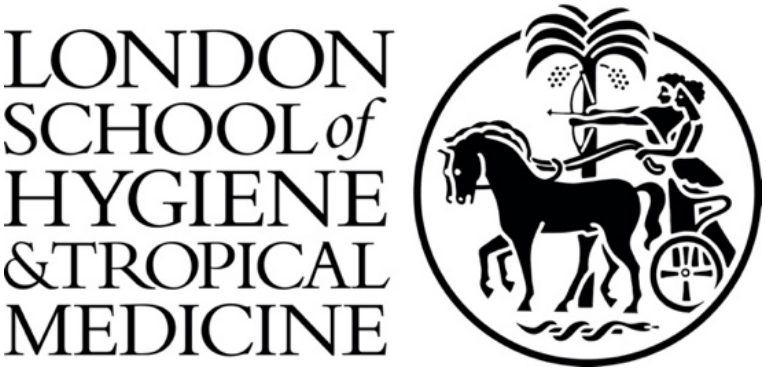

Transmissibility and antimalarial resistance in human malaria parasite *Plasmodium falciparum* in Mali

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I, Leen Vanheer, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The emergence of artemisinin partial resistance (ART-R) in Africa threatens to reverse decades of malaria control progress. In addition, widespread resistance to sulfadoxine-pyrimethamine could jeopardise chemoprevention strategies. Gametocytes, the only *Plasmodium falciparum* life stage capable of infecting mosquitoes, may be present at higher densities and/or exhibit increased infectivity in resistant parasites. Despite this, the development of gametocytocidal drugs receives little attention. Artemisinin-based combination therapies (ACTs) have varying effects on gametocytes and transmission, with primaquine being the only antimalarial currently licensed for *Plasmodium falciparum* that is highly effective against mature gametocytes. The aim of this thesis is to investigate levels of antimalarial drug resistance, their effect on transmissibility of parasites, and the transmission-blocking activity of common antimalarial regimens with or without a gametocytocidal drug.

In this thesis, samples and data from six transmission drug trials conducted between 2013 and 2023 in Ouélessébougou, Mali, were analysed and genotyped. I first establish a detailed characterisation of genome-wide variation and drug resistance profiles in asymptomatic gametocyte carriers from 2019 and 2020 and compare them to publicly available whole-genome data from older Malian (2007-2017) and African-wide *P. falciparum* isolates. The analysis reveals high multiclonality and low relatedness among 2019-2020 isolates, alongside increased frequencies of molecular markers for lumefantrine and sulfadoxine-pyrimethamine resistance, compared to older Malian isolates. Next, using infected mosquito midguts from the drug trials, as well as participant blood samples upon which the mosquitoes were fed, I investigate the relative transmissibility of different parasite clones and the effect of molecular markers of drug resistance on this transmissibility. The results show that parasite transmission dynamics are highly complex, even after treatment with an ACT, and certain molecular markers of drug resistance appear to confer a transmission advantage or disadvantage.

Focusing on blocking transmission with antimalarials, this thesis then presents results from the sixth drug trial conducted at this study site in 2022 testing the effect of the triple artemisinin-based combination therapy artemether-lumefantrine-amodiaquine and the ACT artesunate-amodiaquine, with and without a single low-dose of primaquine, on gametocytes and transmission. We find that artemether-lumefantrine-amodiaquine blocks nearly all mosquito infections within 48 hours, but substantial post-treatment transmission occurs after artesunate-amodiaquine. Adding a single low-

dose of primaquine is a safe and effective addition to both artemether-lumefantrine-amodiaquine and artesunate-amodiaquine for blocking *P. falciparum* transmission. Lastly, a pooled analysis from individual patient data from the six trials is presented, to allow for a direct comparison of the transmission-blocking activity of 15 antimalarial regimens, including different ACTs and non-ACTs, alone or in combination with a single low-dose gametocytocide. We show marked differences in the anti-gametocyte and anti-transmission effects between ACTs, with artemether-lumefantrine being superior in blocking transmission. Moreover, the findings validate the rapid effects of a single low-dose primaquine in clearing gametocytes when used in combination with any ACT. The results from all chapters are discussed in the context of the drug resistance threat in Africa, highlighting the urgency to delay its onward transmission.

Thesis publications and manuscripts

1. **Leen N. Vanheer**, Almahamoudou Mahamar, Emilia Manko, Sidi M. Niambele, Koualy Sanogo, Ahamadou Youssouf, Adama Dembele, Makonon Diallo, Seydina O. Maguiraga, Jody Phelan, Ashley Osborne, Anton Spadar, Merel J. Smit, Teun Bousema, Chris Drakeley, Taane G. Clark, William Stone, Alassane Dicko & Susana Campino. Genome-wide genetic variation and molecular surveillance of drug resistance in *Plasmodium falciparum* isolates from asymptomatic individuals in Ouélessébougou, Mali. Sci Rep. 2023 Jun 12;13(1):9522.

2. **Leen N. Vanheer**, Emilia Manko, Almahamoudou Mahamar, Jody Phelan, Koualy Sanogo, Youssouf Sinaba, Sidi M Niambele, Adama Sacko, Sekouba Keita, Ahamadou Youssouf, Makonon Diallo, Harouna M Soumare, Kjerstin Lanke, Djibrilla Issiaka, Halimatou Diawara, Sekou F Traore, Teun Bousema, Alassane Dicko, Chris Drakeley, Susana Campino, William Stone. Preferential transmission of minority and drug-resistant clones in polyclonal *Plasmodium falciparum* infections in Mali. Submitted, Malaria Journal.

3. Almahamoudou Mahamar, PhD*, **Leen N Vanheer, MD*,** Merel J Smit, MD, Koualy Sanogo, MD, Youssouf Sinaba, MD, Sidi M. Niambele, PharmD, Makonon Diallo, MD, Oumar M Dicko, MD, Richard S. Diarra, MD, Seydina O Maguiraga, MD, Ahamadou Youssouf, PharmD, Adama Sacko, MS, Sekouba Keita, MS, Siaka Samake, Pharm D, Adama Dembele, MS, Karina Teelen, Yahia Dicko, MD, Sekou F. Traore, PhD, Prof Arjen Dondorp, MD, Prof Chris Drakeley, PhD†, William Stone, PhD†, Prof Alassane Dicko, MD†. Artemether-lumefantrine-amodiaquine or artesunate-amodiaquine combined with single low-dose primaquine to reduce Plasmodium falciparum malaria transmission in Ouélessébougou, Mali: a five-arm, phase 2, single-blind, randomised clinical trial. In press, Lancet Microbe.

4. **Leen N Vanheer, MD***, Jordache Ramjith, PhD*, Almahamoudou Mahamar, PhD*, Merel J Smit, MD, Kjerstin Lanke, Michelle E Roh, PhD, Koualy Sanogo, MD, Youssouf Sinaba, MD, Sidi M. Niambele, PharmD, Makonon Diallo, MD, Seydina O Maguiraga, MD, Sekouba Keita, MS, Siaka Samake, PharmD, Ahamadou Youssouf PharmD, Halimatou Diawara, MD, Sekou F. Traore, PhD, Roly Gosling, MD, Joelle M Brown, PhD, Chris Drakeley, PhD, Alassane Dicko, MD†, Will Stone, PhD†, Teun Bousema, PhD†. The transmission blocking activity of artemisinin-combination, non-artemisinin, and 8-aminoquinoline antimalarial therapies: a pooled analysis of individual participant data. Submitted, PLoS Medicine.

* Joint first authors, contributed equally
† Joint last authors, contributed equally

Additional publications and manuscripts

During my PhD I have contributed to other manuscripts which are not included in this thesis.

These are outlined below:

1. Osborne A, Jody E. Phelan, **Leen N. Vanheer**, Alphaxard Manjurano, Jesse Gitaka, Christopher J. Drakeley, Akira Kaneko, Kiyoshi Kita, Susana Campino & Taane G. Clark. High throughput human genotyping for variants associated with malarial disease outcomes using custom targeted amplicon sequencing. Sci Rep. 2023 Jul 26;13(1):12062.

2. Jody E. Phelan, Anna Turkiewicz, Emilia Manko, Joseph Thorpe, **Leen N. Vanheer**, Marga van de Vegte-Bolmer, Nguyen Thi Hong Ngoc, Nguyen Thi Huong Binh, Nguyen Quang Thieu, Jesse Gitaka, Debbie Nolder, Khalid B. Beshir, Jamille G. Dombrowski, Silvia Maria Di Santi, Teun Bousema, Colin J. Sutherland, Susana Campino and Taane G. Clark. Rapid profiling of Plasmodium parasites from genome sequences to assist malaria control. Genome Med. 2023 Nov 10;15(1):96.

3. Matthew Higgins, Mojca Kristan, Emma L. Collins, Louisa A. Messenger, Jamille G. Dombrowski, **Leen N. Vanheer**, Debbie Nolder, Chris J. Drakeley, William Stone, Almahamoudou Mahamar, Teun Bousema, Michael Delves, Janvier Bandibabone, Sévérin N'Do, Chimanuka Bantuzeko, Bertin Zawadi, Thomas Walker, Colin J. Sutherland, Claudio R. F. Marinho, Mary M. Cameron, Taane G. Clark†, Susana Campino†. A Pan Plasmodium lateral flow recombinase polymerase amplification assay for monitoring malaria parasites in vectors and human populations. Sci Rep. 2024 Aug 30;14(1):20165.

4. Nina Billows, Jody Phelan, **Leen N. Vanheer**, Mark KI Tan, Susana Campino†, Taane G. Clark†. Malaria-MOI: A tool for estimating multiplicity of Infection from next-generation sequencing data to inform clinical assessments and transmission intensity in malaria. Submitted, Bioinformatics.

5. Catriona Patterson*, Elin Dumont*, Cesc Bertran-Cobo*, Praveen Sahu, **Leen N. Vanheer**, Ruhi Sikka, Aditi Gupta, Akshaya Mohanty, James Beeson, Chris Drakeley, Anne Kessler, Jane M. Carlton, Sanjib Mohanty, Himanshu Gupta†, Kevin K.A. Tetteh †, Samuel C. Wassmer†. Anti-PfGARP antibody levels do not differ between severe and non-severe malaria patients in India. In preparation.

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Abbreviations

ACT	Artemisinin-based combination therapy
AL	Artemether-lumefantrine
AL-AQ	Artemether-lumefantrine-amodiaquine
AQ	Amodiaquine
ART-R	Artemisinin partial resistance
AS	Artesunate
AS-AQ	Artesunate-amodiaquine
cDNA	Complementary deoxyribonucleic Acid
COI	Complexity of infection
CQ	Chloroquine
DGFA	Dual gamete formation assay
DHA-PPQ	Dihydroartemisinin-piperaquine
DMFA	Direct membrane feeding assay
DNA	Deoxyribonucleic acid
DV	Digestive vacuole
EDTA	Ethylenediaminetetraacetic acid
F_{ws}	Wright's inbreeding coefficient
G6PD	Glucose-6-phosphate dehydrogenase
GTP	Guanosine-5'-triphosphate
GWAS	Genome-wide association study
Hb	Haemoglobin
Hct	Haematocrit
IBD	Identity-by-descent
iHS	Integrated haplotype score
Indel	Insertion/deletion
IPTi	Intermittent preventive treatment in infants
IPTsc	Intermittent preventive treatment in school-aged children
IRS	Indoor residual spraying
ITN	Insecticide treated net
LLIN	Long-lasting insecticidal net
LMF	Lumefantrine
NMCP	National Malaria Control Programmes
mAb	Monoclonal antibody

MACS	Magnetic-activated cell sorting
MAF	Minor allele frequency
MB	Methylene blue
MDA	Mass drug administration
MFA	Membrane feeding assay
MFQ	Mefloquine
MOI	Multiplicity of infection
MPAG	Malaria Policy Advisory Group
MVIP	Malaria Vaccine Implementation Programme
N-J	Neighbour-joining
PY-AS	Pyronaridine-artesunate
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDMC	Post-discharge malaria chemoprevention
P. falciparum	*Plasmodium falciparum*
Pfaat1	*P. falciparum* amino acid transporter 1
Pfcrt	*P. falciparum* chloroquine resistance transporter
Pfcytb	*P. falciparum* cytochrome b
Pfdhfr	*P. falciparum* dihydrofolate reductase
Pfdhps	*P. falciparum* dihydropteroate synthase
PfK13	*P. falciparum* Kelch13
Pfmdr1	*P. falciparum* multidrug resistance 1 protein
PMC	Perennial malaria chemoprevention
PM2/3	Plasmepsin 2/3
PPQ	Piperaquine
PQ	Primaquine
PVM	Parasitophorous vacuole membrane
PYR	Pyrimethamine
PY-AS	Pyronaridine-artesunate
RBC	Red blood cell
RDT	Rapid diagnostic test
RNA	Ribonucleic acid
Rsb	Extended haplotype homozygosity between populations
RT-qPCR	Quantitative reverse transcriptase polymerase chain reaction
SDX	Sulfadoxine
SLD	Single low-dose

SMC	Seasonal malaria chemoprevention
SMFA	Standard membrane feeding assay
SNP	Single nucleotide polymorphism
SP	Sulfadoxine-pyrimethamine
SP-AQ	Sulfadoxine-pyrimethamine plus amodiaquine
sWGA	Selective whole genome amplification
TACT	Triple artemisinin-based combination therapy
TES	Therapeutic efficacy study
TQ	Tafenoquine
WGS	Whole genome sequencing
WHO	World Health Organisation

Chapter 1: Introduction

1.1 Malaria as a global health concern

Despite advances in medical research and public health efforts, malaria continues to be a major global health challenge. In 2022, there were an estimated 249 million malaria cases in 85 malaria endemic countries and areas. The World Health Organisation (WHO)'s African region accounted for approximately 94% of cases and 95% of deaths, with 78.1% of deaths occurring in children under 5 years of age. The disease is transmitted by female *Anopheles* mosquitoes and caused by apicomplexan parasites of the genus *Plasmodium*. Six species are known to infect humans: *Plasmodium falciparum*, *P. vivax, P. malariae, P. ovale curtisi, P. ovale wallikeri*, and *P. knowlesi*. Among these, *P. falciparum* is the causative agent of most malaria cases, particularly in the WHO African region, where it is responsible for approximately 99.7% of the infections (1) (**Figure 1**).

The WHO Global Technical Strategy 2016-2030 aims to reduce the global malaria burden by 90% by 2030. Considerable progress has been achieved, with global estimated malaria deaths having decreased from 864,000 in 2000 to 586,000 in 2015. This achievement was largely due to the introduction of artemisinin-based therapies as standard antimalarial treatment, along with the widespread implementation of indoor residual spraying (IRS) and the use of long-lasting insecticidal nets (LLINs) (2). However, since 2015, the number of worldwide malaria deaths and cases has increased, reaching 608,000 deaths in 2022 (**Figure 2**). Alongside this, rapid population growth has caused the population at risk to increase as well, having nearly doubled in sub-Saharan Africa since the beginning of the century. Taken together, following a decades-long decline in incidence and mortality rates, these rates have now plateaued since 2015 (**Figure 2**). As a result, malaria mortality is currently 53% off track compared to the goals set by the WHO Global Technical Strategy (1).

Reasons for this stagnation are complex and the increase in incidence and mortality rates between 2019 and 2020 has been attributed mostly to disruptions to services and control programmes during the COVID-19 pandemic (3). The plateau in progress is attributable to many factors, including biological and ecological factors that have impacted malaria control programs, such as climate change, invasive mosquito species, mosquito resistance to insecticides, parasite resistance to antimalarials, and the parasite's ability to evade diagnostic tests (4–8).

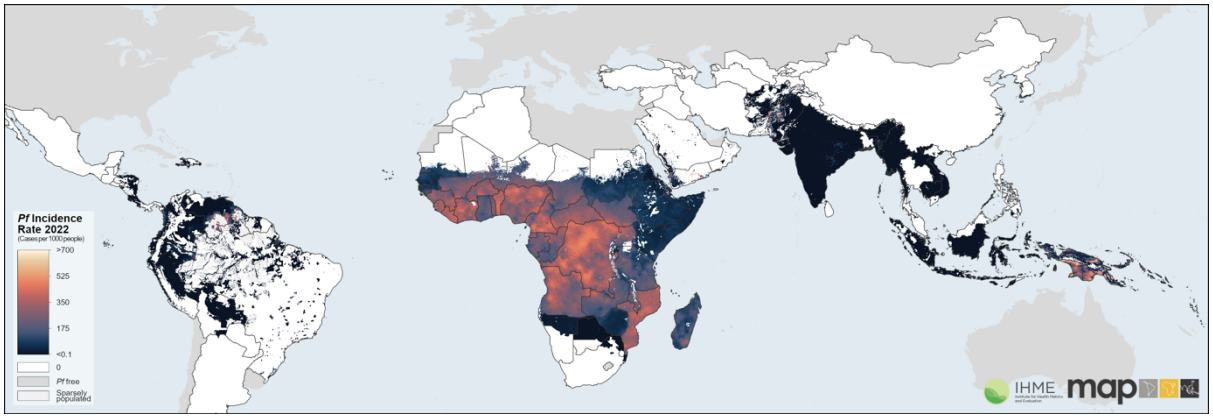

Figure 1. *P. falciparum* incidence rate (clinical cases per 1000 people) in 2022. Reprinted from Malaria Atlas Project (https://data.malariaatlas.org/).

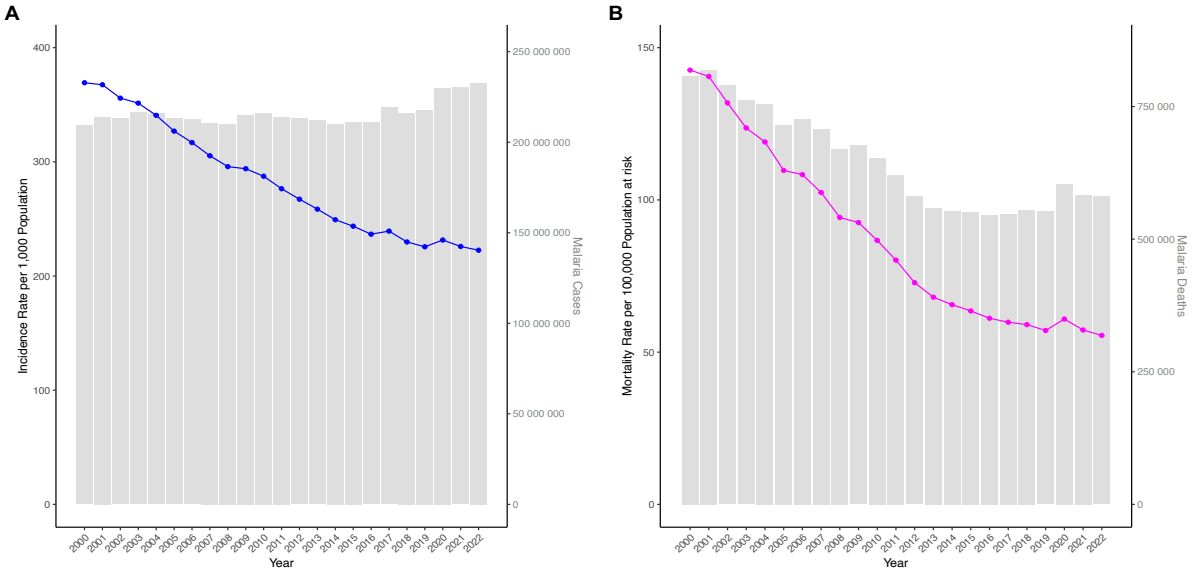

Figure 2. WHO estimated burden of malaria in WHO African region. A. Malaria cases (grey bars) and incidence rates (blue line), and **B.** malaria deaths (grey bars) and mortality rates (purple line) in the WHO African region between 2000 and 2022. Graphs created using data from WHO malaria report 2023 (1).

1.2 *P. falciparum* life cycle

Malaria parasites cycle between the human host and the female anopheline mosquito vector. Throughout their complex life cycle, they differentiate into several morphologically different stages (**Figure 3**). Parasite success depends on a careful balance between asexual replication, to maintain human infection, and gametocyte formation, to achieve transmission to the mosquito and successful spread in the human population.

Human infection starts with the bite of an infected female *Anopheles* mosquito. While the mosquito takes a blood meal, parasite sporozoites are transferred from the mosquito's salivary gland into the human bloodstream. These sporozoites then travel to the liver, where they invade hepatocytes, which marks the beginning of the asymptomatic liver stage infection. Within a period of 6-7 days, parasites mature inside the hepatocytes and eventually form tens of thousands of merozoites, which are then released into the bloodstream to invade erythrocytes. In *P. falciparum*, each asexual blood stage cycle takes about 48 hours, progressing from ring stages to trophozoites and then to schizonts. At the end of this cycle, mature schizonts erupt and the liberated merozoites invade new erythrocytes.

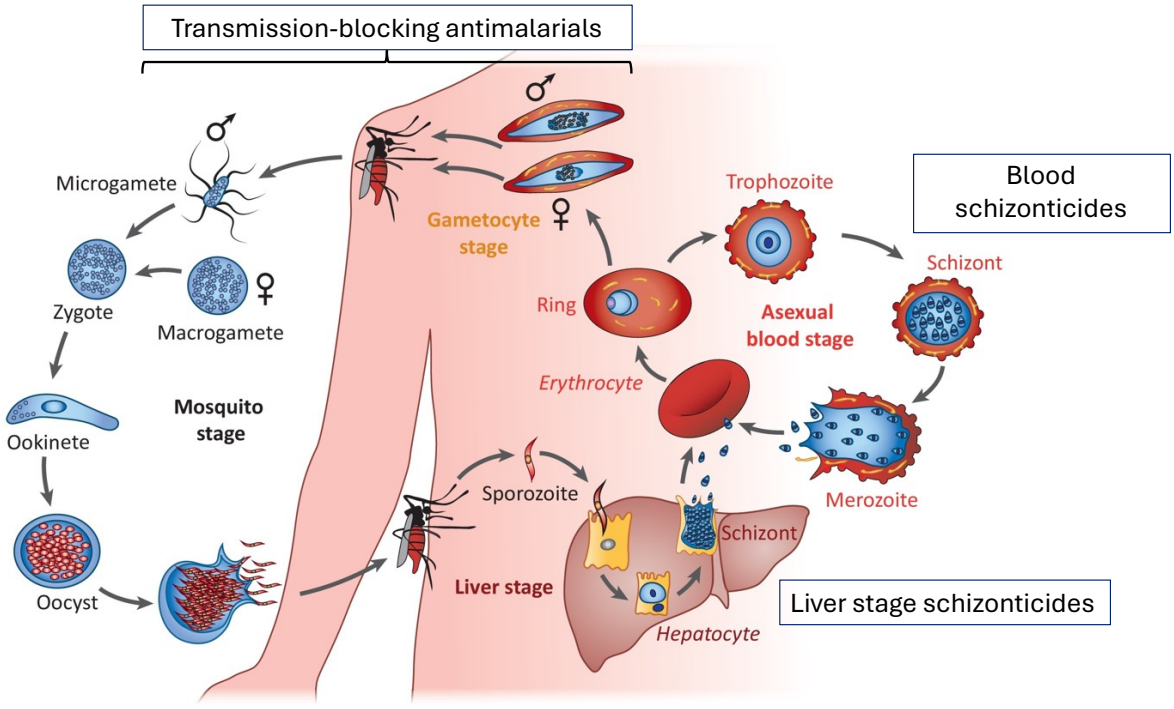

Figure 3. *P. falciparum* life cycle and an indication of which stages are targeted by different antimalarial treatment categories. Adapted from Maier et al. 2019 (9).

A small percentage of asexual blood stage parasites will differentiate into gametocytes, which are the only stages that can be transmitted to the mosquito. This decision to differentiate is also called sexual commitment and involves expression of the *PfAP2-G* locus, which is silenced by heterochromatin in asexual parasites (10). Gametocytes develop during five developmental stages over a 10-12 day maturation period, during which immature stages sequester in the bone marrow, and only mature stage V gametocytes are released back into the bloodstream (11,12).

Upon ingestion of female and male gametocytes by a mosquito, the gametocytes activate into gametes in the mosquito midgut lumen and then fuse to form a diploid zygote. This zygote transforms into an ookinete that attaches to the midgut wall and develops into an oocyst, which after 10–14 days ruptures to release sporozoites that travel to the salivary glands and render the mosquito infectious during the next blood meal. Genetic recombination in the parasite's life cycle takes place exclusively during this sexual phase in the mosquito, leading to the creation of new parasite haplotypes through the reshuffling and re-assortment of genes.

1.3 Infection dynamics

1.3.1 Clinical presentation

The clinical symptoms of malaria primarily result from the rupture of schizonts and the destruction of erythrocytes, with non-specific symptoms such as fever, general malaise, headache, fatigue, nausea and vomiting as common presentations. Fever is the hallmark of malaria, which is often irregular at first but can develop into a cyclic pattern depending on the infecting species of *Plasmodium*, with *falciparum* causing fevers to occur every 48 hours in accordance with the erythrocytic parasite cycle (13). Malaria is typically categorised as asymptomatic, uncomplicated or severe (complicated) (14). Only a small percentage of infections lead to severe malaria, which are almost always caused by *P. falciparum*. Severe malaria cases are often fatal, and can result from various pathological processes, such as haemolysis or microvascular obstruction of infected erythrocytes in the capillaries (15).

In contrast, a large proportion of all *P. falciparum* infections are asymptomatic (16), which are defined as the presence of malaria parasites in the blood in the absence of symptoms, in individuals who have not recently received antimalarial treatment. These asymptomatic infections can either precede a symptomatic infection or can be caused by a non-sterilising adaptive immune response, and are typically characterized by low parasitaemia (17). Asymptomatic individuals with low parasitaemia often remain undetected, and can persist for several months or even years, thereby forming a chronic reservoir for sustained malaria transmission (18,19).

1.3.2 Polyclonal infections

In regions where *P. falciparum* infections are endemic, individuals are often infected by more than one parasite strain at the time, causing polyclonal infections. This can be the result of mosquito

bites that contain several clones (co-transmission), multiple infectious bites (superinfection) or a combination of both (20). The genetic diversity and Multiplicity of Infection (MOI), which indicates the number of clones within an infection, is important for understanding epidemiological patterns and transmission dynamics. Regions with intense malaria transmission tend to have a higher level of multiclonality. Different parasite clones can vary in susceptibility to antimalarial drugs, patterns of recrudescence and clinical course of infection (21,22). As asymptomatic infections often go undetected and untreated for longer, these infections are often associated with a higher MOI (17,23).

1.3.3 Human-to-mosquito transmissibility

The cycle of *Plasmodium* transmission between human and mosquito hosts depends on the parasite's capacity to produce gametocytes. However, the relationship between gametocytes and establishing an infection in the mosquito is not a linear one, and many factors can influence this transmissibility, such as sensitivity to host immune factors, parasite genetic factors and gametocyte viability, senescence and density (24–28) (**Figure 4**). Examples of these factors include naturally acquired anti-gametocyte antibodies against Pfs48/45 and Pfs230, which have been shown to reduce transmission (28). Malaria parasites have evolved to evade mosquito immune responses; one example of this is the interaction with the Pfs47 receptor in the mosquito midgut cells, which renders parasites with a compatible Pfs47 protein haplotype effectively undetectable to the mosquito immune system (26,29). Previous studies found that polyclonal *P. falciparum* infections lead to lower mosquito infection prevalence and intensity, compared to monoclonal infections, though this may also be influenced by gametocyte densities (24,25).

In polyclonal infections, different clones will compete with one another for transmission and the contribution of individual parasite clones in human-to-mosquito transmission remains largely unknown. Although the specific resources that parasite clones compete for are not well understood, evidence of clonal competition is suggested by lower infection intensities that have been observed in polyclonal compared to monoclonal infections (24), and reduced growth rates of certain clones in multiclonal compared to *in vitro* monoclonal infections (30,31). Potential factors influencing competition include the availability of host nutrients like para-aminobenzoic acid (32), preferential invasion of younger erythrocytes, quorum sensing mechanisms (33), and antigenic variation (34), which can lead to clone-specific immune targeting and selective advantages for certain clones. In addition, there may be differences in transmissibility between the infecting clones depending on the time of infection (e.g., clones that infected recently have not had time yet to

produce mature gametocytes) and the parasite density (e.g., clones with higher asexual density may produce a higher number of gametocytes compared to low asexual density clones). This has importance for vaccine studies, as well as drug resistance studies e.g., whether resistant clones are also more transmissible or whether drug resistance comes with a transmission disadvantage (22,35,36). Multiple studies have reported the transmission of clones that were undetectable in the human bloodstream (24,37,38). The reason for this is likely the preferential amplification of asexual parasites, which are the most abundant in circulation, while less abundant mature gametocytes establish infection in the mosquito. In addition, not all clones present in human blood successfully transmit to mosquitoes, which could indicate a new infection that has not yet produced mature gametocytes or a failure to produce gametocytes altogether (32,34). Moreover, some individuals with confirmed gametocytes fail to infect mosquitoes, thereby interrupting the transmission cycle (40–42).

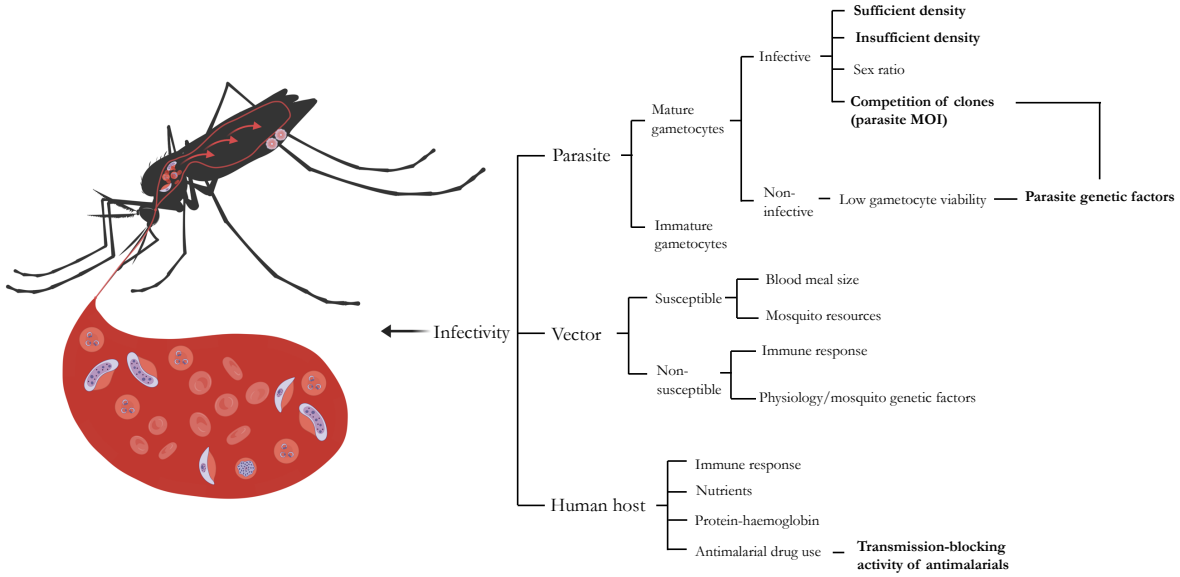

Figure 4. Factors influencing human-to-mosquito transmissibility. A schematic showing an overview of factors that influence the ability of malaria parasites to establish an infection in the mosquito host. Created with BioRender.com. Schematic is adapted from (38). Factors in bold are investigated in this thesis.

1.4 Malaria control measures

1.4.1 Prevention

The WHO recommends preventing malaria through the implementation of vector control strategies, the use of preventive chemotherapies, and the administration of malaria vaccines (44). Vector control measures include the distribution and use of insecticide treated bed nets (ITNs), as well as the implementation of indoor residual spraying (IRS) and larval source management (1). Chemoprevention is another pillar of malaria prevention, administering full therapeutic courses of antimalarials at prescheduled times, irrespective of infection status, to treat existing or prevent new infections. Current WHO recommendations for chemoprevention include perennial malaria chemoprevention (PMC; previously called intermittent preventive treatment in infants), seasonal malaria chemoprevention (SMC), intermittent preventive treatment in pregnant women (IPTp), intermittent preventive treatment in school-aged children (IPTsc) and post-discharge malaria chemoprevention (PDMC) (39). In addition, mass drug administration (MDA) involves providing a complete therapeutic course of an antimalarial to all age groups within a specific geographical area at the same time, and can be timed to coincide with seasonal transmission peaks, respond to epidemic situations, or support targeted campaigns. These chemoprevention recommendations have recently been updated to no longer specify strict age groups, transmission intensity thresholds, numbers of doses or cycles, or specific drugs, with the aim of providing greater flexibility to national malaria programmes to adapt control strategies to suit their settings (44).

In recent years the WHO has recommended two circumsporozoite-based malaria vaccines to prevent malaria in children living in malaria endemic areas: RTS,S/AS01 (RTS,S) and R21/Matrix-M (R21) (1). The RTS,S vaccine was the first malaria vaccine to be approved, and since its introduction in 2019 through the Malaria Vaccine Implementation Programme (MVIP) in Ghana, Kenya, and Malawi, it has reached over 2 million children, and has been shown to be safe and effective. This vaccine has demonstrated significant public health impact, including a 13% reduction in mortality among vaccinated children and a substantial decrease in severe malaria cases (1,45,46). Following successful pilot programs, the RTS,S vaccine has been recommended for broader use across malaria-endemic regions in Africa, and at least 28 countries in the WHO African region have expressed interest in introducing the malaria vaccine. The R21 vaccine has shown high efficacy in clinical trials and has recently become the second vaccine to be recommended by the WHO (1,47,48). The addition of the R21 vaccine, alongside the ongoing rollout of the RTS,S vaccine will enhance the supply of vaccines available for children in malaria endemic areas. Both

vaccines target *P. falciparum* and are designed to be administered in a four-dose schedule starting at five months of age.

1.4.2 Case management

Prompt and accurate diagnosis is essential to ensure swift initiation of treatment. Individuals infected with *P. falciparum* malaria are typically diagnosed using rapid diagnostic tests (RDTs) in field settings. However, the gold standard for diagnosis remains light microscopy (49). This is partly due to the emergence and spread of deletions in the *Pfhrp2/3* gene, which codes for the histidine-rich protein antigen (50,51). These deletions can lead to false negative RDT results, raising concerns about the long-term reliability of these tests (52,53). *Pfhrp2/3* deletions were first identified in Peru in 2010 (54) and have since been documented in Africa, Asia and the Middle East as well. Prevalence estimates vary widely within and between countries, with prevalences as high as 80% being observed among symptomatic patients in Eritrea (55).

Once diagnosed, effective treatment is initiated based on the type and severity of malaria. The WHO recommends artemisinin-based combination therapies (ACTs) for uncomplicated *P. falciparum* malaria. Severe cases require intravenous or intramuscular administration of artesunate, followed by a complete course of oral ACTs. Supportive care, including fluids, antipyretics, and blood transfusion if necessary, is also an integral part of managing severe malaria (1,44).

1.5 Antimalarials

Different categories of antimalarials target various life stages of the parasite (**Figure 3**).

1.5.1 Blood schizonticides

The majority of currently available antimalarials target and clear asexual erythrocytic stages, which are responsible for the clinical symptoms of malaria, thereby offering symptomatic treatment. Common targets of schizonticides are the detoxification of haem into haemozoin (the 4-aminoquinolines chloroquine, amodiaquine, quinine, mefloquine, piperaquine, as well as lumefantrine and pyronaridine) (56), the folate biosynthetic pathway (sulfadoxine, pyrimethamine and proguanil) (57) and the mitochondrial electron transport chain (atovaquone) (58). Artemisinin derivatives are thought to be activated by haem to generate free radicals that damage parasite proteins needed for parasite survival (59). Artemisinin derivatives act exceptionally fast, reducing parasite densities by up to 10,000 fold every 48 hours but their half-lives only range from <1 to 4

hours (60,61). Other blood schizonticides have a slower but longer lasting effect, such as elimination half-lives of 4-6 days, 13-24 days and 33 days in adults for lumefantrine, mefloquine and piperaquine, respectively (61). ACTs are the current first-line treatment for uncomplicated *P. falciparum* malaria globally, combining a fast-acting artemisinin derivative (artesunate, artemether or dihydroartemisinin) with a long-acting partner drug that has a different mode of action (lumefantrine, piperaquine, amodiaquine, pyronaridine or mefloquine). Recently, artemisinin-based combination therapies (TACTs) have been suggested to combat artemisinin partial resistance (ART-R), combining existing ACT regimens with a second partner drug, such as artemether-lumefantrine-amodiaquine (AL-AQ) or dihydroartemisinin-piperaquine-mefloquine (62,63).

The most common adverse events of standard schizonticides are headache, nausea and vomiting, dizziness and itching. Mefloquine can also cause neuropsychiatric effects such as anxiety, psychosis or nightmares, and quinine has been linked to ototoxicity, hypoglycaemia and quinine-induced thrombocytopenia. Lastly, several antimalarials (quinoline or quinoline-like compounds) can cause an elongation of the QTc interval, increasing the risk of cardiac effects in individuals with predisposed long QTc interval (64).

1.5.2 Transmission-blocking antimalarials

Gametocytocidal drugs do not impact the patient's clinical symptoms and recovery but are important for blocking parasite transmission from patients to mosquitoes, thereby reducing subsequent spread in communities. Prior to the transmission trials conducted since 2016 in Mali (40–42,65,66), there was limited data on the anti-gametocyte and anti-transmission activities of antimalarials, and studies often used inconsistent methodologies or did not conduct feeding assays due to their technical challenges (67,68). In recent years, findings from these trials have shown that ACTs have varying efficacy against mature gametocytes and transmission, though artemether-lumefantrine (AL) appears to be more effective than other ACT regimens. In contrast, considerable post-treatment transmission and gametocytaemia after other ACTs such as dihydroartemisinin-piperaquine (DHA-PPQ) and pyronaridine-artesunate (PY-AS) has been observed (40–42).

Primaquine (PQ) and tafenoquine (TQ) are 8-aminoquinolines capable of clearing *P. vivax* liver stages, while also having strong activity against mature *P. falciparum* gametocytes. PQ is the only

drug recommended for the latter indication by the WHO (69), however, its widespread use has been limited by the risk of acute haemolytic anaemia in glucose-6-phosphate dehydrogenase (G6PD) deficient individuals. G6PD is an essential enzyme that replenishes reduced glutathione to combat oxidative stress in red blood cells and other tissues triggered by drugs and infections. G6PD deficiency is an X-linked recessive disorder characterised by over 140 gene mutations, leading to various G6PD-deficient variants that exhibit differing levels of enzyme deficiency (70). Nearly all individuals with this trait have no signs of disease or symptoms, unless they are exposed to an exogenous agent that triggers acute haemolytic anaemia, which may be severe and even life-threatening. Hemizygous males and homozygous females typically experience the most severe effects, while heterozygous females possess a mixed population of normal and deficient G6PD red blood cells. This results in a wide spectrum of enzyme activities and a variable risk of oxidant-induced haemolysis. The prevalence of male G6PD deficiency differs widely across different sub-Saharan countries, from 1.5% in Ethiopia to 20-29% in Mali, Gabon, DRC, Angola and Nigeria (71).

Evidence has now established that a single low-dose PQ (SLD PQ) of 0.25 mg/kg is safe, even for G6PD deficient individuals, while still effective in decreasing mosquito infectivity and gametocyte carriage (72,73). It has been shown to block all transmission within 48 hours at a dose of 0.25 mg/kg, administered in combination with DHA-PPQ, PY-AS and AL (40,42). The WHO recommends a single dose of 0.25 mg/kg of PQ in addition to ACT, without the need for G6PD testing, to prevent human-to-mosquito transmission in areas with low transmission (69,74). The WHO Malaria Policy Advisory Group (MPAG) has recently suggested expanding this recommendation to limit the spread of ART-R (75).

PQ has been found to operate via a two-step mechanism, requiring a conversion into hydroxylated metabolites in the liver through the CPR/CYP2D6 metabolic complex. Afterwards, metabolites undergo oxidation with generation of hydrogen peroxide, which accumulates in sites such as the liver and bone marrow, killing all *Plasmodium* parasites located in these organs (76). As the active metabolites only circulate in the bloodstream for a matter of hours, primaquine has a very fast but short-acting effect, with a median elimination half-life of 4.7 hours (77).

A similar mode of action involving reactive oxygen species has been suggested for TQ, however, the details remain largely unclear (78). Unlike PQ, TQ contains a 3-(trifluoromethyl)phenoxy group that prevents direct oxidation of the compound, rendering TQ's elimination half-life substantially

longer than PQ's (79). Efficacy studies have showed the gametocytocidal and transmission-blocking activity of a SLD TQ of 1.66 mg/kg, both in combination with DHA-PPQ, as well as with SPAQ. However, its effect is delayed, only blocking transmission by day 7 post-treatment initiation (41,42). Despite the promise of a long-lasting gametocytocidal, safety concerns have prevented a single low-dose of TQ being tested in individuals with G6PD deficiency. Therefore, it is currently only in use as a radical cure for *P. vivax*, following G6PD genotyping.

Methylene blue (MB) is the oldest synthetic drug, developed in 1891, to treat malaria (80). It has highly potent gametocytocidal and transmission-blocking effects, is safe and effective against both *P. falciparum* and *P. vivax* asexual parasites, and has shown synergy in combination with artemisinin derivatives (66,81,81). Common side effects include mild urogenital and gastrointestinal symptoms as well as blue coloration of urine (81). Despite the blue urine not adversely affecting health, MB was gradually replaced in the early 20th century by new synthetic antimalarials without colouring properties. Over the past decade there has been a new wave of interest because of its gametocytocidal properties, however, as blue urine coloration is likely to affect compliance, MB is not currently in use as antimalarial.

Different gametocytocidal drugs can exhibit varying effects on gametocytes, such as clearing of gametocytes, distorting their sex-ratio or sterilising gametocytes without clearance from the circulation (82,83). Preferential clearance of female gametocytes upon PQ administration has been observed in drug trials (40,42,66), while for MB, studies consistently find a preferential clearance of male gametocytes (66,82). However, for both drugs, gametocyte densities and sex ratios remain largely unaffected in the first 48 hours, despite an almost complete prevention of transmission within 2 days after treatment, suggesting early sterilisation of gametocytes prior to clearance (84). A significantly male-biased sex ratio, implying preferential clearance of female gametocytes, was also observed after administration of an SLD TQ (1.66 mg/kg) and following AL (41,42).

1.5.3 Liver stage schizonticides

Liver stage schizonticides act on the parasite stages in the hepatocytes. The only drugs having an effect here are atovaquone-proguanil, which are used as prophylactic treatment, as well as PQ and TQ (85–87).

1.6 Genomics to aid malaria control

Plasmodium parasites continuously evolve to adapt to their human and mosquito hosts, exhibiting genetic variation through single nucleotide polymorphisms (SNPs), small genome insertions or deletions (indels), duplications, and large-scale structural variations. Both mutation and recombination generate genetic variation: mutation introduces new differences from the original sequence, while recombination creates novel combinations of these differences (88,89). *P. falciparum's* nuclear genome size is 23-megabase and has an SNP mutation rate of approximately 10^{-10} mutations per base pair per asexual generation (48 hours), generating a large number of mutations, however, most of these will be purged (90,91). Recombination is an obligate step in the malaria parasite life cycle, occurring when gametocytes inside the mosquito transform into gametes and pair to undergo sexual recombination approximately three hours after ingestion. Each pair forms an oocyst, that will later give rise to thousands of haploid sporozoites. Given the high prevalence of multiclonal infections in high-transmission areas, effective recombination of different parasite clones will often take place, leading to novel combinations of existing polymorphisms. This genetic diversity is essential for the parasite's ability to evade drug treatment and immune and vaccine clearance.

Therapeutic efficacy studies (TESs) are considered the gold standard for assessing the effectiveness of antimalarials. In these studies, patients with a malaria diagnosis, validated by microscopy, are given a specific drug under direct supervision. Afterwards, patients are monitored for a defined period of time, to assess the drug's efficacy. However, TESs alone are not sufficient to confirm drug resistance, and must be accompanied by other methods such as (92):

- identification of genetic changes in the parasite genome that are associated with a change in parasite susceptibility to antimalarial drugs.
- *ex vivo* and *in vitro* drug assays to test the sensitivity of cultured parasites to antimalarial drugs, by exposing them to a certain concentration of a drug and observing the effect on parasite survival.
- measurements of drug levels in the blood to determine whether a treatment failure is due to insufficient antimalarial drug exposure or due to resistance.

Whereas drug assays and drug level measurements are costly and resource-intensive, necessitating laboratory infrastructure and specialised personnel, the technology landscape for molecular assays has progressed rapidly over the last decade, and affordable and easy-to-use new techniques have become readily available. Therefore, while TESs remain the gold standard, genomic surveillance has an increasingly important role in monitoring antimalarial resistance (93).

P. falciparum was the first eukaryotic parasite to have a published whole genome sequence (WGS) in 2002, comprising a nuclear genome of 14 chromosomes and two organellar genomes, the mitochondria and the apicoplast (90). Since then, sequencing costs have drastically declined, and sequencing services have become widely available. In addition, the use of leukodepletion methods and selective whole genome amplification (sWGA) has enabled more efficient sequencing and has led to a growth in sequence data, resulting in a large database of over 20,000 *P. falciparum* sequences that are now publicly available (94–96). These data have allowed large-scale studies analysing population dynamics and genome-wide association studies (GWAS), leading to the identification of signatures of selection associated with drug resistance (97–100).

Despite the appeal of WGS for generating extensive data and facilitating large-scale studies, the per-sample cost remains high, and the substantial amount of parasite deoxyribonucleic acid (DNA) required makes it impractical for all sequencing needs. As molecular techniques and sequencing technologies have advanced, lower-cost targeted sequencing methods have become more accessible and attractive for obtaining specific genetic data from parasite samples. Targeted amplicon sequencing, a type of next-generation sequencing that uses polymerase chain reactions (PCR) to create short DNA sequences, has been employed in recent years as an alternative method to screen for *P. falciparum* polymorphisms associated with drug resistance (101,102). The use of sample-specific barcoding allows for multiplexing of a large number of samples, making this method more cost-effective than WGS.

Until recently, Illumina short-read sequencing has been the predominant method for sequencing *P. falciparum* isolates. However, the advent of portable sequencing devices, such as the Oxford Nanopore Technologies MinION platform, has provided a new angle for advancing malaria genomics and its use in control measures (103). Long-read sequence data can be generated in real-time, enabling the rapid identification of parasite species and facilitating rapid and accurate detection of genetic variations, structural variants, and drug resistance markers. The portability and real-time capabilities of platforms like MinION are particularly advantageous for field-based research and surveillance, enabling on-site genomic analysis in malaria-endemic regions.

1.7 Mechanisms and markers of antimalarial resistance

1.7.1 Chloroquine

Chloroquine (CQ) is a drug derived from quinine and was used as a first-line antimalarial drug for *P. falciparum* malaria infections at the start of the 1950s. Unfortunately, in the second half of the 20th century, resistance emerged in both Columbia and the Greater Mekong Region and spread to Africa in the 1970 (104). A high prevalence of CQ resistance prompted its exclusion from treatment guidelines for *P. falciparum* infections in sub-Saharan Africa. The main molecular marker of resistance is a SNP leading to an amino acid substitution of lysine to threonine (K76T) in the chloroquine resistance transporter gene (*Pfcrt*) (105) (**Figure 5, Table 1**). A series of additional mutations in *Pfcrt* have been characterised (C72S, M74I, N75E, A220S, Q271E, N326D, and I356L, R371I), that are associated with various degrees of resistance (106,107). The transporter protein localises to the digestive vacuole membrane and is believed to be involved in transporting peptides released from haemoglobin digestion into the cytosol (108). Resistance mutations in *Pfcrt* were found to mediate CQ efflux out of the digestive vacuole, where haem detoxification takes place (104). This reduces CQ's inhibition of haematin (a toxic product of haem degradation) being converted into haemozoin (non-toxic product of haem degradation), leading to an accumulation of toxic compounds in the parasite digestive vacuole, causing cell death (108). Over the past decades, as ACTs have become the first-line treatment for uncomplicated *P. falciparum* malaria, there have been reports of the prevalence of CQ resistant strains declining around the world (110–113), prompting suggestions of a possible re-introduction of CQ in antimalarial regimens (114).

CQ susceptibility is further modulated by SNPs in the multi-drug resistance transporter gene (*Pfmdr1*), such as the N86Y mutation (115). Like CRT, the MDR1 transporter protein is located on the digestive vacuole membrane, but its transport is inwardly directed towards the vacuole instead of towards the cytoplasm. Transporter isoforms in CQ resistant isolates were found to exhibit reduced capacity to transport CQ into the digestive vacuole (116). Additionally, copy number variations of *Pfmdr1* may also modulate CQ resistance (117). Lastly, a third transporter located on the digestive vacuole membrane, the amino acid transporter (*pfaat1*), was recently identified to modulate CQ sensitivity (98).

1.7.2 Antifolates

Following the spread of CQ resistance, sulfadoxine-pyrimethamine (SP) was implemented as the primary treatment for uncomplicated *P. falciparum* malaria. Although SP was a combination therapy, resistance developed and spread quickly. SP targets two enzymes in the folate biosynthesis pathway: sulfadoxine targets dihydropteroate synthase (*Pfdhps*) and pyrimethamine targets dihydrofolate reductase (*Pfdhfr*) (**Figure 5, Table 1**). Folate derivatives are crucial for DNA replication and protein synthesis, making them essential for cell survival (118). The genetic basis of SP resistance involves an accumulation of mutations in these two genes: a triple mutation in *Pfdhfr* (N51I, C59R and S108N; also called C**IRN**I mutant haplotype) in combination with a double mutation in *Pfdhps* (A437G and K540E; also called S**GE**AA), together called a quintuple mutant, is associated with a risk of SP treatment failure of up to 50% (119,120). A sextuple mutant genotype in which these five mutations are combined with *Pfdhps* A581G (S**GEG**A haplotype) has increasingly been reported as well (121), reaching frequencies of up to 50% in DRC (122).

Because of widespread resistance, this regimen is no longer used to treat malaria infections, but is now exclusively used as IPTp, PMC or SMC (in combination with amodiaquine). Therefore, continued surveillance of resistance markers and efficacy as a preventative chemotherapy is essential.

The K540E mutation in *Pfdhps* frequently serves as an indicator for the presence of the five key mutations linked to SP resistance. The WHO uses this measure to inform decision-making surrounding implementation policy, whereby decisions are to be made at country level or even district level, with PMC implementation only recommended in regions where K540E prevalence is <50% (123,124). In addition, the WHO recommends discontinuation of IPTp with SP when the population prevalence of K540E is greater than 95%, and the prevalence of mutation A581G is greater than 10% (125).

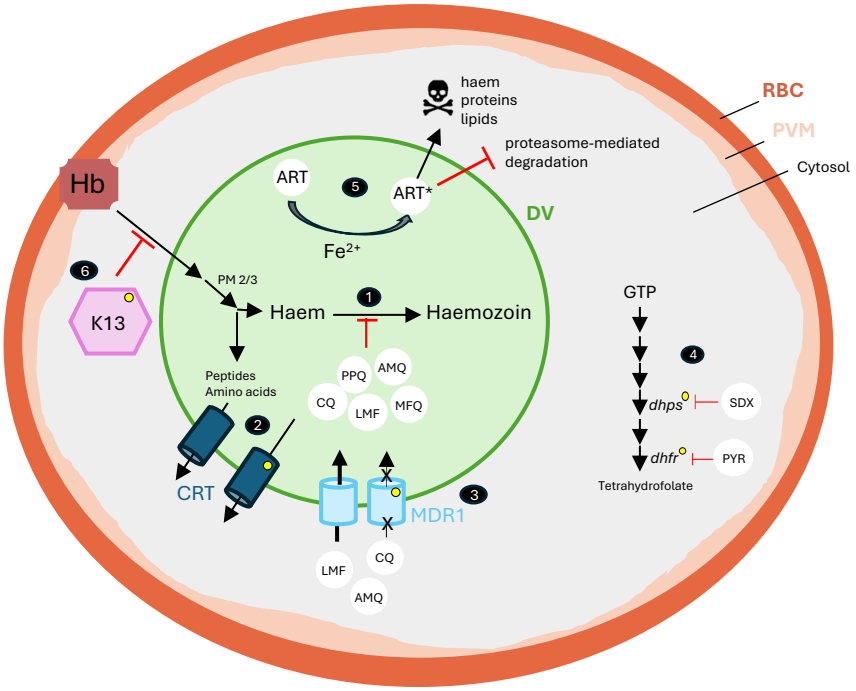

Figure 5. Mechanisms of action and resistance mechanisms of common antimalarials. During its asexual replication, the *P. falciparum* parasite develops within the host red blood cell, enclosed by its parasitophorous vacuole membrane (PVM). **1.** Quinoline-based antimalarials diffuse into the digestive vacuole (DV), where they are protonated and unable to diffuse back to the cytosol. Their protonated forms bind to toxic free haem that results from the degradation of host haemoglobin, thereby preventing its conversion into non-toxic haemozoin crystals. **2.** The CRT transporter on the DV membrane transports peptides released from haemoglobin digestion into the cytosol, however, in drug-resistant parasites, mutations in the gene encoding CRT (indicated by yellow circle) enable the efflux of protonated drug molecules out of the DV. **3.** Mutations in the gene encoding the MDR1 transporter (indicated by yellow circle) on the DV membrane can also regulate transport of drug molecules into the DV. **4.** Folate synthesis in the cytosol: Folate synthetic pathway enzyme dihydropteroate synthase is sensitive to the drug sulfadoxine and dihydrofolate reductase is sensitive to pyrimethamine. Resistance is mediated by mutations in the *dhps* and *dhfr* genes encoding these enzymes (indicated by yellow circles). **5.** Artemisinin drugs are activated by cleavage of their endoperoxide by iron protoporphyrin IX (Fe^{2+}heme), a product of parasite-digested haemoglobin. The asterisk indicates the activated form of artemisinin (ART*), which then damages parasite proteins, haem, lipids and inhibits proteasome-mediated protein degradation. **6.** K13 is a protein required for endocytosis of haemoglobin, and mutations in the propeller domain of the gene encoding K13 (indicated by yellow circle) can cause reduced endocytosis of host haemoglobin. Abbreviations: AMQ, Amodiaquine; ART, artemisinin; CQ, chloroquine; CRT, chloroquine resistance transporter; *dhfr*, dihydrofolate reductase; *dhps*, dihydropteroate synthase; DV, digestive vacuole; GTP, Guanosine-5'-triphosphate; Hb, haemoglobin; K13, Kelch13; LMF, lumefantrine, MDR1, multidrug resistance transporter 1; MFQ, mefloquine; PM2/3, Plasmepsin 2/3; PPQ, piperaquine; PVM, parasitophorous vacuole membrane; PYR, pyrimethamine; RBC, red blood cell; SDX, sulfadoxine.

Table 1. WHO recognised molecular markers of *P. falciparum* resistance to antimalarial drugs. Adapted from (126).

Drug	Gene	Mutations associated with drug resistance
Chloroquine	*Pfcrt*	K76T + different sets of mutations at other codons (including C72S, M74I, N75E, A220S, Q271E, N326S, I356T and R371I)
	Pfmdr1 (in combination with *Pfcrt* mutations only)	N86Y, Y184F, S1034C, N1042D and D1246Y
Amodiaquine	Yet to be validated	Studies show that amodiaquine selects for *Pfmdr1* mutations (86Y)
Piperaquine	*Pfpm2–3*	*Pfpm2–3* increased copy number
	Pfcrt	Detected in vivo: T93S, H97Y, F145I, I218F and C350R Detected in vitro: T93S, H97Y, F145I, I218F, M343L and G353V
Pyrimethamine	*Pfdhfr*	N51I, C59R, S108N and I164L
Sulfadoxine	*Pfdhps*	S436A/F, A437G, K540E, A581G and A613T/S
Proguanil	*Pfdhfr*	A16V, N51I, C59R, S108N and I164L
Lumefantrine	Yet to be validated	Studies show that lumefantrine selects for *Pfmdr1* mutations (N86)
Mefloquine	*Pfmdr1*	*Pfmdr1* increased copy number
Atovaquone	*Pfcytb*	Y268N/S/C
Artemisinin and its derivatives	*PfK13*	Validated: F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, P574L, C580Y Candidate or associated: P441L, G449A, C469F/Y, A481V, R515K, P527H, N537I/D, G538V, V568G, R622I, A675V

1.7.3 Artemisinin derivatives and partner drugs

Since the turn of the century, ACTs have been the first-line treatment for uncomplicated *P. falciparum* malaria. Combining artemisinin derivatives with a partner drug that is slowly eliminated lowers the probability that resistance will emerge to the combination (i.e., the probability is the product of the probabilities for each individual drug). Yet, ART-R emerged in the Greater Mekong region around 2008 (127,128), characterised by delayed *in vivo* parasite clearance after treatment with an artemisinin derivative. The biological basis for this delayed clearance was found to be a reduced susceptibility of ring-stage parasites (129), and mutations in the propeller domain of the *PfKelch13* (*PfK13*) (PF3D7_1343700) gene were determined as the main drivers of ART-R (97) (**Figure 5, Table 1**). Parasites with a resistance-conferring *PfK13* mutation display reduced

endocytosis of haemoglobin, while haemoglobin degradation products are necessary to activate artemisinin derivatives (130). Multiple mutations in the propeller domain can cause resistance, with a list of approved and candidate molecular markers to monitor available from the WHO (126) (**Table 1**). In Thailand, Myanmar and Cambodia, the dominant resistance mutations in *PfK13* are C580Y and F446I (131,132). Over the past few years, *PfK13* mutations and associated delayed clearance of parasites have arisen independently in Africa as well, with reports of resistance-conferring mutations being found in Rwanda (R561H), Uganda (A675V and C469Y), Tanzania (R561H) and Horn of Africa (Ethiopia and Eritrea; R622I) (133–139).

Other candidate genes suggested to be involved in ART-R using *in vitro* experiments include *Pfcoronin*, *Pfatpase6*, *Pfap2mu* and *Pfubp1* (140–142). While some polymorphisms in these genes have been reported in field isolates, it is currently unclear what their contribution is to *in vivo* delayed parasite clearance (143).

In many areas with ART-R, resistance to partner drugs has now emerged as well, leading to a rapid rise in clinical resistance and treatment failure of ACTs in South East Asia (144,145). Resistance to amodiaquine can arise from polymorphisms in *Pfcrt* (76T allele) and *Pfmdr1* (86Y,Y184 and 1246Y alleles), while lumefantrine resistance is associated with the opposite alleles (K76 in *Pfcrt*, N86, 184F and D1246 in *Pfmdr1*) (108,115,146). Piperaquine resistance can be modulated by a number of *Pfcrt* mutations, as well as the presence of the E415G mutation in an exonuclease encoding gene (*Pfexo*) and copy number variants of plasmepsin 2/3 (*Pfpm2, Pfpm3*), proteases involved in the haemoglobin-to-haemozoin conversion pathway (147–150).

At present, ACTs remain widely effective in sub-Saharan Africa. However, there have been reports from Angola, Burkina Faso, the Democratic Republic of Congo, and Uganda indicating that the efficacy of AL has fallen below the 90% threshold recommended for treatment policy change (151–154). It is important to note though, that some of these studies deviated from the WHO-recommended PCR-correction, upon which the 90% efficacy benchmark was based (155).

1.7.4 8-aminoquinolines

Several reports of suspected *P. vivax* PQ resistance exist; however, some findings are confounded by an inability to distinguish between reinfection and relapse, or by instances of incomplete or underdosed therapy. Further obstacles to studying 8-aminoquinoline resistance include the lack of identifiable resistance mechanisms and the challenges associated with propagating *P. vivax in vitro*

(156). The recommended or tested doses of PQ and TQ for *P. falciparum* malaria (0.25 mg/kg and 0.42-1.66 mg/kg, respectively) are substantially lower than those recommended for *P. vivax* malaria (0.5 mg/kg per day for PQ, and 300 mg for TQ which is equivalent to 5 mg/kg in a 60 kg adult). In co-endemic regions, repeated and prolonged exposure of *P. vivax* strains to low doses of 8-aminoquinolines, particularly TQ due to its extended half-life, could potentially drive the development of *P. vivax* resistance (157). In contrast, no reports of 8-aminoquinoline resistance have been observed in *P. falciparum*.

1.8 Impact of drug resistance on transmission

Despite influencing the speed at which drug resistance spreads, the transmissibility of drug-resistant parasites is currently understudied, due to technical challenges in analysing parasite stage composition at a clonal level and complexities in performing mosquito transmission assays. An older study showed increased gametocytaemia and mosquito infectivity in infections with recrudescent CQ resistant *P. falciparum* parasites after CQ treatment, compared to their primary infections pre-treatment (158). For the SP regimen, resistant parasites exhibited increased gametocyte production (159,160), even in the absence of treatment failure (160). It has been hypothesised that the rapid spread of CQ and SP resistance may be due to this transmission advantage (161). In addition, certain drug resistance conferring polymorphisms in the *Pfdhfr* gene were suggested to be host-specific, after observing contrasting SNPs in the *Pfdhfr* gene conferring resistance to either pyrimethamine (high prevalence in human blood samples, low prevalence in mosquito midguts) or cycloguanil, a metabolite of proguanil (low prevalence in human blood samples, high prevalence in mosquito midguts) (35). This can reflect a transmission (dis)advantage or a difference in selective pressures that play a role in human versus mosquito hosts.

For artemisinin resistance, it is currently unknown whether parasites with resistance-conferring mutations in the *PfK13* gene possess similar advantages. Cambodian patients with *PfK13* mutant parasites exhibited a higher gametocyte prevalence upon clinical presentation compared to those with wild-type infections (162), suggesting increased transmission potential. However, this could also reflect a longer duration of infection potentially due to ineffective prior treatment. Additionally, *in vitro* experiments revealed that male gametocytes may preferentially survive artemisinin exposure and may result in enlarged oocysts (163,164). Conversely, another study observed a decreased sexual conversion rate in parasites with *PfK13* mutations and reduced artemisinin susceptibility, though the sample size was small (165).

1.9 Malaria in Mali

In 2022 alone, Mali reported an estimated 7.9 million cases of malaria and 19,716 deaths for a population of around 22 million (1). The entire population of Mali is at risk of malaria, with transmission patterns varying across the country's five geo-climatic zones (166). Transmission is highly seasonal in most areas in Mali, overlapping with the annual rainy season that occurs from June to December. In a study conducted during the high-transmission season over six consecutive years (2016-2021) (167), malaria prevalence from all *Plasmodium* species was 65.20% and 22.41% for passive and active screening, respectively. *P. falciparum* was the most prevalent species encountered in active and passive screening (59.33%, 19.31%), followed by *P. malariae* (1.50%, 1.15%) and *P. ovale* (0.32%, 0.06%). No *P. vivax* was detected. Children aged 5–9 presented the highest frequency of *P. falciparum* infections. Gametocyte prevalences showed highly dependent on seasonality and geography, and highly variable across age groups, with the lowest gametocyte carriage found in children under one year of age (167).

Malaria control efforts in Mali include the distribution of ITNs, the usage of IRS, and administration of IPTp using SP, SMC using SP-AQ, and the treatment of malaria cases with AL (1). Prior to this thesis, drug resistance frequencies reported in isolates sampled between 2015 and 2017, found the *Pfcrt* K76T chloroquine-resistance genotype at a rate of 27-64%, depending on the region, while the amodiaquine-resistance *Pfmdr1* N86Y variant was found at prevalences of 11-29% (168,169). The prevalence of the *Pfdhfr* triple mutant was observed between 50-77%, and the prevalences of the *Pfdhps* S436A and G437A variants were found to be around 42-65% and 47-63%, respectively. No WHO validated or candidate ART-R conferring variants were detected. Population structure analysis of whole-genome sequenced isolates by principal component analysis showed that isolates from different regions clustered into a single genetic cluster with a few outliers, and admixture analysis revealed similar ancestry for all Malian isolates (168).

1.10 Thesis structure

This thesis investigates the genomic diversity and transmissibility in *P. falciparum* infections with the aim of improving our understanding of the parasite transmission biology, drug resistance levels, and the transmission-blocking activity of antimalarials. For this investigation, I use samples and data from six clinical trials conducted in Ouélessébougou, Mali. Published and submitted manuscripts included in this thesis can be found in **Table 2**.

1.10.1 Chapter 2 (published paper)

In chapter 2, I provide a recent assessment of the whole genome diversity of *P. falciparum* isolates from Mali, collected during two clinical trials in Ouélessébougou and surrounding villages in 2019 and 2020. I place these isolates in the context of older publicly available Malian isolates (2007-2017), as well as African-wide *P. falciparum* populations, using Illumina WGS data and population genomic analyses. I hypothesised that antimalarial resistance mutations associated with SP and AL would have increased due to the ongoing use of these drugs in this region, and that CQ resistance would have decreased since this antimalarial is no longer used in Mali. Using sWGA, I sequenced parasite data from asymptomatic infections with low parasitaemia. My analysis revealed high multiclonality and low relatedness among Ouélessébougou isolates, with subclustering observed among Malian isolates based on location and collection date. I found increased frequencies of molecular markers for SP and lumefantrine resistance compared to older isolates while CQ resistance levels remained unchanged, contrary to my hypothesis. Lastly, the analysis identified 21 genes under selective pressure. The data generated in this chapter contributes to the open-source WGS resource, as this reflects a most recent evaluation of genomic diversity and drug resistance levels in Mali.

1.10.2 Chapter 3 (submitted manuscript)

In chapter 3, continuing my work on *P. falciparum* isolates from Ouélessébougou, I focus on the transmission dynamics of these malaria isolates. I investigate this by examining human bloodstream malaria isolates and the malaria infected midguts of mosquitoes that fed on this blood material, both at baseline and at multiple timepoints following ACT treatment. Utilising both Illumina and Oxford Nanopore sequencing technologies, I employ amplicon sequencing to determine the complexity of infection and frequency of molecular markers of drug resistance in both hosts. I hypothesised that before treatment, potentially due to an over-representation of the non-transmissible asexual stages in human blood samples, we may not observe the same parasite clones in human blood and infected mosquito midgut. However, after clearance of asexual parasites by an ACT, because gametocytes persist longer, the same parasite clones should be detectable in both the blood and mosquitoes. Additionally, I hypothesised that molecular markers of CQ and SP resistance may confer a transmission advantage, as this had been suggested by previous studies (159,160). This chapter aims to enhance our understanding of the clonal transmissibility in polyclonal asymptomatic malaria infections, a population that has been largely understudied.

1.10.3 Chapter 4 (accepted manuscript)

Blocking transmission is crucial to slow the spread of malaria, but despite this, few studies have tested the transmission-blocking efficacy of commonly used antimalarials on gametocytes and transmission. TACTs, such as AL-AQ, have recently been proposed to mitigate the spread of ART-R, and have been proven safe and effective against infections with ART-R parasites, however, their transmission-blocking properties remains untested. In chapter 4, I supported the design and implementation of a sixth trial in Ouélessébougou. I report on the results of this trial testing five different combinations of antimalarials for their gametocytocidal and transmission-blocking efficacy, using direct membrane feeding assays. We hypothesised that AL and AL-AQ would block nearly all transmission within a few days, as had been previously observed for AL (42), while gametocytes and transmission after AS-AQ may persist longer (67,170). Treatment groups with an SLD-PQ (0.25 mg/kg) were hypothesised to achieve a complete annulation of transmission within 48 hours. Additionally, with the aim of gaining insights into their mechanism of action, gametocytes were enriched at baseline and at day 2 after treatment initiation and were followed by a second infectivity assay. Notably, this study provides the first assessment of the transmission-blocking efficacy of the TACT regimen AL-AQ and the ACT AS-AQ, alone and in combination with an SLD PQ. In addition, this study adds to the important biobank of samples that can be used to assess interactions between drug pressure, drug resistance and transmissibility.

1.10.4 Chapter 5 (submitted manuscript)

The fifth chapter presents a pooled analysis of individual patient data from the six clinical trials conducted at the same study site in Ouélessébougou between 2013 and 2022, assessing the gametocytocidal and transmission-blocking effect of fifteen different combinations of antimalarials. This allows for direct comparison of the anti-gametocyte and anti-transmission effects between different regimens of antimalarials. We found pronounced differences between ACTs, with AL proving to be the most effective in blocking transmission. SLD PQ rapidly clears and sterilises gametocytes when used with an ACT, while SLD TQ shows a delayed effect. Our analysis also confirms substantially higher post-treatment transmission after SP-AQ compared to most ACTs. The analysis presented in this chapter provides an important comparison of transmission-blocking effects of a plethora of antimalarials, which can be used to inform treatment guidelines.

1.10.5 Chapter 6: Discussion and conclusions

In the final chapter, I discuss the findings from all chapters in the context of the drug resistance threat in Africa, emphasising the urgent need to halt its continued spread. Additionally, I address my studies' limitations and outline directions for future research.

Table 2. Published and submitted manuscripts included in this thesis.

Thesis Chapter	Manuscript Title	Authors	Journal	Status
Chapter 2	Genome-wide genetic variation and molecular surveillance of drug resistance in *Plasmodium falciparum* isolates from asymptomatic individuals in Ouélessébougou, Mali.	**Leen N. Vanheer**, Almahamoudou Mahamar, Emilia Manko, Sidi M. Niambele, Koualy Sanogo, Ahamadou Youssouf, Adama Dembele, Makonon Diallo, Seydina O. Maguiraga, Jody Phelan, Ashley Osborne, Anton Spadar, Merel J. Smit, Teun Bousema, Chris Drakeley, Taane G. Clark, William Stone, Alassane Dicko, Susana Campino	Scientific Reports	Published 12th June 2023
Chapter 3	Preferential transmission of minority and drug-resistant clones in polyclonal infections in Mali.	**Leen N. Vanheer,** Emilia Manko, Almahamoudou Mahamar, Jody Phelan, Koualy Sanogo, Youssouf Sinaba, Sidi M Niambele, Adama Sacko, Sekouba Keita, Ahamadou Youssouf, Makonon Diallo, Harouna M Soumare, Kjerstin Lanke, Djibrilla Issiaka, Halimatou Diawara, Sekou F Traore, Lynn Grignard, Teun Bousema, Alassane Dicko, Chris Drakeley, Susana Campino, William Stone	Malaria Journal	Submitted
Chapter 4	Artemether-lumefantrine-amodiaquine or artesunate-amodiaquine combined with single low-dose primaquine to reduce *Plasmodium falciparum* malaria transmission in Ouélessébougou, Mali: a five-arm, phase 2, single-blind, randomised clinical trial.	Almahamoudou Mahamar, PhD*, **Leen N Vanheer, MD*,** Merel J Smit, MD, Koualy Sanogo, MD, Youssouf Sinaba, MD, Sidi M. Niambele, PharmD, Makonon Diallo, MD, Oumar M Dicko, MD, Richard S. Diarra, MD, Seydina O Maguiraga, MD, Ahamadou Youssouf, PharmD, Adama Sacko, MS, Sekouba Keita, MS, Siaka Samake, Pharm D, Adama Dembele, MS, Karina Teelen, Yahia Dicko, MD, Sekou F. Traore, PhD, Prof Arjen Dondorp, MD, Prof Chris Drakeley, PhD†, William Stone, PhD†, Prof Alassane Dicko, MD†	Lancet Microbe	Accepted July 2024. In Press
Chapter 5	The transmission blocking activity of artemisinin-combination, non-artemisinin, and 8-aminoquinoline antimalarial therapies: a pooled analysis of individual participant data.	**Leen N Vanheer, MD***, Jordache Ramjith, PhD*, Almahamoudou Mahamar, PhD*, Merel J Smit, MD, Kjerstin Lanke, Michelle E Roh, PhD, Koualy Sanogo, MD, Youssouf Sinaba, MD, Sidi M. Niambele, PharmD, Makonon Diallo, MD, Seydina O Maguiraga, MD, Sekouba Keita, MS, Siaka Samake, PharmD, Ahamadou Youssouf PharmD, Halimatou Diawara, MD, Sekou F. Traore, PhD, Roly Gosling, MD, Joelle M Brown, PhD, Chris Drakeley, PhD, Alassane Dicko, MD†, Will Stone, PhD1†, Teun Bousema, PhD2†	PLoS Medicine	Submitted

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Chapter 2: Genome-wide genetic variation and molecular surveillance of drug resistance in *Plasmodium falciparum* isolates from asymptomatic individuals in Ouélessébougou, Mali

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Genome-wide genetic variation and molecular surveillance of drug resistance in *Plasmodium falciparum* isolates from asymptomatic individuals in Ouélessébougou, Mali

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Sequence analysis of *Plasmodium falciparum* parasites is informative in ensuring sustained success of malaria control programmes. Whole-genome sequencing technologies provide insights into the epidemiology and genome-wide variation of *P. falciparum* populations and can characterise geographical as well as temporal changes. This is particularly important to monitor the emergence and spread of drug resistant *P. falciparum* parasites which is threatening malaria control programmes world-wide. Here, we provide a detailed characterisation of genome-wide genetic variation and drug resistance profiles in asymptomatic individuals in South-Western Mali, where malaria transmission is intense and seasonal, and case numbers have recently increased. Samples collected from Ouélessébougou, Mali (2019–2020; n = 87) were sequenced and placed in the context of older Malian (2007–2017; n = 876) and African-wide (n = 711) *P. falciparum* isolates. Our analysis revealed high multiclonality and low relatedness between isolates, in addition to increased frequencies of molecular markers for sulfadoxine-pyrimethamine and lumefantrine resistance, compared to older Malian isolates. Furthermore, 21 genes under selective pressure were identified, including a transmission-blocking vaccine candidate (*pfCelTOS*) and an erythrocyte invasion locus (*pfdblmsp2*). Overall, our work provides the most recent assessment of *P. falciparum* genetic diversity in Mali, a country with the second highest burden of malaria in West Africa, thereby informing malaria control activities.

Malaria was estimated to cause 250 million illnesses worldwide and 619 thousand associated deaths in 2021 alone. Mali is amongst the 9 countries with the highest burden of disease for malaria and its number of malaria cases has increased between 2016 and 2021[1]. Most malaria cases in Mali and in the rest of sub-Saharan Africa are caused by *Plasmodium falciparum,* the most virulent human malaria parasite. Mali is divided into five ecoclimatic zones[2] across which malaria transmission fluctuates. The South-Western zone has the highest *P. falciparum* incidence rates and transmission is highly seasonal, coinciding with the annual rainy season. However, across different regions in South-Western Mali, the degree of perennial and seasonal transmission varies, as does the timing of the rainy season, causing heterogeneity in transmission seasons and malaria epidemiology[2].

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In accordance with WHO guidelines, current malaria intervention strategies in Mali include artemisinin combination therapies (ACTs) for uncomplicated *P. falciparum* malaria, with Artemether-Lumefantrine (AL) as the first-line treatment. Seasonal malaria chemoprevention (SMC) strategies, consisting of sulfadoxine-pyrimethamine with amodiaquine (SPAQ) in children and intermittent preventative treatment for pregnant women (IPTp) with sulfadoxine-pyrimethamine (SP) alone, have been widely implemented in African countries and have been introduced in Mali from 2012 and 2015 onwards, respectively.

In addition to disruption due to the COVID-19 pandemic, increased disease incidence has been linked to the emergence and spread of drug resistant *P. falciparum* parasites[1]. Drug-resistant strains against SP, artemisinin derivatives and partner drugs pose a major challenge in the fight against malaria[3]. Molecular markers of drug resistance are therefore extremely useful in identifying and monitoring drug-resistant *P. falciparum* parasites and have been described for most antimalarial drugs. Mutations in the multidrug resistance (*pfmdr1*) gene for example have been associated with various parasite responses to lumefantrine, chloroquine (CQ), amodiaquine, mefloquine and piperaquine. The genetic basis of SP resistance is well documented and involves an accumulation of mutations in the dihydrofolate-reductase (*pfdhfr*) gene (N51I, C59R, S108N and I164L) and the dihydropteroate-synthase (*pfdhps*) gene (S436A/F, A437G, K540E, A581G and A613S/T). Infections harbouring the triple *dhfr* C**IRN**I mutant (mutations underlined) are common throughout Africa and are pyrimethamine resistant[4,5]. The combination of this triple *dhfr* mutant with the double-mutant dhps (A437G and K540E, S**GE**AA) further increases the risk of SP treatment failure to 50%[5,6].

Mutations in the *pfkelch13* gene associated with decreased artemisinin susceptibility emerged and spread in South-East Asia and have more recently emerged in several countries in East-Africa[7–9]. It is expected that this will spread to other parts of Africa and therefore continuous monitoring of *pfkelch13* genetic variation is critical. A high prevalence of CQ resistance led to its removal from any treatment guidelines for *P. falciparum* infections in sub-Saharan Africa. Decades after this, CQ-sensitive *P. falciparum* parasites have re-emerged in many parts of the world[10–13]. For that reason, a re-introduction of CQ, in combination with other antimalarials, has been proposed[14]. Reports from Mali have not observed a substantial decrease in frequency of mutations in the chloroquine resistance transporter (*pfcrt*) associated with CQ resistance[15], although recently a downwards trend was reported in Malian isolates collected in 2016–2017[16]. Continued assessment is therefore important to determine whether CQ could be reintroduced in the region in future.

The majority of malaria infections in endemic areas are asymptomatic[17], however, such carriers tend to be underrepresented in genome-wide large-scale genetic analyses, due to both the lack of seeking treatment and technical difficulties with sequencing low density infections. Asymptomatic carriers are the main contributors to the infectious reservoir as they can remain infectious for long periods of time without showing any symptoms, meaning they could unknowingly spread the disease to others while remaining unaware they are infected. In addition to this, their frequency in the population and the characteristics of individuals that are more likely to be asymptomatic, such as a higher risk of mosquito bites, further increase their contribution to the infectious reservoir[18,19]. Therefore, understanding the genetic characteristics of the asymptomatic reservoir is key to effectively controlling the spread of malaria.

Advances in Next-Generation Sequencing (NGS) technologies have rendered Whole Genome Sequencing (WGS) more accessible and affordable for use in disease management and malaria control. Along with identifying and monitoring molecular markers of drug resistance, investigating genomic variation is useful for understanding transmission dynamics, selective sweeps, and *P. falciparum* epidemiology. Assessing changes in genomic relatedness within a population, including through using identity-by-descent measures, can provide insights into parasite population demography and transmission intensity over time[20]. In addition, the identification of genes under selection can offer insights into the selective pressure exhibited by drugs or other unknown agents, which is important for developing effective strategies for prevention, control, and treatment of malaria.

In summary, monitoring of *P. falciparum* drug resistance is essential to inform drug policies worldwide, particularly in regions of high malaria transmission, and the WHO recommends regular updating and monitoring of antimalarial resistance to support progress made towards malaria control and elimination[21]. In this report, we provide an in-depth analysis of drug resistance profiles and recent genomic variation, using selection, ancestry and identity-by-descent analysis, in asymptomatic individuals in Ouélessébougou and neighbouring villages, Mali, in 2019 and 2020. Furthermore, we place these in the context of Malian *P. falciparum* isolates collected between 2007 and 2017, as well as African-wide parasite populations.

Results

Genomic population structure of Malian *P. falciparum* populations. Genome-wide SNP analysis of 962 Malian *P. falciparum* isolates, collected between 2007 and 2020 in two different studies and originating in 9 locations (Fig. 1A), revealed differences in multiplicity of infection and population structure. Ouélessébougou isolates collected in 2019–2020 originated from asymptomatic *P. falciparum* infections, while no details on clinical presentation were available for publicly available genomes from the MalariaGEN database. *P. falciparum* incidence rates varied across sample sites and across two decades (Fig. 1A,B). A total of 863,046 high-quality SNPs were identified. Multiclonality was measured using the F_{ws} metric, or in-breeding coefficient, which is indicative of monoclonality if > 0.95, while a lower F_{ws} metric reflects multiclonality. The mean F_{ws} value for the 2019–2020 Mali samples (n = 87) from Ouélessébougou was 0.80, with only 20% of samples harbouring a single clone. We found a higher multiclonality in the 2019–2020 Ouélessébougou isolates compared to isolates from different sites where collection took place between 2015 and 2017 (Fig. 1C). Using the SNP data, a Uniform Manifold Approximation and Projection (UMAP) statistical analysis to cluster isolates revealed little spatial substructure between populations, although some grouping based on location (Fig. 1D) and collection year (Fig. 1E) could be observed and the Ouélessébougou isolates formed an individual subcluster.

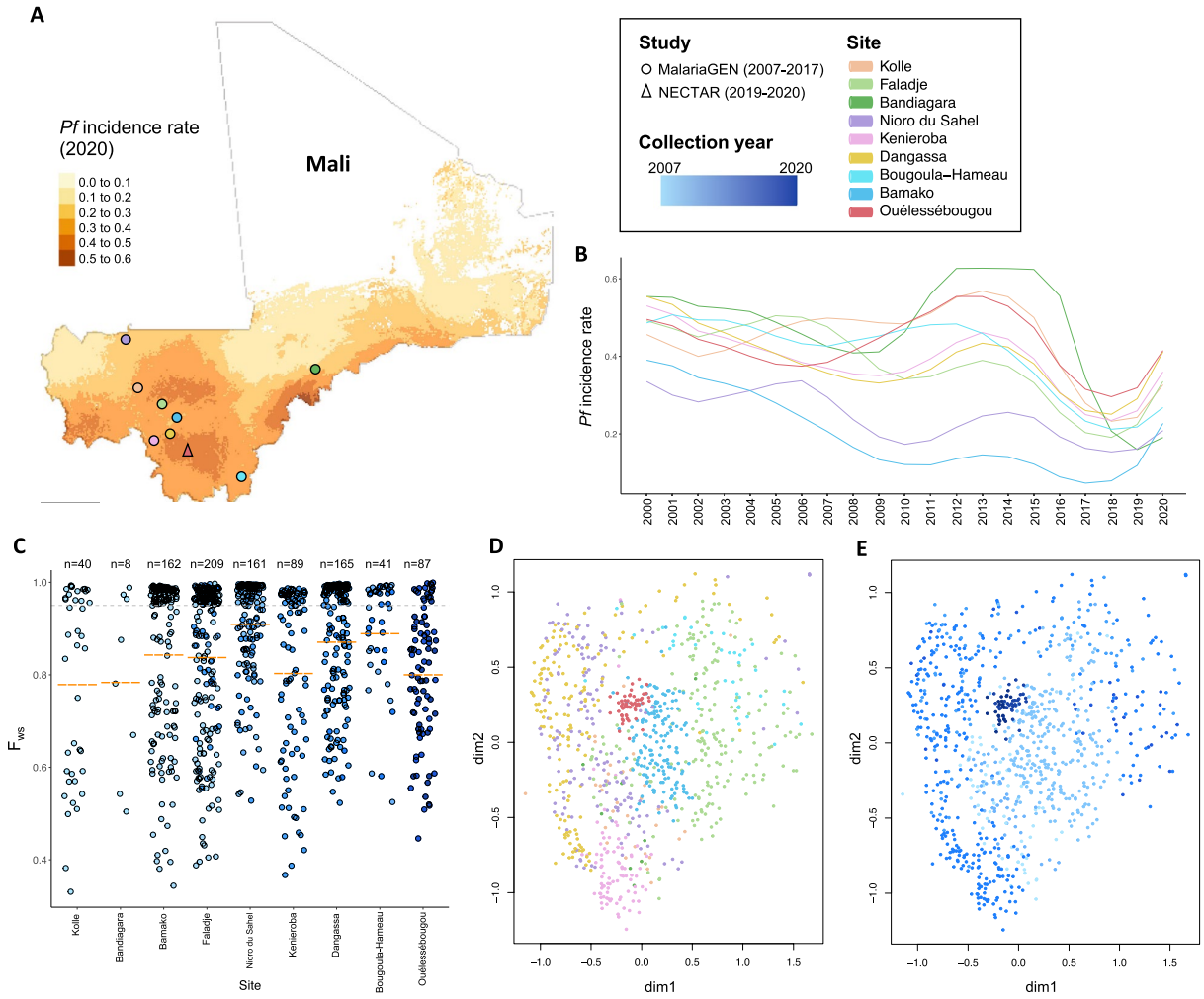

Figure 1. Population structure of *P. falciparum* isolates collected in Mali from 2007 to 2020. **(A)** Map of Mali presenting the number of newly diagnosed *Plasmodium falciparum* cases per 1,000 population in 2020 (colour scale) and indicating collection sites of the MalariaGEN studies (circle) and the New Drug Combinations for *P. falciparum* Transmission Reduction (NECTAR) clinical trials (triangle), coloured by village or city name. This map was generated using the *tmap* R package (version 3.3.3; https://r-tmap.github.io/tmap/) **(B)** *P. falciparum* incidence rate for each collection site (by colour) from 2000 to 2020. **(C)** Complexity of infections estimated by the in-breeding coefficient (F_{ws} metric) per sample, classified per study site, with the colour indicating collection year. Dotted grey trendline is at 0.95, dashed orange marks indicate mean F_{ws} value per group. **(D, E)** Uniform Manifold Approximation and Projection (UMAP) visualisation of 962 Malian *P. falciparum* isolates, coloured by site and collection year, respectively.

Genomic population structure of Malian *P. falciparum* populations in comparison to African-wide populations. A SNP-based UMAP visualisation and maximum likelihood tree show how the most recently collected Ouélessébougou isolates are related to African-wide populations (n = 875) and revealed similarity with West African and Central African isolates (Fig. 2A,B). No distinguishable clusters could be detected, indicating high relatedness, and suggesting movements of genetic information within these populations, which can occur both through human and vector migration. East African, Southeast African and Central African isolates formed separate clusters, while the Horn of Africa appeared to be a distinct cluster within the East African population. Multiclonality assessment showed that the 2019–2020 Malian isolates were relatively more multiclonal compared to the isolates collected in other West African countries (Fig. 2C).

Frequencies of drug resistance molecular markers. Increased frequencies of molecular markers of sulfadoxine-pyrimethamine and persistence of chloroquine resistance markers were observed in the Ouélessébougou 2019–2020 population (n = 87), compared to Malian isolates from 2007 to 2017 (n = 876) (Table ST1). Polymorphisms causing amino acid changes that confer chloroquine resistance in *pfcrt* (K76T, A220S, Q271E, N326S, I356T and R371I) persisted at similar frequencies over time (Fig. 3A,B). The N86Y polymorphism on *pfmdr1* decreased over time from 28.4% in 2007 to 2.6% in 2020. This 86Y allele has been linked to chloroquine

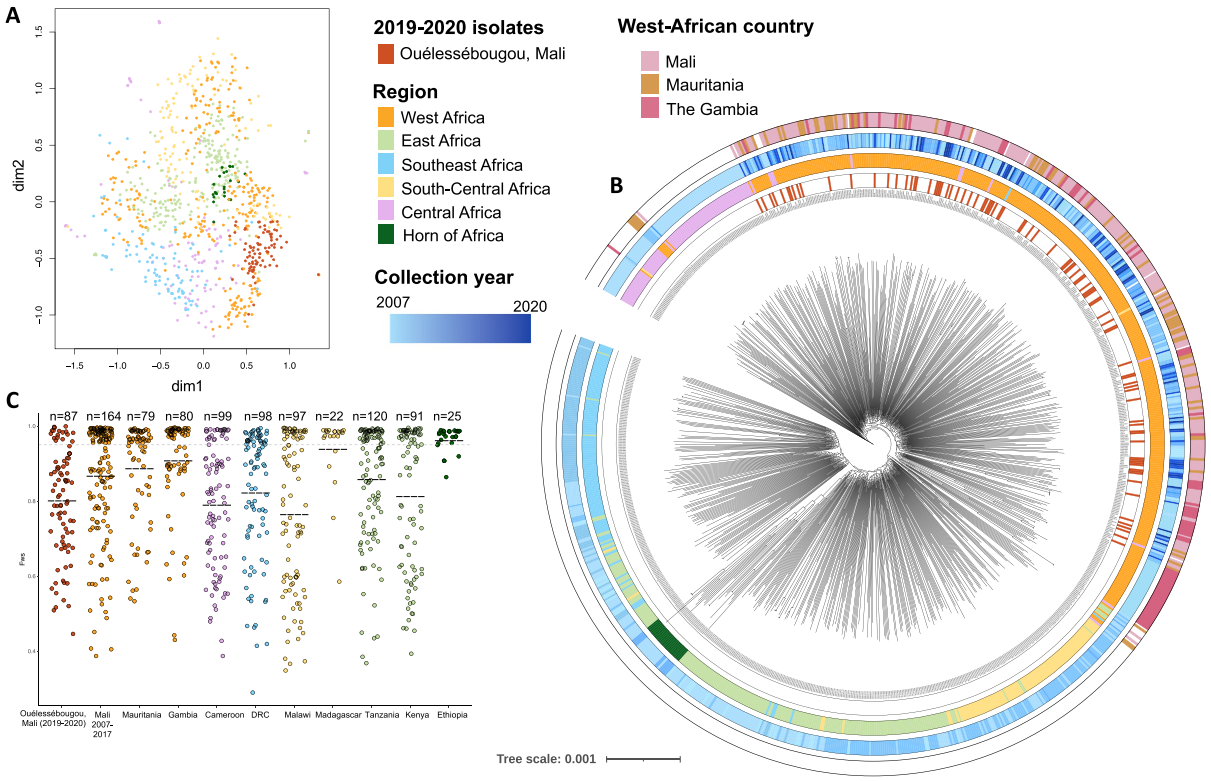

Figure 2. Population structure of Malian *P. falciparum* isolates in the context of African-wide populations. **(A)** Uniform Manifold Approximation and Projection (UMAP) visualisation of 962 African-wide *P. falciparum* isolates, including 87 isolates collected in Mali in 2019–2020 and 164 Malian isolates collected in 2007–2020. African regions where the isolates were collected are indicated by colour. **(B)** Maximum-Likelihood tree of the same dataset, annotated by the 2019–2020 Malian isolates (inner ring), the African region of sample origin (second ring), collection year (third ring) and West-African country of collection (outer ring). **(C)** Complexity of infections (F_{ws} coefficient) per sample, grouped by country and separating the 2019–2020 Malian isolates from the 2007 to 2017 Malian isolates. Dotted grey trendline is at 0.95, dashed lines indicate mean F_{ws} value per group.

and amodiaquine resistance as well as piperaquine resistance (86Y allele in combination with Y184F), while parasites carrying the N86 allele show lower susceptibility to lumefantrine, piperaquine and mefloquine[22,23]. Y184F and D1246Y amino acid changes in MDR1 persisted at comparable frequencies. Three non-synonymous SNPs in *pfmdr1* that were not previously found in Malian isolates, resulting in amino acid changes S400C, D431Y and K503N, were identified in 2.13% of the 2019 isolates and 2.94% of the 2020 isolates, respectively (Table ST1). The frequency of point mutations in *pfdhfr* associated with pyrimethamine resistance (N51I, C59R and S108N) approximately doubled since 2007, reaching alarming frequencies of 92.4%, 93.9% and 92.7%, respectively, in 2020. In addition, the C**IRN**I triple mutant haplotype increased in frequency and made up 82.7% of the parasite population in 2019–2020, while the wild-type haplotype was reduced to 4.9% (Fig. 3C, Table ST2). One mutation in *pfdhps* conferring sulfadoxine resistance (A437G) increased in frequency from 27.7% in 2007 to 74.3% in 2020, while S436A showed a downwards trend (Fig. 3D). The *pfdhps* K540E mutation was found in 4 isolates in 2019–2020 and all of these were combined with the C**IRN**I triple *dhfr* mutant, leading to a quadruple mutant frequency of 2.47%. A non-synonymous SNP at position 748,145 in *pfdhfr* (V20I), was newly identified in Malian isolates in 2019, at 2.04% frequency. No known mutations in *pfkelch13* associated with artemisinin resistance were identified in any of the Malian isolates. The *pfkelch13* mutations R255K, K189N and K189T persisted at similar frequencies to the frequencies observed in 2007 and no new mutations in *pfkelch13* were identified (Table ST1).

Regions under selective pressure in Malian isolates. Determination of genomic regions under directional selection by haplotype structure analysis within the Ouélessébougou 2019–2020 isolates and in comparison to the older Malian populations, revealed a number of genes to be under selective pressure. The integrated haplotype score (*iHS*) metric was used to identify SNPs under selection within the 2019–2020 population (Fig. 4A) and regions of the genome with an elevated number of SNPs under selection (Table ST3). This identified conserved *Plasmodium* protein coding genes with unknown function (PF3D7_0212100 and PF3D7_0425100), predominantly expressed in ookinetes and ring stages, respectively, as well as the *pfCelTOS* gene, which encodes

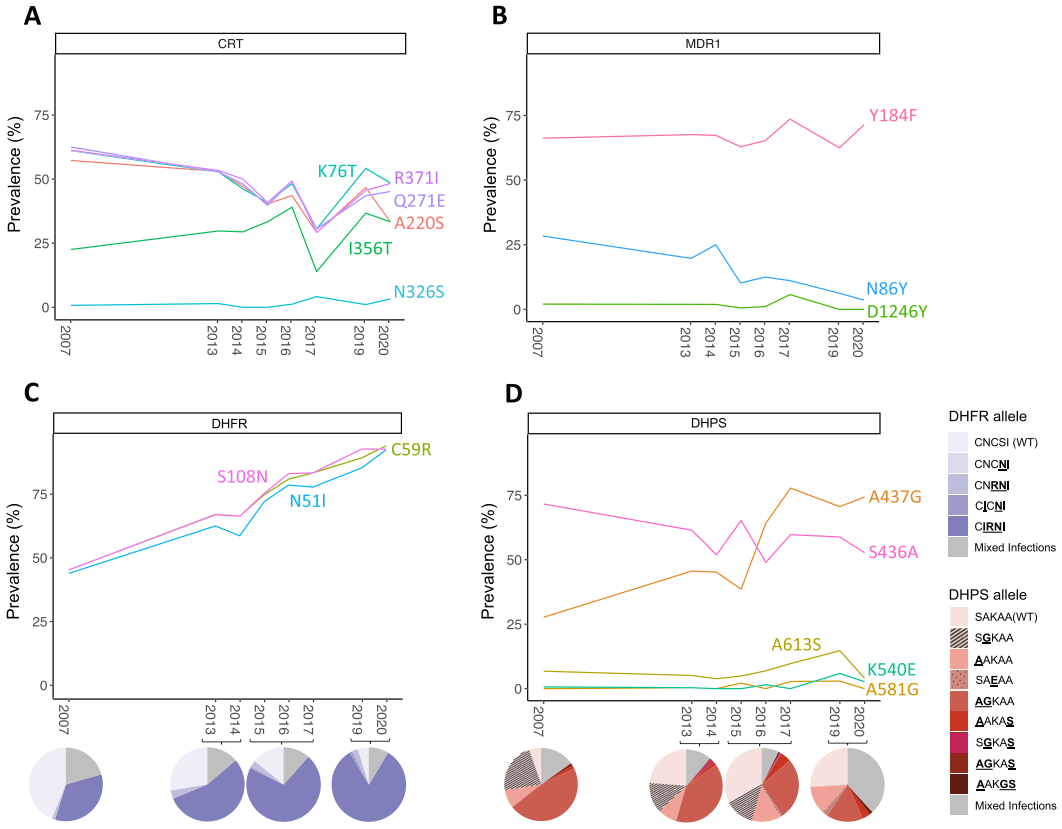

Figure 3. Prevalence of single nucleotide polymorphisms known to cause decreased drug susceptibility. Minor allele frequencies (MAFs) are shown for isolates collected from 2007 to 2020 in Mali for genes associated with drug resistance, including **(A)** *pfcrt*, **(B)** *pfmdr1*, **(C)** *pfdhfr* and **(D)** *pfdhps*. Pie charts in C and D represent the frequencies at which combinations of *pfhdfr* and *pfdhps* mutants were observed in the *P. falciparum* isolates collected in 2007, 2013–2014, 2015–2017 and 2019–2020.

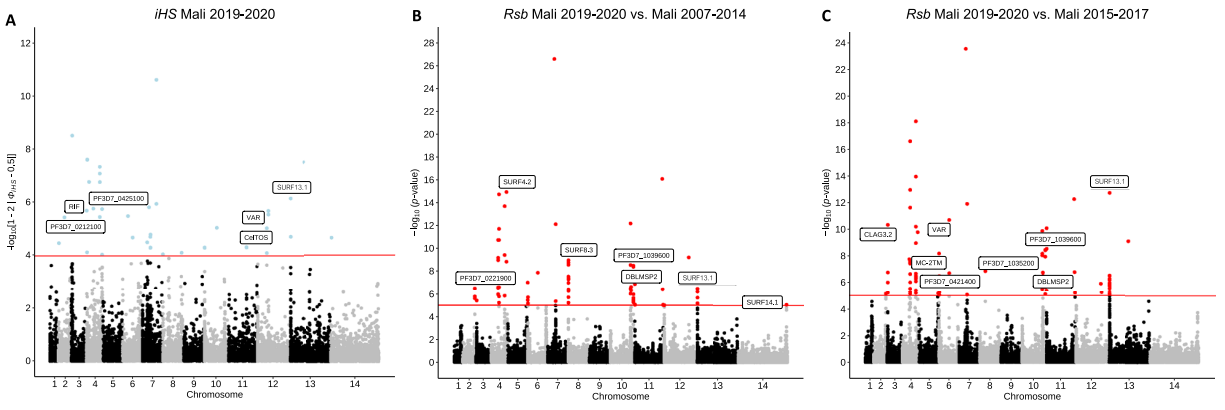

Figure 4. Scan for evidence of recent directional selection. Manhattan plots show analysis of the **(A)** integrated haplotype score (*iHS*) for individual SNPs in the 2019–2020 Mali population and *Rsb* cross-population test for extended haplotypes comparing the 2019–2020 Mali population to the **(B)** 2007–2014 Mali population and the **(C)** 2015–2017 Mali population.

a cell-traversal protein for ookinetes and sporozoites and was suggested as an attractive vaccine candidate antigen[24,25]. The between-population *Rsb* index was used to identify SNPs under selection when comparing the 2019–2020 population with the older Malian isolates collected in 2007–2014 (n = 414) (Fig. 4B) and 2015–2017 (n = 462) (Fig. 4C). Regions with a high number of SNPs under selection were determined as well (Table ST4). This identified genes associated with erythrocyte invasion (*pfdblmsp2, pfmsp3*), protein transport (*pfMC-2TM*), cytoadherence (*pfCLAG3.2*), and a gene encoding RNA of unknown function (Pf3D7_0421400, RUF6).

Ancestral admixture analysis confirms similar ancestry among Malian isolates. Spatial ancestry estimation of Malian isolates along with African-wide populations found similar ancestral origins among all Malian *P. falciparum* isolates. The optimum number of ancestral populations was estimated to be 5 (K = 5; K1–K5), based on eigenvalue decay corresponding to K ranging from 1 to 10. The K1 ancestral population was dominant in Gambian samples (49.4%), while the K2 ancestral population appeared to be linked to South-Central and East African populations (Malawi, 92.3%; Madagascar, 74.4%; Tanzania, 69%; Kenya, 61.9%). Malian isolates, along with Mauritanian isolates, seemed to contain mostly the K3 ancestral population (Mali, 80.3%; Mauritania, 80.5%), in addition to smaller portions of K2 ancestry (Mali, 8.5%; Mauritania, 7.7%) and K4 ancestry (Mali, 10%; Mauritania, 10.1%). Very low fractions of K1 and K5 ancestries were present in Malian and Mauritanian isolates, except for isolates from Bandiagara in Mali that appeared to not contain any K5 ancestry (Fig. 5).

Identity-by-descent analysis reveals highly diverse Ouélessébougou population. As a measure of genetic relatedness within populations, identity-by-descent (IBD) analysis revealed that Ouélessébougou isolates exhibit very low fractions of pairwise IBD across the genome (median = 0, range = 0–0.133), while the Malian isolates collected in 2007–2014 and 2015–2017 showed a slightly higher relatedness (median = 0.021 and median = 0.017, respectively) (Fig. S2). The top 5% of IBD positions (classified in 10 kb windows of the genome) in the Ouélessébougou isolates were distributed across 17 regions on 3 chromosomes (chr. 6, 7 and 13) (Fig. S3, Table ST5). Three regions with high IBD on chromosome 6 included the gene encoding histone methyltransferase SET1 and the *pfcrt gene* on chromosome 7 was also identified as encompassing high IBD.

Discussion

NGS technologies have provided an increasingly feasible method for exploring genome-wide genomic variation and population dynamics of malaria parasites. Here, we have provided a detailed analysis of genome-wide diversity of *P. falciparum* isolates from asymptomatic gametocyte carriers in 11 villages in Ouélessébougou in 2019 and 2020 and have placed them in the context of previously sequenced Malian isolates, as well as African-wide isolates via genome-wide SNP analysis. We found high multiclonality and low relatedness among isolates and identified genes under selective pressure. We also observed increased frequencies of molecular markers for sulfadoxine-pyrimethamine and lumefantrine resistance, compared to older Malian isolates.

Genomes from Malian *P. falciparum* isolates collected between 2007 and 2017 have previously been generated[16,26]. However, a more up-to-date and systematic sampling strategy is required to support efforts toward infection control and elimination. Ouélessébougou is a rural community in the Koulikoro region of South-Western Mali, which is an area with one of the highest *P. falciparum* incidence rates in the country[27]. Ideally, in such settings there should be regular monitoring of changes in genomic variation, to determine epidemiological

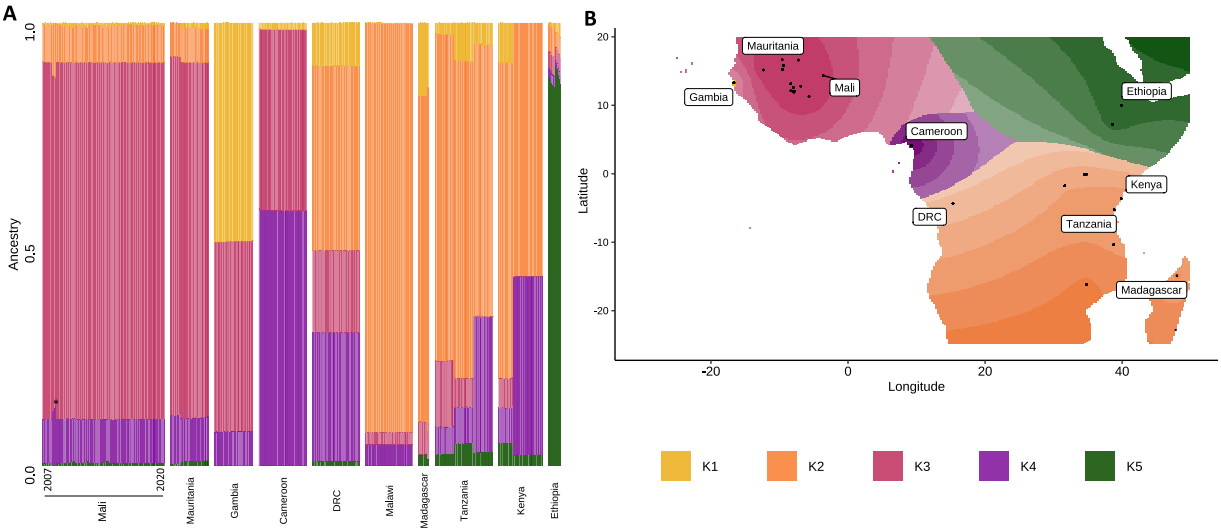

Figure 5. Genome-wide admixture ancestry proportions for *P. falciparum* populations across the African continent. **(A)** Ancestries per isolate (columns) for each country, ranked per country by ascending collection year. Asterisk indicates isolates from Bandiagara in Mali. **(B)** Geographic map of estimated ancestries using K = 5 ancestral populations across the African continent.

patterns and population dynamics, especially among asymptomatic gametocyte carriers, and to assess the impact of infection control measures. For example, continued surveillance of drug resistance molecular markers is necessary to inform malaria chemotherapy approaches.

Malian isolates show minor clustering using UMAP visualisation, with Ouélessébougou isolates forming a subcluster. On a maximum-likelihood tree, Ouélessébougou and other Malian isolates cluster with West-African populations, as expected. Despite a recent report observing decreasing multiplicity of infection from 2007 to 2017 in Mali[16], our findings show a higher multiclonality in 2019–2020 than in isolates from different sites where collection took place between 2015 and 2017. There may be multiple explanations for the highly multiclonal status of the study parasite population, such as the high *P. falciparum* incidence rates in this region (Fig. 1B), the timing of sample collection at peak transmission season (September-December), and the asymptomatic clinical presentation of the individuals. As asymptomatic *P. falciparum* gametocyte carriers are less likely to seek treatment, such infections may be prolonged, increasing the likelihood of reinfection and multiclonality. Despite most available sequencing data originating from incident infections, it is important to genetically characterise asymptomatic *P. falciparum* carriers, as these are the main contributors to the infectious reservoir and carry the infections that escape treatment[18].

Malaria treatment strategies world-wide have been altered over time due to the emergence of resistant parasites to former first-line drugs, with the aim of preserving the efficacy of antimalarial drugs and reducing the global burden of malaria. We found that frequencies of molecular markers conferring CQ resistance have persisted in the 2019–2020 Malian isolates at similar frequencies compared to a decade ago, despite its removal from any *P. falciparum* treatment guidelines since 2006 and a report of a decreasing trend in 2017 in Mali[16]. This is unlike other areas in the world where a return in CQ sensitivity has been observed[11–14,28] and could indicate a low fitness cost associated with maintaining *pfcrt* resistance polymorphisms in the population or a continued over the counter use of CQ[29–31], thereby highlighting the need to investigate and reduce the availability of this drug in Mali and neighbouring countries.

We observed an increase in the N86 variant *pfmdr1* from 71.6% in 2007 to 97.4% in 2020. This N86 allele has been linked to lower susceptibility to lumefantrine, piperaquine and mefloquine[22,23], while the 86Y allele previously showed chloroquine and amodiaquine resistance, as well as piperaquine resistance (86Y allele in combination with Y184F). As a result, AL was previously found to select for the N86 allele, whereas artesunate-amodiaquine and dihydroartemisinin-piperaquine select for the 86Y allele[32–35]. N86Y genotyping could therefore be a useful marker to guide rotation of ACTs in a given geographical area[36]. Thus, the observed increase in the N86 allele may reflect an expanding proportion of isolates with a decreased susceptibility to AL, thereby raising concern for a continued use of AL as the first-line ACT in Mali. A low frequency of the 86Y allele has been reported previously in Mali in 2016, as well as in other African countries where AL is widely used[15,34]. This finding is in contrast with a previously observed increase in the 86Y allele frequency in children in Ouélessébougou between 2014 and 2016, following 3 years of SMC with SPAQ, which was likely due to amodiaquine selecting for the 86Y allele[37]. However, it is important to note that, while a decreased susceptibility to AL in isolates with the N86 allele has been observed in isolates from multiple African countries[22,32,33], this has not been phenotypically assessed in Malian isolates specifically.

High frequencies of triple *pfdhfr* C**IRN**I mutants (82.72%) were observed in the 2019–2020 Ouélessébougou isolates, which is a substantial increase from previous years (34.3%, 55.1% and 70.7% in 2007, 2013–2014 and 2015–2017, respectively). The *pfdhps* K540E mutant, which was quasi absent in the Malian isolates collected between 2007 and 2017, was found in 5.9% of 2019 isolates and 2.7% of 2020 isolates. In addition, all K540E mutant isolates harboured the triple *dhfr* mutant. Continued surveillance is therefore needed to monitor levels of SP resistance and the emergence of quadruple and quintuple (in combination with *dhps* A437G or *dhps* A581G) mutants. Moreover, online mutation prevalence maps such as wwarn.org SP molecular surveyor will become increasingly useful to assess which drug to use for SMC[38]. No SNPs at positions in the *pfkelch13* gene that have been reported to cause decreased susceptibility to artemisinin derivatives were observed in any Malian isolates, however, further monitoring for *pfkelch13* mutants over the next years is needed, as a spread of these alleles from East-Africa or their independent emergence can be expected.

Analysis of haplotype structure in the 2019–2020 Ouélessébougou isolates identified SNPs under selective pressure in the *pfCelTOS* gene, which is involved in sporozoite gliding motility, cell traversal, and is a transmission-blocking vaccine candidate[24,25]. Selective pressure has been observed at this genomic location before[39], indicating a high degree of diversity and thereby rendering *pfCelTOS* a less attractive vaccine candidate. Cross-population analysis comparing parasite populations from 2019–2020 to 2007–2014 and 2015–2017 was performed and, despite increasing frequencies of molecular markers of drug resistance over this 13 year period, we did not find any drug resistance associated SNPs under directional selective pressure. We identified SNPs under selective pressure in genes associated with erythrocyte invasion (*pfdblmsp2)* in both population comparisons and genes associated with protein transport (*pfMC-2TM*), cytoadherence (*pfCLAG3.2*), and a gene encoding RNA of unknown function (Pf3D7_0421400, RUF6) in the comparison between the 2019–2020 and 2015–2017 populations. Selective pressure in *pfdblmsp2* and members of the clag multigene family has been described before and is likely due to their location as surface proteins and the resulting contact with the host immune system[39,40]. IBD analysis revealed very low fractions of pairwise IBD across the genome in isolates from 2019–2020 Ouélessébougou, indicating low relatedness between isolates. The *pfcrt* gene, associated with CQ and piperaquine resistance was found to be in the top 5% of IBD positions, suggesting that this gene is highly conserved among Ouélessébougou isolates. This is in accordance with the observed persistent frequencies of molecular markers for CQ resistance. In addition, the *pfSET1* gene, an import histone lysine methyltransferase, was highly conserved as well. Admixture ancestry analysis showed similar ancestries for all Malian isolates, which is in line with a recent report[16].

This study had several limitations. Firstly, sampling bias cannot be excluded as we only sequenced parasites from asymptomatic infections and from individuals between the ages of 5 and 50, while publicly available datasets do not specify the clinical presentation or individual's age. In addition, copy number analysis of *plasmepsin1-2*, which confers piperaquine resistance, was not performed, due to selective Whole Genome Amplification (sWGA) of parasite DNA prior to sequencing, which prevents adequate analysis of copy number. Lastly, we did not assess phenotypic resistance or any association between genotype and phenotype, however, the molecular markers for drug resistance reported here have been widely proven to predict either in vitro drug resistance levels or patient treatment outcomes[4–6,41–43].

After years of progress towards reducing the global burden of malaria, incidence rates and deaths are now on the rise. This rise could be due to disruptions in malaria control programmes during the COVID-19 pandemic, but antimalarial resistance has been linked to this increase as well. In order to progress towards the goal of reducing the global malaria burden by 90% by 2030[44], we need to generate a comprehensive picture of the genomic variation and the epidemiology of parasite populations. Here, we have provided an updated assessment of genomic diversity of the *P. falciparum* parasite population in South-Western Mali, a region with very intense malaria transmission. Our results showed that the parasites originating from Mali clustered according to their geographic and temporal origin, with the Ouélessébougou isolates forming a separate subcluster. This suggests a high genetic diversity among Malian isolates. Molecular markers of SP resistance were found to be on the rise, which shows a progression towards failure of this drug combination and necessitates continued monitoring. No decline in CQ resistance over time was observed, opposing the idea of a potential CQ re-introduction in Mali in the near future. Our study contributes valuable data regarding the current epidemiological and drug resistance scenario of malaria in Mali and can aid effective malaria control in Mali. Further applications of sequencing approaches, including new portable technologies and amplicon sequencing assays, in malaria endemic countries are needed to assist disease control and inform treatment guidelines.

Methods

Study sites. In 2019 and 2020 a total of 180 individuals with microscopy detectable *P. falciparum* gametocytes in the absence of malaria symptoms were recruited into two clinical trials[45,46] in Ouélessébougou and 11 villages in Ouélessébougou, Mali (Fig. S1). Ouélessébougou is a commune that includes the town of Ouélessébougou and 44 surrounding villages, which have a total of approximately 50,000 inhabitants. The town is located about 80 km south from Bamako, the capital city of Mali. Malaria transmission in Ouélessébougou is highly seasonal occurring during rainy season from July to November. Publicly available WGS data from Malian isolates originated from an additional 8 locations across Southern Mali, largely consisting of rural villages (Bougoula-Hameau, Dangassa, Faladje, Kenieroba, Kolle, Nioro-du-Sahel, Bandiagara) and one urban area (Bamako). All sites have a subtropical climate with dry and rainy seasons, except Nioro-du-Sahel, which is characterised by a desert climate[2,16].

Sample collection and whole genome sequencing. A total of 97 whole blood samples were selected from *P. falciparum* gametocyte carriers, aged between 5 and 50 years, recruited into two previously published clinical trials in Ouélessébougou[45,46]. Permission to conduct this study was obtained from the London School of Hygiene and Tropical medicine Research Ethics Committee (reference numbers 17507 and 21905) and the University of Sciences Techniques and Technologies of Bamako Ethical Committee (reference numbers 2019/67/CE/FMPOS and 2020/96/CE/FMPOS/FAPH) and performed in accordance with relevant guidelines and regulations. The trials were registered on ClinicalTrials.gov (NCT04049916 and NCT04609098). Written informed consent was obtained from all subjects and/or their legal guardians prior to sample collection. For minor participants, informed consent for study participation was obtained from their parent and/or legal guardian. Species identification was carried out by microscopy by trained microscopists at the Malaria Research and Training Centre of the University of Bamako (Bamako, Mali). DNA was extracted from 83.3 µL whole blood using a MagNAPure LC automated extractor (Total Nucleic Acid Isolation Kit High Performance; Roche Applied Science, Indianapolis, IN, USA) and amplified using an established selective whole genome amplification (sWGA) primer set and protocol[47,48]. Whole genome sequencing was performed on an Illumina Novaseq 6000 platform at Eurofins Genomics, Germany, rendering a minimum of 3.75 M paired reads (250 bp reads) per sample.

Data set selection, read mapping, variant detection and quality control. A total of 1701 *P. falciparum* isolates were included in the analysis (Supplementary File S2), including publicly available whole genome sequences Malaria Genetic Epidemiology Network[26] (MalariaGEN) (Pf Community Project, n = 1141; SPOTmalaria project, n = 463) and newly sequenced isolates (n = 97) from asymptomatic *P. falciparum* infected individuals recruited into two previously published clinical trials in Ouélessébougou, Mali[45,46]. All raw sequence data was filtered using *trimmomatic* (version 0.39) and the following parameters: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN:36. Illumina reads were then mapped to the *P. falciparum* (Pf3D7; v3) reference genome using *bwa-mem* software (v0.7.17). SNPs and short insertions and deletions were called using *samtools* (v1.12) and GATK (v4.1.4.1) software. Mixed call SNPs were assigned genotypes determined by a ratio of coverage in which nucleotide calls were 80% or higher. Samples with more than 40% missingness were not included in any analysis. Of the 97 newly sequenced samples, 9 were removed due to missingness and one was removed after species prediction identified *P. malariae* (https://github.com/jodyphelan/malaria-profiler), leaving a total of 87 (89.6%) newly sequenced isolates in the final analyses. Of the publicly available datasets, 8 were removed, resulting a total of 1673 samples included in the analyses, including isolates from Cameroon (n = 99), Democratic Republic of Congo (DRC; n = 98), Gambia (n = 80), Kenya (n = 91), Malawi (n = 97), Mali (n = 962), Mauritania (n = 79), Tanzania (n = 120), Madagascar (n = 22) and Ethiopia (n = 25).

Population genetic analyses. Visualisation of sample site geography was performed using the *ggmap* (version 3.0.0) and *tmap* (version 3.3.3) R packages. *P. falciparum* incidence rates from 2000 to 2020 were accessed from the Malaria Atlas Project[27]. Uniform Manifold Approximation and Projection (UMAP) plots were created using the *uwot* R package with 'hamming' metric and default parameters. A maximum likelihood tree was created by applying *iqtree* software using genome-wide SNPs, and visualisation was performed in *iTOL* (version 6)[49,50]. For population genetic analyses that compare Malian isolates to African-wide populations, a subset of Malian isolates collected between 2007 and 2017 was used (including 10 isolates from each site and each collection year, if available), to obtain comparable number of isolates between populations. Multiclonality was determined by calculating the F_{ws} metric, using an in-house script that utilizes the moimix R package (https://github.com/bahlolab/moimix) to assess within-host diversity in relation to the local population diversity. Only bi-allelic SNPs in coding regions were used for the calculations and Minor Allele frequencies (MAFs) filtering of 0.1% was performed in order to exclusively include robust SNPs. MAFs in drug resistance genes were extracted from the binary matrix and annotated using *Bcftools CSQ* software, which identifies the mutation as non-synonymous, synonymous, or intergenic as well as the codon and protein shift caused by any non-synonymous mutations[51]. Only genomic positions with MAFs of at least 2% were retained in the analysis. Data visualisation was performed using the R-based *ggplot2* package (R version 4.1.2). Regions of the genome under directional selection were identified using the *rehh* R package (version 3.2.2), which uses population-based measures of haplotype diversity within (*iHS*) or between (*Rsb*) populations[52]. The R-based *Tess3r* package (version 1.1.0, using default parameters apart from 'rep = 25'). was used to calculate admixture based on the spatial modelling of allele sharing using geographical coordinates from sampling sites in addition to genome-wide SNP data[53]. The optimal number of ancestries was determined across different numbers of sub-populations (K = 1, 2,…, 10). IBD analysis for isolates with $F_{ws} > 0.85$ was performed to assess connectivity between parasites within populations. This was achieved by estimating the pairwise fraction of shared ancestry between genomic segments, which were inferred to have descended from a recent common ancestor. These IBD fractions were calculated using the *hmmIBD* software with default parameters, which deploys a hidden Markov model-based approach[54]. French translation of the manuscript is available in Supplementary File S3 and was assisted by DeepL Translator (https://www.deepl.com/translator).

Data availability

The datasets presented in this study can be found in European Nucleotide Archives (ENA). The names and sample accession number(s) can be found in the Supplementary File S1. Raw sequences for the isolates sequenced in this study are available from the ENA website (Project accession PRJEB60381).

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Author contributions
A.D., W.S., C.D., M.J.S., T.B., S.C., A.M. and L.N.V conceived and designed the study; A.M., S.M.N., K.S., A.Y., A.D., M.D., S.O. and A.D. coordinated and conducted recruitment and sample collection; L.N.V. processed samples for sequencing. L.N.V., S.C. and C.D. coordinated the sequencing of samples; L.N.V., E.M., J.P., A.O. and A.S. performed the bioinformatic and statistical analysis, under the supervision of S.C. and T.G.C.; L.N.V. wrote the first draft of the manuscript, and the final version included edits from all authors. The final manuscript was read and approved by all authors.

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Competing interests
The authors declare no competing interests.

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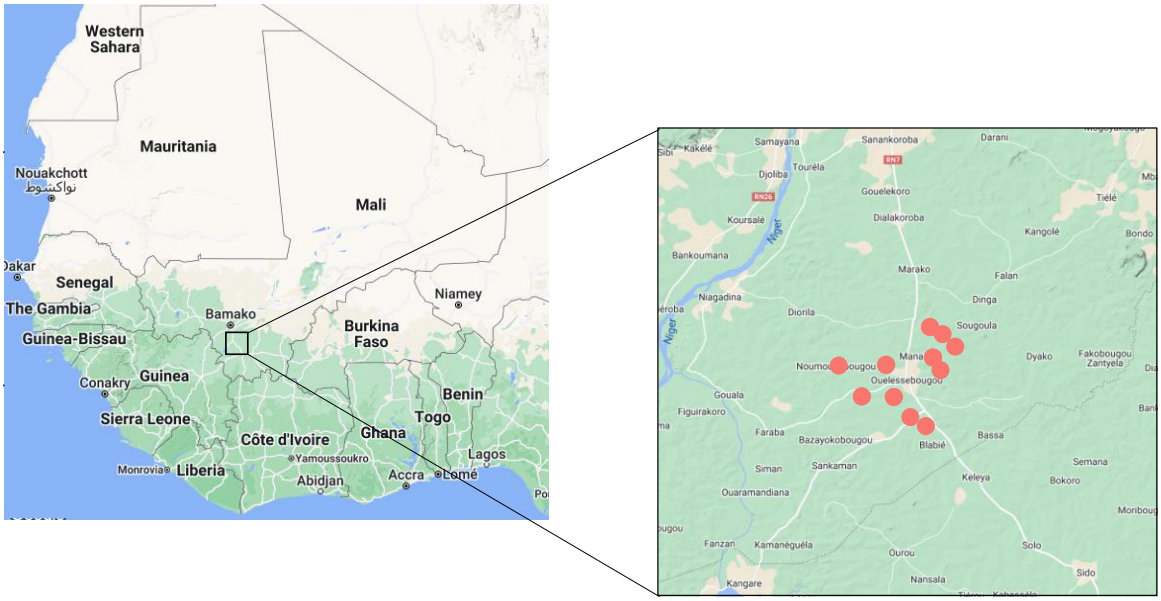
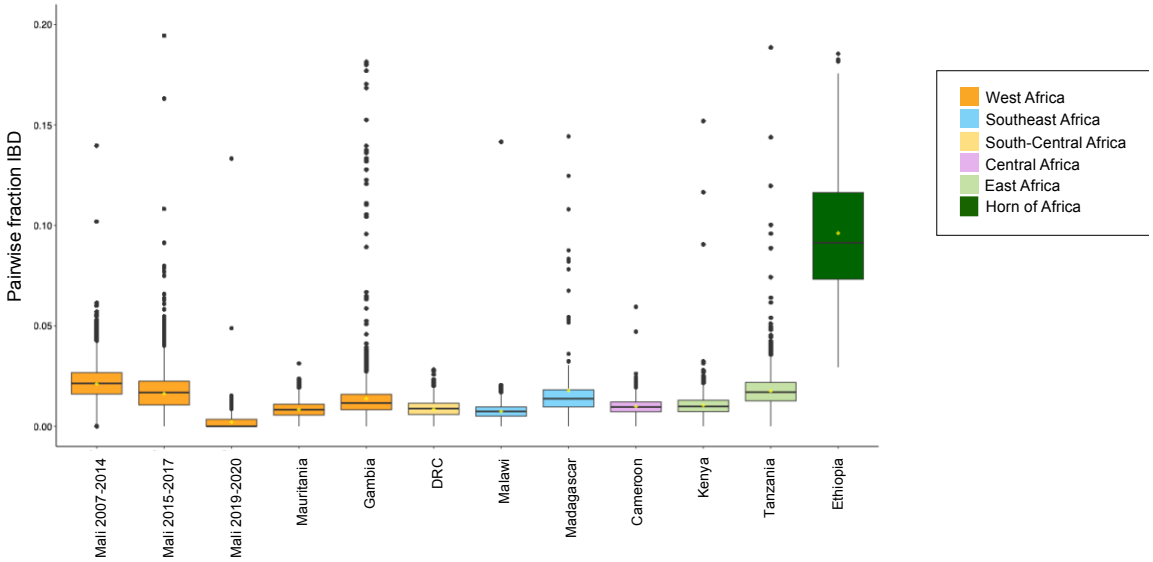
SUPPLEMENTAL FIGURES

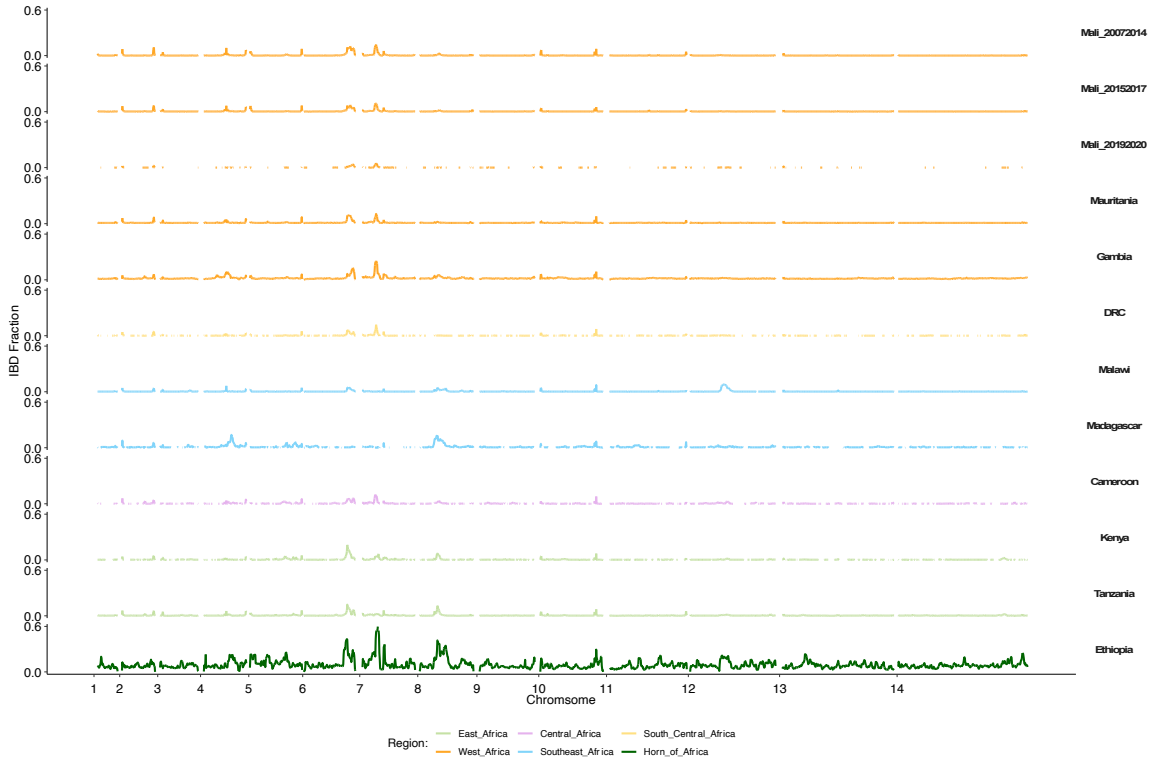
Supplemental Figure S1. Sampling sites in Ouélessébougou. Location of 11 sample sites (red dots) in Ouélessébougou and surrounding villages where *P. falciparum* blood samples were collected in 2019 and 2020. This map was generated using the *ggmap* R package (version 3.0.0; https://github.com/dkahle/ggmap).



Supplemental Figure S2. Pairwise Identity-by-descent (IBD)-fractions across country-level populations of *P. falciparum* isolates. Isolates with F_{ws}>0.85 were incorporated in the analysis and include isolates from Mali 2007-2014 (67), Mali 2015-2017 (97), Mali 2019-2020 (87), Mauritania (79), Gambia (80), Cameroon (99), DRC (98), Malawi (97), Madagascar (22), Tanzania (120), Kenya (91) and Ethiopia (25) populations. All boxplots consist of boxes (median and interquartile range) and whiskers that extend to the most extreme data point which is no more than 1.5 times the interquartile range from the box.



Supplemental Figure S3. Genome-wide distribution of pairwise identity-by-descent (IBD) fractions across country-level populations of *P. falciparum* isolates. Isolates with F_{ws}>0.85 were incorporated in the analysis and include isolates from Mali 2007-2014 (67), Mali 2015-2017 (97), Mali 2019-2020 (87), Mauritania (79), Gambia (80), Cameroon (99), DRC (98), Malawi (97), Madagascar (22), Tanzania (120), Kenya (91) and Ethiopia (25) populations. Top 5% of genomic regions for the Mali 2019-2020 population are presented in Supplemental Table S4.



SUPPLEMENTAL TABLES

Supplemental Table ST1. Non-synonymous single nucleotide polymorphisms in genes associated with drug resistance. Positions with minor allele frequency (MAF) of at least 2% were retained in this table. Frequencies are presented for each sampling year in Mali.

Gene	Gene ID	Chromosome	Nt change	AA change	MAF 2007 (%) n=74	MAF 2012 (%) n=4	MAF 2013 (%) n=284	MAF 2014 (%) n=52	MAF 2015 (%) n=84	MAF 2016 (%) n=342	MAF 2017 (%) n=36	MAF 2019 (%) n=51	MAF 2020 (%) n=36
MDR1	PF3D7_0523000	5	957990A>G	K34R	0.00	33.33	1.06	0.00	1.20	0.44	1.39	2.17	0.00
			958145A>T	N86Y	28.38	0.00	19.72	25.00	11.31	12.17	11.11	6.25	3.57
			958440A>T	Y184F	66.22	66.67	67.61	67.31	66.27	64.56	72.22	62.50	71.43
			958496C>T	P203S	0.00	0.00	0.53	0.00	1.20	0.29	2.86	1.06	0.00
			959087A>T	S400C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.13	0.00
			959180G>T	D431Y	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.13	0.00
			959398A>T	K503N	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.94
			959401T>A	N504K	4.05	0.00	4.05	5.77	1.22	4.33	2.78	3.33	4.41
			960702T>A	F938Y	0.00	0.00	4.05	5.77	0.62	1.55	7.14	3.00	1.56
			961462G>A	M1191I	0.00	0.00	0.35	0.00	0.00	0.30	0.00	3.06	0.00
			961473A>C	Q1195P	0.00	0.00	0.18	0.00	0.00	0.00	2.78	0.00	0.00
			961494A>G	N1202S	0.00	0.00	0.00	0.00	0.00	0.30	0.00	2.00	0.00
			961625G>T	D1246Y	2.03	0.00	1.94	1.92	1.90	0.74	5.71	0.00	0.00
CRT	PF3D7_0709000	7	403625A>C	K76T	61.11	100.00	52.82	46.15	36.31	48.98	31.94	54.17	48.48
			404407G>T	A220S	57.26	100.00	53.01	47.12	36.31	44.41	30.56	46.67	33.33
			404836C>G	Q271E	62.50	100.00	52.82	48.08	36.31	49.71	31.94	43.48	45.16
			404948A>T	Y276F	0.00	0.00	0.00	0.00	0.00	0.00	2.78	0.00	0.00
			405362A>G	N326S	0.74	0.00	1.44	0.00	0.60	1.03	4.17	1.04	3.23
			405600T>C	I356T	22.54	100.00	29.75	29.41	28.57	39.91	15.28	36.73	33.33
			405838G>T	R371I	61.27	100.00	53.35	50.00	36.90	49.85	30.56	45.56	48.28

K13	PF3D7_1343700	13	1726226A>T	L258M	1.35	0.00	1.06	0.00	0.00	0.44	0.00	2.00	0.00
			1726234C>T	R255K	4.05	16.67	2.29	0.00	3.61	2.51	0.00	0.00	1.43
			1726349T>G	N217H	0.00	0.00	0.00	0.00	0.00	0.00	2.78	0.00	0.00
			1726431T>A	K189N	6.76	0.00	4.40	2.88	3.05	3.54	11.43	7.84	6.76
			1726432T>G	K189T	52.70	83.33	49.82	62.50	44.23	54.91	44.29	50.00	56.76
			1726663C>T	G112E	0.00	0.00	1.76	0.00	5.95	1.32	0.00	0.00	0.00
			1726697T>C	R101G	0.00	0.00	0.18	0.00	0.60	0.44	2.78	0.00	0.00
			1726737G>C	N87K	0.00	0.00	0.00	0.00	0.00	0.00	2.78	1.96	0.00
DHFR	PF3D7_0417200	4	748145G>A	V20I	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.04	0.00
			748239A>T	N51I	43.92	83.33	62.50	58.65	82.74	75.88	76.39	85.29	92.42
			748262T>C	C59R	45.27	83.33	67.08	66.35	83.93	78.59	81.94	89.22	93.94
			748410G>A	S108N	45.27	83.33	66.90	66.35	83.33	80.99	81.94	92.71	92.65
			748558T>G	N157K	0.00	0.00	0.00	0.00	0.00	0.00	2.78	0.00	0.00
			748854A>G	K256R	0.00	0.00	0.00	2.88	0.00	0.00	0.00	0.00	0.00
DHPS	PF3D7_0810800	8	548940G>C	E189Q	2.03	0.00	0.53	0.00	0.00	1.46	6.94	0.00	0.00
			549666A>G	I431V	0.00	0.00	0.53	0.00	1.19	0.29	2.78	3.92	0.00
			549681T>G	S436A	71.62	50.00	61.44	51.92	60.12	50.44	61.11	58.82	52.70
			549685G>C	A437G	27.70	100.00	45.60	45.19	38.60	61.76	76.39	70.59	74.32
			549993A>G	K540E	0.68	0.00	0.35	0.00	0.00	1.47	0.00	5.88	2.70
			550117C>G	A581G	0.00	0.00	0.35	0.00	1.19	0.29	2.78	2.94	0.00
			550212G>T	A613S	6.76	0.00	5.11	3.85	4.17	7.04	9.72	14.71	4.05

Nt = Nucleotide; AA = Amino Acid

Supplemental Table ST2. Observed frequencies of *dhfr* and *dhps* haplotypes. Frequencies of combinations of SNPs in *dhfr* and *dhps* known to confer SP resistance are reported. Malian isolates are grouped by collection year.

Gene	Haplotype	Frequency 2007 (%) n=74	Frequency 2013-2014 (%) n=336	Frequency 2015-2017 (%) n=462	Frequency 2019-2020 (%) n=87
DHFR	CNCSI	43.84	27.68	13.97	4.94
	CIRNI	34.25	55.06	70.74	82.72
	CICNI	0.00	0.00	0.66	1.23
	CNRNI	1.37	3.57	2.84	2.47
	CNCNI	0.00	0.00	0.22	0.00
	Mixed Infections	20.55	13.69	11.57	8.64
DHPS	SAKAA	5.48	23.81	32.97	25.93
	AAKAA	8.22	8.63	12.88	12.35
	SGKAA	10.96	6.55	6.33	0.00
	SAEAA	0.00	0.00	0.87	2.47
	AGKAA	46.58	39.29	27.07	16.05
	AAKAS	1.37	1.49	4.59	3.70
	SGKAA	10.96	6.55	6.33	0.00
	SGKAS	0.00	0.89	0.44	0.00
	AGKAS	1.37	0.30	0.00	0.00
	SGKAS	0.00	0.89	0.44	0.00
	AAKGS	0.00	0.30	0.66	1.23
	Mixed Infections	15.07	11.31	7.42	38.27
DHFR+DHPS	CIRNI+SAEAA	0.00	0.00	0.66	2.47

Supplemental Table ST3. Candidate regions in positive selection analysis using the integrated haplotype score (*iHS*) in Ouélessébougou (Mali 2019-2020) population.

Chr	Start	End	Number of markers	Mean iHS markers	Max iHS	Number of iHS extreme markers (iHS p < 1x10-4, two-sided)	Percentage of extreme iHS markers (iHS p < 1x10-4, two-sided)	Mean iHS of extreme markers (iHS p < 1x10-4, two-sided)	Genes in the region	Products
4	110000	140000	93	0.648	7.899	3	3.23	5.571	PF3D7_0402100	Plasmodium exported protein (PHISTb), unknown function
7	590000	620000	166	0.638	5.175	4	2.41	4.489	PF3D7_0713600	ribosomal protein S5, mitochondrial, putative
12	640000	670000	22	1.085	5.628	2	9.09	5.075	PF3D7_1216700(PLP2)	perforin-like protein 2
13	90000	120000	96	0.838	6.023	2	2.08	5.597	PF3D7_1302000(PTP6); PF3D7_1301700(CBP2)	EMP1-trafficking protein; CX3CL1-binding protein 2
13	2490000	2520000	11	2.087	10.641	2	18.18	10.316	PF3D7_1362500(EXO); PF3D7_1362700; PF3D7_1362200(RUVB3)	3'-5' exonuclease, putative; conserved Plasmodium protein, unknown function; RuvB-like helicase 3

Supplemental Table ST4. Candidate regions in positive selection analysis using the between populations (*Rsb*) metric comparing the Ouélessébougou (Mali 2019-2020) population with the Mali 2007-2014 and Mali 2015-2017 populations.

Population comparison	Chr	Start	End	Number of markers	Mean Rsb markers	Max Rsb	Number of Rsb extreme markers	Percentage of extreme Rsb markers	Mean Rsb of extreme markers	Genes in the region	Products
Mali 2007-2014 vs Mali 2019-2020	2	860000	890000	66	0.962	6.486	3	4.55	5.954	PF3D7_0221800	hypothetical protein
Mali 2007-2014 vs Mali 2019-2020	4	530000	560000	54	1.566	10.727	8	14.81	8.08	PF3D7_0412100(mtRPS12)	ribosomal protein S12, mitochondrial
Mali 2007-2014 vs Mali 2019-2020	4	590000	620000	210	0.747	14.738	10	4.76	8.515	PF3D7_0413600(RPT3)	26S protease regulatory subunit 6B, putative
Mali 2007-2014 vs Mali 2019-2020	4	1090000	1120000	178	0.534	14.937	2	1.12	11.893	PF3D7_0424300(EBA165)	erythrocyte binding antigen-165, pseudogene
Mali 2007-2014 vs Mali 2019-2020	6	40000	80000	79	1.383	7.005	4	5.06	5.83	PF3D7_0601500	Plasmodium exported protein (PHISTb), unknown function
Mali 2007-2014 vs Mali 2019-2020	7	590000	620000	294	0.846	12.113	2	0.68	8.736	PF3D7_0713600	ribosomal protein S5, mitochondrial, putative
Mali 2007-2014 vs Mali 2019-2020	10	1380000	1410000	238	0.859	12.178	6	2.52	8.413	PF3D7_1035400(MSP3); PF3D7_1034900(MRScyt)	merozoite surface protein 3; methionine--tRNA ligase
Mali 2007-2014 vs Mali 2019-2020	10	1580000	1610000	23	2.564	8.49	7	30.43	6.37	PF3D7_1040200	stevor
Mali 2007-2014 vs Mali 2019-2020	13	90000	120000	122	1.243	6.454	4	3.28	5.869	PF3D7_1302000(PTP6); PF3D7_1301700(CBP2)	EMP1-trafficking protein; CX3CL1-binding protein 2

Mali 2015-2017 vs Mali 2019-2020	3	110000	140000	206	0.825	10.366	4	1.94	7.117	PF3D7_0302300; PF3D7_0302500(CLAG3.1); PF3D7_0302200(CLAG3.2)	erythrocyte membrane protein 1 (PfEMP1), pseudogene; cytoadherence linked asexual protein 3.1; cytoadherence linked asexual protein 3.2
Mali 2015-2017 vs Mali 2019-2020	4	530000	560000	56	1.019	7.812	2	3.57	7.812	PF3D7_0412100(mtRPS12)	ribosomal protein S12, mitochondrial
Mali 2015-2017 vs Mali 2019-2020	4	590000	620000	205	0.911	16.604	11	5.37	8.429	PF3D7_0413600(RPT3)	26S protease regulatory subunit 6B, putative
Mali 2015-2017 vs Mali 2019-2020	6	50000	80000	59	1.573	6.537	3	5.08	5.614	PF3D7_0601500	Plasmodium exported protein (PHISTb), unknown function
Mali 2015-2017 vs Mali 2019-2020	6	710000	740000	16	2.032	10.73	2	12.5	8.746	PF3D7_0617200); PF3D7_0617600; PF3D7_0617100	BFR1 domain-containing protein, putative; stevor; AP-2 complex subunit alpha, putative
Mali 2015-2017 vs Mali 2019-2020	10	1380000	1410000	239	0.683	9.906	6	2.51	7.723	PF3D7_1035400(MSP3); PF3D7_1034900(MRScyt)	merozoite surface protein 3; methionine--tRNA ligase
Mali 2015-2017 vs Mali 2019-2020	10	1580000	1610000	24	2.476	8.486	7	29.17	6.454	PF3D7_1040200	stevor
Mali 2015-2017 vs Mali 2019-2020	13	90000	120000	119	2.335	12.761	19	15.97	6.226	PF3D7_1302000(PTP6); PF3D7_1301700(CBP2)	EMP1-trafficking protein; CX3CL1-binding protein 2

Supplemental Table ST5. Top 5% of Identity-by-descent (IBD) regions in 2019-2020 Mali isolates from Ouélessébougou.

Chr	Start	End	Fraction	Location	Gene ID	Gene product	Gene name
6	1110001	1120000	0.02074037	1114544-1117537	PF3D7_0627800	acetyl-CoA synthetase, putative	ACS
6	1200001	1210000	0.02281915	1205190-1207781	PF3D7_0629300	phospholipase, putative	PL
6	1210001	1220000	0.02544293	1210420-1212762	PF3D7_0629400	polyadenylate-binding protein 3, putative	PABP3
6	1210001	1220000	0.02544293	1213948-1216005	PF3D7_0629500	amino acid transporter AAT1	AAT1
6	1220001	1230000	0.02996695	1221941-1242922	PF3D7_0629700	SET domain protein, putative	SET1
6	1230001	1240000	0.03621427	1221941-1242922	PF3D7_0629700	SET domain protein, putative	SET1
6	1240001	1250000	0.04015512	1221941-1242922	PF3D7_0629700	SET domain protein, putative	SET1
6	1250001	1260000	0.04146341	1254907-1256940	PF3D7_0630100	alpha/beta hydrolase, putative	/
7	370001	380000	0.0249511	372897-373106	PF3D7_0708100	DNA-directed RNA polymerases I, II, and III subunit RPABC5, putative	RPB10
7	380001	390000	0.03311512	385583-388321	PF3D7_0708500	heat shock protein 86 family protein	/
7	390001	400000	0.04070598	395711-398332	PF3D7_0708800	heat shock protein 110	HSP110c
7	390001	400000	0.04070598	391502-392188	PF3D7_0708700	Cg8 protein	/
7	400001	410000	0.0493389	403222-406317	PF3D7_0709000	chloroquine resistance transporter	CRT
7	420001	430000	0.05203866	428723-429346	PF3D7_0709500	nucleic acid-binding protein, putative	/
7	430001	440000	0.04008451	435089-436195	PF3D7_0709700	prodrug activation and resistance esterase	PARE
7	460001	470000	0.01624402	463105-471837	PF3D7_0710200	conserved Plasmodium protein, unknown function	/
13	100001	110000	0.01553341	99548-100521	PF3D7_1301700	CX3CL1-binding protein 2	CBP2

SUPPLEMENTAL FILES

A list of samples included in the analysis (41598_2023_36002_MOESM2_ESM.xlsx) can be downloaded from https://doi.org/10.1038/s41598-023-36002-w

Chapter 3: Preferential transmission of minority and drug-resistant clones in polyclonal *Plasmodium falciparum* infections in Mali

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Student ID Number	lsh2004630	**Title**	Dr.
First Name(s)	Leen Nele		
Surname/Family Name	Vanheer		
Thesis Title	Transmissibility and antimalarial resistance in human malaria parasite Plasmodium falciparum in Mali		
Primary Supervisor	Chris Drakeley		

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	Traore, Teun Bousema, Alassane Dicko, Chris Drakeley, Susana Campino, William Stone
Stage of publication	**Submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I carried out laboratory work including primer and multiplex optimisation, as well as DNA extraction of infected mosquito midguts, DNA clean-up, and coordinated the sequencing. I then performed the bioinformatic analysis and interpreted the results under the supervision of my supervisors. I wrote the first draft of the manuscript that was then circulated to supervisors and afterwards to co-authors.

SECTION E

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Supervisor Signature	
Date	

Preferential transmission of minority and drug-resistant clones in polyclonal infections in Mali

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ABSTRACT

In polyclonal human infections, the roles of distinct clones in transmission and their relative transmissibility remain poorly understood. In addition, mutations conferring drug resistance can result in a transmission advantage or disadvantage. Using amplicon sequencing of complexity of infection and drug resistance markers, we investigate post-treatment stage-specific malaria parasite dynamics in human blood and in the midguts of mosquitoes that became infected after membrane feeding experiments using the same blood material. Blood samples originated from 50 asymptomatic gametocyte-carrying participants and were collected at five timepoints over 28 days following a three-day artemisinin-based combination therapy (ACT) regimen of dihydroartemisinin-piperaquine or pyronaridine-artesunate at the Ouélessébougou Clinical Research Unit of the Malaria Research and Training Centre of the University of Bamako (Bamako, Mali). We identified 57 *Pfcsp* haplotypes and 53 *Pftrap* haplotypes, indicating high genetic diversity among parasite clones. Prior to treatment, we found that, compared to mosquito infections, human infections were more often polyclonal and had a higher median multiplicity of infection (MOI; 3 (IQR 2-5) in human infections compared to 1 (IQR 1-2) in midguts). At this timepoint, it is likely that some clones detected in human blood are not producing gametocytes and are therefore not contributing to mosquito transmission. We found that minority clones seemed to preferentially transmit, and these same clones often persisted in the human blood samples post-treatment. These observations mirror the rapid decline in asexual parasite density that occurs after ACT treatment initiation, and the more persistent circulation of gametocytes. Our data therefore suggests that asexual gametocyte-non-producing clones outnumber the gametocyte-producing clones at baseline, yet it is these gametocyte-producing minority clones that are transmitted to and surviving in mosquitoes. We also observed that certain haplotypes are more prevalent in human samples compared to mosquito infections, and vice versa, with 12.6% of haplotypes at baseline only ever observed in mosquitoes. Along with this, varying odds of transmission for different parasite clones

were observed, indicating that there are inherent clonal differences in gametocyte productivity or viability. To assess the transmission of drug-resistant clones, we determined the overall prevalence of molecular markers of drug resistance in humans and mosquito hosts and conducted pairwise comparison between human blood infections and paired infected midguts. We found that Asn51Ile and Lys540Glu in *Pfdhfr* may have a transmission advantage under ACT treatment, while Ala613Ser in *Pfdhps* may confer a transmission disadvantage. Overall, our findings indicate that parasite dynamics and clonal transmissibility are highly complex, even after ACT treatment. This complexity may have important epidemiological implications, as it suggests the transmission of minority clones and highlights the impact of drug resistance markers on transmissibility.

Keywords: malaria, *Plasmodium falciparum*, gametocytes, transmission, comparative genomics, genetic diversity, artemisinin-combination therapy, drug resistance

INTRODUCTION

The cycle of *Plasmodium* transmission between human and mosquito hosts relies on the parasite's ability to produce gametocytes. These gametocytes, when ingested by a mosquito, develop into zygotes and eventually oocysts in the mosquito's midgut. Various factors such as gametocyte viability and density, host immune responses, and the complexity of the infection can influence this process (1–5).

In regions where *P. falciparum* infections are endemic, individuals are often infected by more than one parasite strain at the time, leading to polyclonal infections, which may be the result of clonal co-transmission (a single mosquito infection with multiple clones) or serial superinfections (multiple mosquito infections, each with a single or multiple clones). This multiplicity of infection (MOI), indicating the number of clones within an infection, and the complexity of infection (COI), referring to specific genetic characteristics of these clones, are important for understanding *Plasmodium* epidemiological patterns and transmission dynamics. In regions with high malaria transmission and especially among asymptomatic individuals, infections usually exhibit greater multiclonality, and the various parasite clones compete with one another for replication and transmission, though the resources they compete for are not well understood (6–8). Although most parasite stages are haploid, the zygote formed after fertilization in the mosquito midgut is diploid. At this stage, new parasite haplotypes emerge when genetically distinct gametocytes are transmitted to mosquitoes, where recombination occurs during sexual reproduction, increasing genetic diversity in subsequent generations.

In polyclonal human infections, the contribution of distinct clones to transmission and their relative transmissibility have largely remained unexplored, as have the parasite genetic factors that may influence this. In addition, due to the parasite's complex life cycle, many different population

bottlenecks and selective pressures are encountered throughout human host and organ transitions, which may intensify random genetic drift and natural selection (9). Transmission represents a major population bottleneck in the parasite life cycle, as natural mosquito infections typically harbour only about 2–5 oocysts, in contrast to the approximately 10^{11} asexual parasites found in an infected host (10). In a prior study of transmission using naturally-infected paired human and mosquito samples, haplotype diversity was found to be greater in mosquitoes than in humans, establishing the mosquito vector as a reservoir of genetic diversity in the malaria parasite populations (8). This finding is seemingly contradictory to observations that not all clones present in the human blood transmit to mosquitoes. This could indicate a new infection which may not have had time yet to generate mature gametocytes, or a potential failure to produce gametocytes altogether (8,11). On the other hand, transmission of clones that were undetectable in the bloodstream was reported in multiple studies (1,11,12), and it is commonly observed that not all individuals with confirmed gametocytes are able to infect mosquitoes (13–15). Such observations may be more likely to reflect the density dependence of successful transmission, the technical limitations in detecting low density clones, or the activity of human or mosquito immune transmission blockage, rather than genetic unviability.

Artemisinin-based combination therapies (ACT) are the current first-line treatment for uncomplicated *P. falciparum* malaria, however, their gametocytocidal and transmission-blocking effects vary widely. After treatment with commonly used ACTs such as dihydroartemisinin-piperaquine (DHA-PPQ), pyronaridine-artesunate (PY-AS) or artesunate-amodiaquine, gametocytes and transmission can persist up to 28 days after treatment initiation (13,16). A recent study found that post-treatment parasite dynamics of blood stage parasites are highly complex despite efficacious treatment (17). To our knowledge, post-treatment parasite dynamics in both human blood and cognate infected mosquito midguts remain unstudied.

The rise of antimalarial drug resistance has driven the adoption of new therapeutic approaches. Investigating the effect of drug resistance on transmissibility is crucial, as any transmission advantages conferred by resistance linked mutations could expedite the spread of these dangerous parasite strains and compound a developing public health crisis. Studies have shown evidence of increased gametocytaemia and increased transmission in infections with chloroquine (CQ) resistant *P. falciparum* parasites, compared to infections with parasites sensitive to this antimalarial (18). For sulfadoxine-pyrimethamine (SP), it has been found that resistant parasites produce more gametocytes, but their transmission potential remains unclear (19,20). In addition, host-specific selection of drug resistant polymorphisms have been observed, reporting contrasting single nucleotide polymorphisms (SNPs) in the *Pfdhfr* gene in human and mosquito hosts (21).

Here, we characterize parasite clones and investigate the clonal transmissibility of 50 naturally-infected asymptomatic *P. falciparum* gametocyte carriers at different timepoints following a course of ACT (dihydroartemisinin-piperaquine or pyronaridine-artesunate) treatment in South-Western Mali, a region of intense malaria transmission, where high SP resistance and persistent chloroquine resistance have been reported (22,23). Gametocyte carriage was an essential recruitment criterion, allowing for inference of parasite stage identity based on the differential clearance times of sexual and asexual stage parasites after ACT treatment. In addition, we investigate the relatedness of molecular markers of antimalarial drug resistance and transmission potential.

METHODS

Study site, sample collection and feeding assays

Human blood samples of 50 individuals with microscopy detectable *P. falciparum* gametocytes (≥ 1 gametocyte against 500 white blood cells on thick smear, equating to ≥ 16 gametocytes per μL

of blood with an assumed WBC density of 8000/μL blood), and mosquitoes infected by direct membrane feeding assay (DMFA) were obtained from a clinical trial performed in Mali in 2019 (13) (Supplementary Figure 1). Individuals were treated with an ACT, which consisted of a 3-day course of dihydroartemisinin-piperaquine or pyronaridine-artesunate and were followed up at pre-specified days after treatment initiation. Re-treatment with dihydroartemisinin-piperaquine administered at day 21 to prevent re-infection. Blood samples were taken at each study visit for parasite density measurements and mosquito feeding assays. Blood samples for molecular analyses were stored in RNA protect cell reagent (Qiagen, Hilden, Germany) and frozen at -80°C until nucleic acid extraction. At each study visit, 75 insectary-reared *Anopheles gambiae* (*s.l.*) mosquitoes were allowed to feed for 15-20 minutes on whole blood collected from participants. Surviving mosquitoes were dissected 7 days after feeding to allow for oocyst development and the elimination of residual parasite material from the blood meal (24). Midguts were stained with 1% mercurochrome solution and the number of oocysts in the lamina of the midguts was recorded by trained technicians, after which midguts were stored in RNA protect cell reagent at -80°C until extraction. Permission to conduct this study was obtained from the London School of Hygiene and Tropical medicine Research Ethics Committee (reference number 17507) and the University of Sciences Techniques and Technologies of Bamako Ethical Committee (reference number 2019/67/ CE/FMPOS) and performed in accordance with relevant guidelines and regulations. The trial was registered on ClinicalTrials.gov (NCT0404991). Written informed consent was obtained from all subjects and/or their legal guardians prior to sample collection. For minor participants, informed consent for study participation was obtained from their parent and/or legal guardian.

Nucleic acid extraction and parasite quantification

DNA was extracted from 83.3 µL whole blood using a MagNAPure LC automated extractor (Total Nucleic Acid Isolation Kit High Performance; Roche Applied Science, Indianapolis, IN, USA). Ring stage parasitaemia was determined by reverse-transcriptase quantitative PCR (RT-qPCR) targeting skeleton-binding protein 1 (SBP1) (25). Female and male gametocytes were quantified by RT-qPCR targeting *PfCCP4* and *PfMGET,* respectively, as previously described (26). If any number of infected mosquitoes resulted from the infectivity assay at a certain timepoint for a certain individual, a maximum of three infected mosquitoes were selected at random (Supplementary Figure 1) and oocyst DNA was extracted using the Qiagen DNeasy blood & tissue kit with overnight proteinase K lysis and eluted in 50 µL EB.

Genotyping of human blood samples and infected mosquito midguts

Amplicon sequencing to determine complexity of infection was performed as previously described, targeting the circumsporozoite protein (*Pfcsp*) and the thrombosporin-related anonymous protein (*Pftrap*) (27–29) (Supplementary Table 1). Briefly, an approximate 300 base pair (bp) region of each gene was amplified by multiplexed PCR in duplicates and an in-line barcode was added to the primer sequences, allowing pooling of amplicons. Sequencing of amplicon pools was then performed using overlapping 250 bp paired-end MiSeq Illumina reads at Genewiz (Azenta Life Sciences). *In vitro P. falciparum* culture DNA of parasite lines 3D7 and HB3 was used to assess the limit of detection of the assay (Supplementary Figure 2). Amplicon sequencing of *P. falciparum* antimalarial resistance markers using nanopore sequencing was performed as previously described (30), on 50 human and 87 cognate mosquito specimens which fed on blood samples from 35 of these individuals, sampled on day 2 after treatment initiation (Supplementary Figure 1). This timepoint was chosen since nearly all asexual parasites were

removed from circulation by the ACT treatment within 48 hours of treatment commencement, while gametocyte densities were still high (Figure 1). Primer sequences and multiplexed PCR conditions for amplification of the *Pfcrt*, *Pfdhfr*, *Pfdhps*, *Pfmdr1* and *PfKelch13* genes can be found in Supplementary Table 2. Library preparation was carried out according to manufacturer instructions using ONT kit SQK-NBD114.96 following the 'ligation sequencing amplicons-native barcoding' protocol.

Bioinformatics and statistical analysis

The haplotypeR package was used to determine complexity of infection per sample (31), with minor modifications to extend certain functions (https://github.com/leenvh/haplotypR_funs) and using the parameters minMMrate 0.5, minOccGen 2, minCov 3, detectionLimit 1/200, minOccHap 2, MinCovSample 20. Haplotypes were only considered as real if they were present in both technical replicates, thereby minimising the risk of detecting haplotypes caused by amplification or sequencing errors. Correlations between markers and replicates were assessed by Spearman's rank correlation coefficient. Haplotype networks were constructed using the R package *Pegas* (32). Bioinformatics analysis was done by in-house demultiplexing script (https://github.com/LSHTMPathogenSeqLab/amplicon-seq/tree/main) and drug resistant polymorphisms were analysed by the malaria profiler tool (33). Frequencies of molecular markers of drug resistance were compared between human and mosquito populations by Fisher exact test. Visualisations and statistical analyses were performed in R (version 4.3.2) and can be found on https://github.com/leenvh/Amplicons-falciparum-MOI. The sequence data presented in this study can be found in the European Nucleotide Archives (ENA, Project accession PRJEB73503).

RESULTS

Stage-specific infection dynamics and infectivity before and after treatment

Parasite densities were quantified by stage-specific molecular assays before, during and after treatment in both treatment groups (dihydroartemisinin-piperaquine and pyronaridine artesunate). Before treatment initiation, molecular assessment of asexual parasites was successful in 48 out of 50 individuals, and asexual parasites were detectable by RT-qPCR in 91.6% (44/48) of these individuals (median density 396.77 parasites/µL, IQR 54.15-1931.89). All individuals were recruited based on the presence of gametocytes by microscopy and gametocytes were detectable by RT-qPCR in all participants at baseline (median density 77.33 gametocytes/µL, IQR 37.1-124.02). A total of 66% (33/50) of study participants were able to infect mosquitoes at baseline, with a median infection rate of 14.9% in mosquitoes (IQR 3.51-31.4). At 48 hours after treatment initiation, nearly all asexual parasites were cleared in both treatment groups (only densities of ≤7 asexual parasites/µL remained in 20 individuals, median 1.59, IQR 0.55-2.5), while gametocytes densities showed a slow decline (Figure 1A-B). Parasite prevalences and densities were comparable between treatment groups during follow-up (Supplementary table 3, Figure 1A-B). Mosquito infection rates declined after treatment, but transmission persisted until day 28 in some individuals in both groups. The median oocyst density in infected mosquitoes at baseline was 1 (IQR 1-2) (Figure 1C-D, Supplementary table 4).

Majority of infections in asymptomatic gametocyte carriers are polyclonal and highly diverse

To investigate the *P. falciparum* genotypes in human and mosquito samples, a total of 195 human blood samples from 50 trial participants and 315 mosquito midgut samples from DMFAs conducted on these participants were selected. Amplicons for *Pfcsp* and *Pftrap* were successfully

sequenced (median coverage of 1002 and 944, respectively) for 168 (86.2%) human samples and 151 (47.9%) mosquito samples (Supplementary Table 5). Multiplicity of infection was highly correlated between technical replicates of the same sample (*Pfcsp* marker spearman correlation = .76, p < .0001; *Pftrap* marker spearman correlation = .75, p <.0001) and between the two sequenced markers (spearman correlation= .8, p < .0001; Supplementary Figure 3). A total of 1,220 amplicons across both markers were analysed, identifying a total of 57 *Pfcsp* haplotypes and 53 *Pftrap* haplotypes, reflecting 28 and 20 positions with single nucleotide polymorphisms (SNPs), respectively (Figure 2).

Pre-treatment infected human blood samples were successfully genotyped for complexity of infection markers for 43 out of 50 potential study participants. Of these, 88.4% (38/43) of infections were polyclonal, with a median MOI of 3 (IQR 2-5). During the trial, mosquitoes feeding on these participants at the same timepoint were incubated for 7 days to allow parasite establishment and oocyst development. Clonality in established mosquito infections was lower than in matched blood stage parasites, with only 36.5% (19/52) of midguts being polyclonal and a median MOI of 1 (IQR 1-2). At days 2 and 7 after treatment, asexual parasite densities had declined 249-fold and 1,240-fold, respectively, compared to pre-treatment. Gametocytes persisted at densities only 1.2-fold and 1.8-fold lower than pre-treatment at days 2 and 7, respectively (Figure 1A-B, Supplementary table 4). The median MOI of these blood stage parasites, consisting mainly of gametocytes, was 2 (IQR 1-3) and 1 (IQR 1-2) at days 2 and 7 after treatment, respectively. Clonality in paired infected mosquito midguts was lower, with a median MOI of 1 (IQR 1-2) at both timepoints. The clonality in blood stage parasites further declined to 33.3% (9/27) multiclonal human infections and 26.3% (5/19) multiclonal infected midguts at day 14 after treatment initiation, with a median MOI of 1 (IQR 1-2) in human samples and 1 (IQR 1-1) in infected midguts (Supplementary Figures 4, 5, Supplementary Table 6). No substantial variations were observed in gametocyte densities or gametocyte fractions (gametocyte density as a percentage of

total parasite density) between monoclonal and polyclonal infections during study visits. Similarly, there were no significant differences in mosquito infection rates or oocyst densities in monoclonal and polyclonal infections (Supplementary Table 7).

Transmission and persistence of minority clones

Considering clones found in humans and cognate mosquitoes as whole populations, certain haplotypes were found to be more prevalent in human samples compared to mosquito samples and vice versa (Figure 3A, Supplementary Figure 6). To assess transmission between hosts and cognate mosquitoes in more depth, only pairwise human-mosquito groups were considered; human blood samples that did not have any cognate and successfully genotyped infected midguts were excluded and both COI markers were considered together, with percentages representing an average of both markers. Of all clones present in any species at baseline or on day 2/7, 30.1 % was found to be present in baseline human infection while being absent in post-treatment (day 2/7) human infections and absent in mosquito infections (i.e. putative non-gametocyte producers), 13.4% to be present in post-treatment (day 2/7) human infections, while absent in mosquito infections (i.e. putative gametocyte-producers, non-transmitting), and 56.5% to be present in mosquito infections (i.e. gametocyte producers, transmitting). At baseline, prior to treatment initiation, 66.8% of haplotypes detected in participants transmitted to mosquitoes. A total of 82.3% of haplotypes detected in mosquitoes that had fed on baseline blood samples were observed in cognate human samples, while 29% of all haplotypes were found in human samples only and 12.6% of all haplotypes in mosquitoes only. At days 2 and 7, when >99% of asexual parasites were removed but gametocytes persisted, the percentage of haplotypes detected in participants that transmitted to mosquitoes increased to 83.6% and 91.7%, respectively. The percentage of haplotypes observed in mosquitoes that were detected in cognate human samples increased as well to 95.3% and 95.4% at days 2 and 7, respectively. Of all haplotypes found in either species, the

percentage of non-transmitting haplotypes, i.e. haplotypes that were exclusively detected in human samples decreased to 15.8% (day 2), 7.9% (day 7) and 0% (day 14), while the percentage of haplotypes that were only ever observed in mosquitoes decreased from 12.6% at baseline to 3.9% at day 2, and then increased again to 4.3% (day 7) and 7.8% (day 14) (Figure 3B).

Transmitting haplotypes were often a minority clone at baseline, while they represented a higher percentage of sequencing reads at later timepoints (Figure 3C), reflecting the rapid decline of asexual densities after treatment start, and the persistence of gametocytes post-treatment. With the aim of investigating whether the persisting clones are transmitted prior to treatment, the identity of clones in baseline infected mosquitoes were compared to human samples at all timepoints. We found indeed that the haplotypes that are transmitted at baseline match most closely with day 7 and 14 human samples (Figure 3D). These matching haplotypes were often present as majority clones in the human blood samples at these timepoints (Figure 3E). Transmission odds per haplotype showed differences in the likelihood of transmission for each haplotype, indicating that some haplotypes are more likely to transmit than others, at baseline and 48 hours post-treatment (Supplementary Figure 7).

Differential transmission of drug resistance molecular markers

At day 2 after treatment, when only gametocyte-producing clones were remaining, 49/50 (98%) human samples and 73/87 (83.9%) selected cognate mosquito samples were successfully assessed for the presence of antimalarial drug resistance molecular markers with a median coverage of 990-6364 reads (Supplementary Table 5). A total of 610 amplicons were analysed across *Pfcrt*, *Pfmdr1*, *Pfdhfr*, *Pfdhps* and *PfKelch13* genes.

As most infections were polyclonal, we assessed the frequency of the molecular markers in each sample, corresponding to the proportion of sequencing reads that contained the mutation. The prevalence of certain drug resistance polymorphisms was significantly different in blood stage and mosquito stage populations, with Asn51Ile in *Pfdhfr* and Lys540Glu in *Pfdhps* being significantly higher in mosquitoes (75.73% and 2.96% in blood stage parasites and 84.83% and 14.19% in mosquito midguts, $p=0.025$ and $p<0.0001$, respectively) and Ala613Ser in *Pfdhps* higher in blood stage parasites (15.99% in blood stage parasites and 2.13% in mosquito midguts, $p=0.0057$, Figure 4, Supplementary Table 8). In a pairwise comparison of cognate human blood and mosquito samples, the mean difference in frequency showed similarly that Asn51Ile and Lys540Glu may have a transmission advantage, while Ala613Ser shows a transmission disadvantage. One missense mutation in the propeller region of PfK13 was found (Val494Phe) in one infected midgut, however, this polymorphism has not been linked to partial artemisinin resistance (Supplementary Table 8).

DISCUSSION

In this study, we used amplicon sequencing to determine complexity of infection and prevalence of molecular markers of drug resistance, with the aim of investigating pre- and post-treatment parasite dynamics and genetic characteristics in blood stage parasites and oocysts from matched mosquito midguts. Our results highlight the important role of gametocyte complexity and infectivity in creating the extensive diversity of *P. falciparum* genotypes found in infected individuals, in this area of seasonal transmission.

In baseline human blood stage parasites, we observed a prevalence of polyclonal infections of 88.4% (38/43), and frequencies of drug resistance molecular markers consistent with previous reports from this area (22,23,34). Prior to treatment, we found that minority clones preferentially transmitted to mosquitoes. This may be due to majority clones representing new infections that consist predominantly of asexual parasites, which are present at higher densities than the 'minority' gametocytes (35). In addition, new infections may not have had sufficient time to produce gametocytes. Alternatively, this observation aligns with the hypothesis of Berry et al (12) reporting a selective advantage of minority clones in the vector. A possible reason for preferential transmission of minority clones could be to maintain genetic diversity in the parasite population, which may be of epidemiological importance with respect to the spread of drug resistance polymorphisms (12,36). Our findings of clones detectable in the mosquito only confirm previous reports that gametocytes present in blood stage infections at undetectable densities, potentially due to selective amplification of asexual parasites, are infectious for the mosquito vector (1,11,12). In contrast with a study finding higher genetic diversity in mosquitoes (8), we observed a higher clonality in human blood samples compared to infected mosquito midguts. This could be attributed to the naturally infected mosquitoes that were investigated in that study, as compared to experimentally infected. Naturally infected mosquitoes can have taken multiple feeds on

infected hosts, allowing parasite strains to accumulate in the mosquito abdomen. Comparing pre- and post-ACT treatment, we found that clones that persisted in the blood generally shared identity with clones present in cognate mosquitoes, as we would expect based on prior reports showing the persistence of gametocytes after most standard treatments, with the exception of artemether lumefantrine (13–15,37,38). These gametocyte-producing clones can persist until day 28 in some individuals, even after re-treatment with an ACT treatment at day 21, supporting the addition of a single-low dose of primaquine to accelerate gametocyte clearance and preventing transmission.

A transmission advantage caused by a certain genetic variation can be the result of a higher gametocyte production, a higher gametocyte longevity or a more efficient fertilisation. The latter is evidenced by cases of gametocyte-producing clones after treatment that fail to infect mosquitoes, confirming that factors other than gametocyte density play a role in establishing oocyst development. Advantages in human-to-mosquito transmission have previously been observed in chloroquine-resistant parasites strains (18), and more recently in artemisinin-resistant malaria parasites under artemisinin drug pressure (39,40). Sulfadoxine-pyrimethamine resistant isolates were found to produce more gametocytes, but with unknown effect on transmission (19,20). In this study, we used nanopore sequencing to identify molecular markers of drug resistance and assess their prevalence in blood stage parasites at 48 hours after ACT initiation and in matched infected mosquito midguts. We observed that the polymorphisms Asn51Ile in *Pfdhfr,* conferring pyrimethamine resistance*,* and Lys540Glu in *Pfdhps,* conferring sulfadoxine resistance, appeared to have a transmission advantage. We also observed that Ala613Ser in *Pfdhps* was significantly more prevalent in human blood samples than in infected midguts and could therefore be associated with a transmission disadvantage. Notably, the Lys540Glu and Ala613Ser variants in *Pfdhps* were never observed together in the same infection. These mutations may be rendering the parasite intrinsically more or less infectious, in the absence of sulfadoxine-pyrimethamine drug pressure. *In vitro* studies introducing these polymorphisms with gene editing are needed to investigate a

causal relationship between the polymorphisms and a change in parasite transmissibility. Additionally, we observed a small but statistically insignificant transmission disadvantage of drug resistance marker Lys76Thr in *Pfcrt*, linked to chloroquine resistance, which is consistent with a previous report from Zambia showing preferential transmission of the wild-type (Lys76) form of *Pfcrt* compared to the mutant 76Thr (41), while another study found similar frequencies of wild-type and mutant *Pfcrt* alleles in gametocytes and sporozoite samples (12). Monitoring the relative infectivity of drug-resistant mutations, including *PfKelch13* mutations in areas with artemisinin partial resistance, may help model the spread of resistance.

This study had several limitations. Firstly, for many infected midguts we were unable to successfully amplify parasite DNA by PCR in the complexity of infection assay. This could be due to the midgut storage conditions and sensitivity of the assay. Consequently, our data may have incurred a density bias, if midguts with a higher number of oocysts were more likely to be amplified compared to those with a lower number of oocysts. Although the median number of oocysts in infected midguts that amplified was identical to the midguts that did not amplify (median oocyst density of 1), the 75th percentile was higher in the amplified group (IQR 1–4 vs. IQR 1–2), indicating that a certain degree of density bias may have occurred. In addition, the mosquitoes used in the feeding assays were insectary-reared and therefore may be genetically different and have less overall genetic diversity than the natural population of mosquitoes in Mali. As the insectary-reared mosquitoes likely harbour low genetic diversity in midgut receptors for parasite invasion and development, this could affect transmission results (3). Furthermore, the drug resistance polymorphisms assessed may not be selected for by the antimalarial treatments administered in this study, and no artemisinin partial resistance has been reported in Mali to date. Complexity of infection markers and the drug resistance markers only represent a very small portion of the genome, and it is not currently possible to "phase" this genotypic data. Therefore, the complexity of infection data cannot be linked to the observed frequencies of drug resistance

markers for a specific sample. For example, in a blood sample containing four parasite clones, we cannot determine which clones possess drug resistance polymorphisms and which do not. There is a need for bioinformatic tools that enable phasing of this genetic variation in polyclonal infections, as this would offer valuable insights into whether drug resistance is present in the minority or majority parasite clone and whether transmitting or non-transmitting gametocyte producing clones are drug resistant. Parasite sexual recombination takes place inside in the mosquito, leading to the creation of new parasite haplotypes; however, as another consequence of the short segments that were assessed in this study, it is highly unlikely that meiotic recombination occurred in the sequenced portion of the genome (42). This suggests that our analysis may have underestimated the parasite genomic diversity in infected mosquitoes. Finally, a study with a larger sample size and a wider range of infection densities is needed to draw epidemiological conclusions about which age groups have a higher multiplicity of infection and infectivity.

Overall, we find preferential transmission of minority parasite clones and putatively gametocyte-producing clones with SP resistance conferring polymorphisms. Our findings underscore the intricate nature of parasite-parasite and host-parasite interactions in their natural environments. They further stress the need for both fundamental and field studies to assess the importance of genetic and biological parasite and vector characteristics in driving parasite transmission. Molecular characterisation of transmission could prove beneficial in the fight against drug resistance.

FIGURES

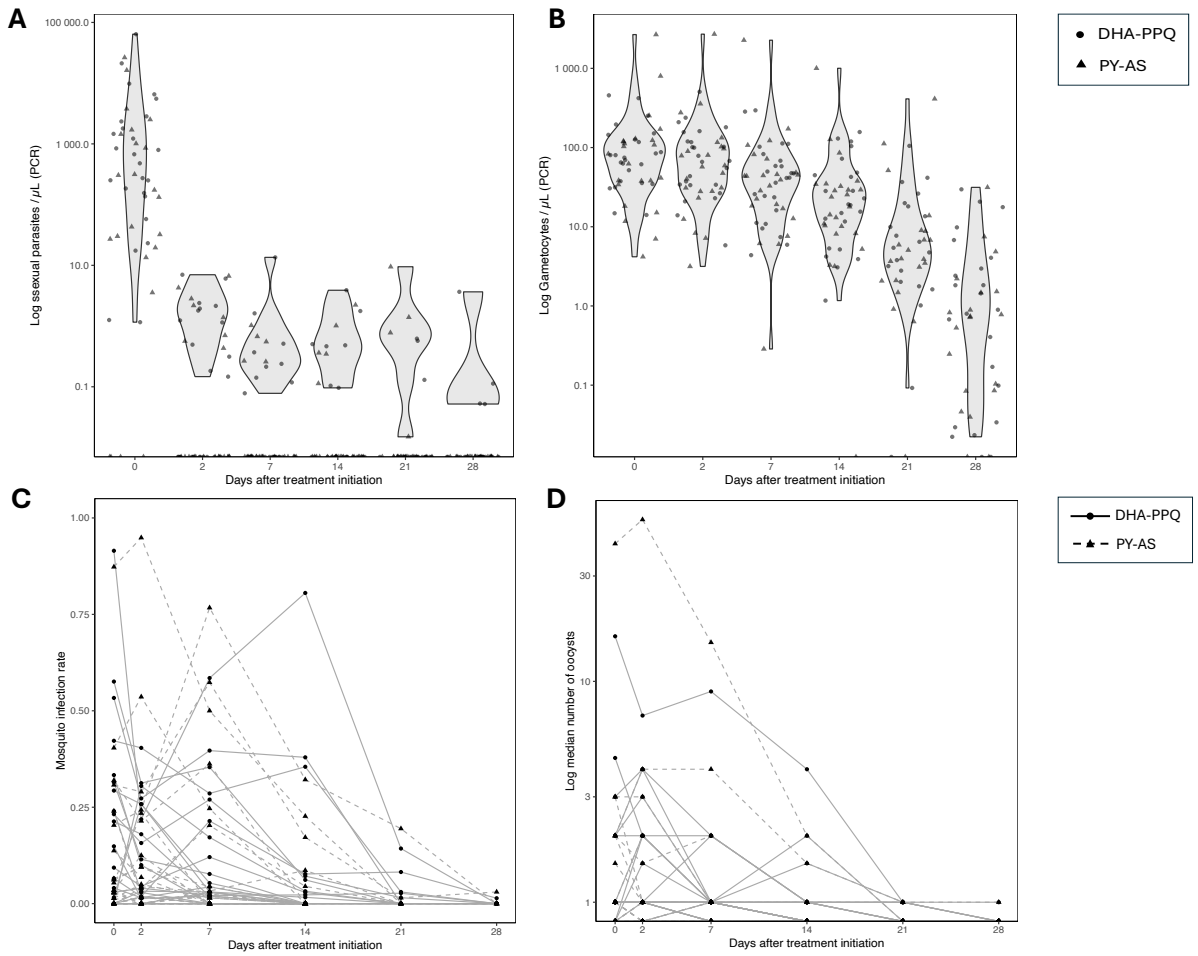

Figure 1. Infection dynamics. Violin plots showing A) Asexual parasite densities (parasites / µL) and B) gametocyte densities measured by qPCR (gametocytes/µL) at each study visit. Each point represents a study participant, with circles indicating participants in the dihydroartemisinin-piperaquine group and triangles indicating participants in the pyronaridine-artesunate group. Line graphs represent C) mosquito infection rate and D) median number of oocyst in infected mosquito midguts. Each line represents one individual, with full lines and circles marking individuals in the dihydroartemisinin-piperaquine group and dashed lines and triangles indicating participants in the pyronaridine-artesunate group. Median number of oocysts in D was set to zero if no mosquitoes were infected at a certain timepoint for a certain participant. DHA-PPQ=dihydroartemisinin-piperaquine, PY-AS=pyronaridine-artesunate

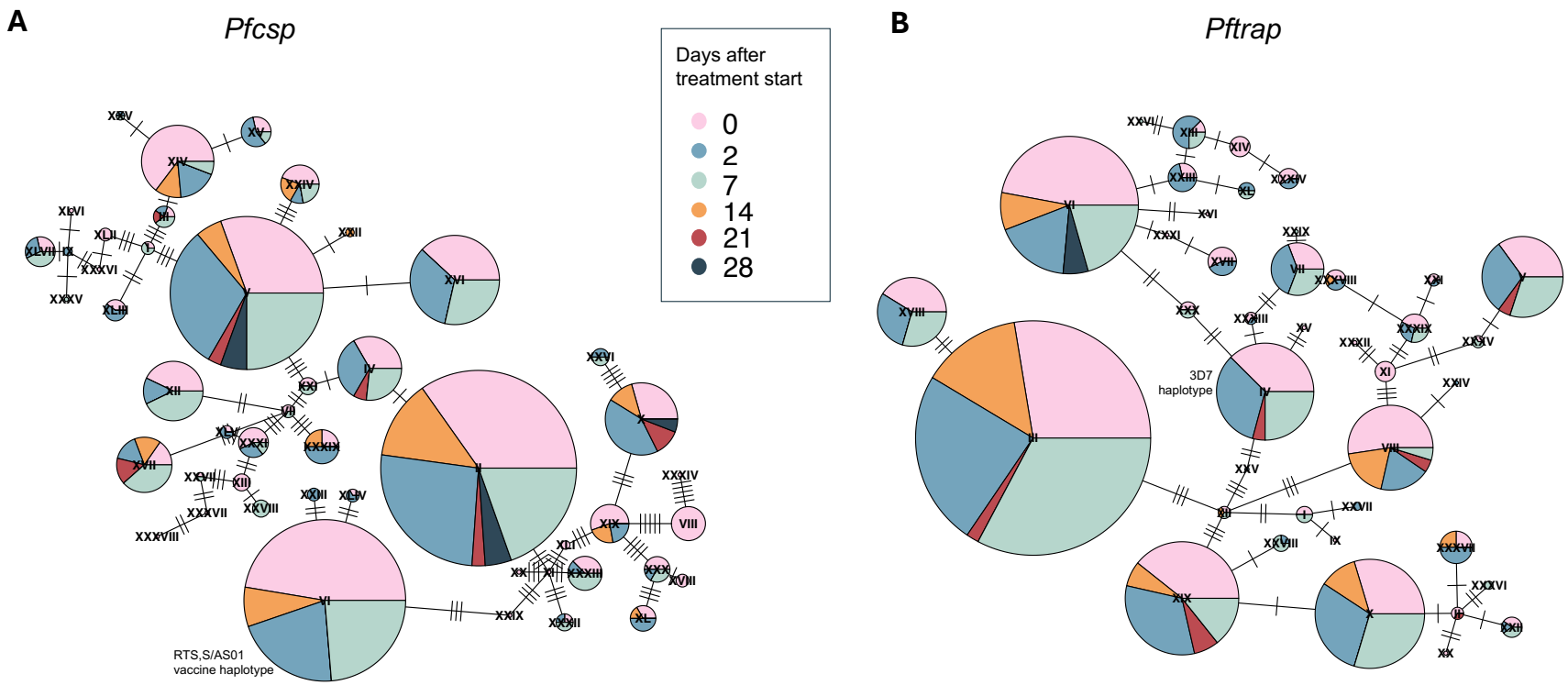

Figure 2. Haplotype network showing genetic diversity. Haplotype or minimal-spanning network constructed using A) *Pfcsp* and B) *Pftrap*. Each node represents a haplotype, each segment within the node represents a study timepoint, and is proportionally sized to the number of sequences present in the segment and node. The number of ticks between nodes represents the number of genetic differences between nodes.

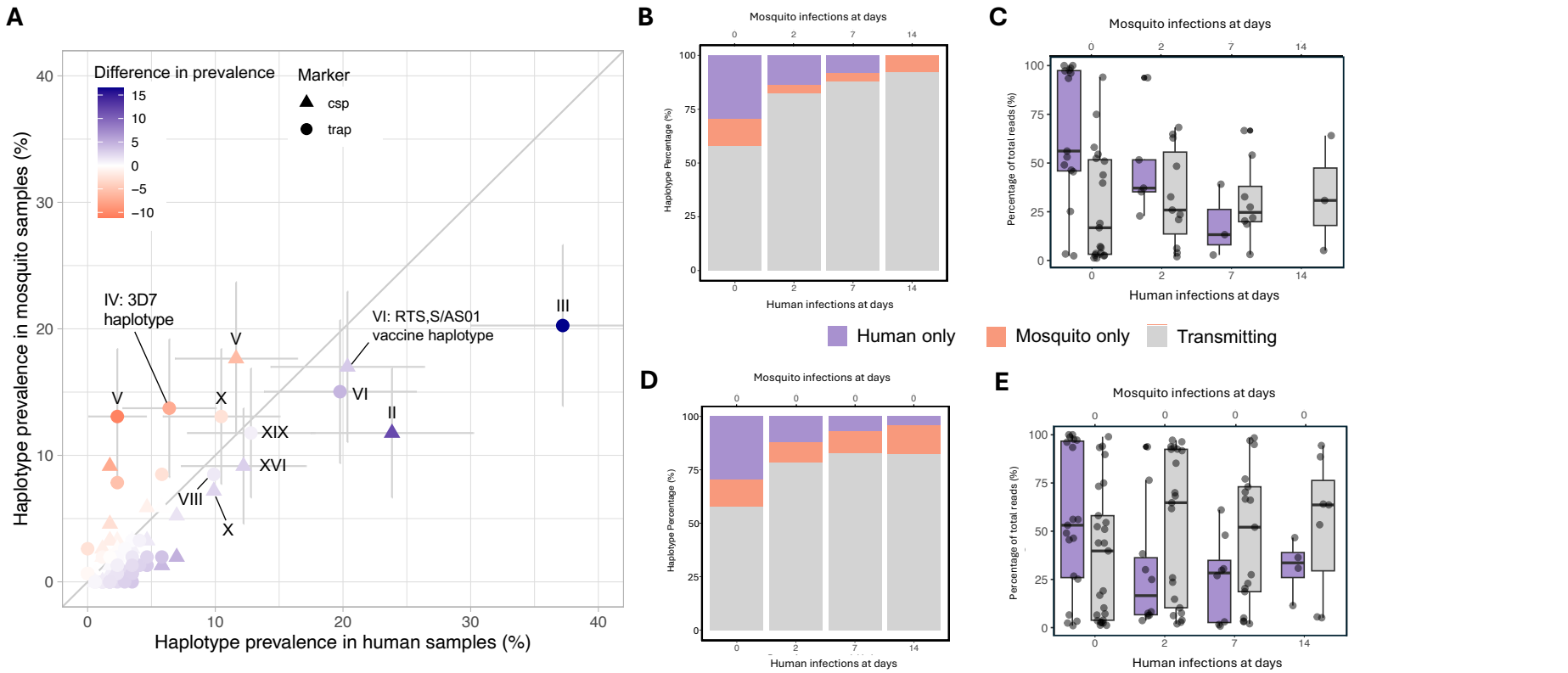

Figure 3. Molecular comparison of parasite clones in human infections and mosquito midguts post-feeding. A) Differential prevalence of the *Pfcsp* and *Pftrap* haplotypes in the human and mosquito samples. Roman numbers represent the haplotypes in Figure 2 haplotype networks. Error bars (representing 95% CI) and annotations are presented for haplotypes with at least 10% prevalence in either population. B) Percentage of transmitting haplotypes and species-exclusive haplotypes when comparing human and mosquito samples at each timepoint (e.g., comparing haplotypes from day 2 human blood samples with haplotypes from infected midguts of mosquitoes that fed on the same day 2 blood material). C) Percentage of transmitting haplotypes and species-exclusive haplotypes when comparing baseline mosquito samples to human samples at each timepoint (e.g., comparing haplotypes from day 2 human blood samples with haplotypes from infected midguts of mosquitoes that fed on day 0 blood material). D) The percentage of total reads that transmitting and non-transmitting haplotypes encompass at each timepoint when comparing human and mosquito samples at each timepoint and E) when comparing baseline mosquito samples to the human blood samples at each timepoint.

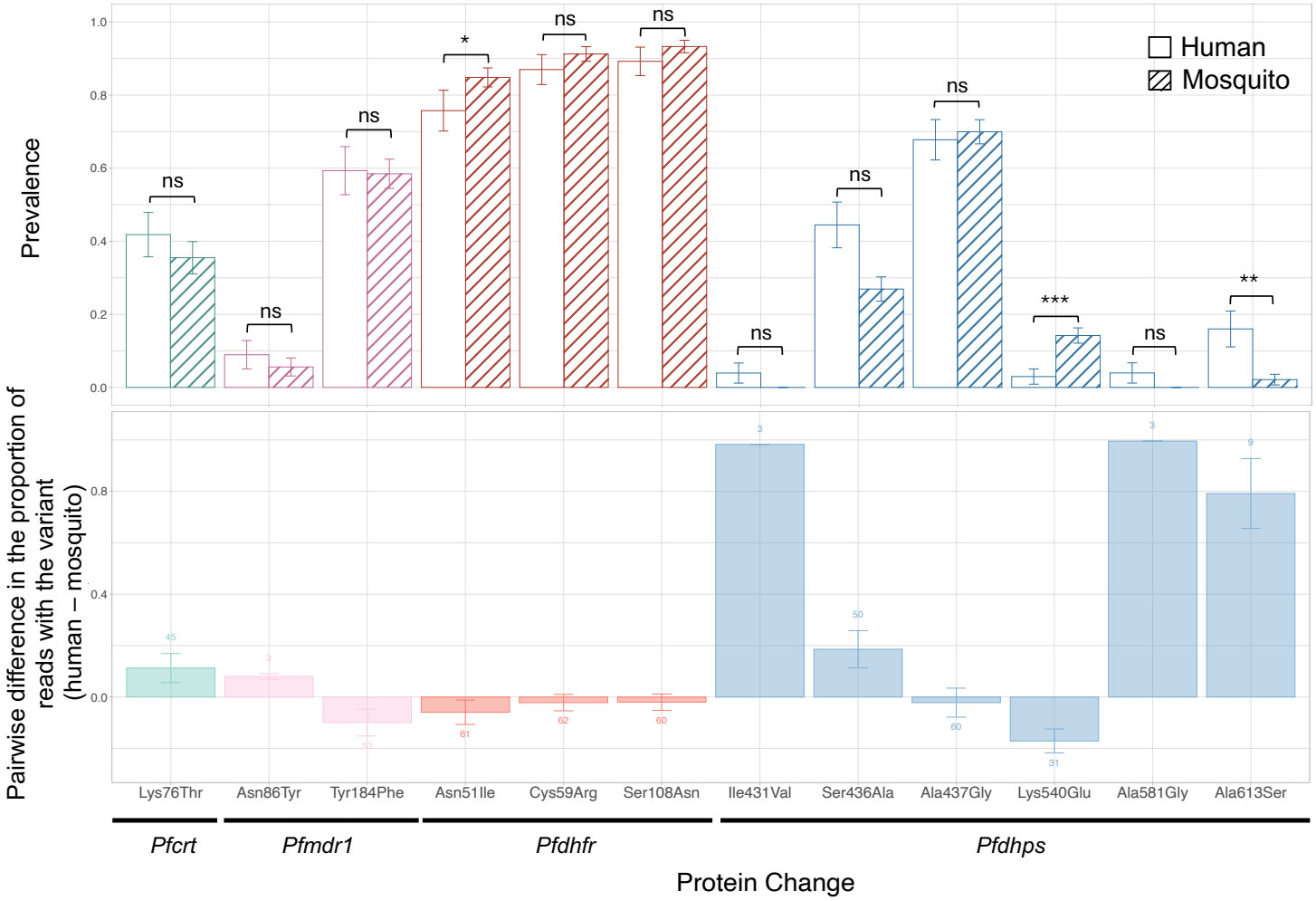

Figure 4. Molecular markers of drug resistance in human and mosquito samples. Prevalence of known single nucleotide polymorphisms linked to drug resistance in both species (upper panel). Pairwise comparison of human sample and cognate infected mosquito midgut, showing the difference in frequency of drug resistance markers between both (lower panel). ns=not significant, * = p <0.05, ** = p< 0.01, *** = p<0.001

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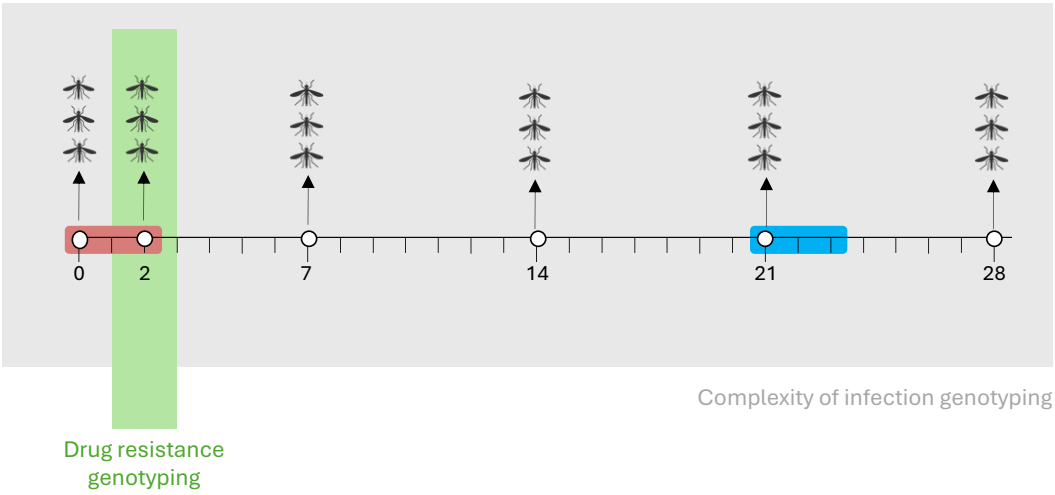
APPENDIX

Supplementary Figure 1. Diagram of sample selection and genotyping



Fifty study participants were treated with dihydroartemisinin-piperaquine or pyronaridine artesunate. Blood samples were collected from participants prior to treatment (d0), during treatment (d2) and days 7, 14, 21 and 28 after treatment initiation, for infectivity assessment with direct membrane feeding assays, as well as for the quantification of asexual parasite and gametocyte densities. All individuals were retreated with dihydroartemisinin-piperaquine at day 21 to prevent re-infection. If the infectivity assays infected any number of mosquitoes at a certain timepoint for a certain individual, a maximum of three infected mosquitoes were selected at random per study participant per timepoint for complexity of infection genotyping (all timepoints) and drug resistance genotyping (day 2 only).

Supplementary Table 1. Primer sequences, barcodes and PCR conditions for complexity of infection markers

Primer name	Sequence
csp F	TTAAGGAACAAGAAGGATAATACCA
csp R	AAATGACCCAAACCGAAATG
trap F	TCCAGCACATGCGAGTAAAG
trap R	AAACCCGAAAATAAGCACGA

Forward barcodes

BC1	CTATCACG	**BC6**	CATCTAAC	**BC21**	AACCAAGG	**BC26**	CAACCATG
BC2	TCCAGTGT	**BC7**	TACAGATC	**BC22**	AAGGTACG	**BC27**	CTTCGAAG
BC3	GATCAGTA	**BC8**	CGTCTTGT	**BC23**	ACCTACCT	**BC28**	CAGAAGTG
BC4	AGTGTCGG	**BC9**	TATGATCA	**BC24**	ACTGGACT	**BC29**	CAGTGACT
BC5	GTAGCGCT	**BC10**	GGTAGCTT	**BC25**	ATATGCCG	**BC30**	CATGTGGT

Reverse barcodes

BC11	ATGGCTAG	**BC16**	GGGACTAC	**BC31**	CGTAGGAA	**BC36**	TCACTCTG
BC12	GACTTGGT	**BC17**	ACGTACTG	**BC32**	GACATCTG	**BC37**	TCTCCAGT
BC13	TCGATCAC	**BC18**	TGATTGCC	**BC33**	GCAATAGG	**BC38**	TGGTTCCT
BC14	ACACGTCA	**BC19**	AACTCTAC	**BC34**	GACACTGT	**BC39**	TGTGACTG
BC15	CAATGTGC	**BC20**	TGACTCAA	**BC35**	GTGAGTCT	**BC40**	GTCTACAG

PCR reaction

Component	Volume per reaction (µL)
Q5 buffer	5
Q5 enzyme	0.25
dNTPs	0.5
ddH_20	9.25
Primers (at 10 µM)	1.25 CSP F, 1.25 CSP R 1.25 TRAP F, 1.25 TRAP R
Template	5
Total	25

PCR programme		
Step	**Temperature**	**Duration**
Initial denature	98	30 sec
For 30 cycles:		
Denature	98	10 sec
Annealing	58	45 sec
Extending	72	45 sec
Final extension	68	2 min

Supplementary Table 2. Primer sequences and PCR conditions for drug resistance markers

Primer name	Sequence	PCR reaction
crt F	TGTCTTGGTAAATGTGCTCA	1
crt R	AGTTGTGAGTTTCGGATGTT	1
dhfr F	GTTTTCGATATTTATGCCATATGTG	1
dhfr R	TGATAAACAACGGAACCTCC	1
dhps F	TTTGTTGAACCTAAACGTGC	2
dhps R	AACATTTTGATCATTCATGCAAT	2
mdr1 F	TGTGTTTGGTGTAATATTAAAGAACA	1
mdr1 R	ACATAAAGTCAAACGTGCATTT	1
kelch13 F	AAGCCTTGTTGAAAGAAGCA	2
kelch13 R	GGGAACTAATAAAGATGGGCC	2

PCR reaction 1

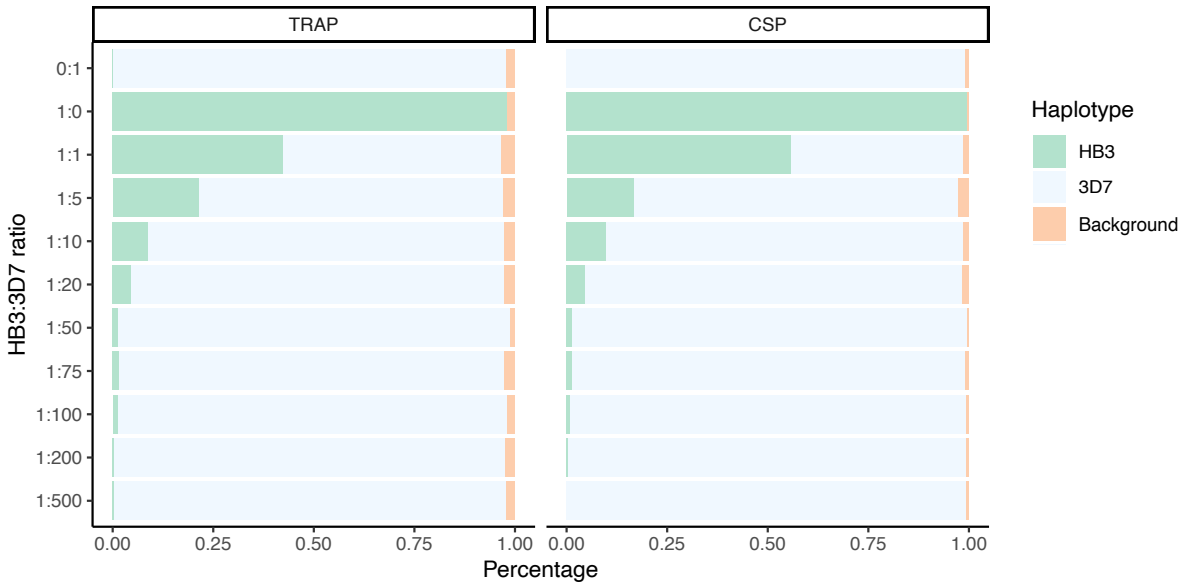
Component	Volume per reaction (µL)
Q5 buffer	10
Q5 enzyme	0.5
dNTPs	2.25
ddH_20	30.05
Primers (at 50 µM)	0.25 CRT F, 0.25 CRT R 0.25 mdr1 F, 0.25 mdr1 R 0.6 dhfr F, 0.6 dhfr R
Template	5
Total	50

PCR reaction 2

Component	Volume per reaction (µL)
Q5 buffer	10
Q5 enzyme	0.5
dNTPs	2.25
ddH_20	27.45
Primers (at 50 µM)	1.2 dhps F, 1.2 dhps R 1.2 K13 F, 1.2 K13 R
Template	5
Total	25

PCR programme		
Step	**Temperature**	**Duration**
Initial denature	98	30 sec
For 35 cycles:		
Denature	98	10 sec
Annealing	60	35 sec
Extending	72	45 sec
Final extension	68	2 min
Store	10	Forever

Supplementary Figure 2. Detection of minority clones



Stacked bar chart displaying the percentage of reads attributed to each haplotype. All values shown are the average of both the replicates that were conducted for each dilution of 3D7/HB3.

Supplementary Table 3. Parasite prevalence and densities

Day of follow-up	Treatment arm	Asexual parasites		Total gametocytes		Ratio median asexual parasites / gametocytes density (%)
		Prevalence % (n/N)	Density Median parasites/µL (IQR)	Prevalence % (n/N)	Density Median parasites/µL (IQR)	
Day 0	*Overall*	91.67% (44/48)	397 (54.2-1932)	100% (48/48)	77.3 (37.1-124)	513.58
	DHA-PPQ	100% (25/25)	673 (156-2342)	100% (25/25)	74.5 (37.6-126)	903.36
	PY-AS	82.61% (19/23)	305 (31.2-1572)	100% (23/23)	83.2 (35.9-122)	366.59
Day 2	*Overall*	41.67% (20/48)	1.59 (0.55-2.5)	100% (48/48)	62.6 (30.2-117)	2.54
	DHA-PPQ	48% (12/25)	1.52 (0.45-2.21)	100% (25/25)	67.9 (33.9-119)	2.24
	PY-AS	34.78% (8/23)	1.78 (0.66-3.19)	100% (23/23)	57.1 (28-99.5)	3.12
Day 7	*Overall*	28.57% (14/49)	0.32 (0.22-0.64)	100% (49/49)	41.6 (17.0-68.2)	0.77
	DHA-PPQ	36% (9/25)	0.24 (0.14-0.51)	100% (25/25)	41.6 (16.1-68.2)	0.58
	PY-AS	20.83% (5/24)	0.55 (0.27-0.68)	100% (24/24)	41.4 (18.0-54.1)	1.33
Day 14	*Overall*	25.53% (12/47)	0.47 (0.29-1.2)	97.87% (46/47)	21 (10.2-37.1)	2.24
	DHA-PPQ	30.43% (7/23)	0.48 (0.29-1.14)	100% (23/23)	19.2 (8.49-46.5)	2.50
	PY-AS	20.83% (5/24)	0.36 (0.35-1.01)	95.83% (23/24)	23.8 (10.2-33.5)	1.51
Day 21	*Overall*	16.67% (7/42)	0.62 (0.35-1.08)	95.24% (40/42)	5.25 (2.62-13.6)	11.81
	DHA-PPQ	14.29% (3/21)	0.57 (0.35-0.6)	95.24% (20/21)	5.94 (2.60-18.5)	9.60
	PY-AS	19.05% (4/21)	1.08 (0.59-3.42)	95.24% (20/21)	4.51 (2.85-8.84)	23.95
Day 28	*Overall*	9.3% (4/43)	0.08 (0.05-1)	90.7% (39/43)	0.83 (0.14-2.69)	9.64
	DHA-PPQ	17.39% (4/23)	0.08 (0.05- 0.99)	91.3% (21/23)	1.44 (0.17-4.06)	5.56
	PY-AS	0% (0/20)	.	90% (18/20)	0.76 (0.14-1.51)	.

DHA-PPQ=dihydroartemisinin-piperaquine, PY-AS=pyronaridine-artesunate

Supplementary Table 4. Infectivity to mosquitoes in infectious individuals

Day of follow-up	Treatment arm	Infectious individuals % (n/N)	Mosquito infection rate* Median % (IQR)	Oocyst density** Median (IQR)
Day 0	*Overall*	33/50	14.9 (3.51-31.4)	1 (1-2)
	DHA-PPQ	16/25	23.6 (8.64-35.6)	1 (1-2)
	PY-AS	17/25	6.15 (2.99-24)	1 (1-2)
Day 2	*Overall*	35/50	10 (3.61-25)	1 (1-2)
	DHA-PPQ	19/25	11.5 (3.06-25.8)	1 (1-2)
	PY-AS	16/25	9.49 (4.26-23.6)	1 (1-1.5)
Day 7	*Overall*	31/50	5.33 (2.7-27.8)	1 (1-1)
	DHA-PPQ	17/25	7.69 (3.03-27)	1 (1-1)
	PY-AS	14/25	3.85 (2.1-33.3)	1 (1-1)
Day 14	*Overall*	15/49	7.69 (3.85-27.4)	1 (1-1.5)
	DHA-PPQ	10/24	6.65 (2.98-28.5)	1 (1-1.38)
	PY-AS	5/25	17.2 (8.62-22.7)	1 (1-1.5)
Day 21	*Overall*	7/23	3.03 (2.12-11.2)	1 (1-1)
	DHA-PPQ	5/15	3.03 (2.7-8.2)	1 (1-1)
	PY-AS	2/8	10.5 (6-15)	1 (1-1)
Day 28	*Overall*	2/18	2.22 (1.81-2.62)	1 (1-1)
	DHA-PPQ	1/12	1.41 (1.41-1.41)	1 (1-1)
	PY-AS	1/6	3.03 (3.03-3.03)	1 (1-1)

*In infectious individuals

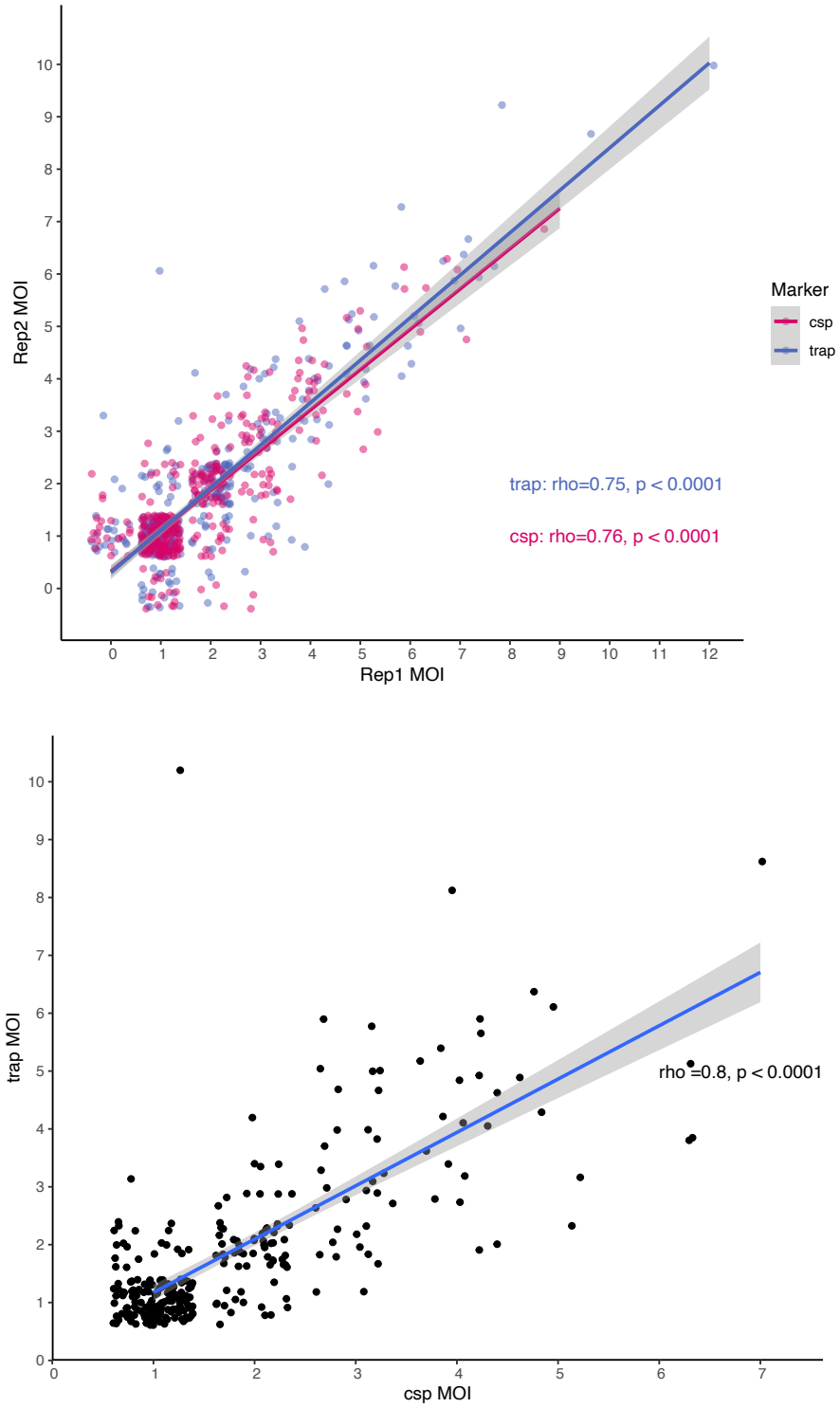
**In infectious individuals, median number of oocysts in infected mosquitoes

DHA-PPQ=dihydroartemisinin-piperaquine, PY-AS=pyronaridine-artesunate

Supplementary Table 5. Coverage across markers

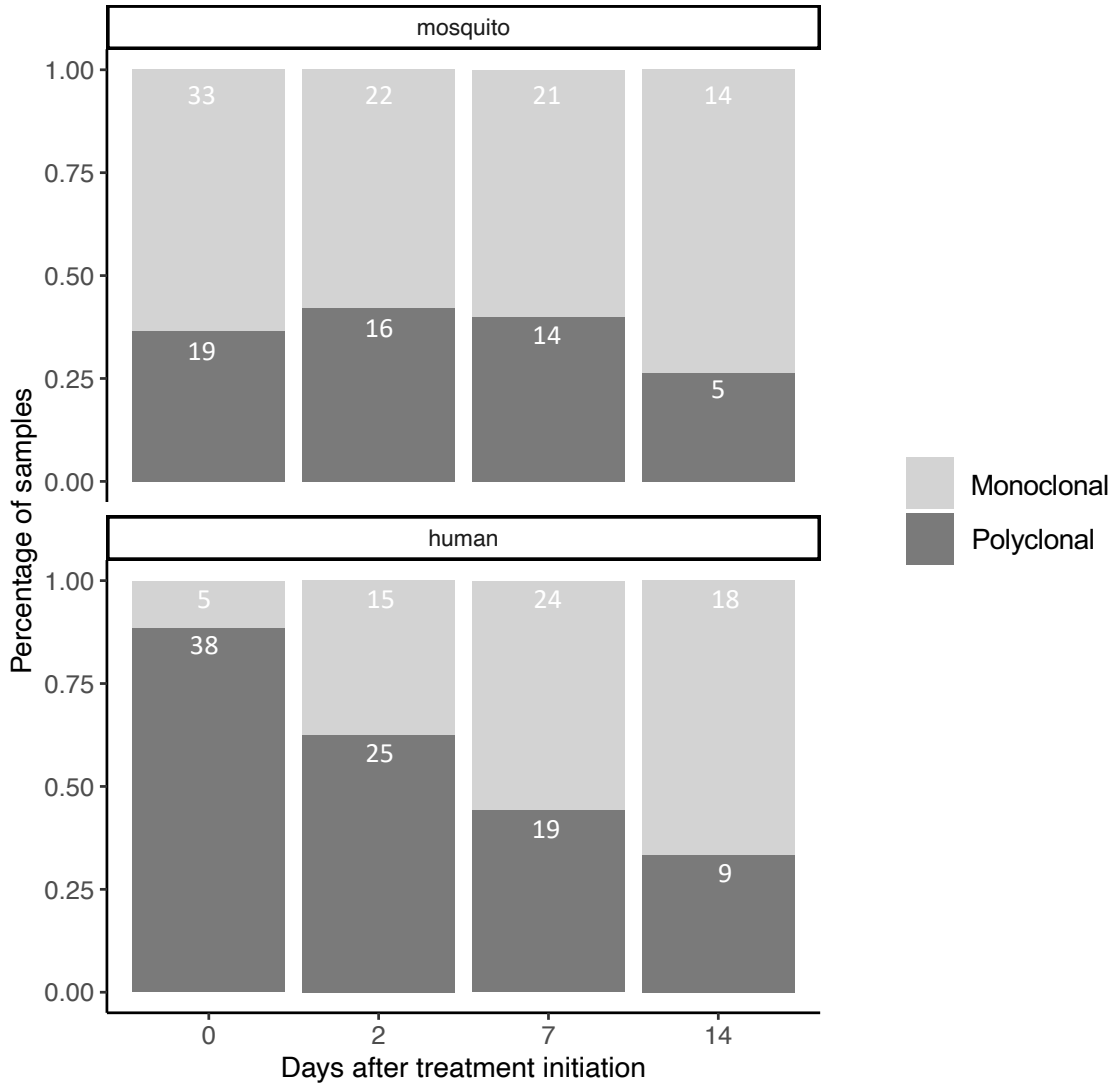
Marker	Genome positions	Median coverage [IQR]
Pfcsp	Pf3D7_03_v3: 221,352-221,640	1002 [287-3360]
Pftrap	Pf3D7_13_v3: 1,465,059-1,465,378	944 [258-3244]
Pfcrt	Pf3D7_07_v3: 403,536-403,673	6364 [772-23033]
Pfmdr1	Pf3D7_05_v3: 958,143-958,459	2812 [70.5-9948]
Pfdhfr	Pf3D7_04_v3: 748,134-748,579	2790 [58-8391]
Pfdhps	Pf3D7_08_v3: 922,591-923,189	3528 [47-11726]
Pfk13	Pf3D7_13_v3: 1,724,874-1,725,701	990 [21-6071]

Supplementary Figure 3. Correlation between replicates and markers

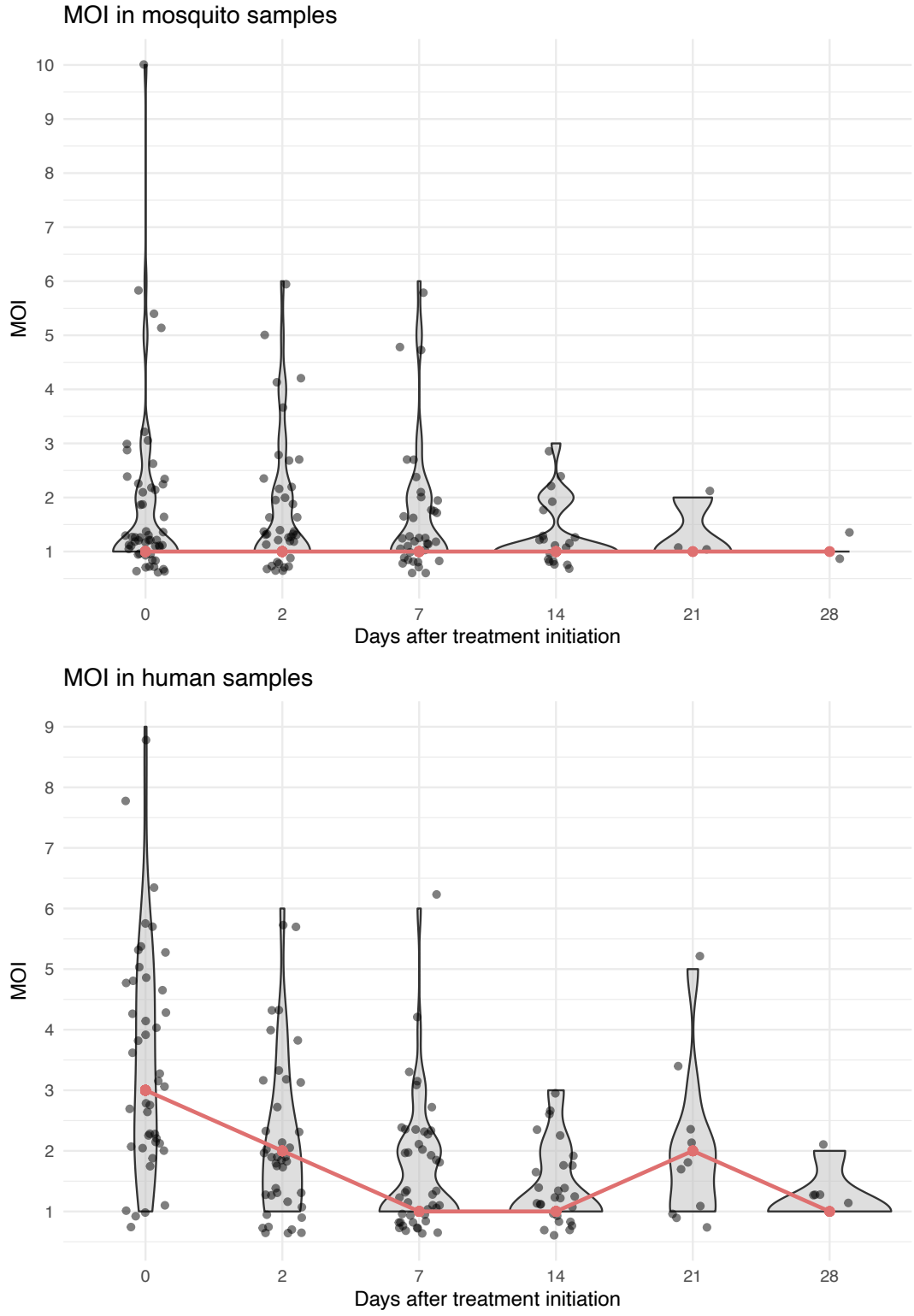


Spearman correlation coefficient was calculated to assess the correlation between markers and replicates. MOI = Multiplicity of infection.

Supplementary Figure 4. Percentage of polyclonal infections



Supplementary Figure 5. Median MOI at all timepoints in both species



MOI = multiplication of infection.

Supplementary Table 6. Percentage of polyclonal infections and median MOI at all timepoints

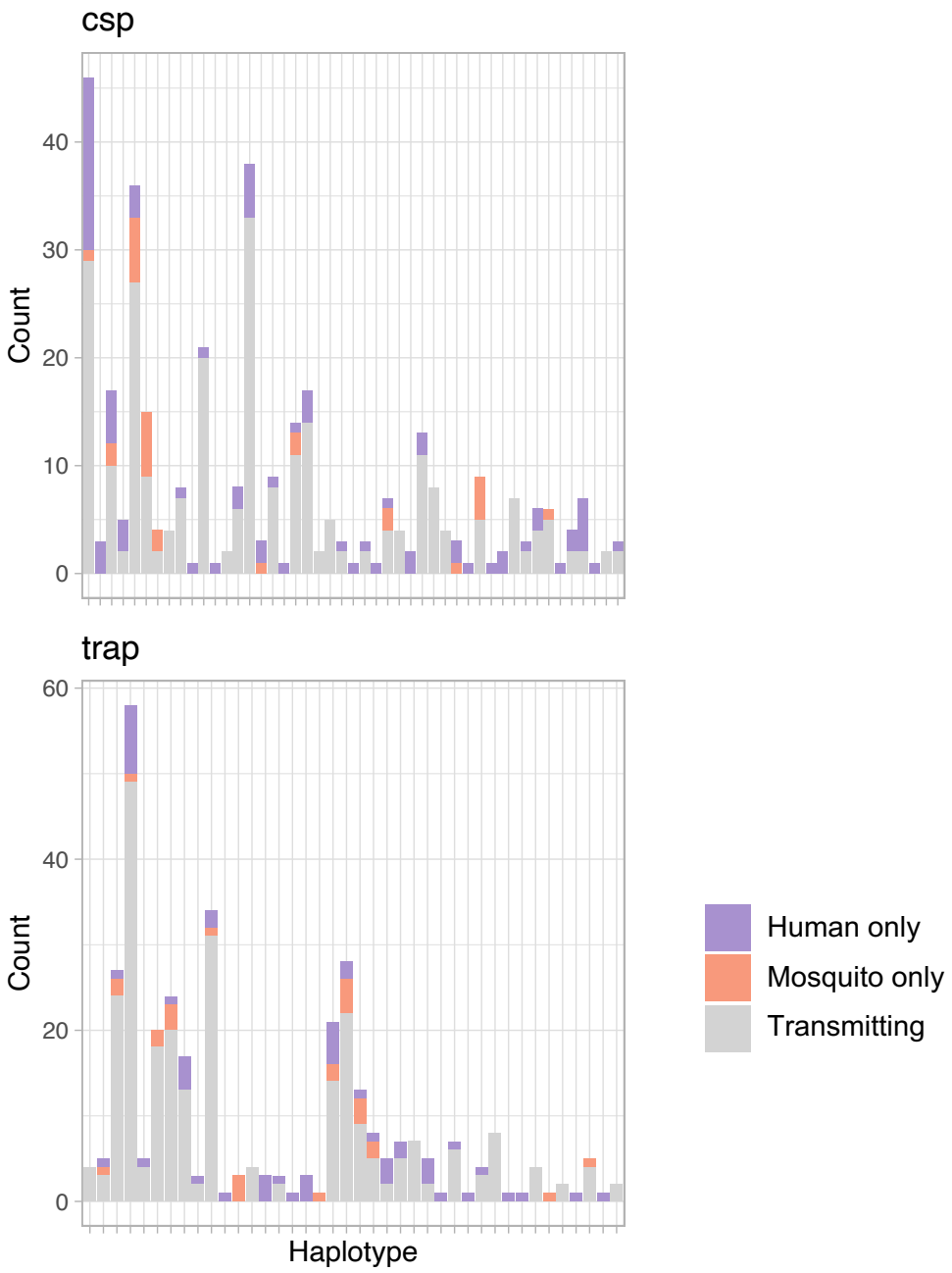
Day of follow-up	Proportion of multiclonal infections in blood stage parasites % (n/N)	Proportion of multiclonal infections in infected midguts % (n/N)	Median MOI blood stage parasites (IQR)	Median MOI infected mosquito midguts (IQR)
Day 0	80.37% (38/43)	36.54% (19/52)	3 (2-5)	1 (1-2)
Day 2	62.5% (25/40)	42.11% (16/38)	3 (1-3)	1 (1-2)
Day 7	37.2% (19/43)	40% (14/35)	1 (1-2)	1 (1-2)
Day 14	33.33% (9/27)	26.32% (5/19)	1 (1-2)	1 (1-2)
Day 21	54.55% (6/11)	33.34% (1/3)	2 (1-2)	1 (1-1.5)
Day 28	20% (1/5)	0% (0/2)	1 (1-1)	1 (1-1)

Supplementary Table 7. Gametocyte densities, gametocyte fraction, mosquito infection rate and oocyst densities in monoclonal versus multiclonal infections

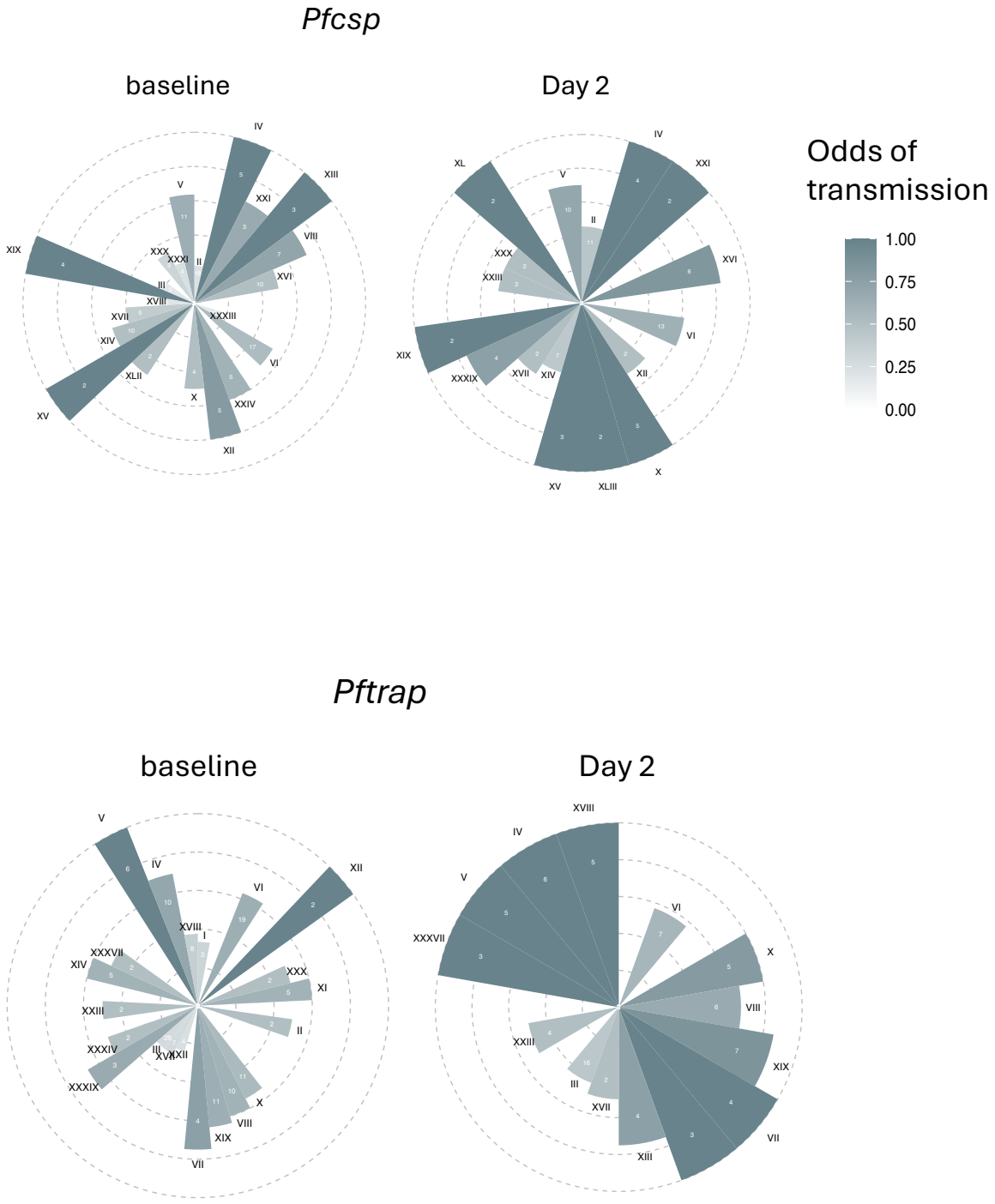
Day of follow-up	MOI	Gametocyte density		Gametocyte fraction (gametocytes/total parasites)		Mosquito infection rate		Oocyst density	
		Median parasites/µL (IQR)	p-value	Median % (IQR)	p-value	Median % (IQR)	p-value	Median % (IQR)	p-value
Day 0	1	80.2 (62.4-145)	ref	77.6 (0.23-82.3)	ref	24 (6.15-31.4)	ref	1 (1-2)	ref
	>1	80.3 (37.8-123)	0.698	20.4 (5.33-58.2)	0.756	2.92 (0-21.1)	0.105	3 (1-10)	<0.001
Day 2	1	66.0 (32.2-102)	ref	100 (99.2-100)	ref	9.52 (1.75-19.7)	ref	1 (1-4)	ref
	>1	96.7 (40.2-157)	0.199	100 (98.7-100)	0.748	4.35 (1.56-21.9)	0.888	2 (1-6)	0.001
Day 7	1	41.3 (17.0-49.8)	ref	100 (99.6-100)	ref	2.13 (0-4.41)	ref	2 (1-4)	ref
	>1	47.5 (34.4-105)	0.0756	100 (99.8-100)	0.989	5.33 (0.76-25.8)	0.199	2 (1-5)	0.27
Day 14	1	25.4 (11.8-43.7)	ref	100 (100-100)	ref	0 (0-6.65)	ref	2 (1-4)	ref
	>1	28.6 (14.9-48.3)	0.555	100 (99.4-100)	0.0434	0 (0-4.48)	0.867	1 (1-2)	0.001
Day 21	1	41.5 (26.2-105)	ref	100 (99.7-100)	ref	1.54 (0-8.2)	ref	1 (1-1)	ref
	>1	28.3 (15.2-47.8)	0.648	100 (100-100)	0.486	0.76 (0-2.41)	0.503	1 (1-1)	0.36
Day 28	1	14.1 (6.63-23.0)	ref	100 (100-100)	ref	0 (0-0.35)	ref	1 (1-1)	ref
	>1	31.4 (31.4-31.4)	0.289	100 (100-100)	nc	3.03 (3.03-3.03)	0.236	1 (1-1)	nc

P-values were calculated by Wilcoxon rank sum test. ref= reference, nc = not calculable, no observations/no observations over the threshold density for analysis

Supplementary Figure 6. Haplotype count in human and mosquito hosts



Supplementary Figure 7. Odds of transmission per haplotype



Haplotypes were only included if their transmissibility was assessed more than once, with the white number representing the occurrence count. Roman haplotype names correspond to the haplotypes in Figure 2 and are only presented in the figure if the odds of transmission > 0.1.

Supplementary Table 8. Non-synonymous single nucleotide polymorphisms in genes associated with drug resistance.

Gene	Gene ID	Nt change	AA change	Human blood *Pf* samples MAF (%) n = 48	Mosquito midgut *Pf* samples MAF (%) n = 73	P value*
Pfcrt	PF3D7_0709000	403625A>C	Lys76Thr	41.81	35.49	1
Pfmdr1	PF3D7_0523000	958145A>T	Asn86Tyr	8.94	5.59	0.506
		958440A>T	Tyr184Phe	59.31	58.45	0.121
Pfdhfr	PF3D7_0417200	748239A>T	Asn51Ile	75.73	84.83	0.025
		748262T>C	Cys59Arg	86.97	91.24	0.384
		748410G>A	Ser108Asn	89.23	93.28	0.300
Pfdhps	PF3D7_0810800	549666A>G	Ile431Val	3.93	0	0.158
		549681T>G	Ser436Ala	44.46	26.91	0.852
		549682C>T	Ser436Phe	1.55	0	nc
		549682C>A	Ser436Tyr	0	0.83	nc
		549685G>C	Ala437Gly	67.76	69.95	0.178
		549993A>G	Lys540Glu	2.96	14.2	<0.001
		550087G>T	Arg571Met	0	0.19	nc
		550098G>T	Asp575Tyr	0	0.43	nc
		550117C>G	Ala581Gly	3.95	0	0.155
		550212G>T	Ala613Ser	15.99	2.13	<0.001
PfK13	PF3D7_1343700	1725518G>T	Val494Phe	0	0.13	nc

MAF = Minor allele frequency; Nt = Nucleotide; AA = Amino Acid
*Comparing human blood *Pf* samples and mosquito midgut *Pf* samples

Chapter 4: Artemether-lumefantrine-amodiaquine or artesunate-amodiaquine combined with single low-dose primaquine to reduce *Plasmodium falciparum* malaria transmission in Ouélessébougou, Mali: a five-arm, phase 2, single-blind, randomised clinical trial

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SECTION A – Student Details

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First Name(s)	Leen Nele		
Surname/Family Name	Vanheer		
Thesis Title	Transmissibility and antimalarial resistance in human malaria parasite Plasmodium falciparum in Mali		
Primary Supervisor	Chris Drakeley		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?			
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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Lancet Microbe
Please list the paper's authors in the intended authorship order:	Almahamoudou Mahamar, PhD*, Leen N Vanheer, MD*, Merel J Smit, MD, Koualy Sanogo, MD, Youssouf Sinaba, MD, Sidi M. Niambele, PharmD, Makonon Diallo, MD, Oumar M Dicko, MD, Richard S. Diarra, MD, Seydina O Maguiraga, MD, Ahamadou Youssouf, PharmD, Adama

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Stage of publication	**In press**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I wrote the first draft of the study protocol for ethics approval, upon which co-authors commented. I managed the ClinicalTrials.gov study registration and procurement of consumables and equipment to be shipped to the study site. I verified and analysed the raw data, and wrote the first draft of the manuscript, which was commented upon by my co-authors.

SECTION E

Student Signature	[redacted]
Date	[redacted]

Supervisor Signature	[redacted]
Date	[redacted]

Artemether-lumefantrine-amodiaquine or artesunate-amodiaquine combined with single low-dose primaquine to reduce *Plasmodium falciparum* malaria transmission in Ouélessébougou, Mali: a five-arm, phase 2, single-blind, randomised clinical trial

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Keywords: Gametocytes, Artemether-lumefantrine, Primaquine, Artemether-lumefantrine-Amodiaquine, Triple Artemisinin-based Combination Therapy, Artesunate-Amodiaquine, Transmission-blocking

Running title: Transmission-blocking efficacy of AL-AQ and AS-AQ with and without PQ

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SUMMARY

Background: Triple artemisinin-based combination therapies, such as artemether-lumefantrine-amodiaquine, can delay the spread of antimalarial drug resistance. Artesunate-amodiaquine is widely used for uncomplicated *Plasmodium falciparum* malaria. We aimed to determine the efficacy of artemether-lumefantrine-amodiaquine and artesunate-amodiaquine with and without single low-dose primaquine for reducing gametocyte carriage and transmission to mosquitoes.

Methods: We conducted a five-arm, single-blind, phase 2, randomised clinical trial at the Ouélessébougou Clinical Research Unit of the Malaria Research and Training Centre of the University of Sciences, Techniques and Technologies of Bamako (Bamako, Mali). Eligible participants aged 10-50 years, with asymptomatic *P. falciparum* microscopy-detected gametocyte carriage, were randomised (1:1:1:1:1) to receive either artemether-lumefantrine, artemether-lumefantrine-amodiaquine, artemether-lumefantrine-amodiaquine plus primaquine, artesunate-amodiaquine, or artesunate-amodiaquine plus primaquine. Treatment allocation was computer randomised and concealed to all study staff other than the trial pharmacist. Participants were unmasked. The primary outcome was the within-person percentage reduction in mosquito infection rate at 48 hours after treatment initiation compared to pre-treatment, assessed by direct membrane feeding assay. Data were analysed per protocol. This study is registered with ClinicalTrials.gov, NCT05550909 (closed).

Findings: Between Oct 16 and Dec 28, 2022, 1249 individuals were screened for eligibility, 100 of which were enrolled and randomly assigned to one of five treatment groups (n=20 per group). Before treatment, 61 (61%) of 100 participants were infectious to mosquitoes, with a median of 7·3% (IQR 3·2-23·5) of mosquitoes becoming infected. Among infectious individuals, the median percentage reduction in mosquito infection rate between pre-treatment and 2 days post-treatment was 100% (IQR 100-100) in the artemether-lumefantrine (range 83·1-100, p=0·0018), artemether-lumefantrine-amodiaquine (range 82·4-100, p=0·0018), and artemether-lumefantrine-

amodiaquine plus primaquine (range 100-100, p=0·0009) treatment groups. In the artesunate-amodiaquine group the median percent reduction in mosquito infection rate was only 32% (IQR -10·9-79.4, range -112.6-100, p=0·20), whereas there was 100% median reduction in the artesunate-amodiaquine plus primaquine group (IQR 100-100, range 100-100, p=0·0009). At day 2, 10% (2/20) of participants in the artemether-lumefantrine group, 11% (2/19) in the artemether-lumefantrine-amodiaquine group, and 75% (15/20) in the artesunate-amodiaquine group infected any number of mosquitoes whilst no infected mosquitoes were observed at this time-point in the primaquine arms. No serious adverse events occurred.

Interpretation: These data support the effectiveness of artemether-lumefantrine alone or as part of triple combination therapy for preventing nearly all human-mosquito malaria parasite transmission within 48 hours. In contrast, substantial transmission was observed following treatment with artesunate-amodiaquine. The addition of a single low-dose of primaquine blocks transmission to mosquitoes rapidly regardless of schizonticide.

Funding: Bill & Melinda Gates Foundation

INTRODUCTION

Malaria morbidity and mortality remains unacceptably high (1) and the emergence and spread of partial resistance against artemisinin derivatives, the main component of artemisinin-based combination therapies (ACTs), in South East Asia (2,3) and East-Africa (4,5) is threatening to increase malaria cases and deaths. There is therefore a clear need for antimalarial treatments designed to slow the spread of resistance, either through novel combinations of existing drugs or supplementation with drugs that have specific effects on gametocytes, the sexual life stages responsible for maintaining parasite transmission. For optimal use, it is essential that we understand how effectively current and future antimalarials combat gametocytes, and how this translates into reductions in transmission to mosquitoes.

Triple artemisinin-based combination therapies (TACT) combine an existing ACT with a second partner drug that is slowly eliminated, to reduce the likelihood of incomplete parasite clearance and thus delay the spread of artemisinin resistance (6). Artemether-lumefantrine-amodiaquine is a TACT that has been proven safe, well-tolerated and efficacious for the treatment of uncomplicated *P. falciparum* malaria, including in areas with artemisinin and partner drug resistance (7,8). The effect of artemether-lumefantrine-amodiaquine on mature gametocytes and infectivity is unknown. Artesunate-amodiaquine is the first-line ACT for uncomplicated *P. falciparum* malaria in many countries (9), but its transmission reducing efficacy has not been tested directly. Studies assessing gametocyte carriage after artesunate-amodiaquine observed persistent gametocyte carriage post-treatment for 21 days or longer, but without transmission assays the infectivity of these persisting gametocytes cannot be confirmed (10–12).

Although artemisinin-based treatments have superior gametocytocidal properties to non-artemisinin's (13), with artemether-lumefantrine being the most potent (14), the transmission reducing activities of ACTs vary widely (14–16). In contrast, the 8-aminoquinoline primaquine is a potent gametocytocidal drug which at a single low-dose (0·25mg/kg) blocks transmission within

48 hours of treatment. Since 2015 the World Health Organization (WHO) recommends the addition of a single low-dose of primaquine (0·25 mg/kg) to ACT to reduce *P. falciparum* transmission (17). The gametocytocidal and transmission reducing activities of single low-dose primaquine have been assessed in combination with dihydroartemisinin-piperaquine, pyronaridine-artesunate and artemether-lumefantrine (14–16,18,19), however, combining artemether-lumefantrine-amodiaquine or artesunate-amodiaquine with a single low-dose primaquine for *P. falciparum* transmission reduction has not yet been tested.

In the current study, we aimed to determine the safety and efficacy of artemether-lumefantrine-amodiaquine and artesunate-amodiaquine with and without single low-dose primaquine for reducing the transmission of *P. falciparum* gametocytes in a cohort of Malian children and adults.

METHODS

Study design and participants

This five-arm, single-blind, phase 2 randomised controlled trial was conducted at the Ouélessébougou Clinical Research Unit of the Malaria Research and Training Centre (MRTC) of the University of Sciences, Techniques and Technologies of Bamako in Mali. Ouélessébougou is a commune that includes the town of Ouélessébougou and 44 surrounding villages, which have a total of approximately 50,000 inhabitants. Malaria transmission is highly seasonal, tied to the rainy season occurring from July to November. The prevalence of *P. falciparum* malaria and gametocytes in children aged over 5 years varies between 50-60% and 20-25%, respectively, during the transmission season. Two days before the start of enrolment, the study team met with community leaders, village health workers, and heads of households from each village, before the commencement of screening, to explain the study and obtain verbal assent to undertake screening. Village health workers then used a door-to-door approach to inform all available households of

the date and location where consenting and screening would take place. Participants were included in the trial if they met the following criteria: positive for *P. falciparum* gametocytes by microscopy (i.e. ≥1 gametocytes observed in a thick film against 500 white blood cells (WBC), equating to 16 gametocytes/μL with a standard conversion of 8000 WBC)/μL blood); absence of other non-*P. falciparum* species on blood film; haemoglobin density of ≥10 g/dL; aged between 10-50 years; bodyweight of ≤80 kg; no clinical signs of malaria, defined by fever (≥37·5°C); no signs of acute, severe or chronic disease. Exclusion criteria included pregnancy (tested at enrolment by urine test) or lactation, allergies to any of the study drugs, use of other medication (except for paracetamol and/or aspirin), use of antimalarial drugs over the past week, history of prolongation of the corrected QT (QTc) interval, documented or self-reported history of cardiac conduction problems or epileptic seizures, and blood transfusion in last 90 days. We chose to recruit only asymptomatic individuals in order to increase the likelihood of observing high gametocyte densities (20). Prior to screening and prior to study enrolment, participants provided written informed consent (≥18 years) or written parental consent (10-17 years). In addition to parental consent, an assent was sought for individuals 10-17 years old. Ethical approval was granted by the Ethics Committee of the University of Sciences, Techniques, and Technologies of Bamako (Bamako, Mali) (No2022/244/CE/USTTB), and the Research Ethics Committee of the London School of Hygiene and Tropical Medicine (London, United Kingdom) (LSHTM Ethics Ref. 28014). The study protocol is provided in appendix 2.

Randomisation and masking

Allocation to five treatment groups (artemether-lumefantrine, artemether-lumefantrine-amodiaquine, artemether-lumefantrine-amodiaquine plus primaquine, artesunate-amodiaquine and artesunate-amodiaquine plus primaquine) was randomised in a 1:1:1:1:1 ratio. Enrolment continued until the prespecified threshold of 100 participants were enrolled (20 individuals assigned to each treatment group). An independent MRTC statistician randomly generated the

treatment assignment using Stata version 16 (StataCorp, College Station, Texas, USA), which was linked to participant ID number. The statistician prepared sealed, opaque envelopes with the participant ID number on the outside and treatment assignment inside which were sent to the MRTC study pharmacist. Study participants were aware of the allocated treatment. The study pharmacist provided treatment and was consequently not blinded to treatment assignment; staff involved in assessing safety, infectivity and laboratory outcomes were blinded.

Procedures

Artesunate-amodiaquine and artemether-lumefantrine treatment (Guilin Pharmaceutical, Shanghai, China) was administered over three days as per manufacturer instructions (supplementary information 1). Participants in the artemether-lumefantrine plus amodiaquine group were treated with standard doses of artemether-lumefantrine and amodiaquine (Guilin Pharmaceutical, Shanghai, China) as per manufacturer instructions. A single dose of 0·25 mg/kg primaquine (ACE Pharmaceuticals, Zeewolde, The Netherlands) was administered on day 0 in parallel with the first dose of ACT or TACT, as described previously (19).

Participants received a full clinical and parasitological examination on days 2, 7, 14, 21, and 28 after receiving the first dose of the study drugs (supplementary figure 1). Giemsa-stained thick film microscopy was performed as described previously (19), with gametocytes counted against 500 WBC and asexual stages counted against 200 WBC. Total nucleic acids were extracted from 83.3 μL whole blood for molecular gametocyte quantification using a MagNAPure LC automated extractor (Total Nucleic Acid Isolation Kit-High Performance; Roche Applied Science, Indianapolis, IN, USA). Female and male gametocytes were quantified in a multiplex reverse transcriptase quantitative PCR (RT-qPCR) assay (supplementary table 1) (21). Samples were classified as negative for a particular gametocyte sex if the qRT-PCR quantified density of gametocytes of that sex was less than 0·01 gametocytes per μL (i.e. one gametocyte per 100 μL of blood sample). Haemoglobin density (grams/decilitre) was measured in finger-prick samples using

a haemoglobin analyser (HemoCue; AB Leo Diagnostics, Helsingborg, Sweden) or using an automatic haematology analyser (HumaCount 5D; Wiesbaden, Germany) in venous blood samples. Additional venous blood samples were taken for biochemical and infectivity assessments on day 0, 2, 7, and 14 in all treatment groups. Levels of aspartate transaminase (AST), alanine transaminase (ALT) and blood creatine were measured using the automatic biochemistry analyser Human 100 (Wiesbaden, Germany). For each assessment of infectivity, ~75 locally insectary-reared female *Anopheles gambiae (s.l.)* mosquitoes were allowed to feed for 15-20 minutes on venous blood samples (Lithium Heparin VACUETTE tube, Greiner Bio-One, Kremsmünster, Austria) through a prewarmed glass membrane feeder system (Coelen Glastechniek, Weldaad, the Netherlands). Mosquitoes that had taken no blood meal or a partial blood meal were discarded; surviving blood-fed mosquitoes were dissected on the 7th day post-feeding. Midguts were stained with 1% mercurochrome and examined for the presence and density of oocysts by expert microscopists.

To investigate whether early post-treatment transmission-blocking was due either to insufficient gametocyte densities or drug-induced sterilization effects, a separate blood sample (from baseline and day 2 only) was processed by magnetic-activated cell sorting (MACS) to enrich its gametocyte content prior to mosquito feeding for transmission assays. Gametocytes in the infected whole blood sample were concentrated by MACS using a QuadroMACS™ separator and LS MACS columns (MiltenyiBiotech, UK) as previously described (22). Briefly, MACS LS columns were equilibrated with 1 mL of warm incomplete medium, followed by 3 mL of infected whole blood and 2 mL medium wash. LS columns were then removed from the magnet, and gametocytes were eluted in 4 mL of warm medium. Flow-through and gametocyte fractions were then centrifuged (2000 RPM, 5 minutes, 37°C). Medium was removed carefully, and the gametocyte pellet was resuspended in 450 μL warm malaria naïve serum and 600 μL of the same participants packed cells. The entire MACS procedure was carried out in a 37°C cabinet incubator.

Outcomes

The primary outcome measure was median percentage change in mosquito infection rate between pre-treatment and 2 days after treatment initiation. Secondary outcomes were mosquito infection metrics (infectious individuals, mosquito infection rate and oocyst density) at prespecified timepoints (days 0, 2, 7 and 14); gametocyte and asexual parasite prevalence, density, gametocyte circulation time, area under the curve (AUC) of gametocyte density over time, and sex ratio (i.e., proportion of gametocytes that were male or female); and safety assessments including incidence of clinical and laboratory adverse events. Differences in all transmission metrics, gametocyte, asexual stages, and safety outcomes were compared between matched treatment groups (ie, artemether–lumefantrine vs artemether–lumefantrine-amodiaquine and artemether–lumefantrine-amodiaquine plus primaquine, artesunate-amodiaquine vs artesunate-amodiaquine plus primaquine) as secondary outcomes. Primary and secondary analyses of mosquito infection rate and oocyst density metrics were performed on individuals infectious at baseline, but are shown for all individuals in the appendix. Exploratory outcomes included mosquito infection metrics after gametocyte enrichment, for within and between treatment group comparison. Gametocyte infectivity was assessed as an exploratory outcome using logistic regression models adjusted for gametocyte density, wherein the shape of the relationship between gametocyte density and mosquito infection rate was estimated using fractional polynomials.

Adverse events were graded by the study clinician for severity (mild, moderate, or severe) and relatedness to study medication (unrelated or unlikely, possibly, probably, or definitely related). A reduction in haemoglobin concentration of 40% or more from baseline was categorised as a haematological severe adverse event. An external data safety and monitoring committee was assembled before the trial. Safety data were discussed after enrolment of 50 participants, and after the final follow-up visit of the last participant.

Statistical analysis

Sample size was informed by previous trials in the study setting using a mixed effects logistic regression model that accounted for correlation between mosquito observations from the same participant (14–16,18,19) and expecting a reduction in infectivity of 90% as previously detected for a single low-dose of primaquine (15,19). When including 20 participants per group and dissecting 50 mosquitoes per participant per timepoint, we calculated 92% empirical power to detect >85% reduction in infectivity with a one-tailed test with an $\alpha=0{\cdot}05$ level of significance. The sample size was not designed for between-group comparisons and any comparison of transmission-blocking effects between groups is secondary and limited to matched treatment groups. Mosquito infectivity was assessed at three levels: the percentage of participants infectious to any number of mosquitoes (i.e., infectious individuals), the proportion of mosquitoes infected with any number of oocysts (i.e., mosquito infection rate), and the mean number of oocysts in a sample of mosquitoes (i.e., oocyst density).

The proportion of infectious individuals and the prevalence of gametocytes and asexual stage parasites were compared between treatment groups using one-sided Fishers exact tests and within groups using McNemar tests. Mosquito infection rate was compared within-groups (relative to baseline) by Wilcoxon sign rank test (z-score) and between-groups by linear regression adjusted for baseline mosquito infection rate (t score, coefficient with 95% CI). For all direct membrane feeding assays (prior and post-gametocyte enrichment), the proportion of infectious individuals was compared between-group (direct membrane feeds prior to gametocyte concentration as reference) and within-group (relative to baseline) using one-sided Fishers exact tests. Haemoglobin levels were compared using paired t tests (t score) for within-group analyses and linear regression adjusted for baseline levels of each measure for between-group analyses (t score, coefficient with 95% CI). Percentage change from baseline was analysed using two-sample t tests for between group analysis and paired t tests (t score) for within-group analysis.

The proportion of gametocytes that were male was analysed for all values with total gametocyte densities of 0·2 gametocytes per μL or more, ensuring accurate quantification of sex ratios. Gametocyte circulation time was calculated to determine the mean number of days that a mature gametocyte circulates in the blood before clearance, using a deterministic compartmental model that assumes a constant rate of clearance and has a random effect to account for repeated measures on individuals, as described previously (23). Differences in circulation time between groups and between gametocyte sexes were estimated in the model. Statistical theory shows that these parameter estimates follow a t-distribution. Area under the curve (AUC) of gametocyte density per participant over time was calculated using the linear trapezoid method and was analysed by fitting linear regression models to the log10 adjusted AUC values, with adjustment for baseline gametocyte density (t score, coefficient with 95% CI). All other analyses of quantitative data were done using Wilcoxon sign rank tests (z-score) and Wilcoxon rank-sum tests (z-score). All comparisons were defined before study completion and analyses were not adjusted for multiple comparisons. For all analyses, the threshold for statistical significance was set at $p<0·05$.

Statistical analysis was conducted using Stata (version 17.0) and SAS (version 9.4). Data visualisation was performed using the R-based *ggplot2* package (R version 4.3.2) and Stata-based graphics (version 17.0). The trial is registered with ClinicalTrials.gov, NCT05550909.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

RESULTS

Between Oct 16 and Dec 28, 2022, 1249 individuals were screened for eligibility, 100 of whom were enrolled and randomly assigned to one of five treatment groups (n=20 per group; figure 1). Participant characteristics were similar between the study groups, although the proportion of

infectious participants at baseline was higher in the artesunate-amodiaquine group (table 1). The primary outcome was recorded on day 2 of follow-up, with 98 (98%) of 100 individuals completing this study visit (one in the artemether-lumefantrine-amodiaquine group and one in the artemether-lumefantrine-amodiaquine plus primaquine group did not complete this visit). 96 (96%) participants completed all visits to day 28 (two in the artemether-lumefantrine-amodiaquine, one in the artemether-lumefantrine-amodiaquine plus primaquine, and one in the artesunate-amodiaquine group did not complete all visits).

The median number of mosquitoes dissected per individual and per timepoint in a mosquito feeding experiment was 60 (IQR 54-64). Before treatment, 61 (61%) individuals were infectious to mosquitoes (17 individuals randomised to artesunate-amodiaquine group and 11 individuals in all other treatment groups), with a median of 7·3% (IQR 3·2–23·5) of mosquitoes becoming infected. The median number of oocysts per infected mosquito was 1·3 (IQR 1-3). At day 2 there was a significant within-person reduction in mosquito infection rate relative to baseline in all groups except for the artesunate-amodiaquine group. At the same timepoint, 2 (10%) of 20 participants in the artemether-lumefantrine group, 2 (11%) of 19 in the artemether-lumefantrine-amodiaquine group, and 15 (75%) of 20 in the artesunate-amodiaquine group infected any number of mosquitoes. No individuals remained infectious to mosquitoes at day 2 in the treatment groups with primaquine (table 2). At all timepoints after day 2, infectious participants were only found in the artesunate-amodiaquine group; 7 (35%) of 20 at day 7, 3 (16%) of 19 at day 14 and 1 (5%) of 19 at days 21 and 28 (figure 2 and supplementary table 2). Mosquito infection data for all individuals, regardless of baseline infectivity, is presented in supplementary table 3.

Gametocyte enrichment by MACS was performed on 95 blood samples collected pre-treatment and 94 blood samples that were collected on the second day after treatment initiation. Overall, gametocyte enrichment increased mosquito infection rates by a mean of 7·29% (supplementary figure 2). Comparing direct membrane feeds prior to enrichment with those after, the percentage

of infectious individuals increased in the enrichment-boosted group in all treatment groups (supplementary figure 2, supplementary table 4), whilst at day 2, the percentage of infectious individuals increased for all treatment groups except for artemether-lumefantrine-amodiaquine plus primaquine, in which all individuals remained non-infectious. In the artesunate-amodiaquine plus primaquine group, two initially non-infectious individuals infected 1-4 mosquitoes following gametocyte enrichment.

Asexual parasite densities, measured by microscopy, decreased rapidly after treatment initiation, with only one individual in both the artemether-lumefantrine and artesunate-amodiaquine groups retaining asexual stages at day 2, whereas in all other treatment groups, no asexual parasites were observed after treatment initiation (supplementary table 5). Gametocyte densities declined over time in all treatment groups, though much more rapidly in those who received primaquine, with median gametocyte densities of 13·25 gametocytes/μL (IQR 5·61-21), 6.89 (IQR 1·05-49·64) and 31.74 (7·27-61·59) at day 7 in the groups without primaquine compared to median densities of 0 (IQR 0-0) and 0·17 (IQR 0-0·87) in the groups with primaquine (supplementary figure 3, supplementary table 6). 18 (95%) of 20 participants treated with artesunate-amodiaquine were still gametocyte positive (> one gametocyte per 100 μL) at the final day of follow-up (day 28), whereas 16 (80%) of 20 in the artemether-lumefantrine and 13 (72%) of 18 in the artemether-lumefantrine-amodiaquine groups remained gametocyte positive at the same timepoint. Only one individual in both primaquine treatment groups had persisting gametocytes at day 28 (1 (6%) of 18 in the artemether-lumefantrine-amodiaquine plus primaquine and 1(5%) of 20 in the artesunate-amodiaquine plus primaquine groups). Total gametocyte circulation time was estimated at 6·1 days (95% CI 5·4-6·9) in the artemether-lumefantrine group, 6·0 days (5·2-6·8) in the artemether-lumefantrine-amodiaquine group and 2·6 (2·1-3·1) in the artemether-lumefantrine-amodiaquine plus primaquine group (supplementary table 7); the same measure was estimated at 7·9 days (6·7-9·3) and 3·3 days (2·8-3·8) in the artesunate-amodiaquine and artesunate-amodiaquine plus

primaquine respectively. Gametocyte sex ratios showed a male bias from day 2 after treatment start in the artemether-lumefantrine, artemether-lumefantrine-amodiaquine and artemether-lumefantrine-amodiaquine plus primaquine groups and from day 7 in the artesunate-amodiaquine plus primaquine group, with significantly more males in the artesunate-amodiaquine plus primaquine group (median proportion male gametocytes of 1 (IQR 0·88-1)) compared to the artesunate-amodiaquine alone group (median proportion male gametocytes of 0·59 (IQR 0·36-0·73), p=0·0002, supplementary tables 6 and 8, supplementary figure 4). Too few gametocytes persisted to make conclusions about absolute per-gametocyte infectivity (supplementary table 9).

There was a statistically significant within-group reduction in mean haemoglobin in all treatment groups at day 2 compared to baseline, however, by day 7 the haemoglobin levels had normalised in all group and were comparable to baseline (supplementary table 10, supplementary figure 5). The greatest reduction in mean haemoglobin density in any treatment group or timepoint was 5·58% (95% CI 3·64 to 7·54) in the artesunate-amodiaquine group at day 2. The were no statistically significant decreases in percent change in haemoglobin compared to baseline between treatment groups at any timepoint. The greatest reduction in haemoglobin density in any individual was 25·2% (from 14·3 g/dL at baseline to 10·7 g/dL at day 21 in an individual in the artemether-lumefantrine group). The lowest observed haemoglobin density in any individual and timepoint was 9 g/dL at baseline in an individual in the artesunate-amodiaquine group. No severe laboratory abnormalities occurred; all possibly drug related laboratory abnormalities normalised on the subsequent visit (supplementary table 11).

Overall, 85 (85%) of 100 participants had a total of 262 adverse events during follow-up, of which 181 (69%) were categorised as mild and 81 (30·1%) as moderate (table 3, supplementary table 12). No severe adverse events or serious adverse events occurred during the trial. The most common treatment-related adverse event was mild or moderate headache, which occurred in 43 (43%) participants (artemether-lumefantrine n=6; artemether-lumefantrine plus amodiaquine n=9;

artemether-lumefantrine plus amodiaquine plus primaquine n=11; artesunate-amodiaquine n=8; and artesunate-amodiaquine plus primaquine n=9). There were no significant differences between treatment groups in the proportion of participants who experienced any mild adverse event (p=0·612), mild (p=0·178) or moderate (p=0·055) treatment-related adverse events at any study visit.

DISCUSSION

To our knowledge, this is the first clinical trial designed to test the gametocytocidal and transmission-blocking properties of the triple artemisinin-based combination therapy artemether-lumefantrine-amodiaquine with and without primaquine and of artesunate-amodiaquine with and without primaquine. Within 48 hours of treatment, transmission was greatly reduced in the artemether-lumefantrine and artemether-lumefantrine-amodiaquine groups, and completely annulled in both treatment groups with primaquine. In contrast, transmission to mosquitoes continued in a minority of individuals until day 28 after treatment with artesunate-amodiaquine alone.

Calls for malaria eradication and the emergence and spread of drug resistance have reinforced the need to assess the effects of antimalarial drugs on gametocytes and their infectiousness (17,24). The addition of a second partner drug to ACTs could significantly delay the emergence and spread of artemisinin resistance and treatment failure. Lumefantrine and amodiaquine provide mutual protection against resistance development, and deployment of the TACT artemether-lumefantrine-amodiaquine is expected to extend the useful lives of artemisinin derivatives and both partner drugs (6). This study was not designed to investigate the clinical efficacy of TACT, nor had the study site recorded any partial artemisinin resistance at the time of the study. We found that both treatments with artemether-lumefantrine; alone and with amodiaquine, greatly reduced transmission by day 2 after treatment. The addition of a single low-dose primaquine only marginally enhanced this transmission-blocking effect. Gametocyte densities minimally differed

between artemether-lumefantrine and artemether-lumefantrine-amodiaquine, but we observed a near complete clearance of gametocytes by day 7 in the group with an added single low-dose of primaquine. These observations align with recent data indicating that artemether-lumefantrine has potent transmission-blocking effects (14).

Artesunate-amodiaquine is the first-line treatment for uncomplicated *P. falciparum* malaria in many countries, yet its effect on mature gametocytes and transmission were unclear. In line with previous studies (10,11), we found that gametocyte carriage persisted in all individuals treated with artesunate–amodiaquine until the end of follow-up (day 28) and three (16%) of 19 individuals (three (18%) of 17 individuals infectious at baseline) were still infectious to mosquitoes 14 days after initiation of treatment, with one individual remaining infectious until the end of follow-up (day 28). Moreover, one individual was infectious on day 2 but not at baseline, and one individual was infectious on day 7, but not at baseline or day 2. Whilst there is an inherent stochastic element in transmission to mosquitoes and observing no infected mosquitoes at one timepoint therefore does not rule out (low levels of) infectivity, this pattern suggests a possible role for differences in the drug susceptibility or exposure at different gametocyte developmental stages i.e. immature gametocytes may be released from sequestration in the bone marrow or spleen after treatment. The addition of a single low-dose of primaquine resulted in an enhanced clearance of gametocytes and achieved in a near-total reduction of transmission potential within 48 hours.

The exploratory assay of magnetic gametocyte enrichment showed that the percentage of infectious individuals increased at day 2 after treatment start in all groups except for the artemether-lumefantrine-amodiaquine plus primaquine group, although this increase was non-significant. This suggests that in all ACT-only groups, the initial lack of infectivity is due to low gametocyte densities or sex ratio distortion rather than the sterilisation of either gametocyte sex. In the primaquine groups, the enrichment results were contradictory: the addition of primaquine to artemether-lumefantrine-amodiaquine blocked all transmission at day 2 even after gametocyte

enrichment, suggestive of primaquine sterilising gametocytes before reducing their numbers significantly (25). Conversely, in the artesunate-amodiaquine plus primaquine group, two individuals who were not infectious in standard feeding assays became infectious after gametocyte concentration. Although these are very small sample sizes and other factors such as variations in primaquine concentration have not been measured, this may indicate varying primaquine efficacy with different artemisinin therapies. Of relevance is that in the process of gametocyte enrichment, human plasma is replaced by malaria-naïve serum, thereby removing potentially transmission-modulating antibodies or drugs that might affect parasite development upon mosquito ingestion.

The emergence of transmission after treatment that was observed in the artesunate-amodiaquine plus primaquine group treatment group in standard feeding assays, has been seen previously after artemisinin (14) and non-artemisinin treatments (14,26). We hypothesise that artesunate-amodiaquine may have lower efficacy on or exposure to immature, developing gametocytes than artemether-lumefantrine, and that those gametocytes released from sequestration after treatment would be unaffected by primaquine's active metabolites which only circulate for a matter of hours. In addition, it is important to consider that, although artemether-lumefantrine-amodiaquine without primaquine prevents nearly all mosquito infections within 48 hours, gametocytes in *PfKelch13* mutant infections may preferentially survive artemisinin exposure and infect mosquitoes (27). Our data supports the suggestion from the WHO malaria policy and advisory group to expand the focus on reducing parasite transmission with a single low-dose of primaquine in areas where partial artemisinin resistance has been detected (28).

Previous studies reported a higher frequency of side effects with the combination of partner drugs lumefantrine and amodiaquine, compared to lumefantrine alone (7,8), including vomiting, nausea and vertigo, and mild bradycardia. We did not see an increase in vomiting or nausea, and only a slight increase in vertigo related to the drug treatment from one adverse event in the artemether-lumefantrine group, to three and six in the artemether-lumefantrine-amodiaquine groups with and

without primaquine, respectively. Overall, all drug regimens were well tolerated, and no instances of cardiac adverse events or severe side effects were reported.

Our study had some limitations. For instance, we assessed many secondary outcomes, and their interpretations therefore require caution due to issues of multiple testing. In addition, we recruited individuals carrying high densities of gametocytes, consistent with previous studies with similar outcomes and at the same study site (15,16,18,19). This allowed us to collect robust data on post-treatment transmissibility but does not represent the average gametocyte-infected individual. Consequently, our estimates of persistence of transmissible gametocytes primarily demonstrate the impact of antimalarial drugs on the transmission potential stemming from a comparatively small subset of highly infectious individuals; though these would be the most important group for the drug regimens to work in. Lastly, it could be argued that the public health significance of these study findings needs to be validated through community trials focused on transmission outcomes. Mass administrations of primaquine or other gametocytocidal compounds (for example, alongside seasonal malaria chemoprophylaxis) may be necessary to achieve reductions in transmission at the community level (29). Conversely, given the negligible cost of primaquine, absence of safety concerns and lack of obvious alternative, there is a compelling argument to add primaquine to slow the transmission of drug-resistant parasites.

In conclusion, our findings show that artemether-lumefantrine-amodiaquine can prevent nearly all mosquito infections but reveal considerable post-treatment transmission after artesunate-amodiaquine. The addition of a single low-dose of primaquine is a safe and effective addition to artemether-lumefantrine-amodiaquine and artesunate-amodiaquine for blocking *P. falciparum* transmission. Enriching the gametocyte content of mosquito blood meals in transmission assays shows that viable male and female gametocytes can persist at densities too low to result in mosquito infection at physiological concentrations, after treatment in the artemether-lumefantrine, artemether-lumefantrine-amodiaquine, artesunate-amodiaquine and artesunate-amodiaquine plus

primaquine groups, but not in the artemether-lumefantrine-amodiaquine plus primaquine group. This strengthens the argument for the addition of a single-low dose of primaquine to block the transmission of artemisinin resistant gametocytes.

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Contributors

LNV, WS, AM, MJS, ADo, CD, and ADi conceived the study and developed the study protocol. AM, WS, LNV, MJS, KS, YSi, SMN, OMD, RSD, MD, SOM, AY, AS, SK, SS, ADe, YD, SFT, CD, and ADi implemented the trial. KT performed molecular analyses. LNV, AM and WS verified the raw data. LNV and WS analysed the data. LNV, AM, WS, MJS, CD, and ADi wrote the first draft of the manuscript. All authors had full access to all the data in the study and accept responsibility for the decision to submit for publication. All authors read and approved the final manuscript.

Declaration of interests

We declare no competing interests.

Data sharing

Anonymised data reported in the manuscript will be made available to investigators who provide a methodologically sound proposal to the corresponding author. The study protocol is available in the supplementary information. The data from this trial will be made accessible on the Clinical Epidemiology Database Resources website (https://clinepidb.org).

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FIGURES

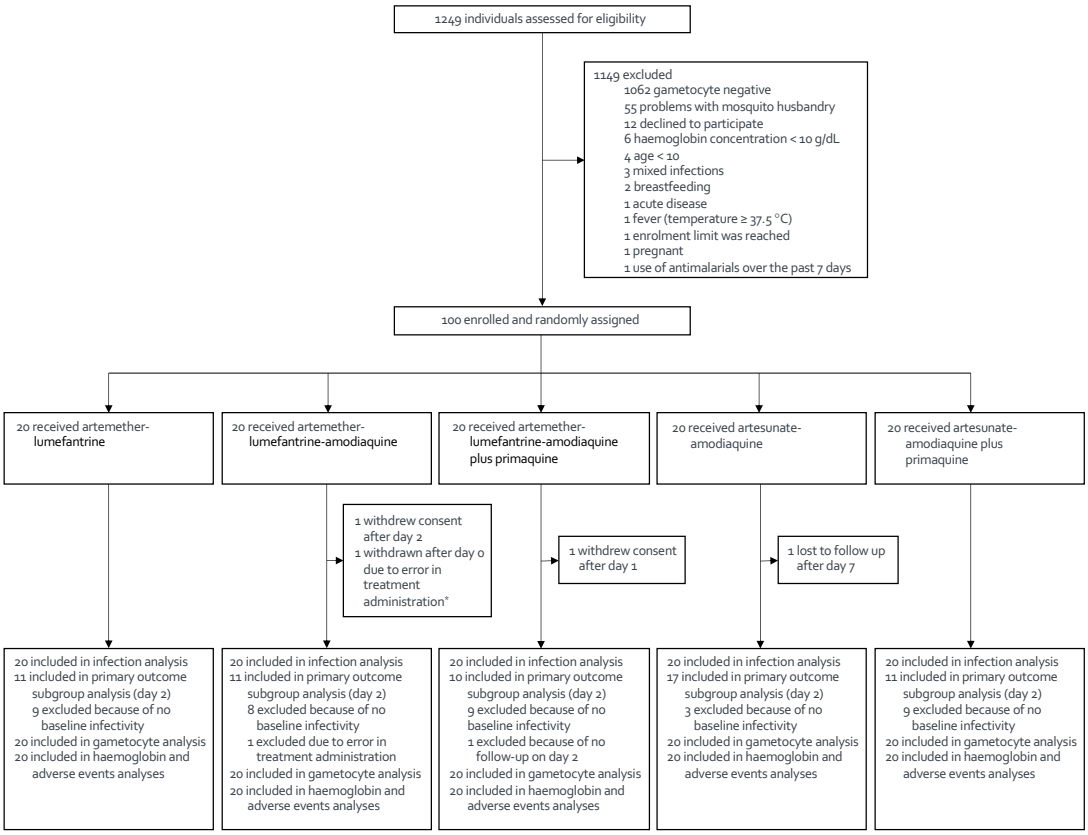

Figure 1. Trial profile. 96 of 100 (96%) participants completed all visits to day 28 (two in the artemether-lumefantrine-amodiaquine, one in the artemether-lumefantrine-amodiaquine plus primaquine, and one in the artesunate-amodiaquine plus primaquine group did not complete all visits). *One participant randomised to the artemether-lumefantrine-amodiaquine group was given erroneous treatment on day 1. All measures following this error were removed from analysis.

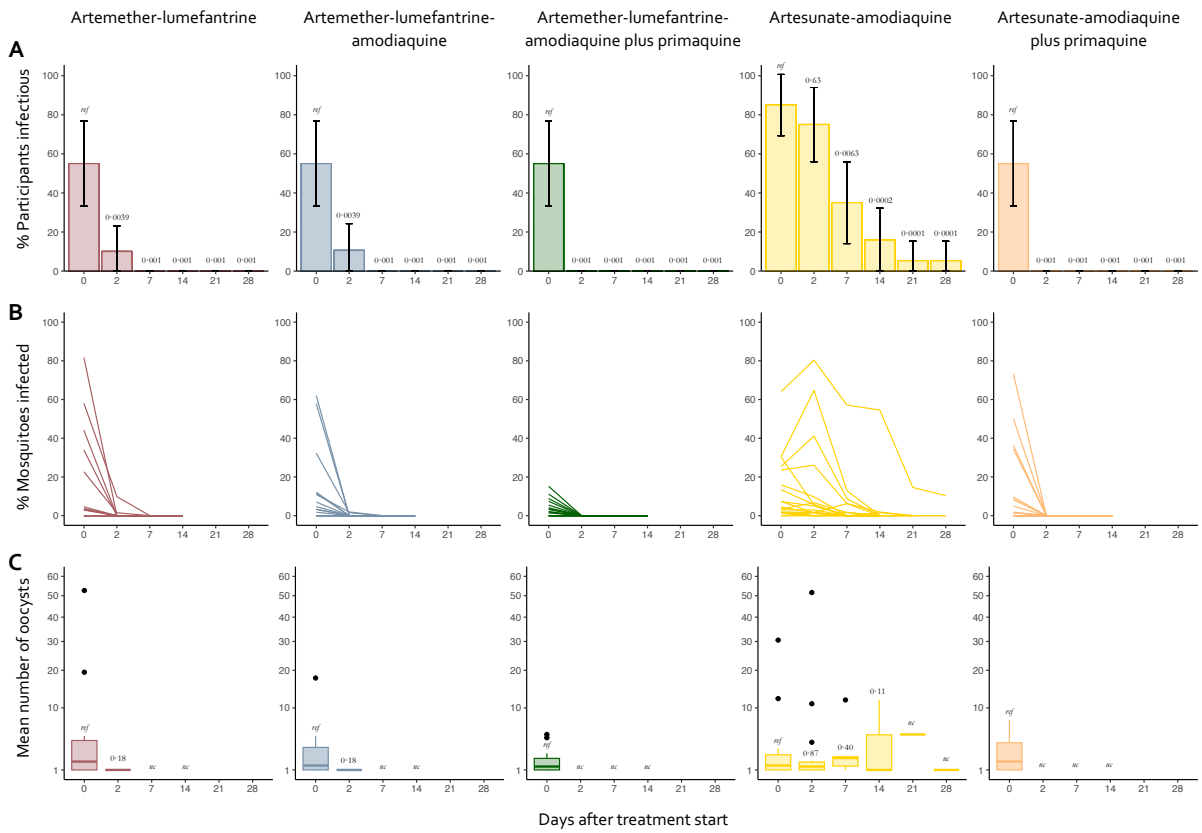

Figure 2. Participant infectivity and proportion of mosquitoes infected in direct membrane feeding assays. (A) Participant infectivity. Error bars show 95% CI. The denominator for participants infectious is the total number of participants still enrolled at the given timepoint, rather than the number tested for infectivity at that timepoint. Infectivity assays were discontinued after 14 days when a participant did not infect any mosquitoes at two subsequent timepoints and were thereafter considered non-infectious. Mosquito feeding assays at days 21 and 28 were only conducted in the artesunate-amodiaquine group (seven at day 21 and three at day 28). The prevalence infectious individuals was compared within treatment groups using McNemar tests. (B) Mosquito infection rate. Each line represents one individual. Statistical analyses are shown in Supplementary Table 2. (C) Oocyst density. Box plots show the median (central line), IQR (box limits), upper and lower quartiles plus 1·5 × IQR (whiskers), and outliers for mean oocyst densities in infected mosquitoes within each participant. The mean number of oocysts was compared to baseline within treatment groups using the Wilcoxon sign-rank test. nc = not calculable. Ref=reference.

TABLES

Table 1. Characteristics of the study participants prior to treatment

	Artemether-lumefantrine (n=20)	**Artemether-lumefantrine-amodiaquine (n=20)**	**Artemether-lumefantrine-amodiaquine plus primaquine (n=20)**	**Artesunate-amodiaquine (n=20)**	**Artesunate-amodiaquine plus primaquine (n=20)**
Age in years (median [IQR])	13·0 (11·0-18·5)	13·0 (11·5-28·0)	13·0 (11·0-15·0)	12·0 (10·0-16·0)	12·5 (11·5-20·0)
Female participants	11 (55%)	10 (50%)	12 (60%)	11 (55%)	7 (35%)
Male participants	9 (45%)	10 (50%)	8 (40%)	9 (45%)	13 (65%)
Haemoglobin (g/dL)	11·8 (11·5-13·8)	12·1 (11·3-12·6)	11·8 (11·1-12·1)	11·4 (10·9-12·3)	11·7 (11·2-12·8)
Gametocyte prevalence (n/% positive)	20 (100%)	20 (100%)	20 (100%)	20 (100%)	20 (100%)
Gametocyte density (parasites/μL)	31·0 (19·9-92·7)	28·6 (11·5-130·5)	42·3 (11·8-97·0)	52·5 (33·6-129·0)	24·8 (10·7-115·2)
Asexual parasite prevalence (n/% positive)	10 (50%)	6 (30%)	5 (25%)	8 (40%)	8 (40%)
Asexual parasite density (parasites/μL)	37·9 (0·0-300·0)	0·0 (0·0-79·8)	0·0 (0·0-37·6)	0·0 (0·0-1654·9)	0·0 (0·0-720·0)

Age, haemoglobin concentration and parasite densities are given as median and interquartile range [IQR]. Gametocyte prevalence and density were calculated from reverse transcriptase quantitative PCR targeting CCP4/PfMGET mRNA (gametocytes). Asexual parasite prevalence and density were assessed by thick film microscopy.

Table 2. Median percent reduction in mosquito infection rate for individuals infectious before treatment

	Infectious individuals %	Median mosquito infection rate % (IQR)	Median % reduction in mosquito infection rate (IQR)	p-value¥	p-value†
Baseline					
Artemether-lumefantrine	55% (11/20)	4·5% (3·3-44·1)	·	*ref*	·
Artemether-lumefantrine-amodiaquine	55% (11/20)	10·9% (3·3-32·3)	·	*ref*	·
Artemether-lumefantrine-amodiaquine plus primaquine	55% (11/20)	4·1% (2·1-8·8)	·	*ref*	·
Artesunate-amodiaquine	85% (17/20)	7·3% (1·9-23·5)	·	*ref*	·
Artesunate-amodiaquine plus primaquine	55% (11/20)	9·3% (1·8-36·2)	·	*ref*	·
Day 2					
Artemether-lumefantrine	10% (2/20)	0% (0-0)	100% (100-100)	0·0018	*ref*
Artemether-lumefantrine-amodiaquine	10·5% (2/19)	0% (0-0)	100% (100-100)	0·0018	1·0000
Artemether-lumefantrine-amodiaquine plus primaquine	0% (0/19)	0% (0-0)	100% (100-100)	0·0009	0·1478
Artesunate-amodiaquine	75% (15/20)	5% (1·5-9·7)	31·7% (-10·87-79·39)	0·1927	*ref*
Artesunate-amodiaquine plus primaquine	0% (0/20)	0% (0-0)	100% (100-100)	0·0009	0·0001

Individuals were classed as infectious if direct membrane feeding assays resulted in at least one mosquito with any number of oocysts. All values are for individuals who were infectious to mosquitoes before treatment. The range of median percentage reduction in mosquito infection rate between pre-treatment and 2 days post-treatment was 83·1-100 in the artemether-lumefantrine group, 82·4-100 in the artemether-lumefantrine-amodiaquine group, 100-100 in the artemether-lumefantrine-amodiaquine plus primaquine group, -112.6-100 in the artesunate-amodiaquine and 100-100 in the artesunate-amodiaquine plus primaquine group. ¥Within-group comparison of median reduction in mosquito infection rate by Wilcoxon signed rank test (day 0 as reference, primary outcome). †Between-group comparison of median reduction in mosquito infection rate (i.e., artemether–lumefantrine vs artemether–lumefantrine-amodiaquine and artemether–lumefantrine-amodiaquine plus primaquine, artesunate-amodiaquine vs artesunate-amodiaquine plus primaquine) by Wilcoxon rank-sum test. Full details of mosquito feeding assay outcomes are in supplementary table 2.

Table 3. Frequency of adverse events

Description	Total (n=100)	Artemether-lumefantrine (n=20)	Artemether-lumefantrine-amodiaquine (n=20)	Artemether-lumefantrine-amodiaquine plus primaquine (n=20)	Artesunate-amodiaquine (n=20)	Artesunate-amodiaquine plus primaquine (n=20)
All	85% (85/100)	90% (18/20)	75% (15/20)	90% (18/20)	80% (16/20)	90% (18/20)
P-value	0·612*	*ref*	0·407**	1·000**	*ref*	0·661**
Mild related AE	47% (47/100)	60% (12/20)	35% (7/20)	40% (8/20)	35% (7/20)	65% (13/20)
P-value	0·178*	*ref*	0·205**	0·343**	*ref*	0·113**
Moderate related AE	26% (26/100)	10% (2/20)	35% (7/20)	35% (7/20)	40% (8/20)	10% (2/20)
P-value	0·055*	*ref*	0·065127**	0·127**	*ref*	0·065**
Severe related AE	0% (0/20)	0% (0/20)	0% (0/20)	0% (0/20)	0% (0/20)	0% (0/20)
P-value	*nc*	*ref*	*nc*	*nc*	*ref*	*nc*

Number of participants per group who experienced adverse events. Classification as 'related to treatment' was defined as probably, possibly or definitely related to treatment, as described in the methods. If there were multiple episodes per participant, the highest grade and most likely related to treatment is presented in this table. P-values are from Fisher's exact tests for differences in proportion of individuals with an AE between all groups* or between AL-AQ or AL-AQ+PQ groups and the AL reference group and AS-AQ+PQ group and the AS-AQ reference group**. ref = reference group, nc = not calculable. AL = artemether-lumefantrine; AL-AQ = artemether-lumefantrine-amodiaquine; AL-AQ+PQ = artemether-lumefantrine-amodiaquine plus primaquine; AS-AQ = artesunate-amodiaquine; AS-AQ+PQ = artesunate-amodiaquine plus primaquine

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Research in context

Evidence before the study

The WHO recommends adding a single-low dose of the gametocytocide primaquine to artemisinin-based combination therapy (ACT) in areas of low transmission or with artemisinin-resistant *P. falciparum*, to facilitate transmission reduction. Primaquine has the potential to further slow the spread of antimalarial drug resistance by blocking the escape of (drug-resistant) parasites from treated individuals. For transmission reduction, the added benefits of primaquine in combination with TACT or artesunate-amodiaquine are currently unknown.

We searched PubMed on 13 November 2023, with no publication date or language restrictions, for studies assessing the post-treatment transmission of artemether-lumefantrine-amodiaquine with the following search terms: ([Artemether-lumefantrine] OR [Coartem] OR [Riamet]) AND ([Amodiaquine] OR [Flavoquine] OR [Triple ACT] OR [TACT] OR [Triple Artemisinin-based Combination therapy] OR [Triple therapy]) AND ([Plasmodium falciparum]) AND ([Gametocytocidal] OR [Gametocytes]) AND ([Transmission]). The following search terms were added to search for studies using artemether-lumefantrine-amodiaquine plus primaquine: AND ([Primaquine] OR [Jasoprim] OR [Malirid] OR [Neo-Quipenyl] OR [Pimaquin] OR [Primachina] OR [Primacin] OR [Primaquina] OR [Remaquin]) A second search was done for studies assessing the post-treatment transmission of artesunate-amodiaquine, with search terms: ([Artesunate-amodiaquine] OR [Camoquin] OR [Coarsucam] OR [Artesunat Plus] OR [TesquinCare]) AND ([Plasmodium falciparum]) AND ([Gametocytocidal] OR [Gametocytes]) AND ([Transmission]). The aforementioned search terms for primaquine were added to search for studies using artesunate-amodiaquine plus primaquine.

The initial search (without the primaquine search terms) yielded 9 studies: seven did not assess the combination of artemether-lumefantrine-amodiaquine and two were non-clinical trials. After addition of primaquine search terms, only one study was found, which assessed the effectiveness and post-treatment gametocyte density of four ACTs with or without primaquine, however, the combination of artemether-lumefantrine-amodiaquine was not tested. The second search found 26 studies: seven that assessed safety and gametocyte carriage after artesunate-amodiaquine treatment but did not include mosquito feeding assays, 15 that only tested artesunate and amodiaquine separately or in combination with other drugs, and four that were non-clinical trials. The duration of gametocyte carriage, determined by microscopy, observed in these seven trials post-treatment with artesunate-amodiaquine ranged from 21 days to persisting past 28 days after

treatment start. Narrowing this search to include studies assessing artesunate-amodiaquine in combination with primaquine identified only two relevant trials, both of which assessed safety and efficacy against gametocytes determined by microscopy, but neither performed mosquito feeding assays. Both studies found that after artesunate-amodiaquine plus primaquine treatment, gametocyte densities decreased to reach zero within 21-28 days.

Added value of this study

To formulate precise treatment guidelines, understanding the impact of antimalarial treatments on transmission to mosquitoes is essential. Sole reliance on gametocyte presence for assessing post-treatment transmission risk is insufficient, as viable gametocytes at densities below microscopic detection limits may be transmitted after treatment. Moreover, some antimalarial medications may render gametocytes non-infectious before they are cleared from the bloodstream. Therefore, evaluations necessitate the use of mosquito-feeding assays.

Our study provides valuable data on the extent of transmission after artemether-lumefantrine, artemether-lumefantrine-amodiaquine, and artesunate-amodiaquine in a highly infectious population sample. This is the first assessment of artemether-lumefantrine-amodiaquine with and without single low-dose primaquine on gametocyte densities and transmission, using mosquito feeding assays. We show that artemether-lumefantrine-amodiaquine has potent transmission-blocking activity, even in the absence of primaquine. However, gametocyte densities declined more rapidly when primaquine is added and this combination blocks transmission even after gametocyte enrichment of the mosquito blood meal. We also provide the first evidence of continued transmission up until day 28 after artesunate-amodiaquine. Notably, we provide the first data demonstrating that the addition of a single-low dose of primaquine to artesunate-amodiaquine completely annules transmission by day 2 post-treatment. Lastly, we concentrated gametocytes in the mosquito blood meal to gain insights into the transmission-blocking mechanisms of antimalarial drugs.

Implications of all the available evidence

Our study findings are consistent with previous evidence that artemether-lumefantrine has potent transmission-blocking activity. We found that the addition of amodiaquine to artemether-lumefantrine did not influence gametocyte densities or transmission. However, the addition of a single-low dose primaquine to the TACT achieved a near-complete clearance of gametocytes by day 7, which is a more rapid clearance than previously observed after artemether-lumefantrine plus

primaquine. In line with this, gametocyte enrichment enhanced transmission in the artemether-lumefantrine and artemether-lumefantrine-amodiaquine groups at day 2, but not in the group with added primaquine. This provides evidence that all gametocytes and transmission are completely annulled post-artemether-lumefantrine-amodiaquine plus primaquine treatment.

In addition, this study's findings contribute to the expanding body of research supporting the incorporation of a single low-dose primaquine regimen with ACT therapy as an immediate measure to halt the further transmission of *Plasmodium falciparum* malaria. The available data on the effects of artesunate-amodiaquine alone provide a resource to policy makers considering treatment options, and our findings on the efficacy of artesunate-amodiaquine with a single-low does primaquine support the WHO recommendation of combining ACTs with single low-dose primaquine to prevent transmission in areas aiming to eliminate malaria or fighting the spread of antimalarial drug resistance.

APPENDIX 1

Supplementary Information 1. Antimalarial treatment dosing

1. Artemether-Lumefantrine (AL)

Participants in the AL, AL-AQ or AL-AQ+PQ groups were treated with standard doses of AL (Guilin Pharmaceutical, Shanghai, China) from day 0-2. Tablets containing 20 mg artemether and 120 mg lumefantrine will be administered per manufacturer guidelines as shown below:

Bodyweight (kg)	**20/120 mg tablet**		
	D0	**D1**	**D2**
5 to < 15 kg	1 disp tab x 2	1 disp tab x 2	1 disp tab x 2
15 to < 25 kg	2 disp tab x 2	2 disp tab x 2	2 disp tab x 2
25 to < 35 kg	3 tab x 2	3 tab x 2	3 tab x 2
≥ 35 kg	4 tab x 2	4 tab x 2	4 tab x 2

2. Primaquine (PQ)

Participants in the AL-AQ+PQ and AS-AQ+PQ groups received PQ (ACE Pharmaceuticals, Zeewolde, The Netherlands) at a single low dose of 0.25mg/kg as is currently recommended by the World Health Organization. The single dose of PQ was given on day 0 together with the first dose of AL or AS-AQ, administered in an aqueous solution, according to a standard operating procedure (SOP) provided Sanofi as previously done at the study site when PQ was combined with DP, PA or AL (1–3).

3. Amodiaquine (AQ)

Participants in the AL-AQ and AL-AQ+PQ groups were given AQ as tablets of 153 mg (Guilin Pharmaceutical, Shanghai, China). The weight-based treatment schedule as shown below aims for a dosage of approximately 10 mg (7.7-15.3mg)/kg/day, given once or twice daily (together with artemether–lumefantrine) for three days:

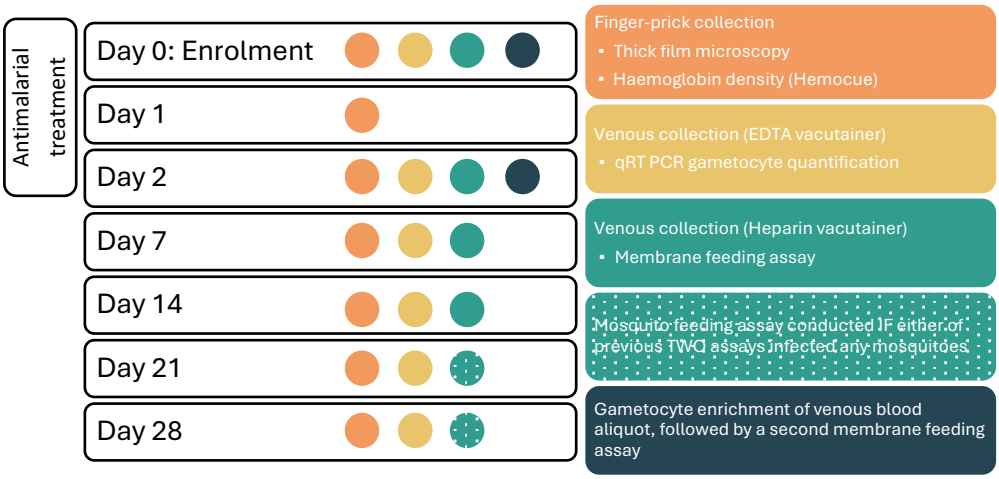
Bodyweight (kg)	**153 mg tablet**					
	D0 (0hr)	**D0 (8hr)**	**D1 (24hr)**	**D1 36hr)**	**D2 (48hr)**	**D2 (60hr)**
10 to 19.9	1 tab	0 tab	1 tab	0 tab	1 tab	0 tab
20 to 29.9	1 tab	1 tab	1 tab	1 tab	1 tab	1 tab
30 to 54.9	2 tab	1 tab	2 tab	1 tab	2 tab	1 tab
55 to 80	3 tab	2 tab	3 tab	2 tab	3 tab	2 tab

4. Artesunate-Amodiaquine (AS-AQ)

Participants in the AS-AQ and AS-AQ+PQ groups received fixed-dose combination tablets containing 50mg/135 mg or 100mg/270 mg of artesunate/amodiaquine (Guilin Pharmaceutical, Shanghai, China). Tablets were administered according to manufacturer guidelines, as shown below:

Weight	**Tablets**	**D0**	**D1**	**D2**
9 to < 18 kg	50 mg AS/135 mg AQ base	1 tab	1 tab	1 tab
18 to < 36 kg	100 mg AS/270 mg AQ base blister pack of 3 tab	1 tab	1 tab	1 tab
≥ 36 kg	100 mg AS/270 mg AQ base blister pack of 6 tab	2 tab	2 tab	2 tab

Supplementary Figure 1. Schematic representation of sample collection and analysis pipeline



Supplementary Table 1. Primer sequences and qPCR conditions for PfMGET CCp4 assay

PfMGET Primer/Probe Sequences

Primers	Sequence
Primer-FW (5'-3')	CGGTCCAAATATAAAAATCCTG
Primer-RV (5'-3')	TGTG TAACG TATG ATTCATTTTC
Probe (5'-3')	FAM-CAGCTCCAG CATTAAAACAC-BHQ1

CCp4 Primer/Probe Sequences

Primers	Sequence
Primer-FW (5'-3')	CACATGAATATGAGAATAAAATTG
Primer-RV (5'-3')	TAGGCGAACATGTGGAAAG
Probe (5'-3')	TexasRed-AGCAACAACGGTATGTGCCTTAAAACG-BHQ2

Male and female gametocyte quantification was performed as described previously, using a multiplex RT-qPCR assay (4). Assays were run using commercial RT-qPCR mixes (Luna® Universal Probe One-Step RT-qPCR Kit, New England Biolabs, Ipswich, MA, USA). FW = Forward primer. RV = Reverse primer.

Supplementary Table 2. Infectivity to mosquitoes for individuals infectious at baseline

Day of follow-up	Treatment arm	Infectious individuals % (n/N) *	p-value¥	p-value†	Mosquito infection rate Median % (IQR) **	p-value¥	p-value†	Oocyst density Median (IQR) ***	p-value¥	p-value†
Day 0	**Overall**	61% (61/100)	·	·	7·3% (3·2-23.5)	·	·	1·3 (1-3·0)	·	·
	AL	55% (11/20)	*ref*	*ref*	4·5% (3·3-44·1)	*ref*	*ref*	1·7 (1-4·8)	*ref*	*ref*
	AL-AQ	55% (11/20)	*ref*	1·000	10·9% (3·3-32·3)	*ref*	0·646	1·3 (1-3·5)	*ref*	0·756
	AL-AQ+PQ	55% (11/20)	*ref*	1·000	4·1% (2·1-8·8)	*ref*	0·045	1·2 (1-2·5)	*ref*	0·535
	AS-AQ	85% (17/20)	*ref*	*ref*	7·3% (1·9-23·5)	*ref*	*ref*	1·3 (1-2·3)	*ref*	*ref*
	AS-AQ+PQ	55% (11/20)	*ref*	0·082	9·3% (1·8-36·2)	*ref*	0·370	1·7 (1-3·9)	*ref*	0·716
Day 2	**AL**	10% (2/20)	0·0039	*ref*	0% (0-0)	0·0033	*ref*	1·0 (1-1)	0·1797	*ref*
	AL-AQ	10·5% (2/19)	0·0039	0·678	0% (0-0)	0·0033	0·523	1·0 (1-1)	0·1797	*nc*
	AL-AQ+PQ	0% (0/19)	0·001	0·256	0% (0-0)	0·0033	0·788	*nc*	*nc*	*nc*
	AS-AQ	75% (15/20)	0·625	*ref*	5% (1·5-9·7)	0·6192	*ref*	1·2 (1-1·8)	0·8744	*ref*
	AS-AQ+PQ	0% (0/20)	0·001	<0·0001	0% (0-0)	0·0033	0·006	*nc*	*nc*	*nc*
Day 7	**AL**	0% (0/20)	0·001	*ref*	0% (0-0)	0·0033	*ref*	*nc*	*nc*	*ref*
	AL-AQ	0% (0/18)	0·001	*nc*	0% (0-0)	0·0033	*nc*	*nc*	*nc*	*nc*
	AL-AQ+PQ	0% (0/19)	0·001	*nc*	0% (0-0)	0·0033	*nc*	*nc*	*nc*	*nc*
	AS-AQ	35% (7/20)	0·0063	*ref*	0% (0-6·2)	0·001	*ref*	2·0 (1·6-2·2)	0·3991	*ref*
	AS-AQ+PQ	0% (0/20)	0·001	0·004	0% (0-0)	0·0033	0·045	*nc*	*nc*	*nc*
Day 14	**AL**	0% (0/20)	0·001	*ref*	0% (0-0)	0·0033	*ref*	*nc*	*nc*	*ref*
	AL-AQ	0% (0/18)	0·001	*nc*	0% (0-0)	0·0033	*nc*	*nc*	*nc*	*nc*
	AL-AQ+PQ	0% (0/19)	0·001	*nc*	0% (0-0)	0·0033	*nc*	*nc*	*nc*	*nc*
	AS-AQ	15·8% (3/19)	0·0002	*ref*	0% (0-0)	0·0004	*ref*	1 (1-11·9)	0·1088	*ref*
	AS-AQ+PQ	0% (0/20)	0·001	0·106	0% (0-0)	0·0033	0·146	*nc*	*nc*	*nc*
Day 21	**AL**	0% (0/20)	0·001	*ref*	0% (0-0)	·	*ref*	·	·	*ref*
	AL-AQ	0% (0/18)	0·001	*nc*	0% (0-0)	·	·	·	·	·
	AL-AQ+PQ	0% (0/18)	0·001	*nc*	0% (0-0)	·	·	·	·	·
	AS-AQ	5·3% (1/19)	0·0001	*ref*	0% (0-0)	0·0277	*ref*	5 (5-5)	*nc*	*ref*
	AS-AQ+PQ	0% (0/20)	0·001	*nc*	0% (0-0)	·	·	·	·	·
Day 28	**AL**	0% (0/20)	0·001	*ref*	0% (0-0)	·	*ref*	·	·	*ref*
	AL-AQ	0% (0/18)	0·001	*nc*	0% (0-0)	·	·	·	·	·
	AL-AQ+PQ	0% (0/19)	0·001	*nc*	0% (0-0)	·	·	·	·	·
	AS-AQ	5·3% (1/19)	0·0001	*ref*	0% (0-10·3)	0·1088	*ref*	1 (1-1)	*nc*	*ref*
	AS-AQ+PQ	0% (0/20)	0·001	*nc*	0% (0-0)	·	·	·	·	·

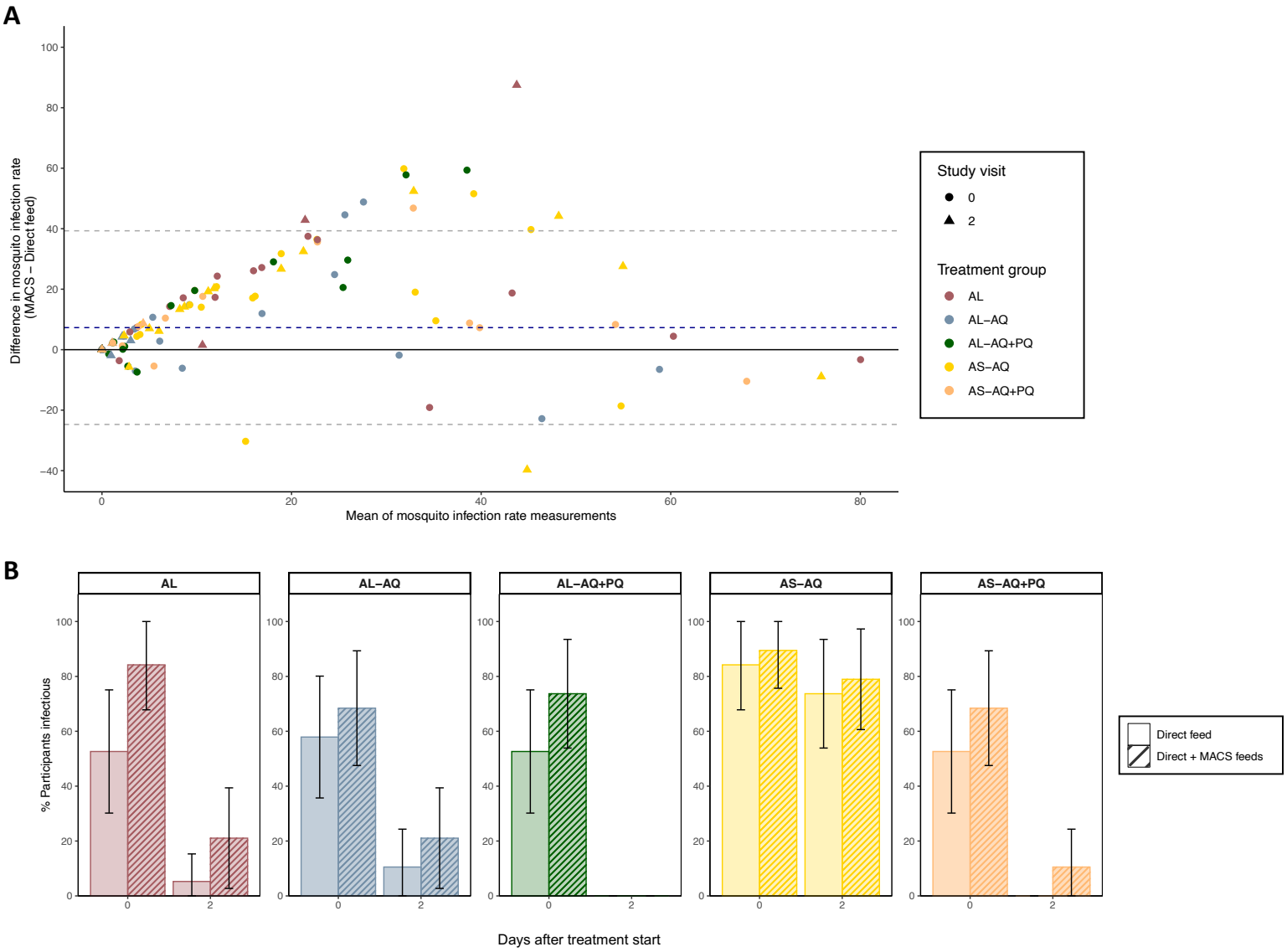
*Percentage of infectious individuals. Individuals were classed as infectious if direct membrane feeding assays (DMFA) resulted in at least one mosquito with any number of oocysts. Mosquito infection measures (mosquito infection rate and oocyst density) are presented for all participants who were infectious at baseline, and oocyst densities are from all infected mosquitoes **Mosquito infection rate is the median percentage of mosquitoes infected by each participant, where for each participant mosquito infection rate the number of mosquitoes infected as a percentage of all mosquitoes surviving to dissection. Mosquito infection rate was compared within-groups (relative to baseline) by Wilcoxon sign rank test (z-score) and between-groups by linear regression adjusted for baseline mosquito infection rate (t score, coefficient with 95% CI). ***The average oocyst density for each participant was calculated as the mean number of oocysts in infected mosquitoes (i.e., with at least one oocyst). The value presented in the table is the median of all individuals' average oocyst intensities (a composite figure of all oocysts/all infected mosquitoes is not statistically valid). P-value¥ = Within group comparison. P-value† = Between group comparison (artemether–lumefantrine vs artemether–lumefantrine-amodiaquine and artemether–lumefantrine-amodiaquine plus primaquine, artesunate-amodiaquine vs artesunate-amodiaquine plus primaquine). nc = not calculable, no positive observations. · = not tested, ref = reference group. AL = artemether-lumefantrine; AL-AQ = artemether-lumefantrine-amodiaquine; AL-AQ+PQ = artemether-lumefantrine-amodiaquine plus primaquine; AS-AQ = artesunate-amodiaquine; AS-AQ+PQ = artesunate-amodiaquine plus primaquine

Supplementary Table 3. Infectivity to mosquitoes for all individuals

Day of follow-up	Treatment arm	Mosquito infection rate Median % (IQR) *	p-value¥	p-value†	Oocyst density Median (IQR) **	p-value¥	p-value†	Median % reduction in mosquito infection rate (IQR) ***	p-value¥	p-value†
Day 0	**Overall**	2·0% (0-9·4)	·	·	1·3 (1-3·0)	·	·	·	·	·
	AL	3·0% (0-13·6)	*ref*	*ref*	1·7 (1-4·8)	*ref*	*ref*	·	*ref*	·
	AL-AQ	2·5% (0-11·2)	*ref*	0·686	1·3 (1-3·5)	*ref*	0·7563	·	*ref*	·
	AL-AQ+PQ	1·6% (0-4·8)	*ref*	0·071	1·2 (1-2·5)	*ref*	0·5349	·	*ref*	·
	AS-AQ	3·9% (1·6-19·8)	*ref*	*ref*	1·3 (1-2·3)	*ref*	*ref*	·	*ref*	·
	AS-AQ+PQ	1·5% (0-9·4)	*ref*	0·955	1·7 (1-3·9)	*ref*	0·7158	·	*ref*	·
Day 2	**AL**	0% (0-0)	0·0012	*ref*	1·0 (1-1)	0·1797	*ref*	88·19 (0-100)	0·001	*ref*
	AL-AQ	0% (0-0)	0·0012	0·513	1·0 (1-1)	0·1797	*nc*	95·30 (0-100)	0·001	0·8644
	AL-AQ+PQ	0% (0-0)	0·0012	0·750	*nc*	*nc*	*nc*	100 (0-100)	0·0009	0·6003
	AS-AQ	2·3% (0·7-8·2)	0·614	*ref*	1·2 (1-1·8)	0·8744	*ref*	7·40 (-9·40-72·91)	0·1901	*ref*
	AS-AQ+PQ	0% (0-0)	0·0012	0·006	*nc*	*nc*	*nc*	100 (0-100)	0·0009	0·0465
Day 7	**AL**	0% (0-0)	0·0012	*ref*	*nc*	*nc*	*ref*	100 (0-100)	0·0009	*ref*
	AL-AQ	0% (0-0)	0·0013	*nc*	*nc*	*nc*	*nc*	100 (0-100)	0·0009	0·707
	AL-AQ+PQ	0% (0-0)	0·0012	*nc*	*nc*	*nc*	*nc*	100 (0-100)	0·0009	0·8573
	AS-AQ	0% (0-4)	0·0011	*ref*	2·0 (1·0-2·2)	0·3991	*ref*	100 (32·38-100)	0·0018	*ref*
	AS-AQ+PQ	0% (0-0)	0·0012	0·070	*nc*	*nc*	*nc*	100 (0-100)	0·0009	0·5878
Day 14	**AL**	0% (0-0)	0·0012	*ref*	*nc*	*nc*	*ref*	100 (0-100)	0·0009	*ref*
	AL-AQ	0% (0-0)	0·0013	*nc*	*nc*	*nc*	*nc*	100 (0-100)	0·0009	0·707
	AL-AQ+PQ	0% (0-0)	0·0012	*nc*	*nc*	*nc*	*nc*	100 (0-100)	0·0009	0·8573
	AS-AQ	0% (0-0)	0·0002	*ref*	1 (1-11·9)	0·1088	*ref*	100 (90·23-100)	0·0001	*ref*
	AS-AQ+PQ	0% (0-0)	0·0012	0·236	*nc*	*nc*	*nc*	100 (0-100)	0·0009	0·2022
Day 21	**AL**	0% (0-0)	·	*ref*	·	·	*ref*	·	·	*ref*
	AL-AQ	0% (0-0)	·	·	·	·	·	·	·	·
	AL-AQ+PQ	0% (0-0)	·	·	·	·	·	·	·	·
	AS-AQ	0% (0-0)	0·022	*ref*	5 (5-5)	*nc*	*ref*	100 (77·24-100)	0·0174	*ref*
	AS-AQ+PQ	0% (0-0)	·	·	·	·	·	·	·	·
Day 28	**AL**	0% (0-0)	·	*ref*	·	·	*ref*	·	·	*ref*
	AL-AQ	0% (0-0)	·	·	·	·	·	·	·	·
	AL-AQ+PQ	0% (0-0)	·	·	·	·	·	·	·	·
	AS-AQ	0% (0-10·3)	0·1088	*ref*	1 (1-1)	*nc*	*ref*	100 (83·85-100)	0·1025	*ref*
	AS-AQ+PQ	0% (0-0)	·	·	·	·	·	·	·	·

Mosquito infection measure (mosquito infection rate, oocyst density and reduction in mosquito infection rate) are presented for all individuals regardless of baseline infectivity. *Mosquito infection rate is the median percentage of mosquitoes infected by each participant, where for each participant mosquito infection rate the number of mosquitoes infected as a percentage of all mosquitoes surviving to dissection. Mosquito infection rate was compared within-groups (relative to baseline) by Wilcoxon sign rank test (z-score) and between-groups by linear regression adjusted for baseline mosquito infection rate (t score, coefficient with 95% CI). **The average oocyst density for each participant was calculated as the mean number of oocysts in infected mosquitoes (i.e., with at least one oocyst). The value presented in the table is the median of all individuals' average oocyst intensities (a composite figure of all oocysts/all infected mosquitoes is not statistically valid). *** Median within-person (relative to baseline) reduction in mosquito infection, including individuals not infectious at baseline. P-value¥ = Within group comparison. P-value† = Between group comparison (artemether–lumefantrine vs artemether–lumefantrine-amodiaquine and artemether–lumefantrine-amodiaquine plus primaquine, artesunate-amodiaquine vs artesunate-amodiaquine plus primaquine). nc = not calculable, no positive observations. · = not tested, ref = reference group. AL = artemether-lumefantrine; AL-AQ = artemether-lumefantrine-amodiaquine; AL-AQ+PQ = artemether-lumefantrine-amodiaquine plus primaquine; AS-AQ = artesunate-amodiaquine; AS-AQ+PQ = artesunate-amodiaquine plus primaquine

Supplementary Figure 2. Difference in mosquito infection rate and person infectivity before and after gametocyte enrichment



A. Difference in mosquito infection rate comparing direct feeds to gametocyte enriched feeds (after MACS). Each point represents the difference between a pair of measurements plotted against the average of the two measurements. Colour indicates treatment group and shape indicates the study visit. Blue dashed line indicates the mean of the difference between measurements across all samples. Grey dashed lines represent ± 1·96 standard deviations (SD) of the mean. **B.** Person infectivity by direct membrane feeding assay compared to direct membrane feeding assay boosted by gametocyte enrichment at baseline and at day 2 post-treatment. Error bars are 95% CI. AL = artemether-lumefantrine; AL-AQ = artemether-lumefantrine-amodiaquine; AL-AQ+PQ = artemether-lumefantrine-amodiaquine plus primaquine; AS-AQ = artesunate-amodiaquine; AS-AQ+PQ = artesunate-amodiaquine plus primaquine

Supplementary Table 4. Participant infectivity after gametocyte enrichment.

	Direct membrane feed	Direct and gametocyte enriched membrane feeds		
	Infectious individuals % (n/N)	**Infectious individuals % (n/N)**	**p-value¥**	**p-value†**
Day 0				
AL	53% (10/19)	84% (16/19)	0·039	*ref*
AL-AQ	58% (11/19)	68% (13/19)	0·369	0·224
AL-AQ+PQ	53% (10/19)	74% (14/19)	0·157	0·695
AS-AQ	84% (16/19)	89% (17/19)	0·500	*ref*
AS-AQ+PQ	53% (10/19)	68% (13/19)	0·254	0·232
Day 2				
AL	5% (1/19)	21% (4/19)	0·170	*ref*
AL-AQ	11% (2/19)	21% (4/19)	0·330	0·654
AL-AQ+PQ	0% (0/18)	0% (0/18)	nc	0·059
AS-AQ	74% (14/19)	79% (15/19)	0·500	*ref*
AS-AQ+PQ	0% (0/19)	11% (2/19)	0·243	<0·0001

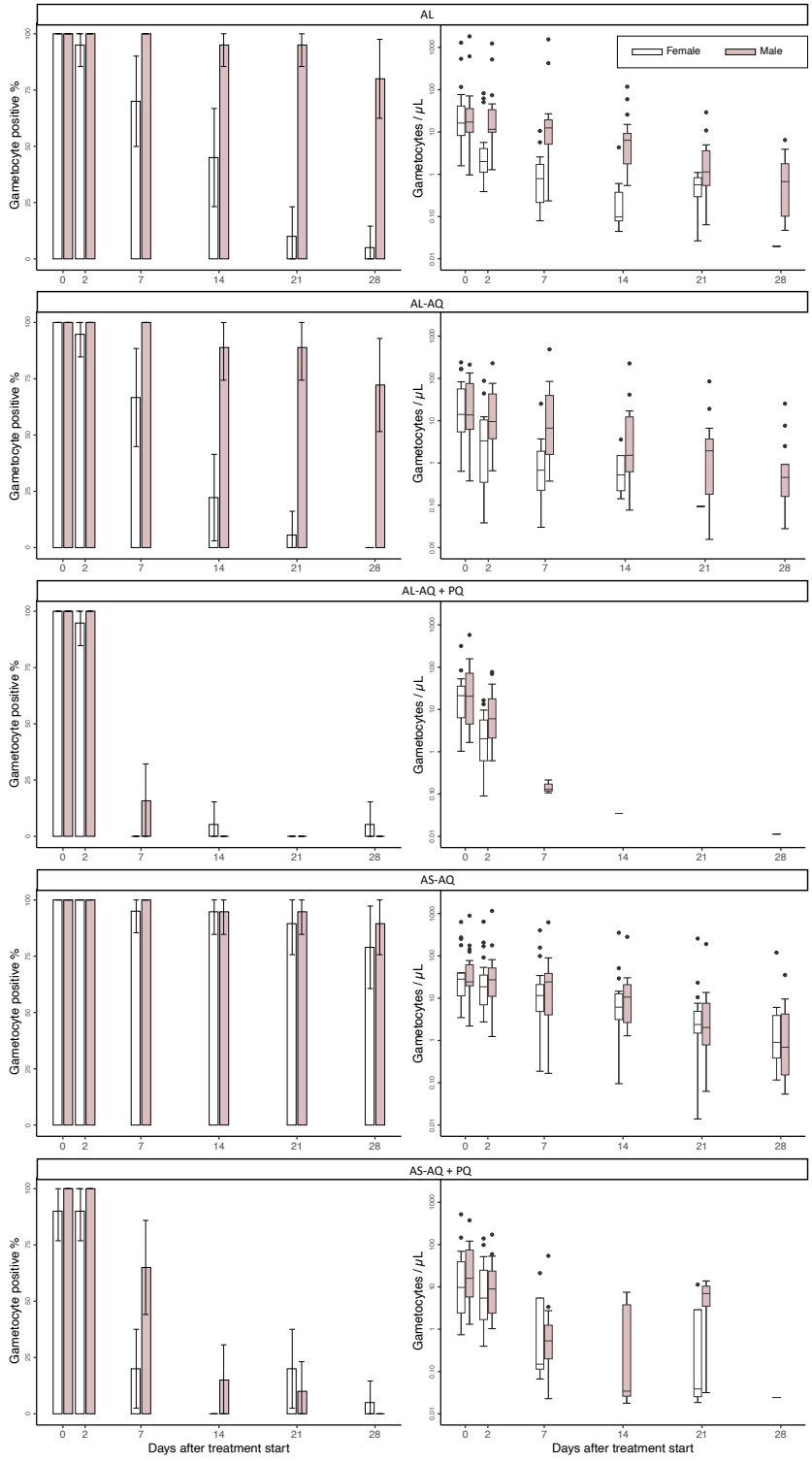
Participant infectivity after direct membrane feeding assay and gametocyte enrichment-boosted direct membrane feeding assays at baseline and day 2 after treatment initiation. ¥Within-group comparison (direct feed as reference). †Between artemisinin-based combination therapy matched group comparison (i.e., artemether–lumefantrine vs artemether–lumefantrine-amodiaquine and artemether–lumefantrine-amodiaquine plus primaquine, artesunate-amodiaquine vs artesunate-amodiaquine plus primaquine) by Fishers exact test. Nc = not calculable, ref = reference group. AL = artemether-lumefantrine; AL-AQ = artemether-lumefantrine-amodiaquine; AL-AQ+PQ = artemether-lumefantrine-amodiaquine plus primaquine; AS-AQ = artesunate-amodiaquine; AS-AQ+PQ = artesunate-amodiaquine plus primaquine

Supplementary Table 5. Asexual parasite density by microscopy

Day of follow-up	Treatment arm	Median asexual parasites/µL (IQR)	p-value¥	p-value†	Prevalence n/N (%)	p-value¥	p-value†
Day 0	**Overall**	0·00 (0·00-241·99)	.	.	37% (37/96)	.	.
	AL	37·9 (0·0-300·0)	*ref*	*ref*	50% (10/20)	*ref*	*ref*
	AL-AQ	0·0 (0·0-79·8)	*ref*	0·1193	30% (6/20)	*ref*	0·167
	AL-AQ+PQ	0·0 (0·0-37·6)	*ref*	0·0871	25% (5/20)	*ref*	0·095
	AS-AQ	0·0 (0·0-1654·9)	*ref*	*ref*	40% (8/20)	*ref*	*ref*
	AS-AQ+PQ	0·0 (0·0-720·0)	*ref*	0·5824	40% (8/20)	*ref*	0·626
Day 2	**AL**	0·0 (0·0-0·0)	0·0031	*ref*	5% (1/20)	0·004	*ref*
	AL-AQ	0·0 (0·0-0·0)	0·0148	0·3297	0% (0/19)	0·031	0·513
	AL-AQ+PQ	0·0 (0·0-0·0)	0·0459	0·3297	0% (0/19)	0·125	0·513
	AS-AQ	0·0 (0·0-0·0)	0·0051	*ref*	5% (1/20)	0·016	*ref*
	AS-AQ+PQ	0·0 (0·0-0·0)	0·0051	0·3173	0% (0/20)	0·008	0·500
Day 7	**AL**	0·0 (0·0-0·0)	0·0019	*ref*	0% (0/20)	0·002	*ref*
	AL-AQ	0·0 (0·0-0·0)	0·0149	nc	0% (0/18)	0·031	nc
	AL-AQ+PQ	0·0 (0·0-0·0)	0·0459	nc	0% (0/19)	0·125	nc
	AS-AQ	0·0 (0·0-0·0)	0·0051	*ref*	0% (0/20)	0·008	*ref*
	AS-AQ+PQ	0·0 (0·0-0·0)	0·0051	nc	0% (0/20)	0·008	nc
Day 14	**AL**	0·0 (0·0-0·0)	0·0019	*ref*	0% (0/20)	0·002	*ref*
	AL-AQ	0·0 (0·0-0·0)	0·0149	nc	0% (0/18)	0·031	nc
	AL-AQ+PQ	0·0 (0·0-0·0)	0·0459	nc	0% (0/19)	0·125	nc
	AS-AQ	0·0 (0·0-0·0)	0·0087	*ref*	0% (0/19)	0·008	*ref*
	AS-AQ+PQ	0·0 (0·0-0·0)	0·0051	nc	0% (0/20)	0·008	nc
Day 21	**AL**	0·0 (0·0-0·0)	0·0019	*ref*	0% (0/20)	0·002	*ref*
	AL-AQ	0·0 (0·0-0·0)	0·0149	nc	0% (0/18)	0·031	nc
	AL-AQ+PQ	0·0 (0·0-0·0)	0·0836	nc	0% (0/18)	0·125	nc
	AS-AQ	0·0 (0·0-0·0)	0·0087	*ref*	0% (0/19)	0·008	*ref*
	AS-AQ+PQ	0·0 (0·0-0·0)	0·0051	nc	0% (0/20)	0·008	nc
Day 28	**AL**	0·0 (0·0-0·0)	0·0139	*ref*	5% (1/20)	0·002	*ref*
	AL-AQ	0·0 (0·0-0·0)	0·0149	0·3428	0% (0/18)	0·031	0·526
	AL-AQ+PQ	0·0 (0·0-0·0)	0·0459	0·3297	0% (0/19)	0·125	0·513
	AS-AQ	0·0 (0·0-0·0)	0·0087	*ref*	0% (0/19)	0·008	*ref*
	AS-AQ+PQ	0·0 (0·0-0·0)	0·0051	nc	0% (0/20)	0·008	nc

Asexual parasite density (asexual parasites / µL) and prevalence of asexual parasites at all time points, measured by thick film microscopy (counted against 200 WBC). ¥Within-group comparisons †Between artemisinin-based combination therapy matched group comparison (i.e., artemether–lumefantrine vs artemether–lumefantrine-amodiaquine and artemether–lumefantrine-amodiaquine plus primaquine, artesunate-amodiaquine vs artesunate-amodiaquine plus primaquine). Nc = not calculable, ref = reference group. AL = artemether-lumefantrine; AL-AQ = artemether-lumefantrine-amodiaquine; AL-AQ+PQ = artemether-lumefantrine-amodiaquine plus primaquine; AS-AQ = artesunate-amodiaquine; AS-AQ+PQ = artesunate-amodiaquine plus primaquine

Supplementary Figure 3. Gametocyte density and prevalence by gametocyte sex



AL = artemether-lumefantrine; AL-AQ = artemether-lumefantrine-amodiaquine; AL-AQ+PQ = artemether-lumefantrine-amodiaquine plus primaquine; AS-AQ = artesunate-amodiaquine; AS-AQ+PQ = artesunate-amodiaquine plus primaquine

Supplementary Table 6. Total gametocyte density, prevalence and sex ratio

Day of follow-up	Total gametocytes (CCP4 & PfMGET)						
	Treatment arm	Median gametocytes/µL (IQR)	p-value	Prevalence n/N (%)	p-value	Median proportion male (IQR)	p-value
Day 0	Overall	38·01 (13·55-113·01)	·	100% (100/100)	·	0·54 (0·42-0·65)	·
	AL	30·96 (19·90-92·72)	*ref*	100% (20/20)	*ref*	0·50 (0·42-0·65)	*ref*
	AL-AQ	28·58 (11·49-130·45)	0·908	100% (20/20)	*nc*	0·53 (0·42-0·62)	0·9784
	AL-AQ+PQ	42·30 (11·79-97·04)	0·838	100% (20/20)	*nc*	0·52 (0·43-0·70)	0·3302
	AS-AQ	52·52 (33·59-128·97)	*ref*	100% (20/20)	*ref*	0·52 (0·37-0·64)	*ref*
	AS-AQ+PQ	24·82 (10·74-115·22)	0·105	100% (20/20)	*nc*	0·63 (0·49-0·68)	0·1231
Day 2	AL	15·43 (10·30-43·69)	*ref*	100% (20/20)	*ref*	0·88 (0·77-0·94)	*ref*
	AL-AQ	11·39 (4·12-59·23)	0·684	100% (19/19)	*nc*	0·82 (0·77-0·92)	0·4397
	AL-AQ+PQ	7·77 (3·43-25·28)	0·066	100% (19/19)	*nc*	0·77 (0·68-0·93)	0·1559
	AS-AQ	45·56 (19·18-100·32)	*ref*	100% (20/20)	*ref*	0·50 (0·44-0·67)	*ref*
	AS-AQ+PQ	12·64 (5·11-49·64)	0·011	100% (20/20)	*nc*	0·54 (0·41-0·71)	0·7251
Day 7	AL	13·25 (5·61-21)	*ref*	100% (20/20)	*ref*	0·98 (0·91-1)	*ref*
	AL-AQ	6·89 (1·05-49·86)	0·937	100% (18/18)	*nc*	0·99 (0·93-1)	0·6775
	AL-AQ+PQ	0 (0-0)	0·002	15·8% (3/19)	<0·0001	1 (1-1)	0·2389
	AS-AQ	31·74 (7·27-61·59)	*ref*	100% (20/20)	*ref*	0·59 (0·36-0·73)	*ref*
	AS-AQ+PQ	0·17 (0-0·87)	<0·0001	70·0% (14/20)	0·010	1 (0·88-1)	0·0002
Day 14	AL	5·61 (1·41-9·30)	*ref*	95·0% (19/20)	*ref*	1 (0·98-1)	*ref*
	AL-AQ	1·44 (0·37-11·63)	0·899	88·9% (16/18)	0·459	1 (1-1)	0·2648
	AL-AQ+PQ	0 (0-0)	0·246	5·3% (1/19)	<0·0001	*nc*	*nc*
	AS-AQ	13·65 (4·93-32)	*ref*	94·7% (18/19)	*ref*	0·49 (0·38-0·68)	*ref*
	AS-AQ+PQ	0 (0-0)	0·014	15·0% (3/20)	<0·0001	1 (1-1)	0·1003
Day 21	AL	1·02 (0·50-3·60)	*ref*	95·0% (19/20)	*ref*	1 (1-1)	*ref*
	AL-AQ	1·16 (0·09-3·44)	0·212	88·9% (16/18)	0·459	1 (1-1)	0·7063
	AL-AQ+PQ	0 (0-0)	*nc*	0% (0/18)	<0·0001	*nc*	*nc*
	AS-AQ	4·25 (0·91-13·39)	*ref*	94·7% (18/19)	*ref*	0·47 (0·37-0·72)	*ref*
	AS-AQ+PQ	0 (0-0)	0·500	20·0% (4/20)	<0·0001	0·55 (0·55-0·55)	0·6299
Day 28	AL	0·21 (0·06-1·48)	*ref*	80·0% (16/20)	*ref*	1 (1-1)	*ref*
	AL-AQ	0·21 (0-0·63)	0·307	72·2% (13/18)	0·427	1 (1-1)	*nc*
	AL-AQ+PQ	0 (0-0)	0·579	5·3% (1/19)	<0·0001	*nc*	*nc*
	AS-AQ	1·22 (0·22-7·23)	*ref*	94·7% (18/19)	*ref*	0·42 (0·27-0·90)	*ref*
	AS-AQ+PQ	0 (0-0)	0·543	5·0% (1/20)	<0·0001	*nc*	*nc*

P-values are for differences between artemisinin-based combination therapy matched group comparison (i.e., artemether–lumefantrine vs artemether–lumefantrine-amodiaquine and artemether–lumefantrine-amodiaquine plus primaquine, artesunate-amodiaquine vs artesunate-amodiaquine plus primaquine). Density was compared using regression analyses of log10 transformed density values, with adjustment for baseline densities. Prevalence was compared with one sided Fishers exact tests. For males and females, proportion male is given for participants/time-points with total gametocyte densities of 0·2/µL and over, as described previously (1). For the calculation of gametocyte prevalence, samples were classified as negative for a particular gametocyte sex if the estimated density of in gametocytes of that sex was less than 0·01/µL (i.e. one gametocyte per 100 µL of blood sample). P-value = between group comparison. nc = not calculable, no observations/no observations over the threshold density for analysis, ·= not tested, *ref* = reference group, AL = artemether-lumefantrine; AL-AQ = artemether-lumefantrine-amodiaquine; AL-AQ+PQ = artemether-lumefantrine-amodiaquine plus primaquine; AS-AQ = artesunate-amodiaquine; AS-AQ+PQ = artesunate-amodiaquine plus primaquine

Supplementary Table 7. Gametocyte circulation time and area under the curve

	Treatment group	Total gametocytes (CCP4 & PfMGET)	p-value*	Female gametocytes (CCP4)	p-value*	Male gametocytes (PfMGET)	p-value*	p-value♂♀
Circulation time Days (95% CI)	**AL**	6·13 (5·36-6·90)	*ref*	3·19 (2·69-3·70)	*ref*	6·83 (5·95-7·72)	*ref*	<0·0001
	AL-AQ	6·00 (5·20-6·79)	0·8071	2·75 (2·17-3·32)	0·2410	6·75 (5·82-7·68)	0·8951	<0·0001
	AL-AQ+PQ	2·60 (2·06-3·13)	<0·0001	3·27 (2·31-4·24)	0·8824	1·31 (1·00-1·63)	<0·0001	0·0006
	AS-AQ	7·99 (6·70-9·28)	*ref*	9·07 (7·16-10·98)	*ref*	7·77 (6·63-8·91)	*ref*	0·0066
	AS-AQ+PQ	3·30 (2·79-3·81)	<0·0001	4·81 (3·45-6·17)	0·0005	3·43 (2·76-4·11)	<0·0001	0·1191
AUC Median (IQR) gametocytes per uL/day	**AL**	9·36 (5·31-21·91)	*ref*	0·88 (0·61-2·21)	*ref*	7·79 (3·96-17·55)	*ref*	<0·0001
	AL-AQ	5·35 (2·21-40·44)	0·323	1·11 (0·33-3·02)	0·925	3·55 (1·54-26·71)	0·071	<0·0001
	AL-AQ+PQ	4·42 (1·22-14·86)	<0·0001	1·15 (0·31-2·69)	0·057	2·42 (0·75-10·78)	<0·0001	<0·0001
	AS-AQ	26·39 (12·80-51·70)	*ref*	7·23 (5·03-23·10)	*ref*	19·64 (4·83-27·47)	*ref*	0·0337
	AS-AQ+PQ	6·03 (2·84-18·41)	0·002	1·35 (0·23-5·84)	0·001	3·19 (2·27-11·11)	0·007	0·0003

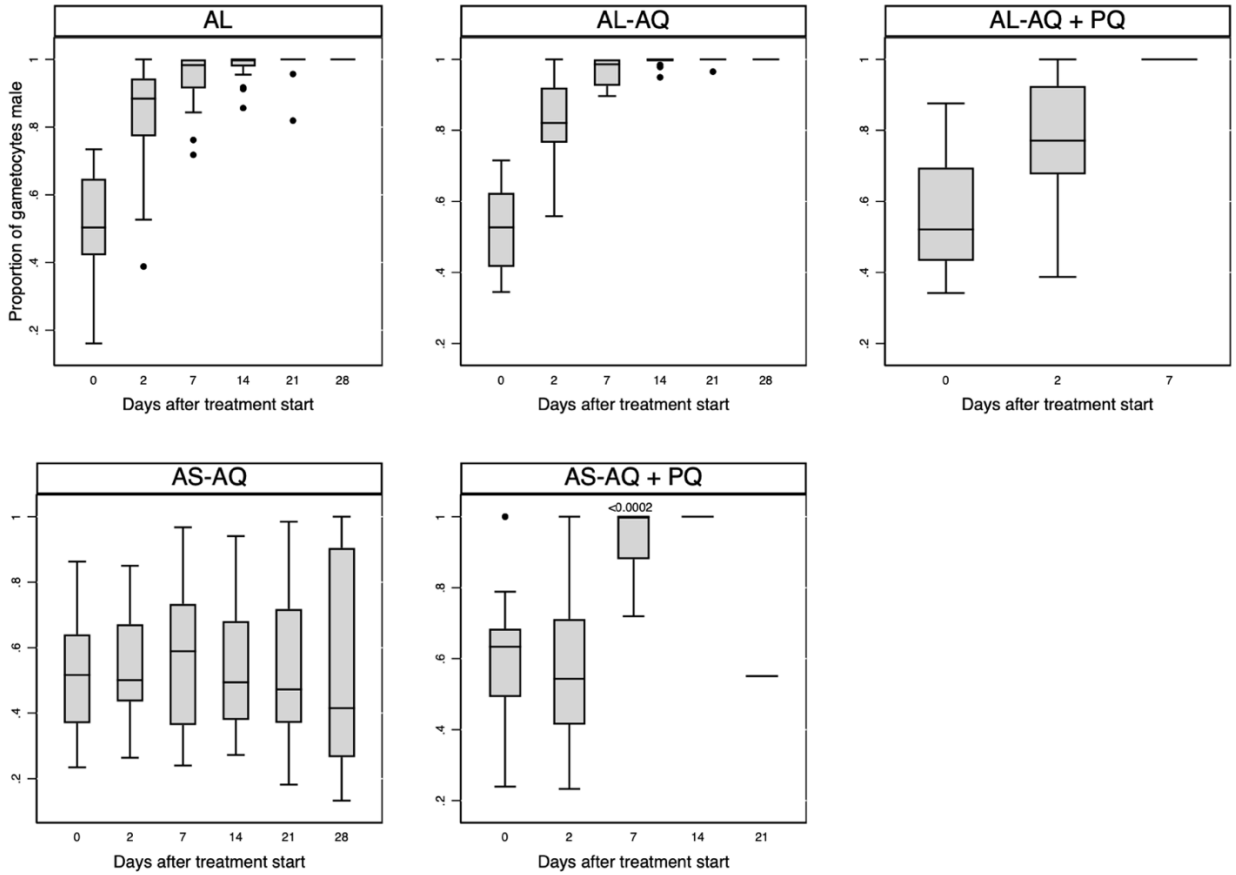
Gametocyte circulation time was calculated using a deterministic compartmental model (5), and is presented as the model estimate (mean days) with 95% CI. Area under the curve (AUC) of gametocyte density per participant over time was calculated using the linear trapezoid method (6), and is presented as the median and IQR of individual AUC values by treatment arm. P-values are for differences in the t-statistic between AL-AQ, AL-AQ+PQ and the AL reference group, and between AS-AQ+PQ and the AS-AQ reference group (*), and for between sexes within treatment groups (♂♀). *Ref* = reference, AL = artemether-lumefantrine; AL-AQ = artemether-lumefantrine-amodiaquine; AL-AQ+PQ = artemether-lumefantrine-amodiaquine plus primaquine; AS-AQ = artesunate-amodiaquine; AS-AQ+PQ = artesunate-amodiaquine plus primaquine.

Supplementary Table 8. Female (CCP4) and male (PfMGET) gametocyte density and prevalence

Day of follow-up	Treatment arm	Female gametocytes (CCP4)				Male gametocytes (PfMGET)			
		Median/µL (IQR)	p-value	Prevalence n/N (%)	p-value	Median/µL (IQR)	p-value	Prevalence n/N (%)	p-value
Day 0	Overall	15·63 (6·12-39·20)	·	98% (98/100)	·	19·72 (8·52-69·39)	·	100% (100/100)	·
	AL	16·26 (7·83-47·60)	*ref*	100% (20/20)	*ref*	17·19 (9·52-39·56)	*ref*	100% (20/20)	*ref*
	AL-AQ	14·07 (4·87-61·79)	0·890	100% (20/20)	*nc*	13·70 (6·20-77·32)	0·825	100% (20/20)	*nc*
	AL-AQ+PQ	21·19 (5·73-35·53)	0·935	100% (20/20)	*nc*	20·54 (4·44-75·63)	0·637	100% (20/20)	*nc*
	AS-AQ	28·10 (11·26-39·20)	*ref*	100% (20/20)	*ref*	24·18 (19·48-66·89)	*ref*	100% (20/20)	*ref*
	AS-AQ+PQ	7·47 (1·43-38·49)	0·090	90% (18/20)	0·244	15·98 (5·08-76·78)	0·234	100% (20/20)	*nc*
Day 2	AL	1·81 (0·98-4·05)	*ref*	95% (19/20)	*ref*	11·56 (9·69-35·11)	*ref*	100% (20/20)	*ref*
	AL-AQ	2·35 (0·29-10·90)	0·207	94·7% (18/19)	0·744	9·55 (3·60-48·33)	0·701	100% (19/19)	*nc*
	AL-AQ+PQ	1·95 (0·46-6·23)	0·880	94·7% (18/19)	0·744	6·03 (1·94-18·23)	0·040	100% (19/19)	*nc*
	AS-AQ	18·61 (6·51-41·24)	*ref*	100% (20/20)	*ref*	27·25 (10·53-57·52)	*ref*	100% (20/20)	*ref*
	AS-AQ+PQ	4·35 (1·36-22·11)	0·018	90% (18/20)	0·244	8·94 (2·29-28·44)	0·024	100% (20/20)	*nc*
Day 7	AL	0·26 (0-1·23)	*ref*	70% (14/20)	*ref*	12·46 (4·87-20·28)	*ref*	100% (20/20)	*ref*
	AL-AQ	0·18 (0-1·17)	0·566	66·7% (12/18)	0·550	6·68 (1·05-46·15)	1·000	100% (18/18)	*nc*
	AL-AQ+PQ	0 (0-0)	*nc*	0% (0/19)	<0·0001	0 (0-0)	0·003	15·8% (3/19)	<0·0001
	AS-AQ	11·07 (4·06-21·03)	*ref*	95% (19/20)	*ref*	24·15 (3·78-39·47)	*ref*	100% (20/20)	*ref*
	AS-AQ+PQ	0 (0-0)	0·064	20% (4/20)	<0·0001	0·17 (0-0·87)	0·00042	65% (13/20)	0·004
Day 14	AL	0 (0-0·10)	*ref*	45% (9/20)	*ref*	5·61 (1·41-9·26)	*ref*	95% (19/20)	*ref*
	AL-AQ	0 (0-0)	0·668	22·2% (4/18)	0·128	1·44 (0·37-11·38)	0·908	88·9% (16/18)	0·459
	AL-AQ+PQ	0 (0-0)	0·729	5·3% (1/19)	0·005	0 (0-0)	*nc*	0% (0/19)	<0·0001
	AS-AQ	5·61 (2·99-12·68)	*ref*	94·7% (18/19)	*ref*	10·23 (1·68-21·99)	*ref*	94·7% (18/19)	*ref*
	AS-AQ+PQ	0 (0-0)	*nc*	0% (0/20)	<0·0001	0 (0-0)	0·115	15% (3/20)	<0·0001
Day 21	AL	0 (0-0)	*ref*	10·0% (2/20)	*ref*	1·02 (0·50-3·6)	*ref*	95% (19/20)	*ref*
	AL-AQ	0 (0-0)	*nc*	5·6% (1/18)	0·541	1·16 (0·09-3·44)	0·199	88·9% (16/18)	0·459
	AL-AQ+PQ	0 (0-0)	*nc*	0% (0/18)	0·270	0 (0-0)	*nc*	0% (0/18)	<0·0001
	AS-AQ	2·24 (0·28-4·89)	*ref*	89·5% (17/19)	*ref*	2·01 (0·72-7·79)	*ref*	94·7% (18/19)	*ref*
	AS-AQ+PQ	0 (0-0)	0·832	20·0% (4/20)	<0·0001	0 (0-0)	0·512	10% (2/20)	<0·0001
Day 28	AL	0 (0-0)	*ref*	5·0% (1/20)	*ref*	0·21 (0·05-1·48)	*ref*	80% (16/20)	*ref*
	AL-AQ	0 (0-0)	*nc*	0% (0/18)	0·526	0·21 (0-0·63)	0·310	72·2% (13/18)	0·427
	AL-AQ+PQ	0 (0-0)	*nc*	5·3% (1/19)	0·744	0 (0-0)	*nc*	0% (0/19)	<0·0001
	AS-AQ	0·45 (0·12-3·21)	*ref*	78·9% (15/19)	*ref*	0·44 (0·13-4·21)	*ref*	89·5% (17/19)	*ref*
	AS-AQ+PQ	0 (0-0)	0·663	5·0% (1/20)	<0·0001	0 (0-0)	*nc*	0% (0/20)	<0·0001

P-values are for differences between artemisinin-based combination therapy matched group comparison (i.e., artemether–lumefantrine vs artemether–lumefantrine-amodiaquine and artemether-lumefantrine-amodiaquine plus primaquine, artesunate-amodiaquine vs artesunate-amodiaquine plus primaquine). Density was compared using regression analyses of log10 transformed density values, with adjustment for baseline densities. Prevalence was compared with one sided Fishers exact tests. For the calculation of gametocyte prevalence, samples were classified as negative for a particular gametocyte sex if the estimated density of in gametocytes of that sex was less than 0·01 gametocytes per µL (i.e. one gametocyte per 100 µL of blood sample). · = not tested. *ref* = reference group, *nc* = not calculable. AL = artemether-lumefantrine; AL-AQ = artemether-lumefantrine-amodiaquine; AL-AQ+PQ = artemether-lumefantrine-amodiaquine plus primaquine; AS-AQ = artesunate-amodiaquine; AS-AQ+PQ = artesunate-amodiaquine plus primaquine

Supplementary Figure 4. Proportion of gametocytes that were male



The proportion of gametocytes that were male was calculated for all values with total gametocyte densities of 0.2/μL and over, as described previously.(1) P-values (<0.05) for differences between treatment groups AL-AQ, AL-AQ+PQ and the AL reference group, and between AS-AQ+PQ and the AS-AQ reference group, were calculated using Wilcoxon rank sum tests. AL = artemether-lumefantrine; AL-AQ = artemether-lumefantrine-amodiaquine; AL-AQ+PQ = artemether-lumefantrine-amodiaquine plus primaquine; AS-AQ = artesunate-amodiaquine; AS-AQ+PQ = artesunate-amodiaquine plus primaquine.

Supplementary Table 9. Gametocyte infectivity

Day of follow-up	Treatment arm	Odds ratio (95% CI)	P-value
Day 0	AL	1	*ref*
	AL-AQ	0·64 (0·47-0·89)	0·008
	AL-AQ+PQ	0·19 (0·13-0·28)	<0·0001
	AS-AQ	1	*ref*
	AS-AQ+PQ	1·71 (1·29-2·27)	<0·0001
Day 2	AL	1	*ref*
	AL-AQ	1·3 (0·15-11·27)	0·808
	AL-AQ+PQ	*nc*	*nc*
	AS-AQ	1	*ref*
	AS-AQ+PQ	*nc*	*nc*

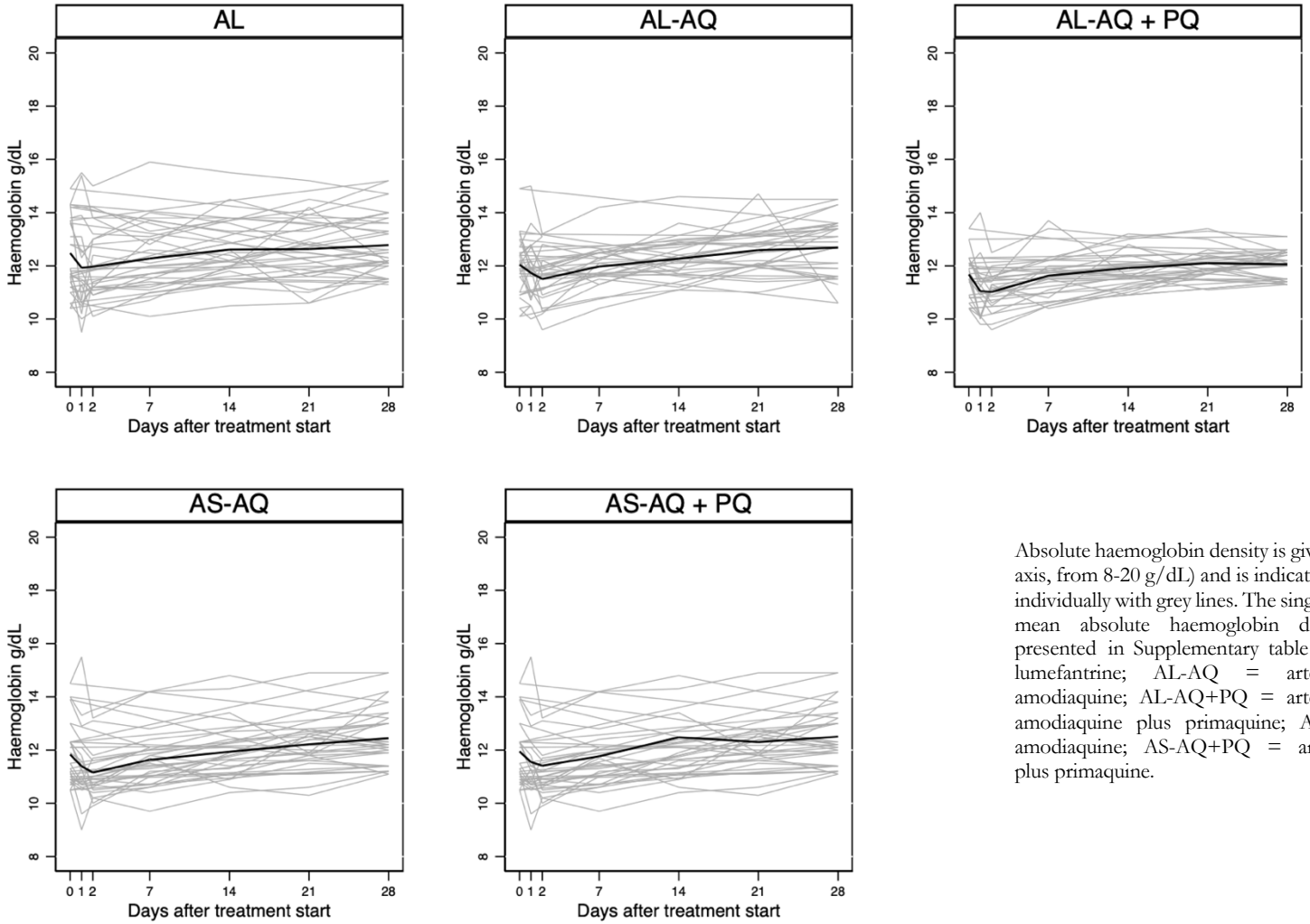
Odds ratios are for the change in mosquito infection rate in the AL-AQ and AL-AQ+PQ groups compared to the reference (AL) group and the AS-AQ+PQ group compared to the reference (AS-AQ) group with adjustment for gametocyte densities. At later timepoints, there were too few infected mosquitoes to calculate the odd ratios. *nc* = not calculable, no observations (too few infected mosquitoes for convergence), *ref* = reference, AL = artemether-lumefantrine; AL-AQ = artemether-lumefantrine-amodiaquine; AL-AQ+PQ = artemether-lumefantrine-amodiaquine plus primaquine; AS-AQ = artesunate-amodiaquine; AS-AQ+PQ = artesunate-amodiaquine plus primaquine.

Supplementary Table 10. Haemoglobin density

Day of follow-up	Treatment arm	Mean g/dL (range)	p-value¥	p-value†	Percent change from day 0			
					Mean (lower/upper 95% CI)	Range	p-value¥	p-value†
Day 0	**Overall**	12·0 (10·1-14·9)	·	·	·	·	·	·
	AL	12·5 (10·4-14·9)	*ref*	*ref*	·	·	·	·
	AL-AQ	12 (10·1-14·9)	*ref*	0·283	·	·	·	·
	AL-AQ+PQ	11·7 (10·4-13·4)	*ref*	0·031	·	·	·	·
	AS-AQ	11·8 (10·5-14·5)	*ref*	*ref*	·	·	·	·
	AS-AQ+PQ	11·9 (10·1-13·8)	*ref*	0·748	·	·	·	·
Day 1	**AL**	11·9 (9·5-15·5)	0·0354	*ref*	-4·41% (-8·28 / -0·54)	-25·17/8·65	0·0278	*ref*
	AL-AQ	11·7 (10-15)	0·1068	0·530	-2·40% (-5·52 / 0·71)	-12·03/8·18	0·1232	0·4038
	AL-AQ+PQ	11·1 (9·8-14)	0·0005	0·761	-5·36% (-7·93 / -2·79)	-15·97/4·48	0·0003	0·6719
	AS-AQ	11·4 (9-15·5)	0·0069	*ref*	-3·79% (-6·42 / -1·17)	-14·29/7·62	0·0070	*ref*
	AS-AQ+PQ	11·5 (9·6-13·6)	0·0408	0·821	-3·19% (-6·35 / -0·04)	-13·79/8·13	0·0474	0·7617
Day 2	**AL**	12 (10·1-15)	0·0006	*ref*	-4·02% (-6·02 / -2·02)	-14·50/4·20	0·0005	*ref*
	AL-AQ	11·5 (9·6-13·2)	0·0007	0·411	-4·83% (-7·25 / -2·41)	-12·71/4·55	0·0005	0·5890
	AL-AQ+PQ	11 (9·6-12·5)	<0·00001	0·167	-5·42% (-7·18 / -3·66)	-12·31/3·67	<0·00001	0·2779
	AS-AQ	11·2 (9·9-13·5)	<0·00001	*ref*	-5·59% (-7·54 / -3·64)	-14·63/1·74	<0·00001	*ref*
	AS-AQ+PQ	11·4 (10·2-13·6)	0·0005	0·288	-4·36% (-6·50 / -2·23)	-13·33/4·27	0·0004	0·3797
Day 7	**AL**	12·3 (10·1-15·9)	0·1394	*ref*	-1·52% (-3·66 / 0·62)	-11·40/6·71	0·1543	*ref*
	AL-AQ	12 (10·4-14·2)	0·5061	0·947	-0·55% (-3·40 / 2·31)	-7·52/16·35	0·6917	0·5649
	AL-AQ+PQ	11·6 (10·4-13·7)	0·7899	0·629	-0·23% (-2·30 / 1·84)	-12·61/5·50	0·8165	0·3715
	AS-AQ	11·6 (9·7-14·2)	0·1751	*ref*	-1·53% (-4·05 / 0·99)	-13·01/8·33	0·2191	*ref*
	AS-AQ+PQ	11·8 (10·2-13·3)	0·1723	0·802	-1·31% (-3·61 / 0·99)	-10·53/6·25	0·2478	0·8933
Day 14	**AL**	12·6 (10·5-15·5)	0·4351	*ref*	1·37% (-1·50 / 4·23)	-10·29/13·21	0·3309	*ref*
	AL-AQ	12·3 (11·1-14·6)	0·2260	0·787	1·86% (-0·85 / 4·57)	-8·46/11·54	0·1657	0·7948
	AL-AQ+PQ	11·9 (10·9-13·2)	0·1487	0·568	2·40% (-0·58 / 5·38)	-10·77/11·30	0·1082	0·6030
	AS-AQ	11·9 (10·4-14·8)	0·2327	*ref*	1·19% (-0·63 / 3·00)	-4·07/7·62	0·1869	*ref*
	AS-AQ+PQ	12·5 (10·5-13·8)	0·0026	0·021	4·64% (1·90 / 7·37)	-6·67/19·27	0·0021	0·0357
Day 21	**AL**	12·6 (10·6-15·2)	0·5576	*ref*	1·65% (-2·79 / 6·09)	-10·92/21·70	0·4462	*ref*
	AL-AQ	12·6 (11-14·7)	0·0208	0·481	4·60% (0·98 / 8·21)	-4·17/25·64	0·0157	0·2938
	AL-AQ+PQ	12·1 (11·1-13·4)	0·0092	0·943	4·34% (1·41 / 7·27)	-9·23/12·50	0·0062	0·3075
	AS-AQ	12·2 (10·3-14·9)	0·0429	*ref*	3·77% (0·48 / 7·07)	-14·39/18·10	0·0272	*ref*
	AS-AQ+PQ	12·3 (10·3-13·6)	0·0087	0·923	3·06% (0·89 / 5·23)	-2·68/11·93	0·0083	0·7026
Day 28	**AL**	12·8 (11·3-15·2)	0·0838	*ref*	2·75% (-0·05 / 5·55)	-7·69/16·04	0·0535	*ref*
	AL-AQ	12·7 (10·6-14·5)	0·0103	0·436	5·33% (1·50 / 9·17)	-9·40/18·18	0·0093	0·2540
	AL-AQ+PQ	12·1 (11·3-13·1)	0·0233	0·266	3·71% (0·62 / 6·80)	-5·04/21·15	0·0213	0·6328
	AS-AQ	12·4 (11·1-14·9)	0·0009	*ref*	5·67% (2·64 / 8·71)	-1·63/19·64	0·0010	*ref*
	AS-AQ+PQ	12·5 (10·8-14·3)	0·0029	0·850	4·86% (1·83 / 7·88)	-4·31/15·60	0·0033	0·6914

Haemoglobin density and percent reduction in haemoglobin density (relative to baseline) were compared within treatment arms (p-value¥) using paired t-tests (with day 0 as reference for percent change) and between treatment arms (p-value†) using linear regression (for density, adjusted for baseline Hb density) or two-way t-tests (for percent reduction). · = not tested. *ref* = reference group, AL = artemether-lumefantrine; AL-AQ = artemether-lumefantrine-amodiaquine; AL-AQ+PQ = artemether-lumefantrine-amodiaquine plus primaquine; AS-AQ = artesunate-amodiaquine; AS-AQ+PQ = artesunate-amodiaquine plus primaquine.

Supplementary Figure 5. Absolute haemoglobin density



Absolute haemoglobin density is given in grams per dL (y-axis, from 8-20 g/dL) and is indicated for each participant individually with grey lines. The single black line shows the mean absolute haemoglobin density. P-values are presented in Supplementary table 8. AL = artemether-lumefantrine; AL-AQ = artemether-lumefantrine-amodiaquine; AL-AQ+PQ = artemether-lumefantrine-amodiaquine plus primaquine; AS-AQ = artesunate-amodiaquine; AS-AQ+PQ = artesunate-amodiaquine plus primaquine.

Supplementary Table 11. Biochemistry

Day of follow-up	Treatment arm	Mean ALT U/L (range)	p-value¥	p-value†	Mean AST U/L (range)	p-value¥	p-value†	Mean creatinine mg/dL (range)	p-value¥	p-value†
Day 0	**Overall**	23·45 (8-157)	·	·	29·24 (4-215)	·	·	0·64 (0·2-1·2)	·	·
	AL	20·90 (12-37)	*ref*	*ref*	23·95 (4-40)	*ref*	*ref*	0·63 (0·2-1·2)	*ref*	*ref*
	AL-AQ	19·15 (10-27)	*ref*	0·387	25·45 (13-35)	*ref*	0·562	0·77 (0·3-1·2)	*ref*	0·055
	AL-AQ+PQ	21·50 (8-67)	*ref*	0·881	30·30 (16-62)	*ref*	0·065	0·66 (0·2-1·2)	*ref*	0·650
	AS-AQ	25·50 (7-117)	*ref*	*ref*	27·25 (2-63)	*ref*	*ref*	0·58 (0·2-1·0)	*ref*	*ref*
	AS-AQ+PQ	24·75 (5-118)	*ref*	0·921	26·75 (6-42)	*ref*	0·881	0·66 (0·2-1·2)	*ref*	0·303
Day 2	**AL**	18·35 (9-40)	0·0694	*ref*	21·80 (4-40)	0·4851	*ref*	0·77 (0·1-1·4)	0·0280	*ref*
	AL-AQ	22·00 (8-57)	0·2813	0·125	28·37 (5-48)	0·1114	0·045	0·77 (0·2-1·3)	0·9404	0·279
	AL-AQ+PQ	29·16 (7-164)	0·2216	0·099	31·32 (11-94)	0·8206	0·226	0·61 (0·3-1·0)	0·3205	0·017
	AS-AQ	30·10 (12-126)	0·5847	*ref*	34·80 (8-163)	0·3167	*ref*	0·64 (0·3-1·0)	0·3306	*ref*
	AS-AQ+PQ	28·10 (7-105)	0·1422	0·821	28·90 (11-52)	0·5094	0·484	0·63 (0·2-1·2)	0·6319	0·573
Day 7	**AL**	20·40 (11-33)	0·7623	*ref*	24·55 (10-37)	0·8239	*ref*	0·61 (0·3-1·0)	0·6154	*ref*
	AL-AQ	22·00 (7-44)	0·1483	0·276	28·94 (8-44)	0·1364	0·143	0·74 (0·4-1·2)	0·6901	0·259
	AL-AQ+PQ	26·05 (8-129)	0·4681	0·380	29·84 (8-110)	0·8915	0·628	0·62 (0·4-0·8)	0·4581	0·915
	AS-AQ	26·45 (9-92)	0·8855	*ref*	27·30 (10-38)	0·9835	*ref*	0·65 (0·2-1·0)	0·2183	*ref*
	AS-AQ+PQ	25·20 (13-57)	0·9068	0·810	29·75 (12-49)	0·1975	0·293	0·72 (0·2-1·0)	0·1907	0·476
Day 14	**AL**	19·45 (10-38)	0·2991	*ref*	24·95 (9-39)	0·7192	*ref*	0·64 (0·2-1·0)	0·8152	*ref*
	AL-AQ	28·72 (10-157)	0·2054	0·137	36·17 (19-215)	0·2846	0·300	0·70 (0·2-1·0)	0·2615	0·861
	AL-AQ+PQ	25·11 (8-112)	0·6021	0·354	33·95 (15-86)	0·4069	0·140	0·60 (0·3-0·8)	0·3105	0·407
	AS-AQ	25·47 (15-65)	0·9046	*ref*	26·89 (4-42)	0·9360	*ref*	0·62 (0·2-1·2)	0·3620	*ref*
	AS-AQ+PQ	19·20 (9-30)	0·2683	0·043	25·05 (5-35)	0·3725	0·520	0·64 (0·4-1·2)	0·7263	0·742

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine were compared within treatment arms (p-value¥) using paired t-tests (with day 0 as reference) and between treatment arms (p-value†) using linear regression (adjusted for baseline levels). *Ref* = reference, · = not tested. AL = artemether-lumefantrine; AL-AQ = artemether-lumefantrine-amodiaquine; AL-AQ+PQ = artemether-lumefantrine-amodiaquine plus primaquine; AS-AQ = artesunate-amodiaquine; AS-AQ+PQ = artesunate-amodiaquine plus primaquine.

Supplementary Table 12. All adverse events

Description	Total (n=100)	AL (n=20)	AL-AQ (n=20)	AL-AQ+PQ (n=20)	AS-AQ (n=20)	AS-AQ+PQ (n=20)
Abdominal pain	23^{21}(5)	3^{3}	5^{5}(1)	4^{4}(1)	5^{5}(3)	6^{4}
Acute respiratory infection	26^{1}(17)	6(2)	2(2)	7(4)	6^{1}(4)	5(5)
Allergic contact dermatitis	1(1)	0	0	1(1)	0	0
Anemia	1^{1}	0	1^{1}	0	0	0
Asthenia	16^{16}(5)	2^{2}	4^{4}(2)	3^{3}	4^{4}(2)	3^{3}(1)
Chills	2^{2}	1^{1}	0	1^{1}	0	0
Conjunctivitis	3(3)	1(1)	1(1)	1(1)	0	0
Cough	9^{2}(4)	2^{1}(1)	1(1)	1	4^{1}(1)	1(1)
Diarrhea	4^{4}(1)	0	2^{2}(1)	0	1^{1}	1^{1}
Drowsiness	5^{5}(1)	1^{1}	1^{1}	2^{2}(1)	1^{1}	0
Dyspnea	1^{1}	0	0	1^{1}	0	0
Eczema	1(1)	0	1(1)	0	0	0
Elevation of ALT/ GPT	5^{2}(1)	0	1	2^{1}(1)	2^{1}	0
Elevation of ASAT/GOT	5^{1}	0	1	3^{1}	1	0
Elevation of creatinemia	3^{2}	2^{1}	0	0	0	1^{1}
Fatigue	4^{3}	2^{1}	0	2^{2}	0	0
Fever	2^{1}(1)	1^{1}(1)	0	0	0	1
Food indigestion	1(1)	0	0	0	1(1)	0
Headaches	51^{38}(23)	8^{7}(3)	10^{8}(5)	12^{10}(7)	8^{6}(4)	13^{7}(4)
Hyperleukocytosis	4^{1}	1	0	2^{1}	0	1
Leucopenia	3^{3}	0	0	0	2^{2}	1^{1}
Liquid diarrhea	1^{1}(1)	0	1^{1}(1)	0	0	0
Localized left arm pain	1	0	0	1	0	0
Loss of appetite	10^{10}	0	3^{3}	2^{2}	5^{5}	0
Low back pain	1	0	1	0	0	0
Muscular pain	10^{8}(1)	1(1)	1^{1}	3^{3}	3^{2}	2^{2}
Nausea	17^{17}(2)	3^{3}	3^{3}(1)	3^{3}	6^{6}(1)	2^{2}
Rhinitis	3(1)	1(1)	2	0	0	0
Rhinorrhea	13(1)	3	3	2	3	2(1)
Traumatic wound right food	1(1)	0	1(1)	0	0	0
Uncomplicated malaria	1(1)	1(1)	0	0	0	0
Vertigo	19^{18}(6)	2^{1}	3^{3}(1)	6^{6}(4)	4^{4}(1)	4^{4}
Vomiting	15^{14}(3)	4^{4}(1)	4^{3}(1)	3^{3}	3^{3}(1)	1^{1}
ALL	*262^{172}*	*45^{26}*	*52^{35}*	*62^{43}*	*59^{42}*	*44^{26}*
MILD	*181^{129}*	*33^{21}*	*33^{22}*	*42^{32}*	*41^{30}*	*32^{24}*
MODERATE	*81^{43}*	*12^{5}*	*19^{13}*	*20^{11}*	*18^{12}*	*12^{2}*

85/100 participants experienced a total of 262 adverse events over the course of the trial; 181 categorised for severity by the study clinician (in accordance with the study protocol and data safety and monitoring charter) as 'mild' and 81 as 'moderate'. No severe adverse events or serious adverse events (SAE) occurred during the trial. The frequency of all AEs is given outside parentheses, with the frequency of moderate AEs in parentheses. The frequency of AEs that were related to drug treatment (defined as probably, possibly or definitely related to treatment) is given in superscript. 172 of the 262 AEs were classified as possibly, probably or definitely related to the study drug; of these, 129/169 were mild and 43/169 were moderate.

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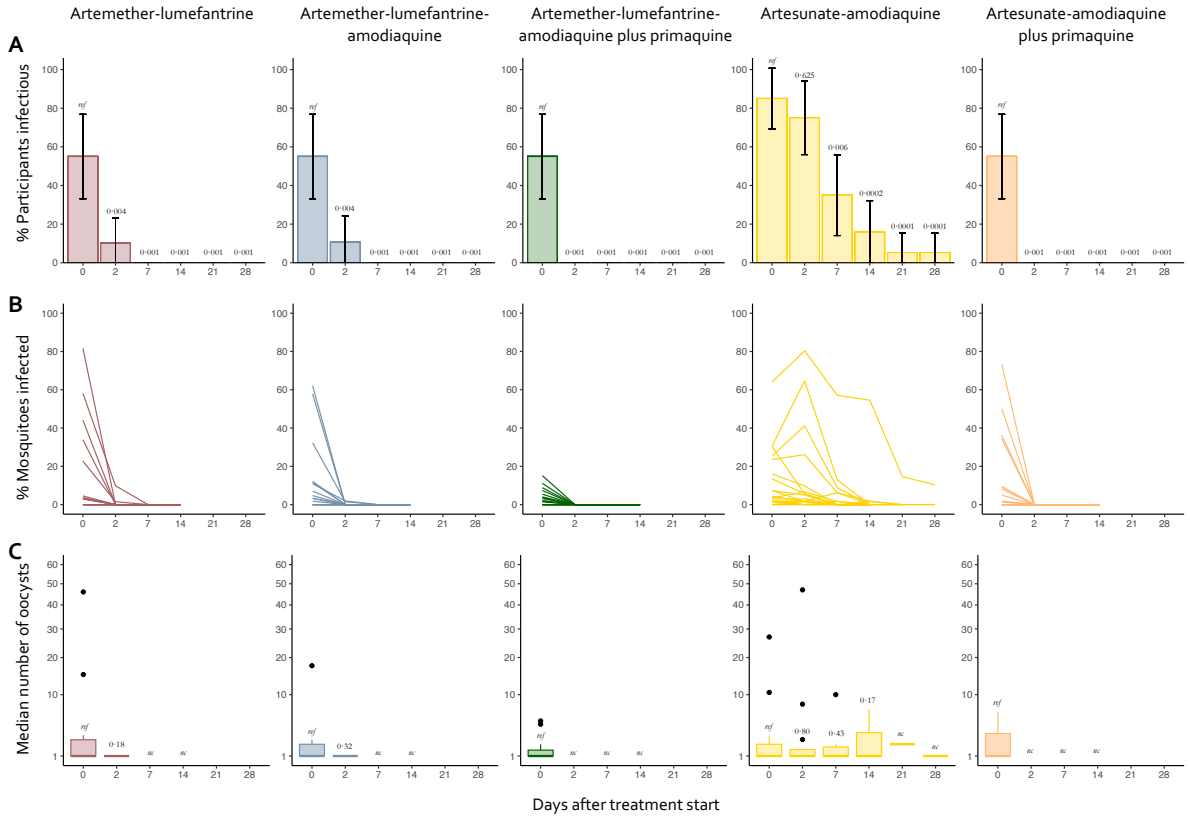
6. Méndez F, Muñoz Á, Plowe CV. Use of area under the curve to characterize transmission potential after antimalarial treatment. Am J Trop Med Hyg. 2006 Oct 1;75(4):640–4.

APPENDIX 2

Study protocol can be found on
https://www.medrxiv.org/content/10.1101/2024.02.23.24303266v1.supplementary-material.

APPENDIX 3: Median oocyst densities



Updated figure 2. Participant infectivity and proportion of mosquitoes infected in direct membrane feeding assays. (A) Participant infectivity. Error bars show 95% CI. The denominator for participants infectious is the total number of participants still enrolled at the given timepoint, rather than the number tested for infectivity at that timepoint. Infectivity assays were discontinued after 14 days when a participant did not infect any mosquitoes at two subsequent timepoints and were thereafter considered non-infectious. Mosquito feeding assays at days 21 and 28 were only conducted in the artesunate-amodiaquine group (seven at day 21 and three at day 28). The prevalence infectious individuals was compared within treatment groups using McNemar tests. (B) Mosquito infection rate. Each line represents one individual. Statistical analyses are shown in Supplementary Table 2. (C) Oocyst density. Box plots show the median (central line), IQR (box limits), upper and lower quartiles plus 1·5 × IQR (whiskers), and outliers for median oocyst densities in infected mosquitoes within each participant. The median number of oocysts was compared to baseline within treatment groups using the Wilcoxon sign-rank test. nc = not calculable. Ref=reference.

Updated Supplementary table 2: Infectivity to mosquitoes for individuals infectious at baseline

Day of follow-up	Treatment arm	Infectious individuals % (n/N) *	p-value¥	p-value†	Mosquito infection rate Median % (IQR) **	p-value¥	p-value†	Oocyst density Median (IQR) ***	p-value¥	p-value†
Day 0	**Overall**	61% (61/100)	·	·	7·3% (3·2-23.5)	·	·	1 (1-1)	·	·
	AL	55% (11/20)	*ref*	*ref*	4·5% (3·3-44·1)	*ref*	*ref*	1 (1-3)	*ref*	*ref*
	AL-AQ	55% (11/20)	*ref*	1·000	10·9% (3·3-32·3)	*ref*	0·646	1 (1-2)	*ref*	0·7563
	AL-AQ+PQ	55% (11/20)	*ref*	1·000	4·1% (2·1-8·8)	*ref*	0·045	1 (1-2)	*ref*	0·5349
	AS-AQ	85% (17/20)	*ref*	*ref*	7·3% (1·9-23·5)	*ref*	*ref*	1 (1-2)	*ref*	*ref*
	AS-AQ+PQ	55% (11/20)	*ref*	0·082	9·3% (1·8-36·2)	*ref*	0·370	1 (1-4)	*ref*	0·7158
Day 2	**AL**	10% (2/20)	0·0039	*ref*	0% (0-0)	0·0033	*ref*	1 (1-1)	0·1797	*ref*
	AL-AQ	10·5% (2/19)	0·0039	0·678	0% (0-0)	0·0033	0·523	1 (1-1)	0·3173	*nc*
	AL-AQ+PQ	0% (0/19)	0·001	0·256	0% (0-0)	0·0033	0·788	*nc*	*nc*	*nc*
	AS-AQ	75% (15/20)	0·625	*ref*	5% (1·5-9·7)	0·6192	*ref*	1 (1-1·5)	0·7921	*ref*
	AS-AQ+PQ	0% (0/20)	0·001	<0·0001	0% (0-0)	0·0033	0·006	*nc*	*nc*	*nc*
Day 7	**AL**	0% (0/20)	0·001	*ref*	0% (0-0)	0·0033	*ref*	*nc*	*nc*	*ref*
	AL-AQ	0% (0/18)	0·001	*nc*	0% (0-0)	0·0033	*nc*	*nc*	*nc*	*nc*
	AL-AQ+PQ	0% (0/19)	0·001	*nc*	0% (0-0)	0·0033	*nc*	*nc*	*nc*	*nc*
	AS-AQ	35% (7/20)	0·0063	*ref*	0% (0-6·2)	0·001	*ref*	1·25 (1-2)	0·425	*ref*
	AS-AQ+PQ	0% (0/20)	0·001	0·004	0% (0-0)	0·0033	0·045	*nc*	*nc*	*nc*
Day 14	**AL**	0% (0/20)	0·001	*ref*	0% (0-0)	0·0033	*ref*	*nc*	*nc*	*ref*
	AL-AQ	0% (0/18)	0·001	*nc*	0% (0-0)	0·0033	*nc*	*nc*	*nc*	*nc*
	AL-AQ+PQ	0% (0/19)	0·001	*nc*	0% (0-0)	0·0033	*nc*	*nc*	*nc*	*nc*
	AS-AQ	15·8% (3/19)	0·0002	*ref*	0% (0-0)	0·0004	*ref*	1 (1-7)	0·1655	*ref*
	AS-AQ+PQ	0% (0/20)	0·001	0·106	0% (0-0)	0·0033	0·146	*nc*	*nc*	*nc*
Day 21	**AL**	0% (0/20)	0·001	*ref*	0% (0-0)	·	*ref*	·	·	*ref*
	AL-AQ	0% (0/18)	0·001	*nc*	0% (0-0)	·	·	·	·	·
	AL-AQ+PQ	0% (0/18)	0·001	*nc*	0% (0-0)	·	·	·	·	·
	AS-AQ	5·3% (1/19)	0·0001	*ref*	0% (0-0)	0·0277	*ref*	2 (2-2)	*nc*	*ref*
	AS-AQ+PQ	0% (0/20)	0·001	*nc*	0% (0-0)	·	·	·	·	·
Day 28	**AL**	0% (0/20)	0·001	*ref*	0% (0-0)	·	*ref*	·	·	*ref*
	AL-AQ	0% (0/18)	0·001	*nc*	0% (0-0)	·	·	·	·	·
	AL-AQ+PQ	0% (0/19)	0·001	*nc*	0% (0-0)	·	·	·	·	·
	AS-AQ	5·3% (1/19)	0·0001	*ref*	0% (0-10·3)	0·1088	*ref*	1 (1-1)	*nc*	*ref*
	AS-AQ+PQ	0% (0/20)	0·001	*nc*	0% (0-0)	·	·	·	·	·

*Percentage of infectious individuals. Individuals were classed as infectious if direct membrane feeding assays (DMFA) resulted in at least one mosquito with any number of oocysts. Mosquito infection measures (mosquito infection rate and oocyst density) are presented for all participants who were infectious at baseline, and oocyst densities are from all infected mosquitoes **Mosquito infection rate is the median percentage of mosquitoes infected by each participant, where for each participant mosquito infection rate the number of mosquitoes infected as a percentage of all mosquitoes surviving to dissection. Mosquito infection rate was compared within-groups (relative to baseline) by Wilcoxon sign rank test (z-score) and between-groups by linear regression adjusted for baseline mosquito infection rate (t score, coefficient with 95% CI). ***The median oocyst density for each participant was calculated as the median number of oocysts in infected mosquitoes (i.e., with at least one oocyst). The value presented in the table is the median of all individuals' median oocyst. P-value¥ = Within group comparison. P-value† = Between group comparison (artemether–lumefantrine vs artemether–lumefantrine-amodiaquine and artemether–lumefantrine-amodiaquine plus primaquine, artesunate-amodiaquine vs artesunate-amodiaquine plus primaquine). nc = not calculable, no positive observations. · = not tested, ref = reference group. AL = artemether-lumefantrine; AL-AQ = artemether-lumefantrine-amodiaquine; AL-AQ+PQ = artemether-lumefantrine-amodiaquine plus primaquine; AS-AQ = artesunate-amodiaquine; AS-AQ+PQ = artesunate-amodiaquine plus primaquine

Updated Supplementary table 3: Infectivity to mosquitoes for all individuals

Day of follow-up	Treatment arm	Mosquito infection rate Median % (IQR) *	p-value¥	p-value†	Oocyst density Median (IQR) **	p-value¥	p-value†	Median % reduction in mosquito infection rate (IQR) ***	p-value¥	p-value†
Day 0	**Overall**	2·0% (0-9·4)	·	·	1 (1-2)	·	·	·	·	·
	AL	3·0% (0-13·6)	*ref*	*ref*	1 (1-3)	*ref*	*ref*	·	*ref*	·
	AL-AQ	2·5% (0-11·2)	*ref*	0·686	1 (1-2)	*ref*	0·7461	·	*ref*	·
	AL-AQ+PQ	1·6% (0-4·8)	*ref*	0·071	1 (1-2)	*ref*	0·3803	·	*ref*	·
	AS-AQ	3·9% (1·6-19·8)	*ref*	*ref*	1 (1-2)	*ref*	*ref*	·	*ref*	·
	AS-AQ+PQ	1·5% (0-9·4)	*ref*	0·955	1 (1-4)	*ref*	0·9787	·	*ref*	·
Day 2	**AL**	0% (0-0)	0·0012	*ref*	1 (1-1)	0·1797	*ref*	88·19 (0-100)	0·001	*ref*
	AL-AQ	0% (0-0)	0·0012	0·513	1 (1-1)	0·3173	*nc*	95·30 (0-100)	0·001	0·8644
	AL-AQ+PQ	0% (0-0)	0·0012	0·750	*nc*	*nc*	*nc*	100 (0-100)	0·0009	0·6003
	AS-AQ	2·3% (0·7-8·2)	0·614	*ref*	1 (1-1·5)	0·7921	*ref*	7·40 (-9·40-72·91)	0·1901	*ref*
	AS-AQ+PQ	0% (0-0)	0·0012	0·006	*nc*	*nc*	*nc*	100 (0-100)	0·0009	0·0465
Day 7	**AL**	0% (0-0)	0·0012	*ref*	*nc*	*nc*	*ref*	100 (0-100)	0·0009	*ref*
	AL-AQ	0% (0-0)	0·0013	*nc*	*nc*	*nc*	*nc*	100 (0-100)	0·0009	0·707
	AL-AQ+PQ	0% (0-0)	0·0012	*nc*	*nc*	*nc*	*nc*	100 (0-100)	0·0009	0·8573
	AS-AQ	0% (0-4)	0·0011	*ref*	1 (1-2)	0·4250	*ref*	100 (32·38-100)	0·0018	*ref*
	AS-AQ+PQ	0% (0-0)	0·0012	0·070	*nc*	*nc*	*nc*	100 (0-100)	0·0009	0·5878
Day 14	**AL**	0% (0-0)	0·0012	*ref*	*nc*	*nc*	*ref*	100 (0-100)	0·0009	*ref*
	AL-AQ	0% (0-0)	0·0013	*nc*	*nc*	*nc*	*nc*	100 (0-100)	0·0009	0·707
	AL-AQ+PQ	0% (0-0)	0·0012	*nc*	*nc*	*nc*	*nc*	100 (0-100)	0·0009	0·8573
	AS-AQ	0% (0-0)	0·0002	*ref*	1 (1-7)	0·1655	*ref*	100 (90·23-100)	0·0001	*ref*
	AS-AQ+PQ	0% (0-0)	0·0012	0·236	*nc*	*nc*	*nc*	100 (0-100)	0·0009	0·2022
Day 21	**AL**	0% (0-0)	·	*ref*	·	·	*ref*	·	·	*ref*
	AL-AQ	0% (0-0)	·	·	·	·	·	·	·	·
	AL-AQ+PQ	0% (0-0)	·	·	·	·	·	·	·	·
	AS-AQ	0% (0-0)	0·022	*ref*	2 (2-2)	0·3173	*ref*	100 (77·24-100)	*nc*	*ref*
	AS-AQ+PQ	0% (0-0)	·	·	·	·	·	·	·	·
Day 28	**AL**	0% (0-0)	·	*ref*	·	·	*ref*	·	·	*ref*
	AL-AQ	0% (0-0)	·	·	·	·	·	·	·	·
	AL-AQ+PQ	0% (0-0)	·	·	·	·	·	·	·	·
	AS-AQ	0% (0-10·3)	0·1088	*ref*	1 (1-1)	0·3173	*ref*	100 (83·85-100)	*nc*	*ref*
	AS-AQ+PQ	0% (0-0)	·	·	·	·	·	·	·	·

Mosquito infection measure (mosquito infection rate, oocyst density and reduction in mosquito infection rate) are presented for all individuals regardless of baseline infectivity. *Mosquito infection rate is the median percentage of mosquitoes infected by each participant, where for each participant mosquito infection rate the number of mosquitoes infected as a percentage of all mosquitoes surviving to dissection. Mosquito infection rate was compared within-groups (relative to baseline) by Wilcoxon sign rank test (z-score) and between-groups by linear regression adjusted for baseline mosquito infection rate (t score, coefficient with 95% CI). **The median oocyst density for each participant was calculated as the median number of oocysts in infected mosquitoes (i.e., with at least one oocyst). The value presented in the table is the median of all individuals' average oocyst *** Median within-person (relative to baseline) reduction in mosquito infection, including individuals not infectious at baseline. P-value¥ = Within group comparison. P-value† = Between group comparison (artemether–lumefantrine vs artemether–lumefantrine-amodiaquine and artemether–lumefantrine-amodiaquine plus primaquine, artesunate-amodiaquine vs artesunate-amodiaquine plus primaquine). nc = not calculable, no positive observations. · = not tested, ref = reference group. AL = artemether-lumefantrine; AL-AQ = artemether-lumefantrine-amodiaquine; AL-AQ+PQ = artemether-lumefantrine-amodiaquine plus primaquine; AS-AQ = artesunate-amodiaquine; AS-AQ+PQ = artesunate-amodiaquine plus primaquine

Chapter 5: The transmission blocking activity of artemisinin-combination, non-artemisinin, and 8-aminoquinoline antimalarial therapies: a pooled analysis of individual participant data

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Student ID Number	lsh2004630	**Title**	Dr.
First Name(s)	Leen Nele		
Surname/Family Name	Vanheer		
Thesis Title	Transmissibility and antimalarial resistance in human malaria parasite Plasmodium falciparum in Mali		
Primary Supervisor	Chris Drakeley		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

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Where is the work intended to be published?	PLoS Medicine
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Stage of publication	**Submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I verified and analysed the data and interpreted the results. I wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and read and approved the final manuscript.

SECTION E

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The transmission blocking activity of artemisinin-combination, non-artemisinin, and 8-aminoquinoline antimalarial therapies: a pooled analysis of individual participant data.

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Abbreviations

ACT, artemisinin-based combination therapy; AL, Artemether-lumefantrine; AL-AQ; Artemether-lumefantrine-amodiaquine; AQ, Amodiaquine; AS-AQ, Artesunate-Amodiaquine; DHA-PPQ, Dihydroartemisinin-Piperaquine; G6PD; glucose-6-phosphate dehydrogenase; PY-AS, Pyronaridine-Artesunate; PQ, Primaquine; SLD, Single low-dose; RT-qPCR, reverse-transcriptase quantitative PCR; SP-AQ, Sulfadoxine-pyrimethamine plus Amodiaquine; TACT, Triple artemisinin-based combination therapy; TQ, Tafenoquine; WHO, World Health Organisation.

ABSTRACT

Background: Interrupting human-to-mosquito transmission is important for malaria elimination strategies as it can reduce infection burden in communities and slow the spread of drug resistance. Antimalarial medications differ in their efficacy in clearing the transmission stages of *Plasmodium falciparum* (gametocytes) and in preventing mosquito infection. Here we present a combined analysis of six trials conducted at the same study site with highly consistent methodologies that allows for a direct comparison of the gametocytocidal and transmission-blocking activities of fifteen different antimalarial regimens or dosing schedules.

Methods and findings: Between January 2013 and January 2023, six clinical trials with transmission endpoints were conducted at the Clinical Research Centre of the Malaria Research and Training Centre of the University of Bamako in Mali. These trials tested Artemisinin-based Combination Therapies (ACTs), non-ACT regimens and combinations with 8-aminoquinolines. Participants were males and non-pregnant females, between 5-50 years of age, who presented with *P. falciparum* mono-infection and gametocyte carriage by microscopy. Blood samples were taken before and after treatment for thick film microscopy, infectivity assessments by mosquito feeding assays and molecular quantification of gametocytes. Mixed-effects generalized linear models were fit with individual-specific random effects and fixed effects for time points, treatment groups and their interaction. Models quantified changes in mosquito infection rates and gametocyte densities within treatment arms over time and between treatments. In a pooled analysis of 422 participants, we observed substantial differences between ACTs in gametocytocidal and transmission-blocking activities, with artemether-lumefantrine (AL) being significantly more potent at reducing mosquito infection rates within 48 hours than dihydroartemisinin-piperaquine (DHA-PPQ), artesunate-amodiaquine (AS-AQ) and pyronaridine-artesunate (PY-AS) ($p<0.0001$). The addition of single low dose primaquine (SLD PQ) accelerated gametocyte clearance and led to a significantly greater reduction in mosquito infection rate within 48-hours of treatment for each ACT, while an SLD of

the 8-aminoaquinoline tafenoquine (TQ) showed a delayed but effective response compared to SLD primaquine. Finally, our findings confirmed considerably higher post-treatment transmission after sulfadoxine-pyrimethamine plus amodiaquine (SP-AQ) compared to most ACTs, with a significantly lower relative reduction in mosquito infection rate at day 7 compared to DHA-PPQ, AS-AQ, and AL ($p<0.0001$). Therefore, adding an SLD PQ to SP-AQ may be beneficial to block malaria transmission in community treatment campaigns.

Conclusions: We found marked differences among ACTs and single low-dose 8-aminoquinoline drugs in their ability and speed to block transmission. The findings from this analysis can support treatment policy decisions for malaria elimination and be integrated into mathematical models to improve the accuracy of predictions regarding community transmission and the spread of drug resistance under varying treatment guidelines.

INTRODUCTION

The primary aim in the therapeutic management of malaria is the clearance of pathogenic asexual blood-stage parasites, using antimalarials with schizonticidal properties. Artemisinin-based combination therapies (ACTs) are the first-line treatment for uncomplicated *Plasmodium falciparum* malaria across the world and are highly potent against asexual parasites, capable of reducing the circulating asexual parasite biomass ~10,000 fold per 48 hour cycle (1). Gametocytes are distinct parasite stages that are not associated with symptoms but are the only parasite life stage that can be transmitted to and establish infection in mosquitoes. In *P. falciparum*, gametocytes develop during five developmental stages over a prolonged 10–12-day maturation period. Importantly, *P. falciparum* gametocytes have markedly different sensitivity profiles to antimalarial drugs than asexual parasites. Assessing and comparing the gametocytocidal and transmission-blocking properties of different antimalarial regimens is important for informing treatment guidelines that aim to contribute to transmission reduction.

Artemisinins are active against developing gametocytes but less so against the mature forms (2). Although gametocyte density generally decreases more after treatment with ACTs compared to non-ACTs (3), there is considerable variation in gametocyte-clearing potential between ACTs (4). For example, current artemisinin partner drugs such as piperaquine, lumefantrine and amodiaquine have limited activity against *P. falciparum* gametocytes at clinically relevant concentrations (2,5–7). *In vitro* data for the partner drug pyronaridine are contradictory; one study found a strong effect against mature gametocytes (8), whereas others only observed activity against mature gametocytes at concentrations above the therapeutic threshold (2,6,9). Furthermore, a disconnect exists between the detection of gametocytes and infectivity to mosquitoes; on the one hand, gametocytes can be infectious at sub-microscopic densities (10), whereas on the other, antimalarials can have a parasite-inhibiting effect, active after ingestion by mosquitoes (2,11) or sterilise gametocytes so that these are detectable but not transmissible (12). Gametocyte quantification is therefore an

imperfect measure of post-treatment transmission potential, and mosquito feeding assays are considered more informative (13).

With the threat of artemisinin partial resistance in sub-Saharan Africa (14), various strategies have been proposed to counter the spread of resistant parasites. One of the most promising approaches, according to modelling simulations, is the use of Triple Artemisinin-based Combination Therapies (TACT), that combine existing ACTs with a second partner drug. This approach may offer protection against partner drug resistance and maintain high treatment efficacy in areas where resistance against artemisinins and one of the partner drugs is present (15). Other suggested strategies include multiple first-line ACT therapies or cycling between different ACTs (16,17). An alternative or complementary strategy is to supplement treatment with gametocytocidal compounds. Primaquine (PQ) and its long-lasting analogue tafenoquine (TQ) are 8-aminoquinolines that can clear *P. vivax* liver stages (18) and have *P. falciparum* gametocytocidal activity. The WHO recommends supplementing first-line ACTs with a single low-dose (0.25mg/kg) PQ (SLD PQ) in low transmission areas (19), and the WHO malaria policy and advisory group has now suggested expanding this recommendation to other regions (20). TQ holds the promise of long-lasting transmission blocking activity and recently a single-low dose of TQ (SLD TQ) in combination with ACTs and non-ACT treatments was found to effectively block transmission within 7 days at a dose of 1.66 mg/kg (21,22). To date, no trials directly comparing SLD TQ to SLD PQ have been conducted, largely due to the impracticality of trials involving mosquito feeding assays.

In order to make informed decisions regarding optimal antimalarial regimens, it is necessary to understand and compare the transmission-blocking abilities of different ACTs and TACTs. We conducted a pooled analysis of individual-level data from six clinical trials to compare the transmission-blocking effects of 15 different antimalarials and combinations. All six trials measured mosquito transmission endpoints, used sensitive gametocyte quantification, and were conducted

using the same mosquito feeding protocols and assays in Ouélessébougou, Mali, between 2013 and 2023 (21–26). Results from this analysis can be used in mathematical models to more accurately predict community transmission and the spread of drug resistance under different treatment guidelines, and will inform malaria control programmes.

METHODS

Study design and participants

Between January 2013 and January 2023, six clinical trials (21–26) (Table 1) (appendix 2) involving a total of 521 participants were performed at the Clinical Research Centre of the Malaria Research and Training Centre (MRTC) of the University of Bamako (Bamako, Mali). Study participants were recruited in the commune of Ouélessébougou, which includes the central town of Ouélessébougou and 44 surrounding villages, with an estimated 50,000 inhabitants and located approximately 80 km south of Bamako, the capital of Mali. Malaria transmission in this region is hyperendemic and highly seasonal with incidence peaking during and following the rainy season from July to November. In all six trials, participants were males and non-pregnant females, between 5-50 years of age, with a body weight less than 80 kg, who presented with *P. falciparum* mono-infection and at least 1 (study acronyms PQ03, NECTAR 1-4) or 2 (study PQ01) gametocytes per 500 white blood cells (WBC) detected by blood smear. This minimum gametocyte density corresponds to an approximate minimum of 16-32 gametocytes per μL of blood when assuming 8000 WBC per μL. Participants in all studies, except for the 2013-2014 PQ01 study, were exclusively asymptomatic, and four studies (study acronyms PQ01, PQ03, NECTAR2, NECTAR3) required participants to have normal glucose-6-phosphate dehydrogenase (G6PD) production, as determined by OSMMR-D-D calorimetric test (R&D Diagnostics, Aghia Paraskevi, Greece; studies PQ01, NECTAR2 and NECTAR3), CareStart G6PD rapid diagnostic test (Access Bio, Somerset, NJ,USA; study PQ03) and/or STANDARD G6PD quantitative enzyme activity test (SD Biosensor, Suwon, South Korea; studies NECTAR2 and NECTAR3; table 1, appendix 2). Other study-specific inclusion criteria are presented in appendix 2.

Ethical approvals for the individual studies were obtained from the Ethics Committee of the Faculty of Medicine, Pharmacy, and Dentistry of the University of Science, Techniques, and Technologies of Bamako (Bamako, Mali). In addition, the studies were approved by the

Committee on Human Research at the University of California (San Francisco, CA, USA), and/or the Research Ethics Committee of the London School of Hygiene and Tropical Medicine (London, UK).

Procedures

Of the antimalarial treatments evaluated in the included studies, the following were included in this analysis: i) Dihydroartemisinin-piperaquine (DHA-PPQ; Eurartesim; Sigma Tau, Gaithersburg, MD, USA); ii) Pyronaridine-artesunate (PY-AS; Pyramax; Shin Poong Pharmaceutical, Seoul, South Korea; iii) Artemether-lumefantrine (AL; Coartem; Novartis, Basel, Switzerland or Guilin Pharmaceutical, Shanghai, China); iv) Artesunate-amodiaquine (AS-AQ; Guilin Pharmaceutical, Shanghai, China); v) Sulfadoxine-pyrimethamine plus amodiaquine (SP-AQ; Guilin Pharmaceutical, Shanghai, China); vi) Any of the ACTs listed above plus a single low-dose of primaquine (ACT-PQ; 0.25 or 0.5 mg/kg; Sanofi, Laval, QC, Canada or ACE Pharmaceuticals, Zeewolde, the Netherlands); vii) Non-ACT (SP-AQ) plus a single low-dose of primaquine (Non-ACT-PQ; Sanofi, Laval, QC, Canada); viii) ACT (DHA-PPQ) plus a single low dose of tafenoquine (ACT-TQ; 0.83 or 1.66 mg/kg; 60° Pharmaceuticals Ltd, USA) (Table 1). Antimalarial treatments were administered as per manufacturer's instructions (supplementary Information 1 for dosing tables and supplementary Table 1 for manufacturers, appendix 1, pp 3-6) under direct supervision. ACT treatments and sulfadoxine-pyrimethamine plus amodiaquine were administered over 3 days (days 0, 1, and 2). Primaquine and tafenoquine were administered as a single dose immediately after the first dose of ACT. In the NECTAR1 study, participants were treated with a full course of DHA-PPQ at day 21 of follow-up, to prevent re-infection. For the current analysis, we did not consider study arms within the included studies that are currently not considered as treatment regimens, such as arms with PQ doses below 0.25 mg/kg (n=33 participants) and with TQ doses below 0.83 mg/kg (n=20). Likewise, treatment arms involving

methylene blue (n=20) or the combination of SP-AQ with an SLD TQ (n=20) were not considered in the analysis.

In all studies, blood samples were taken before treatment and on days 2 and 7 following treatment for thick film microscopy, infectivity assessments and molecular analysis of gametocyte density, as outlined in Figure 1 and described in detail in appendix 2. For infectivity assessments in all studies, 75-90 insectary-reared *Anopheles gambiae (s.l.)* females were allowed to feed for 15–20 min on venous blood samples collected in Lithium Heparin tubes and offered in a water-jacketed membrane feeder system, as previously described (27). Fully fed mosquitoes were kept in a temperature and humidity-controlled insectary until day 7 post-feeding, and then dissected in 1% mercurochrome to detect and quantify *P. falciparum* oocysts. Giemsa-stained blood slides were double read by expert research microscopists with asexual stages counted against 200 white blood cells and gametocytes counted against 500 white blood cells. For molecular gametocyte quantification in all studies, venous or finger-prick blood collected in EDTA tubes was aliquoted into L6 buffer (Severn Biotech, Kidderminster, UK) or RNA protect cell reagent (Qiagen, Hilden, Germany) and stored at ≤−70°C until total nucleic acid extraction using a MagNAPure LC automated extractor (Total Nucleic Acid Isolation Kit High Performance; Roche Applied Science, Indianapolis, IN, USA). Male and female gametocytes were quantified in a multiplex reverse-transcriptase quantitative PCR (RT-qPCR) assay targeting Pfs25 or CCP4 transcripts for female gametocytes and PfMGET for male gametocyte quantification, as specified in appendix 2. Molecular gametocyte quantification was repeated for the PQ03 study, after the original density estimates of this study (but not of other studies) showed marked disagreement with their respective microscopy density measurements (supplementary figure 1, appendix 1, p 7). Samples were classified as negative for a specific gametocyte sex if the RT-qPCR quantified gametocyte density was less than 0.01 gametocytes per μL (equivalent to one gametocyte per 100 μL of the blood sample).

Statistical analysis

Asexual parasite and microscopy gametocyte density distributions at baseline were presented per study by violin plots. Six individuals from the PQ01 study that lacked baseline infectivity measures or microscopy gametocyte densities were excluded from analysis. The association between gametocyte density (gametocytes / μL) on a log10 scale and the proportion of infected mosquitoes was determined by a mixed-effects logistic regression with a random effect for study. The association between (log10) gametocyte density estimates by microscopy and molecular methods was determined by a linear model with an interaction by study.

To quantify reductions in key output parameters (gametocyte prevalence and densities, the proportion of infected mosquitos, mean oocyst density) within treatments over time (days 2, 7 and 14) and between treatments at each time point, mixed-effects generalized linear models were fit with individual-specific random effects and fixed effects for time points, treatment groups and their interaction. A Poisson family with log-link was used for gametocyte prevalence (since a binomial family with log-link failed to converge), a Gamma family with log-link was used for gametocyte densities assessed by microscopy and RT-qPCR, a binomial family with log-link was used for proportion of infected mosquitos, while a negative binomial family with log-link was used for oocyst density.

The models were of the form:

$$y_i = \exp\left(\beta_0 + \sum_{k=1}^{8} \beta_{1k} \times arm_{ik} + \sum_{j=0}^{3} \beta_{2j} \times t_{ij} + \sum_{k=1}^{8}\sum_{j=0}^{3} \beta_{3kj} \times arm_{ik} \times t_{ij} + Z_i\right),$$

where the study arms were DHA-PPQ, SP-AQ, PY-AS, AS-AQ, AL, Non-ACT-PQ, ACT-PQ and ACT-TQ, and the time points were t_0=day 0, t_1=day 2, t_2=day 7 and t_3=day 14, the β's are the regression parameters to be estimated, Z_i are the individual specific random effects, and y_i is one of the outcomes of interest: gametocyte prevalence or densities, the proportion of infected

mosquitos, oocyst density. The beta (β) coefficients estimated from the model were used to calculate the relative reductions at each time point for each treatment arm.

Kaplan-Meier survival curves were generated with the *survfit* function from the R-based *survival* package to display the cumulative probability of remaining uncleared of gametocytes detected by microscopy, gametocytes detected by RT-qPCR and any infectivity to mosquitoes. The Cox proportional hazards model was used to estimate hazard ratios and to draw comparisons between treatment arms for each of the clearance events. Statistical analyses and visualisations were conducted in R (version 4.3.2).

The original studies were registered with ClinicalTrials.gov (NCT01743820, NCT02831023, NCT04049916, NCT04609098, NCT05081089 and NCT05550909).

RESULTS

A total of 422 study participants (42-100 participants per study) and 15 different treatment regimens or dosing schedules were evaluated across the six included trials (20-79 participants per regimen/dosing schedule, Table 1, Appendix 2). Parasite density was broadly similar between studies that recruited asymptomatic parasite carriers (2016-2022) but higher in the PQ01 study conducted in 2013-2014 where clinical malaria patients were eligible (Figure 2A). Although participants were recruited based on microscopically detected gametocyte carriage, at the time of enrolment (up to 24 hours after the initial screening), microscopy gametocyte prevalence was 99.5% (420/422) with a median gametocyte density of 55 (IQR 32-112) gametocytes/μL among gametocyte carriers (Figure 2B). Gametocyte density by microscopy was highly skewed with a range of 15.5-2720 gametocytes/μL (supplementary table 2, appendix 1, p 8). Prior to treatment, 68.8% (290/422) of individuals were infectious to mosquitoes (Figure 2C). The proportion of mosquitoes each participant infected at this time point ranged from 0.75% to 94.3% and was positively associated with microscopically determined gametocyte density (Figure 2D). In the four studies where oocyst numbers were recorded (NECTAR studies 1-4), the proportion of mosquitoes infected was strongly positively associated with mosquito infection burden (oocyst density) (supplementary figure 2, appendix 1, p 9).

Acknowledging that densities below the microscopic threshold for detection (16 gametocytes/ μL) can result in mosquito infections, gametocyte density was also quantified by molecular methods. For all studies, gametocyte prevalence by molecular methods was 100% at enrolment and there was a strong positive association between gametocyte density by microscopy and by molecular methods (Figure 3A). At enrolment, two study participants were positive for gametocytes by molecular methods but not by microscopy; both became gametocyte positive by microscopy one day after baseline.

Following treatment, gametocyte prevalence and densities declined in all treatment groups. The extent and speed at which this occurred depended strongly on the treatment provided (Figure 3B-D, supplementary figure 3, appendix 1, p 10). Two days after treatment, relative reductions compared to baseline in microscopically detected gametocyte prevalence were non-significant in all arms. However, at seven days after treatment initiation, this reduction was statistically significant in the AL group (51.12%, 95% CI 3.85% -75.15%, p=0.0381), but not after the other schizonticidal regimens without addition of a gametocytocide (p=0.2258, p=0.3892, p=0.5731 and p=0.7997 after DHA-PPQ, SP-AQ, PY-AS and AS-AQ treatment, respectively). The reduction in gametocyte prevalence was more rapid for treatment groups that included a single low-dose gametocytocide, with reductions of 68.31% (95% CI 10.02% -88.84%, p=0.0309) in the non-ACT-PQ group, 90.52% (95% CI 78.52% -95.81%, p<0.0001) in the ACT-PQ group and 61.53% (95% CI 16.07% -82.37%, p=0.0164) in the ACT-TQ group, at day 7 compared to baseline. Relative reductions in gametocyte prevalence assessed by microscopy and molecular methods at days 2, 7 and 14 post-treatment are presented in Figure 3B and supplementary tables 3-8 (appendix 1, pp 11-16). We combined different ACTs with PQ for this analysis; the findings per study arm are given in supplementary figure 4 (appendix 1, p17).

Reductions in gametocyte prevalence were predictably slower than reductions in gametocyte densities, which showed significant relative reductions at day 2 compared to baseline by both microscopy and molecular measures in the AL (p=0.0116 and p<0.0001, respectively) and ACT-PQ (p=0.0003 and p<0.0001, respectively) groups (Figure 3 C-D, supplementary tables 9-14, supplementary figure 5, appendix 1, pp 18-24). Reductions in PCR-determined gametocyte density on day 2 after initiation of treatment were more pronounced for AL compared to DHA-PPQ (p=0.0036), SP-AQ (p=0.0308), PY-AS (p=0.0302) (supplementary table 12, appendix 1, p 21) with similar patterns at day 7 and 14 (supplementary tables 13 and 14, appendix 1, p 22-23). AS-AQ also resulted in a smaller reduction in gametocyte density compared to AL, which was not

significant at day 2 (p=0.1085) or day 7 (p=0.0617), but became significant at day 14 (p=0.0013). ACT-PQ resulted in the fastest reductions in molecular gametocyte density and this reduction was significantly larger than the best performing ACT, AL, on days 7 and 14 (p<0.0001; supplementary tables 13 and 14, appendix 1, pp 22-23). Non-ACT-PQ and ACT-TQ regimens showed reductions of nearly 30% in gametocyte density by PCR on day 2, which was significantly less than in the ACT-PQ group (p=0.0400 and p=0.0043, respectively). Across all post-treatment timepoints, the proportion of gametocyte infections that were submicroscopic was 28.63% (777/2714).

A valuable feature of the studies included was the availability of mosquito infection data before and after initiation of treatment. In all groups, 58.3% to 95% of participants infected mosquitoes before treatment (supplementary table 2, appendix 1, p 8), with 99.7% (289/290) of infectious individuals having gametocytes detected by microscopy at this timepoint. Following treatment, as gametocyte densities declined, the contribution of submicroscopic infections to transmission increased (Figure 4A). At day 2, 8.33% (12/144) of all infectious individuals had submicroscopic gametocyte infections. At days 7 and 14, these percentages were 6.59% (6/91) and 3.70% (1/27), respectively. The experiments in which mosquitoes became infected after feeding on samples with submicroscopic gametocyte densities typically resulted in low proportions of infected mosquitoes.

Transmission blocking effects per treatment group were quantified as averages of individual level effects; that is, within-person reductions in the proportion of mosquitoes infected at post-treatment timepoints compared to baseline (Figure 4B, supplementary figures 6 and 7, appendix 1, pp 28-29). All treatment groups showed a statistically significant relative reduction in mosquito infection rates by day 14 (Figure 4B, supplementary tables 15-17, appendix 1, pp 25-27). At earlier time-points, we observed a modest but statistically significant reduction in mosquito infection prevalence on day 2 for DHA-PPQ (20.35%, 95% CI 12.70-27.33, p<0.0001) and for non-ACT-PQ (83.26%, 95% CI 78.11-87.21, p<0.0001) whilst >90% transmission reduction was observed for AL and for ACTs with SLD PQ. For AS-AQ and ACT with SLD TQ, a statistically significant

reduction in mosquito infection rates was only observed at day 7, while for SP-AQ and PY-AS, this was only observed by day 14. For these treatment groups, relative differences in infection rates at day 2 ranged from reductions of 2.17% in the SP-AQ arm (95% CI -12.80% -15.14%) to increases in infectivity in the AS-AQ arm ('reduction' of -17.32%, 95% CI -42.92-3.69); neither of these findings were statistically significant. Taken together, AL performed significantly better at reducing mosquito infection rate within 48 hours of treatment, compared to the other ACT regimens DHA-PPQ, PY-AS and AS-AQ at day 2 ($p<0.0001$ compared to AL) (Figure 5A). The differences in reductions in infectivity became overall smaller at day 7 after treatment (Figure 5B), however, SP-AQ and PY-AS still only showed a <10% reduction, while the reduction in mosquito infection was increased in the DHA-PPQ and AS-AQ groups to 41.51% (95% CI 34.98% - 47.37%) and 55.32% (95% CI 39.21% - 67.16%), respectively

Over all treatment groups, 27 individuals became infectious to mosquitoes after treatment while initially not infecting mosquitoes; 13.92% (11/79) of participants in DHA-PPQ groups, 12.5% (5/40) in SP-AQ groups, 16% (4/25) in the PY-AS group, 10% (2/20) in the AS-AQ group, 1.67% (1/60) in AL groups, 0.72% (1/138) in the ACT plus primaquine groups and 7.5% (3/40) in ACT plus tafenoquine groups. The probability of infecting at least one mosquito was reduced predictably slower than the mosquito infection rate, with only the groups with PQ reaching >90% reduction in this probability at day 2, followed by AL that achieved >80% reduction at day 7 and ACT-TQ that achieved this at day 14 (supplementary Figure 8, supplementary Tables 18-20, appendix 1 pp 30-33). Relative reductions in oocyst density in dissected mosquitoes preceded the reduction in mosquito infection rates in certain treatment groups, such as DHA-PPQ, SP-AQ, PY-AS and AS-AQ, where a larger reduction in oocyst density was found prior to observing the same level of reduction in mosquito infection rate (supplementary Figure 9, supplementary tables 21-23, appendix 1 pp 34-37).

The clearance of infectivity (i.e. no longer infecting any mosquitoes) preceded the clearance of gametocytes assessed by both microscopy and RT-qPCR (Figure 6). The treatment groups with SLD PQ exhibited the fastest infectivity clearance, followed by those treated with AL (hazard ratios 0.62 ($p<0.0001$) and 0.66 ($p=0.0006$) compared to non-ACT-PQ and ACT-PQ, respectively). Infectivity was annulled by day 7 in the AL, ACT-PQ and non-ACT-PQ groups, and by day 14 in the ACT-TQ group, while this effect was slower in the SP-AQ (day 28), DHA-PPQ (day 35) and PY-AS (day 35) groups. Not all infectivity was cleared by the end of follow up (day 28) in the AS-AQ group (4.55 % of individuals still infectious, 95% CI 0.67-30.85). Hazard ratios and between-arm comparisons for the clearance of infectivity, adjusted by baseline PCR gametocyte densities, can be found in supplementary table 24 (appendix 1 p 38). ACT-PQ was significantly faster than all other regimens at gametocyte clearance ($p<0.0001$ in all comparisons; supplementary tables 25-26, appendix 1 pp 39-40). Gametocytes detectable by microscopy persisted after day 28 in the DHA-PPQ, SP-AQ, PY-AS, AL and non-ACT-PQ groups, while gametocytes were microscopically undetectable by day 14 in the ACT-PQ group and by day 21 in the ACT-TQ group. Gametocytes measured by RT-qPCR (limit of detection of 0.01 gametocytes/μL) persisted in a subset of individuals in all treatment groups until the end of follow-up.

DISCUSSION

This pooled analysis from individual patient data compares the transmission-blocking activity of different regimens of schizonticidal drugs with or without a single low-dose gametocytocide in *P. falciparum* gametocyte carriers in Mali. We found a marked difference between the different ACTs, with AL causing the largest reduction in mosquito infection rate (97.10%) within 48 hours of treatment initiation. The impact of DHA-PPQ, PY-AS and AS-AQ on transmission were significantly less than AL and led to prolonged gametocyte carriage and infectivity post-treatment. Adding a single low-dose of primaquine (0.25 or 0.5 mg/kg) to any ACT accelerated the clearance of gametocytes and led to a significantly greater reduction in mosquito infection rate within 48-hours of treatment.

The effectiveness and utility of the treatments in the six studies analysed here have been discussed in depth in the original trial reports (21–26). This combined analysis represents the first synthesis of this data and direct comparison of these treatments. A number of treatments were tested in multiple studies (DHA-PPQ, DHA-PPQ plus 0.25 mg/kg PQ, AL and SP-AQ) and our results were highly concordant across years (supplementary figure 7, appendix 1, p 29), evidence of the value of established transmission testing facilities and the appropriateness of a pooled analysis. Prior to these trials, there was sparse evidence of the gametocytocidal and transmission reducing efficacy for many of the included treatments. The available data frequently originated from single studies, used disparate and insensitive methodologies for gametocyte quantification, or did not conduct transmission assays to determine the transmissibility of gametocytes (28–32). This was the rationale for including only studies with highly similar enrolment criteria and assessments, including transmission assays before and after initiation of treatment.

This pooled analysis confirms SP-AQ's poor ability to clear gametocytes and reduce transmission, with a significantly lower relative reduction in mosquito infection rate at day 7 compared to DHA-

PPQ, AS-AQ, and AL. As the only non-artemisinin-based antimalarial recommended for systematic distribution via seasonal malaria chemoprevention (SMC) (33), SP-AQ's poor performance against gametocytes may reduce the overall community impact of SMC; adding a single low-dose of primaquine to SP-AQ for chemoprevention, would likely improve this.

This pooled analysis provides the first direct comparison of SLD primaquine and SLD tafenoquine with the same partner drug. Our analysis indicates a delayed but highly effective response of SLD tafenoquine compared to SLD primaquine when combined with ACTs, achieving a 99.37% reduction in mosquito infection rate at day 7 from baseline. Although the transmission-blocking efficacy of tafenoquine is dose-dependent, our pooled analysis combined the two highest dose groups (0.83 mg/kg and 1.66 mg/kg) for simplicity, possibly obscuring the effects of the higher dose. Despite these delayed transmission-blocking properties of TQ compared with SLD primaquine in our findings, its long half-life could be a major advantage and could be of relevance to prevent the transmission of drug resistant parasites, which may have an increased gametocyte conversion rate and transmission potential (34,35).

Before ACTs were introduced, several antimalarials including chloroquine and sulfadoxine-pyrimethamine were reported to increase gametocytaemia and/or infectivity after treatment (36,37). Our analysis, almost exclusively examining ACTs, did not find evidence for post-treatment enhancement of infectivity although we did observe that a non-negligible proportion of individuals became infectious after treatment (this proportion being ≥10% of participants in the DHA-PPQ, SP-AQ, PY-AS and AS-AQ groups); this percentage was below 2% in the remaining treatment groups, including AL. The fact that very few individuals become gametocyte positive or infectious after AL suggests that, among ACT regimens, AL may be superior at targeting immature or sequestered gametocytes.

The presence of gametocytes after treatment at densities that normally permit transmission does not make transmission an inevitability, because gametocytes may be sterilised or become non-infectious for other reasons before being cleared (12). In this pooled analysis, this discordance was most pronounced in the AL group and groups with SLD PQ, where post-treatment effects on infectivity preceded gametocyte clearance. In all other treatment groups, reductions in gametocyte density and mosquito infection rate followed more similar patterns. The combination of an ACT with SLD PQ was significantly faster than all other regimens at gametocyte clearance (100% of microscopical gametocytes by day 14), while ACT-TQ was the second fastest regimen to clear gametocytes (100% of microscopical gametocytes by day 21). Infectivity clearance was the fastest in the treatment groups with SLD PQ, followed by AL.

A few limitations in our study warrant consideration. Firstly, we established transmission-blocking activity in highly infectious individuals. This population allows a detailed assessment of transmission-blocking properties; however, post-treatment transmission potential is likely to be smaller in the majority of malaria patients who predominantly have submicroscopic gametocyte densities or may even be free of gametocytes at clinical presentation. A different study would be needed to determine the relative importance of sub- vs microscopic gametocyte carriage for malaria transmission in clinical patients though these would likely require larger sample sizes for mosquito infectivity endpoints. Secondly, our study utilized direct membrane feeding assays rather than direct skin feeding, which may have resulted in reduced infectivity, despite no compelling evidence of gametocytes sequestering in the skin (38). In addition, we measured oocyst prevalence instead of sporozoite prevalence and some infected mosquitoes may not have become infectious (39). Nevertheless, comparisons between groups remain valid, and we observed a strong association between oocyst prevalence and oocyst densities, indicating that a higher proportion of infected mosquitoes reflects a greater transmission potential. Lastly, this pooled analysis set out to assess the ability of antimalarial treatment to prevent the transmission of drug-resistant parasites.

Considering that gametocytes in *PfKelch13* mutant infections might preferentially survive artemisinin exposure and infect mosquitoes (35), our findings regarding ACTs may not be generalisable to areas where artemisinin partial resistance is present. In addition, increased gametocytaemia was found to be an early indicator of resistance emergence against previous non-ACT first-line treatments (34). Consequently, while our results indicate that AL is nearly as effective as ACT-PQ in reducing mosquito infection rates, it remains unclear whether this would extend to settings with artemisinin-resistant malaria infections. Our data on SLD gametocytocides in combination with ACTs therefore supports the 2023 advice from the WHO malaria policy and advisory group to expand the focus on reducing parasite transmission with SLD PQ in areas where partial artemisinin resistance has been detected (20). Additionally, we observed high transmission-blocking efficacy of AL when administered as a directly observed therapy (DOT); its efficacy may be lower in real-life settings where treatment adherence may be lower.

In conclusion, utilising individual patient-level data from six clinical trials conducted at the same study site with highly consistent transmission experiments across trials, we showed pronounced differences in anti-gametocyte and anti-transmission effects between ACTs, with AL showing the most effectiveness in blocking transmission. Additionally, our findings confirm the rapid effects of SLD PQ in clearing and sterilising gametocytes when used in combination with an ACT, while the addition of an SLD TQ to ACTs has a delayed transmission-blocking effect compared to SLD PQ. Lastly, our analysis confirms considerably higher post-treatment transmission after SP-AQ compared to most ACTs, and adding an SLD PQ to SP-AQ may be beneficial to block malaria transmission in community treatment campaigns.

Data Availability Statement

All trial data are available through a novel open-access clinical epidemiology database resource, ClinEpiDB; and code for analysis and the selection of data used for this analysis can be found on https://github.com/leenvh/NECTAR-pooled-analysis.

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Competing interests

The authors have declared that no competing interests exist

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Author contributions

LNV, JR, AM, CD, AD, WS and TB conceived the study. AM, MJS, MER, LNV, KS, YS, SMN, MD, SM, SK, SS, AY, HD, SFT, RG, JMB, CD, AD, WS and TB conceived and undertook

individual studies. KL performed molecular analyses. JR, LNV, AM, RG, JMB, CD, AD, WS and TB analysed the data and interpreted results. LNV, JR, WS and TB wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and read and approved the final manuscript.

TABLES

Table 1. Overview of the included studies. Rows in grey indicate treatment groups that were not included in the analyses.

Study	Year	Treatment group	Number of participants	Treatment category	Study population	Reference
PQ01	2013-2014	DHA-PPQ	16	DHA-PPQ	G6PD+ males, 5-50 years, ≥32 gametocytes/μL	(24)
		DHA-PPQ + 0.0625 mg/kg PQ	16	/		
		DHA-PPQ + 0.125 mg/kg PQ	17	/		
		DHA-PPQ + 0.25 mg/kg PQ	15	ACT-PQ		
		DHA-PPQ + 0.5 mg/kg PQ	17	ACT-PQ		
PQ03	2016	SP-AQ	20	SP-AQ	G6PD+ males, 5-50 years, asymptomatic, ≥16 gametocytes/μL	(23)
		SP-AQ + 0.25 mg/kg PQ	20	Non-ACT-PQ		
		DHA-PPQ	20	DHA-PPQ		
		DHA-PPQ + MB	20	/		
NECTAR1	2019	DHA-PPQ	25	DHA-PPQ	Males and non-pregnant females, 5-50 years, asymptomatic, ≥16 gametocytes/μL	(25)
		DHA-PPQ + 0.25 mg/kg PQ	25	ACT-PQ		
		PY-AS	25	PY-AS		
		PY-AS + 0.25 mg/kg PQ	25	ACT-PQ		
NECTAR2	2020	DHA-PPQ	20	DHA-PPQ	G6PD+ males and non-pregnant females, 12-50 years, asymptomatic, ≥16 gametocytes/μL	(22)
		DHA-PPQ + 0.42 mg/kg TQ	20	/		
		DHA-PPQ + 0.83 mg/kg TQ	20	ACT-TQ		
		DHA-PPQ + 1.66 mg/kg TQ	20	ACT-TQ		
NECTAR3	2021	AL	20	AL	G6PD+ males and non-pregnant females, 10-50 years, asymptomatic, ≥16 gametocytes/μL	(21)
		AL + 0.25 mg/kg PQ	20	ACT-PQ		
		SP-AQ	20	SP-AQ		
		SP-AQ + 1.66 mg/kg TQ	20	/		
NECTAR4	2022	AL	20	AL	Males and non-pregnant females, 10-50 years, asymptomatic, ≥16 gametocytes/μL	(26)
		AL-AQ	20	AL		
		AL-AQ + 0.25 mg/kg PQ	20	ACT-PQ		
		AS-AQ	20	AS-AQ		
		AS-AQ + 0.25 mg/kg PQ	20	ACT-PQ		

FIGURES

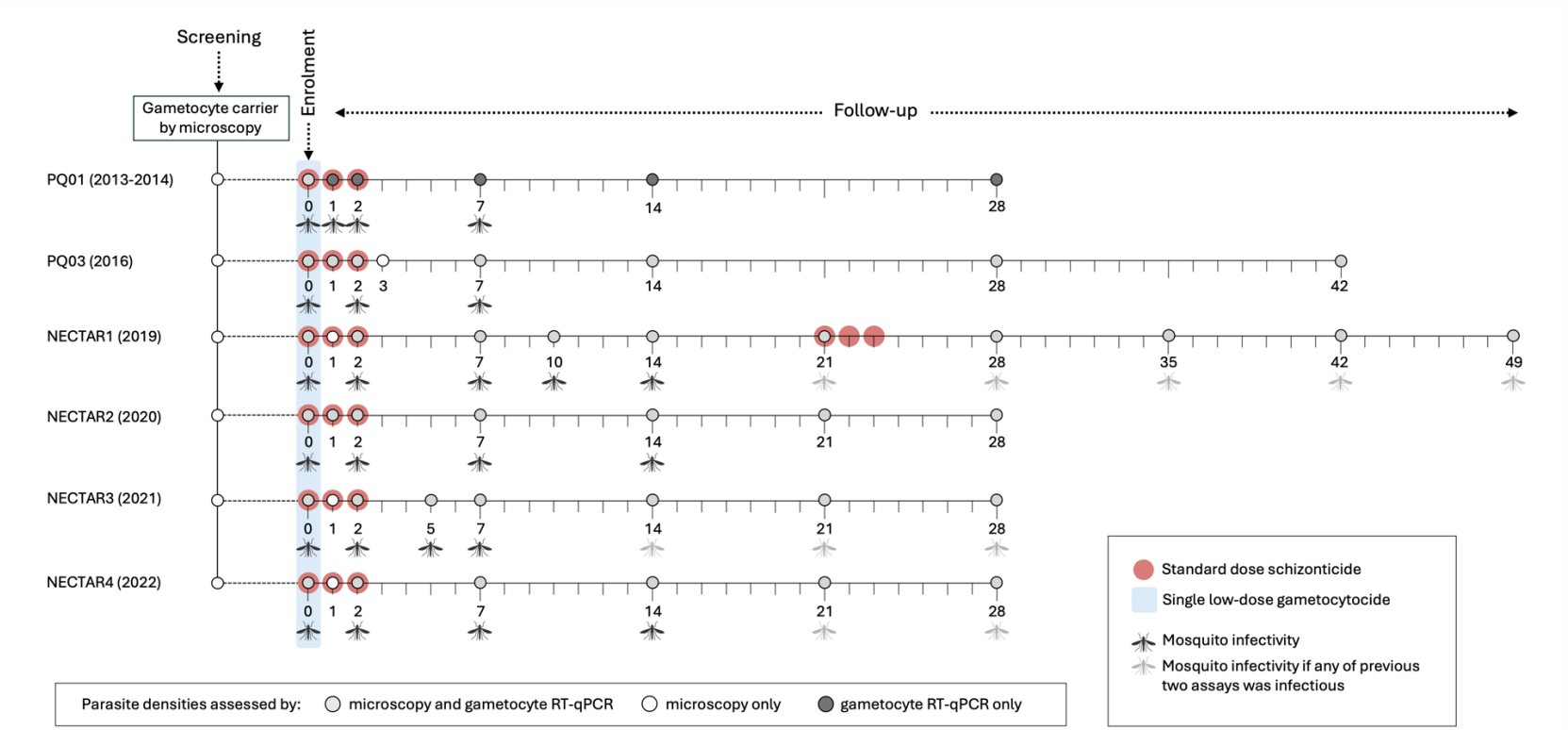

Figure 1. Study design of included trials. All studies assessed infectivity to mosquitoes before (d0), during (d2) and one and two weeks after initiation of treatment (d7 and d14) with additional time-points that differed between studies. Tick marks represent days; circles indicate sampling/screening time points. Circles that are encompassed by larger red circles indicate that a standard dose schizonticide was administered at these study visits, while a single low-dose gametocytocide was administered at the time points with a larger blue circle. Grey coloured circles indicate that parasite densities were assessed by both microscopy (asexual parasite and gametocyte densities) and RT-qPCR (gametocyte densities). At other study visits, parasite densities were determined by microscopy only (white circles) or RT-qPCR only (dark grey circles). Mosquito infectivity assays were conducted at the study visits marked with a black mosquito symbol, while a grey mosquito symbol indicates that mosquito infectivity assays were only conducted at that time point if any of the previous two assays resulted in at least one infected mosquito.

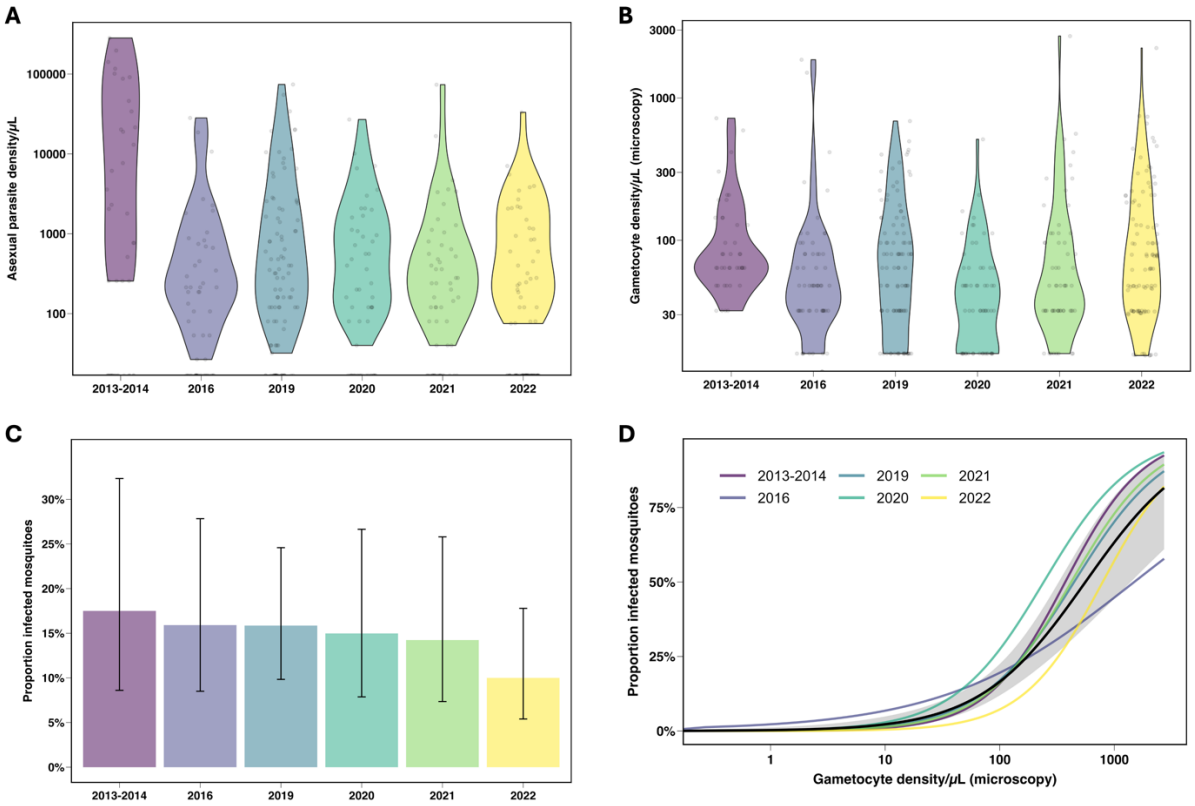

Figure 2. Baseline study characteristics. **A.** Violin plot of microscopical asexual parasite density (parasites/µL) distribution per study. **B.** Violin plot of microscopical gametocyte density (gametocytes/µL) distribution per study. **C**. The mean proportion of mosquitoes that became infected after feeding on venous blood collected at enrolment, prior to treatment, per study. Vertical bars represent 95% CIs estimated from a logistic regression model. **D**. Results from a logistic regression between microscopically determined gametocyte densities (gametocytes / µL) on a log10 scale and the proportion of infected mosquitoes over the different study years shown by the different colours. Mosquito feeding assays in this analysis were conducted before treatment was initiated. The black line indicates the overall trend averaged across all years with the shaded area showing the 95% confidence interval for this overall fit. Visualisations represent a total of 422 observations, from 42 (2013-2014), 60 (2016), 100 (2019), 60 (2020), 60 (2021) and 100 (2022) study participants. The median number of dissected mosquitoes per study participant (panel C) was 71.8 (IQR 65.6-77) for 2013-2014, 79 (IQR 72-84) for 2016, 64 (IQR 57-70) for 2019, 60 (IQR 51.8-66.5) for 2020, 62 (IQR 53.8-64.2) for 2021 and 61 (IQR 55-66) for 2022.

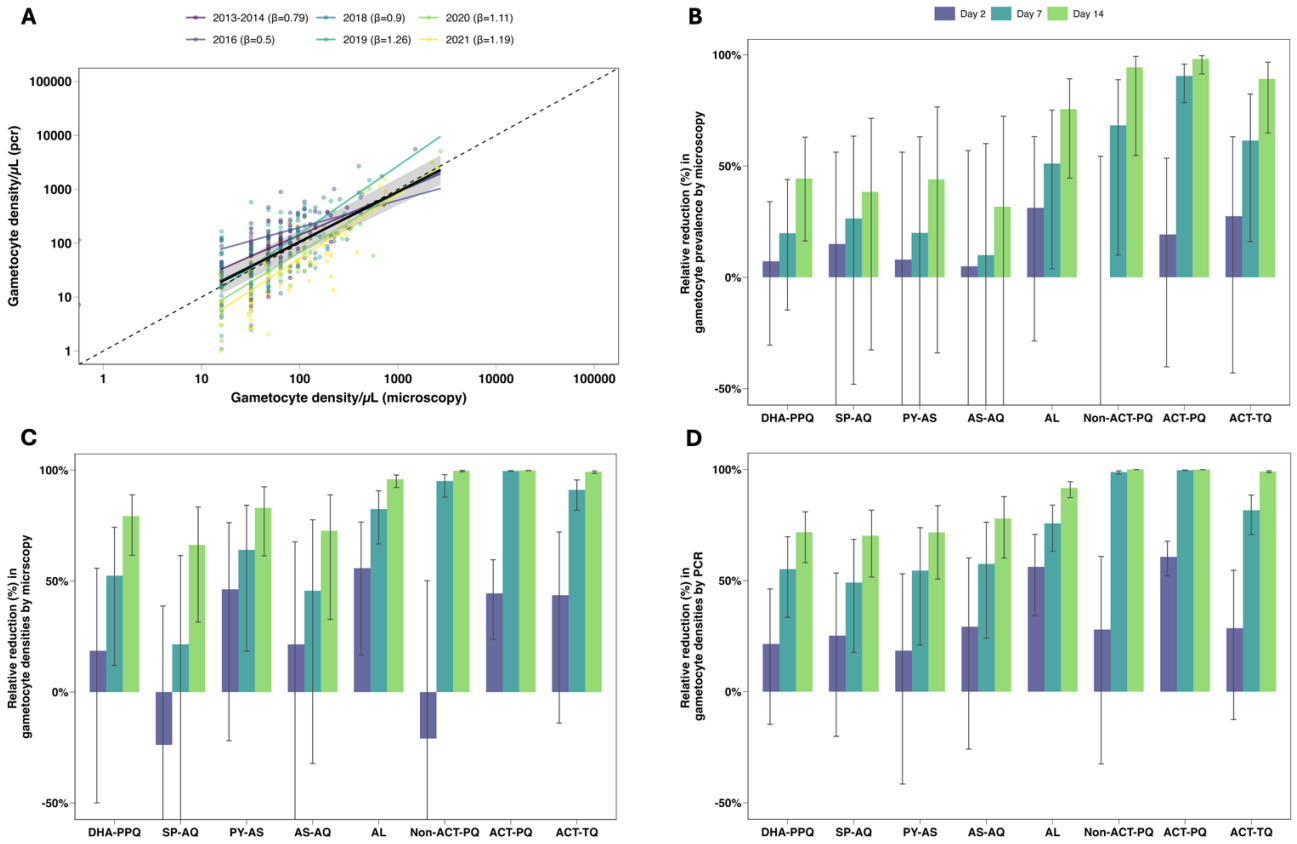

Figure 3. Gametocyte prevalence and densities. **A.** scatter plot of the association between gametocyte density measured by microscopy (x-axis) and RT-qPCR (y-axis) pre-treatment, both on a log10 scale. A linear model with an interaction by year was used to estimate the study specific trend lines (shown in colour with estimated slopes (β) indicated in the legend). A linear mixed effects model was used to estimate the trend line averaged across the study years (β=0.94) shown by the black line, and the corresponding 95% CI, shown by the grey shaded area. The dashed line represents the line of equality where measurements by microscopy and PCR would be equal. **B-D.** Bar charts illustrating the relative reduction compared to baseline in gametocyte prevalence by microscopy (**B**), and gametocyte densities measured by microscopy (**C**) and molecular methods (**D**), by treatment arm over three time points (Day 2 - indigo, Day 7 - turquoise, and Day 14 - green). Vertical bars depict the 95% confidence intervals for these estimates. Due to inflated standard errors the y-axis was cut off below -50. Visualisations represent data from 422 individuals at baseline (79, 40, 25, 20, 60, 20, 138 and 40 individuals from the DHA-PPQ, SP-AQ, PY-AS, AS-AQ, AL, non-ACT-PQ, ACT-PQ and ACT-TQ groups, respectively). At day 2, 369 individuals were included (65, 39, 23, 20, 58, 19, 105 and 40 individuals from the DHA-PPQ, SP-AQ, PY-AS, AS-AQ, AL, Non-ACT-PQ, ACT-PQ and ACT-TQ groups, respectively). Data from 357 individuals at day 7 is shown (57, 38, 24, 20, 57, 19, 104 and 38 individuals from the DHA-PPQ, SP-AQ, PY-AS, AS-AQ, AL, Non-ACT-PQ, ACT-PQ and ACT-TQ groups, respectively). Day 14 includes data from 357 individuals (60, 38, 24, 19, 57, 18, 105 and 36 individuals from the DHA-PPQ, SP-AQ, PY-AS, AS-AQ, AL, Non-ACT-PQ, ACT-PQ and ACT-TQ groups, respectively

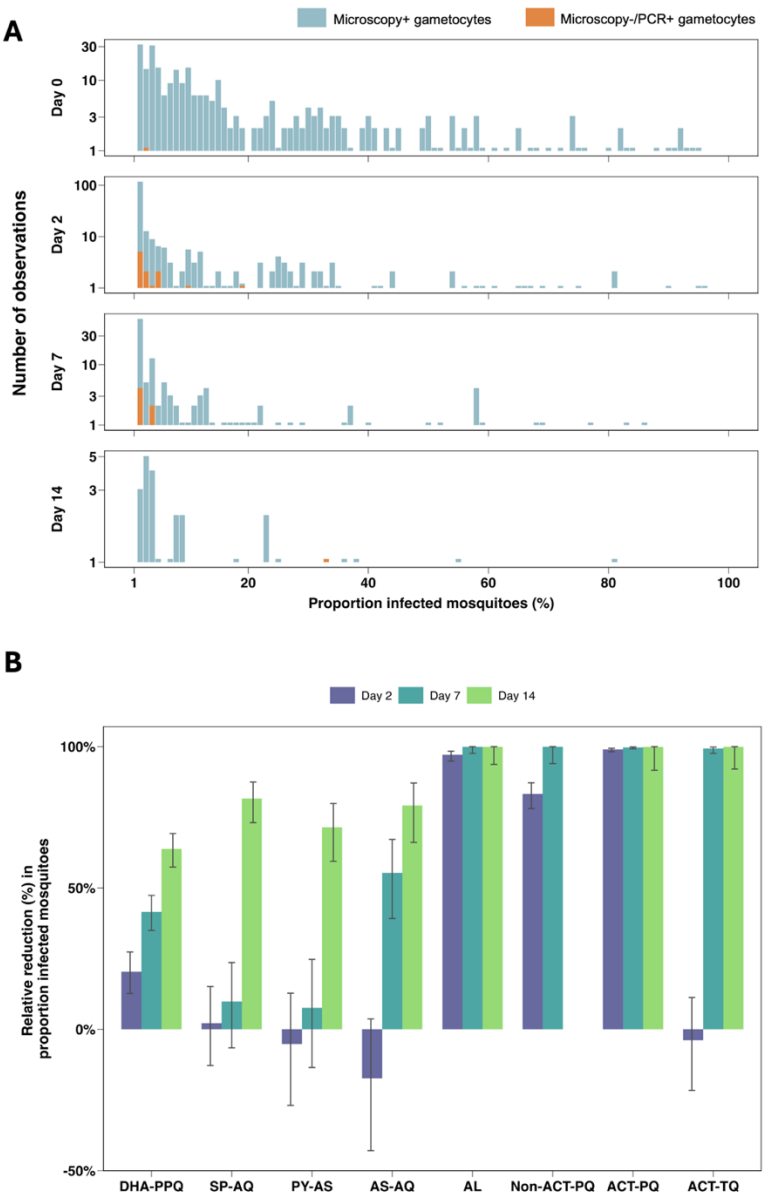

Figure 4. Infectivity of submicroscopic gametocyte infections and the relative reduction in the proportion of infected mosquitoes compared to baseline per treatment category. A. Stacked bar chart representing the number of observations for each proportion of infected mosquitoes (rounded to the nearest integer) at baseline and at days 2,7 and 14 post-treatment initiation. For each day of follow-up, individual study participants contribute a single observation. Bars are coloured by the presence of gametocytes by microscopy (blue) or by PCR only (orange). Baseline visualisations represent a total of 422 study participants; per study 42 (2013-2014), 60 (2016), 100 (2019), 60 (2020), 60 (2021) and 100 (2022) participants were enrolled and presented here. At day 2, data from 375 individuals are presented (60, 99, 60, 58, 98 participants from the 2016, 2019, 2020, 2021 and 2022 studies, respectively). At day 7 post-treatment initiation, 367 participants are presented (56, 98, 59, 58 and 97 participants from the 2016, 2019, 2020, 2021 and 2022 studies, respectively). Finally, at day 14, data from 218 individuals are presented (47, 56, 17 and 96 participants from the 2019, 2020, 2021 and 2022 studies, respectively). **B.** Bar chart illustrating the relative reduction compared to baseline in the proportion of infected mosquitoes by treatment arm over three time points (Day 2 - indigo, Day 7 - turquoise, and Day 14 - green). Vertical bars depict the 95% confidence intervals for these estimates. Visualisations represent data from 416 individuals at baseline (79, 40, 25, 20, 60, 20, 139 and 40 individuals from the DHA-PPQ, SP-AQ, PY-AS, AS-AQ, AL, non-ACT-PQ, ACT-PQ and ACT-TQ groups, respectively). At day 2, 416 individuals were included (78, 40, 25, 20, 58, 20, 135 and 40 individuals from the DHA-PPQ, SP-AQ, PY-AS, AS-AQ, AL, Non-ACT-PQ, ACT-PQ and ACT-TQ groups, respectively). Data from 409 individuals at day 7 is shown (76, 39, 25, 20, 57, 19, 134 and 39 individuals from the DHA-PPQ, SP-AQ, PY-AS, AS-AQ, AL, Non-ACT-PQ, ACT-PQ and ACT-TQ groups, respectively). Day 14 includes data from 218 individuals (43, 15, 25, 19, 39, 40 and 37 individuals from the DHA-PPQ, SP-AQ, PY-AS, AS-AQ, AL, ACT-PQ and ACT-TQ groups, respectively).

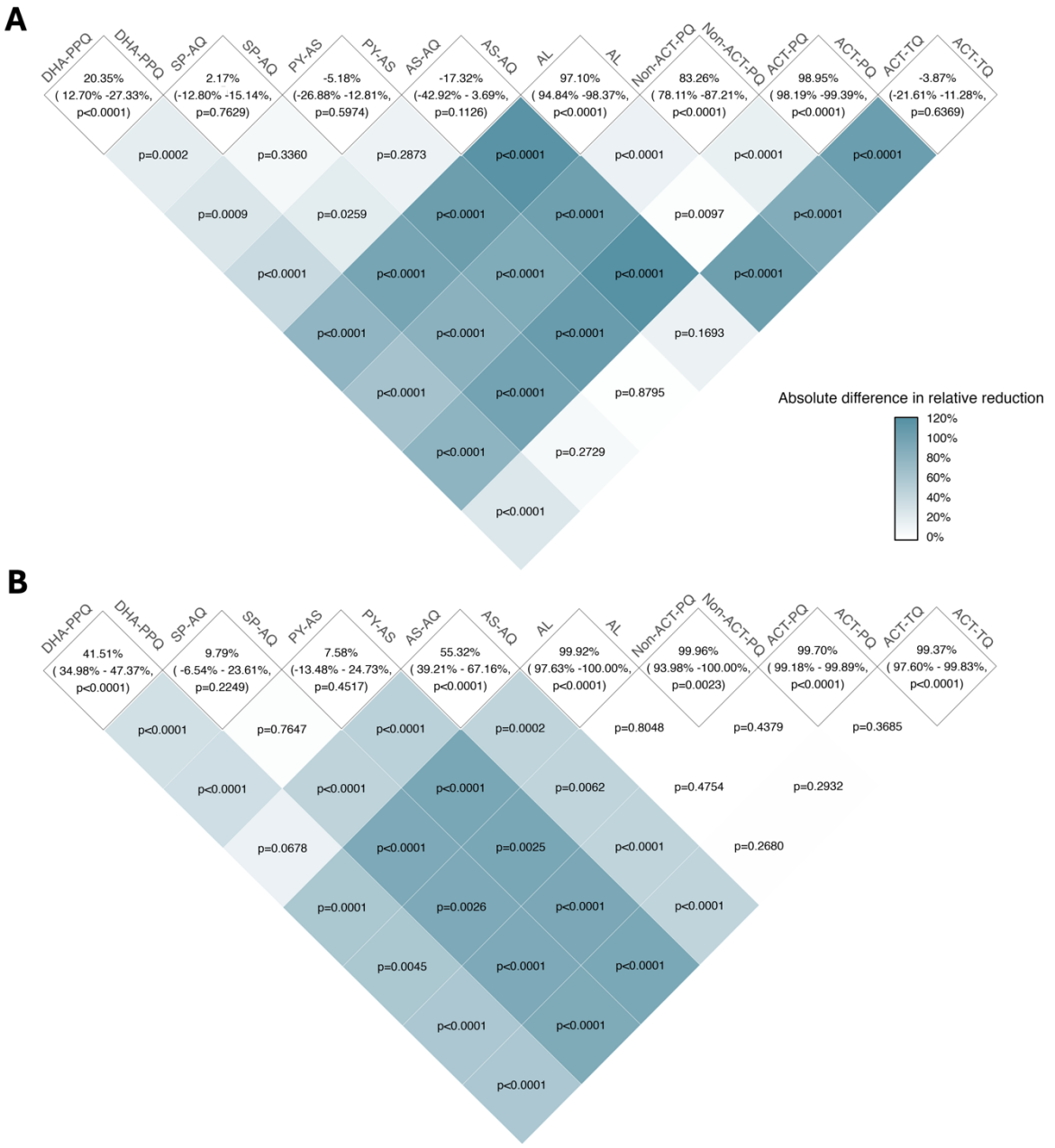

Figure 5. Between-group comparison of the reduction in proportion infected mosquitoes at days 2 and 7 post-treatment, compared to baseline. Heatmaps representing the percentage reduction in the proportion infection mosquitoes per treatment category at day 2 (**A**) and day 7 (**B**) compared to baseline in the top cells, with 95% confidence intervals and p-value comparing to baseline. Other cells in the heatmap represent the p-values comparing treatment groups. Heatmap cells are coloured by the absolute difference between treatment groups in the relative reduction in proportion infected mosquitoes at days 2 and 7 post-treatment, compared to baseline. For example, DHA-PPQ achieves 20.35% reduction in the proportion of infected mosquitoes by day 2 compared to 97.10% for AL; the difference between these arms (76.75% lower reduction for DHA-PPQ) is statistically significant ($p<0.0001$).

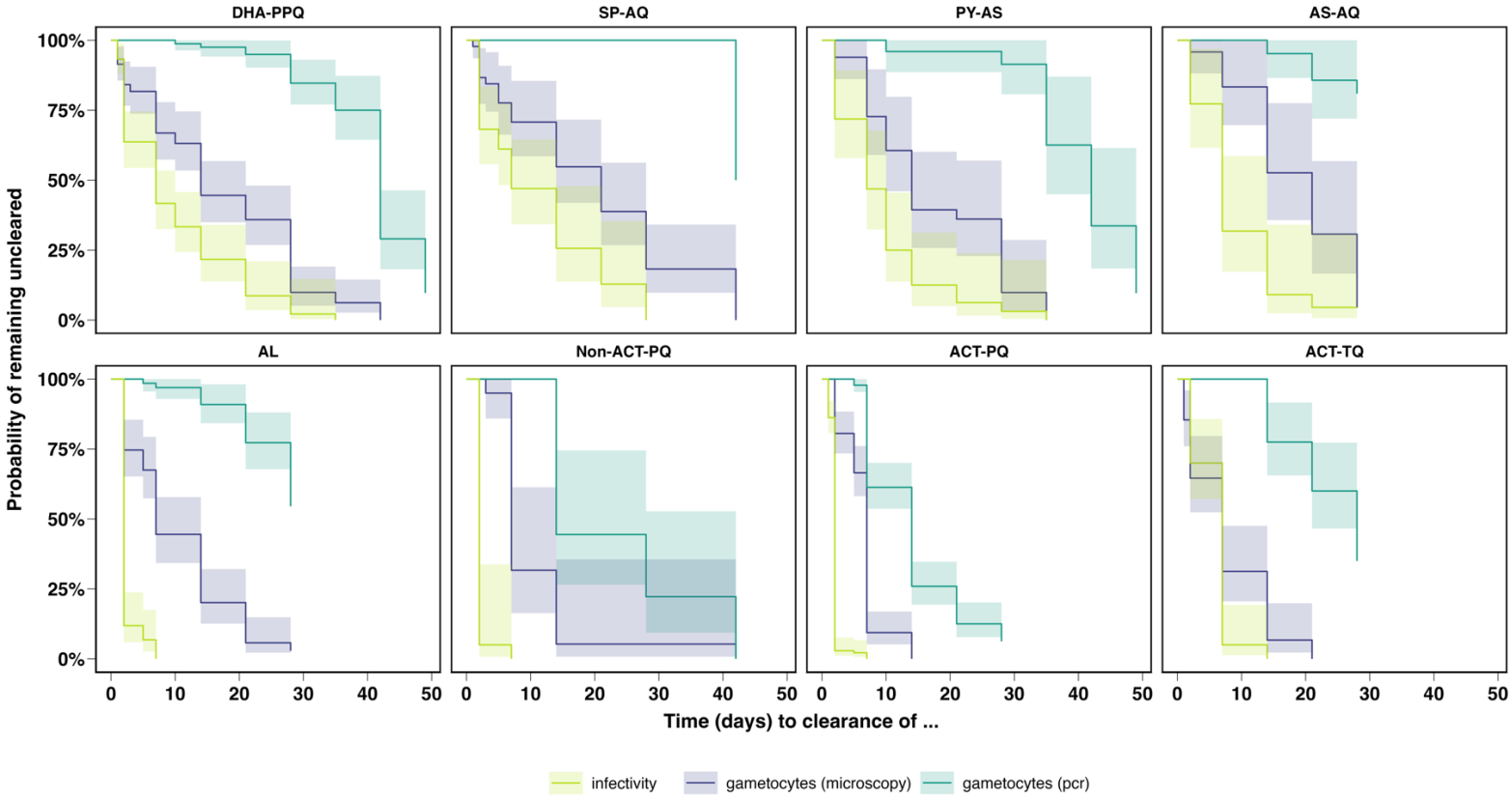

Figure 6. Time to clearance of infectivity and gametocytes per treatment category. Kaplan-Meier survival curves showing the cumulative probability of remaining uncleared of gametocytes detected by microscopy (purple), gametocytes detected by PCR (turquoise) and infectivity to mosquitos (green) over time stratified across different antimalarial treatment categories (DHA-PPQ, SP-AQ, PY-AS, AS-AQ, AL, Non-ACT-PQ, ACT-PQ, ACT-TQ). Shaded areas indicate 95% confidence intervals for the Kaplan-Meier survival estimates. Survival curves showing infectivity represent data from 417 individuals (79, 40, 25, 20, 58, 20, 135 and 40 individuals from the DHA-PPQ, SP-AQ, PY-AS, AS-AQ, AL, Non-ACT-PQ, ACT-PQ and ACT-TQ groups, respectively). Survival curves visualising gametocytes by microscopy show data from 375 individuals (65, 40, 25, 20, 58, 20, 107 and 40 individuals from the DHA-PPQ, SP-AQ, PY-AS, AS-AQ, AL, Non-ACT-PQ, ACT-PQ and ACT-TQ groups, respectively) and 417 individuals (79, 40, 25, 20, 58, 20, 135 and 40 individuals from the DHA-PPQ, SP-AQ, PY-AS, AS-AQ, AL, Non-ACT-PQ, ACT-PQ and ACT-TQ groups, respectively) for gametocyte assessment by RT-qPCR.

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APPENDIX 1

Supplementary Information 1. Antimalarial treatment dosage

Sulphadoxine-Pyrimethamine plus Amodiaquine

SP tablets containing 500 mg sulfadoxine and 25 mg pyrimethamine and AQ tablets containing 150 mg amodiaquine were administered according to weight as per manufacturer guidelines shown below:

Body weight	**500/50 mg sulfadoxine/pyrimethamine tablet**		
	Day 0	**Day 1**	**Day 2**
11 to 20 kg	1x 1 tablet	1x 1 tablet	1x 1 tablet
21 to 30 kg	1x 1·5 tablets	1x 1·5 tablets	1x 1·5 tablets
31 to 45 kg	1x 2 tablets	1x 2 tablets	1x 2 tablets
> 45 kg	1x 3 tablets	1x 3 tablets	1x 3 tablets
Body weight	**150 mg amodiaquine tablet**		
	Day 0	**Day 1**	**Day 2**
15 to 18 kg	1x 1·5 tablets	1x 1 tablet	1x 1 tablet
19 to 24 kg	1x 1·5 tablets	1x 1·5 tablets	1x 1·5 tablets
25 to 35 kg	1x 2·5 tablets	1x 2·5 tablets	1x 2 tablets
36 to 50 kg	1x 3 tablets	1x 3 tablets	1x 3 tablets
> 50 kg	1x 4 tablets	1x 4 tablets	1x 3 tablets

Artemether-lumefantrine

AL treatment tablets containing 20/120 mg artemether/lumefantrine or 80/480 mg artemether/lumefantrine were administered according to weight as per manufacturer guidelines shown below:

Body weight (kg)	**20/120 mg artemether/lumefantrine tablet**			**80/480 mg artemether/lumefantrine tablet**		
	Day 0	**Day 1**	**Day 2**	**Day 0**	**Day 1**	**Day 2**
5 to < 15 kg	2x 1 tablet	2x 1 tablet	2x 1 tablet	-	-	-
15 to < 25 kg	2x 2 tablets	2x 2 tablets	2x 2 tablets	-	-	-
25 to < 35 kg	2x 3 tablets	2x 3 tablets	2x 3 tablets	-	-	-
≥ 35 kg	2x 4 tablets	2x 4 tablets	2x 4 tablets	2x 1 tablet	2x 1 tablet	2x 1 tablet

Dihydroartemisinin-Piperaquine

Treatment tablets containing 160/320 mg piperaquine with 20/40 mg dihydroartemisinin tablets were administered according to weight as per manufacturer guidelines shown below:

Body weight (kg)	**Total daily dose (mg) (1x/day for 3 days)**		**Tablet strength and number of tablets per dose**
	Piperaquine	DHA	
5 to <7	80	10	½ x 160mg / 20mg
7 to <13	160	20	1 x 160mg / 20mg
13 to <24	320	40	1 x 320mg / 40mg
24 to <36	640	80	2 x 320mg / 40mg
36 to <75	960	120	3 x 320mg / 40mg
75 to 80	1,280	160	4 x 320mg / 40mg
>80	Not eligible		

Artesunate-Amodiaquine

Tablets contained 50mg/135 mg or 100mg/270 mg of artesunate/amodiaquine and were administered according to manufacturer guidelines, as shown below:

Weight	**Tablets**	**D0**	**D1**	**D2**
9 to < 18 kg	50 mg AS/135 mg AQ base	1 tab	1 tab	1 tab
18 to < 36 kg	100 mg AS/270 mg AQ base blister pack of 3 tab	1 tab	1 tab	1 tab
≥ 36 kg	100 mg AS/270 mg AQ base blister pack of 6 tab	2 tab	2 tab	2 tab

Pyronaridine-Artesunate

PY-AS granules containing 60 mg pyronaridine-tetraphosphate/20mg artesunate were administered to children <20kg, and PY-AS tablets containing 180 mg pyronaridine-tetraphosphate/60mg artesunate were administered to children and adults >20kg, according to weight as per manufacturer guidelines shown below:

Granules (Children <20kg)			
Body weight (kg)	**Total daily dose (mg) (1x/day for 3 days)**		**Sachet strength and number of tablets per dose**
	Pyronaridine-tetraphosphate	Artesunate	
5 - <8kg	60	20	1 x 60mg/20mg
8 - <15kg	120	40	2 x 60mg/20mg
15 - <20kg	180	60	3 x 60mg/20mg

Tablets (Children and adults >20kg)			
Body weight (kg)	**Total daily dose (mg) (1x/day for 3 days)**		**Tablet strength and number of tablets per dose**
	Pyronaridine-tetraphosphate	Artesunate	
20 - <24kg	180	60	1 x 60mg/20mg
24 - <45kg	360	120	2 x 60mg/20mg
45-<65kg	540	180	3 x 60mg/20mg
>65kg	720	240	4 x 180mg/60mg

Primaquine

Primaquine tablets were dissolved to a 1 mg/mL solution in distilled water and administered orally to the nearest mL, according to bodyweight at 0·25 mg/kg. Primaquine was administered as a single dose immediately after the first dose of ACT.

Tafenoquine

100mg Tafenoquine tablets were available for this study, and were prepared into a 1mg/mL solution in water for weight-based dosing in 5 kg bands as follows:

1.66 mg/kg Tafenoquine

Weight min	Weight max	TQ 1mg/mL total (mL)	Water (mL)	Masking solution (mL)
30	35	54.0	136.1	10
35.01	40	62.3	127.7	10
40.01	45	70.6	119.4	10
45.01	50	78.9	111.1	10
50.01	55	87.2	102.8	10
55.01	60	95.5	94.5	10
60.01	65	103.8	86.2	10
65.01	70	112.1	77.9	10
70.01	75	120.4	69.6	10
75.01	80	128.7	61.3	10

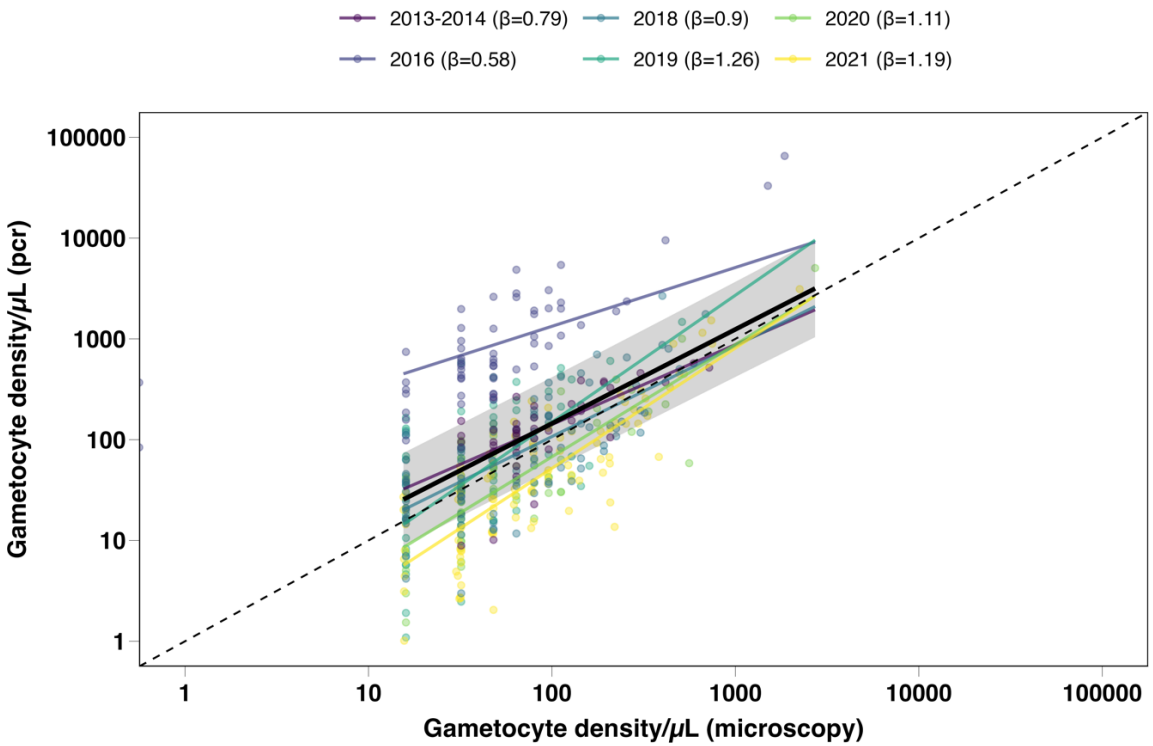
0.83 mg/kg Tafenoquine

Weight min	Weight max	TQ 1mg/mL total (mL)	Water (mL)	Masking solution (mL)
30	35	27.0	163.0	10
35.01	40	31.1	158.9	10
40.01	45	35.3	154.7	10
45.01	50	39.4	150.6	10
50.01	55	43.6	146.4	10
55.01	60	47.7	142.3	10
60.01	65	51.9	138.1	10
65.01	70	56.0	134.0	10
70.01	75	60.2	129.8	10
75.01	80	64.3	125.7	10

Supplementary Table 1. Antimalarial treatment suppliers

Study	Study drug	Supplier
PQ01	Primaquine	Sanofi, Laval, QC, Canada
	Dihydroartemisinin-piperaquine (Eurartesim)	Sigma-Tau, Pomezia, Italy
PQ03	Primaquine	Sanofi, Laval, QC, Canada
	Sulfadoxine-pyrimethamine (Fansidar)	Guilin Pharmaceutical, Shanghai, China
	Amodiaquine	Guilin Pharmaceutical, Shanghai, China
	Dihydroartemisinin-piperaquine (Eurartesim)	Sigma-Tau, Pomezia, Italy
NECTAR1	Pyronaridine-artesunate (Pyramax)	Shin Poong Pharmaceutical, Seoul, South Korea
	Primaquine	ACE Pharmaceuticals, Zeewolde, the Netherlands
	Dihydroartemisinin-piperaquine (Eurartesim)	Sigma Tau, Gaithersburg, MD, USA
NECTAR2	Dihydroartemisinin-piperaquine (Eurartesim)	Sigma Tau, Gaithersburg, MD, USA
	Tafenoquine	60° Pharmaceuticals Ltd, USA
NECTAR3	Artemether-lumefantrine (Coartem)	Novartis, Basel, Switzerland
	Primaquine	ACE Pharmaceuticals, Zeewolde, the Netherlands
	Sulfadoxine-pyrimethamine plus amodiaquine	Guilin Pharmaceutical, Shanghai, China
	Tafenoquine	60° Pharmaceuticals Ltd, USA
NECTAR4	Artemether-lumefantrine	Guilin Pharmaceutical, Shanghai, China
	Amodiaquine	Guilin Pharmaceutical, Shanghai, China
	Primaquine	ACE Pharmaceuticals, Zeewolde, The Netherlands
	Artesunate-amodiaquine	Guilin Pharmaceutical, Shanghai, China

Supplementary Figure 1. Original PQ03 molecular gametocyte densities



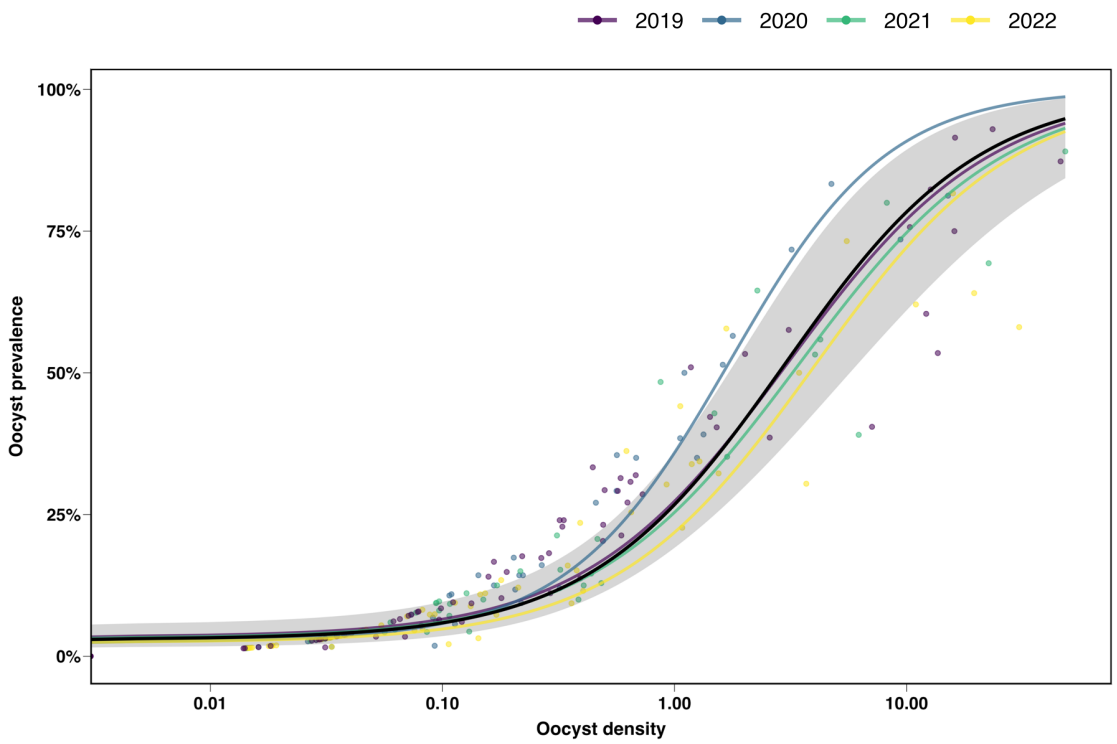
Scatter plot of the association between gametocyte density measured by microscopy (x-axis) and RT-qPCR (y-axis) pre-treatment, both on a log10 scale. The original PQ03 (2016) RT-qPCR data is presented here, which showed that the association with microscopy gametocyte density for this study was an outlier compared to the other studies. Following this, the molecular gametocyte quantification was repeated for this study.

A linear model with an interaction by year was used to estimate the study specific trend lines (shown in colour with estimated slopes (β) indicated in the legend). A linear mixed effects model was used to estimate the trend line averaged across the study years (β=0.94) shown by the black line, and the corresponding 95% CI, shown by the grey shaded area. The dashed line represents the line of equality where measurements by microscopy and PCR would be equal.

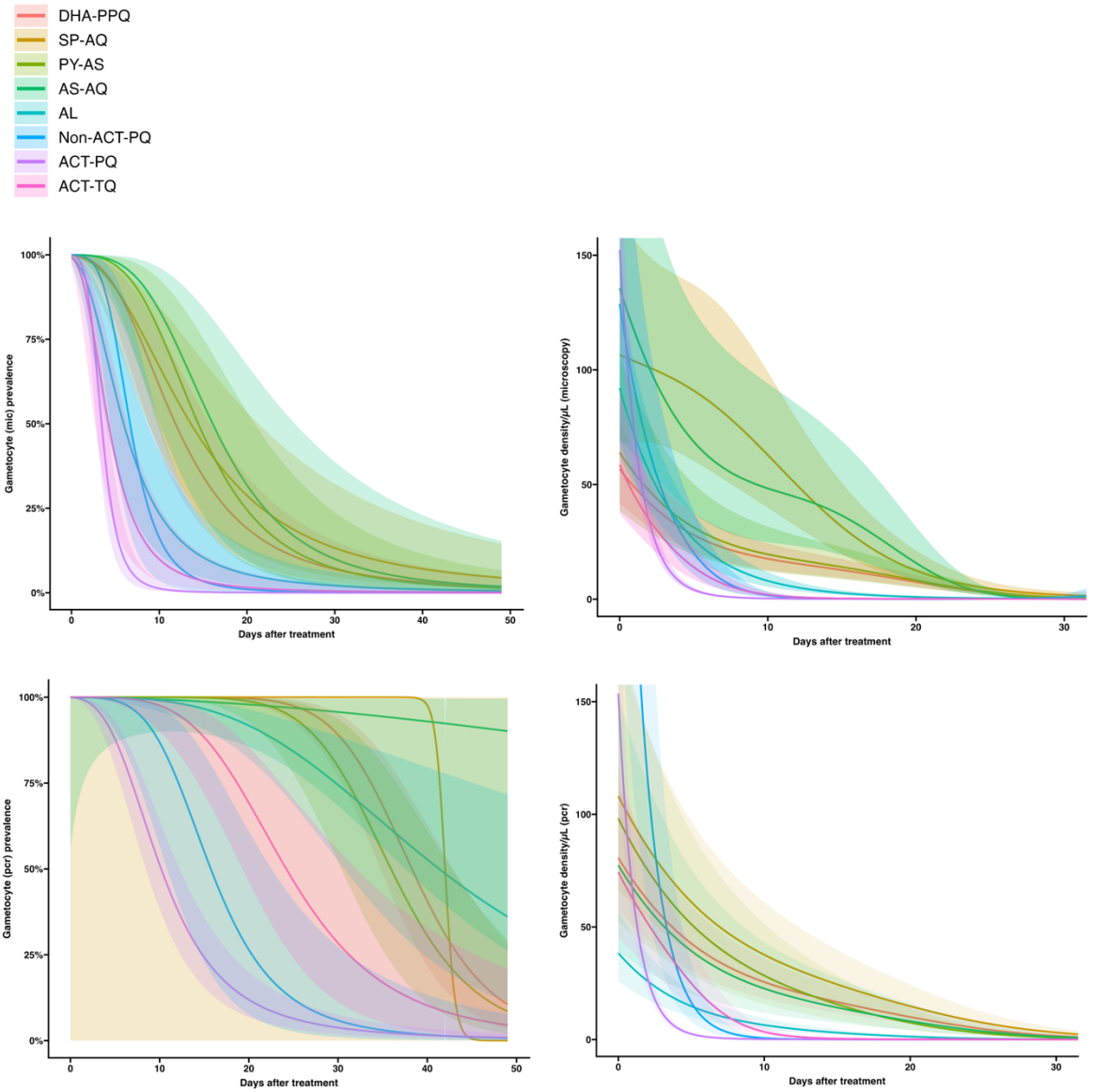
Supplementary Table 2. Baseline descriptives per treatment group

Treatment	N (%)	Males	Age	Temperature	Total parasites/uL	Gametocytes/uL (microscopy)	Gametocytes/uL (pcr)	Female gametocytes/uL (pcr)	Proportion infected	Gametocyte prevalence	Asexual parasite prevalence	Proportion participants infectious
DHA-PPQ	79 (18.8%)	59 (74.7%)	16.5 (10.3)	36.5 (0.4)	224.0 (76.0 - 840.0)	48.0 (16.0 - 80.0)	80.3 (35.6 - 128.4)	60.0 (20.8 - 115.7)	3.0 (0.0 - 14.7)	77 (97.5%)	53 (67.1%)	50 (63.3%)
SP-AQ	40 (9.5%)	29 (72.5%)	17.2 (11.4)	36.6 (0.5)	288.0 (106.0 - 868.0)	48.0 (32.0 - 112.0)	65.2 (30.3 - 183.3)	44.5 (15.1 - 129.2)	5.2 (0.0 - 10.4)	40 (100.0%)	26 (65.0%)	28 (70.0%)
PY-AS	25 (5.9%)	16 (64.0%)	13.0 (7.7)	36.5 (0.6)	216.0 (64.0 - 704.0)	64.0 (32.0 - 96.0)	83.2 (35.9 - 121.7)	46.0 (25.6 - 74.5)	3.0 (0.0 - 13.7)	25 (100.0%)	13 (52.0%)	17 (68.0%)
AS-AQ	20 (4.7%)	9 (45.0%)	14.4 (6.3)	36.4 (0.3)	386.3 (162.0 - 1498.4)	79.0 (41.5 - 232.1)	52.5 (34.2 - 107.3)	28.1 (11.3 - 38.9)	3.9 (1.6 - 17.9)	20 (100.0%)	8 (40.0%)	17 (85.0%)
AL	60 (14.2%)	27 (45.0%)	18.9 (11.1)	36.5 (0.4)	154.5 (48.0 - 411.0)	48.0 (31.7 - 124.8)	32.2 (12.8 - 95.8)	15.6 (6.6 - 38.6)	3.1 (0.0 - 11.7)	60 (100.0%)	29 (48.3%)	35 (58.3%)
Non-ACT-PQ	20 (4.7%)	20 (100.0%)	10.3 (3.6)	36.8 (0.4)	112.0 (80.0 - 233.3)	72.0 (32.0 - 96.0)	198.8 (63.3 - 435.7)	171.0 (55.1 - 332.6)	21.6 (4.3 - 33.1)	20 (100.0%)	12 (60.0%)	19 (95.0%)
ACT-PQ	138 (32.7%)	81 (58.7%)	15.0 (10.0)	36.4 (0.4)	328.0 (96.0 - 1473.9)	70.5 (32.0 - 144.0)	66.3 (24.8 - 165.6)	35.5 (12.7 - 109.3)	5.2 (0.0 - 16.3)	138 (100.0%)	83 (60.1%)	96 (69.6%)
ACT-TQ	40 (9.5%)	28 (70.0%)	20.2 (10.9)	36.5 (0.4)	156.0 (64.0 - 1092.0)	48.0 (32.0 - 64.0)	45.2 (19.1 - 204.2)	22.3 (11.0 - 88.5)	5.2 (0.0 - 18.8)	40 (100.0%)	23 (57.5%)	28 (70.0%)

Supplementary Figure 2. Relation between oocyst density and prevalence



Supplementary Figure 3. Gametocyte prevalence and density during follow up per treatment category



Supplementary Table 3. Relative reduction in gametocyte prevalence by microscopy at day 2 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	7.21% (-30.47% - 34.01%, p=0.6668)	p=0.7632	p=0.9799	p=0.9484	p=0.2631	p=0.8356	p=0.5300	p=0.4102
SP-AQ	p=0.7632	15.00% (-65.17% - 56.26%, p=0.6316)	p=0.8312	p=0.7790	p=0.4931	p=0.6792	p=0.8491	p=0.6374
PY-AS	p=0.9799	p=0.8312	8.00% (-93.53% - 56.27%, p=0.8261)	p=0.9407	p=0.4098	p=0.8457	p=0.6830	p=0.5287
AS-AQ	p=0.9484	p=0.7790	p=0.9407	5.00% (-109.65% - 56.95%, p=0.8989)	p=0.3942	p=0.9093	p=0.6406	p=0.5020
AL	p=0.2631	p=0.4931	p=0.4098	p=0.3942	31.27% (-28.59% - 63.26%, p=0.2407)	p=0.3191	p=0.5131	p=0.8667
Non-ACT-PQ	p=0.8356	p=0.6792	p=0.8457	p=0.9093	p=0.3191	0.00% (-119.28% - 54.40%, p>0.9999)	p=0.5348	p=0.4207
ACT-PQ	p=0.5300	p=0.8491	p=0.6830	p=0.6406	p=0.5131	p=0.5348	19.27% (-40.22% -53.52%, p=0.4474)	p=0.7008
ACT-TQ	p=0.4102	p=0.6374	p=0.5287	p=0.5020	p=0.8667	p=0.4207	p=0.7008	27.50% (-42.94% - 63.23%, p=0.3532)

Diagonal cells indicate percentage relative reduction at day 2 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline.

Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

Supplementary Table 4. Relative reduction in gametocyte prevalence by microscopy at day 7 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	19.86% (-14.66% - 43.98%, p=0.2258)	p=0.7789	p=0.9959	p=0.7557	p=0.0913	p=0.0636	p<0.0001	p=0.0379
SP-AQ	p=0.7789	26.47% (-48.06% -63.48%, p=0.3892)	p=0.8280	p=0.6201	p=0.2246	p=0.1101	p<0.0001	p=0.0969
PY-AS	p=0.9959	p=0.8280	20.00% (-73.84% - 63.18%, p=0.5731)	p=0.7900	p=0.1916	p=0.0945	p<0.0001	p=0.0858
AS-AQ	p=0.7557	p=0.6201	p=0.7900	10.00% (-103.06% - 60.11%, p=0.7997)	p=0.1245	p=0.0660	p<0.0001	p=0.0556
AL	p=0.0913	p=0.2246	p=0.1916	p=0.1245	51.12% (3.85% - 75.15%, p=0.0381)	p=0.4036	p<0.0001	p=0.5279
Non-ACT-PQ	p=0.0636	p=0.1101	p=0.0945	p=0.0660	p=0.4036	68.31% (10.02% - 88.84%, p=0.0309)	p=0.0340	p=0.7273
ACT-PQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p=0.0340	90.52% (78.52% - 95.81%, p<0.0001)	p=0.0017
ACT-TQ	p=0.0379	p=0.0969	p=0.0858	p=0.0556	p=0.5279	p=0.7273	p=0.0017	61.53% (16.07% - 82.37%, p=0.0164)

Diagonal cells indicate percentage relative reduction at day 7 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline.

Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

Supplementary Table 5. Relative reduction in gametocyte prevalence by microscopy at day 14 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	44.37% (16.36% - 63.00%, p=0.0048)	p=0.7602	p=0.9864	p=0.6191	p=0.0233	p=0.0281	p<0.0001	p=0.0037
SP-AQ	p=0.7602	38.45% (-32.58% -71.42%, p=0.2151)	p=0.8227	p=0.8134	p=0.0189	p=0.0233	p<0.0001	p=0.0029
PY-AS	p=0.9864	p=0.8227	44.00% (-33.96% -76.59%, p=0.1926)	p=0.6845	p=0.0634	p=0.0326	p<0.0001	p=0.0081
AS-AQ	p=0.6191	p=0.8134	p=0.6845	31.71% (-68.92% -72.39%, p=0.4091)	p=0.0267	p=0.0211	p<0.0001	p=0.0036
AL	p=0.0233	p=0.0189	p=0.0634	p=0.0267	75.56% (44.56% -89.23%, p=0.0007)	p=0.1672	p=0.0009	p=0.1754
Non-ACT-PQ	p=0.0281	p=0.0233	p=0.0326	p=0.0211	p=0.1672	94.40% (54.76% - 99.31%, p=0.0069)	p=0.3857	p=0.5681
ACT-PQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p=0.0009	p=0.3857	98.10% (91.41% -99.58%, p<0.0001)	p=0.0492
ACT-TQ	p=0.0037	p=0.0029	p=0.0081	p=0.0036	p=0.1754	p=0.5681	p=0.0492	89.20% (64.90% - 96.68%, p=0.0002)

Diagonal cells indicate percentage relative reduction at day 14 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline.

Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

Supplementary Table 6. Relative reduction in gametocyte prevalence by RT-qPCR at day 2 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	-0.02% (-37.32% - 27.14%, p=0.9989)	p=0.9998	p=0.9987	p=0.9995	p=0.9971	p=0.9983	p=0.9995	p=0.9993
SP-AQ	p=0.9998	-0.02% (-93.77% - 48.37%, p=0.9996)	p=0.9987	p=0.9997	p=0.9979	p=0.9983	p=0.9998	p=0.9996
PY-AS	p=0.9987	p=0.9987	-0.08% (-110.22% - 52.35%, p=0.9983)	p=0.9985	p=0.9967	p=0.9996	p=0.9983	p=0.9983
AS-AQ	p=0.9995	p=0.9997	p=0.9985	0.00% (-114.87% -53.46%, p>0.9999)	p=0.9986	p=0.9982	p=0.9998	p>0.9999
AL	p=0.9971	p=0.9979	p=0.9967	p=0.9986	0.06% (-77.68% -43.79%, p=0.9982)	p=0.9965	p=0.9973	p=0.9982
Non-ACT-PQ	p=0.9983	p=0.9983	p=0.9996	p=0.9982	p=0.9965	-0.11% (-126.54% -55.76%, p=0.9980)	p=0.9979	p=0.9980
ACT-PQ	p=0.9995	p=0.9998	p=0.9983	p=0.9998	p=0.9973	p=0.9979	-0.01% (-66.31% - 39.86%, p=0.9997)	p=0.9997
ACT-TQ	p=0.9993	p=0.9996	p=0.9983	p>0.9999	p=0.9982	p=0.9980	p=0.9997	0.00% (-87.91% - 46.78%, p>0.9999)

Diagonal cells indicate percentage relative reduction at day 2 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline.

Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

Supplementary Table 7. Relative reduction in gametocyte prevalence by RT-qPCR at day 7 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	0.08% (-38.11% - 27.71%, p=0.9960)	p=0.9957	p=0.9972	p=0.9981	p=0.9991	p=0.9970	p=0.0123	p=0.9987
SP-AQ	p=0.9957	-0.08% (-95.15% -48.68%, p=0.9982)	p=0.9942	p=0.9985	p=0.9952	p=0.9998	p=0.0532	p=0.9973
PY-AS	p=0.9972	p=0.9942	0.20% (-109.67% -52.50%, p=0.9957)	p=0.9962	p=0.9979	p=0.9953	p=0.0947	p=0.9965
AS-AQ	p=0.9981	p=0.9985	p=0.9962	0.00% (-116.09% - 53.72%, p>0.9999)	p=0.9976	p=0.9989	p=0.1096	p=0.9992
AL	p=0.9991	p=0.9952	p=0.9979	p=0.9976	0.11% (-79.11% -44.29%, p=0.9970)	p=0.9965	p=0.0191	p=0.9980
Non-ACT-PQ	p=0.9970	p=0.9998	p=0.9953	p=0.9989	p=0.9965	-0.06% (-127.64% - 56.02%, p=0.9988)	p=0.1393	p=0.9980
ACT-PQ	p=0.0123	p=0.0532	p=0.0947	p=0.1096	p=0.0191	p=0.1393	42.91% (1.55% - 66.89%, p=0.0438)	p=0.0414
ACT-TQ	p=0.9987	p=0.9973	p=0.9965	p=0.9992	p=0.9980	p=0.9980	p=0.0414	0.04% (-89.91% - 47.38%, p=0.9991)

Diagonal cells indicate percentage relative reduction at day 7 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline. Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

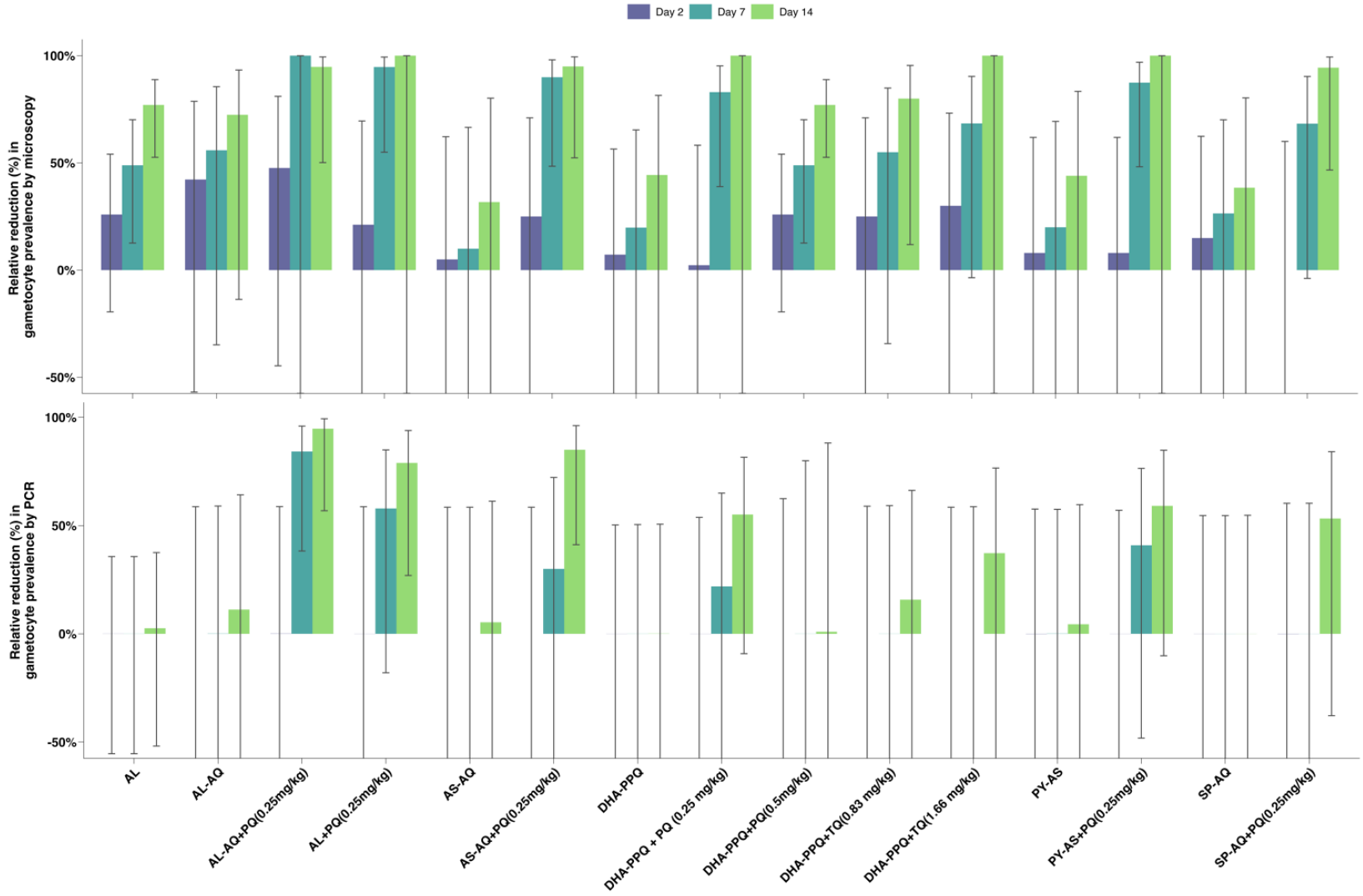
Supplementary Table 8. Relative reduction in gametocyte prevalence by RT-qPCR at day 14 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	0.15% (-37.69% - 27.59%, p=0.9926)	p=0.9939	p=0.8986	p=0.8834	p=0.8295	p=0.1125	p<0.0001	p=0.3296
SP-AQ	p=0.9939	-0.08% (-94.71% -48.56%, p=0.9982)	p=0.9062	p=0.8917	p=0.8573	p=0.1380	p<0.0001	p=0.4032
PY-AS	p=0.8986	p=0.9062	4.43% (-101.51% - 54.67%, p=0.9053)	p=0.9827	p=0.9778	p=0.1863	p=0.0007	p=0.5252
AS-AQ	p=0.8834	p=0.8917	p=0.9827	5.34% (-106.97% -56.71%, p=0.8906)	p=0.9994	p=0.2031	p=0.0015	p=0.5590
AL	p=0.8295	p=0.8573	p=0.9778	p=0.9994	5.37% (-69.78% - 47.25%, p=0.8532)	p=0.1474	p<0.0001	p=0.4455
Non-ACT-PQ	p=0.1125	p=0.1380	p=0.1863	p=0.2031	p=0.1474	53.25% (-25.90% - 82.64%, p=0.1325)	p=0.3048	p=0.3673
ACT-PQ	p<0.0001	p<0.0001	p=0.0007	p=0.0015	p<0.0001	p=0.3048	71.76% (48.60% - 84.48%, p<0.0001)	p=0.0026
ACT-TQ	p=0.3296	p=0.4032	p=0.5252	p=0.5590	p=0.4455	p=0.3673	p=0.0026	25.59% (-45.84% - 62.03%, p=0.3893)

Diagonal cells indicate percentage relative reduction at day 14 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline.

Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

Supplementary Figure 4. Relative reduction in gametocyte prevalence per study arm (ungrouped)



Supplementary Table 9. Relative reduction in gametocyte density by microscopy at day 2 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	18.59% (-49.99% - 55.81%, p=0.5094)	p=0.2270	p=0.3077	p=0.9348	p=0.0489	p=0.3718	p=0.1502	p=0.2895
SP-AQ	p=0.2270	-23.83% (-150.43% - 38.77%, p=0.5519)	p=0.0607	p=0.3406	p=0.0040	p=0.9612	p=0.0124	p=0.0439
PY-AS	p=0.3077	p=0.0607	46.29% (-21.88% - 76.33%, p=0.1371)	p=0.4686	p=0.6389	p=0.1212	p=0.9317	p=0.9138
AS-AQ	p=0.9348	p=0.3406	p=0.4686	21.49% (-90.80% -67.70%, p=0.5933)	p=0.2033	p=0.4335	p=0.4125	p=0.4882
AL	p=0.0489	p=0.0040	p=0.6389	p=0.2033	55.84% (16.68% - 76.59%, p=0.0116)	p=0.0260	p=0.4143	p=0.4958
Non-ACT-PQ	p=0.3718	p=0.9612	p=0.1212	p=0.4335	p=0.0260	-20.98% (-194.03% -50.22%, p=0.6742)	p=0.0657	p=0.1103
ACT-PQ	p=0.1502	p=0.0124	p=0.9317	p=0.4125	p=0.4143	p=0.0657	44.49% (23.64% - 59.64%, p=0.0003)	p=0.9622
ACT-TQ	p=0.2895	p=0.0439	p=0.9138	p=0.4882	p=0.4958	p=0.1103	p=0.9622	43.64% (-13.98% - 72.13%, p=0.1105)

Diagonal cells indicate percentage relative reduction at day 2 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline.

Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

Supplementary Table 10. Relative reduction in gametocyte density by microscopy at day 7 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	52.44% (12.02% - 74.29%, p=0.0179)	p=0.1550	p=0.4917	p=0.7638	p=0.0015	p<0.0001	p<0.0001	p<0.0001
SP-AQ	p=0.1550	21.53% (-59.92% -61.49%, p=0.5045)	p=0.0810	p=0.4450	p<0.0001	p<0.0001	p<0.0001	p<0.0001
PY-AS	p=0.4917	p=0.0810	64.10% (18.46% - 84.19%, p=0.0144)	p=0.4285	p=0.0872	p=0.0002	p<0.0001	p=0.0018
AS-AQ	p=0.7638	p=0.4450	p=0.4285	45.64% (-32.20% -77.65%, p=0.1788)	p=0.0127	p<0.0001	p<0.0001	p=0.0002
AL	p=0.0015	p<0.0001	p=0.0872	p=0.0127	82.43% (66.76% - 90.72%, p<0.0001)	p=0.0056	p<0.0001	p=0.0591
Non-ACT-PQ	p<0.0001	p<0.0001	p=0.0002	p<0.0001	p=0.0056	95.08% (87.89% - 98.00%, p<0.0001)	p<0.0001	p=0.2228
ACT-PQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	99.64% (99.50% - 99.74%, p<0.0001)	p<0.0001
ACT-TQ	p<0.0001	p<0.0001	p=0.0018	p=0.0002	p=0.0591	p=0.2228	p<0.0001	91.11% (81.94% - 95.62%, p<0.0001)

Diagonal cells indicate percentage relative reduction at day 7 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline.

Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

Supplementary Table 11. Relative reduction in gametocyte density by microscopy at day 14 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	79.32% (61.63% - 88.85%, p<0.0001)	p=0.1648	p=0.6312	p=0.5357	p<0.0001	p<0.0001	p<0.0001	p<0.0001
SP-AQ	p=0.1648	66.29% (31.53% -83.40%, p=0.0026)	p=0.1249	p=0.6658	p<0.0001	p<0.0001	p<0.0001	p<0.0001
PY-AS	p=0.6312	p=0.1249	83.01% (61.42% - 92.52%, p<0.0001)	p=0.3675	p=0.0007	p<0.0001	p<0.0001	p<0.0001
AS-AQ	p=0.5357	p=0.6658	p=0.3675	72.65% (32.79% -88.87%, p=0.0047)	p<0.0001	p<0.0001	p<0.0001	p<0.0001
AL	p<0.0001	p<0.0001	p=0.0007	p<0.0001	95.88% (92.20% - 97.82%, p<0.0001)	p<0.0001	p<0.0001	p<0.0001
Non-ACT-PQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	99.70% (99.25% - 99.88%, p<0.0001)	p=0.1110	p=0.0778
ACT-PQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p=0.1110	99.85% (99.79% - 99.89%, p<0.0001)	p<0.0001
ACT-TQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p=0.0778	p<0.0001	99.28% (98.53% - 99.65%, p<0.0001)

Diagonal cells indicate percentage relative reduction at day 14 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline.

Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

Supplementary Table 12. Relative reduction in gametocyte density by RT-qPCR at day 2 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	21.48% (-14.69% - 46.25%, p=0.2109)	p=0.8363	p=0.8914	p=0.7188	p=0.0036	p=0.7777	p<0.0001	p=0.6758
SP-AQ	p=0.8363	25.22% (-20.10% - 53.44%, p=0.2292)	p=0.7818	p=0.8642	p=0.0308	p=0.9113	p=0.0035	p=0.8649
PY-AS	p=0.8914	p=0.7818	18.46% (-41.54% - 53.03%, p=0.4682)	p=0.6888	p=0.0302	p=0.7359	p=0.0056	p=0.6644
AS-AQ	p=0.7188	p=0.8642	p=0.6888	29.25% (-25.84% - 60.22%, p=0.2390)	p=0.1085	p=0.9630	p=0.0335	p=0.9756
AL	p=0.0036	p=0.0308	p=0.0302	p=0.1085	56.18% (34.26% - 70.79%, p<0.0001)	p=0.1154	p=0.5477	p=0.0398
Non-ACT-PQ	p=0.7777	p=0.9113	p=0.7359	p=0.9630	p=0.1154	27.99% (-32.47% - 60.86%, p=0.2910)	p=0.0400	p=0.9811
ACT-PQ	p<0.0001	p=0.0035	p=0.0056	p=0.0335	p=0.5477	p=0.0400	60.70% (52.17% - 67.71%, p<0.0001)	p=0.0043
ACT-TQ	p=0.6758	p=0.8649	p=0.6644	p=0.9756	p=0.0398	p=0.9811	p=0.0043	28.56% (-12.53% -54.65%, p=0.1469)

Diagonal cells indicate percentage relative reduction at day 2 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline.

Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

Supplementary Table 13. Relative reduction in gametocyte density by RT-qPCR at day 7 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	55.14% (33.47% - 69.75%, p<0.0001)	p=0.5973	p=0.9593	p=0.8502	p=0.0026	p<0.0001	p<0.0001	p=0.0001
SP-AQ	p=0.5973	49.12% (17.61% - 68.58%, p=0.0060)	p=0.7179	p=0.5761	p=0.0029	p<0.0001	p<0.0001	p=0.0002
PY-AS	p=0.9593	p=0.7179	54.51% (20.93% - 73.83%, p=0.0052)	p=0.8445	p=0.0271	p<0.0001	p<0.0001	p=0.0029
AS-AQ	p=0.8502	p=0.5761	p=0.8445	57.54% (23.99% -76.28%, p=0.0039)	p=0.0617	p<0.0001	p<0.0001	p=0.0085
AL	p=0.0026	p=0.0029	p=0.0271	p=0.0617	75.71% (63.18% -83.98%, p<0.0001)	p<0.0001	p<0.0001	p=0.2454
Non-ACT-PQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	98.87% (97.91% -99.39%, p<0.0001)	p<0.0001	p<0.0001
ACT-PQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	99.67% (99.60% - 99.73%, p<0.0001)	p<0.0001
ACT-TQ	p=0.0001	p=0.0002	p=0.0029	p=0.0085	p=0.2454	p<0.0001	p<0.0001	81.64% (70.69% - 88.50%, p<0.0001)

Diagonal cells indicate percentage relative reduction at day 7 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline.

Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

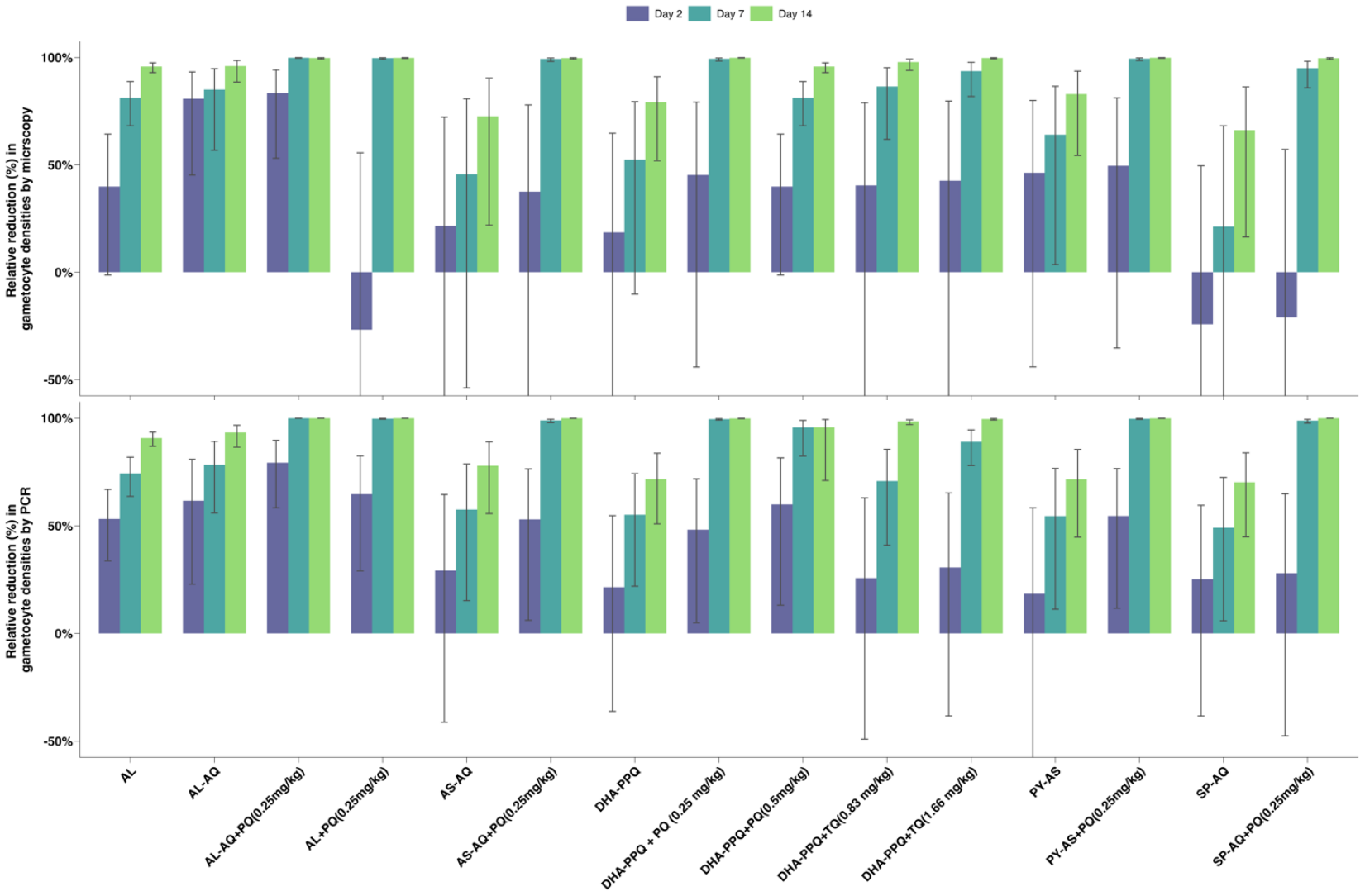
Supplementary Table 14. Relative reduction in gametocyte density by RT-qPCR at day 14 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	71.75% (58.07% - 80.96%, p<0.0001)	p=0.8226	p=0.9919	p=0.4020	p<0.0001	p<0.0001	p<0.0001	p<0.0001
SP-AQ	p=0.8226	70.20% (51.58% - 81.65%, p<0.0001)	p=0.8705	p=0.3599	p<0.0001	p<0.0001	p<0.0001	p<0.0001
PY-AS	p=0.9919	p=0.8705	71.67% (50.66% - 83.73%, p<0.0001)	p=0.4825	p<0.0001	p<0.0001	p<0.0001	p<0.0001
AS-AQ	p=0.4020	p=0.3599	p=0.4825	77.92% (60.11% -87.78%, p<0.0001)	p=0.0013	p<0.0001	p<0.0001	p<0.0001
AL	p<0.0001	p<0.0001	p<0.0001	p=0.0013	91.67% (87.34% -94.52%, p<0.0001)	p<0.0001	p<0.0001	p<0.0001
Non-ACT-PQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	99.97% (99.95% -99.99%, p<0.0001)	p=0.0020	p<0.0001
ACT-PQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p=0.0020	99.94% (99.92% - 99.95%, p<0.0001)	p<0.0001
ACT-TQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	99.10% (98.56% - 99.44%, p<0.0001)

Diagonal cells indicate percentage relative reduction at day 14 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline.

Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

Supplementary Figure 5. Relative reduction in gametocyte densities per study arm (ungrouped)



Supplementary Table 15. Relative reduction in mosquito infection rate at day 2 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	20.35% (12.70% - 27.33%, p<0.0001)	p=0.0002	p=0.0009	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
SP-AQ	p=0.0002	2.17% (-12.80% - 15.14%, p=0.7629)	p=0.3360	p=0.0259	p<0.0001	p<0.0001	p<0.0001	p=0.2729
PY-AS	p=0.0009	p=0.3360	-5.18% (-26.88% - 12.81%, p=0.5974)	p=0.2873	p<0.0001	p<0.0001	p<0.0001	p=0.8795
AS-AQ	p<0.0001	p=0.0259	p=0.2873	-17.32% (-42.92% - 3.69%, p=0.1126)	p<0.0001	p<0.0001	p<0.0001	p=0.1693
AL	p<0.0001	p<0.0001	p<0.0001	p<0.0001	97.10% (94.84% - 98.37%, p<0.0001)	p<0.0001	p=0.0097	p<0.0001
Non-ACT-PQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	83.26% (78.11% - 87.21%, p<0.0001)	p<0.0001	p<0.0001
ACT-PQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p=0.0097	p<0.0001	98.95% (98.19% - 99.39%, p<0.0001)	p<0.0001
ACT-TQ	p<0.0001	p=0.2729	p=0.8795	p=0.1693	p<0.0001	p<0.0001	p<0.0001	-3.87% (-21.61% - 11.28%, p=0.6369)

Diagonal cells indicate percentage relative reduction at day 2 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline.

Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

Supplementary Table 16. Relative reduction in mosquito infection rate at day 7 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	41.51% (34.98% - 47.37%, p<0.0001)	p<0.0001	p<0.0001	p=0.0678	p=0.0001	p=0.0045	p<0.0001	p<0.0001
SP-AQ	p<0.0001	9.79% (-6.54% - 23.61%, p=0.2249)	p=0.7647	p<0.0001	p<0.0001	p=0.0026	p<0.0001	p<0.0001
PY-AS	p<0.0001	p=0.7647	7.58% (-13.93% - 24.73%, p=0.4517)	p<0.0001	p<0.0001	p=0.0025	p<0.0001	p<0.0001
AS-AQ	p=0.0536	p<0.0001	p<0.0001	55.32% (39.21% - 67.16%, p<0.0001)	p=0.0002	p=0.0062	p<0.0001	p<0.0001
AL	p=1e-04	p<0.0001	p<0.0001	p=2e-04	99.92% (97.63% - 100.00%, p<0.0001)	p=0.8048	p=0.4754	p=0.2680
Non-ACT-PQ	p=0.0044	p=0.0026	p=0.0025	p=0.0062	p=0.8048	99.96% (93.98% - 100.00%, p=0.0023)	p=0.4379	p=0.2932
ACT-PQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p=0.4754	p=0.4379	99.70% (99.18% - 99.89%, p<0.0001)	p=0.3685
ACT-TQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p=0.2680	p=0.2932	p=0.3685	99.37% (97.60% - 99.83%, p<0.0001)

Diagonal cells indicate percentage relative reduction at day 7 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline.

Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

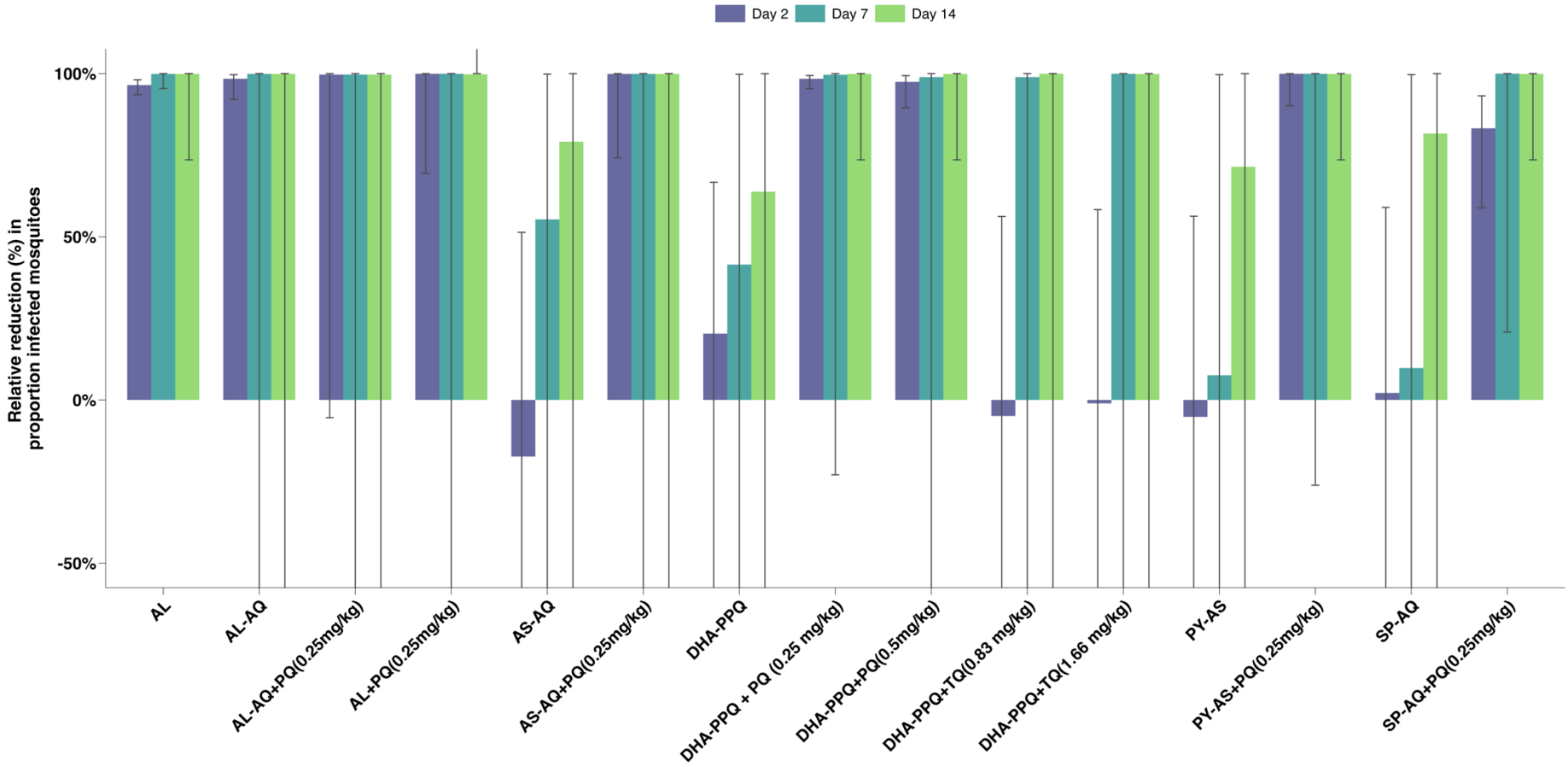
Supplementary Table 17. Relative reduction in mosquito infection rate at day 14 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	64.05% (57.68% - 69.46%, p<0.0001)	p=1e-04	p=0.1469	p=0.0194	p=0.0058	p>0.9999	p=0.0078	p=0.0098
SP-AQ	p=1e-04	81.65% (73.10% - 87.48%, p<0.0001)	p=0.0320	p=0.6313	p=0.0144	p>0.9999	p=0.0197	p=0.0213
PY-AS	p=0.1469	p=0.0320	71.44% (59.42% - 79.91%, p<0.0001)	p=0.2203	p=0.0081	p>0.9999	p=0.0109	p=0.0129
AS-AQ	p=0.0194	p=0.6313	p=0.2203	79.14% (66.13% - 87.15%, p<0.0001)	p=0.0125	p>0.9999	p=0.0170	p=0.0187
AL	p=0.0058	p=0.0144	p=0.0081	p=0.0125	99.91% (93.71% - 100.00%, p=0.0013)	p>0.9999	p=0.8763	p=0.9430
Non-ACT-PQ	p=0.0098	p=0.0213	p=0.0129	p=0.0187	p=0.9430	64.05% (57.68% - 69.46%, p<0.0001)	p=0.8259	p>0.9999
ACT-PQ	p=0.0078	p=0.0197	p=0.0109	p=0.0170	p=0.8763	p>0.9999	99.86% (91.65% - 100.00%, p=0.0017)	p=0.8259
ACT-TQ	p=0.0098	p=0.0213	p=0.0129	p=0.0187	p=0.9430	p>0.9999	p=0.8259	99.93% (92.06% - 100.00%, p=0.0027)

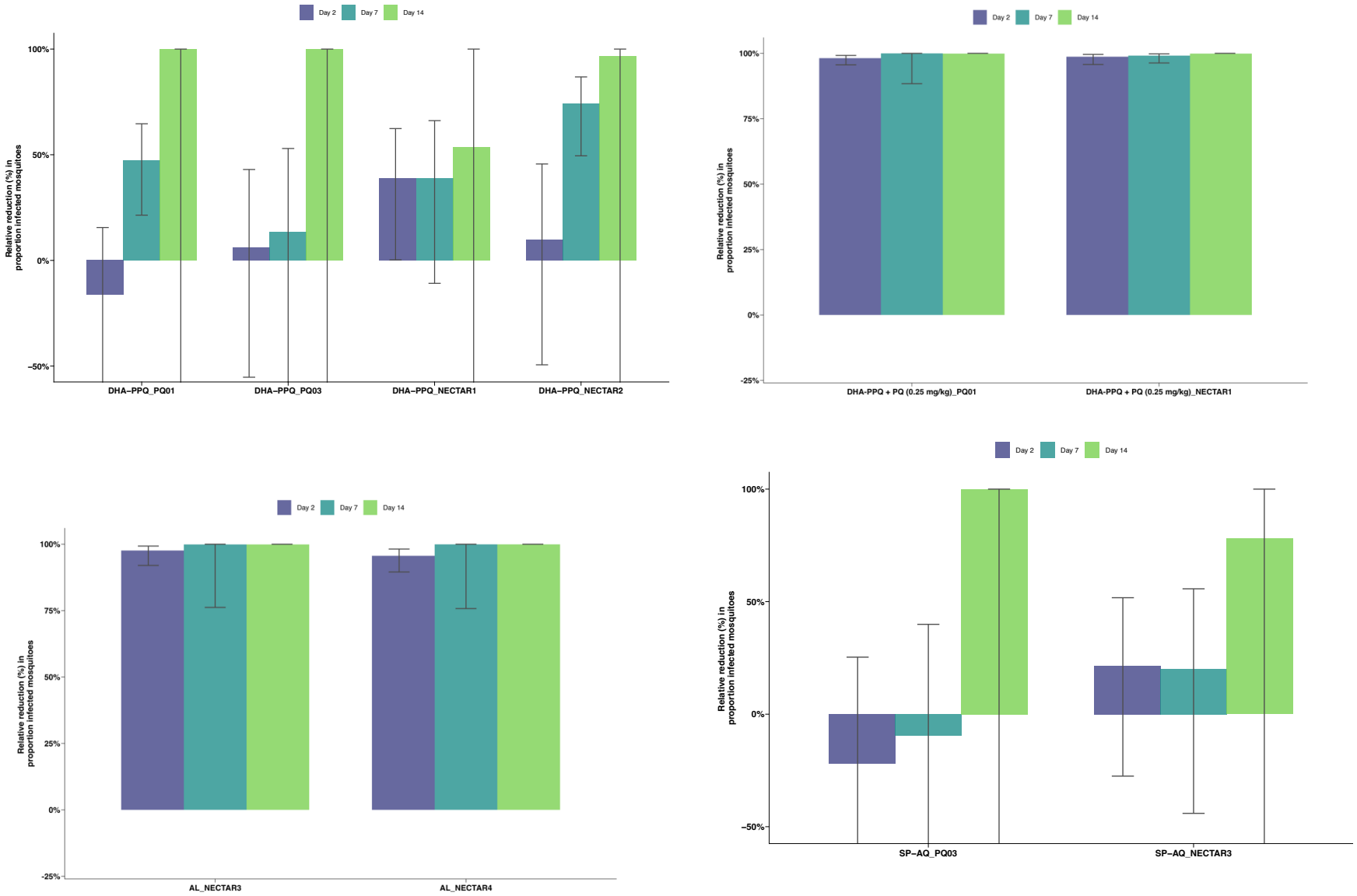
Diagonal cells indicate percentage relative reduction at day 14 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline.

Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

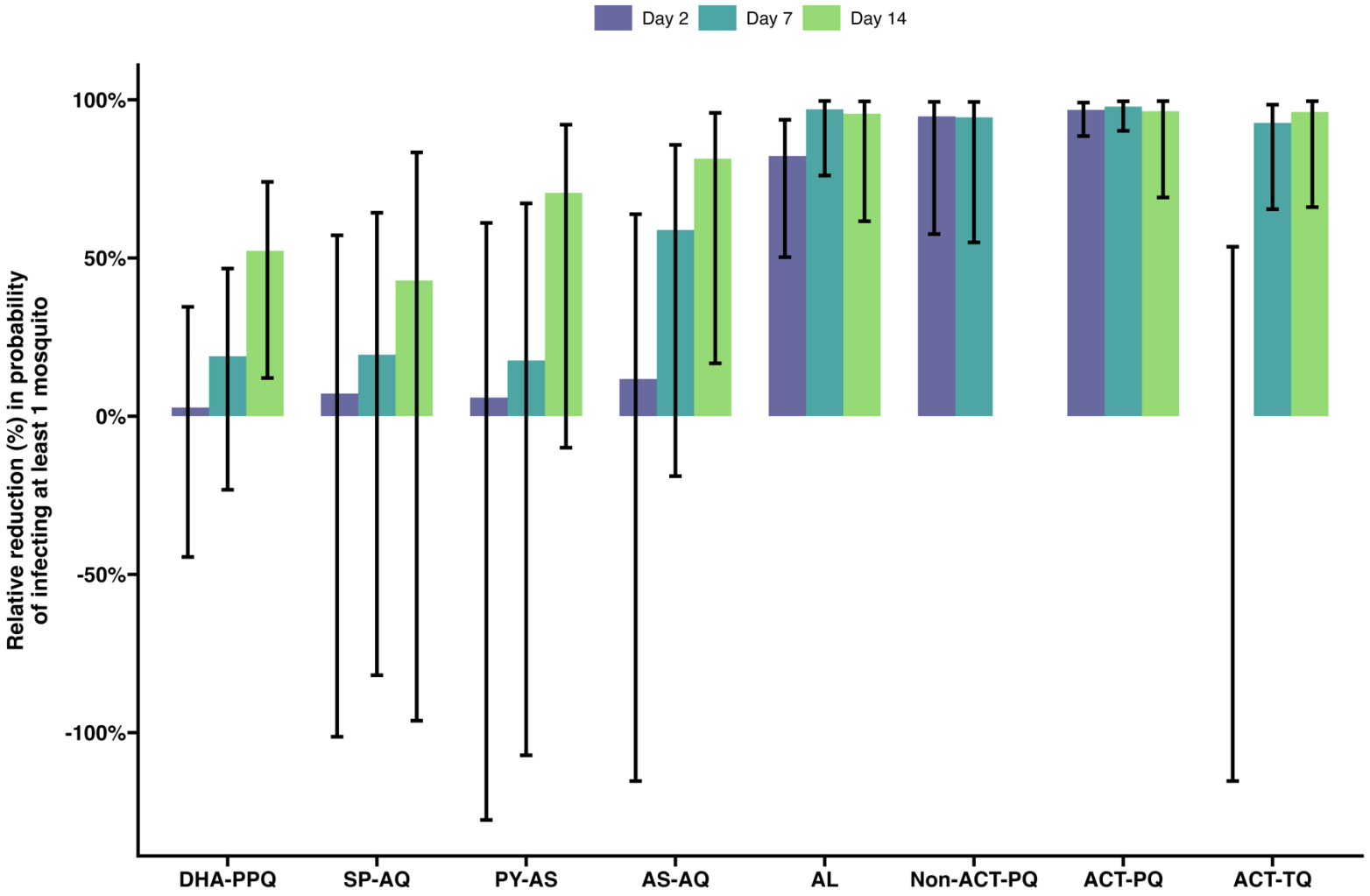
Supplementary Figure 6. Relative reduction in mosquito infection rate per study arm (ungrouped)



Supplementary Figure 7. Relative reduction in mosquito infection rate comparing the same study arms across different studies



Supplementary Figure 8. Relative reduction in the probability of infecting at least 1 mosquito



Supplementary Table 18. Relative reduction in the probability of infecting at least 1 mosquito at day 2 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	2.77% (-44.48% - 34.57%, p=0.8895)	p=0.8920	p=0.9356	p=0.8118	p=0.0005	p=0.0053	p<0.0001	p=0.9332
SP-AQ	p=0.8920	7.14% (-101.30% -57.17%, p=0.8511)	p=0.9757	p=0.9090	p=0.0014	p=0.0069	p<0.0001	p=0.8460
PY-AS	p=0.9356	p=0.9757	5.88% (-127.61% - 61.08%, p=0.8930)	p=0.8966	p=0.0030	p=0.0078	p<0.0001	p=0.8902
AS-AQ	p=0.8118	p=0.9090	p=0.8966	11.76% (-115.31% - 63.84%, p=0.7833)	p=0.0046	p=0.0094	p<0.0001	p=0.7779
AL	p=0.0005	p=0.0014	p=0.0030	p=0.0046	82.27% (50.26% - 93.68%, p=0.0010)	p=0.2768	p=0.0196	p=0.0008
Non-ACT-PQ	p=0.0053	p=0.0069	p=0.0078	p=0.0094	p=0.2768	94.74% (57.56% - 99.35%, p=0.0057)	p=0.6726	p=0.0055
ACT-PQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p=0.0196	p=0.6726	96.81% (88.53% - 99.11%, p<0.0001)	p<0.0001
ACT-TQ	p=0.9332	p=0.8460	p=0.8902	p=0.7779	p=0.0008	p=0.0055	p<0.0001	0.00% (-115.31% - 53.55%, p>0.9999)

Diagonal cells indicate percentage relative reduction at day 2 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline.

Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

Supplementary Table 19. Relative reduction in the probability of infecting at least 1 mosquito at day 7 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	18.92% (-23.24% -46.66%, p=0.3262)	p=0.9863	p=0.9703	p=0.1731	p=0.0015	p=0.0105	p<0.0001	p=0.0016
SP-AQ	p=0.9863	19.41% (-81.86% - 64.29%, p=0.6032)	p=0.9624	p=0.2068	p=0.0018	p=0.0119	p<0.0001	p=0.0023
PY-AS	p=0.9703	p=0.9624	17.65% (-107.17% -67.26%, p=0.6800)	p=0.2289	p=0.0021	p=0.0131	p<0.0001	p=0.0030
AS-AQ	p=0.1731	p=0.2068	p=0.2289	58.82% (-18.95% - 85.75%, p=0.1011)	p=0.0183	p=0.0733	p=0.0005	p=0.0444
AL	p=0.0015	p=0.0018	p=0.0021	p=0.0183	96.99% (76.07% - 99.62%, p=0.0009)	p=0.6720	p=0.7854	p=0.4766
Non-ACT-PQ	p=0.0105	p=0.0119	p=0.0131	p=0.0733	p=0.6720	94.46% (54.93% - 99.32%, p=0.0068)	p=0.4480	p=0.8245
ACT-PQ	p<0.0001	p<0.0001	p<0.0001	p=0.0005	p=0.7854	p=0.4480	97.85% (90.19% - 99.53%, p<0.0001)	p=0.2299
ACT-TQ	p=0.0016	p=0.0023	p=0.0030	p=0.0444	p=0.4766	p=0.8245	p=0.2299	92.67% (65.42% - 98.45%, p=0.0010)

Diagonal cells indicate percentage relative reduction at day 7 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline.

Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

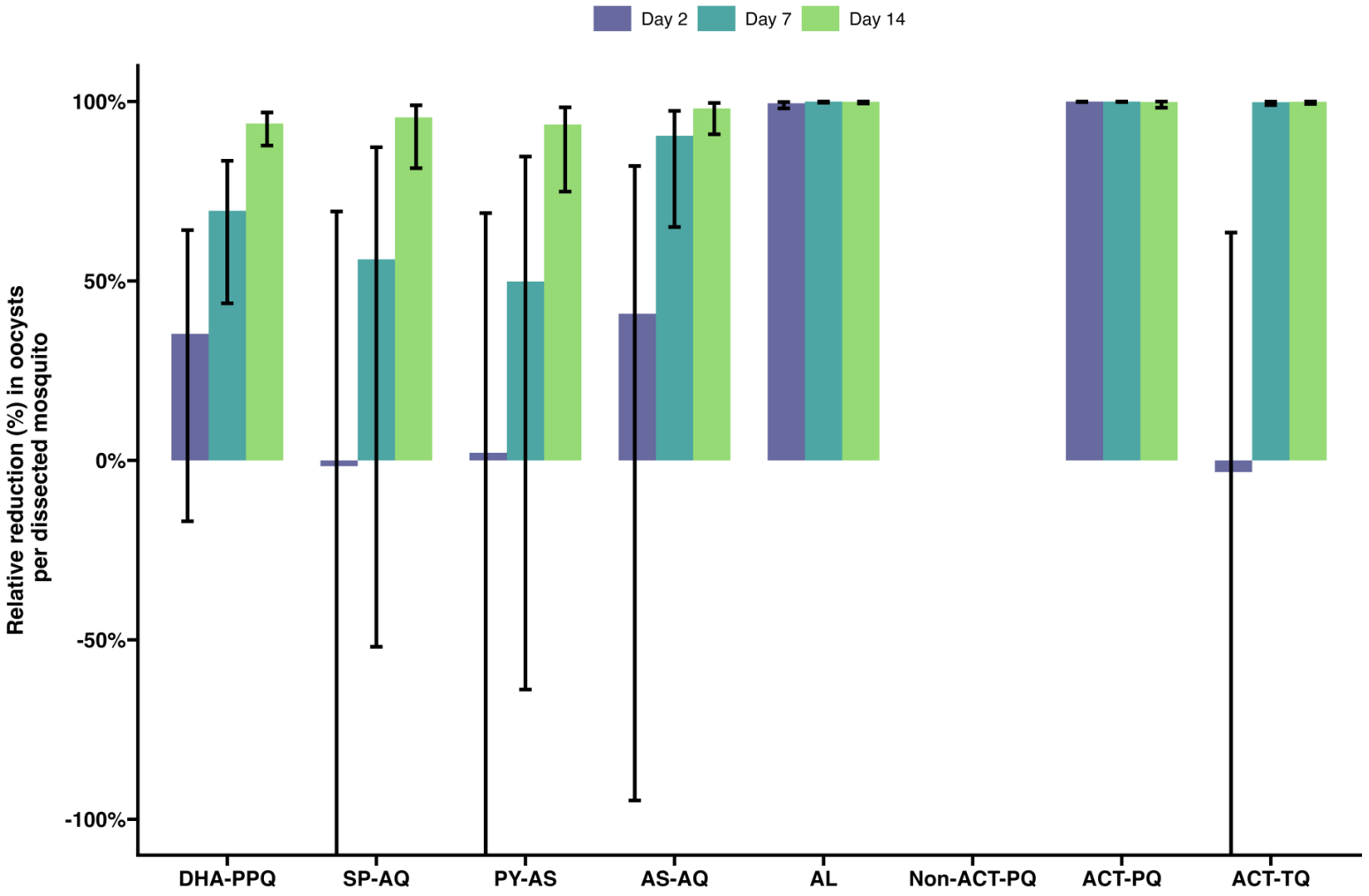
Supplementary Table 20. Relative reduction in the probability of infecting at least 1 mosquito at day 14 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ	52.23% (12.07% - 74.05%, p=0.0176)	p=0.7432	p=0.4162	p=0.1768	p=0.0245	p>0.9999	p=0.0139	p=0.0181
SP-AQ	p=0.7432	42.86% (-96.23% - 83.36%, p=0.3740)	p=0.3281	p=0.1450	p=0.0208	p>0.9999	p=0.0120	p=0.0154
PY-AS	p=0.4162	p=0.3281	70.59% (-9.96% - 92.13%, p=0.0689)	p=0.5690	p=0.0939	p>0.9999	p=0.0620	p=0.0743
AS-AQ	p=0.1768	p=0.1450	p=0.5690	81.42% (16.72% - 95.86%, p=0.0279)	p=0.2266	p>0.9999	p=0.1654	p=0.1886
AL	p=0.0245	p=0.0208	p=0.0939	p=0.2266	95.60% (61.62% - 99.50%, p=0.0047)	p>0.9999	p=0.8878	p=0.9281
Non-ACT-PQ	p=0.0181	p=0.0154	p=0.0743	p=0.1886	p=0.9281	52.23% (12.07% - 74.05%, p=0.0176)	p=0.9600	p>0.9999
ACT-PQ	p=0.0139	p=0.0120	p=0.0620	p=0.1654	p=0.8878	p>0.9999	96.41% (69.12% - 99.58%, p=0.0024)	p=0.9600
ACT-TQ	p=0.0181	p=0.0154	p=0.0743	p=0.1886	p=0.9281	p>0.9999	p=0.9600	96.14% (66.07% - 99.56%, p=0.0033)

Diagonal cells indicate percentage relative reduction at day 14 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline.

Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

Supplementary Figure 9. Relative reduction in mean oocyst density in dissected mosquitoes



Supplementary Table 21. Relative reduction in mean oocyst density in dissected mosquitoes at day 2 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	ACT-PQ	ACT-TQ
DHA-PPQ	35.26% (-16.96% - 64.16%)	p=0.3969	p=0.4096	p=0.8643	p<0.0001	p<0.0001	p=0.2845
SP-AQ	p=0.3969	-1.59% (-236.76% -69.35%)	p=0.9499	p=0.3799	p<0.0001	p<0.0001	p=0.9763
PY-AS	p=0.4096	p=0.9499	2.13% (-208.08% - 68.91%)	p=0.3931	p<0.0001	p<0.0001	p=0.9166
AS-AQ	p=0.8643	p=0.3799	p=0.3931	40.84% (-94.75% - 82.03%)	p<0.0001	p<0.0001	p=0.2982
AL	p<0.0001	p<0.0001	p<0.0001	p<0.0001	99.47% (98.09% - 99.85%)	p=0.0019	p<0.0001
ACT-PQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p=0.0019	99.97% (99.83% - 99.99%)	p<0.0001
ACT-TQ	p=0.2845	p=0.9763	p=0.9166	p=0.2982	p<0.0001	p<0.0001	-3.23% (-191.81% - 63.48%)

Diagonal cells indicate percentage relative reduction at day 2 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline. Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

Supplementary Table 22. Relative reduction in mean oocyst density in dissected mosquitoes at day 7 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	ACT-PQ	ACT-TQ
DHA-PPQ	93.88% (87.73% - 96.94%)	p=0.6129	p=0.9439	p=0.1035	p<0.0001	p=0.0019	p<0.0001
SP-AQ	p=0.6129	95.57% (81.42% - 98.94%)	p=0.6115	p=0.3065	p=0.0003	p=0.0071	p=0.0004
PY-AS	p=0.9439	p=0.6115	93.61% (74.89% - 98.37%)	p=0.1264	p<0.0001	p=0.0024	p<0.0001
AS-AQ	p=0.1035	p=0.3065	p=0.1264	98.07% (90.87% - 99.59%)	p=0.0041	p=0.0490	p=0.0064
AL	p<0.0001	p=0.0003	p<0.0001	p=0.0041	99.95% (99.44% - 99.99%)	p=0.5060	p=0.8712
ACT-PQ	p=0.0019	p=0.0071	p=0.0024	p=0.0490	p=0.5060	99.85% (98.27% - 99.99%)	p=0.6067
ACT-TQ	p<0.0001	p=0.0004	p<0.0001	p=0.0064	p=0.8712	p=0.6067	99.93% (99.32% - 99.99%)

Diagonal cells indicate percentage relative reduction at day 7 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline. Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

Supplementary Table 23. Relative reduction in mean oocyst density in dissected mosquitoes at day 14 compared to baseline

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	ACT-PQ	ACT-TQ
DHA-PPQ	69.52% (43.76% - 83.48%)	p=0.5041	p=0.3361	p=0.0468	p<0.0001	p<0.0001	p<0.0001
SP-AQ	p=0.5041	56.00% (-51.93% - 87.26%)	p=0.8313	p=0.0224	p<0.0001	p<0.0001	p<0.0001
PY-AS	p=0.3361	p=0.8313	49.87% (-63.86% - 84.67%)	p=0.0099	p<0.0001	p<0.0001	p<0.0001
AS-AQ	p=0.0468	p=0.0224	p=0.0099	90.44% (65.03% - 97.39%)	p<0.0001	p<0.0001	p<0.0001
AL	p<0.0001	p<0.0001	p<0.0001	p<0.0001	99.96% (99.66% - 100.00%)	p=0.8497	p=0.1893
ACT-PQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p=0.8497	99.97% (99.83% - 100.00%)	p=0.0699
ACT-TQ	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p=0.1893	p=0.0699	99.81% (99.01% - 99.96%)

Diagonal cells indicate percentage relative reduction at day 14 compared to baseline (95% CI) per treatment category, with corresponding p-value compared to baseline. Other cells in the table contain p-values of between-arm comparisons of the relative reductions.

Supplementary Table 24. Hazard ratios for infectivity survival curves (adjusted by baseline PCR gametocyte densities)

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ		0.91 (0.50- 1.66) p=0.7526	1.04 (0.69- 1.56) p=0.8541	1.13 (0.74- 1.74) p=0.5667	5.26 (3.12- 8.86) p<0.0001	8.52 (4.50-16.13) p<0.0001	7.93 (5.69-11.04) p<0.0001	2.01 (1.26- 3.22) p=0.0037
SP-AQ	1.10 (0.60- 2.01) p=0.7526		1.14 (0.76- 1.72) p=0.5179	1.25 (0.82- 1.89) p=0.2940	5.79 (3.24-10.36) p<0.0001	9.38 (4.96-17.75) p<0.0001	8.73 (5.13-14.87) p<0.0001	2.22 (1.40- 3.50) p=0.0007
PY-AS	0.96 (0.64- 1.45) p=0.8541	0.87 (0.58- 1.32) p=0.5179		1.09 (1.05- 1.14) p<0.0001	5.06 (3.92- 6.53) p<0.0001	8.20 (5.54-12.12) p<0.0001	7.63 (6.37- 9.14) p<0.0001	1.94 (1.75- 2.14) p<0.0001
AS-AQ	0.88 (0.57- 1.35) p=0.5667	0.80 (0.53- 1.21) p=0.2940	0.92 (0.88- 0.96) p<0.0001		4.64 (3.66- 5.88) p<0.0001	7.51 (5.26-10.73) p<0.0001	6.99 (5.85- 8.36) p<0.0001	1.77 (1.66- 1.90) p<0.0001
AL	0.19 (0.11-0.32) p<0.0001	0.17 (0.10-0.31) p<0.0001	0.20 (0.15-0.25) p<0.0001	0.22 (0.17-0.27) p<0.0001		1.62 (1.29-2.03) p<0.0001	1.51 (1.19-1.91) p=0.0006	0.38 (0.32-0.46) p<0.0001
Non-ACT-PQ	0.12 (0.06-0.22) p<0.0001	0.11 (0.06-0.20) p<0.0001	0.12 (0.08-0.18) p<0.0001	0.13 (0.09-0.19) p<0.0001	0.62 (0.49-0.78) p<0.0001		0.93 (0.64-1.36) p=0.7097	0.24 (0.17-0.32) p<0.0001
ACT-PQ	0.13 (0.09-0.18) p<0.0001	0.11 (0.07-0.19) p<0.0001	0.13 (0.11-0.16) p<0.0001	0.14 (0.12-0.17) p<0.0001	0.66 (0.52-0.84) p=0.0006	1.07 (0.74-1.57) p=0.7097		0.25 (0.21-0.30) p<0.0001
ACT-TQ	0.50 (0.31-0.80) p=0.0037	0.45 (0.29-0.71) p=0.0007	0.52 (0.47-0.57) p<0.0001	0.56 (0.53-0.60) p<0.0001	2.61 (2.18-3.14) p<0.0001	4.23 (3.10-5.78) p<0.0001	3.94 (3.29-4.72) p<0.0001	

Hazard ratios for between-arm comparisons of infectivity clearance, with corresponding 95% CI and p-value.

E.g. Hazard ratio of infectivity clearance for AL compared to DHA-PPQ is 5.26, meaning that clearance is 5.26 times more likely to take place in the AL group compared to the DHA-PPQ group, and this is significantly different (p<0.0001).

Supplementary Table 25. Hazard ratios for gametocytes by microscopy survival curves

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ		1.01 (0.60- 1.71) p=0.9598	1.35 (0.87- 2.10) p=0.1822	1.42 (0.88- 2.28) p=0.1492	2.86 (1.73- 4.71) p<0.0001	2.23 (1.47- 3.37) p=0.0002	6.50 (3.81-11.09) p<0.0001	3.67 (2.19- 6.17) p<0.0001
SP-AQ	0.99 (0.59- 1.66) p=0.9598		1.33 (0.81- 2.19) p=0.2600	1.40 (0.85- 2.31) p=0.1886	2.82 (1.77- 4.49) p<0.0001	2.20 (1.34- 3.60) p=0.0018	6.41 (3.97-10.34) p<0.0001	3.62 (2.20- 5.97) p<0.0001
PY-AS	0.74 (0.48-1.15) p=0.1822	0.75 (0.46-1.24) p=0.2600		1.05 (0.99-1.12) p=0.1146	2.12 (1.89-2.37) p<0.0001	1.65 (1.55-1.75) p<0.0001	4.81 (3.72-6.23) p<0.0001	2.72 (2.27-3.26) p<0.0001
AS-AQ	0.70 (0.44-1.13) p=0.1492	0.71 (0.43-1.18) p=0.1886	0.95 (0.89-1.01) p=0.1146		2.02 (1.89-2.15) p<0.0001	1.57 (1.43-1.72) p<0.0001	4.58 (3.56-5.90) p<0.0001	2.59 (2.29-2.93) p<0.0001
AL	0.35 (0.21-0.58) p<0.0001	0.35 (0.22-0.56) p<0.0001	0.47 (0.42-0.53) p<0.0001	0.50 (0.47-0.53) p<0.0001		0.78 (0.67-0.90) p=0.0008	2.27 (1.75-2.96) p<0.0001	1.29 (1.15-1.44) p<0.0001
Non-ACT-PQ	0.45 (0.30-0.68) p=0.0002	0.46 (0.28-0.75) p=0.0018	0.61 (0.57-0.64) p<0.0001	0.64 (0.58-0.70) p<0.0001	1.28 (1.11-1.49) p=0.0008		2.92 (2.34-3.64) p<0.0001	1.65 (1.37-1.98) p<0.0001
ACT-PQ	0.15 (0.09-0.26) p<0.0001	0.16 (0.10-0.25) p<0.0001	0.21 (0.16-0.27) p<0.0001	0.22 (0.17-0.28) p<0.0001	0.44 (0.34-0.57) p<0.0001	0.34 (0.28-0.43) p<0.0001		0.57 (0.44-0.72) p<0.0001
ACT-TQ	0.27 (0.16-0.46) p<0.0001	0.28 (0.17-0.45) p<0.0001	0.37 (0.31-0.44) p<0.0001	0.39 (0.34-0.44) p<0.0001	0.78 (0.70-0.87) p<0.0001	0.61 (0.50-0.73) p<0.0001	1.77 (1.39-2.25) p<0.0001	

Hazard ratios for between-arm comparisons of microscopical gametocyte clearance, with corresponding 95% CI and p-value.

E.g. Hazard ratio of microscopical gametocyte clearance for AL compared to DHA-PPQ is 2.86, meaning that clearance is 2.86 times more likely to take place in the AL group compared to the DHA-PPQ group, and this is significantly different (p<0.0001).

Supplementary Table 26. Hazard ratios for gametocytes by PCR survival curves

reference	DHA-PPQ	SP-AQ	PY-AS	AS-AQ	AL	Non-ACT-PQ	ACT-PQ	ACT-TQ
DHA-PPQ		0.42 (0.18- 0.96) p=0.0395	1.07 (0.73- 1.56) p=0.7219	1.27 (0.76- 2.10) p=0.3606	2.91 (1.47- 5.76) p=0.0021	6.62 (3.88-11.29) p<0.0001	24.94 (12.19-51.02) p<0.0001	6.01 (3.61- 9.99) p<0.0001
SP-AQ	2.38 (1.04-5.44) p=0.0395		2.55 (1.38- 4.71) p=0.0028	3.02 (1.23- 7.41) p=0.0161	6.93 (2.16- 22.25) p=0.0011	15.76 (8.13- 30.56) p<0.0001	59.39 (19.90-177.26) p<0.0001	14.31 (5.79- 35.32) p<0.0001
PY-AS	0.93 (0.64- 1.36) p=0.7219	0.39 (0.21- 0.72) p=0.0028		1.18 (0.81- 1.72) p=0.3795	2.72 (1.47- 5.04) p=0.0015	6.18 (5.00- 7.64) p<0.0001	23.29 (13.10-41.41) p<0.0001	5.61 (3.85- 8.17) p<0.0001
AS-AQ	0.79 (0.48- 1.31) p=0.3606	0.33 (0.13- 0.81) p=0.0161	0.85 (0.58- 1.23) p=0.3795		2.30 (1.41- 3.74) p=0.0008	5.23 (3.97- 6.89) p<0.0001	19.69 (13.06-29.70) p<0.0001	4.74 (4.53- 4.96) p<0.0001
AL	0.34 (0.17- 0.68) p=0.0021	0.14 (0.04- 0.46) p=0.0011	0.37 (0.20- 0.68) p=0.0015	0.43 (0.27- 0.71) p=0.0008		2.27 (1.26- 4.11) p=0.0064	8.57 (4.69-15.63) p<0.0001	2.06 (1.27- 3.36) p=0.0037
Non-ACT-PQ	0.15 (0.09-0.26) p<0.0001	0.06 (0.03-0.12) p<0.0001	0.16 (0.13-0.20) p<0.0001	0.19 (0.15-0.25) p<0.0001	0.44 (0.24-0.79) p=0.0064		3.77 (2.26-6.27) p<0.0001	0.91 (0.69-1.20) p=0.4959
ACT-PQ	0.04 (0.02-0.08) p<0.0001	0.02 (0.01-0.05) p<0.0001	0.04 (0.02-0.08) p<0.0001	0.05 (0.03-0.08) p<0.0001	0.12 (0.06-0.21) p<0.0001	0.27 (0.16-0.44) p<0.0001		0.24 (0.17-0.35) p<0.0001
ACT-TQ	0.17 (0.10-0.28) p<0.0001	0.07 (0.03-0.17) p<0.0001	0.18 (0.12-0.26) p<0.0001	0.21 (0.20-0.22) p<0.0001	0.48 (0.30-0.79) p=0.0037	1.10 (0.83-1.46) p=0.4959	4.15 (2.86-6.02) p<0.0001	

Hazard ratios for between-arm comparisons of molecular gametocyte clearance, with corresponding 95% CI and p-value.

E.g. Hazard ratio of molecular gametocyte clearance for AL compared to DHA-PPQ is 2.91, meaning that clearance is 2.91 times more likely to take place in the AL group compared to the DHA-PPQ group, and this is significantly different (p=0.0021).

APPENDIX 2

Study	PUBMED ID	Year	Treatment arms	N	Inclusion criteria	G6PD testing	Primary outcome studyvisit	#Mosquitoes fed in DMFA	DMFA timepoints	Venous blood collection	Finger-prick collection	Molecular assessment of gametocyte densities
PQ01	26906747	2013-2014	DHA-PPQ DHA-PPQ + 0.0625 mg/kg PQ DHA-PPQ+ 0.125 mg/kg PQ DHA-PPQ + 0.25 mg/kg PQ DHA-PPQ + 0.5 mg/kg PQ	16 16 17 15 17	G6PD+ males 5-50 years ≥2 gametocytes/500 WBC by microscopy	OSMMR2000-D G-6-PD, R&D Diagnostics, Aghia Paraskevi, Greece	Day 2	90	Days 0,1,2,7	Days 0,1,2,3,7,14,28	Day 1	Pfs25 RT-qPCR
PQ03	29422384	2016	SP-AQ SP-AQ + 0.25 mg/kg PQ DHA-PPQ DHA-PPQ + MB	20 20 20 20	G6PD+ males 5-50 years asymptomatic ≥1 gametocyte/500 WBC by microscopy	Carestart G6PD rapid diagnostic test (Access Bio, Somerset, NJ, USA)	Days 2 and 7	70	Days 0,2,7	Days 0,1,2,7,14,28,42	Days 0,1,2,3,7,14,28,42	PfMGET + CCP4 RT-qPCR
NECTAR1	35028628	2019	DHA-PPQ DHA-PPQ + 0.25 mg/kg PQ PY-AS PY-AS + 0.25 mg/kg PQ	25 25 25 25	males & non-pregnant females 5-50 years asymptomatic ≥1 gametocyte/500 WBC by microscopy	/	Day 2	75	Days 0,2,7,10*,14*, 21*,28*,35*,42*,49*	Days 0,2,7,10*,14,21, 28,35*,42*,49*	Days 0,1,2,7,10*,14,21, 28,35,42,49	PfMGET + CCP4 RT-qPCR
NECTAR2	35544095	2020	DHA-PPQ DHA-PPQ + 0.42 mg/kg TQ DHA-PPQ + 0.83 mg/kg TQ DHA-PPQ + 1.66 mg/kg TQ	20 20 20 20	G6PD+ males & non-pregnant females 12-50 years asymptomatic ≥1 gametocyte/500 WBC by microscopy	OSMMR-D G-6-PD Test, R&D Diagnostics, Aghia Paraskevi, Greece and STANDARD G6PD Test, SD Biosensor, Suwon, South Korea	Day 7	75	Days 0,2,7,14	Days 0,1,2,5,7,14,21,28	Days 0,1,2,7,14,21,28	PfMGET + CCP4 RT-qPCR
NECTAR3	38705163	2021	AL AL + 0.25 mg/kg PQ SP-AQ SP-AQ + 1.66 mg/kg TQ	20 20 20 20	G6PD+ males & non-pregnant females 10-50 years asymptomatic ≥1 gametocyte/500 WBC by microscopy	OSMMR-D G-6-PD Test, R&D Diagnostics, Aghia Paraskevi, Greece and STANDARD G6PD Test, SD Biosensor, Suwon, South Korea	Day 2 (AL groups) or 7 (SP-AQ groups)	75	Days 0,2,5,7,14,21,28	Days 0,2,5,7,14,21,28	Days 0,1,2,5,7,14,21,28	PfMGET + CCP4 RT-qPCR
NECTAR4	Unpublished MedRxiv	2022	AL AL-AQ AL-AQ + 0.25 mg/kg PQ AS-AQ AS-AQ + 0.25 mg/kg PQ	20 20 20 20 20	Males & non-pregnant females 10-50 years asymptomatic ≥1 gametocyte/500 WBC by microscopy	/	Day 2	75	Days 0,2,7,14,21,28	Days 0,2,7,14,21,28	Days 0,1,2,7,14,21,28	PfMGET + CCP4 RT-qPCR

Abbreviations:
DHA-PPQ, Dihydroartemisinin-piperaquine; PQ, Primaquine; SP-AQ, Sulfadoxine-pyrimethamine plus amodiaquine; PY-AS, Pyronaridine-Artesunate; TQ, Tafenoquine; AL, Artemether-lumefantrine; AL-AQ, Artemether-lumefantrine-amodiaquine; AS-AQ, Artesunate-amodiaquine; MB, Methylene-Blue
N, number of patients; DMFA, Direct membrane feeding assay; G6PD, Glucose-6-Phosphate Dehydrogenase; RT-qPCR, Reverse Transcriptase quantitative Polymerase Chain Reaction

Underline: infectivity assays were only conducted at that time point if any of the previous two assays resulted in at least one infected mosquito

* only in the non-primaquine treatment groups

Chapter 6: Discussion and conclusion

6.1 Discussion

6.1.1 Molecular markers to monitor drug efficacy

6.1.1.1 Drug resistance in Mali

Malaria control efforts in Mali currently utilise a variety of methods aimed at both the malaria parasite and its mosquito carriers. These methods include the distribution of ITNs, the usage of IRS, the administration of IPTp using SP, SMC using SP-AQ, and the treatment of malaria cases with AL (1).

As interventions are introduced, an evolutionary arms race occurs between the malaria parasite and antimalarial drugs, vaccines and diagnostic tests, as well as between mosquito vectors and insecticides. Monitoring the evolution of resistance is essential for effective malaria management. TESs are the gold standard to ensure treatment efficacy, however, genomic surveillance of drug resistance markers has an increasingly important role in monitoring antimalarial resistance (2). Molecular resistance data can inform treatment guidelines on which control measures are most appropriate and where they should be applied for maximum effectiveness.

Prior to this thesis, data on molecular markers of antimalarial resistance and whole-genome sequences of *P. falciparum* isolates from Mali was available, but only up to 2017 (3–5). In a dynamic and rapidly evolving landscape of antimalarial resistance, the whole genome sequences generated and analysed in **chapter 2** (6) from *P. falciparum* isolates collected in 2019-2020 provide the most up-to-date antimalarial drug resistance and genome-wide genetic variation profile from Mali. This past decade has been especially important for molecular surveillance in Africa, with ART-R resistance emerging in multiple countries across the continent (7). In addition, the comparison to older isolates from the same country allows for an evaluation of temporal changes in genomic variation. The infections sequenced in this study originate from asymptomatic individuals, who are typically underrepresented in genomic analyses due to the need for active sampling, despite constituting a significant portion of the infectious reservoir (8–10). In a country where transmission seasons and malaria epidemiology are highly heterogeneous across different regions (3,11), these data provide the first whole genome sequences from the Ouélessébougou commune.

We found that these 2019-2020 Ouélessébougou isolates formed a subcluster within older Malian isolates, and that they had a higher multiclonality (80% of samples were polyclonal) compared to older Malian isolates (48% and 56% of samples were polyclonal in 2007-2014 and 2015-2017 samples, respectively) (3,5) and compared to another study reporting complexity of infection in Malian isolates from 2016 (11-34% of samples were polyclonal, depending on study site) (4). Several factors may explain the highly multiclonal nature of the parasite population in our study. These include the region's high *P. falciparum* incidence rates, sample collection during peak transmission season, and the asymptomatic presentation of participants, while previous studies involving Malian *falciparum* infections did not provide information on their clinical presentation. Since asymptomatic gametocyte carriers are less likely to seek treatment, infections may have persisted longer, increasing the likelihood of reinfection and multiclonality. Assessing and genotyping the asymptomatic reservoir of parasites is important, as upwards of 75% of all infections that are detected during community surveys are asymptomatic (12,13). Adding to the complexity, ~30% of infectious individuals may carry submicroscopic parasites, which can remain undetected even in active sampling methods (14). The role of asymptomatic *P. falciparum* infections in the evolution and spread of antimalarial drug resistance in unclear. Studies have suggested that asymptomatic infections constitute a reservoir of submicroscopic resistant parasites that remain after incomplete treatment (15), while others hypothesized that they can select for drug susceptible parasites, especially during therapy-free windows in the dry seasons (16). The latter may be due to fitness costs that are associated with drug resistance mutations, resulting in reduced growth and/or transmission (17,18). More refined surveillance strategies beyond passive case detection are necessary, incorporating resistance monitoring to better define the asymptomatic reservoir of parasites.

I hypothesised that antimalarial resistance mutations associated with SP and AL may have increased due to the ongoing use of these drugs in Mali, and that CQ resistance may have decreased since this antimalarial is no longer used in this region. In line with the former, we observed widespread molecular markers of resistance to SP, which likely reflects the ongoing use of SP in IPTp. This included a high prevalence of triple *pfdhfr* N51I-C59R- S108N mutant (82.72%), as well as high allelic frequency of *pfdhps* A437G (70.59-74.32%) and S436A (52.70-58.82%). While nearly absent in the WGS Malian data collected between 2007 and 2017 (3,5), the *pfdhps* K540E mutation was detected in 5.88% of the 2019 isolates and 2.70% of the 2020 isolates, which was in line with two other studies conducting targeted sequencing of drug resistance markers in Mali (4,19). In addition, all K540E mutant isolates harboured the triple *dhfr* mutant. The WHO uses the K540E mutation as a measure to inform decision-making surrounding implementation policy,

recommending discontinuation of IPTp with SP when the population prevalence of K540E is greater than 95%, and the prevalence of mutation A581G is greater than 10% (20). This is supported by a meta-analysis concluding that IPTp did not reduce the risk of low birth weight in infants in studies in East Africa where K540E exceeded 50% (21). Another study reported that even in areas where *pfdhps*540E prevalence exceeds 90%, modest reductions in risk of low birth weight remain, if *pfdhps*581G prevalence is below 10% (22). While K540E prevalences have started to reach this threshold in East-Africa (23,24), the prevalences found in our study along with ones reported previously in Mali and other countries in West-Africa are still far below this threshold (5,23). Tools such as the WorldWide Antimalarial Resistance Network (WWARN) SP Molecular Surveyor, providing up-to-date spatial information on the spread of antimalarial resistance, are increasingly important for health organizations to prioritise surveillance efforts and effectively plan control and elimination strategies (23).

Prior to the introduction of AL in Mali in 2006, CQ was the first-line therapy for uncomplicated malaria. We found that frequencies of molecular markers conferring CQ resistance have persisted in the 2019–2020 Malian isolates at similar frequencies compared to a decade ago (K76T prevalence of ~50%). This is in contrast with other countries in sub-Saharan Africa observing CQ sensitive parasites again (25–28) and may reflect continued off-label use, which often involves the sale of low-quality CQ products (29). It is important to note that since the publishing of this study, another gene involved in CQ resistance has now been identified. The putative amino acid transporter (*pfaat1*) was found to manipulate the balance between amino acid and drug transport in the food vacuole, with the variants S258L and F313S influencing CQ resistance (30), thereby adding to the complexity in CQ resistance.

The first-line treatment for uncomplicated malaria in Mali is AL (1). We found the N86 variant in *mdr1,* associated with resistance to lumefantrine, to be present in 97.4% of the 2020 isolates, showing an increase from 71.6% in 2007. We did not detect any mutations in *K13* associated ART-R in the 2019-2020 isolates from Ouélessébougou, consistent with previous studies in West-Africa (3,5,31). The efficacy of AL for treating uncomplicated malaria was still high in the most recent efficacy study conducted in Mali (PCR-corrected efficacy at day 28 of 91.0%), however this study was conducted between 2015 and 2016 (32), and no updated TES data have been made available. A TES in the neighbouring country Burkina Faso conducted in 2017-2018 found that the PCR-corrected 28-day therapeutic efficacy of AL had decreased to 74% in certain study sites. Continued monitoring of therapeutic efficacy levels are necessary to inform treatment guidelines (33). To my knowledge, no updated reports on drug resistance frequencies in Mali have been published since

our study. It is likely that the prevalence of antifolate resistance markers will continue to rise in the coming years. Historical patterns of resistance to earlier first-line antimalarials, which first emerged in Southeast Africa, then spread through East Africa and eventually reached West Africa, suggest that markers of ART-R are likely to spread or independently emerge in West Africa in the coming years as well (34). Samples collected since the study in **chapter 2**, including during the trial conducted in **chapter 4**, can be used for continued molecular surveillance of resistance markers in this region.

In summary, the emergence of ART-R in Africa highlights the critical need for intensified monitoring. This includes tracking K13 mutations across the continent, testing for *ex vivo* resistance to artemisinin, lumefantrine, and other partner drugs, and routinely evaluating the efficacy of leading ACTs.

6.1.1.2 Surveillance methodology

Although WGS provides a wealth of data, this technique is expensive and may be cost-prohibitive, while drug resistance profiles can also be assessed by targeted sequencing, offering a cheaper and more high-throughput alternative (35). In addition, the recent development of portable sequencing devices, such as the Oxford Nanopore Technologies MinION platform, allows for whole-genome or targeted sequencing with the added advantage of minimal infrastructural requirements, enabling in-house sequencing. In **chapter 3**, nanopore sequencing is used to conduct targeted sequencing of drug resistance markers, using a protocol adapted from Girgis et al (31). Whilst nanopore sequencing currently has a higher error rate than Illumina and other competitor sequencing platforms, the technology is advancing rapidly, with continuous improvements in accuracy, affordability, and accessibility. The longer sequence reads generated by nanopore sequencing can provide additional advantages, such as the ability to characterise highly polymorphic or repetitive regions, as well as complex structural rearrangements, which are often difficult to resolve using short-read sequencing technologies (36,37).

Although important for an antimalarial policy change, TES may take years to plan, conduct, analyse and disseminate the findings. Nanopore sequencing has the potential to be implemented in endemic settings for real-time genomic surveillance of malaria infections and integrated into clinical and public health applications. Population surveys with nanopore surveillance monitoring could, for example, be conduction on top of TES. Molecular markers are currently not routinely monitored by national malaria control programmes (NMCP) and are primarily assessed in research

studies rather than integrated into standard surveillance efforts. Incorporating molecular markers into routine surveillance for antimalarial drug resistance could significantly enhance the ability to monitor drug efficacy, enabling the early detection of resistance before it leads to treatment failures. Without surveillance, resistance is only detected when treatment failure occurs, at which point options to respond are limited.

6.1.1.3 ART-R in Africa

The first evidence of ART-R in Southeast Asia was published in 2008 (38). Since then, ART-R has become widespread in Southeast Asia and partner drug efficacy has deteriorated as well, leading to increasing reports of ACT treatment failure (39,40). As malaria incidence was low in these regions and a large proportion of infections were already resistant to ACTs, the best strategy to combat drug resistance was to aim for complete elimination in these regions. The Regional Artemisinin-resistance Initiative (RAI) worked together with NMCPs to accelerate progress towards elimination, using improved surveillance systems, with active case detection, chemoprophylaxis and mass drug administrations (41).

Recently, ART-R has emerged independently in Africa (42,43) and is associated with several mutations in *PfK13* that are distinct from those in Southeast Asia (7). As most regions in Africa are high-transmission settings, a focus on malaria elimination remains premature. However, history shows that rapid action is needed, and that molecular surveillance as part of a multifaceted approach is essential to contain the spread of resistance (44). More specifically, validated and standardised protocols with agreed quality control thresholds are needed for the transition from research settings to public health application. In addition, standardised data analysis and data sharing platforms for rapid dissemination of surveillance data are needed. The WHO recognised this need to validate methods and approaches in the 2024 MPAG meeting (45). A key lesson from the drug resistance spread in Southeast Asia is that once ART-R emerges, resistance to the partner drugs in combination therapies is likely to follow. Therefore, WGS is needed in clinical trials to continue the search for new markers of resistance, especially for partner drugs such as lumefantrine, for which no good molecular marker of resistance is currently available. Mutations in *PfK13* do not fully explain level of ART-R either, since resistance phenotypes can vary based on the genetic background of the *PfK13* mutations (46) and genetic polymorphisms that contribute to ART-R in addition to *PfK13* have been identified (47,48). Moreover, certain therapeutic efficacy studies conducted in Angola, the Democratic Republic of the Congo, Burkina Faso, and Uganda have reported genotype-corrected efficacies below 90% for AL at some sites (33,49–51). Notably,

these studies were conducted in areas where *PfK13* were not highly prevalent, confirming that additional molecular markers for ART-R are needed.

The dynamics of ART-R in Africa are likely to differ from those observed in Southeast Asia. Unlike Southeast Asia, which is characterised by low malaria transmission, most African regions experience high *P. falciparum* transmission, resulting in multiclonal infections with high competition of clones, significant population-level immunity, and a lower proportion of symptomatic cases (9). Furthermore, the use of antimalarials is poorly quantified in African populations, and the large reservoir of asymptomatic infections could lead to relatively low drug pressure (52), potentially slowing the spread of *PfK13* mutant parasites. These factors suggest a slower spread of ART-R in Africa and may indicate that the situation is less urgent than widely perceived. However, it remains uncertain whether *PfK13* mutant parasites in Africa might possess a transmission advantage (53,54), which could accelerate their spread and counterbalance the slower progression expected under current conditions.

6.1.2 The complex nature of parasite infectivity

While the first-line antimalarial therapy in Mali focuses on clearance of asexual malaria parasites, which are responsible for the clinical symptoms of the disease, human-to-mosquito transmission is mediated by the sexual blood stages, or gametocytes. Interrupting human-to-mosquito transmission is important for malaria elimination strategies as it can reduce infection burden in communities. In addition, blocking transmission is crucial to slow the spread of drug resistance. This is especially important since reports have observed that drug-resistant parasites can be more transmissible, as has been observed for CQ-resistant parasites (55), and more recently has been observed for ART-R parasites under artemisinin drug pressure (53). A transmission advantage can be the result of an increased production of gametocytes (higher commitment rate), increased longevity of gametocytes or other factors leading to a more efficient fertilisation. A higher gametocyte production was previously observed in SP-resistant parasites, without assessment of transmission efficiency (56,57). Overall, studies assessing human-to-mosquito transmissibility are limited, mainly due to the technical difficulties performing membrane feeding assays or sampling naturally-infected mosquitoes along with blood material from the infectious individual. **In chapter 3**, I investigate the complexity of infection and drug resistance molecular markers in human blood samples and infected mosquito midguts of mosquitoes that fed on the same blood material.

We observed that parasite transmission dynamics were highly complex, with minority clones preferentially transmitting before treatment (**Figure 1**). These minority clones appeared to persist post-treatment as well. Moreover, even after treatment, when only gametocyte-producing clones remained, certain clones still failed to transmit to the mosquito. Additionally, at all observed timepoints (before, during, and after treatment), parasite clones were observed transmitting that could not be detected in blood samples (mosquito-only clones). These findings expand on previous reports about the complex interactions between parasites and their hosts in natural environments and during treatment (58–62). However, they also highlight that much remains unknown in this area of study, as well as the complexities involved in studying these interactions.

We aimed to assess any transmission advantages or disadvantages of drug-resistant parasite clones in polyclonal infections, by genotyping for molecular markers of drug resistance at 48 hours after treatment initiation. This timepoint was chosen as it reflects a time when almost all asexual blood stages have been killed by the ACT, while gametocyte levels have barely declined. As no resistance-conferring *PfK13* mutants were detected in the study samples, and the antimalarial regimens included in this study were ACTs (DHA-PPQ and PY-AS), the only drug resistance markers assessed that were under drug selection in our study were mutations in *Pfmdr1* or *Pfcrt* conferring piperaquine or pyronaridine resistance. We found a transmission dis(advantage) associated with mutations in *Pfdhfr* and *Pfdhps* conferring SP-resistance, which suggests an intrinsic variation in transmissibility without any drug selection (**Figure 1**). As mutations in *Pfdhfr* and *Pfdhps* are often detected in combinations (forming triple to sextuple mutants), it is difficult to envision how individual mutations could lead to changes in transmissibility. For the K540E polymorphism in *Pfdhps*, it is perhaps more straightforward to entertain the possibility of a transmission advantage, since this mutant is present in high frequencies in East Africa, as discussed above, whilst also being on the rise in West Africa. The fact that we find a transmission advantage associated with this polymorphism could have implications for the speed of its spread and the speed at which SP containing regimens may lose effectiveness.

Many laboratory assay and bioinformatics challenges were encountered during the work described in **chapter 3**. A high percentage of infected midguts did not amplify in the complexity of infection assays. This is likely due to the low concentrations of parasite DNA in the extract, the sensitivity of the assay, or, most likely, a combination of both. Many different methods were tested to optimise the DNA extraction of the infected midguts, such as the Qiagen DNeasy blood and tissue kit, phenol-chloroform extraction, Qiagen investigator kit, Quanta bio Extracta, Qiasymphony Qiagen robot extraction and Dynabeads SILANE Genomic DNA kit, however, these did not

improve the extraction outcome. The RNA protect cell reagent (Qiagen, Hilden, Germany) as storage buffer is a plausible cause of this suboptimal DNA storage or extraction. A better alternative could have been oocyst lysis buffer (NaCl 0.1 M: EDTA 25 mM: Tris–HCl 10 mM), which showed successful results for the storage and sequencing of infected mosquito midguts recently (63). The mercurochrome that was used to stain the midguts for oocyst detection prior to storage is another potential culprit, since mercurochrome was found to intercalate into DNA, which could inhibit or alter the DNA polymerase's ability to bind to the DNA template and replicate it in a PCR reaction (64). Many attempts were made to optimise the sensitivity of the assay, such as optimisation of PCR annealing temperatures, extension temperature, and number of PCR cycles. The latter increased the likelihood of amplification, however, upon bioinformatics analysis, many chimeric haplotypes were found as a result of chimera formation during the PCR reaction (65).

In addition to these challenges, we found that there was a certain level of contamination among samples in the sequencing data, evidenced by barcode combinations that were not sequenced being present in the data. This was likely caused by the formation of chimeric sequences in the PCR reaction during library preparation, or by a small amount of primer sequences from the first PCR reaction carrying over to the PCR reaction during library preparation. This issue was resolved by switching to a library preparation by adaptor ligation, which does not involve an additional amplification step.

Two highly heterozygous regions were assessed in the complexity of infection assay, although a greater number of markers would have been preferable. Better assay alternatives are available in the meantime, such as the Multiplex Amplicons for Drug, Diagnostic, Diversity, and Differentiation Haplotypes via Targeted Resequencing (MAD4HatTeR), a highly multiplexed amplicon sequencing panel for *P. falciparum* consisting of 165 highly diverse microhaplotypes (https://github.com/EPPIcenter/mad4hatter) (66). In addition, while assessing haplotypes within amplicon sequences is informative, for polyclonal infections, the ability to phase genetic variation across amplicon sequences would provide deeper insights. For instance, it would enable the exploration of whether certain drug resistance mutations predominantly occur in minority or majority clones, and whether these are being selected for under drug pressure. Current bioinformatics tools that have been developed for this purpose such as DEploid and DEploidIBD (67,68) are not designed to handle highly polyclonal infections, and a second generation of tools are being developed, using advanced Markov chain Monte Carlo (MCMC) algorithms to ensure

that results are accurate even for high complexity of infection (https://github.com/mrc-ide/Tapestry).

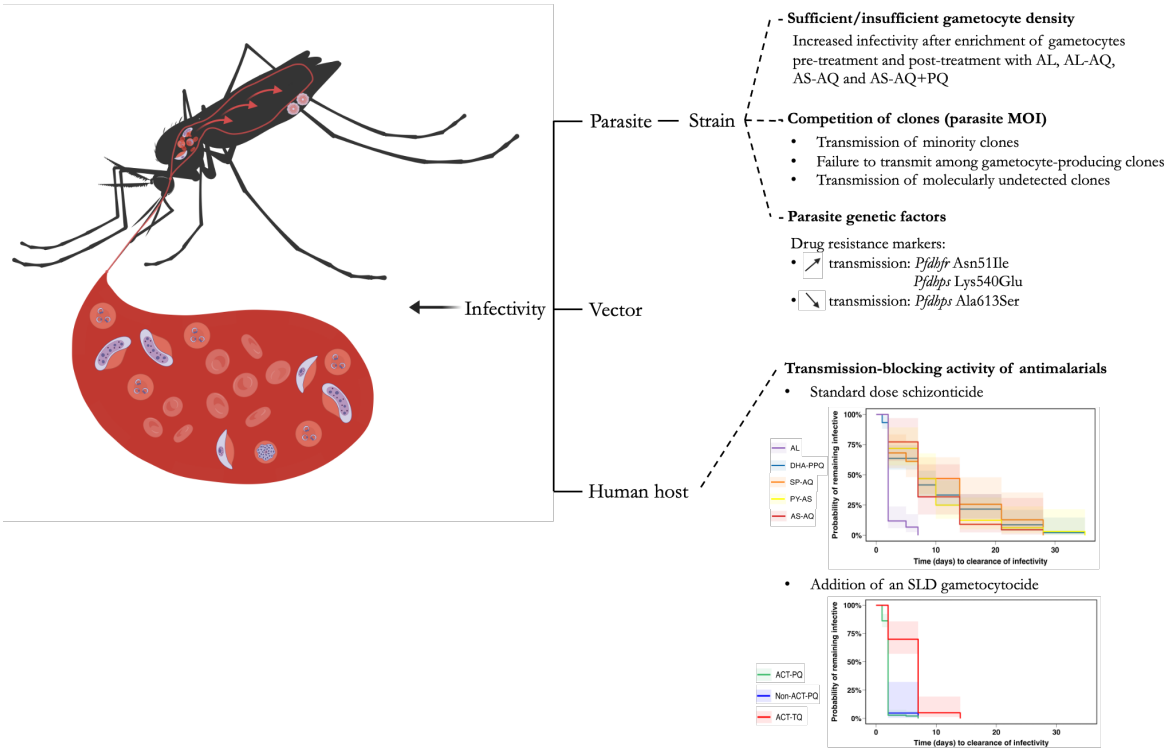


Figure 1. Factors and drugs influencing human-to-mosquito transmissibility. A schematic showing an overview of factors that influence the ability of malaria parasites to establish an infection in the mosquito host, with results from different chapters annotated. Created with BioRender.com.

As demonstrated by the transmission of molecularly undetectable clones (mosquito-only clones) in this chapter, and consistent with previous studies (58,59), sequencing limitations can hinder the capture of very low-density infections. In some cases, transmission of molecularly undetectable clones even persisted after treatment, when only gametocytes were remaining. This suggests an inherent sequencing bias in polyclonal infections, meaning that low-density infections may consistently be missed, leaving their genetic variation unassessed (69,70). Moreover, there is a lack of bioinformatics tools capable of accommodating highly polyclonal infections in population genomics analyses.

Finally, in this thesis chapter, complexity of infection was assessed in human blood material and paired infected mosquito midguts before and after ACT treatment, to allow investigation of stage-specific parasite dynamics and infectivity. It would be preferable to investigate this prior to any

treatment, to more accurately reflect natural infections. A gametocyte-specific genotyping marker was recently identified, enabling the assessment of infection complexity and the relative frequency of mature gametocyte parasite clones in natural *P. falciparum* infections. This was achieved by targeting a region of the *pfs230* gene in cDNA synthesized from extracted RNA (71).

6.1.3 The importance of blocking transmission

The development of resistance to past and present antimalarial drugs highlights the need for continued research to ensure that antimalarial drug development stays one step ahead of parasite evolution. New drugs are needed, especially those with novel mechanisms of action to prevent the overlap of resistance pathways with existing treatments. New antimalarials are in development, such as the elongation factor inhibitor M5717 (72), the *PfATP4* inhibitor Cipargamin (KAE609) (73), Acetyl-Coa synthetase inhibitor GSK701 (MMV367) and drugs with unknown targets such as Ganaplacide (KAF156) (74) and INE963 (75). However, these compounds are currently in phase 1-3 clinical trials and even if their development continues successfully, they may not become available for many years. As ART-R is already emerging and spreading across Sub-Saharan Africa, there is an urgent need for effective interventions. In the meantime, different approaches can be taken with existing antimalarials, such as cycling of ACTs or multiple first-line treatments (76). The latter could be achieved by administering different first-line therapies based on age group, region, a random factor (such as the day of the week), or true randomisation. This would reduce the constant exposure to one type of drug and thereby decrease the likelihood of resistance forming against any single treatment. The addition of a third partner drug to ACTs (TACTs), such as AL-AQ, is an alternative approach (77,78). In ACT regimens, after the third day of treatment, the slowly eliminated partner drug is unprotected by the rapidly eliminated artemisinin component of the regimen. The addition of a second slowly eliminated partner drug provides mutual protection for the partner drugs in TACTs (79). Furthermore, combining lumefantrine and amodiaquine may leverage opposing resistance mechanisms (80,81). Mathematical models suggest that the implementation of TACTs would delay the emergence and spread of drug resistance and extend the useful therapeutic life of the existing antimalarials (82).

However, a key determinant in this is the transmission-blocking activity of TACTs, which was unknown prior to this thesis. In **Chapter 4** (83), we test the transmission-blocking effect of AL-AQ alone and in combination with SLD PQ. We found that AL-AQ prevents nearly all infectivity within 48 hours and has comparable activity to AL alone. The addition of SLD PQ showed a small added benefit, blocking all transmission within 48 hours. With the aim of differentiating between

a lack of infectivity due to low gametocyte densities and gametocyte sterilisation, gametocytes were enriched in our study pre-treatment and at day 2, followed by another membrane feeding assay. In the AL and AL-AQ groups, the percentage of infectious individuals increased at day 2 following gametocyte enrichment, indicating that viable gametocytes were still present in the circulation after 48 hours, albeit at densities too low to establish a mosquito infection under normal conditions (**Figure 1**). However, in the group in which AL-AQ was combined with an SLD PQ, transmission remained completely blocked at day 2, even after gametocyte enrichment. This suggests that adding an SLD PQ to AL or AL-AQ still offers a benefit. Additionally, it is important to note that while AL-AQ without PQ effectively prevents most mosquito infections within 48 hours, gametocytes in *PfK13* mutant infections may preferentially survive artemisinin exposure, allowing them to remain infectious (53).

In this study we also tested the transmission-blocking activity of AS-AQ, which is an ACT regimen that is a first-line treatment for uncomplicated *falciparum* malaria in 19 African countries (1). Despite this widespread use, its effect on gametocytes and transmission remained unclear prior to this thesis. We observed substantial transmission after AS-AQ, with some participants remaining infectious until the end of follow-up (day 28). Adding an SLD of PQ to AS-AQ effectively blocked all transmission within 48 hours.

The trial in **chapter 4** was the sixth transmission study done to assess transmission-blocking activities of antimalarial regimens at the same study site in Mali (84–88). The findings of these trials provided insights into the transmission-blocking activity of many different antimalarial regimens; however, temporal and inter-trial variation makes it difficult to compare treatment groups from different trials. In **chapter 5**, transmission-blocking efficacies of 15 antimalarial regimens and dosing schedules that were tested in these six transmission trials were analysed together from individual patient data, to allow for comparisons between treatment regimens (**Figure 1**). We found that AL was more effective than other ACTs (DHA-PPQ, AS-AQ, PY-AS) at blocking transmission, with a significant difference in the reduction of mosquito infection rate at day 2 compared to baseline. Our data also showed that SLD PQ effectively annulled transmission in combination with any ACT, while SLD TQ had a delayed transmission-blocking effect compared to SLD PQ. SP-AQ had a lower transmission-blocking efficacy than most ACTs, causing substantial post-treatment transmission after SP-AQ. Therefore, it may be beneficial to add an SLD PQ to SP-AQ in chemoprevention strategies to block malaria transmission in community treatment campaigns. Overall, the findings from our pooled analysis support the suggestion by the WHO Malaria Policy Advisory Group (MPAG) meeting report to expand the use of SLD PQ with

the aim of slowing the spread of drug resistance (89). While ART-R has only been detected in a subset of African countries so far, there is an argument for extending the use of SLD gametocytocides across the African continent (90), as there is an inherent lag in detecting and reporting the emergence of drug resistance, while it may already be present and disseminating undetected.

Mathematical models are often used in malaria research to guide decisions on which deployment strategies to explore and which populations to target (91). The accuracy of a mathematical model's predictions is proportional to the precision and quality of the input parameters used (92). The work in **chapter 5** provides a first direct comparison of the effects of many different antimalarial regimens on infectivity. The findings from this analysis can be integrated into mathematical models, to simulate malaria transmission using different ACTs as first-line treatments, or to predict the spread of drug resistance with and without the addition of SLD gametocytocides to standard antimalarial treatment.

Since the initial WHO recommendation in 2012 to add 0.25 mg/kg of PQ to ACT treatment (93,94), there have been numerous studies to further prove the efficacy and safety of SLD PQ doses up to 0.5 mg/kg, even in G6PD deficient individuals (83–86,88,95–102). Despite this, the incorporation of an SLD PQ into antimalarial treatment regimens has been slow (1). Remaining challenges in administering an SLD of PQ in combination with ACTs include the need for a paediatric formulation, as PQ tablets currently need to be crushed or dissolved in order to administer the correct dose to children. This brings out the bitter taste and thereby affects compliance (103). Paediatric formulations including tablets of different sizes that reflect different doses, and acceptable flavouring, are in development (104). Perhaps the ideal formulation would be flavoured PQ granules, which allow flexible dosing and easy administration, such as direct swallowing without the need for drinking water (which is not always available in the field). Another challenge lies in the unregulated market for uncomplicated malaria treatment in many African settings, where the use of substandard medications, non-recommended antimalarial monotherapies and incomplete treatment regimens is frequently reported. This was recently recognised in the WHO Strategy to respond to antimalarial drug resistance in Africa (2). Additional challenges include the overall availability of PQ, perceptions of its safety, and the availability of good pharmacovigilance guidelines for local health facilities.

PQ is rapidly metabolised, and its active metabolites only circulate in the blood stream for a matter of hours (105). While this short half-life (4-9 hours) contributes to the safety of a SLD PQ in

G6PD deficient individuals, the downside is that the effect of SLD PQ may be limited to the killing and sterilisation of currently circulating gametocytes. Any maturing gametocytes that are released from bone marrow later in the infection or that arise from recrudescence may not be affected. We saw evidence of this in the gametocyte enrichment data in our study in **chapter 4**, where we could see an increase in infectivity at day 2 after gametocyte enrichment in the AS-AQ with SLD PQ group. We hypothesized that AS-AQ may not be as effective as some other ACTs, such as AL, at killing or accessing maturing gametocytes, and that PQ's active metabolites may no longer be present in the blood stream at efficacious concentrations when these now-matured gametocytes are being released from the bone marrow. TQ is an analogue of PQ that has a longer half-life (15 days) and could therefore offer a major advantage by preventing the transmission of infections that are acquired after initiation of treatment, although the duration of TQ's transmission-blocking activity is still unclear. While this extended half-life can be a benefit, it also carries the risk of prolonged haemolysis in G6PD deficient individuals, and no studies have been conducted to date to assess the safety of an SLD TQ in this population.

While the results of our trial support existing evidence that SLD gametocytocides can effectively reduce the infectiousness of treated individuals, the impact of these drugs in accelerating transmission reduction at the population level relies on their ability to reach a substantial portion of the infectious reservoir. The effectiveness of introducing transmission-blocking antimalarials will be limited unless the approximately 30% of infectious individuals that harbour submicroscopic gametocytes, as well as those with detectable densities, can be reached (14,106). Gametocytocides can also be included in MDA campaigns to eliminate the gametocyte reservoir in asymptomatic individuals within a population (107). Transmission-blocking vaccines or monoclonal antibodies (mAbs) could provide an alternative strategy for targeting the infectious reservoir by inducing antibodies against functionally important proteins that are expressed on transmission stages (108–110). The main vaccine candidates currently being developed include Pfs25 (111,112), Pfs48/45 (113,114), Pfs230 (115) and more recently Pfs47 (116). An ideal transmission-blocking vaccine or monoclonal antibody would be able to annul infectiousness for an entire transmission season, which in combination with natural clearance of lingering gametocytes, has the potential to vastly reduce the infectious reservoir in both human and mosquito hosts. The mAb TB31F is a humanised version of rat mAb 85RF45.1, which was derived from rats immunised with *P. falciparum* gametocytes and targets a highly conserved epitope on the Pfs48/45 surface protein (117,118). In a phase 1 clinical trial, a single dose of 10 mg/kg of TB31F showed an estimated potential to block >80% of transmission for up to 160 days (110). A phase 1/2a trial is currently

ongoing to assess the transmission reducing ability of TB31F in Mali over an 84-day follow-up period (NCT06413108).

Due to their ability to reduce human infectivity and transmission at the population level, as well as their potential to slow the spread of drug resistance, there is significant interest in drugs that exhibit transmission-blocking activity. Once mature gametocytes leave the bone marrow to re-enter the peripheral blood, they maintain a quiescent state and await uptake into the mosquito. This arrested state leads to an insensitivity to most current antimalarials (119). In addition, the complexity of screening drugs with transmission outcomes is a major challenge in the development of transmission-blocking drugs. *In vitro* assays are available to assess activity against mature gametocytes, using various indicators of metabolic activity to determine gametocyte viability (120–122). Functional assays such as the dual gamete formation assay (DGFA) and standard membrane feeding assays (SMFA) have also been developed; however, these methods are technically demanding and only a limited number of high-throughput screens have been performed (123,124).

Despite these challenges, there are a handful of drugs in the antimalarial development pipeline that have shown gametocytocidal and transmission-blocking properties. Perhaps the two most promising candidates are M5717 and Ganaplacide. On top of excellent *in vitro* activity against asexual parasites (IC50 of 1 nM), M5717 showed highly active against all gametocyte stages with IC50 values in the low-nanomolar range (IC50 values for stages I–V of 3, 5, 5, 1, and 9 nM, respectively (119)). Moreover, the compound had low nanomolar IC50 values in both direct (drug added at the moment of feeding) and indirect (gametocytes are pre-incubated for 24 hours with the drug prior to feeding) SMFA (125). M5717 is being developed in combination with pyronaridine and is currently in a phase 2a trial to evaluate its safety and efficacy to clear current infection and protect against recurrent infections in asymptomatic individuals with *falciparum* malaria infection (NCT05974267). Ganaplacide was shown to have low-nanomolar activity against both asexual blood and hepatic stage parasites and to inhibit the maturation of stage II gametocytes, with >75% and 100% reductions in stage V gametocytes at 5 and 50 nM, respectively. In an indirect SMFA, treatment with 500 nM Ganaplacide resulted in a 90% decrease in oocyst numbers. Additionally, *P. berghei*-infected mice treated with a single oral dose at 100 mg/kg were found to be non-infectious to *Anopheles* mosquitoes during blood feeding, thus confirming the compound's transmission-blocking potential. (126,127). Ganaplacide is currently in a phase 3 trial in a fixed dose combination with lumefantrine, to confirm its safety, efficacy and tolerability and non-inferiority to AL (NCT05842954).

Our findings from the gametocyte enrichment assay in **chapter 4** add to the existing evidence that PQ has gametocyte sterilising activity (128,129). It also suggests that AL may not sterilise gametocytes, or to a lesser extent, since two participants became infectious after gametocyte enrichment at day 2 after treatment, indicating low gametocyte densities post-treatment rather than sterilisation. However, our sample size was relatively small, making it difficult to draw any robust conclusions regarding mechanism of action from these finding. An important unresolved question is why AL is more effective at clearing gametocytes and blocking transmission compared to other ACTs. Since all artemisinin derivatives are believed to share the same target, interacting with iron in haem to generate reactive oxygen species, the difference may instead lie in the lumefantrine partner drug. Contrary to this, studies have demonstrated that lumefantrine has limited activity against mature gametocytes (120,130). Gametocyte sex ratios, as assessed in the individual transmission studies in Mali (83,86–88), showed that AL was the only ACT that preferentially cleared female gametocytes, resulting in a male-biased sex ratio from day 2 after treatment initiation. DHA-PPQ, PY-AS and AS-AQ did not significantly increase or decrease the sex ratio post-treatment. At baseline, before any treatment, gametocyte sex ratios were more male-biased than expected, with the proportion of male gametocytes close to 0.5, though highly variable sex ratios have been previously observed in natural infections (131). In addition, multiclonal infections were previously found to favour a more balanced proportion of male and female gametocytes, which may explain our findings, as most infections in our study are likely multiclonal, given that participants are asymptomatic gametocyte carriers over the age of 10 (132,133).

Antimalarial drugs are a key component of a comprehensive malaria control strategy, which also includes measures like vector control and vaccines. Malaria control may be evolving towards a lifetime protection approach, where interventions may vary by age group (e.g., PMC, vaccines, and SMC targeting specific at-risk populations). Combining antimalarial drugs with other strategies such as vaccines and vector control can enhance the effectiveness of these interventions, prolong their efficacy, and help delay the emergence and spread of drug resistance.

6.2 Future studies

In **chapter 3**, I investigated whether drug-resistant parasites showed an altered transmissibility in *P. falciparum* isolates from Mali and found two polymorphisms in the *pfdhfr* and *pfdhps* genes that were associated with a transmission advantage, without any SP drug pressure. A follow-up study should be conducted to investigate whether this increase in transmission is consistent or perhaps

even enhanced under drug pressure of antimalarial regimes that involve SP. In addition, *in vitro* commitment assays with parasite lines in which these mutations have been introduced can validate any intrinsic changes in gametocyte conversion rates.

Findings from Southeast Asia have suggested that gametocyte commitment (134) and mature gametocyte prevalence (40,135) may be higher in ART-R parasites. This can cause an intrinsic transmission advantage that is apparent prior to treatment, however, evidence suggests that it is likely to be more pronounced after treatment. Studies have shown that *PfK13* mutant male gametocytes are less sensitive to artemisinins compared to wild-type gametocytes (53,54), are more likely to continue transmission under artemisinin drug pressure and may result in enlarged oocysts (53). No data exists to date on the relative transmissibility of *PfK13* mutants in Africa, although this factor determines the speed at which drug resistance is spreading across the African continent. Therefore, studies examining the human-to-mosquito transmissibility, before and after ACT treatment, of *falciparum* infections in areas with high prevalence of resistance-conferring *PfK13* mutations in Africa are needed to address this knowledge gap.

The transmission trials in **chapters 4 and 5** tested the transmission-blocking activity of 15 antimalarial regimens in Mali, where no ART-R has been detected to date. As parasites with *PfK13* mutants may be more transmissible, it is unclear whether the findings would be consistent in regions where ART-R is common. Studies testing the efficacy of ACTs and an SLD PQ on gametocytes and infectivity need to be conducted in areas with ART-R in Africa, as this will inform treatment guidelines with the aim of slowing the spread of drug resistance. Sample size would be a key factor to consider in such studies, in the absence of a rapid molecular test to screen for the presence of *PfK13* mutations.

Additionally, while these transmission trials were designed to test the transmission-blocking efficacy of antimalarials and we saw significant differences in transmission outcomes, the public health implications of these findings need validation through community treatment trials that focus on community benefits such as parasite prevalence, incidence and clinical incidence. There is an urgent need for these community trials to determine the benefits of incorporating SLD gametocytocides into first-line antimalarial treatments and in mass treatment campaigns to reduce the transmission of (drug-resistant) malaria. Very recently, a cluster randomised controlled trial in Senegal found that three cycles of MDA with DHA-PPQ plus SLD PQ was associated with a 55% lower incidence of malaria during the transmission season of the intervention year, compared to the standard-of-care, which consisted of three cycles of SMC with SP-AQ. The authors

hypothesised that this was partly due to the MDA's additional effect on transmission, however, no comparison with DHA-PPQ alone was made in this study (136).

Genomic surveillance of drug resistance as was done in **chapters 2 and 3** will continue to be of high importance, and is to be included in national control programmes, in addition to TESs. Similarly, monitoring for vaccine resistance is important to ensure long-term effectiveness of vaccine deployment (137). On top of monitoring known markers of drug resistance, GWAS studies comparing malaria isolates with *in vivo* treatment failure or *in vitro* resistance with sensitive isolates can identify novel markers associated with drug resistance (47,138,139). While amplicon sequencing approaches only monitor what is already known, genome-wide data is needed for progress towards elimination. Identifying clinically relevant genotypes requires more thorough planning compared to opportunistic sequencing approaches. It necessitates integrating genomic strategies into clinical trial designs with sufficient statistical power to ensure meaningful results.

However, resistance is often not solely driven by one or a few genetic mutations, but instead involves a complex interplay of various metabolic and cellular factors. In addition, most genetic polymorphisms associated with complex traits have been found in non-coding regions, which often function as regulatory sequences. These regions influence gene expression, through which the phenotype is manifested (140,141). As a result, transcriptome-wide association studies (TWAS) have gained prominence as a method for identifying causative genetic variants underlying complex traits across diverse biological systems (142,143). In a study investigating the transcriptome of Southeast Asian ART-R *Plasmodium falciparum* isolates, an 'artemisinin resistance-associated transcriptional profile' was identified, which included 69 significantly upregulated and 87 downregulated transcripts, involved in biological processes such as proteotoxic stress, host cytoplasm remodelling, and REDOX metabolism (134). As ART-R parasites in Africa have emerged independently (42,43) and harbour *PfK13* mutations that are distinct from those in Southeast Asian isolates (42,43,144), it is unclear whether this transcriptional profile will be similar. Analysing the transcriptome of isolates with resistance-conferring *PfK13* mutations in Africa can identify the transcriptional responses and biological mechanisms involved in ART-R on the African continent.

To gain insights on a more detailed level, single-cell RNA sequencing (scRNA-seq) of malaria parasites allows for in-depth analysis of various aspects of parasite variation, including differences in gene expression, genetic mutations, relatedness, and transcriptional variation across strains at different stages of the parasite life cycle (145–147). This technique has recently enabled the creation

of the Malaria Cell Atlas (MCA) which represents a comprehensive resource of gene expression profiles of parasite stages in the human and mosquito host, at a single-cell resolution (147–149). Despite the great potential of scRNA-seq in malaria research, its use has been mostly limited to parasites from *in vitro* cultures or animal models, with only one published study so far analysing cells from four natural *P. falciparum* infections (147,150). Although expensive and technically challenging, recent advances in scRNA-seq technologies have made it increasingly feasible to study individual parasites in field isolates (151).

Analysing parasites at a single-cell level in natural infections offers the potential to address numerous questions, especially in multiclonal or mixed-species infections, where it was previously impossible to separate their transcriptomes. This approach allows for the exploration of how drug-resistant strains differ transcriptionally from sensitive parasites, even within the same infection, and throughout the parasite's development. In addition, this technique could also be used to gain insights into the mechanisms of antimalarial drugs by comparing transcriptional profiles before and after treatment, enhancing our understanding of their effects (152).

In recent years, the extensive application of multi-omics technologies, including genomics, proteomics, metabolomics, transcriptomics and epigenomics, has offered a more comprehensive understanding of the functional principles and dynamics underlying biological mechanisms (153–157). Multi-omics data can also allow us to identify novel drug targets by elucidating pathways essential for parasite survival and pathogenicity (152,157–159). However, most multi-omics data currently available has been derived from parasites that have been in culture for decades, and likely exhibit substantial differences to parasite strains from natural infections (150). Multi-omics approaches of natural infections combined with machine-learning approaches for data integration hold the promise of enhancing our understanding of the parasite molecular mechanisms and host interactions, and can guide the development of novel drugs and vaccines (158–160).

Historically, genomics research involving data derived from malaria-endemic regions has often involved exporting samples to institutions in the Global North, where the sequencing and analysis is conducted (161,162). This pattern can reinforce existing global health inequalities. While some progress has been made towards equity, looking ahead, it is essential for health research funders to commit to sustainable capacity strengthening in the Global South and to foster enhanced knowledge exchange between institutions in the Global North and South (163,164). Emerging technologies like portable sequencing devices, such as Oxford Nanopore Technologies MinION platform, enable real-time insights into drug resistance in resource-limited settings to improve local

research capabilities, and have the potential to help decentralise genomics. However, aside from global health funding systems, multiple challenges remain, such as procurement of sequencing reagents, maintenance of sequencing infrastructure and access to adequate computing power.

6.3 Conclusion

As sequencing technologies continue to advance, genomic techniques can help assess the effects of malaria interventions on the genetic diversity, population structure, and resistance patterns of *P. falciparum*. Worryingly, ART-R has been detected in several countries in Africa in the past few years, with high prevalences of *PfK13* mutants in certain regions in Uganda and Ethiopia. Assessing the transmissibility of these resistant parasites is crucial to determine the future of malaria prevention and treatment in Africa. The efficacy of artemisinin derivatives could be prolonged by combining them with multiple partner drugs (TACT) and/or with gametocytocidal drugs to slow the spread of drug-resistant parasites. In the meantime, the search for antimalarials with novel drug targets must continue, as well as the pursuit of safe and effective gametocytocides and transmission-blocking vaccines or antibodies to target the infectious reservoir and halt transmission for a substantial period of time. At the advent of the Big Data era, the generation of vast amounts of biological and chemical information can offer the scientific community new opportunities to connect drugs to diseases. *In silico* drug discovery using artificial intelligence to translate multi-omics data into potential druggable targets could provide a valuable aid in the fight against malaria, keeping the goal of elimination within reach.

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