiting (*caguba*) as typical symptoms. Weakness, body pain, back pain, stomachache, lack of appetite, shivering and trembling were also mentioned.

> *What happens now is that you just feel headaches, feel body aches and cold, it´s malaria, caused by mosquito.*
> FGD1_MT17 Traditional healers

Perceived symptoms of childhood malaria were convulsions, abdominal swelling, stomachache, fever, and weakness.

> *I have been teaching the population about malaria… I met along the way two people going to the healers with a child who was convulsed with a swollen belly. I told them to go to the hospital [for malaria treatment] before going to the healers.* FGD2_LC3 community leaders

Perceived forms of malaria

Two forms of malaria illness emerged from the FGDs. One involved signs of unusual behaviour or confused thinking. Another was termed "bile" and, especially female respondents, described as vomiting of greenish fluid accompanied by other malaria symptoms, such as weakness and shivering.

> *Malaria goes up to the head [it gets worse] and you start talking nonsense… you start having problems with the neighbors while you're not doing it on purpose… it's malaria that went up, so this is not a good [form] of malaria… the only way is to go to the hospital.*
> FGD1_LC1 community leaders

> *I was saying it was Mututumelo (malaria) and if we vomited they said it was bile.*
> FGD4_MIR12 women of reproductive age

Fever or "heating up of body" was described consistently as a symptom that accompanied malaria illness and characterized by an increase in body temperature. It was interpreted as indication that an immediate 'hospital' visit was needed. 'Fever' sometimes was used as a generic term for a group of symptoms that resembled malaria. 'Malaria' and 'fever' were not however viewed as being the same, but rather distinguished through a malaria test and medication, such as 'paracetamol'.

> *When the person is in this [malaria] situation, the body heats up a lot with fevers…we first wet a cloth, put it in the armpits, on the neck and the feet to be able to take him or her quickly to the hospital.*
> FGD2_HA4 adult men

> *[Fever and Malaria] is not the same thing because I can tremble, have headaches and vomiting but when I take paracetamol it stops. While in malaria, I can take paracetamol from 1 to 30 [tablets] and I still feel hot …it will not pass without first taking malaria pills.*
> FGD1_MIR6 women of reproductive age

Few participants attributed some of the malaria symptoms to cholera, tuberculosis, and HIV/AIDS. They emphasized that test confirmation from the healthcare provider is needed to diagnose malaria.

Perceived causes of malaria

Participants identified mosquitoes as the main cause of malaria and perceived transmission as occurring through direct and indirect pathways. Malaria-causing mosquitoes were reportedly female with habitats that included water, animals, and plants. Women reported poor hygiene and sanitation as the reason for the presence of mosquitoes. And respondents viewed mosquitoes and water as vital factors for the occurrence of malaria illness in the community. Mosquitoes were viewed as abundant and likely to bite during the rainy season, close to ditches, streams, and boreholes; or generally in the presence of dirty and stagnant waters.

> *[Bouts of malaria] are caused by mosquitoes that stay in the water where they [live] and reproduce. They come out and bite people inside the houses… if [people] do not close [the windows] the mosquitoes appear inside the houses and bite people.*
> FGD5_LC14 community leader

Respondents mentioned that animals played a role in malaria transmission, either as carriers of the disease or as enhancers of mosquito numbers. 'Malaria' was viewed as caused by an agent that came from animals and transferred to human through mosquito bites and animal waste (e.g., urine). In other scenarios, the presence of livestock near houses attracted malaria-causing mosquitoes to enter nearby households.

> *If someone starts getting sick near the barn, a mosquito bites an animal and then it bites me. I end up getting malaria.*
> FGD3_HA7 adult men

Malaria illness was also linked to the consumption of fruit and vegetables. Also, participants identified unpruned trees, grasses, and areas near plantations as the lairs of malaria-causing mosquitoes.

Malaria parasites (e.g., *Plasmodium falciparum*) were not mentioned explicitly as the causative agent of the disease. However, participants hinted at an additional component necessary for malaria illness to occur that involved transfer of biological materials carried by mosquitoes. These materials were mainly infected blood, fecal matter, urine, and dirt. Less frequently, they mentioned 'virus', 'poison', and 'venom'.

> *In another case, when [a mosquito] bites someone with malaria and as soon as it bites me, I will get the disease. The blood of that person comes to infect mine.*
> FGD3_HA7 adult men

Flies and various insects were also identified as carriers of dirt that causes malaria, especially among children. In addition, bad-smelling, witchcraft, and sorcery were mentioned as perceived causes of malaria.

Those big [flies] that go into those latrines because we don't have improved latrines there… the person digs a pit and [defecates]. Those flies can get out of there and they will land on the food and the children eat that food and contract malaria.
FGD1_LC1 community leaders

Prevention of malaria transmission

Preventive measures reported by the community against malaria transmission could be systematically divided into three main groups: (1) mosquito avoidance, (2) chemoprophylaxis, and (3) other measures of 'malaria' prevention.

Mosquito avoidance

Mosquito avoidance encompassed practices linked to mosquito-human contact evasion and actions that target places or objects associated with mosquito breeding and resting sites (although these two concepts were often intertwined). Using a bed net while sleeping emerged as the most common practice to avoid mosquito bites. Bed net ownership indicated an assurance to reduce chances of getting malaria. In fact, women underscored bed net as the only option to avoid malaria.

[To avoid malaria] let's sleep under 'Mutchiquitelo' [mosquito net]. There is nothing else, it's just 'mutchiquitelo'… you do nothing else, just stretch [the mosquito net].
FGD2_MIR2 women of reproductive age

Keep the spaces clean and use mosquito nets. When you go to sleep, stretch the net and sleep inside it so that the mosquito does not bite you.
FGD3_HA7 adult men

It's the mosquito net, it's necessary in the afternoon when it's already dark, to stretch the mosquito net, to close it here in bed, so that the mosquito does not enter, or if there is a small child, the child must be there before we sleep while talking. So when we get back, we, husband and wife, are going to get in there and close it.
FGD3_LC8 community leaders

On the other hand, several respondents described bed net non-use and misuse. Community leaders reported that many of the bed nets distributed in the past were still 'in the package' and unused by the community. The non-use of bed nets was explained in terms of discomfort while sleeping. Participants also reported that bed nets were used as fishing nets and/or to protect crops.

The mosquito nets that we talked about, so far most of it are still in the plastic and they have not been used yet.
FGD2_LC3 community leaders

We use a mosquito net but you find people in those places where they were told to use a mosquito net… [They] received a mosquito net and heard that they have to sleep inside it, and reply yes. When you leave and they take it (bed net) home and say that they cannot sleep in a mosquito net, some use it for fishing.
FGD4_HA10 adult men

Other methods of mosquito avoidance included closing windows and using insect repellants or coils. Also, participants perceived that cleanliness or improving hygiene and sanitation conditions will repel malaria-causing mosquitoes.

In our houses, when the night falls we need to close the windows to avoid mosquitoes from entering. We need to clean and cover the pits with water, so you can prevent yourself from having malaria by avoiding mosquitoes from landing.
FGD5_LC14 community leaders

When I am seated if it's not sprayed, I stay in the room and I buy that coil that is burned, how is it called? It's called dragon. I burn it because it kills the mosquito for sure… you won't get bitten by mosquito when you're chatting. You cannot chat within a mosquito net. You can just chat in the seating room.
FGD1_LC1 community leaders

In contrast to the revealed knowledge about mosquito avoidance, their discourse on actual practices suggests that people open the windows at night, sit in communal areas during biting hours, sleep late, and do not use insect repellants or insecticidal coils. Women participants acknowledged the importance of mosquito repellants to avoid mosquito bites outside bed net protection zones. However, they reported to never or rarely use protective methods aside from bed net due to lack of purchasing capacity.

We need to use a mosquito net… but even in the day the mosquito bites you. Even when you are seated they can come and bite you, they can bite you before sleeping in the net.
FGD5_MIR13 women of reproductive age

Actions that target places or objects associated with mosquito breeding and resting sites were described as a fundamental control measure to reduce malaria-causing mosquitoes. Most practices described for breeding

site elimination included disposal of stagnant water or dirty water and containers that capture rainwater. Adult men also mentioned the treatment of water bodies near houses to eliminate mosquitoes.

We need to clean our homes because there are places where we store water, the bowls keep rainwater... It happens that mosquitoes live in those places.
FGD1_MT17 traditional healers

Get rid of bottles that usually retain water because the mosquitoes usually live in waters inside the cafulo (coconut shell).
FGD1_LC1 community leaders

The water has to be far from houses. Inside houses there can be holes with water where mosquitoes lay eggs and reproduce.
FGD5_MIR13 women of reproductive age

There is tlhive, what we call a pond, I do not know how to say but, the great help for those tlhives that we call lagoons, if we have medicine to put there, maybe the mosquito can disappear because that water is too much and you cannot cover the tlhive. We are close to it, we build close to it, we will not be able to cover it.
FGD3_HA7 adult men

Additional practices targeting mosquitoes comprised the acceptance of IRS, removal of garbage or clutter, pruning plants, and avoidance of animal pens near houses.

We have to accept [IRS] at home. Do not refuse so that we can scare away that existing mosquitoes.
FGD1_MT17 Traditional healers

We have to avoid hoarding garbage in our homes. We take care of the trash because it will cause us mosquitoes... malaria. Malaria comes from mosquitoes.
FGD2_HA4 adult men

At home, we need to take care of ourselves, clean the house and clear the grass near the house, leave the space clean so that the mosquito cannot find a place to stay.
FGD3_HA7 community leaders

[To prevent malaria], lessen the nearby shrubs and prune the trees.
FGD1_MIR6 women of reproductive age

However, participants also reported refusals to IRS. Community leaders described IRS as triggering cough, promoting cockroach infestation, and making houses dirty. Aside from the perceived side effects, those with concrete houses and air-conditioned rooms refused IRS because of the apparent absence of mosquitoes.

They will accept mosquito nets but not spraying because it causes coughing.
FGD2_LC3 community leaders

It's the younger ones who deny [IRS] and will say this brings cockroaches and makes houses dirty.
FGD3_LC8 community leaders

Chemoprophylaxis

Chemoprophylaxis emerged as a hypothetical solution in the form of protective pills that participants reported would avoid mosquito bites and initiation of malaria symptoms. Respondents were aware of the concept of malaria chemoprophylaxis as a result of the community engagement campaigns that were already underway during the study period, as well as through their experience in South Africa where malaria prophylaxis is offered to travelers going to or coming from endemic areas.

I can spray the house, sleep under the mosquito net, but many of us study at night class. At school there are no mosquito nets and it has not been sprayed, there the person is bitten and can get malaria, whereas if there is a pill that the hospital has created that protects us, the mosquito can bite but it will not do anything.
FGD1_MIR6 women of reproductive age

For the prevention of this disease, malaria, it is necessary that the government brings us...tablets to take. Similar to South Africa...there are tablets that have been distributed and people have taken and got the body drugged so that when the mosquito bites, they do not get sick.
FGD3_HA7 adult men

During the FGDs, women did not mention IPTp as a form of malaria prevention. However, a few adult men hinted that the hospital offered IPTp and a community leader identified *Fansidar* (Sulfadoxine–Pyrimethamine) as an antimalarial drug, although he did not associate this drug to IPTp.

[Pregnant women] must go to the hospital. There are what they give [pregnant women] to prevent this evil that comes from the mosquito.
FGD2_HA4 adult men

In the hospital when they test you and confirm Malaria, they give you a number of pills to be taken… of which I believe I'm right in recalling right now, that are called Fansidar.
FGD1_LC1 community leaders

Other measures of 'malaria' prevention

Few participants mentioned practices that they believed would prevent malaria illness. These practices were handwashing, drinking clean water, cleaning the air, and practices related to avoidance of fecal contamination.

To prevent malaria, it's necessary to have the residences cleaned up. When the children go to defecate… quickly go clean the feces… it reduces [malaria]. That's what makes …no illnesses in that household.
FGD1_LC5 community leaders

Clean up the house and keep it always neat, because the air we breathe can also create diseases here inside the house.
FGD1_LC5 community leaders

Perceived risk of residual malaria transmission

Participants acknowledged that the existing malaria control program targeting mosquitoes offers limited protection against residual transmission. For example, free bed net distribution and yearly IRS were perceived as not protecting them all the time, hence the demand for a new intervention that they called "the pills that protect".

How did I get sick while the house was sprayed? I sleep inside the mosquito net… this remedy doesn't work then. While [IRS and bed net] work… but the mosquito has bitten you when you were outside. So when I hear this opinion of the pills, I'm happy because I'm already protected, so that's what I say, the main cause is the mosquito.
FGD1_LC1 community leaders

In addition, contradictory notions emerged among participants when expressing concepts of residual malaria transmission or outdoor mosquito biting. Respondents described the indoor home environment as mosquito feeding ground where biting occurs and the outdoor environment as their lair and site of reproduction. While this general concept may indicate low awareness of outdoor transmission, other groups, especially women, showed high awareness on the risk of getting malaria outdoor.

Many of us study at night class. At school there are no mosquito nets and it has not been sprayed, there the person is bitten and can get malaria.
FGD1_MIR6 women of reproductive age

Overall, participants identified several activities that they believed could put people at risk of malaria infection outdoors but not necessarily facilitated by mosquito biting. These included harvesting vegetables or fruits, working in cane plantation, working with livestock, going to the forest, defecating in the bush, fetching water in the river, washing clothes in the river, walking near dumpsites, and chatting in the backyard.

Perception of malaria case management

Respondents described how suspected patients who accessed the health facility normally received a rapid diagnostic test (RDT) to be appropriately treated against malaria, which was generally well accepted. Malaria treatments were viewed as measures to prevent the progression of malaria symptoms and averting death. The perceived treatment options could be divided into home remedy, western medicine, and traditional medicine. Home remedy implied self-treatment or practices to abate fever. Participants reported cooling the body with wet clothes and taking 'paracetamol' to control fever.

The body heats up a lot, with fevers, we first wet a cloth, put it in the armpits, put it on the neck and the feet to be able to take him or her quickly to the hospital, we have no other way of prevention.
FGD2_HA4 adult men

Participants argued that malaria can only be cured by medicines prescribed by healthcare providers wherein malaria case management included diagnosis through RDT and artemisinin-based combination therapy (ACT). A few participants were aware of the anti-malarial drugs used in the health facility by mentioning Coartem (Artemether–Lumefantrine).

While in the hospital they make analysis and discover the number of crosses (parasite density) that indicates that you have malaria. Then they give the medicine to take the way they tell you.
FGD4_LC11 community leaders

When a child or an adult has fever…take the person to the hospital to be tested, what they call lab tests, in which they make a small cut in the finger to test, to check out if he has the Malaria virus, there is no other thing, in my opinion.
FDG1_LC1 community leaders

For the treatment of malaria it is necessary that when the person feels any of these symptoms, such as pains in the joints, lack of appetite and [fever], you have to go to the hospital to do malaria test. If the disease is found they give you Coartem to take.
FGD4_HA10 adult men

In contrast, several community members, especially older people, feared and doubted western medicines. This arose from perceived drug-related side effects. They particularly recalled drug side effects after taking tablets from a population-wide deworming campaign.

> *There were tablets that we have taken for some time and after consuming the tablets some people got dizzy, and some people did not clarify anything about the side effects.*
> FGD4_HA10 adult men

> *Another one when taking tablet, says that it does not pass here (indicating the throat).*
> —FGD4_LC11 community leaders

Participants from Mahele (a remote administrative post/locality on the northwestern part of Magude) described that locals who do not visit the hospital tend to self-medicate using traditional (non-biomedical) medicine. Such medicines, in the form of plant mixture prepared in a clay pot (*ximbitane*), were used to prevent and treat diseases such as malaria and measles. However, respondents reported that traditional medication was ineffective against malaria. Participants, even traditional healers, reported that malaria has never been and can never be cured by traditional medicines since it's a "non-traditional disease".

> *In the old days, every year we take a mixture of plants called Mpondzo to avoid diseases that are still to come. Also, if you have the habit of eating kakana (Momordica balsamina) without putting peanuts, it is because you have something that you want to avoid. There's a disease we call Xitsinana (measles)… What's the name? Sarampo (measles)!*
> FGD4_HA10 adult men

> *There is a tree that most people said had a positive effect on the cure of malaria. I saw several times in Namaacha when someone has malaria, boils it and drinks. It was healing [them], but lately it was no longer used. We now use CoArtem… In Namaacha, when people wander in the hills, there were many of these trees. Many people from the hidden areas never had the habit of going to the hospital, people self-medicated via traditional medicine.*
> FGD4_HA10 adult men

> *Traditional remedies do not cure, because this is not a traditional disease, it doesn't combine with roots, it needs pills.*
> FGD1_LC5 community leaders

Several barriers were reported to accessing health facilities for malaria treatment in Magude District (Fig. 1). Participants described that the long distance from their houses to health facilities and the inaccessible roads delayed—or completely prevented—them from seeking malaria treatment. The figure of the Community Health Workers or *Agentes Polivalentes Elementares* (APE) who act as a bridge between the community and the health facility appeared to provide a temporary solution.

> *For me who lives in Makunanine [Magude Sede administrative post], the hospital is far away, but there are [health] post agents. I will go to one of them, he is always available because it can be at night, and the disease won't wait for the morning. You go and get there before taking pills, because once you take the pills, the agent won't diagnose you, even if he tests you nothing will be detected. They do the tests and if you are malaria infected, he starts giving you pills, if you see that you won't make it, he will give me a document that says I went to see him and then I came to the hospital.*
> FGD1_LC1 community leaders

> *The government has trained people who have the responsibility of helping the population in different places. If the leader has the phone number of the APE, if someone does not feel well, he or she can call the APE for help. The APE must monitor and count with enough tablets to help. If he cannot, the APE can write a letter and send the person to the hospital (referral health centre).*
> FGD3_HA7 adult men

Community members from remote areas only reported seeking medical help when disease progressed to severe condition. This delayed health-seeking behaviour appeared to be associated with distance to health facility and a lack of information.

> *People here when they do not feel well, are in the habit of going to the forest to look for roots to boil, to take and to feel better. When you talk about taking pills it's difficult. He needs to be serious so he can go to the hospital if he feels that he cannot do anything even open a pit to put their rubbish or do anything else. It's only in this state that he agrees to go to the hospital.*
> FGD2_LC3 community leaders

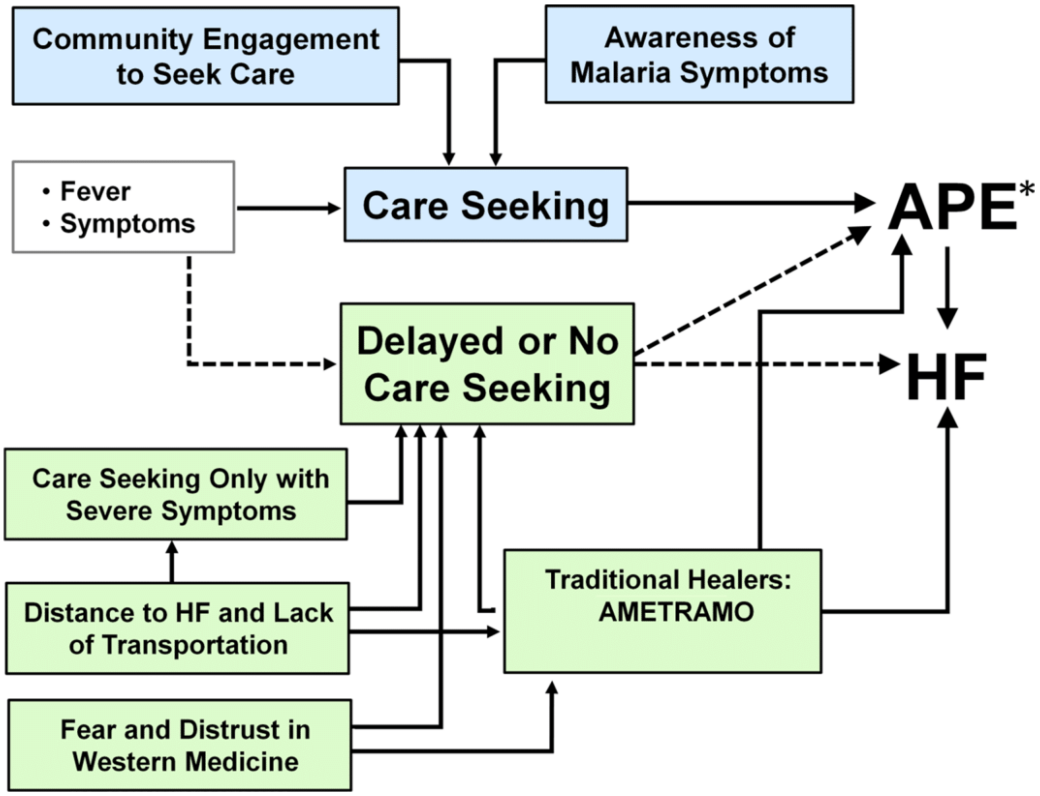

Fig. 1 Facilitators and barriers to the early phase of malaria treatment. *APE* Agentes Polivalentes Elementares (Community Health Workers), *HF* Health Facility. *APEs can also be the end of the care seeking chain if symptoms are not severe

Discussion

FGDs prior to the initiation of the malaria elimination project in Magude, indicate that the community was aware of malaria, trusted formal healthcare institutions, and identified gaps in prevention provided by the available vector control tools. However, it also reveals limited knowledge and misunderstandings about the concepts of malaria transmission and preventive measures. Additional significant findings include the varied awareness about asymptomatic carriers, the risk of outdoor malaria transmission, and the openness of the participants to new interventions. The possible basis to these notions and their implication to the on-going malaria elimination campaign are discussed below.

In Magude, malaria remains a prevalent disease with around 195 cases per 1000 inhabitants in 2014 [16]. The area has seen malaria prevention and control initiatives combined with Information, Education, and Communication (IEC) campaigns [4, 8]. These efforts are reflected in the respondents' awareness of malaria and its prevention and offer promise for planned elimination campaigns because, as in other endemic settings, malaria knowledge and awareness promotes case reporting and care seeking when a malaria-like symptom arises [17, 18]. For community members, the ambiguousness of malaria symptoms (e.g., fever vs malaria) can pose serious challenges to prevention and control [19, 20]. In contrast, respondents from Magude viewed 'fever' and 'malaria' as separate phenomena, with the latter encompassing the former. Fever alone could trigger care-seeking at health facility or to an APE. In addition, two distinct symptoms were associated with malaria and bouts of severe disease that also led to care-seeking: 'confused thinking' is likely to describe delirium; whereas "bile" may imply persistent vomiting, which is particularly common in children with severe malaria [21, 22].

The high awareness of malaria symptomatology and care-seeking could have the following implications for the transition to elimination. First, the implementation of MDAs and vector control in Magude is expected to drastically reduce transmission in a short period of time [23], after which reactive interventions will be implemented to sustain the gains. These reactions will likely be triggered by malaria infections that are passively detected at health facilities or by the APE, and its success is highly dependent on the level of awareness of clinical malaria presentation and will to seek care at community level. Second, as transmission decreases, communities should

be informed of the existence of other causes of fever; and febrile patients should be screened for alternative sources of fever such as viral infections [24] and pneumonia [25].

Few participants alluded to the concept of sub-clinical infections that can still act as sources of transmission. This has been previously reported in other areas where villagers seemed unaware and having difficulties understanding the concept of asymptomatic carriers [26]. This lack of awareness of the existence of asymptomatic infection reinforces the need to deliver specific messages about the concept and importance of symptomless infections prior to the implementation of interventions, such as MDA, which target this parasite reservoir at a population level [27]. However, this study also revealed that the community is aware of the protection gaps around vector control tools, and seemed to welcome chemoprophylaxis as an additional prevention measure. A thorough review of the acceptability to MDAs and its potential barriers in Magude will be presented in a separate publication.

In contrast to their familiarity with the symptoms of malaria, respondents' awareness of malaria aetiology is not comprehensive and fragmented with concepts extrapolated from vector control programmes. Perceived causes of malaria infection were diverse with many underlying agents, including animals and plants, but generally mosquito habitats and behaviour. In Mozambique, *Anopheles arabiensis* and *Anopheles funestus* are the primary vectors of malaria with tendencies to feed on animals and humans indoors and outdoors [28, 29]. Of all the perceived causes, mosquitoes and water emerged as vital causal elements for malaria infection. These responses reflect the extensive efforts at malaria prevention and control through vector control programmes (i.e., IRS and bed net distribution). However, this also suggests that the IEC that accompanied these (and other) programmes did not effectively communicate the role of anopheline mosquito in transmitting the parasite. For instance, several reported measures are not technically linked to malaria prevention, and vector-directed practices may not be specific to anopheline mosquito behaviour. Identifying the mosquito as the direct causative agent of malaria could represent a challenge when promoting preventive interventions that do not directly target mosquitoes.

In Magude, the existing malaria-risk avoiding practices focused mainly on mosquito avoidance, which includes bed net use, IRS, and the elimination of mosquito breeding and resting sites. Nationwide, from 2007 to 2015, household bed net ownership ranged from 16 to 66%, with reported usage between 7 and 52% among pregnant women and children under 5 years of age [4, 5]. Respondents described their reasons for using bed nets as being based on a desire to protect themselves from malaria rather than avoiding nuisance bites, as reported elsewhere [30]. Anecdotal reports of misusing bed nets into a livelihood tool (e.g., fishing net) reiterate how poverty hinders the full potential of malaria control [31, 32]. FGD participants reported accepting IRS because of strong community leadership and general compliance with the 'law'—a socially constructed concept present in Mozambique [33, 34]. But, as in the neighboring district of Manhiça, acceptance was affected by rumored side effects and socioeconomic status [33].

Some participants, particularly women, listed activities in which outdoor mosquito-human contact took place, and in a few instances associated these activities to the risk of transmission. Similarly, the UNFPA [35] reported that drought in Maputo Province resulted in new and prolonged outdoor activities among women, who tend to gather woods and reed plants for daily subsistence, and may spend 10 to 12 h outside, including at dawn and during the night, to collect water. Despite insufficient evidence from the areas to quantify ongoing malaria transmission outdoors, these findings highlight the potential limitations of vector control interventions that focus on the indoor environment and open the space for tools targeting residual forms of transmission. Positive opinions about targeting mosquito breeding sites (e.g., treating water bodies) could indicate openness to try new vector control tools, such as larval source management.

IPTp was not mentioned by respondents. This reflects the generally low IPTp awareness and uptake throughout Mozambique (16–43%) [4, 5] and the need for focused messages to address this gap. Low levels of IPTp uptake has been explained in terms of the complexities of antenatal care (including delayed first visits, negative attitudes of health professionals towards the intervention, and lack of supporting infrastructure), household decision making, perceived drug side effects, poor awareness, and lack of education, among others [6, 36, 37]. Considering the emergent positive views on protective pills captured through this study as a result of the ongoing IEC to promote MDA participation, the low level of awareness of IPTp coverage may be associated with insufficient messaging to the community about the functionality and availability of this chemoprevention measure.

Malaria treatment means abating symptoms and averting deaths through western medicine or healthcare providers. In Magude, visible malaria programs and related community engagement positively influence the decision to seek and access treatment but structural factors constrained the early phase of the treatment pathway. In particular, transportation and distance delay malaria treatment while efforts in installing localized APEs offer a relative solution to the multifaceted problem. The traditional approach to treating malaria was described as

rarely taken, even by some traditional healers. This finding conflicts with that of many other studies, which have highlighted traditional medicine as the first source of assistance for malaria treatment in sub-Saharan Africa [38]. Mozambican policy towards licensing traditional healers through the *Associação de Médicos Tradicionais de Moçambique* (AMETRAMO) [39] might have driven inclusivity of healers into different malaria-related campaigns, which in effect influence their beliefs about malaria. This way, traditional healers constructively contribute to malaria control and case referral. On the other hand, healers' perception towards malaria treatment should be interpreted cautiously due to overrepresentation and potential desirability bias of trained healers and underrepresentation of sorcerers or healers who are not part of AMETRAMO.

Strengths and limitations

This study is based on self-reported information and recalled experience. However, drawing respondents from across different population groups and conducting FDGs across the community allowed researchers to triangulate the results and minimize potential bias. There is potential desirability bias among participants, particularly traditional healers. Two participants outside the desired inclusion criteria of the group 'women of reproductive age' participated in two of the FGDs—one in Panjane and one in Motaze. This did not result in exceptional responses during the respective FGDs and they had no apparent influence on overall responses from the concerned group. The two-stage translation process and the deployment of multiple interviewers were additional limitations of the study. Nevertheless, transcripts translations were conducted by certified local (Mozambican) translators and validated by in-house/local researchers. Interviewers were trained professionals and underwent workshops on the FGD materials used in this study.

Conclusion

The residents of Magude showed relatively high awareness of malaria, trusted the health institutions, and identified gaps in prevention offered by the vector control tools. These favourable perceptions and behaviours emerged prior to the elimination campaign and may suggest the community's openness to new intervention. However, a lack of awareness of asymptomatic carriers, inadequate understanding of residual transmission, and several barriers to seeking care might affect the overall strategy of malaria elimination in Magude. Continuous improvement of IEC is needed to emphasize the importance and benefits of the on-going control programmes (especially IPTp, bed net, and IRS) while integrating the concepts related to community-based chemoprophylaxis to maximize MDA uptake. Altogether, these findings suggest that malaria elimination efforts in southern Mozambique will strongly benefit from the intensified community engagement campaigns that reinforce prevention and treatment concepts while including messages to promote new interventions based on the local context.

Additional file

Additional file 1. Tabulated summary of the key results from the Focus Group Discussions in each community group.

Abbreviations

ACT: artemisinin-based combination therapy; AMETRAMO: Associação de Médicos Tradicionais de Moçambique; APE: Agentes Polivalentes Elementares; FGD: focus group discussion; HF: Health Facility; IPTp: Intermittent Preventive Treatment of Pregnant Women; IRS: indoor residual spraying; LLIN: long-lasting insecticidal nets; MDA: mass antimalarial drug administration; NMCP: National Malaria Control Program; RDT: rapid diagnostic test; UNFPA: United Nations Population Fund; WHO: World Health Organization; WMR: World Malaria Report.

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Authors' contributions

HPP organized the data, performed data coding/analysis and interpretation, and drafted the manuscript. BG contributed in study design, data analysis, interpretation, and writing of the manuscript. HN, HD, IM, participated in the study design, data collection and transcription. FS, PA participated in the conception of study design, supported in study implementation, and contributed to the writing of the manuscript. CP directly supervised HPP throughout the study and contributed to data analysis, interpretation, and manuscript writing. KM conceptualized the study design, trained field researchers, and supervised data collection and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data are within the manuscript and the Additional file 1.

Ethics approval and consent to participate

This study is part of a larger study for which ethical clearance was obtained from the Institutional Review Group of the Centro de Investigação em Saúde de Manhiça, the Ministry of Health National Bioethics Committee of Mozambique (IRB00002657), and the Hospital Clinic of Barcelona Ethics Review Committee. Written and verbal informed consents were obtained from participants before the start of the discussion. The verbal consent to participate was audio-recorded in all FGDs. Participants anonymity and confidentiality were guaranteed throughout the research process.

Consent for publication

Consent was gained from all participants to publish anonymized data.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

Supplementary data

Examining community perceptions of malaria to inform elimination efforts in Southern Mozambique: a qualitative study

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Supplementary Table 1. List of Focus Group Discussions

Group	Location	Time	Participants
Adult men			
FGD1_M16	Magude Center	38 min	6
FGD2_HA4	Motaze	90 min	5
FGD3_HA7	Panjane	58 min	12
FGD4_HA10	Mahele	97 min	12
FGD5_HA15	Mapulanguene	55 min	4
Community leaders			
FGD1_LC1	Magude Center	76 min	7
FGD1_LC5	Magude Center	-	3
FGD2_LC3	Motaze	70 min	12
FGD3_LC8	Panjane	62 min	12
FGD4_LC11	Mahele	63 min	10
FGD5_LC14	Mapulanguene	55 min	8
Women of reproductive age			
FGD1_MIR6	Magude Center	43 min	7
FGD2_MIR2	Motaze	31 min	12
FGD3_MIR9	Panjane	45 min	12
FGD4_MIR12	Mahele	50 min	12
FGD5_MIR13	Mapulanguene	30 min	10
Healer			
FGD1_MT17	Mixed location	58 min	10

Supplementary Table 2. Clinical signs and symptoms associated with different malaria terminologies mentioned by participants

Participants	Malaria	Musothoto	Dze-dze-dze
Community leader	Fever, headache, joint pain, weakness, cold, vomiting, back pain, confused thinking Childhood malaria: severe stomach ache, swollen belly, convulsion, weakness	Signs same with malaria	Vomiting, diarrhoea, trembling
Adult Men	Weakness, headache, fever, shivering, chilly trembling, vomiting (*caguba*), stomach ache, diarrhoea, lack of appetite, afternoon cold, body pain	Headaches, body pain, diarrhoea, vomiting, headache, disease of falling ("epilepsy"), joint pain, fevers, discouraged and unhappy, weakness, shivering, warm body, feel cold, wants to sleep, quiet (*Ku zinga*)	Vomiting, trembling, feel cold, binding of limbs, diarrhoea, headaches
Women of Reproductive Age	Body pain, weakness, headache, joint pain, fever, vomiting	Vomiting (bile)	Cold, vomiting bile, joint pain, chills, headaches, body pain
Healer	Fever, headache, body pain, cold, shivering, vomiting		Signs same with malaria

Supplementary Table 3. Community perceptions of the cause of malaria

Perceived cause of malaria	Community leaders	Adult men	Women of reproductive age	Healer
Mosquito	*Mosquito names* • Female mosquito • Mosquito • Malaria mosquito *Mosquito biting* • Mosquito reproduces in water, enters houses and bites people • Mosquito bites dogs, snakes, and sick person, and then bites a healthy person. • Mosquito stings and litters the person with dirt *Mosquito associated with water* • Mosquito walks in ditches • Malaria mosquito from dirty water • Mosquito from stagnant water • Mosquito multiplies in streams • Mosquito reproduces in water, enters houses and bites people *Mosquito associated with animals* • Mosquito bites dogs, snakes, and sick person, and then bite a healthy person. • Mosquito proliferates in the presence of animals and animal pens *Mosquito associated with plants* • Mosquito from cane-field • Mosquito from grass around houses • Mosquito from pumpkin and watermelon *Mosquito that transfers biological materials* • Mosquito stings and litters the person with dirt • Mosquito transfers poison. • Mosquito has venom.	*Mosquito names* • Mosquito • Anopheles *Mosquito biting* • Mosquito bites a person with malaria and bites another person • Mosquito bites an animal and bites a person • Mosquito eats dirt and bites a person • Mosquito bites when spraying agents have gone • Mosquito bite transfers blood from infected person • Mosquito that come into houses during nightfall to bite a person • Mosquito becomes the parasite that sucks on human blood *Mosquito associated with water* • Mosquito exists in abundance during rainy season • Mosquito reproduces in stagnant water or rain water. • Mosquito multiplies in the trash and dirty water *Mosquito associated with animals* • Mosquito bites an animal and bites a person *Mosquito that transfers biological materials* • Mosquito lands on dirt (e.g. feces) and then goes to a person to transfer the dirt • Mosquito eats dirt and bites a person • Mosquito bite transfers blood from infected person	*Mosquito names* • Mosquito *Mosquito biting* • Mosquito bites a person with malaria and transmits to another person • Mosquito bites unprotected persons *Mosquito associated with water* • Mosquito in bathroom dirty water • Mosquito stays in dirty water *Mosquito associated with hygiene* • Mosquito comes when you leave trash in the house • Mosquito comes when we gather dirt or not take care of ourselves	*Mosquito names* • Female mosquito *Mosquito biting* • Mosquito bites a person with malaria and then bites another person

Others: *Dirty water, flies, insects, food, bad-smelling, virus, witchcraft and sorcery*	*Water* • Drinking filthy water from Incomate river where person wash clothes • Drinking dirty water • Drinking river water with bacteria *Flies and insects* • Flies land on human feces and land on children food • Flies that land on peeled mango fruit during summer • Dirt at home • Bites from different insects *Food* • Eating uncooked food like salad and tomatoes causes malaria and cholera *Bad-smelling* • Inhaling "bad air" during defecation *Malaria virus* *Witchcraft and Sorcery*	*Water* • Dirty water where children play • Water where mosquito develops • Dirty water used for cooking • Rains cause malaria • Drinking water from boreholes has mud and mosquito dirt *Flies and insects* • Flies at home due to lack of hygiene • Flies land on dirt (human feces) and land on food *Food* • Eating fresh food like pumpkins and coots *Malaria virus* *Witchcraft*		*Water* • Water urinated by ox collected by a person • Littering the river • Drinking dirty water causes malaria or cholera *Food* • Eating dirty food • Eating plenty of watermelon *Sorcery*

Supplementary Table 4. Community perceptions of malaria preventive measures

Community leaders	Adult men	Healers	Women of reproductive age
Vector-human contact avoidance			
• Sleeping at night under the bed net • Child sleeps under the bed net • Burning insecticidal coils (Dragon and Baygon) • Closing windows at dusk and night • Personal hygiene and sanitation	• Sleeping at night under the bed net (Mutchiquitelo) • Burning insecticidal coils (Dragon and Baygon) • Personal hygiene and sanitation	• Personal hygiene and sanitation	• Sleeping at night under the bed net (Mutchiquitelo) (only and most important way to avoid malaria-causing mosquito) • Use of insect repellents • Use of coils
Mosquito breeding and resting sites elimination			
• Eliminate containers that retain water (e.g. bottles, coconut shell [cafulo]) • Eliminate stagnant water from old tires • Clean the water pits in the bathroom • Eliminate trash • Burn and burry trash • Pruning trees • Avoid pile of clothes • Clean houses • Move animal pens far from houses • Cleaning children feces • IRS -scare away mosquito (children don't know to scare mosquito away) -kill mosquito	• Eliminate puddle of water • Eliminate dirty water • Throw empty cans properly • Medicine to treat ponds (tlhive) and lagoons against mosquito (you cannot cover the ponds) • Clean and cover water holes and wells to avoid dirt (e.g. tree leaves) • Cover the pits to avoid rain water from entering and rotting that will attract mosquito breeding • Weed plants in the backyard • Avoid hoarding garbage • Eliminate trash • Clean yards • Clean house and bathroom	• Eliminate water from rainwater bowls • Remove rainwater from tires and cisterns • Eliminate stagnant water • Prune trees • Cleaning homes • Clean dropped tree leaves • IRS -scare away mosquito	• Remove dirty water • Cleaning homes • Eliminate holes with water • Burn trashes • Water far from houses • Prune trees and shrubs • Clean houses • Eliminate trash • Burry and burn dirt • IRS
Chemoprophylaxis or disruption of symptoms initiation			
• Protective pills -Avoid mosquito bites -Way of Immunization -Prevent mosquito from	• Protective pills - Avoid getting sick when mosquito bites -Intermittent preventive		• Protective pills - perceived to mainly avoid mosquito bites

transmitting malaria - Avoid getting sick when mosquito bites	treatment for pregnant		
Other measures of prevention			
• Hygiene and sanitation related to toilet and defecation. • Clean air to breath by cleaning houses • Hand washing	• Hygiene and sanitation related to toilet and defecation • Drinking clean water		

Supplementary Table 5. Malaria risk-taking behavior in Magude district

Community leaders	**Adult men**	**Healers**	**Women of reproductive age**
Indoor malaria risk-taking behaviour and activities			
• Not using bed net -Still in plastic • Misusing bed net -use for fishing • Refusing IRS -causes cough -cockroaches appear -have air-con. -house can get dirty • Not closing windows at night	• Not using bed net -Cannot sleep • Misusing bed net -use for fishing • Refusing IRS	• Misusing bed net -use to encircle cabbage	• Not using bed net • Sitting in common areas • Sleeping late • Not using insect repellent and coil
Outdoor malaria risk-taking behaviour and activities			
• Defecating in the bushes • Visiting and near cane plantation • Going to the forest to look for roots (herbal medicine) • Harvesting fruits and vegetables • Fetching drinking water from the river • Chatting in the backyard • Passing at garbage site	• Bathroom and toilet in the bushes • Harvesting fruits and vegetables • Children play in water areas • Near animal barn		• Attending night class • Diurnal mosquito biting • Not using insect repellent

Chapter 6

Discussion

1. Overview: Targeting malaria transmission

To achieve malaria elimination, great emphasis must be put on targeting the parasite's transmissible stages, so that, along with effective treatment of clinical malaria caused by asexual stages, complete interruption of the life cycle can be achieved. Unfortunately, the sustainable goal of interrupting malaria transmission is more complex than previously thought, as demonstrated by the many failed attempts to eradicate the parasite (35). One of the various reasons why interrupting malaria transmission is challenging is that the transmissible gametocytes are resilient and complex in nature. First, gametocytes do not cause clinical malaria, creating an undetected human infectious reservoir. The inherently low gametocyte numbers make them barely visible under diagnostic microscopy, so that most gametocyte carriers are left undetected. Low gametocyte numbers are also associated with less antigenicity resulting in an insufficient host immune response (276). Hence, gametocytes remain in circulation for days and weeks thereby increasing the chances of uptake by a mosquito vector. Lastly, almost all classes of antimalarial drugs are ineffective against mature gametocytes. This is also compounded by the widespread resistance to clinically relevant antimalarial drugs (47,73,274). Hence, to counter the complex nature of gametocytes, one must go back to the very beginning of gametocyte biology and disentangle how gametocytes are formed and why. By doing so, we can hit hard where malaria transmission is most vulnerable and thus prevent future transmission.

The adaptable nature of the malaria parasite suggests that enhanced gametocyte production is a response to adverse environments (242). Although gametocytes are constitutively formed at a very low frequency (72,74), mounting evidence supports that external factors modulate the

rate of sexual conversion by increasing or decreasing gametocyte production (135). However, whether the most effective antimalarial drug artemisinin can stimulate sexual conversion, which would result in increased production of functional gametocytes in *P. falciparum*, remains to be clarified both under laboratory culture conditions and in field settings. We addressed this research question by first creating a robust assay that measures the sexual conversion rate (**Chapter 2**), and finally tested the impact of artemisinin on sexual conversion and the transmissibility of artemisinin-induced gametocytes to mosquitoes (**Chapter 3**). We extended the scope of our study beyond the laboratory format by looking into how treatment affects the sexual conversion of parasites from naturally-infected patients. We speculate that the current form of malaria treatment, which is artemisinin-based combination therapy (ACT) and artesunate monotherapy followed by ACT, may stimulate the expression of the sexual commitment gene *pfap2-g* and other sexual ring biomarkers. Hence, this would result in a subsequent increase in the production of circulating mature gametocytes (**Chapter 4**). Our *in vitro* study permitted a detailed dissection of the effect of artemisinin exposure on parasite sexual conversion, while our field studies probed how translatable our *in vitro* results are to real human malaria infections, which are naturally complex due to interaction of multiple factors (e.g., immunity and LysoPC levels) inside the human body. Our study designs are therefore complementary to address the impact of artemisinin on parasite's investment on transmission. Lastly, we believe that by understanding the community perceptions of malaria, including its malaria-related interventions, we will be able to tailor the right approach for site-specific malaria elimination campaigns. In this way, we may gain new insights on how a new tool, such as mass drug administration (MDA), can be potentially harnessed to completely interrupt malaria transmission (**Chapter 5**).

2. Impact of artemisinin on *P. falciparum* sexual conversion

2.1. A new *in vitro* assay to measure the sexual conversion rate

One of the main research limitations in malaria transmission biology is that early forms of gametocytes are impossible to distinguish by morphology from asexual parasites, in addition to the low numbers produced at every cycle. This is particularly relevant when we assess the impact of external stimuli on the rate of sexual conversion. To address this concern, we successfully generated different transgenic lines based on different early gametocyte gene promoters that drive the expression of the fluorescent marker tdTomato (i.e., *NF54-gexp02-Tom*, *E5-gexp02-Tom*, *NF54-10.3-Tom*, *E5-10.3-Tom*, and *E5-pfs16-Tom*). In contrast to previously reported parasite lines (150,151,156,159,277–279), we integrate the fluorescent marker in the parasite genome using the CRISPR/Cas9 technology, thus making our system more stable compared to transgenic lines carrying the transgenes episomally.

Although all our transgenic lines can specifically distinguish sexual stages in a flow cytometry-based assay, we found a remarkable advantage of using *gexp02*-based lines over other parasite lines. The *gexp02*-based lines are the first reported transgenic lines that can specifically differentiate sexual rings as early as 10-15 hpi at a single-cell level. Sexual rings are morphologically undistinguishable from asexual rings. In the classical way of conducting sexual conversion assay, one must perform gametocyte culture for at least 4 days to distinguish sexual stages from asexual stages (136). Therefore, the major advantage of our *gexp02*-based lines is that it significantly shortens the time for the sexual conversion assay, dispensing the need for anti-asexual replication compounds.

Our *gexp02*-based lines retain a sexual conversion phenotype that is responsive to external stimuli, which is evident by our choline induction data. The plasticity of their sexual conversion phenotype is important for our new sexual conversion assay as it offers a flexible approach to probe the impact of external stimuli. In other words, it can be used to inform on

how different metabolic states (choline levels) and additional external factors (e.g., antimalarial drugs) are interacting to enhance or repress the rate of sexual conversion. Indeed, we were able to apply this approach when we evaluated the impact of artemisinin, chloroquine, and heat shock on sexual conversion in the *NF54-gexp02-Tom* line (**Chapter 3**).

So far, our new parasite lines have been used to assess the effect of antimalarial drugs on the sexual conversion rate and recently for developing a new approach to diagnose gametocytes (280). Nonetheless, these lines have tremendous potential for the screening of new and old drugs against different sexual stages, particularly against sexual rings (**Figure 19**). Even with the currently used antimalarial drugs, there is a lack of research on their impact on sexual rings due to the unavailability of the right system; thus, most antimalarial drug studies on transmission are directed to mature gametocytes (281–284). Moreover, in the case of artemisinin, it would be interesting to know whether drug-induced latency (264,285) is biased toward sexual rings and if these latent rings play a significant contribution to mosquito transmission. One of the advantages of the *NF54-gexp02-Tom* line is its ability to produce functional gametocytes that are infectious to mosquitoes, making this parasite line suitable to address such types of questions.

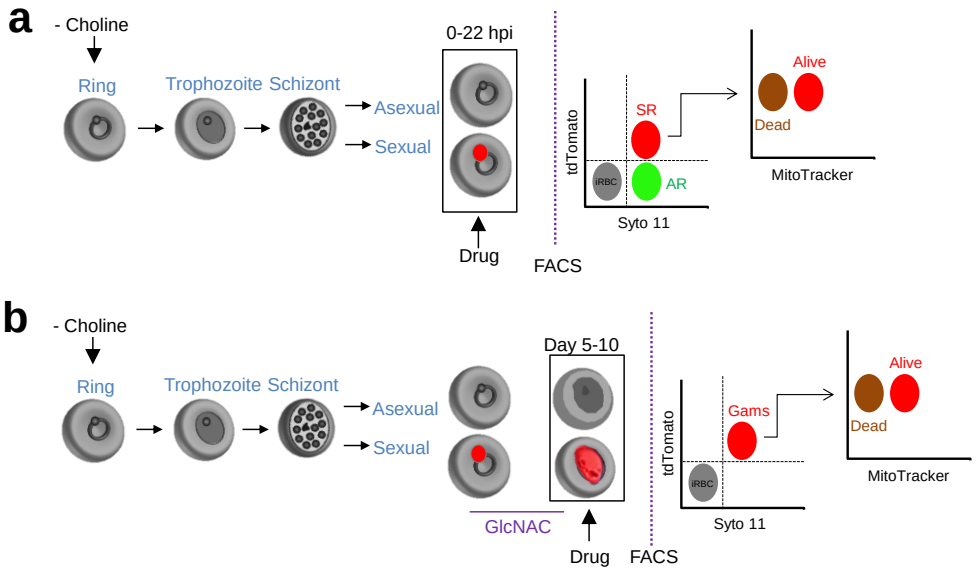

Figure 19. Experimental design to screen antimalarial drugs against different sexual stages using the *NF54-gexp02-Tom* line. (a) Schematic of drug screening for sexual rings. The proposed assay would first involve performing choline induction in the

initial generation of rings to produce large numbers of sexual forms in the next cycle. In the next cycle, the next step would be drug screening on ring stages. After 24 hours, an assessment of the viability of sexual rings could be done using a flow cytometry assay with the MitoTracker dye. A Syto 11 stain can be used to measure the total parasitemia. **(b)** Similar to Panel A, but drug treatment can be done at Stage I to V gametocytes. Abbreviations: SR (sexual ring); AR (asexual ring); GlcNAC (N-acetyl-d-glucosamine); Fluorescence-activated cell sorting (FACS); Gams (gametocytes).

2.2. Artemisinin enhances *P. falciparum* sexual conversion upon exposure at the trophozoite stage but not at the ring stage

To address the impact of artemisinin on sexual conversion independent of its gametocytocidal property, we performed a short drug pulse using our new transgenic lines and the newly developed sexual conversion protocol. Our results reveal that sexual conversion is affected by a complex interplay between artemisinin concentration, parasite stage, and parasite metabolic state related to phosphatidylcholine synthesis. We observed a clear induction of sexual conversion upon exposure of the trophozoite stage to subcurative artemisinin doses, resulting in a higher gametocytemia and a successful mosquito infection. We further demonstrated that artemisinin exposure at the ring stage does not enhance sexual conversion but rather results in a reduction of sexual conversion rate and gametocytemia. Our results, together with previously reported studies on artemisinin, imply that different parasite stages may have different responses to artemisinin: (i) artemisinin exposure at the trophozoite stage enhances sexual conversion (**Chapter 3**); (ii) artemisinin exposure at the ring stage induces temporary latency (264,285) rather than sexual conversion; and (iii) mature Stage V gametocytes are metabolically resistant to artemisinin (272). In the following subsections, I discuss the importance of our results in clarifying the effect of antimalarial drugs on gametocyte production and its potential contribution to understanding the mechanism of sexual conversion.

2.2.1. Clarifying the effect of antimalarial drugs on the sexual conversion rate of malaria parasites

The impact of antimalarial drugs on sexual conversion is generally ambiguous. There is no consensus on whether antimalarial drugs may

enhance or reduce the rate of sexual conversion in malaria parasites, as shown in previous reports (178,240,243,244,274,282). However, based on the evolutionary theory for life history, it is predicted that subcurative doses of antimalarial drugs would result in the reduction of sexual conversion rates, in which parasites undergo a state of 'reproductive restraint'. Here, resource allocation is directed to the parasite's asexual multiplication, thereby ensuring survival within the human host. By contrast, when parasites encounter fatal circumstances, such as treatment with lethal doses of antimalarials, they are predicted to redirect all of their resources to a short-term reproductive act by dramatically enhancing the rate of sexual conversion, also known as 'terminal investment' (183). 'Terminal investment' may seem to be a 'gamble' of survival since the gametocyte is a dead-end stage, meaning it will die naturally in human circulation if not transmitted to mosquito vectors. The timing of gametocyte production must therefore coincide with mosquito breeding season for successful transmission (286). As such, the antimalarial treatment itself is a disruptor of the natural oscillation of transmission timing. Overall, for successful transmission and perpetuation of species, malaria parasites are selected to maximize fitness through a balance between asexual replication and the levels and timing of gametocyte production (287).

The evolutionary assumptions of sexual conversion were recently illustrated in a rodent malaria model using the antimalarial drug pyrimethamine, in which repression and enhancement of sexual conversion rates were observed upon treatment with subcurative and lethal doses, respectively (178). Our results on artemisinin and chloroquine do not completely follow these assumptions. Although we observed reproductive restraint after exposing the ring stage to subcurative doses, we also found a dramatic increase in the sexual conversion rate after exposing the trophozoite stage to similar subcurative doses. Moreover, sexual conversion rates after exposure to lethal doses of antimalarial drugs at either the trophozoite or ring stages were relatively lower than after subcurative doses, so that terminal investment did not seem to occur. Of note, lethal drug doses that kill the majority of parasites

may affect the accuracy of measuring sexual conversion rates, which may be applicable in our study and also in previous studies (178,282), and therefore should be interpreted with caution.

We also hypothesize that the discrepancy between our results and previously reported studies are attributable to (i) parasites' stage-dependent response, (ii) the methods used to assess sexual conversion rate, and (ii) the use of different parasite species. Our study is the first to demonstrate a stage-dependent response of *P. falciparum* upon drug treatment. In many studies, antimalarial drugs are added to *P. falciparum* culture within a 48-hour treatment starting from ring stages, obscuring a potential parasite stage-dependent effect. In our case, a short drug pulse was helpful to assess a stage-directed impact of antimalarial drugs on sexual conversion, independent from their effects on the viability of different parasite stages. A 48-hour long exposure to any antimalarial drugs may decimate those parasites that have already been sexually converted, which may lead to the miscalculation of gametocyte numbers in the final readout.

We also found that artemisinin-induced sexual conversion does not occur when parasites are already induced by another factor, such as choline depletion. Typically, parasites can be cultured with either human serum or Albumax as a supplement. Using a human serum is equivalent to adding choline in our set-up, whereas using Albumax is equivalent to removing choline (135). Therefore, when parasites are already maintained in Albumax, sexual conversion cannot be induced by another stimulus, including antimalarial drugs. This confounding factor has never been considered before when addressing the effect of external stimuli on sexual conversion.

Lastly, although AP2-G is conserved across *Plasmodium* species, it is possible that different species of malaria parasites adopt different mechanisms of adjusting sexual conversion rates. When compared to *P. falciparum*, rodent malaria parasites (*P. yoelii*, *P. berghei*, *P. chabaudi,* and *P. vinckei*) show higher rates of baseline sexual conversion,

unresponsiveness to LysoPC induction, and they lack a *gdv1* ortholog (see also below) (154).

With the very low levels of *P. falciparum* sexual conversion in natural infections (72,74), an additional restraint on its sexual conversion upon exposure to subcurative drug doses would not provide an advantage. Our new data may therefore aid in the recalculation of the predictions of the evolutionary theory for life history in human malaria parasites.

2.2.2. Insight on the molecular mechanism of induced sexual conversion

Our results have generated a few hypotheses on the mechanism of induced sexual conversion. We hypothesize that induced sexual conversion by different stimuli is a consequence of signal transduction associated with moderate parasite stress, suggesting that it is not solely a general response to a 'state of dying' (183) but rather involves a specific molecular pathway. This explains the parasite stage-dependent induction, the inducing effect of low artemisinin doses even without considerable parasite mortality, and the saturation of sexual conversion rate upon multiple stimuli. It is likely that this signal triggers the expression of the GDV1 protein, which has been shown to activate *pfap2-g* at the trophozoite stage, the only stage responsive to induction of sexual conversion. Filarsky and colleagues (2018) demonstrated that the *gdv1* locus is regulated by a long non-coding *gdv1* anti-sense RNA, which is responsive to choline as an external stimulus (97). Therefore, GDV1 creates a link between the external stimulus and *pfap2-g*. Interestingly, rodent malaria parasites lack the *gdv1* orthologue (154), suggesting a unique mechanism of *pfap2-g* activation in the rodent lineage of the parasite, or a potential absence of inducible sexual conversion as they already have a higher baseline sexual conversion rate.

Our results also reveal that adding two simultaneous stimuli, which are both proven to induce sexual conversion, cannot further induce the rate of sexual conversion. This is evident by our data under choline depletion conditions where artemisinin, chloroquine, and heat shock exposure at the trophozoite stage did not cause any additive or synergistic

effects on sexual conversion rates. At the molecular level, this finding suggests that different stimuli converge into the same mechanism that activates *pfap2-g*. In addition, the molecular mechanism of *pfap2-g* silencing may secure that there are always some parasites that remain in a proliferative state to maintain the infection. Of note, even with artificially inducible *P. falciparum* parasite lines, sexual conversion rates never attain a 100% (104,288), meaning that there remains a parasite subpopulation that will continue to replicate asexually. Llorà-Batlle and colleagues (2020) demonstrated that the small fraction of asexual parasites, which did not convert into gametocytes upon artificial induction, have heterochromatin marks in the upstream region of *pfap2-g*, which is likely responsible for continuous asexual replication. They also show that heterochromatin marks are retained at the *pfap2-g* coding sequence even in sexually induced parasites. They proposed that this heterochromatin in the *pfap2-g* coding sequence is constitutive and may expand and retract across the *pfap2-g* locus, suggesting a flexible switching between activation and repression (104). This constitutive heterochromatin, which seems to be difficult to destabilize by inducing factors, may partly explain why sexual conversion rates never reach 100%. Notably, it is more common to encounter parasites with the 'asexual replication rate' of >99% (i.e., sexual conversion rate <1%) (72,74) than the other way around.

Although we do not have answers on the complete mechanism of how artemisinin triggers sexual conversion, there are general cues on which direction to look at. First, it is possible that artemisinin treatment results in the depletion of LysoPC or choline as a result of lipid membrane damage (289) or a metabolic perturbation of the phosphatidylcholine synthesis (290). A metabolic LysoPC deprivation has been well demonstrated to increase the rate of sexual conversion in *P. falciparum*. It also leads to the upregulation of the PMT pathway to compensate for the absence of choline (135). It is predicted that the PMT pathway utilizes essential elements needed also for heterochromatin methylation and maintenance, which might explain the derepression of the heterochromatic *pfap2-g* locus (194,195). One of the major caveats of this proposed

mechanism is that it may assume a loss of heterochromatin not only in the *pfap2-g* locus but also throughout the 14 chromosomes, which has never been reported in *P. falciparum*.

Second, the endoplasmic reticulum (ER) stress response has been previously linked to a higher gametocytemia in *P. falciparum* (232). Artemisinin is known to trigger severe ER stress in *P. falciparum*, especially at the trophozoite stage (258–260). Choline depletion may also trigger an ER stress response (291), although this has to be demonstrated in malaria parasites. ER stress is a result of accumulations of unfolded proteins in the ER, which induce a persistent eIF2α phosphorylation that stops protein translation (259). Several studies showed that eIF2α phosphorylation consistently occurs during protozoan stage transitions, as shown in *Toxoplasma gondii* (292), *Trypanosoma cruzi* (293), *Leishmania infantum* (294), and *Entamoeba histolytica* (295). Usually, it marks a transition from a proliferative stage to the latent stage under stressful conditions, such as heat shock and drug treatment (296). Future works should therefore focus on how eIF2α phosphorylation contributes to heterochromatin destabilization in the *pfap2-g* locus.

2.3. Sexual ring biomarkers (SRBs) as a tool to assess sexual conversion in human malaria infections

Although immature gametocytes are sequestered in the hematopoietic niche, it was previously uncertain whether sexual rings follow a similar pattern of tissue sequestration. In a blood sample of patient with malaria, it is typical to observe ring stages and Stage V gametocytes by diagnostic microscopy, but it is impossible to differentiate sexual rings from asexual rings based on morphology. Moreover, previously available biomarkers for early sexual stages can cross-react with mature gametocytes (e.g., *pfs16*). Hence, the fundamental question of whether sexual rings circulate freely or sequestered in the tissue was left unanswered for many years (131–133,145). Now, new biomarkers *pfap2-g*, *gexp02*, and *gexp5* are available for the detection of sexual rings, with mRNA transcripts expressed at negligible levels in Stage V gametocytes (95,143,146). Previous reports showing the presence of abundant

transcript levels of *pfap2-g* and *gexp5* in human blood samples with malaria infection suggest that sexual rings can be found in the circulation (91,132,275). This is also demonstrated by our field cohort data from Vietnam, Burkina Faso, and Mozambique (**Chapter 4**).

In this thesis, we characterized *gexp02* as a potential sexual ring biomarker candidate for field studies. It has expression levels much higher than *pfap2-g* and is more specific than *gexp5*. A previous study revealed that *gexp5* mRNAs are highly promiscuous in asexual rings (95,132), which may explain the consistently high transcript levels in our field samples. Specific detection of sexual rings is relevant in identifying signs of malaria transmission even before the appearance of mature gametocytes. Our field cohort data demonstrated that some SRBs may correlate to the peak of mature gametocytes 7 to 14 days later, in some patients. A previous study also observed that *pfap2-g* transcript levels correlate to the subsequent circulation of mature gametocytes in human infections (275). While mature gametocyte detection signals a real-time transmission potential that will likely occur in the presence of the mosquito vector, sexual ring detection may predict malaria transmission at a later time (**Figure 20**). The application of the two is clearly advantageous to define the epidemiology of transmission in endemic settings and probably to create an accurate prediction model for seasonal outbreaks. Apart from that, a combination of both biomarkers is expected to properly interrogate the impact of external stimuli, such as antimalarial drugs, on the sexual conversion of malaria parasites in field settings, which has been proven difficult to perform previously due to the absence of specific SRBs (274) (**Chapter 4**).

One of the main characteristics of SRBs is that they are expressed at different levels and at different times of the sexual ring development. For example, *pfap2-g* is highly expressed in very early rings while *gexp02* and *gexp5* expressions start in mid-late rings (**Chapter 2**). Field studies that aim to quantify the age dynamics of sexual rings can take advantage of this, which can be used to identify the dominant age window of asexual rings in an infection. Determination of specific ring age window has been

employed as a predictor of severe malaria and parasite clearance upon artemisinin treatment (297), which may also benefit from additionally incorporating sexual-ring detection to predict transmissibility. However, some SRBs may not be very specific to sexual rings: *gexp5* and *pfs16* transcripts are abundantly detected in asexual rings while *pfg14-744* and *pfs16* in mature gametocytes (95). Hence, these characteristics should be taken into account when choosing SRBs for field applications.

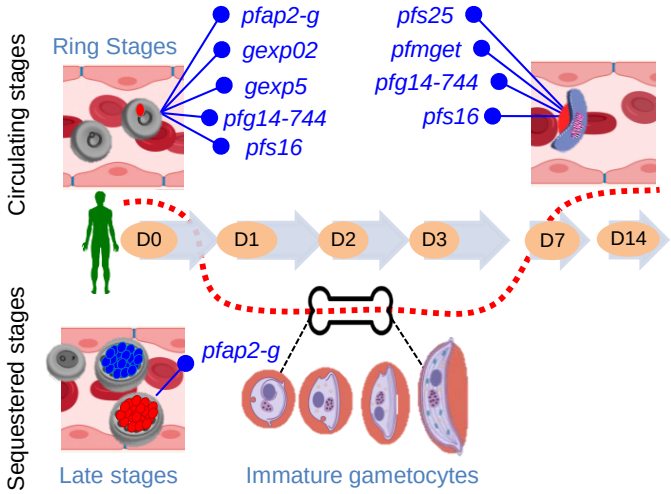

Figure 20. Sexual ring circulation and subsequent release of mature gametocytes on days 7 to 14. Sexual rings (red dot) circulate in the bloodstream and are marked by sexual-stage specific biomarkers (e.g., *pfap2-g* and *gexp02*). Sexual rings sequester in the bone marrow to develop from Stage I to IV gametocytes. Stage V mature gametocytes are released 1 to 2 weeks later and are characterized by mature gametocyte biomarkers (e.g., *pfs25* and *pfmget*). Late-stage parasites, including trophozoite, asexual schizont (blue nuclei), and committed schizont (PfAP2-G-positive, red nuclei) are sequestered in tissues by attaching to the endothelium. Figure created partly using Biorender.

2.4. The association between artemisinin-induced sexual conversion and PfK13 mutations in human malaria infections

The relationship between artemisinin resistance and sexual conversion is not well understood. Our field cohort data surprisingly reveal a reverse association between PfK13 mutations and an increase in *pfap2-g* expression levels immediately upon artemisinin treatment. It shows that resistant parasites may allocate more resources to asexual replication than gametocyte production upon ACT or artesunate monotherapy. This is supported by significant downregulation of *pfap2-g* and other SRBs in the Vietnam cohort, where most patients carried PfK13 mutant parasites. When parasites have a fast-clearing phenotype, expression levels of *pfap2-g* and other SRBs are mostly upregulated immediately after artemisinin treatment. With PfK13 mutant parasites (i.e., ring stages)

showing a higher survival rates than wild-types (298,299), the degree of sensitivity to the drug may explain why the PfK13 mutant appears refractory to artemisinin-induced sexual conversion. However, it does not completely explain the trend at the population level by which most PfK13 mutant parasites tend to downregulate sexual conversion even upon multiple artemisinin exposures that end up killing all parasites.

One of the limitations in field studies is the inability to determine the proportion of sequestered trophozoites and schizonts with respect to the circulating rings. This is particularly relevant in our case because our *in vitro* data clearly shows a stage-dependent induction of sexual conversion, where exposure of rings to artemisinin downregulates sexual conversion and exposure of trophozoites upregulates sexual conversion. It is plausible that the reason why PfK13 mutant parasites downregulate sexual conversion in Vietnam is that they already have an altered life cycle with a delayed ring stage phenotype. Developmental prolongations of ring stages have been well demonstrated in PfK13 mutants even in the absence of artemisinin exposure (260,300,301). The altered developmental characteristic of malaria parasites in Vietnam could be attributed to decades of artemisinin exposure that results in a shift of the vulnerability window in favor of their advantage: the prolongation of ring-stage development and shortening of the trophozoite stage development (302). Based on the model proposed by Khoury and colleagues (2020), when parasites are sensitive to artemisinin, all three artemisinin doses will more likely cover the more vulnerable late stages than the ring stage (**Figure 21**); this could explain the observed upregulation of sexual conversion in Burkina Faso and Mozambique, where all parasites are sensitive to artemisinin. By contrast, in patients with artemisinin-resistant parasites, there is a much lesser probability that all three doses cover the late stage because the majority of parasites have an extended ring-stage development. If we focus only on the first treatment dose, which is the one that determines the SRBs levels at the most informative time points in our study (12-24h post first treatment), the probability of the drug encountering trophozoites would also be lower in parasites with prolonged ring stage.

The next explanation is based on our findings that sexually induced parasites (e.g., by choline depletion) cannot be additionally or synergistically induced by another external factor (e.g., artemisinin). This suggests a state of saturation on the rate of sexual conversion. We observe that basal levels of SRBs in the Vietnam cohort are significantly higher than in African cohorts, suggesting that parasites from Vietnam are already somehow sexually induced even before treatment. The high basal level of SRBs in Vietnam could be associated with the low transmission intensity, which previously was associated with high gametocyte density and prevalence in the field (20,217). The level of plasma LysoPC in all patients, which is not available in our study, could also explain the difference in the relative sexual ring density between study sites. In Ghanaian patients, low LysoPC levels have been shown to correlate with an increase in sexual rings density (91).

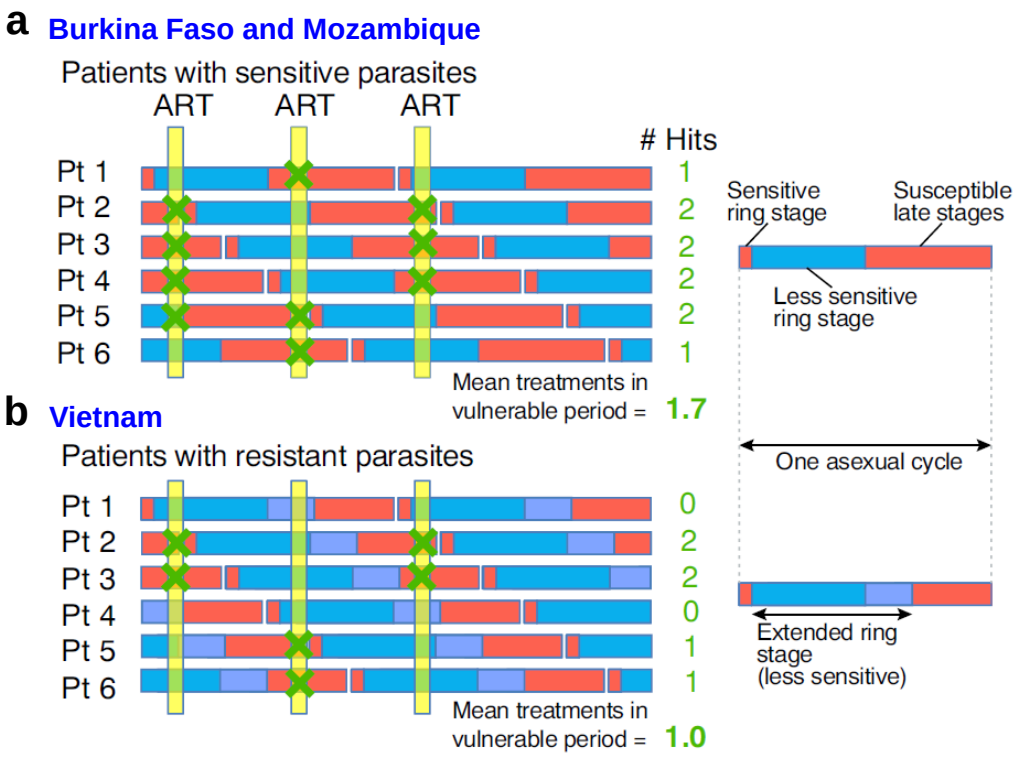

Figure 21. Model for the differences between PfK13 and wild-type parasite life cycle in patients. (a) Three doses regimen (yellow bars) can target vulnerable trophozoite stages (orange bars) at 50-70% likelihood. **(b)** Three doses regimen is more likely to miss vulnerable trophozoites in infections with resistant parasites because of the prolonged development of resistant ring stages. Figures and concept by Khoury et al., 2020 (302).

In relation to the spread of PfK13 mutations, previous work demonstrated that the delayed parasite clearance phenotype shows a high genetic heritability, indicating that artemisinin-resistant parasites are more likely to spread in the human population than wild-types (303). PfK13 mutant parasites were previously described in malaria patients with higher levels of gametocytemia in the field (20). This is specifically true in low transmission settings where low malaria immunity at the population level was found to associate with parasite delayed clearance and high gametocyte prevalence (22). These results are consistent with our observation of higher basal SRBs levels in delayed clearance parasites from Vietnam. Moreover, mature gametocytes produced from PfK13 mutants have been shown to be equally infectious with wild-types (304), and demonstrated to infect varied species of mosquito vector from Southeast Asia and Africa (305). Its male gametocytes have the ability to exflagellate even under artemisinin pressure (306). Altogether, while the main driver of the spread of artemisinin-resistant parasites in the Greater Mekong Subregion is their selective advantage in artemisinin-treated patients, their ability to transmit as efficiently as wild-type parasites may have contributed to their rapid expansion.

Although artemisinin resistance plus high transmission potential are a perfect combination for a malaria disaster, several other traits associated with K13 mutations and host factors may also be at play in balancing infectiousness and pathogenicity. For example, a population with naturally acquired immunity against malaria is associated with low gametocyte prevalence and less likelihood of PfK13 mutant spreading (22). Overall, our data suggest that artemisinin induction on sexual conversion will likely depend on the parasite stage dynamics. The probability of artemisinin-induced sexual conversion will be much higher in patients with predominant trophozoite stages. By contrast, in patients with predominant ring stages or in conditions where parasites have prolonged ring stage development, the artemisinin-induced sexual conversion is unlikely to occur. The diverse parasite stages at the individual patient level may also explain the variation of induction within each study cohort.

3. Public health relevance of induced sexual conversion

The public health implication of our results on the sexually inducing effect of artemisinin is twofold: (i) a paradox where subcurative artemisinin, the crucial component of the frontline antimalarial treatment, contributes to malaria transmission in certain events, and (ii) a more compelling reason to promote a strict policy on the proper use of antimalarial drugs. The efficacy of artemisinin has been historically compromised by incorrect dosing, poor treatment compliance, and the proliferation of poor quality drugs (26,307). These conditions can all contribute to subcurative drug plasma concentrations, which will likely induce sexual conversion in patients with parasites predominantly at the trophozoite stage, in addition to enabling the survival of some of the parasites. A study in Southeast Asia conducted between 2008 and 2009 revealed that 32% of antimalarial drugs sold in the market are of substandard quality (308). Consumption of counterfeit antimalarial drugs was motivated by their low price, which is also linked to poverty. In addition, adherence to a three-day ACT regimen has been shown to be up to only 40% in several malaria-endemic settings, as in the case of Tanzania and Ethiopia (26). These sub-optimal treatment-related behaviors are associated with low socioeconomic status, poor knowledge of malaria treatment, and the young age of caregiver and patient (26). In light of our new data on sexual conversion, it is reasonable to expect that such events may also promote malaria transmission. In fact, even without considering the inducing effect of artemisinin, a recent mathematical model predicted that the likelihood of malaria transmission from patients with inadequate adherence to the ACT regimen is more than twice that of completely-adherent patients, assuming it leads to treatment failure (309).

It is important to note that the impact of a complete ACT regimen on malaria transmission is overwhelmingly positive, as a result of its fast-clearing efficacy against asexual stages and partial efficacy against immature gametocytes (198,272,310–313). However, as we observed in our field study and also indicated in other studies, ACT never fully eliminates mature gametocyte carriage (314–317). Based on our *pfap2-g*

and *pfs25* data, we speculate that a fraction of the circulating mature gametocytes observed one week after the first treatment dose may come from the inducing effect of artemisinin on sexual conversion. It is also very likely that these induced gametocytes are physiologically capable of establishing mosquito infection, thereby contributing to onward malaria transmission (314,318), as also evident in our mosquito feeding assay.

One of the policy recommendations that may help ensure ACT adherence would be the Directly Observed Therapy (DOT), which has been proven effective in completing a treatment regimen for tuberculosis (TB) (319). It is also worth recognizing that DOT for TB does not come without complications and may be surrounded by concerns on ethics, the patient's burden to complete the regimen, and additional cost for the health system (320). In malaria treatments, DOT has already been practiced when administering primaquine (PQ) and therefore would also be feasible when giving ACT. PQ is the only antimalarial drug available with a gametocytocidal effect but is cautiously administered to patients with G6PD deficiency because of severe adverse reactions. There is no consensus on the proper timing of its administration, although it is commonly given after the ACT regimen to avoid compounding side effects (321). In relation to this, the application of specific SRBs may be used as a guide in targeted treatment approaches at the patient level or at the clustered population level, such as identifying which group shall receive a single-dose of PQ treatment on the following days (e.g., 5 to 7 days later). SRBs would also be very useful in the early detection and mapping of transmission hotspots (high-risk areas) and hotpops (high-risk population), which are of particular interest in targeted MDA and IRS during malaria elimination campaigns (322). It could also offer a direct surrogate method to evaluate new drugs and vaccines designed to block the earliest phase of sexual development, along with mature gametocyte analysis and mosquito feeding assays. Further research is however needed to determine the predictive value of SRBs for transmission potential, and to decide if SRBs should be used to inform policies in the fight against malaria.

Meanwhile, policymakers propose adopting ACT as a substitute for SP as chemoprevention during pregnancy because of serious widespread resistance to the latter (29). However, poor adherence to chemoprevention among pregnant women is well-documented in many countries (27) (**Chapter 5**), indicating that when ACT is adopted, it will suffer the same fate as SP. In the worst-case scenario, we will be dealing with both artemisinin-resistant and highly transmissible parasites. With no new antimalarial drug compounds in the pipeline, artemisinin-based treatment protocols, including parenteral artesunate monotherapy, remain the only antimalarial drugs that have very high efficacy against all forms of malaria infections. Unless a strict antimalarial drug treatment policy is implemented along with a strong community engagement, the adoption of ACT as chemoprevention may only be recommended in areas where parasites display resistance to other antimalarial compounds. Investments in and fast-tracking of antimalarial drug discovery and development are keys to solving these issues. Until no new drugs are available, expert opinions suggest increasing the dosage of ACT, extending the treatment period (323), and using triple combination as a stop-gap therapy (324), of course all taking into consideration the proper treatment use and adherence.

4. The role of community in malaria elimination initiatives

We consider the role of community members as essential actors and contributors of knowledge in successfully attaining the goal of malaria elimination. By examining community perceptions on malaria, including its prevention and control, we identify potential constraints and opportunities prior to the deployment of interventions designed for the malaria elimination initiative in the Magude district of Southern Mozambique. Here, malaria-related knowledge and practices are centered on vector control programmes, which have been the main malaria control interventions in the past years. We observed varied perceptions and behavior that will potentially influence the implementation of the malaria elimination campaign, and particular findings that were relevant because they

highlight some limitations of vector control tools and offer an opportunity for new interventions such as MDA.

First, participants are unaware of asymptomatic infections. Low awareness of the possibility of asymptomatic infection, whereby 'healthy' individuals carry malaria parasites, has been described as a major challenge in implementing MDA. To observe a significant result, MDA must reach at least 80% of the target population (50,51). Past studies have revealed that a no-symptom notion is likely to result in poor adherence and refusal to treatment (325,326). This has also been the case in the Magude project, although the failure to eliminate malaria in the district cannot be solely explained by one factor (36).

Second, participants described some events of residual transmissions, such as outdoor mosquito biting, which underscores the limitations of vector control focusing mainly on indoor settings. We consider this notion as an opportunity to highlight the advantage of using MDA, with messaging directed to its overall protection even against outdoor or residual malaria transmission. Indeed, some participants were open to new forms of chemoprophylaxis as an additional malaria preventive measure. Hence, MDA is expected to cover both residual transmission and asymptomatic individuals in the Magude. However, it is worth noting that a successful implementation of MDA may cause a negative impact on the natural protective immunity amongst asymptomatic individuals. While multiple MDAs can accelerate the reduction of parasite burden, it will also lessen the exposure of the population to the malaria parasite, which is essential to maintain protective immunity (327). This might lead to an increased risk of symptomatic malaria. During and after the elimination phase, an increased prevalence of clinical malaria cases must therefore be anticipated (86) and so the need to reinforce logistical support in health facilities.

Third, adherence to malaria chemoprevention and treatment was suboptimal for various reasons, which were mainly linked to structural barriers, such as transportation and distance. This implies the need to strengthen grassroots mobilization, focal messaging on the availability of a

chemopreventive program, addressing underlying causes of poor adherence, and a strict policy on monitoring antimalarial treatment regimen. Our findings also suggest the need for general strengthening of primary care that is accessible to the general population at risk. Overall, because malaria elimination efforts require a long-term commitment and a flexible strategy in the community, it is important to note that its success relies greatly on positive community acceptance and participation to any programmatic interventions (35,37,328).

5. The transdisciplinary approach in malaria elimination

"*No discipline knows more than all disciplines*" - Francois Taddai. Transdisciplinarity is defined as the amalgamation of different perspectives in order to identify, formulate, and resolve a shared problem (329). Transdisciplinary research is an ideal approach to grasp the complexity of a given problem that is usually 'persistent' or 'wicked' in nature (330). Malaria is considered a persistent problem embedded in an impoverished society as no single solution can solve its devastating impact on human health and economy; therefore, multilevel collaborations are needed to eliminate malaria (328). As malaria elimination becomes an immediate priority, achieving such a goal requires interventions that do not only cure the disease *per se* but also stop the spread of the causative agent (37). The spreading of malaria in a community relies on the presence of transmissible forms (gametocytes) so that understanding which factors affect gametocyte production would lay down the foundation of an improved version of a malaria elimination strategy. Here, our laboratory and field studies contribute to the knowledge gap on the impact of treatment on malaria transmission (**Chapter 3 and 4**), which has the potential to improve antimalarial drug treatment policy (**Figure 22**). However, it does not end there as previous experiences in malaria elimination efforts demonstrate that no new tool or policy has been completely successful due to unexpected shortcomings at the grassroots level. Therefore, this encourages transdisciplinary collaborations where both lay and scientific knowledge are equally taken together to improve

certain tools or strategies for a successful malaria elimination efforts. Our findings on the community perception study (**Chapter 5**) have contributed to the successful implementation of the malaria elimination project in the Magude district by identifying possible barriers of the new intervention (i.e., MDA) and finding the opportunity to promote the elimination project itself and the MDA intervention.

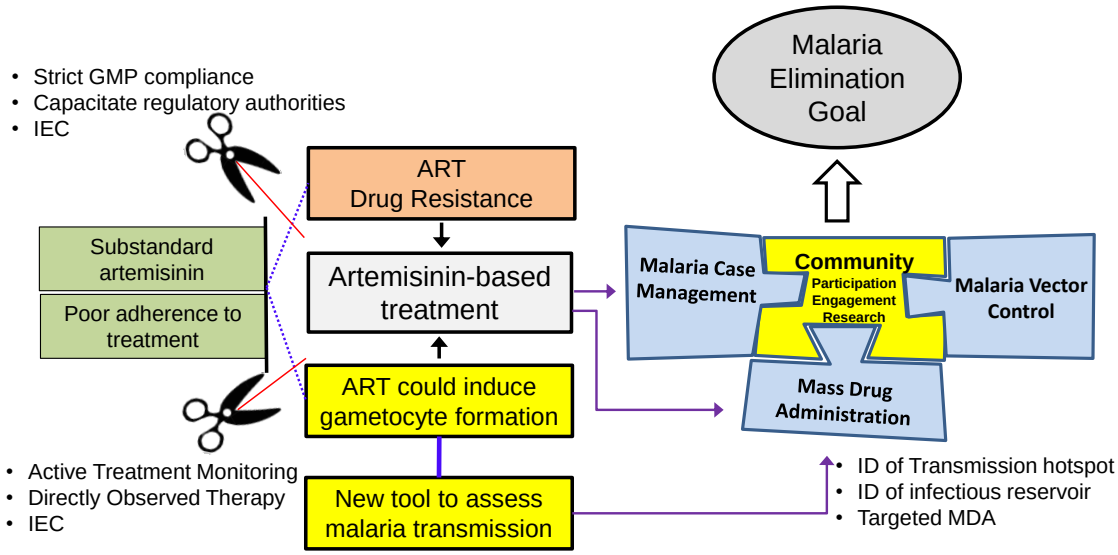

Figure 22. The contribution of this thesis to malaria elimination efforts. Malaria elimination efforts involve a tailored intervention to stop malaria transmission. The yellow boxes represent the potential contribution of this thesis to understanding malaria transmission and antimalarial drug treatment, and on the role of the community in malaria elimination initiative. Abbreviations: Good Manufacturing Practices (GMP); Information, Education and Communication (IEC) campaign; Artemisinin (ART); Identification (ID); Mass Drug Administration (MDA).

6. Strengths and limitations of the studies

Our *in vitro* results are based on multiple validated assays that apply both classical and modern molecular and cellular techniques. Our experiments on sexual conversion rates are tested in parasite lines of different sexual conversion phenotypes (i.e., NF54 and E5 genetic backgrounds). This makes our conclusions robust and reproducible. The impact of artemisinin on the enhanced sexual conversion rate was reproduced by another group in Leiden University Medical Center (Prof. Dr. C.J. Janse). Hence, our hypothesis for the field cohorts was based on our solid *in vitro* data. In field cohorts, we performed multiple sample

collections from three countries, which represent different transmission settings. This makes our results more complementary and informative. Similarly, our qualitative study included different respondents from different population groups, allowing the maximum representation of community members.

For the limitation of our *in vitro* study, there are some hypothesis-generating results that had to be set aside because they were beyond the scope of this thesis or due to time constraints. Some of the examples are the detailed molecular mechanisms underlying stage-dependent artemisinin-induced sexual conversion and the connection between artemisinin resistance and sexual conversion. As for the former, we have an on-going collaboration with a group from Monash University (Prof. Dr. D.J. Creek) to explore the mechanism of induced sexual conversion. One biological replicate of samples for metabolomics and proteomics analyses has already been collected after setting up the experimental protocol in ISGlobal. However, we need at least 3 biological replicates and send them to Creek Lab for metabolomics and proteomics analyses. It is also worth mentioning that the NF54 in our study has higher conversion rates than what is observed in natural infections.

In the field study, one of the main limitations is the absence of the untreated group aside from the treated group because of ethical considerations. Nevertheless, our sample collections are sufficient to address the main hypothesis. We also have limited samples of patients carrying wild-type parasites in Vietnam. This is the first exploratory study on sexual conversion in field conditions; thus, power and sample size estimations may not be properly calculated due to lack of a precedent study. Moreover, the accurate estimation of the number of sequestered parasites and/or parasite age in human infections was proven difficult to perform. This is relevant to address our hypothesis on stage-dependent sexual conversion.

Conclusions

1. We developed a new sexual conversion assay that is robust and time-efficient based on new transgenic lines that can accurately distinguish sexual forms of the malaria parasite *P. falciparum*.

2. Reporter parasite lines with the *gexp02* promoter driving the expression of a fluorescent marker can specifically distinguish sexual ring stages as early as 10-15 hours post-invasion. To the best of our knowledge, this is the first parasite line that can specifically distinguish sexual ring stages from the morphologically similar asexual ring stages.

3. The *gexp02* mRNA provides a new biomarker for sexual ring stages, and along with *pfap2-g* mRNA, shows great potential as a sexual ring biomarker in the field.

4. The impact of dihydroartemisinin treatment on the sexual conversion of *P. falciparum* is affected by artemisinin concentration, parasite stage, and parasite metabolic state related to LysoPC or choline levels.

5. Artemisinin exposure at the trophozoite stage to subcurative artemisinin doses upregulates the expression of *pfap2-g*, the master regulator of sexual commitment, and increases the rate of sexual conversion.

6. Artemisinin-induced sexual forms progress normally, are physiologically functional, and establish a patent mosquito infection.

7. Exposure of the trophozoite stage to subcurative chloroquine doses or after heat shock increases the rate of sexual conversion.

8. Ring stages are refractory to inducible sexual conversion. Choline depletion and exposure of the ring stage to artemisinin and chloroquine did not result in the induction of sexual conversion.

9. Our field cohort data illustrate the crucial role of biomarkers targeting sexual rings and mature gametocytes when characterizing the impact of antimalarial drugs on malaria transmission dynamics in naturally infected patients.

10. In our field cohort studies, there is a clear upregulation of the master regulator of sexual commitment *pfap2-g* and its select target genes (i.e., *gexp02*, *gexp5*, *pfg14-744*, and *pfs16*), suggesting an induced parasite sexual conversion immediately after artemisinin treatment in human infections.

11. Where artemisinin-sensitive parasites predominate, as in the case of Burkina Faso and Mozambique cohorts, an immediate upregulation of sexual ring biomarkers is generally evident after treatment.

12. Where artemisinin-resistant parasites predominate, as in the case of the Vietnam cohort, artemisinin-based treatment generally results in an immediate downregulation of sexual conversion. This suggests that PfK13 mutations influence the effect of artemisinin on parasite sexual conversion.

13. Our study on community perceptions of malaria contributes to the implementation of the malaria elimination initiative in the Magude District.

14. In the Magude district, potential barriers in implementing malaria elimination campaigns would include the lack of community awareness on asymptomatic carriers, insufficient knowledge on residual transmission, and poor care-seeking behavior.

15. In the Magude district, factors that may facilitate the malaria elimination program comprise excellent awareness of malaria disease, overwhelming trust in healthcare institutions, and openness to new chemoprophylaxis against malaria.

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Addendum

Summary in English

Introduction

The malaria disease caused by *Plasmodium falciparum* remains a major threat to human life (9). In 2018, 405 thousand people died from malaria out of ~228 million cases worldwide, and 67% of those who died were children below the age of 5 years (10). The disease burden of malaria is a result of repeated rounds of intraerythrocytic asexual replication, involving the parasite stages ring, trophozoite, and schizont. Ring stages circulate freely in the bloodstream while trophozoites and schizonts are sequestered in tissues, causing capillary blockage and organ failure (171).

To initiate malaria transmission, a small fraction of asexual parasites commit to sexual development in every intraerythrocytic cycle (74). Sexual differentiation is regulated by the transcription factor PfAP2-G (100,101). The expression of PfAP2-G signals the sexual commitment process, a cell state where parasites are programmed to undergo sexual development. Sexual commitment is followed by sexual conversion, which is marked by the expression of gametocyte-specific proteins absent from any replicating asexual stages (95). Sexual rings are the first stage after sexual conversion. They circulate in the bloodstream, and are characterized by the expression of the biomarkers GEXP02, GEXP5, Pfg14-744, and Pfs16. PfAP2-G is also expressed in sexual rings through Stage I gametocytes (104,118,143,146). Sexual rings develop inside the bone marrow as Stage I-IV gametocytes. They are released in the bloodstream as mature male and female Stage V gametocytes after 1 to 2 weeks. Mature gametocytes are the only parasite stage that can be transmitted to a female anopheline mosquito vector, and thus a priority target to eradicate malaria (171).

Malaria eradication means a malaria-free world that can be achieved through regionalized malaria elimination efforts (37). In light of this, understanding malaria transmission dynamics is relevant to the current malaria elimination efforts, with special emphasis on increasing coverage levels of vector control and effective antimalarial drug treatment. To date, artemisinin is

the only reliable antimalarial drug for malaria, and is the main drug component of the frontline treatment artemisinin-based combination therapy (ACT) (331). As one of the elimination tools, ACT has now been deployed in the field to interrupt malaria transmission through multiple rounds of mass drug administration (MDA) (36). The transmission-reducing potential of ACT underscores its outstanding efficacy against asexual stages, as well as its partial efficacy against Stage I to IV gametocytes (198,272,310–313). However, artemisinin and its derivatives are now failing in the Greater Mekong Subregion because of parasite resistance characterized by delayed parasite clearance and nonsynonymous mutations in the PfK13 propeller domain protein (261,263,265,266). In Africa, PfK13 mutations may also occur at a very low prevalence rate, but are not accompanied by delayed parasite clearance or treatment failure (266,268–270).

Antimalarial drugs have been proposed to increase gametocytemia in *P. falciparum in vitro* cultures (159,240–242) and rodent malaria parasites (178,243,244). However, different studies showed conflicting results and many of which did not address the impact of antimalarials on the sexual conversion rate, which is the proportion of parasites that convert into sexual forms at each round of replication. In natural infections, the impact of antimalarial drugs on the parasite's sexual conversion rate remains poorly understood. Reports of drug-induced sexual conversion on the basis of mature gametocyte carriage and density (237,245–247) do not necessarily reflect changes in sexual conversion rate upon treatment because of the inherent temporal dynamics of gametocyte development. Therefore, biomarkers independent of the dynamics of mature gametocytes could be valuable in evaluating the impact of antimalarial drugs on sexual conversion under field conditions (274); specific sexual ring biomarkers are under this category.

In this thesis, we combined both laboratory and field studies to investigate the impact of treatments on sexual conversion and to examine how community understanding of malaria could guide the utilization of new interventions. We first generated a robust sexual conversion assay and tested clinically relevant antimalarial drugs *in vitro* using this assay. Second, we examined the impact of frontline treatment against malaria on sexual conversion in naturally infected

patients. Last, we examined how community perceptions of malaria affect the deployment of interventions intended for malaria elimination efforts.

Results

Article 1: Reporter lines based on the *gexp02* promoter enable early quantification of sexual conversion rates in the malaria parasite *Plasmodium falciparum*

We describe new *Plasmodium falciparum* transgenic lines with fluorescent biomarkers under the control of different early gametocyte promoters, inserted in the genome using CRISPR-Cas9 technology. Our new gametocyte-reporter lines based on the *gexp02* promoter display clear advantages over previously available reporter lines. Firstly, the fluorescent reporter is detectable as early as 10–15 hpi, thereby distinguishing sexual rings from asexual rings and shortening the sexual conversion assay. Secondly, our genome-integrated constructs are stable and do not require drug selection pressure that can confound drug-related experiments. Lastly, our new transgenic lines are responsive to external conditions, such as choline depletion, making them suitable to study sexual conversion upon environmental stimuli. Here, we also propose a robust flow-cytometry-based sexual conversion assay using our parasite lines to measure basal and induced sexual conversion. We also validated *gexp02* mRNA as a highly specific biomarker for sexual stages, with potential application as a sexual ring biomarker in human infection studies.

Article 2: Artemisinin exposure at the ring or trophozoite stage impacts *Plasmodium falciparum* sexual conversion differently

We examine the impact of artemisinin on sexual conversion using our new reporter lines and an improved sexual conversion assay. We found that the sexual conversion rate increases by at least four folds in cultures exposed to a subcurative (5 or 10 nM) dihydroartemisinin (DHA) pulse at the trophozoite stage. This sexual induction operates by *pfap2-g* activation. Artemisinin-induced gametocytes are infectious to mosquito vectors and produce significantly more parasites inside the mosquito midgut. By contrast, a DHA pulse (5 or 10 nM) at the ring stage does not enhance sexual conversion, resulting in reduction of gametocyte density. This stage-dependent effect is also observed using other stimuli: chloroquine (CQ) exposure, choline depletion, and heat shock (HS).

Furthermore, in cultures where sexual conversion is already stimulated at the metabolic level by depletion of choline, exposure to DHA, CQ or HS does not result in an additional or synergistic enhancement of the sexual conversion rate.

Article 3: Expression dynamics of early markers of *Plasmodium falciparum* sexual conversion in naturally infected patients treated with artemisinin

We determine the impact of artemisinin-based combination therapy (ACT) and artesunate (AS) monotherapy on the sexual conversion of *P. falciparum* in naturally infected malaria patients from three independent longitudinal studies in Vietnam (n = 34), Burkina Faso (n = 30), and Mozambique (n = 45). We perform a time-course analysis of different sexual ring biomarkers (i.e., *pfap2-g*, *gexp02*, *gexp5*, *pfg14-744*, and *pfs16*) and mature gametocyte biomarkers (i.e., *pfs25* and *pfmget*) to evaluate the direct impact of ACT or AS. Our results show clear upregulation of *pfap2-g* and its select target genes immediately after artemisinin treatment, suggesting induction of sexual conversion. By comparing the three independent cohorts, we observe that parasites carrying PfK13 mutations or exhibiting a delayed clearance phenotype show lower upregulation or even downregulation of *pfap2-g* expression upon treatment. By contrast, PfK13 wild-types or parasites that display a fast clearance phenotype have high increases in *pfap2-g* expression levels. In the Vietnam cohort, induction of *pfap2-g* levels after treatment is associated with fast parasite clearance and a peak of mature gametocytes one week after treatment. Our results also show that several patients remain gametocyte carriers, and that female gametocytes may be more vulnerable to artemisinin than male gametocytes.

Article 4: Examining community perceptions of malaria to inform elimination efforts in Southern Mozambique: a qualitative study

Here we examine the local understanding of malaria and its prevention and control prior to the deployment of interventions in the Magude district of Southern Mozambique. Knowledge of malaria is connected to participants' awareness of malaria clinical presentation and to the on ongoing vector control programmes. However, community perceptions of malaria aetiology are fragmented and mainly linked to the mosquito-mediated transmission. Preventive measures reported mostly involve mosquito control, although

participants are aware of the limitations of vector control tools to protect against malaria. Fever and malaria-like symptoms trigger immediate care-seeking behavior by going to health facilities. However, awareness of asymptomatic malaria carriers and the risk of outdoor transmission are varied. Several opportunities that may influence the malaria elimination initiative include awareness of malaria, trust in health institutions, and openness for chemoprophylaxis. Factors that could jeopardize uptake include a lack of awareness of asymptomatic carriers, inadequate understanding of residual transmission, and barriers to care-seeking.

Discussion

To attain malaria elimination, gametocytes must be targeted to block further malaria transmission. The adaptive nature of malaria parasites suggests that enhanced gametocyte production is a response to adverse environments (242), with mounting evidence showing that external factors could modulate the parasite's sexual conversion rate (135). However, whether artemisinin can stimulate sexual conversion, which would result in increased production of functional gametocytes in *P. falciparum*, remains to be clarified. Using our newly developed sexual conversion assay and reporter parasite lines (**Article 1**), we demonstrated that sexual conversion is affected by a complex interplay between artemisinin concentration, parasite stage, and parasite metabolic state (**Article 2**). Our results imply that different parasite stages adapt to artemisinin differently: (i) artemisinin exposure at the trophozoite stage enhances sexual conversion; (ii) artemisinin exposure at the ring stage induces temporary latency (264,285) and 'reproductive restraint'; and (iii) mature Stage V gametocytes are metabolically resistant to artemisinin (272).

Based on the evolutionary theory for life history, it is predicted that subcurative doses of antimalarial drugs would result in the reduction of sexual conversion rates (reproductive restraint). Conversely, lethal doses of antimalarials are predicted to redirect all of the parasite resources to a maximum sexual conversion (terminal investment) (183). Our results do not completely follow these assumptions. Although reproductive restraint is observed after exposing the ring stage to subcurative doses, we also found a dramatic increase in the sexual conversion rate after exposing the trophozoite

stage to similar subcurative doses. Meanwhile, sexual conversion rates after exposure to lethal doses were relatively lower than after exposure to the subcurative doses, so that terminal investment did not seem to occur. We suspect that an additional reduction of its sexual conversion upon exposure to subcurative drug doses would not provide an advantage to the parasites, because *P. falciparum* sexual conversion rate is already low in natural infections (72,74). Our new data may therefore aid in the modification of the model predicted by the evolutionary theory for life history in human malaria parasites.

Our results also have generated a few hypotheses on the mechanism of induced sexual conversion. They suggest that induced sexual conversion by different stimuli is a consequence of signal transduction associated with moderate parasite stress rather than a 'state of dying' (183). This explains the parasite stage-dependent induction, the inducing effect of low artemisinin doses even without substantial parasite mortality, and the saturation of sexual conversion rates upon multiple stimuli. Our results may then provide clues on the possible mechanism of induced sexual conversion. First, parasites treated with DHA may have depleted its LysoPC or choline levels as a result of lipid membrane damage (289) and metabolic perturbation (290). LysoPC deprivation has been shown to upregulate the PMT pathway (135), which is believed to compete with the heterochromatin formation pathway by snatching essential elements (i.e., S-adenosylmethionine) needed for heterochromatin methylation and maintenance. This may cause *pfap2-g* activation and sexual conversion (194,195). One of the major caveats of this proposed mechanism is that it may assume a global loss of heterochromatin (not only in the *pfap2-g* locus), which has never been reported in *P. falciparum*. Second, the endoplasmic reticulum (ER) stress response has been previously linked to a higher gametocytemia in *P. falciparum* (232). Artemisinin is known to trigger severe ER stress in *P. falciparum* (258–260). Choline depletion may also trigger an ER stress response (291). ER stress is a result of accumulations of unfolded proteins in the ER, which induce persistent eIF2α phosphorylation (259). Several studies demonstrated that eIF2α phosphorylation is connected to protozoan stage transition, usually from proliferative stages to the latent stage under stressful conditions (296). Future work should therefore focus on how eIF2α

phosphorylation contributes to heterochromatin destabilization at the *pfap2-g* locus and sexual conversion.

Our field cohort data (**Article 3**) reveal a reverse association between PfK13 mutations and an increase in expression levels of *pfap2-g* immediately upon artemisinin treatment. The reason why PfK13 mutant parasites downregulate sexual conversion in our Vietnam cohort is possibly that they have already altered their developmental progression by producing parasite progenies with a delayed ring stage phenotype. Developmental prolongation of the ring stage have been well demonstrated as a constitutive trait of artemisinin-resistant parasites (260,300,301). The altered developmental characteristics of malaria parasites in Vietnam could be attributed to decades of artemisinin exposure that results in a shift of the vulnerability: the prolongation of ring-stage development and shortening of the trophozoite stage development (302). Ring stages are refractory to induction of sexual conversion and an extended ring stage in human infections could mean much higher chances that they are exposed to artemisinin than trophozoite stages. Hence, we observed downregulation of *pfap2-g* expression levels in PfK13 mutants (Vietnam cohort) and upregulation in PfK13 wild-types (African cohorts). Another possible explanation is based on our findings that sexually induced parasites cannot be additively or synergistically induced by different external factors. This suggests a state of saturation on the rate of parasite sexual conversion. We observe that basal levels of SRBs in the Vietnam cohort are significantly higher than in African cohorts, implying that parasites from Vietnam are already in a partially induced state of sexual conversion even before treatment. The high basal level of SRBs in Vietnam could be associated with being a low transmission setting and with the presence of PfK13 mutations, both previously linked to high gametocyte density and prevalence in the field (20,217).

Our results on the sexually inducing effect of artemisinin have two important public health implications: (i) a paradox where subcurative artemisinin may contribute to malaria transmission under certain conditions, and (ii) a compelling reason to promote a strict policy on the proper use of antimalarial drugs. Historically, the efficacy of artemisinin has been compromised by incorrect dosing, poor treatment compliance, and the proliferation of poor quality

drugs (26,307). Notably, the impact of a complete ACT regimen on malaria transmission is overwhelmingly positive. This is a result of ACT's fast-clearing efficacy against asexual stages and partial efficacy against immature gametocytes (198,272,310–313). However, ACT never fully eliminates mature gametocyte carriage (314–317). Based on our data, it is likely that a fraction of the circulating mature gametocytes observed one week after the first treatment dose may come from the inducing effect of artemisinin on sexual conversion (**Article 3**). It is also very likely that these artemisinin-induced gametocytes are physiologically capable of establishing mosquito infection, thereby contributing to onward malaria transmission (314,318), as also evident in our mosquito feeding assay.

Here we consider the role of community members in attaining the goal of malaria elimination (**Article 4**). In Magude, participants are unaware of asymptomatic infections. This is relevant in mass drug administration (MDA) as this strategy must reach at least 80% of the target population (50,51). Previous reports have shown that a no-symptom notion is likely to result in poor adherence and refusal to treatment (325,326). This has also been the case in the Magude project, although the failure to eliminate malaria in the district cannot be solely explained by one factor (36). Some of the participants' responses underscore the limitations of vector control focusing mainly on indoor settings. We consider this notion as an opportunity to highlight the advantage of using MDA, with messaging directed to its overall protection even against outdoor malaria transmission. Moreover, in Magude, adherence to malaria chemoprevention and treatment was suboptimal for various reasons. This implies the need to strengthen grassroots mobilization, focal messaging on the availability of a chemopreventive program, addressing underlying causes of poor adherence, and a strict policy on monitoring antimalarial treatment regimen. Our findings also suggest the need for general strengthening of primary care that is accessible to the general population at risk. Overall, because malaria elimination efforts require a long-term commitment and a flexible strategy in the community, it is important to note that its success relies greatly on positive community acceptance and participation in any programmatic interventions (35,37,328).

Resumen en Español (Summary in Spanish)

Introducción

La malaria, una enfermedad causada por el parásito *Plasmodium falciparum*, sigue siendo una gran amenaza para la vida humana (9). En 2018, 405 mil personas murieron de malaria entre ~ 228 millones de casos en todo el mundo; el 67% de los que murieron eran niños menores de 5 años (10). Los síntomas de la malaria son el resultado de rondas repetidas de replicación del parásito dentro de los eritrocitos, que involucran los siguientes estadios de desarrollo del parásito: anillo, trofozoíto y esquizonte. Los estadios de anillo circulan libremente en el torrente sanguíneo, mientras que los trofozoítos y los esquizontes son secuestrados en los tejidos, lo que puede causar bloqueo capilar e insuficiencia orgánica (171).

Para iniciar la transmisión de la malaria, en cada ciclo de crecimiento intraeritrocítico una pequeña fracción de los parásitos asexuales se "compromete" con el desarrollo sexual (74). La diferenciación sexual está regulada por el factor de transcripción PfAP2-G (100,101). La expresión de PfAP2-G inicia el proceso de compromiso sexual, un estado celular en el que los parásitos están programados para desarrollarse de manera sexual. El compromiso sexual va seguido de la conversión sexual, que está marcada por la expresión de proteínas específicas de los gametocitos ausentes de cualquier etapa asexual de replicación (95). Los anillos sexuales son el primer estadio de desarrollo del parásito después de la conversión sexual. Circulan en el torrente sanguíneo y se caracterizan por la expresión de los biomarcadores GEXP02, GEXP5, Pfg14-744 y Pfs16. PfAP2-G también se expresa en los anillos sexuales (104,118,143,146). Los anillos sexuales se desarrollan dentro de la médula ósea como gametocitos de estadio I a IV. Después de su maduración durante 1 a 2 semanas, se liberan al torrente sanguíneo como gametocitos maduros de estadio V masculinos o femeninos. Los gametocitos maduros son el único estadio del parásito que puede transmitirse a un mosquito vector

hembra anofelino y, por lo tanto, una diana prioritaria para erradicar la malaria (171).

La erradicación de la malaria en todo el planeta se puede lograr mediante esfuerzos regionalizados de eliminación de la enfermedad (37). Comprender la dinámica de transmisión de la malaria es relevante para el objetivo de eliminar la enfermedad, ya que actualmente las actividades para este fin se basan en mejorar el control de vectores y el tratamiento eficaz con medicamentos antimaláricos. Hasta la fecha, la artemisinina es el fármaco más fiable contra la malaria, y es el principal componente farmacológico del tratamiento de primera línea actual, la terapia combinada basada en artemisinina (ACT por sus siglas en inglés) (331). Como una de las herramientas de eliminación, ACT ahora se ha implementado en algunas zonas para interrumpir la transmisión de la malaria a través de múltiples rondas de administración masiva de medicamentos (MDA por sus siglas en inglés) (36). El potencial de ACT para reducir la transmisión se basa en su extraordinaria eficacia contra los estadios asexuales, así como en su eficacia parcial contra los gametocitos de estadios I a IV (198,272,310–313). Sin embargo, la artemisinina y sus derivados ahora están fallando en la subregión del Gran Mekong debido a que los parásitos han desarrollado resistencia, caracterizada por la eliminación retardada del parásito y mutaciones no sinónimas en la proteína PfK13 (261,263,265,266). En África, también se han observado mutaciones en PfK13, pero con una tasa de prevalencia muy baja, y además no van acompañadas de un retraso en la eliminación del parásito ni de fracaso del tratamiento (266,268–270).

Se ha propuesto que los fármacos antimaláricos aumentan la gametocitemia en cultivos *in vitro* de *P. falciparum* (159,240–242) y en modelos murinos de malaria (178,243,244), pero muchos estudios mostraron resultados contradictorios. En las infecciones naturales, el impacto de los medicamentos antimaláricos sobre la conversión sexual del parásito sigue siendo poco conocido. Algunos estudios han reportado que tras el tratamiento con antimaláricos aumenta la densidad de gametocitos maduros (237,245–247), pero esto no puede interpretarse como que inducen la tasa de conversión sexual (proporción de parásitos que se convierten en formas sexuales en cada ciclo de replicación), debido a la compleja dinámica temporal inherente de los

gametocitos. Por lo tanto, sería muy valioso disponer de biomarcadores que no dependan de la compleja dinámica de los gametocitos maduros, para poder evaluar el impacto de los fármacos antimaláricos en la conversión sexual en condiciones naturales (274). En este sentido, biomarcadores específicos de anillos sexuales podrían ser muy útiles, ya que son las fase más tempranas del desarrollo sexual y se encuentran en la circulación sanguínea.

En esta tesis, combinamos estudios de laboratorio y de campo para investigar el impacto de los tratamientos antimaláricos en la conversión sexual, y también examinamos cómo la comprensión comunitaria de la malaria podría guiar la utilización de nuevas intervenciones. Primero desarrollamos un ensayo de conversión sexual robusto y probamos el efecto de medicamentos antimaláricos clínicamente relevantes sobre la conversión sexual *in vitro*, utilizando este ensayo. En segundo lugar, examinamos el impacto del tratamiento de primera línea contra la malaria sobre la conversión sexual en pacientes infectados de manera natural. Por último, examinamos cómo las percepciones de la comunidad sobre la malaria afectan el despliegue de intervenciones destinadas a eliminar la malaria.

Resultados

Artículo 1: Reporter lines based on the *gexp02* promoter enable early quantification of sexual conversion rates in the malaria parasite *Plasmodium falciparum*

En este artículo describimos nuevas líneas transgénicas de *Plasmodium falciparum* que expresan biomarcadores fluorescentes bajo el control de diferentes promotores de gametocitos tempranos, insertados en el genoma mediante la tecnología CRISPR-Cas9. Nuestras nuevas cepas basadas en el promotor *gexp02* presentan claras ventajas sobre las líneas disponibles anteriormente. En primer lugar, el biomarcador fluorescente es detectable a las 10-15 h después de la invasión, lo que distingue los anillos sexuales de los asexuales y acorta el ensayo de conversión sexual. En segundo lugar, nuestras construcciones integradas en el genoma son estables y no requieren presión selectiva con fármacos, que podría alterar los resultados. Por último, nuestras nuevas líneas transgénicas responden a las condiciones externas, como falta

de colina en el medio de cultivo, lo que las hace adecuadas para estudiar la conversión sexual en respuesta a los estímulos ambientales. Además de desarrollar las cepas transgénicas, también describimos un ensayo de conversión sexual robusto, basado en citometría de flujo, para medir niveles de conversión sexual basales e inducidos. Por último, validamos el mRNA de *gexp02* como un biomarcador altamente específico para los estadios sexuales, con una posible aplicación como biomarcador de anillos sexuales para estudios epidemiológicos.

Artículo 2: Artemisinin exposure at the ring or trophozoite stage impacts *Plasmodium falciparum* sexual conversion differently

En este artículo examinamos el impacto de la artemisinina sobre la conversión sexual utilizando nuestras nuevas cepas de parásitos transgénicos y un ensayo de conversión sexual mejorado. Encontramos que la tasa de conversión sexual aumenta en al menos cuatro veces en cultivos expuestos a un pulso de dihidroartemisinina (DHA) subcurativo (5 o 10 nM) en el estadio de trofozoíto. Esta inducción sexual opera mediante la activación de *pfap2-g*. Los gametocitos inducidos por artemisinina son infecciosos para los mosquitos vectores, que cuando se alimentan con cultivos inducidos con DHA tienen un mayor nivel de parásitos dentro del intestino medio que cuando se alimentan con cultivos control. Por el contrario, un pulso de DHA (5 o 10 nM) en el estadio de anillo no aumenta la conversión sexual, sino que la disminuye. Esta situación, en que el efecto de DHA sobre la tasa de conversión sexual depende del estadio del parásito, también se observa con otros estímulos: exposición a cloroquina (CQ), falta de colina y choque térmico. Además, en cultivos donde la conversión sexual ya está estimulada a nivel metabólico por falta de colina, la exposición a DHA, CQ o choque térmico no da como resultado un aumento aditivo o sinérgico de la tasa de conversión sexual.

Artículo 3: Expression dynamics of early markers of *Plasmodium falciparum* sexual conversion in naturally infected patients treated with artemisinin

En este artículo determinamos el impacto de tratamientos que contienen artemisinina sobre la conversión sexual de *P. falciparum*, en pacientes con malaria infectados de manera natural que participaron en tres estudios

longitudinales independientes en Vietnam (n = 34), Burkina Faso (n = 30) y Mozambique (n=45). Realizamos un análisis temporal de diferentes biomarcadores de anillos sexuales (*pfap2-g*, *gexp02*, *gexp5*, *pfg14-744* y *pfs16*) y biomarcadores de gametocitos maduros (*pfs25* y *pfmget*) para evaluar el impacto directo de ACT o AS. Nuestros resultados muestran un claro aumento de los transcritos de *pfap2-g* y otros marcadores de anillos sexuales inmediatamente después del tratamiento con artemisinina, lo que sugiere que el tratamiento ha inducido la conversión sexual del parásito. Al comparar las tres cohortes independientes, observamos que los parásitos portadores de mutaciones en la proteína PfK13, que exhiben un fenotipo de eliminación lenta por artemisinina, no muestran activación de *pfap2-g* sino más bien silenciamiento. Por el contrario, los parásitos sin mutaciones en PfK13, que son eliminados por artemisinina de manera rápida, tienen un aumento de la expresión de *pfap2-g* tras el tratamiento. En la cohorte de Vietnam, la inducción de la expresión de *pfap2-g* después del tratamiento se asocia con el fenotipo de eliminación rápida del parásito, y con un pico de gametocitos maduros al cabo de una semana. Nuestros resultados también muestran que varios pacientes siguen siendo portadores de gametocitos bastantes días después del tratamiento, y que los gametocitos femeninos pueden ser más vulnerables a la artemisinina que los gametocitos masculinos.

Artículo 4: Examining community perceptions of malaria to inform elimination efforts in Southern Mozambique: a qualitative study

Aquí examinamos los conocimientos locales sobre la malaria y su prevención y control, antes del despliegue de intervenciones en el distrito de Magude en el sur de Mozambique. El conocimiento sobre la malaria de los participantes está relacionado con su preocupación por la presentación clínica, y se ha adquirido en parte a través de los programas en curso de control de vectores. Sin embargo, las percepciones de la comunidad sobre la etiología de la malaria están fragmentadas y principalmente vinculadas a la transmisión mediada por mosquitos. Las medidas preventivas contra la malaria implican principalmente el control de mosquitos, aunque los participantes son conscientes de las limitaciones de las herramientas de control de vectores para protegerse contra la malaria. La fiebre y los síntomas parecidos a la malaria

desencadenan un comportamiento de búsqueda inmediata de atención médica, llevándolos a acudir a los centros de salud. Sin embargo, el conocimiento sobre la existencia de portadores de malaria asintomáticos y del riesgo de transmisión al aire libre son limitados. Se ha identificado varias oportunidades para influir de manera positiva en las actividades de eliminación de la malaria, que incluyen la alta concienciación sobre la malaria, la confianza en las instituciones de salud y la buena disposición a tomar quimioprofilaxis. Los factores que podrían poner en peligro la participación en campañas de eliminación de la malaria incluyen la falta de conocimiento sobre la malaria asintomática, la poca comprensión de la transmisión residual y las dificultades para buscar atención médica.

Discusión

Para lograr la eliminación de la malaria, se debe atacar a los gametocitos para así bloquear la transmisión. La capacidad adaptativa de los parásitos de la malaria sugiere que los aumentos en la producción de gametocitos son una respuesta a entornos adversos (242), y existen cada vez más evidencias de que los factores externos pueden modular la tasa de conversión sexual del parásito (135). Sin embargo, queda por determinar si el tratamiento con artemisinina puede estimular la conversión sexual, lo cual podría dar lugar a una mayor producción de gametocitos funcionales en *P. falciparum*. Usando nuestro ensayo de conversión sexual recientemente desarrollado y nuevas líneas de parásitos transgénicos (**Artículo 1**), demostramos que la conversión sexual se ve afectada por una interacción compleja entre la concentración de artemisinina, el estadio de desarrollo del parásito y el estado metabólico del parásito (**Artículo 2**). Nuestros resultados implican que parásito en distintos estadios de desarrollo responden a la artemisinina de manera diferente: (i) la exposición a artemisinina en el estadio de trofozoíto aumenta la conversión sexual; (ii) la exposición a la artemisinina en el estadio de anillo induce latencia temporal (264,285) y disminuye la conversión sexual; y (iii) los gametocitos maduros en estadio V son metabólicamente resistentes a la artemisinina (272).

Según la teoría de la evolución para las historias de vida, se predice que las dosis subcurativas de medicamentos antimaláricos darían como resultado la

reducción de las tasas de conversión sexual (restricción reproductiva). Por el contrario, se prevé que las dosis letales de fármacos hagan que el parásito redirija todos sus recursos hacia una conversión sexual máxima (inversión terminal) (183). Nuestros resultados no siguen completamente estas predicciones teóricas. Aunque se observa restricción reproductiva (disminución de la tasa de conversión sexual) después de exponer parásitos en el estadio de anillo a dosis subcurativas de DHA, también observamos un aumento dramático en la tasa de conversión sexual después de exponer parásitos en el estadio de trofozoíto a estas dosis subcurativas del fármaco. Por otro lado, las tasas de conversión sexual después de exposición a dosis letales de DHA fueron más bajas que tras exposición a dosis subcurativas, por lo que la inversión terminal no pareció ocurrir. *P. falciparum* tiene de manera natural unas tasas de conversión sexual muy bajas; sospechamos que una restricción adicional en su tasa de conversión sexual tras exposición a dosis subcurativas de fármacos no proporcionaría una ventaja selectiva al parásito, (72,74). Por lo tanto, nuestros nuevos datos pueden ayudar a modificar las predicciones de la teoría de la evolución para historias de vida de los parásitos de la malaria humana.

A partir de los resultados de esta tesis, hemos podido generar algunas hipótesis sobre el mecanismo molecular de la conversión sexual inducida. Nuestros resultados sugieren que la conversión sexual inducida por diferentes estímulos es un proceso basado en transducción de señales asociadas a estrés moderado para el parásito, y no a un "estado de muerte inminente" (183). Este escenario concuerda con que la inducción dependa del estadio del parásito, con el efecto inductor de una dosis baja de artemisinina que prácticamente no produce mortalidad del parásito, y con la saturación de la tasa de conversión sexual ante múltiples estímulos. Nuestros resultados también dan algunas pistas sobre posibles rutas involucradas en la conversión sexual inducida. En primer lugar, los parásitos tratados con DHA podrían tener niveles bajos de LysoPC o colina como consecuencia de daños en la membrana lipídica (289) y perturbación metabólica (290) producidos por el fármaco. Se ha demostrado que la privación de LysoPC regula al alza la vía PMT (135), que se cree que compite con la formación de heterocromatina al

arrebatarle elementos esenciales (S-adenosilmetionina) necesarios para la metilación de histonas y mantenimiento de la heterocromatina, lo que provocaría la activación de *pfap2-g* y subsecuente conversión sexual (194,195). Una de las principales limitaciones de este modelo es que se esperaría que conllevara una pérdida global de heterocromatina (no solo en el locus *pfap2-g*), que no se ha observado en *P. falciparum*. En segundo lugar, la respuesta al estrés en el retículo endoplásmico (RE) se ha relacionado previamente con un aumento de la gametocitemia en *P. falciparum* (232). Se sabe que la artemisinina desencadena un estrés grave en el RE en *P. falciparum* (258–260). La falta de colina también puede desencadenar esta respuesta de estrés (291). El estrés del RE es el resultado de la acumulación de proteínas desplegadas en el RE, que inducen una fosforilación persistente de eIF2α (259). Varios estudios en otros protozoos han demostrado que la fosforilación de eIF2α está relacionada con la transición de la etapa proliferativa a la etapa latente, bajo condiciones de estrés (296). Por lo tanto, estudios futuros deberían investigar si la fosforilación de eIF2α contribuye a la desestabilización de la heterocromatina en el locus *pfap2-g* y a la conversión sexual.

Los datos que hemos obtenido en el estudio epidemiológico (**Artículo 3**) revelan una asociación inversa entre mutaciones en PfK13 y un aumento en los niveles de expresión de *pfap2-g* inmediatamente después del tratamiento con artemisinina. Una posible razón por la que los parásitos mutantes para PfK13 regulan negativamente la conversión sexual después del tratamiento en la cohorte de Vietnam es que han alterado su progresión a lo largo del ciclo asexual, de manera que el estadio de anillo se ve prolongado. La prolongación del estadio de anillo en parásitos resistentes a artemisinina está bien demostrada (260,300,301), y puede ser una adaptación del parásito tras décadas de exposición a la artemisinina: los estadios de anillo son menos sensibles a artemisinina, por lo tanto, al extender la duración del estadio de anillo y reducir la del estadio de trofozoíto (muy sensible a artemisinina), disminuye la ventana temporal en que el parásito es más susceptible a artemisinina (302). En los estadios de anillo la artemisinina no induce la conversión sexual, sino al contrario, y un aumento de la duración de este estadio en infecciones humanas aumenta las posibilidades de que la droga (de

corta duración en la sangre) se encuentre con anillos, y no con trofozoítos. Por lo tanto, esto puede explicar que en mutantes de PfK13 (cohorte de Vietnam) observamos una disminución de los niveles de expresión de *pfap2-g* tras el tratamiento, y en cambio en parásitos sin mutaciones en PfK13 (cohortes africanas) observamos una activación. Otra explicación para la distinta respuesta entre parásitos con o sin mutaciones en PfK13 se basa en nuestros hallazgos de que los parásitos inducidos sexualmente no pueden ser inducidos de manera aditiva o sinérgica por otro factor externo. Esto sugiere que existe un nivel de saturación en la tasa de conversión sexual del parásito. Observamos que los niveles basales de SRB en la cohorte de Vietnam son significativamente más altos que en las cohortes africanas, lo que implica que en los parásitos de Vietnam la conversión sexual está parcialmente inducida de forma constitutiva, incluso antes del tratamiento, y esto podría inhibir la inducción posterior por artemisinina. El alto nivel basal de SRB en Vietnam podría ser debido al entorno de baja transmisión y a la presencia de mutaciones de PfK13; ambos factores han sido previamente asociados a una alta densidad y prevalencia de gametocitos (20,217).

Nuestros resultados mostrando un efecto de la artemisinina sobre conversión sexual tienen dos importantes implicaciones para la salud pública: (i) es paradójico que la artemisinina, en dosis subcurativas, podría contribuir a aumentar la transmisión de la malaria en ciertas condiciones, y (ii) nuestros resultados resaltan la importancia de promover una política estricta sobre el uso adecuado de antimaláricos. Históricamente, la eficacia de la artemisinina se ha visto comprometida por la dosificación incorrecta, el mal cumplimiento de las pautas de tratamiento y la proliferación de fármacos de mala calidad (26,307). Cabe destacar que el impacto de un régimen completo de ACT sobre la transmisión de la malaria es claramente beneficioso, ya que los ACTs eliminan los parásitos asexuales de manera eficaz, y también tienen un efecto parcial sobre los gametocitos inmaduros (198,272,310–313). Sin embargo, los ACTs nunca eliminan por completo todos los gametocitos maduros (314–317). Según nuestros datos, es probable que una fracción de los gametocitos maduros circulantes observados una semana después de la primera dosis de tratamiento con ACTs pueda atribuirse al efecto inductor de la artemisinina

sobre la conversión sexual. Según nuestros resultados alimentando mosquitos con gametocitos formados tras estimulación por artemisinina, también es muy probable que estos gametocitos inducidos por artemisinina sean fisiológicamente capaces de infectar mosquitos, contribuyendo así a la transmisión de la malaria (314,318).

También hemos estudiado el papel de los miembros de la comunidad en el objetivo de eliminar la malaria (**Artículo 4**). En Magude, los participantes tienen muy poco conocimiento sobre las infecciones asintomáticas. Esto es relevante para la administración masiva de medicamentos (MDA), ya que esta estrategia debe llegar al menos al 80% de la población para contribuir de manera eficaz al objetivo de eliminar la malaria (50,51). Informes anteriores han demostrado que una noción de ausencia de síntomas probablemente resulta en una mala adherencia y rechazo al tratamiento (325,326). Este también ha sido el caso del proyecto Magude, aunque el fracaso en los intentos de eliminar la malaria en el distrito no puede explicarse únicamente por un factor (36). Algunas de las respuestas de los participantes subrayan las limitaciones del control de vectores, que se centra principalmente en entornos de interior. Consideramos esta noción como una oportunidad para resaltar las ventajas de usar MDA, ya que puede proporcionar protección general incluso contra la transmisión de la malaria al aire libre. Además, en Magude, el cumplimiento del tratamiento profiláctico y el tratamiento clínico no fue óptimo por varias razones. Esto implica la necesidad de fortalecer la concienciación de la población, de explicar con mensajes focalizados la disponibilidad de los tratamientos profilácticos, de abordar las causas subyacentes de la mala adherencia a los tratamientos, y de poner en marcha una política estricta de monitoreo del régimen de tratamiento contra la malaria. Nuestros hallazgos también sugieren la necesidad de un fortalecimiento general de la atención primaria para hacerla más accesible a la población. En general, debido a que los esfuerzos de eliminación de la malaria requieren un compromiso a largo plazo y una estrategia flexible en la comunidad, es importante señalar que su éxito depende en gran medida de la aceptación y participación con actitud positiva de la comunidad, como en cualquier intervención programática (35,37,328).

Samenvatting in het Nederlands (Summary in Dutch)

Inleiding

De ziekte malaria, veroorzaakt door *Plasmodium falciparum*, blijft een grote bedreiging voor de mensheid (9). In 2018 stierven 405 duizend mensen aan malaria op een totaal van 228 miljoen gevallen wereldwijd, 67% van de doden waren kinderen jonger dan 5 jaar (10). De ziektelast van malaria is het resultaat van herhaalde rondes van erytrocytaire replicatie, waarbij de ringstadia van de parasiet, trofozoïeten en schizonten betrokken zijn. Ringstadia circuleren vrij in de bloedbaan, terwijl trofozoïeten en schizonten worden gesequestreerd in weefsels waar zij capillaire blokkering en orgaanfalen veroorzaken (171).

Om malaria-overdracht op gang te brengen, zet in elke erytrocytaire cyclus een klein deel van de aseksuele parasieten zich in voor seksuele ontwikkeling (74). Seksuele differentiatie wordt gereguleerd door de transcriptiefactor PfAP2-G (100,101). De expressie van PfAP2-G geeft het proces van seksuele toewijding aan, een celtoestand waarin parasieten zijn geprogrammeerd om seksuele ontwikkeling te ondergaan. Seksuele toewijding wordt gevolgd door seksuele conversie, die wordt gekenmerkt door de expressie van gametocyt-specifieke eiwitten die afwezig zijn in replicerende aseksuele stadia (95). Seksuele ringen zijn de eerste seksueel geconverteerde parasieten die in de bloedbaan circuleren en worden gekenmerkt door de expressie van de biomarkers GEXP02, GEXP5, Pfg14-744 en Pfs16. PfAP2-G komt ook tot expressie vanaf seksuele ringen tot stadium I-gametocyten (104,118,143,146). Seksuele ringen ontwikkelen zich in het beenmerg als gametocyten van stadium I-IV. Ze komen na 1 tot 2 weken vrij in de bloedbaan als volwassen mannelijke en vrouwelijke stadium V-gametocyten. Rijpe gametocyten zijn het enige parasietstadium dat kan worden overgedragen op een vrouwelijke Anopheles vector, en dus een prioritair doelwit om malaria uit te roeien (171).

Uitroeiing van malaria betekent een malariavrije wereld die kan worden bereikt door middel van regionaal gerichte inspanningen om malaria te elimineren (37). In het licht hiervan is het begrijpen van de dynamiek van malaria-overdracht relevant voor de huidige pogingen tot eliminatie van malaria, met name het vergroten van de dekkingsgraad van vectorcontrole en effectieve antimalariamiddelen. Tot op heden is artemisinine het enige betrouwbare anti-malariageneesmiddel, en de belangrijkste component van eerstelijnsbehandeling met op artemisinine gebaseerde combinatietherapie (ACT) (331). ACT wordt thans in het veld ingezet als eliminatiemiddel teneinde de overdracht van malaria te onderbreken middels meerdere rondes van mass drug administration (MDA) (36). Het transmissieverminderend potentieel van ACT berust op de uitstekende werkzaamheid tegen aseksuele stadia, evenals zijn gedeeltelijke werkzaamheid tegen gametocyten van stadium I tot IV (195,269,311-314). Artemisinine en zijn derivaten schieten nu echter tekort in de Greater Mekong Subregion vanwege parasitaire resistentie die wordt gekenmerkt door vertraagde parasitaire klaring en niet-nonieme mutaties in het PfK13-propellerdomein (198,272,310–313). In Afrika kunnen PfK13-mutaties ook voorkomen met een zeer lage prevalentie, maar deze gaan niet gepaard met een vertraagde klaring van parasieten of falen van de behandeling (266,268–270).

Onderzoeken suggereren dat antimalariamiddelen de gametocytenvorming stimuleren in in-vitro kweken van *P. falciparum* (159,240–242) en bij malariaparasieten bij knaagdieren (178,243,244), hoewel de resultaten deels tegenstrijdig zijn. Bij natuurlijke infecties blijft de impact van antimalariamiddelen op de seksuele conversie van de parasiet slecht begrepen. Bevindingen van door geneesmiddelen geïnduceerde seksuele conversie op basis van dragerschap en dichtheid van rijpe gametocyten (237,245–247) weerspiegelen niet noodzakelijkerwijs veranderingen in seksuele conversiesnelheid bij behandeling, dit vanwege de inherente temporele dynamiek van gametocyt. Daarom kunnen biomarkers die onafhankelijk zijn van de dynamiek van volwassen gametocyten waardevol zijn bij het evalueren van de impact van antimalariamiddelen op seksuele conversie onder

veldomstandigheden (274): specifieke seksuele ringbiomarkers vallen onder deze categorie.

In dit proefschrift hebben we laboratorium- en veldstudies gecombineerd om de impact van behandelingen op seksuele conversie te onderzoeken en om te onderzoeken hoe het begrip van malaria door de gemeenschap het gebruik van nieuwe interventies bepaalt. We hebben eerst een robuuste seksuele conversietest gegenereerd en met behulp daarvan *in vitro* klinisch relevante antimalariamiddelen getest. Vervolgens onderzochten we de impact van eerstelijnsbehandeling van malaria op seksuele conversie bij natuurlijk geïnfecteerde patiënten. Ten slotte onderzochten we hoe de perceptie van malaria door de gemeenschap de inzet van interventies die bedoeld zijn om malaria te elimineren, beïnvloedt.

Resultaten

Artikel 1: Reporterlijnen op basis van de *gexp02*-promotor maken vroege kwantificering mogelijk van seksuele conversiepercentages bij de malariaparasiet *Plasmodium falciparum*

We beschrijven nieuwe transgene lijnen van *Plasmodium falciparum* met fluorescerende biomarkers onder de controle van verschillende vroege gametocytpromoters, ingebracht in het genoom via de CRISPR-Cas9-technologie. Onze nieuwe lijnen gebaseerd op de *gexp02* promotor vertonen duidelijke voordelen ten opzichte van eerder beschikbare parasietlijnen. Ten eerste is de fluorescerende biomarker al detecteerbaar vanaf 10–15 hpi, waardoor seksuele ringen worden onderscheiden van aseksuele ringen en de seksuele conversietest wordt verkort. Ten tweede zijn onze genoom-geïntegreerde constructen stabiel en vereisen ze geen selectiedruk op geneesmiddelen die geneesmiddel-gerelateerde experimenten kunnen verstoren. Ten slotte passen onze nieuwe transgene lijnen zich aan externe omstandigheden aan, zoals cholinebeperking, waardoor ze geschikt zijn om het effect van omgevingsstimuli op seksuele conversie te bestuderen. Hier stellen we ook een robuuste, op flowcytometrie gebaseerde, seksuele conversietest voor om met behulp van onze parasietlijnen basale en geïnduceerde conversies te meten. We hebben ook *gexp02*-mRNA gevalideerd als een zeer specifieke

biomarker voor seksuele stadia, met mogelijke toepassing als seksuele ringbiomarker in onderzoeken naar menselijke infectie.

Artikel 2: Blootstelling aan artemisinine in het ring- dan wel het trofozoietstadium heeft verschillende invloed op de seksuele conversie van *Plasmodium falciparum*

We onderzochten de impact van artemisinine op seksuele conversie met behulp van onze nieuwe parasietlijnen en verbeterde seksuele conversietest. We ontdekten dat de seksuele conversiesnelheid) in het trofozoietstadium minstens vier keer toeneemt in kweken na blootstelling aan een subcuratieve (5 of 10 nM) dihydroartemisinine (DHA) puls. Deze seksuele inductie werkt door activering van *pfap2-g*. Door artemisinine geïnduceerde gametocyten zijn hoger in aantal dan in de controlegroep. Ze zijn ook besmettelijk voor muggenvectoren en produceren significant grote aantallen infectieuze stadia in de middendarm van de mug. Daarentegen verbetert een DHA-puls (5 of 10 nM) in het ringstadium de seksuele conversie niet, en resulteert deze in een vermindering van de gametocytendichtheid. Deze fase-afhankelijke inductie wordt ook waargenomen op basis van van andere stimuli: blootstelling aan chloroquine (CQ), uitputting van choline en hitteschok (HS). Bovendien resulteert blootstelling aan DHA of CQ of HS niet tot een aanvullende of synergetische verbetering van de seksuele conversie in kweken waarin seksuele conversie al op metabool niveau wordt gestimuleerd door uitputting van choline.

Artikel 3: Expressiedynamiek van seksuele biomarkers van *Plasmodium falciparum* na behandeling met artemisinine bij natuurlijk geïnfecteerde patiënten

We bepaalden de impact van op artemisinine gebaseerde combinatietherapie (ACT) en artesunaat (AS) monotherapie op de seksuele conversie van *P. falciparum* bij natuurlijk geïnfecteerde malariapatiënten uit drie onafhankelijke longitudinale onderzoeken in Vietnam (n = 34), Burkina Faso (n = 30) en Mozambique (n = 45). We voerden een tijdsverloopanalyse uit van verschillende biomarkers voor seksuele ringen (d.w.z. *pfap2-g, gexp02, gexp5, pfg14-744* en *pfs16*) evenals volwassen gametocytenbiomarkers (d.w.z. *pfs25* en *pfmget*) om de directe impact van ACT of AS te evalueren. Onze resultaten

tonen een duidelijke opregulatie van *pfap2-g* en geselecteerde doelwitgenen onmiddellijk na behandeling met artemisinine, wat duidt op inductie van seksuele omzetting van parasieten. Door de drie onafhankelijke cohorten te vergelijken, zien we dat parasieten die PfK13-mutaties dragen of een fenotype met vertraagde klaring vertonen lagere *pfap2-g*-expressieniveaus vertonen. Daarentegen hebben PfK13-wildtypen of parasieten die een fenotype met snelle klaring vertonen hoge *pfap2-g*-expressieniveaus. In alle drie de cohorten is inductie van *pfap2-g*-spiegels na behandeling geassocieerd met een snelle klaring van parasieten en AS monotherapie. Onze resultaten laten ook zien dat verscheidene patiënten gametocytendragers blijven, en dat vrouwelijke gametocyten mogelijk kwetsbaarder zijn voor artemisinine dan mannelijke gametocyten.

Artikel 4: Onderzoek naar de perceptie van malaria door de gemeenschap om de eliminatie-inspanningen in Zuid-Mozambique vorm te geven: een kwalitatieve studie

Hier onderzochten we de lokale opvattingen over malaria en de preventie en bestrijding ervan voorafgaand aan de inzet van interventies in het Magude-district in Zuid-Mozambique. Kennis van malaria is verbonden met het besef van deelnemers over de klinische presentatie van malaria, men name met betrekking tot lopende vectorbestrijdingsprogramma's. De percepties van de gemeenschap over de etiologie van malaria zijn echter gefragmenteerd en houden voornamelijk verband met de door muggen veroorzaakte overdracht. De preventieve maatregelen die men kent of toepast hebben vooral betrekking op muggenbestrijding, hoewel de deelnemers zich bewust zijn van de beperkte bescherming tegen malaria van vectorbestrijdingsmaatregelen. Bij koorts en malaria-achtige symptomen zoekt men onmiddellijk zorg door naar gezondheidsinstellingen te gaan. Het besef over asymptomatische malariadragers en het risico op overdracht buitenshuis is echter wisselend. Factoren die het malaria-eliminatie-initiatief kunnen beïnvloeden, zijn onder meer besef over malaria, vertrouwen in gezondheidsinstellingen en het openstaan voor chemoprofylaxe. Factoren die de toepassing van interventies in gevaar kunnen brengen, zijn onder meer gebrekkig besef over asymptomatische dragers, onvoldoende begrip van resterende overdracht en belemmeringen voor het zoeken naar zorg.

Discussie

Eliminatie van malaria vereist het gerichte aanpak van gametocyten teneinde verdere malaria-overdracht te blokkeren. De adaptieve aard van malariaparasieten suggereert dat de productie van gametocyten een reactie is op ongunstige omgevingsomstandigheden (242), met steeds meer bewijs dat aantoont dat externe factoren de mate van seksuele conversie van de parasiet kunnen moduleren (135). Of artemisinine seksuele conversie kan stimuleren, wat zou resulteren in een verhoogde productie van functionele *P. falciparum* gametocyten, moet echter nog worden opgehelderd. Met behulp van onze nieuw ontwikkelde seksuele conversie-assay en reporterparasietlijnen (**Artikel 1**), hebben we aangetoond dat seksuele conversie wordt beïnvloed door een complex samenspel tussen artemisinineconcentratie, parasietstadium en metabole toestand van de parasiet (**Artikel 2**). Onze resultaten impliceren dat verschillende parasietstadia zich op verschillende manieren aanpassen aan artemisinine: (i) blootstelling aan artemisinine in het trofozoïetenstadium bevordert de seksuele conversie; (ii) blootstelling aan artemisinine in het ringstadium leidt tot tijdelijke latentie (264,285) en 'reproductieve beperking'; en (iii) rijpe gametocyten van stadium V zijn metabool resistent tegen artemisinine (272).

Op grond van de evolutietheorie voor *life history* wordt voorspeld dat subcuratieve doses antimalariamiddelen zouden resulteren in een verlaging van de seksuele conversie (reproductieve beperking). Omgekeerd wordt voorspeld dat dodelijke doses antimalariamiddelen alle de parasiet ter beschikking staande middelen zullen omleiden naar een maximale seksuele conversie (terminale investering) (183). Onze resultaten volgen deze aannames niet volledig. Hoewel we aanwijzingen zagen voor reproductieve beperking na blootstelling van ringstadia aan subcuratieve doses, vonden we ook een dramatische toename van de seksuele omzettingssnelheid na blootstelling van het trofozoietstadium aan vergelijkbare subcuratieve doses. Tegelijkertijd waren de seksuele conversiepercentages relatief lager na blootstelling aan dodelijke doses dan aan subcuratieve doses, zodat er geen terminale investering leek plaats te vinden. We vermoeden dat een extra beperking van de seksuele conversie bij blootstelling aan subcuratieve geneesmiddeldoses geen voordeel

zou opleveren omdat de seksuele conversie van *P. falciparum* bij natuurlijke infecties reeds laag is (72,74). Onze nieuwe gegevens kunnen daarom helpen bij de wijziging van het voorspellingsmodel van de evolutietheorie voor de *life history* van menselijke malariaparasieten.

Onze resultaten hebben ook enkele hypothesen opgeleverd over het mechanisme van geïnduceerde seksuele conversie. Onze resultaten suggereren dat geïnduceerde seksuele conversie door verschillende stimuli een gevolg is van signaaltransductie geassocieerd met matige parasitaire stress in plaats van een 'staat van sterven' (183). Dit verklaart de fase-afhankelijke inductie van parasieten, het inducerende effect van een lage dosis artemisinine, zelfs zonder substantiële sterfte door parasieten, en de verzadiging van de seksuele conversiesnelheid na meerdere stimuli. Onze resultaten kunnen dan inzichten geven in het mogelijke mechanisme van geïnduceerde seksuele conversie. Ten eerste kunnen parasieten die met DHA zijn behandeld hun LysoPC- of cholinegehalte hebben uitgeput als gevolg van lipidemembraanbeschadiging (289) en metabole verstoring (290). Van LysoPC-deprivatie is aangetoond dat het de PMT-route opreguleert (135), waarvan wordt aangenomen dat het in competitie is met de heterochromatinevorming door het wegpikken van essentiële elementen (bijv. S-adenosylmethionine) die nodig zijn voor heterochromatinemethylering en -onderhoud, hetgeen *pfap2-g*-activering en seksuele conversie veroorzaakt (194,195). Een van de belangrijkste kanttekeningen bij dit voorgestelde mechanisme is dat het mogelijk een globaal verlies van heterochromatine (niet alleen in de *pfap2-g* locus) veronderstelt, wat nooit is gerapporteerd in *P. falciparum*. Ten tweede is in eerdere studies de stressrespons van het endoplasmatisch reticulum (ER) bij *P. falciparum* in verband gebracht met toegenomen gametocytemie (232). Van artemisinine is bekend dat het bij *P. falciparum* ernstige ER-stress veroorzaakt (258–260). Choline-uitputting kan ook een ER-stressreactie veroorzaken (291). ER-stress is een gevolg van opeenhopingen van ongevouwen en meervoudig geubiquineerde eiwitten in het ER, die een aanhoudende eIF2α-fosforylering induceren (259). Verschillende onderzoeken hebben aangetoond dat eIF2α-fosforylering verband houdt met protozoaire stadiumtransitie, meestal van het proliferatieve naar het latente stadium onder stressvolle omstandigheden (296).

Toekomstig onderzoek zou daarom moeten focussen op hoe eIF2α-fosforylering bijdraagt aan heterochromatine-destabilisatie in het *pfap2-g*-locus.

Onze cohortgegevens laten een omgekeerde associatie zien tussen PfK13-mutaties en toename in expressieniveaus van *pfap2-g* onmiddellijk na behandeling met artemisinine. De reden waarom PfK13-mutante parasieten in Vietnam hun seksuele conversie neer-reguleren is mogelijk dat ze hun ontwikkelingsprogressie al hebben veranderd door het produceren van parasitaire nakomelingen met een vertraagd ringstadium fenotype. Verlenging van de ontwikkelingsduur van ringstadia zijn aangetoond, zelfs na blootstelling aan artemisinine (260,300,301). Het veranderde ontwikkelingspatroon van malariaparasieten in Vietnam zou kunnen worden toegeschreven aan decennia van blootstelling aan artemisinine. Die blootstelling heeft dan geresulteerd in een verschuiving van het *window of vulnerability* in hun voordeel: verlenging van de ontwikkeling van het ringstadium en verkorting van de ontwikkeling van het trofozoietstadium (302). Omdat ringstadia ongevoelig zijn voor het induceren van seksuele conversie, kan een langere duur van ringstadia bij humane infecties een veel grotere kans op blootstelling aan artemisinine inhouden in vergelijking tot trofozoietstadia. Dat verklaart onze waarneming van neerwaartse regulering van pfap2-g-expressieniveaus in PfK13-mutanten (Vietnam-cohort) en opwaartse regulering in PfK13-wildtypen (Afrikaanse cohorten). Een laatste verklaring is gebaseerd op onze bevindingen dat seksueel geïnduceerde parasieten niet aanvullend of synergetisch kunnen worden geïnduceerd door een andere externe factor. Dit suggereert een verzadigingstoestand van de snelheid van parasitaire seksuele conversie. We zien dat de basale niveaus van SRB's in het Vietnam-cohort significant hoger zijn dan in Afrikaanse cohorten, wat impliceert dat parasieten uit Vietnam zelfs vóór de behandeling al seksueel worden geïnduceerd. Het hoge basale niveau van SRB's in Vietnam zou in verband kunnen worden gebracht met een lage transmissie setting en de aanwezigheid van PfK13-mutaties; beide zijn eerder in verband gebracht met hoge gametocytendichtheid en prevalentie (20,217).

Onze resultaten met betrekking tot het seksueel inducerende effect van artemisinine hebben twee belangrijke implicaties voor de volksgezondheid: (i) een paradox waarin onder zommige omstandigheden subcuratieve artemisinine

bijdraagt aan de overdracht van malaria, en (ii) een dwingende reden om een strikt beleid voor het juiste gebruik van antimalariamiddelen te promoten. Historisch gezien is de werkzaamheid van artemisinine in het gedrang gekomen door onjuiste dosering, slechte therapietrouw en de proliferatie van geneesmiddelen van slechte kwaliteit (26,307). Met name volledige ACT behandeling heeft een overweldigend positieve impact op de overdracht van malaria. Dit is een gevolg van snelle klaring door ACT van aseksuele stadia en de gedeeltelijke werkzaamheid tegen onrijpe gametocyten (198,272,310–313). ACT elimineert echter nooit volledig het dragerschap van rijpe gametocyten (314–317). Op basis van onze gegevens is het waarschijnlijk dat een fractie van de circulerende rijpe gametocyten die een week na de eerste behandelingsdosis worden waargenomen, afkomstig kan zijn van het inducerende effect van artemisinine op seksuele conversie (**Artikel 3**). Het is ook zeer waarschijnlijk dat deze geïnduceerde gametocyten fysiologisch in staat zijn muggeninfecties te veroorzaken, waardoor ze bijdragen aan de verdere overdracht van malaria (314,318), zoals ook blijkt uit onze muggenvoedingstest.

Hier bekijken we de rol van de mens als lid van de gemeenschap bij het bereiken van het doel van malaria-uitroeiing (**Artikel 4**). In Magude zijn de deelnemers zich niet bewust van asymptomatische infecties. Dit is relevant bij massale toediening van geneesmiddelen (MDA), aangezien deze strategie ten minste 80% van de doelgroep (50,51) moet bereiken. Eerdere rapporten hebben aangetoond dat de notie van het ontbreken van symptomen waarschijnlijk zal resulteren in een slechte therapietrouw en weigering van behandeling (325,326). Dit is ook het geval geweest in het Magude-project, hoewel het niet uitroeien van malaria in het district niet alleen kan worden verklaard door één factor (36). Sommige reacties van de deelnemers onderstrepen de beperkingen van vectorbestrijding die zich voornamelijk op binnenomgevingen richt. We beschouwen deze notie als een gelegenheid om het voordeel van het gebruik van MDA te benadrukken, met berichten gericht op de algehele bescherming ervan, zelfs tegen malaria-overdracht buitenshuis. Bovendien was de therapietrouw voor malaria-chemopreventie en behandeling in Magude om verschillende redenen niet optimaal. Dit impliceert een noodzaak

om de grassroots-mobilisatie te versterken, aandacht te besteden aan de beschikbaarheid van een chemopreventieprogramma, de onderliggende oorzaken van slechte therapietrouw aan te pakken, en een strikt beleid te voeren ten aanzien van het monitoren van malariabehandeling. Onze bevindingen suggereren ook een noodzaak van algemene versterking van de eerstelijnszorg die toegankelijk is voor de gehele risicopopulatie. Over het algemeen is het belangrijk om op te merken dat het succes ervan in hoge mate afhangt van positieve acceptatie door de gemeenschap en deelname aan programmatische interventies, dit omdat inspanningen voor het elimineren van malaria een langdurige inzet en een flexibele gemeenschapsstrategie vereisen (35,37,328).

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H.P.P.

Ph.D. Portfolio

Ph.D. student: **Harvie P. Portugaliza**
Ph.D. Period: February 2017- November 2020
Ph.D. Supervisors: Prof. Dr. Alfred Cortés-Closas
Prof. Dr. Anna Rosanas-Urgell
Dr. Christopher Pell
Ph.D. Academic Supervisor: Prof. Dr. Frank Cobelens
Ph.D. Tutor: Prof. Dr. Jordi Vila

Ph.D. Training Information

Conference and Symposium	Organizer	Venue	Date	Role	ECTS
68th Annual Meeting of the American Society for Tropical Medicine and Hygiene	American Society for Tropical Medicine and Hygiene	Gaylord National Resort and Convention Center in National Harbor, Maryland, USA	November 20 - 24, 2019	Oral presenter, "Effect of artemisinin on the sexual conversion of the malaria parasite *Plasmodium falciparum*"	2.5
6th ISGlobal Ph.D. Symposium	Barcelona Institute for Global Health (ISGLobal)	Faculty of Medicine, University of Barcelona	November 13, 2019	Oral presenter, "Effect of artemisinin on the sexual conversion of *Plasmodium falciparum*"	1.5
Ph.D. day IDIBAPS	Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS)	CEK, Hospital Clinic, University of Barcelona	June 28, 2019	5 minutes pitch presenter, "Effect of artemisinin on the sexual conversion of *Plasmodium falciparum*"	1.5
2018 Transdisciplinary Global Health annual meeting	Trans Global Health - Erasmus Mundus Joint Doctorate Program and ISGlobal-University of Barcelona	Faculty of Medicine, University of Barcelona	November 5, 2018	Presenter, update of the project Targeting malaria transmission: a transdisciplinary approach	1.5
EMBL Conference: BioMalPar XIV: Biology and Pathology of the Malaria Parasite	European Molecular Biology Laboratory (EMBL)	Meyerhofstraße 1, 69117 Heidelberg, Germany	May 23-25, 2018	Poster Presenter: Effect of antimalarial treatment on *pfap2-g* activation: a pilot study in patients with uncomplicated falciparum malaria in Vietnam	1.5
VIII Annual Chromatin and Epigenetics symposium	Chromatin and Epigenetics section of the Catalan Society of Biology (SCB) and the Barcelona Chromatin Club (BCC)	Prat de La Riba hall, Carrer del Carme, 47, Barcelona, Spain	March 16, 2018	Participant	1
5th ISGlobal Ph.D. Symposium	Barcelona Institute for Global Health (ISGLobal)	Campus Mar, Barcelona Biomedical Research Park (PRBB)	November 6, 2017	Poster presenter ´Transmission Potential of Malaria Parasites Treated	1.5

		Doctor Aiguader, 88 Barcelona 08003, Spain		with Artemisinin´	
4th ISGlobal Ph.D. Symposium	Barcelona Institute for Global Health (ISGLobal)	Campus Clinic, Carrer de Casanova, 143, Barcelona, Spain	November 28, 2017	Poster presenter ´Targeting malaria transmission: a transdisciplinary approach´	1.5
2017 Transdisciplinary Global Health annual meeting	Trans Global Health - Erasmus Mundus Joint Doctorate Program and Amsterdam Institute for Global Health & Development (AIGHD)	AIGHD, Paasheuvelweg 25, 1105 BP Amsterdam-Zuidoost, Netherlands	September 21, 2017	Poster presenter ´Targeting malaria transmission: a transdisciplinary approach´	1.5
VII Annual Chromatin and Epigenetics symposium	Chromatin and Epigenetics section of the Catalan Society of Biology (SCB) and the Barcelona Chromatin Club (BCC)	Prat de La Riba hall, Carrer del Carme, 47, Barcelona, Spain	March 24, 2017	Participant	1
TGH Annual Meeting 2016 / Global Health Symposium	Trans Global Health - Erasmus Mundus Joint Doctorate Program and Amsterdam Institute for Global Health & Development (AIGHD)	Rosarium, Amstelpark 1, Europaboulevard, 1083HZ Amsterdam	September 23, 2016	Participant	0.5

Courses Completed	Organizer	Venue	Date	Grade	ECTS
Research Ethics Training Curriculum	FHI 360	On-line	September 5, 2019	93%	1
Course on Basic Flow Cytometry	La Plataforma de Citometria de IDIBAPS	CEK Building, Barcelona	June 4-8, 2018	Passed	1
STATA course	ISGlobal	C. Rosello 132, ISGlobal, Barcelona	June 7, 2018- June 22, 2018	Passed	1
Fundamentals of Qualitative Health Research	Barcelona Institute for Global Health – University of Barcelona	Faculty of Medicine, University of Barcelona	December 11-20, 2017	9/10	3
Development and Applications of Vaccines in Global Health	Barcelona Institute for Global Health – University of Barcelona	Faculty of Medicine, University of Barcelona	May 24- June 2, 2017	9.3/10	3
Infectious Diseases and Global Health	Barcelona Institute for Global Health – University of Barcelona	Faculty of Medicine, University of Barcelona	May 11-23, 2017	7.7/10	3

Inter- and Transdisciplinary Research for Global Health Research	Athena Institute, VU-University Amsterdam	VU-University Amsterdam	September 19-30, 2016	NA	6

Institutional Presentation	Organizer	Venue	Date	Presentation	ECTS
System Biology and Epidemiology Meeting	ITM	ITM, Antwerp, Belgium	November 21, 2017	Inducing *pfap2-g* expression and gametocytogenesis in *Plasmodium falciparum*	0.5
	ITM	On-line via ITM	May 10, 2020	Effect of artemisinin on the sexual conversion of *Plasmodium falciparum*	0.5
Malaria Coffee Talk	ISGlobal	CEK, Campus Clinic, Barcelona, Spain	Feb 5, 2018	Could antimalarial drugs induce gametocytogenesis in naturally infected malaria patients? *pfap2-g* might know the answer	0.5
	ISGlobal	CEK, Campus Clinic, Barcelona, Spain	June 17, 2019	New reporter lines for accurate quantification of sexual conversion rates in the malaria parasite *Plasmodium falciparum*	0.5

Total ECTS: 35.5

TGH Required ECTS: 30

List of Publications

This Thesis

Portugaliza, H.P., Miyazaki, S., Geurten, F.J., Pell, C., Rosanas-Urgell, A., Janse, C.J. and Cortés, A., 2020. Artemisinin exposure at the ring or trophozoite stage impacts *Plasmodium falciparum* sexual conversion differently. Elife, 9, p.e60058.

Portugaliza, H.P., Llorà-Batlle, O., Rosanas-Urgell, A. and Cortés, A., 2019. Reporter lines based on the gexp02 promoter enable early quantification of sexual conversion rates in the malaria parasite *Plasmodium falciparum*. Scientific reports, 9(1), pp.1-12.

Portugaliza, H.P., Galatas, B., Nhantumbo, H., Djive, H., Murato, I., Saúte, F., Aide, P., Pell, C. and Munguambe, K., 2019. Examining community perceptions of malaria to inform elimination efforts in Southern Mozambique: a qualitative study. Malaria journal, 18(1), p.232.

Others

Portugaliza, H.P., Balaso, I.M.C., Descallar, J.C.B. and Lañada, E.B., 2019. Prevalence, risk factors, and spatial distribution of *Fasciola* in carabao and intermediate host in Baybay, Leyte, Philippines. Veterinary Parasitology: Regional Studies and Reports, 15, p.100261.

Cabardo Jr, D.E. and **Portugaliza, H.P.,** 2017. Anthelmintic activity of *Moringa oleifera* seed aqueous and ethanolic extracts against *Haemonchus contortus* eggs and third stage larvae. International journal of veterinary science and medicine, 5(1), pp.30-34.

Short Bio

Harvie P. Portugaliza is a Filipino veterinarian working in academe and research. He received his degree in Doctor of Veterinary Medicine (cum laude) from the Visayas State University and the license to practice as a veterinarian in the Philippines (First rank in the 2011 Philippine Licensure Examination for Veterinarian). He completed his master's degree in tropical animal health at the Institute of Tropical Medicine in Antwerp through the Belgian DGD scholarship program. His master thesis focused on the molecular epidemiology of five malaria parasites in Cambodia, under the supervision of Prof. Dr. Anna Rosanas-Urgell and Dr. Annette Erhart. He received the Laureate of Antwerp Provincial Prize for Development Cooperation in 2014. His Ph.D. research on malaria transmission is funded by the Erasmus Mundus Program of the European Union. Key findings of his Ph.D. project were presented in local and international fora and published in peer-reviewed journals.