Plasmodium falciparum sexual conversion rates can be affected by artemisinin-based treatment in naturally infected malaria patients

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eBioMedicine 2022;83: 104198
Published online 9 August 2022
https://doi.org/10.1016/j.ebiom.2022.104198

Summary

Background Artemisinins (ART) are the key component of the frontline antimalarial treatment, but their impact on *Plasmodium falciparum* sexual conversion rates in natural malaria infections remains unknown. This is an important knowledge gap because sexual conversion rates determine the relative parasite investment between maintaining infection in the same human host and transmission to mosquitoes.

Methods The primary outcome of this study was to assess the impact of ART-based treatment on sexual conversion rates by comparing the relative transcript levels of *pfap2-g* and other sexual ring biomarkers (SRBs) before and after treatment. We analysed samples from previously existing cohorts in Vietnam, Burkina Faso and Mozambique (in total, *n*=109) collected before treatment and at 12 h intervals after treatment. As a secondary objective, we investigated factors that may influence the effect of treatment on sexual conversion rates.

Findings In the majority of infections from the African cohorts, but not from Vietnam, we observed increased expression of *pfap2-g* and other SRBs after treatment. Estimated parasite age at the time of treatment was negatively correlated with the increase in *pfap2-g* transcript levels, suggesting that younger parasites are less susceptible to stimulation of sexual conversion.

Interpretation We observed enhanced expression of SRBs after ART-based treatment in many patients, which suggests that in natural malaria infections sexual conversion rates can be altered by treatment. ART-based treatment reduces the potential of a treated individual to transmit the disease, but we hypothesise that under some circumstances this reduction may be attenuated by ART-enhanced sexual conversion.

Funding Spanish Agencia Estatal de Investigación (AEI), European Regional Development Fund (ERDF, European Union), Belgium Development Cooperation (DGD), Canadian University Health Network (UHN), TransGlobal-Health−Erasmus Mundus (European Union).

Key words: *Plasmodium falciparum*; Sexual conversion; Artemisinin; Malaria transmission; *pfap2-g*

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Research in context

Evidence before this study

All clinical symptoms of malaria are produced by asexual parasite replication in the human blood. However, at each cycle of replication, a small fraction of the parasites converts into sexual forms termed gametocytes, which are the only parasite stages that can infect a mosquito vector. The conditions of the environment can modulate the proportion of parasites that convert into sexual forms, probably as a parasite response to stress conditions. In recent years, impressive research progress has started to unravel the molecular mechanisms driving sexual conversion in *P. falciparum*, and has identified several conditions that enhance sexual conversion rates under culture conditions. However, the regulation of sexual conversion rates in natural human *P. falciparum* infections is less well understood. We recently demonstrated that, *in vitro*, the antimalarial drug artemisinin (ART) can enhance *P. falciparum* sexual conversion rates in a dose and stage-dependent manner, but the impact of ART on sexual conversion in natural human infections is unknown.

Added value of this study

Determining the effect of ART on sexual conversion rates in natural malaria infections poses important challenges, because it requires disentangling the effect of the drug on sexual conversion rates from its effect on sexual and asexual parasite viability. To overcome these difficulties, we used recently described very early molecular markers of sexual conversion and frequent sampling after treatment. With this approach, we could measure the direct impact of ART-based treatment on *P. falciparum* sexual conversion rates in natural infections. We analysed samples collected in three previously existing, independent cohorts from Vietnam, Burkina Faso and Mozambique. We found that ART-based treatment generally resulted in an increase in sexual conversion rates in the Mozambique cohort and to a lesser extent in the Burkina Faso cohort, but not in the Vietnam cohort. This indicates that the effect of ART-based treatment on sexual conversion rates is not always the same. Because multiple host, parasite and treatment conditions differed between cohorts, we could not disentangle which specific factor was responsible for the differences between cohorts. However, the analysis of variability within individual cohorts identified ART-resistance status and parasite age at the time of treatment as factors that may influence the effect of treatment on sexual conversion rates.

Implications of all the available evidence

These results suggest that ART-based treatment can result in enhanced sexual conversion *in vivo*. The net effect of ART-based treatment on transmission is usually a reduced transmission potential, because ART is highly effective in killing asexual parasites, which are the source of new gametocytes, and directly eliminates some gametocytes. However, under some circumstances, an ART-induced increase in sexual conversion rates may attenuate the net reduction in the potential of an infected individual to transmit the disease after treatment. This may be especially relevant in individuals receiving suboptimal treatment. In addition, our findings demonstrate the feasibility of using early sexual biomarkers to assess how the conditions of the human host environment impact sexual conversion rates *in vivo*.

Introduction

The malaria parasite *P. falciparum* causes over half a million deaths per year, mainly in young children from low- and middle-income countries.[1] Malaria disease results from repeated cycles of intraerythrocytic asexual replication, which involves circulating ring stages and tissue-sequestered mature trophozoites and schizonts. At each asexual replicative cycle, a minority of the parasites convert into non-replicating sexual forms (gametocytes), which are essential for the continuation of the parasite's natural life cycle by allowing transmission from humans to anopheline mosquito vectors.[2,3]

The sexual conversion rate is defined as the proportion of parasites that convert into sexual forms at a given cycle of intraerythrocytic replication. The basal level of sexual conversion involves spontaneous commitment to sexual development of a small fraction of the parasites (typically ~1%),[4,5] which secures constant transmissibility of the infections. However, the conditions of the environment can enhance sexual conversion rates, possibly as an adaptive response to stress conditions.[2,6–11] Parasite commitment to sexual development depends on the transcription factor PfAP2-G, which is the master regulator of the process.[12–16] In asexually growing parasites, the *pfap2-g* gene is silenced by epigenetic mechanisms. A transition from a transcriptionally repressive to a permissive chromatin state at this locus results in expression of the gene and sexual commitment.[12,17] This transition is triggered by the parasite protein gametocyte development 1 (GDV1).[18] After sexual commitment, PfAP2-G progressively activates a new transcriptional program[12,14–16] and parasites differentiate into sexual forms either directly or after an additional cycle of replication.[19] The first stage of sexual development is the sexual ring stage, which recent work has shown to be present in the circulation.[20–23] Sexual rings develop into gametocyte stages I to V in a process that lasts ~10 days and occurs away from the blood circulation because maturing gametocytes are sequestered in tissues such as the bone marrow.[24] Once mature, male and female stage V gametocytes are released back into the peripheral blood, where they can be ingested during a mosquito bloodmeal.

The density of circulating stage V gametocytes is positively associated with the potential of a malaria patient to infect mosquitoes.[25–29] However, *P. falciparum* mature stage V gametocytes reflect sexual conversion events that occurred ~10 days earlier. Sexual rings, which are formed within hours after sexual commitment, are the only immature sexual stage present in the circulation,[20–23] but they are morphologically indistinguishable from asexual rings. Therefore, molecular markers for sexual rings are essential to immediately assess the effect of host conditions on sexual conversion rates. Currently, *pfap2-g* and two recently described new markers, *surfin13.1* and *surfin1.2*,[23] are the only known molecular markers for very young sexual rings. All of these genes show increased expression already during the sexual commitment cycle that precedes the sexual ring stage.[12,15] Older sexual rings express a small number of additional markers, including the well-established *gexp02, gexp5, pfg14-744* and *pfs16* markers.[14,15,19,30–32]

Artemisinin and derivatives (hereafter referred to as ART) are extremely potent antimalarial drugs. ART-based combination therapies (ACTs), consisting of ART, which have a very short plasma half-life (~ 1 h), plus a partner drug with a longer half-life, are the frontline treatment against *P. falciparum* infection globally.[33,34] However, the efficacy of ART against *P. falciparum* is threatened by the emergence of parasite resistance, which currently manifests in the form of delayed parasite clearance and is defined as the presence of microscopy-detectable asexual parasites on day 3 after adequate treatment. Resistance is associated with non-synonymous mutations in the gene encoding the *P. falciparum* Kelch domain protein on chromosome 13 (*K13*).[35–37] ART resistance first emerged in Cambodia and has rapidly spread throughout Southeast Asia, which together with resistance to the partner drugs, has resulted in numerous cases of overt treatment failure.[38–44] The eventual spread of ART resistance across high-transmission areas in Africa[42,45,46] poses a major threat for malaria control globally.

In addition to their use to treat malaria disease, ACTs are being employed in mass drug administration campaigns aimed at interrupting transmission to eliminate malaria.[47–49] ACTs were selected for this goal because of their high efficacy against asexual stages[33,34] and partial efficacy against gametocytes. While treatment with drugs such as chloroquine or sulfadoxine-pyrimethamine results in an apparent increase in circulating gametocytes, treatment with ACTs typically reduces gametocyte density and infectiousness to mosquitoes.[10,50–56] However, circulating gametocytes are not fully eliminated, and patients treated with ACTs can remain infectious to mosquitos for several days, contributing to malaria transmission.[57–60] Observations of changes in gametocyte carriage soon after antimalarial treatment do not inform on how the drug affects sexual conversion rates, but rather reflect the ability of the drug to kill immature and mature gametocytes, or to induce the release of gametocytes sequestered in the bone marrow.[10,61–63]

The effect of antimalarial drugs on sexual conversion rates has been directly addressed using *in vitro* parasite cultures[64–67] or rodent malaria models,[68–70] but the results were often discordant between studies. This may be explained by a complex parasite response to antimalarial drugs that depends on multiple factors that differed between specific studies. In a recent carefully conducted study in which parasites were exposed to different drugs for the duration of a full asexual replication cycle (48 h), no effect of ART or the majority of other drugs tested on sexual conversion rates was observed.[11] However, the long exposure time implied that even very low ART concentrations resulted in high parasite mortality. We recently measured the impact of chloroquine and dihydroartemisinin (DHA), the active metabolite of all ARTs, on *P. falciparum* sexual conversion. We used gametocyte-reporter lines that enable quantification of very early sexual forms[30] and a new *in vitro* assay with a short drug pulse (3 h) to measure the effect of the drugs on sexual conversion independently from their gametocytocidal activity.[71] Indeed, we found that the effect of the drugs on sexual conversion rates depends on multiple factors, including parasite stage at the time of exposure and drug dose. Subcurative doses of ART administered at the trophozoite stage increased sexual conversion rates, whereas exposure at the ring stage had the opposite effect. Furthermore, enhanced conversion was not observed in parasites in which sexual conversion was metabolically stimulated by depletion of choline.[71]

To elucidate whether ART-based treatment results in changes in sexual conversion rates in natural infections, it is necessary to disentangle the effect of the drugs on sexual conversion rates from their effects on asexual parasites (the source of new gametocytes) and on gametocyte viability and release from the bone marrow. This requires the use of markers of sexual conversion with a very early onset of expression during sexual development that are present in sexual rings,[61] and frequent sampling after treatment. Here we investigated the temporal changes in the transcript levels of *pfap2-g* and other sexual ring biomarkers (SRBs) after ART-based treatment as a proxy for changes in sexual conversion rates. We used samples collected as part of three previously existing independent cohorts in which blood was obtained from malaria patients just before treatment and then every 12 h after treatment. We also did an exploratory analysis of factors that may influence the impact of treatment on sexual conversion rates.

Methods

Information of reagents and tools is available in Supplementary Table 1.

Patient samples

We analysed samples collected as part of three previously existing independent longitudinal cohorts conducted in Vietnam (Krong Pa District)[72] (ClinicalTrials.gov registration ID NCT02604966), Burkina Faso (Nanoro Department) and Mozambique (Manhiça District) (ClinicalTrials.gov registration ID NCT02694874) (Supplementary Figure 1). In the three cohorts, samples were collected before treatment, at 12 h intervals after treatment and also 7-14 days after treatment. The aim of our study was to compare the expression of SRBs before and after treatment, as a proxy for changes in sexual conversion rates, and as a secondary objective to explore factors potentially associated with the effect of treatment on sexual conversion rates. The reason for analysing samples from three different cohorts was to assess if the effect of ART-based treatment on sexual conversion rates was similar in cohorts with very different epidemiological, demographical, clinical and treatment characteristics, informing about the generalisability of the findings, and to increase the total sample size. In the three studies, informed consent was obtained before enrolment from all participants or their parents or guardians (<18 years old participants). The main characteristics of the study cohorts, including demographical, parasitological and clinical data, is provided in Table 1, Supplementary Dataset 1 and Supplementary Figure 2. Publicly available previous evidence on the effect of drug treatment on *Plasmodium* spp. sexual conversion was systematically compiled by regular automated Pubmed searches before and during the study.

In the Vietnam cohort (*N*=57), patients with uncomplicated *P. falciparum* mono-infection attending the Chu R'Cam, Ia R'Sai or Ia R'Suom health centres were recruited for a two-arm randomised open-label ART efficacy study between April 2015 and September 2017, as previously described.[72] Randomization, generated by the researchers, was carried out in blocks of 10 with an allocation ratio of 1:1. Each enrolled patient chose randomly a sealed opaque envelope which contained that patient's treatment group. Following randomization, only the medical doctor was aware of the treatment assignment while both the patient and the non-medical staff (nurses, microscopists, technicians) remained blinded to the treatment allocation. Patients in arm 1 were treated with oral DHA-piperaquine (PPQ) (Eurartesim®, 40 mg+320 mg/tablet) using a weight-based daily dose for 3 days, whereas those enrolled in arm 2 were treated with oral artesunate (AS) (Co-Artesun®, 50 mg/tablet) also using a weight-based daily dose for 3 days, followed by a standard DHA-PPQ treatment for another 3 days. Samples were collected before treatment and every 12 h until 84 h after treatment. The main inclusion criteria were: at least 1 year of age, single infection with *P. falciparum* as determined by light microscopy [and later confirmed by diagnostic quantitative PCR (qPCR)] and parasite density higher than 500 parasites/µL. The main exclusion criteria were severe malaria symptoms, other diseases, taking other medication or being unable to remain in the research site region for the follow up period. The primary outcome of the study was to evaluate the *in vivo* and *ex vivo* susceptibility of *P. falciparum* to ART monotherapy and to DHA-PPQ in Central Vietnam.[72] Studies on parasite-related factors were a secondary objective. Samples from the 34 patients with highest before treatment parasitaemia, irrespectively of which study arm they belonged to (arm 1 or arm 2), were used for the SRBs analysis presented here.

The cohort in Burkina Faso was a village-based longitudinal observational study (*N*=870) in which the primary outcome, which has not yet been published, was to investigate host, parasite and environmental factors associated with gametocyte carriage. Selection of study participants was based on the existing health demographic surveillance system in Nanoro district. Children < 5 years were randomly selected according to a computer-generated list. For each selected child, another participant from the same household was considered for enrolment: either an individual 5-19 years old or aged 20 years or more (half of the households of each type). Analysing the effect of standard oral artemether-lumefantrine (AL) treatment on sexual conversion was a secondary outcome. For this, a nested sub-cohort study (the samples used here) was conducted in which patients enrolled in the main cohort that attended the Nanoro hospital (unscheduled visit) with uncomplicated *P. falciparum* malaria with parasite density ≥ 1000 parasites/µL were recruited between September 2019 and February 2020. Exclusion criteria were severe malaria, having received an antimalarial drug in the last 15 days and being unable to remain in the research site region during the follow up period. Patients were confined in the hospital for 3 days for clinical and diagnostic assessment, and treated with the standard oral AL (Coartem®, 20 mg / 120 mg) twice a day for 3 days. 3 mL of venous blood were collected on day 0 (before treatment) and 500 µL at 12 h, 24 h, 36 h, 48 h, 60 h, 72 h and 8-10 days after first dose of treatment, when the patients returned to the hospital for additional clinical examination. All patients attending the hospital that met the inclusion criteria and were willing to participate were enrolled in the sub-cohort until a sample size of 30 patients was reached.

In Mozambique, patients with severe *P. falciparum* malaria (*N*=180) were recruited at the Manhiça district hospital from March 2016 to December 2019 in a hospital-based double-blind placebo-controlled Phase IIb clinical trial in which rosiglitazone (insulin sensitizer drug) was tested as an adjunctive therapy for children with severe malaria. The Phase IIa clinical trial has been reported elsewhere.[73] In Phase IIb, with severe malaria cases, the standard treatment regimen was parenteral AS (Artesun®, 2.4 mg/kg) administered by intravenous route at the time of hospital admission, 12 h and 24 h later, and repeated every 12 hours until patients could tolerate oral treatment, at which time a complete AL

	Vietnam	Burkina Faso	Mozambique
Study area	Krong Pa district (Gia Lai province)	Nanoro department (Boulkiemdé province)	Manhiça district (Maputo province)
Baseline malaria epidemiology	- Perennial transmission (peaks May–June and Sep.–Oct.) - 2,191 microscopy positive cases in Krong Pa in 2014 (1,051 *P. falciparum* / 1,124 *P. vivax*) - Main malaria vectors: A. dirus, A. minimus	- Perennial transmission (peak July–Dec) - Case incidence in 2018: 655/1,000 - Main malaria vectors: *A. gambiae*, *A. arabiensis*, *A. funestus*	- Perennial transmission (peak Nov.–April) - Case Incidence (July 2016 to June 2017): 186/ 1,000 - Main malaria vectors: *A. funestus*, *A. arabiensis*
Sample size included/total in the original study	34/57	30/35	45/180
Proportion of females	14.71%	46.66%	46.66%
Age median (quartiles)	25 (16.75-30.75)	5 (4.25-6)	4 (3-6)
Ethnicity	JaRai (86.9%), Kinh (13.2%)	Mossi (87.1%), Gourounsi (11.3%), Fulani (1.3%)	Information not collected, majority Shangaan in the region
Disease severity for inclusion	uncomplicated malaria	uncomplicated malaria	severe malaria requiring hospitalization
Other inclusion criteria	*P. falciparum* only > 500 P/µl, Age > 1 y	*P. falciparum* >1,000 P/µl	*P. falciparum* > 2,500 P/µl, Age > 1-12 y
Main exclusion criteria	Severe malaria, underlying illness, other medication or unable to remain in the research site region	Severe malaria, antimalarial drug in the last 15 days or unable to remain in the research site region	Uncomplicated malaria, severe malaria anaemia (Hb < 50g/L), underlying illness or unable to remain in the research site region
Treatment (arms) and proportion of individuals by arm	ARM1: 58.8%, DHA-PPQ (3 days); ARM2: 41.2%, Artesunate (3 days) + DHA-PPQ (3 days)	AL (3 days)	Parenteral artesunate (at least at 0h, 12h and 24h) + AL (3 days)
Additional drugs used	none	none	53.3% patients received rosiglitazone
Dates of sample collection	April 2015 – Sep. 2017	Sep. 2019 – Feb. 2020	June 2018 - May 2019
Original study design	Two-arm randomized open-label ART efficacy study [72] (Clinical Trials.gov ID: NCT02604966)	Nested sub-cohort study (see Methods)	Phase IIb clinical trial (Clinical Trials.gov ID: NCT02694874)

***Table 1*: Patients sample information.**
Description of the samples that were selected from the cohort studies in Vietnam, Burkina Faso and Mozambique. Sample collection dates, demographic and inclusion criteria are for the samples included in the analysis presented in this manuscript. DHA-PPQ: dihydroartemisinin-piperaquine; AL: artemisinin-lumefantrine.

(Coartem®, 20 mg/120 mg) regimen was given to all patients. In addition, a sub-group of patients received oral rosiglitazone as an adjunct therapy, whereas the others received placebo. Simple randomization (1:1) was employed using a computer-generated randomization list. Treatment allocation, to either rosiglitazone or placebo, was recorded on paper and kept in sequentially numbered sealed opaque envelopes, which were drawn for each randomized participant by an unblinded investigator who was not responsible for patient care, laboratory or data analysis. Rosiglitazone and the placebo were packaged and labeled identically to ensure blinding of both treating medical personnel and hospital staff. All staff involved in the clinical care of the participants, laboratory analyses, and data analysis remained blinded to treatment assignment. The primary outcome of this Phase IIb clinical study, which has not been published yet, was to determine whether supplemental rosiglitazone in addition to standard of care anti-malarial treatment accelerates the rate of decline in angiopoietin-2 (Ang-2) from admission levels in children with severe malaria. Studies on parasite-related factors were a secondary objective. The main inclusion criteria were: 1 to 12 years of age, *P. falciparum* positive using a rapid diagnostic test and confirmed by microscopy with parasite density greater than 2,500 parasites/µL, at least 1 sign of severe malaria (i.e., at least 2 episodes of generalised seizure within 24 h, prostration, impaired consciousness or respiratory distress), and requiring hospitalisation. Exclusion criteria were uncomplicated malaria infection, presence of severe malaria anaemia (Hb < 50 g/L), known underlying illness, and being unable to remain in the research site region for the follow up period. Samples were collected before treatment

and every 12 h until 108 h after treatment. Samples from all patients recruited between June 2018 and May 2019, irrespectively of which study arm they belonged to (rosiglitazone or placebo), were used for gene expression analysis and included in our study.

The number of individuals that were selected from each cohort for the gene expression analysis presented here was 34 in Vietnam, 30 in Burkina Faso and 45 in Mozambique, for a total of 109 individuals. Because this is an exploratory study, we decided to analyse a minimum sample size of 30 individuals per cohort, based on feasibility of the molecular analyses (involving analysis of 5 SRBs and 3 normalizing genes for samples collected at least at 5 time points for each patient, in addition to the analysis of markers of mature male and female gametocytes at multiple time points) and availability of samples with sufficient material for transcriptional analysis in the previously existing cohorts.

Blood sample collection and preparation of RNA and DNA

For the Vietnam and Burkina Faso cohorts, a venous blood sample was collected on day 0 before treatment, from which a 100 µL aliquot was preserved for RNA extraction in 500 µL of RNAprotect Cell Reagent (Qiagen, cat. no. 76526) and a 200 µL aliquot was preserved in an EDTA tube for DNA extraction using the FavorPrep™ 96-well Genomic DNA kit (Favorgen) (Vietnam) or the QIAamp® 96 DNA blood Kit (Qiagen, cat. no. 51161) (Burkina Faso). Then, every 12 h, finger-prick blood samples were collected and the same volumes as on day 0 used for RNA and DNA extraction. All samples collected in RNAprotect were frozen at -80°C until use. In the Mozambique cohort, a 500 µL whole blood aliquot was immediately processed for RNA extraction in Trizol reagent (Invitrogen, cat. no. 15596026), both before treatment and at 12 h intervals. Briefly, after spinning, the erythrocytes pellet was lysed and homogenised with 4.5 mL Trizol and frozen directly at -80°C until RNA extraction.

Total RNA was extracted from Vietnam samples following the protocol of the RNeasy Plus 96 Kit (Qiagen, cat. no. 74192).[72] For the samples from Burkina Faso, 900 µL of Trizol was added to the samples in RNAprotect. Total RNA was extracted from samples from Burkina Faso and Mozambique in Trizol using a previously described protocol based on the RNeasy® Mini Kit (Qiagen no. 74104) and optimised for samples with a low amount of RNA.[74,75] All RNA samples were subjected to on-column DNase I treatment (Qiagen no. 79254). In all RNA samples, the first-strand cDNA was synthesised by using the AMV Reverse Transcription System (Promega, cat. no. A3500), with both oligo (dT) and random primers included in the reaction.[75]

qPCR analysis

To quantify transcript levels, qPCR analysis of the cDNAs was performed in triplicate wells using the relative standard curve method, in which Ct values for samples are interpolated against a standard curve (included for each primer pair in every plate) prepared with serial dilutions of parasite genomic DNA unless stated otherwise.[75] No template controls (negative controls) were also included for each primer pair in every plate. Reactions with no detectable amplification or a Ct value higher than 36 were considered negative. Transcript levels of the SRBs *pfap2-g* (PF3D7_1222600), *gexp02* (PF3D7_1102500), *gexp5* (PF3D7_0936600), *pfg14-744* (PF3D7_1477300) and *pfs16* (PF3D7_0406200) were normalised against transcript levels of the housekeeping genes *ubiquitin-conjugating enzyme* (*uce*) (PF3D7_0812600), *serine-tRNA ligase* (*serrs*) (PF3D7_0717700) and *18S rRNA* (PF3D7_1148600, PF3D7_0112300 and PF3D7_1371000) to obtain their relative transcript levels.[30] Samples negative for amplification of the normalizing genes were excluded from further analysis. The female gametocyte marker *pfs25* (PF3D7_1031000) and the male gametocyte marker *pfmget* (PF3D7_1469900) were also analysed. Transcript levels of the mature female and male gametocyte markers were expressed as copy numbers per µl, because the standard curves for these genes were based on plasmids with a known copy number of the *pfs25* or *pfmget* genes.[72,76] Total parasite density was estimated by RT-qPCR using the *18S rRNA* (PF3D7_1148600; PF3D7_0112300; PF3D7_1371000) transcript levels for Vietnam samples,[72] qPCR analysis of genomic DNA for the same gene for Mozambique samples and *varATS* qPCR analysis of genomic DNA for Burkina Faso samples.[77,78] All primers used for qPCR and RT-qPCR analysis are listed in Supplementary Table 2. Normalised transcript levels for all samples are presented, together with parasitological data, in Supplementary Dataset 2.

Data analysis, missing values and statistics

The main outcome variables used to evaluate the effect of treatment on sexual conversion were the changes in relative transcript levels of SRBs from before the first dose of ART-based treatment until 48 h after this first dose. These changes were expressed as fold-change (FC), calculated as the ratio of relative (normalized against a housekeeping gene) SRB expression levels in the post-treatment period (12 h to 48 h) to the relative expression levels in the pre-treatment time point (0 h). In all analyses, we used the median transcript levels FC from 12 h to 48 h (median $FC^{12\text{-}48h}$) to represent the central fold-change value observed after treatment. In selected analyses, we grouped the patient samples using cut-off values of 1 or 2 median $FC^{12\text{-}48h}$. Time points beyond 48 h were excluded from the final analysis because of very low parasite density or absence of parasites as a result of ACT treatment. In some samples, parasitaemia was very low at some of the time points

after treatment (mainly at the 36 h or 48 h time points) and the relative expression of some of the markers was not quantifiable (Supplementary Dataset 2). In these samples, the median $FC^{12\text{-}48h}$ was calculated from the after-treatment time points available.

When the pre-treatment sample or all of the post-treatment samples were RT-qPCR-negative for an SRB or a normalizing gene, the median $FC^{12\text{-}48h}$ could not be calculated. In the Burkina Faso cohort, 2 out of 30 samples had missing values for the *pfap2-g* median $FC^{12\text{-}48h}$ using *serrs* as normalizing genes, and 1 using *uce* or *18S rRNA*; 1 sample had missing values for the *gexp02, gexp5* or *pfs16* median $FC^{12\text{-}48h}$ using *serrs* as normalizing gene; and 6 samples had missing values for the *pfg14-744* median $FC^{12\text{-}48h}$ using *uce* or *18S rRNA* as normalizing genes, and 7 using *serrs*. In the Vietnam cohort, 3 out of 34 samples had missing values for the *pfap2-g* median $FC^{12\text{-}48h}$ using either of the normalizing genes; 5 samples had missing values for the *gexp02* median $FC^{12\text{-}48h}$ using *serrs* or *uce* as normalizing gene, and 4 using *18S rRNA*. Additionally, the analysis of *gexp5, pfg14-744* and *pfs16* was excluded from all samples from Vietnam because many samples had missing values, suggestive of a possible technical problem with the qPCR analysis batch (and there was not sufficient material to repeat the analysis). Data about the *K13* mutation status was missing from one patient from Vietnam, and data for D7-14 female (*pfs25* RT-qPCR analysis) and male (*pfmget*) mature gametocyte levels was missing from 4 patients from Burkina Faso and 4 from Mozambique. In addition, because most patients from the Vietnam cohort were negative for the expression of *pfmget*, data for this marker was excluded for the full cohort and male to female ratios were not analysed in this cohort. The missing data in the three cohorts is summarized in Supplementary Dataset 2.

Relative transcript levels and fold changes were $\log_2$ transformed to meet the assumption of data normality, as assessed using the Shapiro–Wilk test. Welch's ANOVA with Games-Howell post-hoc test was used to compare the median FC of more than two variables with unequal variance (which was confirmed by Levene's test), whereas Welch's t-test (two-tailed) was used to compare two variables with unequal variance. Pearson's correlation coefficient (r) was calculated to assess the linear correlations between SRBs. Spearman's correlation coefficient (r_s) was used to evaluate the correlation between SRBs and mature gametocyte density on days 7 to 14. Univariate and backwards stepwise multivariate linear regression analysis was performed with the $\log_2$(*pfap2-g* median $FC^{12\text{-}48h}$) as the dependent (outcome) variable. Parasite or patient/clinical factors were included as independent variables: sex, age, haemoglobin, parasite clearance (hours), gametocyte proportion at 0 h and at 12-48 h, gametocyte density at 0 h and parasitaemia at 0 h. In addition to these independent variables included for the three cohorts, other variables included only in specific cohorts were: fever, treatment arm, presence of gametocytes at day 7 and presence of *K13* mutations in the Vietnam cohort; fever and presence of gametocytes at day 8-10 in the Burkina Faso cohort; and glucose and lactate concentration, rosiglitazone or placebo, presence of gametocytes at day 7-14 and *pfs25/pfmget* ratio in the Mozambique cohort. The variables were selected for each cohort based on availability of data.

In all analyses, only statistically significant p values ($p < 0.05$) are shown; when no p value is shown (in a figure in which statistical analysis was performed, as indicated in the legend), differences were statistically non-significant. All data was processed and stored using Microsoft Excel 2019. Statistical analyses were performed using GraphPad Prism v.9 and SPSS v.12. All figures were generated using GraphPad Prism v.9 and assembled using Microsoft PowerPoint 2019.

Ethical approval and clinical trials registration

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

Role of funding source

The funding source did not play any role in study design, data collection and analyses, manuscript preparation or decision to submit the work for publication.

Results

Study design

We analysed the impact of ART on *P. falciparum* sexual conversion rates in naturally infected malaria patients from three previously existing independent longitudinal

studies conducted in Vietnam (*N*=34), Burkina Faso (*N*=30) and Mozambique (*N*=45). The key features of all three cohorts were ART-based treatment, collection of samples for gene expression analysis from before treatment (0 h) to at least 48 h after treatment at 12 h intervals, and an additional sample between days 7 and 14 for mature gametocyte analysis (Figure 1 and Supplementary Figure 1). The specific antimalarial drug treatment and clinical presentation of the patients varied between cohorts, with oral ACT (DHA-PPQ, treatment arm 1) or oral AS monotherapy for 3 days followed by 3 days DHA-PPQ (treatment arm 2) and uncomplicated malaria cases in Vietnam, oral ACT (AL) and uncomplicated malaria cases in Burkina Faso, and parenteral AS followed by oral AL and severe malaria cases in Mozambique. Of note, all ARTs produce the same active metabolite, DHA. Most study participants from Vietnam were 15–30 years old males, which in this area are at high risk of malaria infection due to forest-related activities,[79] whereas all participants in Burkina Faso and Mozambique were children 1 to 11 years old. The delayed parasite clearance phenotype, defined as light microscopy-detectable asexual parasitaemia on day 3 after treatment, was only observed in the Vietnam cohort (22 of 34 patients). Demographic, parasitological and haematological parameters for each cohort are provided in Table 1, Supplementary Figure 2 and Supplementary Dataset 1.

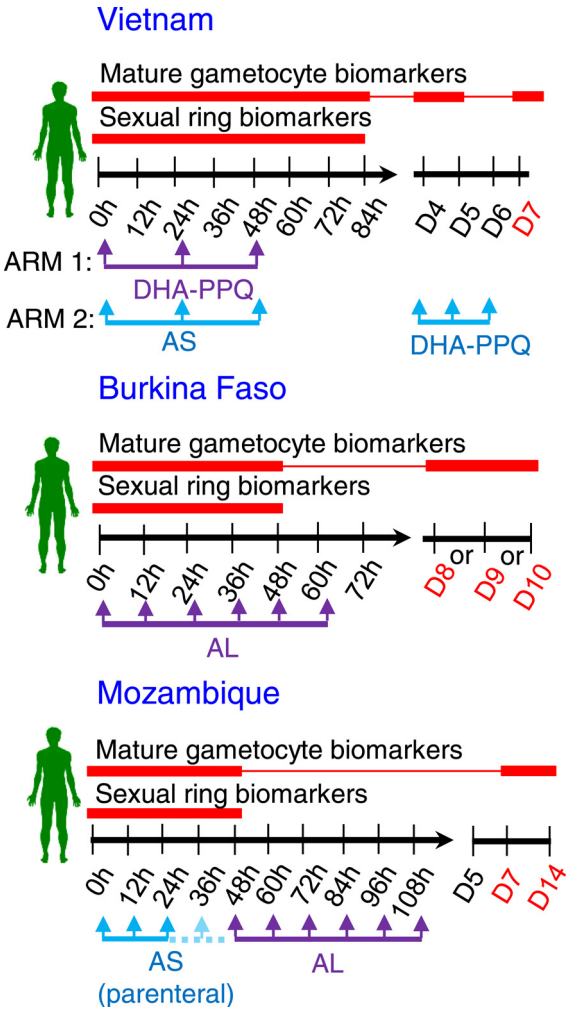

Figure 1. Study design of the three cohorts. Red lines indicate the time of blood sample collection for RNA analysis. Blue and violet arrows indicate the time of treatment with dihydroartemisinin-piperaquine (DHA-PPQ), artesunate (AS) or artemether-lumefantrine (AL). Administration was by oral route except for AS in Mozambique. In Mozambique, parenteral AS was administered every 12 h until patients tolerated oral administration, at which time a complete AL regimen was given. While most patients received AS at 0, 12 and 24 h, some patients received more than 5 doses before AL treatment.

Changes in *pfap2-g* transcript levels after ART-based treatment

Using RT-qPCR analysis, we observed a rapid increase in *pfap2-g* relative transcript levels (normalised to the housekeeping gene *uce*) after ART-based treatment in the majority of patients from the Mozambique cohort. In the Burkina Faso cohort, we also observed predominantly an increase in *pfap2-g* relative transcript levels, although it was less marked, and in the Vietnam cohort we observed predominantly a decrease (Figure 2a and Supplementary Dataset 2). The *pfap2-g* median $FC^{12\text{-}48h}$, defined as the median of the fold-change in normalised transcript levels across the four time points after treatment relative to before treatment levels, was >1 (increased) in the majority of isolates from the African cohorts (~90% from Mozambique and ~70% from Burkina Faso), but only in ~30% of the isolates from the Vietnam cohort. Likewise, a *pfap2-g* median $FC^{12\text{-}48h}$ >2 was observed much more frequently in isolates from the African than the Vietnam cohorts (Figure 2b). These results suggest that ART-based treatment predominantly resulted in stimulation of sexual conversion in the African but not the Vietnam cohorts.

The majority of isolates from Vietnam (87.5%) carried mutations in the *K13* gene associated with ART resistance,[72] 62.5% of them the C580Y mutation that is dominant in Vietnam and the Greater Mekong subregion[80] (Figure 2c). Therefore, we hypothesised that differences in the dynamics of *pfap2-g* transcripts after treatment between the African and Vietnam cohorts may be influenced by the presence of *K13* mutations and reduced ART susceptibility only in Vietnam isolates.[72] Indeed, the $\log_2$(*pfap2-g* median $FC^{12\text{-}48h}$) was statistically significantly higher in Vietnam wild-type (population mean: +0.65, 95% C.I. -0.79 to +2.09) than *K13* mutant (population mean: -1.46, 95% C.I. -2.31 to -0.61) isolates (Figure 2d). The $\log_2$(*pfap2-g* median $FC^{12\text{-}48h}$) was also higher in the Burkina Faso (population mean: +0.77, 95% C.I. +0.15 to +1.40) and the

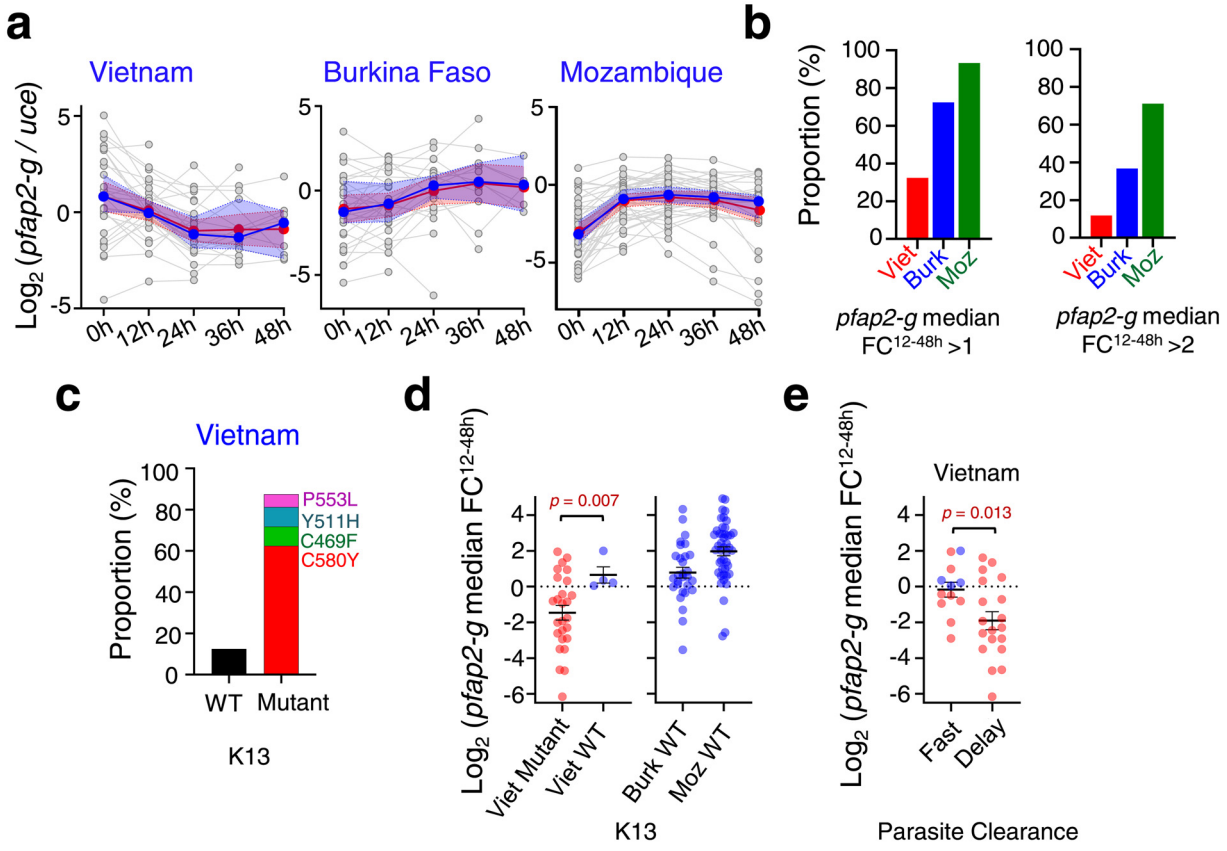

Figure 2. Changes in *pfap2-g* transcript levels after artemisinin-based treatment. (a) Time-course analysis of *pfap2-g* relative transcript levels before (0 h) and after treatment (12-48 h). Transcript levels were normalised against *uce*. The average (red) and median (blue) of all samples, with 95% confidence intervals (CI) (shaded areas), are shown. Individual values are represented as grey dots. **(b)** Proportion (%) of patients with *pfap2-g* median fold-change (relative to before treatment levels) between 12-48 h (median $FC^{12\text{-}48h}$) >1 or >2. **(c)** Proportion (%) of isolates from Vietnam with wild-type (WT) and mutant *K13* alleles. The coloured bar indicates the proportion of different *K13* mutations. **(d)** Comparison of the *pfap2-g* median $FC^{12\text{-}48h}$ between *K13* mutant and WT isolates from the Vietnam, Burkina Faso and Mozambique cohorts. Blue dots indicate *K13* WT isolates, whereas red dots indicate *K13* mutants. Mean and s.e.m. are shown. The *p* value was calculated using Welch's t-test (only for the mutant vs WT comparison; no statistical analysis was performed for comparisons between cohorts because of the disparity in their clinical, demographical and parasitological characteristics). **(e)** Comparison of the *pfap2-g* median $FC^{12\text{-}48h}$ between patients from the Vietnam cohort with fast or delayed parasite clearance. Blue dots indicate *K13* WT isolates, whereas red dots indicate *K13* mutants. Mean and s.e.m. are shown. The *p* value was calculated using Welch's t-test. For panel c, *N*=33; for all other panels, Vietnam, *N*=31, of which *K13* WT, *N*=4, *K13* mutant, *N*=26 (*K13* data not available for one patient), fast clearance, *N*=12, delayed clearance, *N*=19; Burkina Faso, *N*=29; Mozambique, *N*=45. The mean and 95% CI for the data presented in panels a, d and e is available in Supplementary Dataset 2.

Mozambique (population mean: +1.97, 95% C.I. +1.47 to +2.47) cohorts when compared with *K13* mutant isolates from the Vietnam cohort. Among *K13* wild-type samples, the *pfap2-g* median $FC^{12\text{-}48h}$ was similar between the Vietnam and Burkina Faso cohorts, but higher in the Mozambique cohort (Figure 2d). Similarly, Vietnam isolates with fast parasite clearance (indicative of ART sensitivity) had a statistically significantly higher $\log_2$(*pfap2-g* median $FC^{12\text{-}48h}$) than isolates with delayed clearance (fast clearance, population mean: -0.17, 95% C.I. -1.09 to +0.75; delayed clearance, population mean: -1.91, 95% C.I. -2.96 to -0.86) (Figure 2e). Of note, 8 isolates with *K13* mutant alleles had a fast clearance phenotype, but 6 of them still showed a reduction in *pfap2-g* levels (i.e., fold-change < 1) after treatment. Similar results were obtained using the *serrs* or *18s rRNA* genes for normalisation, indicating that the choice of the normalising gene did not affect the conclusions (Supplementary Figure 3).

Overall, these results suggest that ART-based treatment generally resulted in an increase in sexual conversion rates in ART-sensitive parasites, but this was not observed in ART-resistant isolates. However, a link between ART resistance and the effect of treatment on sexual conversion rates could not be unambiguously established because the number of *K13* wild-type

samples in the Vietnam cohort was very low. We observed differences in the *pfap2-g* median $FC^{12\text{-}48h}$ between the cohorts, but the contribution of specific factors to these differences could not be disentangled because several demographic, parasitological clinical and treatment characteristics were different between the cohorts (Table 1, Figure 1 and Supplementary Figure 2), in addition to the *K13* status. We speculate that characteristics unique to the Mozambique cohort such as parenteral drug administration or severe malaria may potentially underly the higher *pfap2-g* median $FC^{12\text{-}48h}$ observed in this cohort.

Changes in the expression levels of other SRBs after ART-based treatment

We also assessed changes in the expression of other SRBs (*gexp02, gexp5, pfg14-744* and *pfs16*) in the same set of samples (but in the Vietnam cohort, *gexp5, pfg14-744* and *pfs16* were excluded from the analysis because of too many missing values). The activation of all of these genes is PfAP2-G-dependent,[12,14,15,19] but there are differences in their degree of specificity for sexual versus asexual rings and in the precise time during sexual development at which their expression starts (Figure 3a). While *pfap2-g* is expressed in sexual rings of all ages because the gene is already active in the preceding sexually committed schizonts, expression of *gexp02* and *gexp5* starts at ~10 hpi, and expression of *pfg14-744* and *pfs16* starts later during the sexual ring stage (~20 hpi). Only *pfap2-g, gexp02* and *pfg14-744* transcripts appear to be completely absent from asexual parasites. Transcripts for these markers may be present in mature gametocytes, which are found in the circulation, but at much lower levels than in sexual rings.[14,15,19,20,30,81]

Correlation analysis of the changes in the expression of the different SRBs (including *pfap2*-g) after treatment (median $FC^{12\text{-}48h}$) generally showed a moderate positive correlation (median r : Burkina Faso, 0.31; Mozambique, 0.51) (Figure 3b). The limited correlation likely reflects differences in the temporal expression dynamics of the SRBs across the sexual ring stage.

Despite the differences between the SRBs at the individual sample level, time-course analysis of *gexp02, gexp5, pfg14-744*, and *pfs16* after ART treatment showed a similar overall dynamic to *pfap2-g*. Relative transcript levels of all the SRBs predominantly increased after treatment in the two African cohorts, and the proportion of infections showing a median $FC^{12\text{-}48h} > 2$ of the different SRBs was highest in Mozambique, similar to *pfap2-g* (Figure 3c-d and Supplementary Data Set 2). Increased expression of the SRBs after treatment in the African cohorts was already observed at the initial time points after treatment (Figure 3c and Supplementary Figure 4), when parasitaemias were still high. We also compared basal transcript levels of SRBs in the three cohorts using samples collected before treatment (0 h), and found that generally *pfg14-744* transcripts showed the lowest abundance and *gexp5* and *pfs16* the highest abundance (Supplementary Figure 5).

Infections with younger rings at the time of ART treatment are associated with less induction of *pfap2-g* expression

The large dispersion observed in the median $FC^{12\text{-}48h}$ of *pfap2-g* and other SRBs within each cohort suggests that factors that vary within each cohort may influence the effect of treatment on sexual conversion. Since we previously observed that, under culture conditions, exposure to DHA at different stages impacts sexual conversion rates differently,[71] we assessed the effect of parasite age at the time of drug exposure on the *pfap2-g* median $FC^{12\text{-}48h}$. To estimate the age of sexual rings at the time of ART exposure (time 0 h), we calculated the ratio of *pfap2-g* to *gexp02* transcripts. Higher values of this ratio correspond to younger sexual rings and lower levels to older rings, because *pfap2-g* expression peaks in very early sexual rings (i.e., 0-5 hpi) whereas high *gexp02* expression starts later during sexual ring development (at ~10-15 hpi).[19,30,71] Assuming that sexual and asexual rings develop at the same pace, the age of sexual rings is expected to reflect the age of all circulating rings (including asexual rings). Indeed, in a published large multicentre study in South-East Asia,[82] the *pfap2-g/gexp02* transcripts ratio showed a weak to moderate but statistically highly significant negative correlation with parasite age statistically estimated from the full transcriptomes, in six different study sites (average r between the sites -0.35, range -0.19 to -0.45, $p < 0.05$ in all sites) (Supplementary Figure 6).

In the Vietnam cohort, a strong statistically significant negative correlation (r = -0.81, $p < 0.001$) was observed between the *pfap2-g/gexp02* ratio at the time of ART exposure and the *pfap2-g* median $FC^{12\text{-}48h}$, suggesting that in isolates with younger rings (higher *pfap2-g/gexp02* ratio) there was less enhancement of sexual conversion (Figure 4a). In patients from the Burkina Faso and Mozambique cohorts, the same trend was observed, but the correlation was weaker and marginally statistically significant in Burkina Faso (r = -0.38, p = 0.040) and not statistically significant in Mozambique (r = -0.24). Furthermore, parasite clearance time tended to be higher in isolates with a higher *pfap2-g/gexp02* ratio, although this was statistically significant (r = 0.42, p = 0.018) only in the Vietnam cohort (Figure 4b). Lastly, the *pfap2-g/gexp02* ratio before treatment was higher in parasites from the Vietnam cohort showing delayed clearance or carrying K13 mutations than in parasites from the African or Vietnam cohorts with non-delayed clearance (Figure 4c). This result indicates that ART resistance was associated with younger estimated parasite age, which probably reflected slower progression through the ring stage. This is consistent with

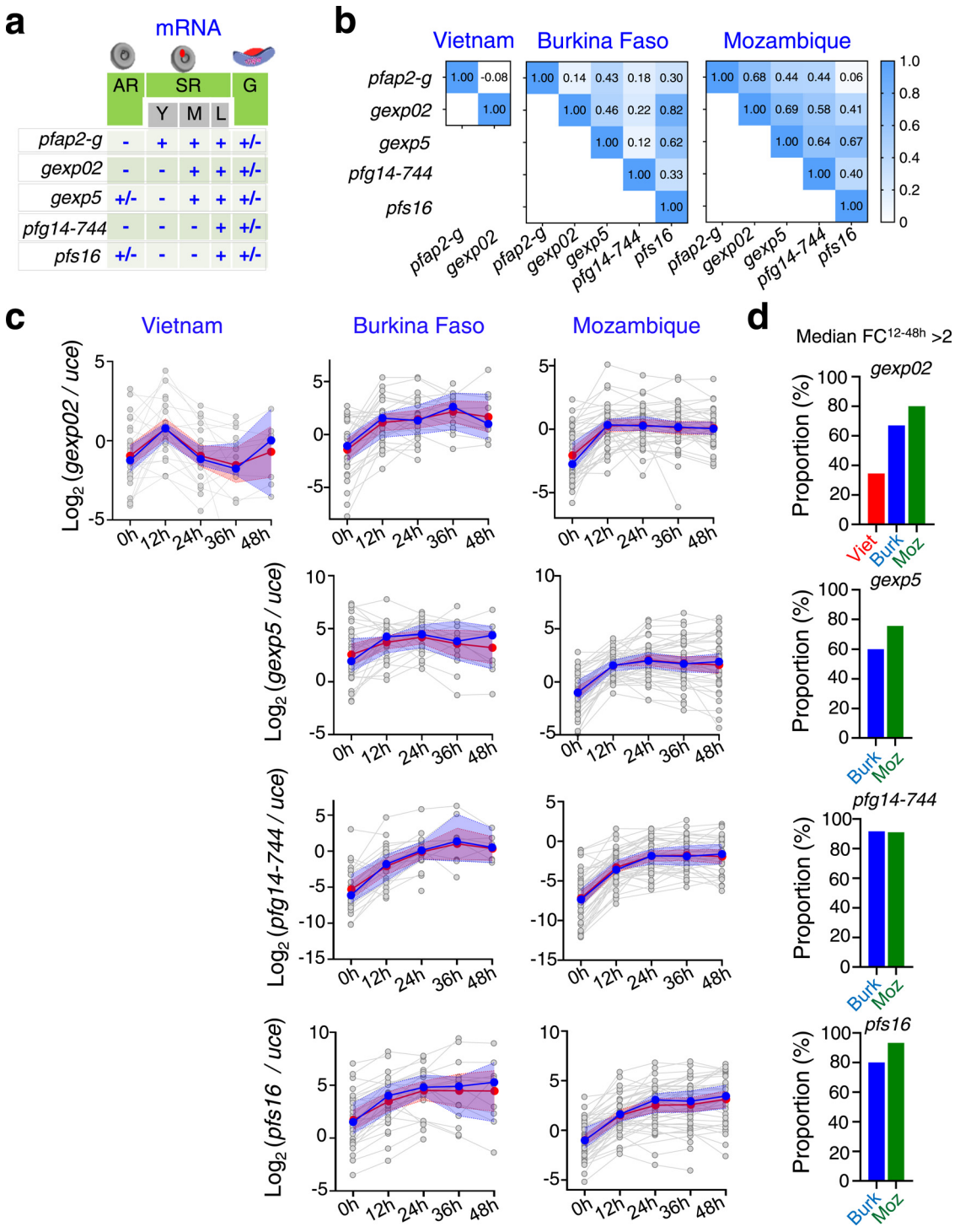

Figure 3. Comparison of transcript levels after artemisinin-based treatment for different sexual ring biomarkers (SRBs). (a) Expression of SRBs at parasite stages found in the circulation, based on published studies.[19,30,31,81] Stages: asexual rings (AR); sexual rings (SR), which can be young sexual rings (Y), e.g., 0-5 hours post-invasion (hpi); mid sexual rings (M), e.g., 10-15 hpi; or late sexual rings (L), e.g., 20-25 hpi; and mature gametocytes (G). **(b)** Linear correlation between the median $FC^{12\text{-}48h}$ for different SRBs (using *uce*-normalised transcript levels). Pearson correlation coefficients (*r*) are shown. **(c)** Time-course analysis of SRBs relative transcript levels (normalised against *uce*) before (0 h) and after treatment (12-48 h). The average (red) and median (blue) of all samples, with 95% confidence intervals (CI) (shaded areas), are shown. Individual values are represented as grey dots. **(d)** Proportion (%) of

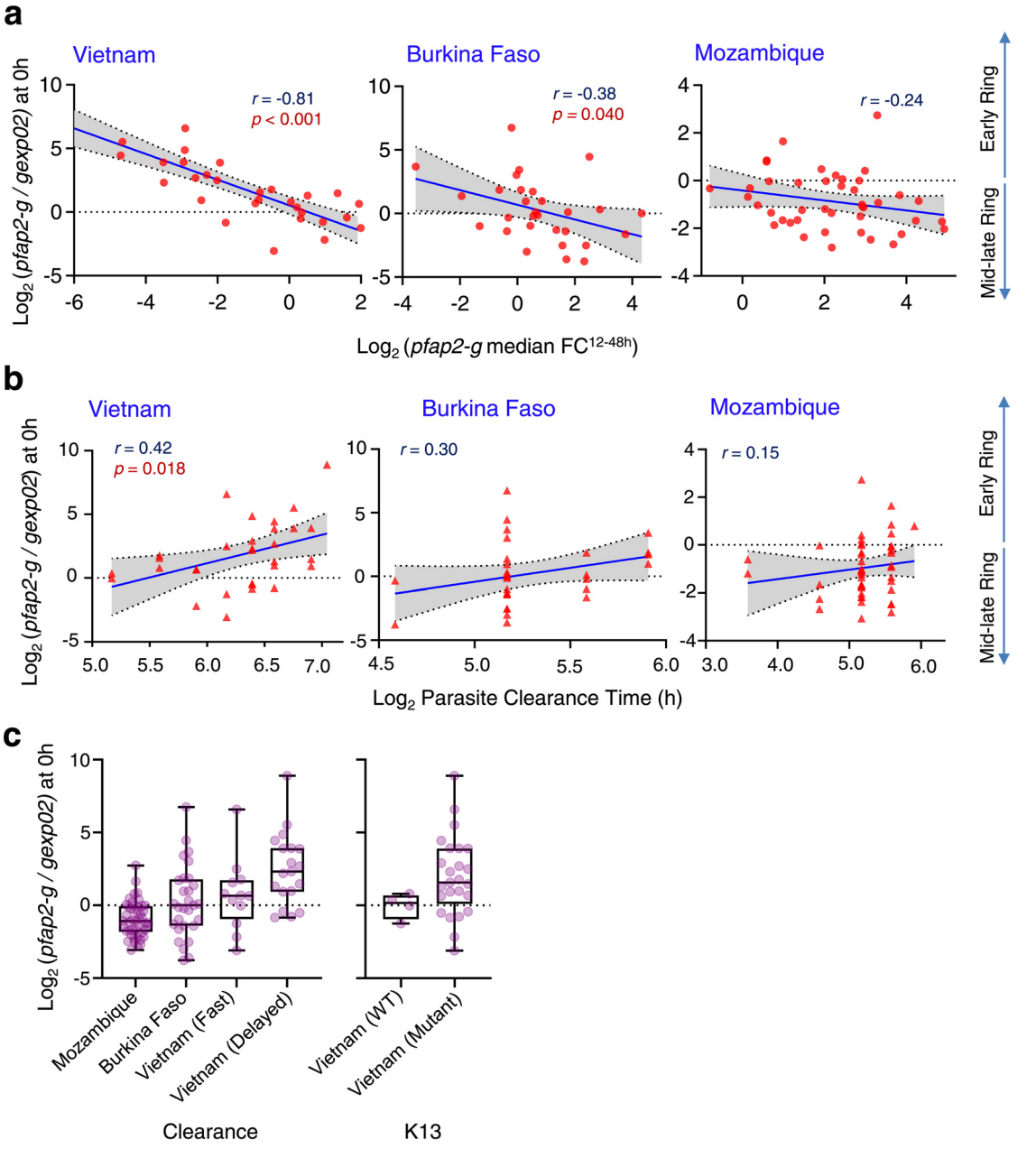

Figure 4. Association of estimated rings age before treatment with *pfap2-g* fold-change after treatment and parasite clearance time. Rings age before treatment (in arbitrary units) was estimated from the ratio of *pfap2-g* to *gexp02* transcript levels (log_2-transformed). Higher values of this ratio indicate younger rings. **(a-b)** Association between estimated rings age before treatment (0 h) and the *pfap2-g* median fold-change between 12 and 48 h after treatment (log_2-transformed) **(a)** or with parasite clearance time **(b)**. The blue line represents the linear prediction with 95% confidence interval (CI, grey shaded area), calculated from linear regression analysis, with Pearson correlation coefficient (*r*) and *p* value. Only *p* values < 0.05 are shown. Individual values are represented as red dots. **(c)** Estimated relative rings age before treatment in the different cohorts, with samples from Vietnam divided in delayed or non-delayed (fast) parasite clearance (left panel) and in *K13* wild-type (WT) or mutant isolates (right panel). Boxes show median and quartiles, and whiskers the range. The mean and 95% CI for the data presented in panel c is available in Supplementary Dataset 2.

patients with a > 2 value for the *gexp02, gexp5, pfg14-744,* and *pfs16* median $FC^{12\text{-}48h}$. In panels b-d, data for *gexp5, pfg14-744* and *pfs16* was excluded from the Vietnam cohort analysis because many values were missing. For the number of samples included in the analysis of each gene in each cohort, please see Methods and Supplementary Data Set 2. The mean and 95% CI for the data presented in panel c is available in Supplementary Dataset 2.

previous reports showing that ART-resistant parasites have a longer ring stage and a shorter trophozoite/schizont stage, in spite of a similar overall duration of the IDC,[85,86] and with ART resistance involving decreased drug susceptibility specifically in young rings.[36,37,83,84]

pfap2-g expression is a rather poor predictor of future mature gametocyte carriage in drug-treated patients

We quantified mature gametocyte densities from before treatment until 7 to 14 days after treatment, when gametocytes resulting from sexual conversion events occurring immediately after treatment are expected to be back in the circulation, together with older gametocytes formed before drug exposure. For this, we used the *pfs25* marker, which is expressed in mature female gametocytes and correlates with overall mature gametocyte density and mosquito infection rates.[25,61,87] In the three cohorts, the prevalence and density of gametocytes decreased progressively after treatment, but 25−50% of the participants still had detectable gametocytes one to two weeks after treatment (Figure 5a-b). In some patients, mainly from the Vietnam cohort, an increase in gametocyte density was observed about one week after treatment (Figure 5a). While in most patients the gametocyte density at days 7-14 was orders of magnitude lower than before treatment, it has been shown that mosquitoes can be infected by patients with very low gametocyte densities and that a substantial fraction of patients who received ART-based treatment continue to transmit malaria.[57−60] Therefore, many patients in our cohorts remained potentially infective at the end of the follow-up period. In samples from the Mozambique and Burkina Faso cohorts, we also assessed changes in the gametocyte sex ratio based on the relative transcript levels of *pfs25* and *pfmget* (male gametocyte marker). We observed a pronounced decrease of *pfs25* relative to *pfmget* transcripts with time after treatment, suggesting that female gametocytes may be more vulnerable to ART than male gametocytes, resulting in a progressive reduction of the female to male ratio (Supplementary Figure 7).

The density of sexual rings, estimated from the transcript levels of *pfap2-g* or other SRBs, predicts the future density of mature gametocytes if an infection is left untreated.[28,88] To estimate the relative (between samples) density of sexual rings in our cohorts before treatment or 12 h after treatment, we multiplied the total parasitaemia (quantified by qPCR) by the relative *pfap2-g* transcript levels (*pfap2-g*/*uce*). There was no statistically significant correlation between mature gametocyte density (day 7-14) and estimated relative density of sexual rings before or just after treatment (Supplementary Figure 8), except for the Mozambique cohort before treatment and day 7 gametocytes ($r = 0.39$, $p = 0.013$). Therefore, sexual ring density before or soon after treatment are a poor predictor of future gametocyte carriage in ACT-treated patients.

Although the relative transcript levels of SRBs (reflecting the proportion of sexual to asexual rings) before treatment or their changes after treatment (median $FC^{12\text{-}48h}$) are not expected to directly correlate with future mature gametocyte carriage measured as an absolute density per microliter of blood, the relative levels of commitment may influence gametocyte carriage. Therefore, we assessed possible associations between these parameters. The pre-treatment relative transcript levels of *pfap2-g* and the other SRBs were generally similar between patients with or without mature gametocytes on days 7-14 (Supplementary Figure 9a). There was a positive weak to moderate correlation between pre-treatment relative transcript levels of some SRBs and *pfs25* transcripts per µl on days 7-14, but it was of borderline statistical significance and only observed for some of the SRBs (not for *pfap2-g*) in African cohorts (Supplementary Figure 9b).

In the Vietnam cohort, the *pfap2-g* median $FC^{12\text{-}48h}$ was statistically significantly higher in infections with gametocytes detected on day 7 than in those without gametocytes on that day (Figure 5c), and there was also a moderate statistically significant positive correlation ($r = 0.43$, $p = 0.016$) between the *pfap2-g* median $FC^{12\text{-}48h}$ and *pfs25* transcript copies per µl one week later (Figure 5d). Indeed, in the Vietnam cohort, a distinct mature gametocytes peak that reversed the decreasing trend in mature gametocyte density with time after treatment was apparent on day 7 only in patients with an increase in *pfap2-g* transcripts after treatment (*pfap2-g* median $FC^{12\text{-}48h} > 1$), but not in those with a decrease (*pfap2-g* median $FC^{12\text{-}48h} < 1$) (Figure 5e). However, there was no association between the *pfap2-g* median $FC^{12\text{-}48h}$ and mature gametocyte carriage 7-10 days later in the Burkina Faso and Mozambique cohorts (Figure 5c-e), and the median $FC^{12\text{-}48h}$ of the other SRBs did not correlate with mature gametocyte carriage in any of the three cohorts, except for *gexp02* (negative correlation) in Mozambique (Supplementary Figure 10). Therefore, an association between increased SRB expression after treatment and future mature gametocyte carriage was only observed for *pfap2-g* in the Vietnam cohort and needs to be interpreted with caution.

Host and parasite factors that may influence the effect of ART on *pfap2-g* expression

While we could not disentangle which specific factor(s) were responsible for the differences between cohorts, because multiple host, parasite and treatment conditions differed between them, we performed univariate and backwards stepwise multivariate linear regression analyses to identify factors within each cohort associated with the expression changes in *pfap2-g* after treatment (*pfap2-g* median $FC^{12\text{-}48h}$, dependent variable) (Supplementary Figure 11). Demographical, parasitological and clinical variables available were included in the analysis

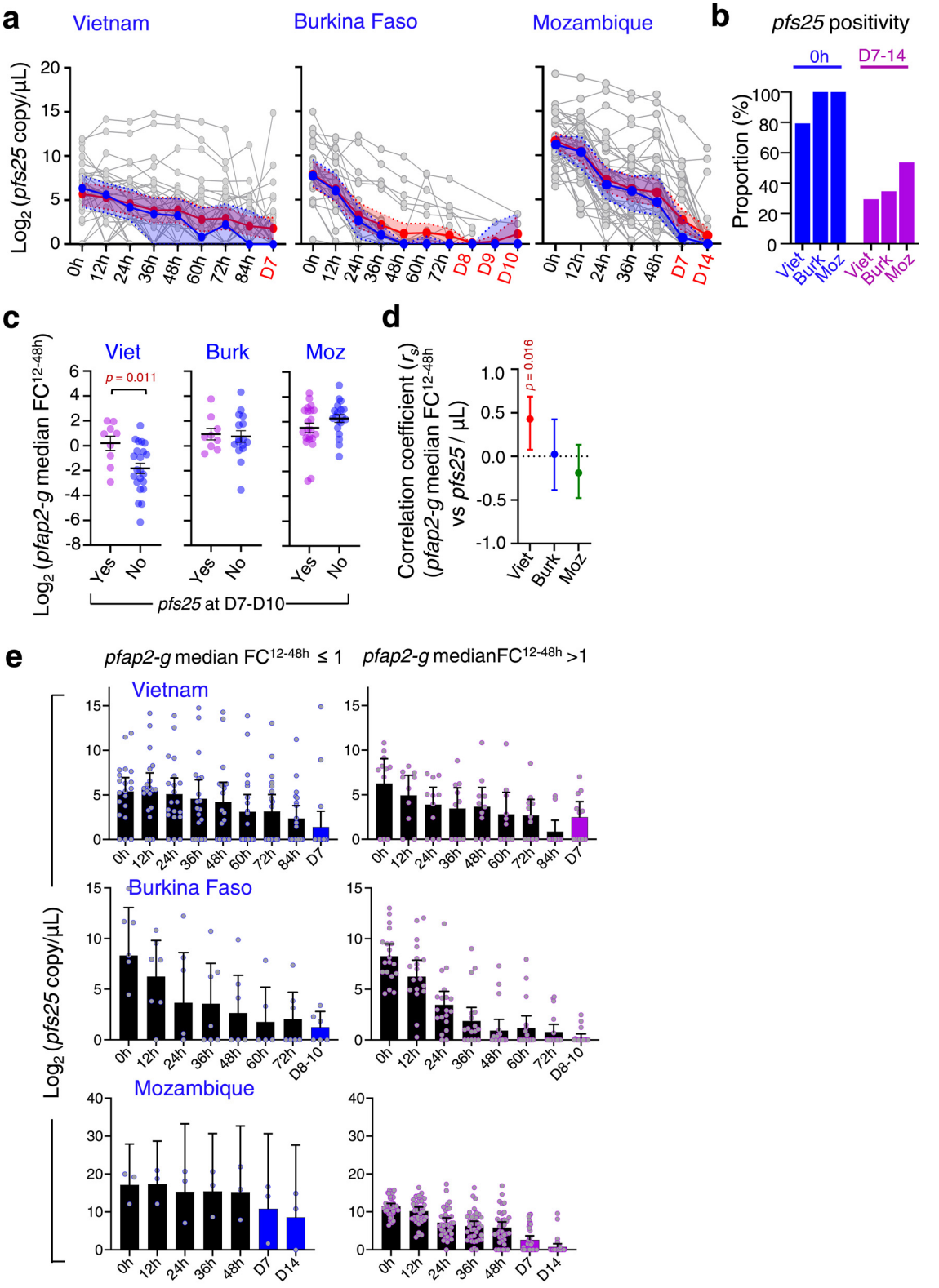

Figure 5. Association of *pfap2-g* expression changes after treatment with mature gametocyte carriage 1 to 2 weeks later. (a) Temporal dynamics of the mature female gametocyte marker *pfs25* transcript levels. The average (red) and median (blue) of all

as independent variables. In Vietnam, multivariate analysis confirmed the positive association between increased *pfap2-g* median $FC^{12\text{-}48h}$ and mature gametocytes on day 7 (reg. coef. 1.36, 95% CI 0.59 to 2.13, $p = 0.001$) and also suggested that younger patients were more likely to have enhanced *pfap2-g* median $FC^{12\text{-}48h}$ (reg. coef. -0.04, 95% CI -0.07 to -0.01, $p = 0.019$). We also performed the analysis excluding from the model the 'day 7 gametocyte' variable that has a dominant effect, which confirmed that carrying a *K13* wild-type allele was statistically significantly associated with increased *pfap2-g* median $FC^{12\text{-}48h}$ (reg. coef. -1.69, 95% CI -2.89 to -0.65, $p = 0.006$), and revealed host male sex and low pre-treatment or 12-48 h post-treatment mature gametocyte to total parasites ratio as factors associated with increased *pfap2-g* transcript levels after treatment (the latter two with borderline statistical significance). The sex variable should be interpreted with caution because female participants were underrepresented in the Vietnam cohort.

In the Burkina Faso cohort, we did not observe any statistically significant association between explanatory variables and changes in *pfap2-g* median $FC^{12\text{-}48h}$. In the Mozambique cohort, a higher blood glucose concentration on day 0 was associated with a higher increase in *pfap2-g* median $FC^{12\text{-}48h}$ (reg. coef. 1.23, 95% CI 0.16 to 2.30, $p = 0.025$). In contrast, treatment with rosiglitazone, an insulin sensitizer tested as an adjunctive treatment for severe malaria in the clinical trial from which the Mozambique samples were obtained, and the 12-48 h post-treatment mature gametocyte to total parasites ratio were associated with a lower increase in *pfap2-g* median $FC^{12\text{-}48h}$ (with borderline statistical significance).

Overall, the results of the multivariate analysis, which takes into account many of the potential confounders, support our previous observations that the presence of mature gametocytes at day 7 and carrying wild-type *K13* alleles were associated with increased *pfap2-g* expression in the Vietnam cohort. However, as discussed above, this could not be conclusively demonstrated because the number of *K13* wild-type allele samples in the Vietnam cohort was low. The multivariate analysis also identified factors such as blood glucose levels before treatment (determined only in Mozambique) and rosiglitazone treatment of severe malaria patients, and patient sex (only in Vietnam) that may modulate the effect of ART-based treatment on sexual conversion rates.

Discussion

The impact of drug treatment on malaria transmission is complex, as it depends on the killing of asexual parasites, which are the source of new gametocytes, the direct killing of gametocytes, and drug-induced alterations of sexual conversion rates. While the dynamics of *P. falciparum* asexual parasites and mature gametocyte levels after ACT treatment have been described in detail,[10,50–60] studies addressing the direct impact of treatment on parasite sexual conversion rates in natural infections are lacking. Previous studies reported changes in mature gametocyte carriage after drug treatment, but the effect of the drugs on sexual conversion rates could not be disentangled from their effect on other processes.[10,61–63] Here we characterised the impact of ART treatment on sexual conversion rates using samples from three different cohorts with regular sampling every 12 h after treatment, and taking advantage of the recent validation of transcripts of *pfap2-g* and several other genes as *in vivo* markers for sexual rings,[21,23,28,88] the only early sexual stage present in the circulation.[20–23,61] Our results for this primary outcome of the study revealed increased relative transcript levels of *pfap2-g* and other SRBs soon after ART-based treatment in many patients, suggesting that parasites can enhance their sexual conversion rates in response to treatment. However, in other patients, SRBs relative transcript levels decreased after treatment, indicating that the effect of ART on sexual conversion is complex and is likely influenced by patient, parasite and/or treatment characteristics. The differences observed between cohorts also indicate that the trends of the impact of ART on sexual conversion observed in any particular cohort cannot be generalized.

We found that increased expression of *pfap2-g* upon ART treatment occurred in the majority of infections with wild-type *K13* alleles and fast clearance, whereas it was rare in ART-resistant parasites from Vietnam. This raises the possibility that the ART resistance status may influence the effect of the drug on sexual conversion, but the low number of wild-type samples in the Vietnam cohort and the differences in parasitological, clinical and demographical factors between cohorts precluded unambiguously establishing this association. Larger studies including wild-type and *K13* mutant parasites from the same population, conducted in regions where *K13* mutations occur with intermediate frequencies, will be needed to confirm if sexual conversion is

samples, with 95% confidence intervals (CI) (shaded area), are shown. Individual values are represented as grey dots. **(b)** Proportion (%) of infections carrying gametocytes (*pfs25*-positive) before treatment (0 h) and 1-2 weeks after treatment. **(c)** *pfap2-g* median fold-change between 12 and 48 h after treatment (*pfap2-g* median $FC^{12\text{-}48h}$) in patients with detected (Yes) or undetected (No) *pfs25* transcripts on days 7-10. Transcript levels were normalised against *uce*. Mean, s.e.m. and individual data points are shown. The *p* value was calculated using the Welsch's t-test. Only *p* values < 0.05 are shown. **(d)** Spearman correlation coefficient (r_s) of *pfap2-g* median $FC^{12\text{-}48h}$ with *pfs25* transcripts/µl on day 7 to 10, with 95% CI and *p* value. Only *p* values < 0.05 are shown. **(e)** *pfs25* transcript levels in patients with *pfap2-g* median $FC^{12\text{-}48h} > 1$ or ≤ 1. Bars show the mean, with 95% CI. The mean and 95% CI for the data presented in panels a, c and e is available in Supplementary Dataset 2.

generally enhanced after ART treatment in infections with wild-type but not mutant parasites. If this was confirmed, a possible explanation would be that *K13* mutations may influence the effect of ART on sexual conversion rates because they result in altered life cycle progression, with a prolongation of the ring stage that is less sensitive to ART.[37,85,89,90] Indeed, estimation of parasite age at the time of treatment based on the relative levels of *pfap2-g* and *gexp02* transcripts revealed that parasites with *K13* mutations or delayed clearance tended to be younger than wild-type parasites. Recently, we demonstrated *in vitro* a stage-dependent impact of ART on sexual conversion rates, with increased conversion upon drug exposure at the trophozoite stage, but reduced conversion upon exposure at the early ring stage.[71] Therefore, it is possible that in natural infections ART-based treatment generally led to reduced sexual conversion rates in *K13* mutant isolates because the majority of parasites were at the early ring stage when they encountered the drug, whereas in wild type isolates parasites were generally older, at stages susceptible to ART-mediated stimulation of sexual conversion. The observation of a higher increase in *pfap2-g* expression in isolates with older parasites at the time of treatment is consistent with this hypothesis.

Our hypothesis fits with a recently proposed model[86] that postulates that in *K13* mutant isolates, with an extended ring stage, a short-lived ART dose may overlap with the highly vulnerable trophozoite stage in a lower proportion (~30%) of patients than in wild type isolates (>50%), contributing to resistance. It also fits with previous observations showing that the stage distribution of parasites before treatment is the main determinant of the parasite clearance profile in the first hours after treatment.[91] Our data suggest that as a consequence of the extended ring stage, *K13* mutants may be not only less sensitive to killing by ART, but also less susceptible to stimulation of sexual conversion.

Identifying the relative contribution of different groups of human individuals (e.g., asymptomatic cases, clinical cases, drug-treated patients, etc.) to *P. falciparum* transmission to mosquitoes is important to guide malaria control and elimination activities. Until recently, the potential of a malaria-infected individual to transmit malaria could only be predicted using mosquito feeding assays, which requires testing fresh blood samples and availability of mosquito colonies, or from the density of mature gametocytes.[25–28] These assays provide a valuable estimator of the potential to infect a mosquito at the time of collecting the blood sample. On the other hand, SRBs have been shown to predict the future infectious potential of an individual, if not receiving antimalarial treatment.[28,88] The discovery of SRBs expressed by circulating sexual rings also enables the assessment of the effect of human host conditions or drug treatment on sexual conversion rates. Two recent studies showed that the relative transcript levels of *pfap2-g* and SRBs such as *surfin13.1* and *surfin1.2* correlate with sexual conversion rates in natural infections,[21,23] and a study using controlled human malaria infection (CHMI) showed that *pfap2-g* relative transcript levels correlate with peak mature gametocytes density one to two weeks later.[88] Furthermore, in untreated naturally infected asymptomatic patients, *pfap2-g* and *gexp5* relative transcript levels were positively associated with mature gametocyte carriage two weeks later.[28] Here we report the use of *pfap2-g* and other SRBs to study the effect of drugs on sexual conversion rates in natural infections. However, the levels of the SRBs immediately before treatment or soon after the first dose did not correlate with mature gametocyte carriage one to two weeks later. The reason is that all participants in the three cohorts were administered a complete ART-based treatment course over three to six days, which eventually resulted in the elimination of all asexual parasites and the majority of gametocytes, even in the Vietnam cohort where most parasites carried *K13* mutant alleles. Many immature sexual parasites are also expected to be killed by the treatment.[92,93] Therefore, in natural infections, SRBs can predict future mature gametocyte carriage only for untreated (typically asymptomatic) patients, but not in drug-treated patients.

We compared the expression of five different SRBs, which revealed a moderate positive correlation between their changes in expression after treatment at the individual isolate level, but similar dynamics at the population level. The limited correlation between the changes of the different SRBs in individual isolates is likely attributable to their different timing of expression throughout sexual development. Together with the new SRBs described in a recent study[23] and several *in vitro* time-course expression analyses,[14,15,17–19,81] a large number of validated SRBs are now available for epidemiological studies aimed at characterising the prevalence and abundance of sexual rings in malaria patients. Several factors need to be considered for the selection of SRBs: i) the specificity for sexual rings, as some of the SRBs are also expressed in other circulating stages such as asexual rings (*gexp5* and *pfs16*) or mature gametocytes, albeit at lower levels than in sexual parasites[19,20,30,31,81]; ii) the fine temporal expression dynamics during the sexual ring stage. So far, *pfap2-g*[19] and possibly *surfin13.1* and *surfin1.2*[23] appear to be the only SRBs expressed from the beginning of the sexual ring stage; iii) the transcript abundance. High transcript levels are an important factor for the sensitivity of the analysis when a low volume of blood is available and parasite density is low, as often occurs in epidemiological studies. In our samples, we observed that *gexp5* and *pfs16* have the highest transcript levels. Considering all of these factors, *pfap2-g* may be the most informative SRB, but a combination of several markers can help to define the dynamics of early sexual forms in field settings. For instance, the relative transcript levels of

different markers can be used to estimate the age of sexual rings, as we did.

Studying the effect of drugs on sexual conversion would be irrelevant (beyond the academic interest) if *all* parasites were killed by the drugs and treated patients were unable to transmit the infection further, but this is clearly not the case. The net effect of ART-based treatment on transmission potential is a clear reduction, because ART kills asexual parasites, which are the source of new gametocytes, and directly eliminates some gametocytes.[10,50–56] Immature gametocytes are also sensitive to ART *in vitro*,[92,93] although *in vivo* they develop in haematopoietic tissues, where they may be less sensitive to drug treatment.[94] Consistent with previous reports,[51,57–60] our results show that while gametocyte density drops after ART treatment, many patients remain gametocyte carriers for several days after treatment. A fraction of these gametocytes likely originated from sexual conversion events after treatment: in some patients, we observed an increase in mature gametocyte densities approximately one week after treatment, when the levels of pre-treatment mature gametocytes are expected to decline. Previous studies demonstrated that gametocytes from individuals taking ART-based treatment,[60,95] as well as ART-induced gametocytes,[71] are capable of infecting *Anopheles* mosquitos. Thus, the mature gametocytes observed after treatment can contribute to malaria transmission. Furthermore, in non-controlled settings, many patients take incomplete treatment or suboptimal drugs,[96] which may result in increased survival of gametocytes and continuous production of new gametocytes some days after treatment. Consequently, if conversion rates are enhanced, such treated individuals may contribute more to transmission. Notwithstanding the overall reduction of transmission potential generally associated with ART-based treatment, the contribution of treated individuals to the malaria infectious reservoir should not be neglected. Indeed, in areas with ACT-resistance or aiming to eliminate malaria, WHO recommends combining ACTs with a single-dose primaquine to rapidly clear gametocytes from the blood circulation and prevent transmission, despite the risk of haemolysis associated with the use of this drug in G6PD-defficient individuals.[97] The combination of ACTs with a gametocidal drug would counteract any enhancement of sexual conversion associated with ART-based treatment.[98,99] Otherwise, ART-induced changes in sexual conversion rates may in some cases attenuate the reduction in transmission potential associated with ART-based parasite killing.

This study has some limitations. First, multiple characteristics of the patients and the specific ART-based treatment differed between the three cohorts, making it impossible to disentangle the contribution of specific factors to the different effects of treatment on sexual conversion between cohorts. Second, the number of patients in each cohort was relatively small, and future studies with larger sample size will be needed to unambiguously establish the factors that influence the effect of ART-based treatment on sexual conversion rates. Third, the number of infections with wild-type *K13* alleles in the Vietnam cohort was low (4 infections), limiting the statistical power to compare *K13* wild-type and mutant parasites from the same cohort. Obtaining additional wild-type parasites from Vietnam was not possible, and currently the prevalence of *K13* mutations in the region where the samples were collected is approaching fixation. Fourth, parasite densities were low in samples collected after treatment, because many parasites were killed by the drug. The lower parasitaemia in samples collected after treatment is a possible confounder, because the main outcome of this study was the comparison of relative SRBs expression levels between pre-treatment and after treatment samples. However, our RT-qPCR analysis included a standard curve in every plate, which is a robust approach that minimizes the effect of variability in amplification efficiency and the potential quantification bias between samples with different parasitaemia levels.[75] Also related, in some patients, parasite density was very low after treatment, especially at the latest time points, and relative expression values could not be determined at these time points. Calculating the median $FC^{12\text{-}48h}$ from only some of the time points for some samples may result in a bias (e.g. related with the speed of parasite clearance), but it is important to note that in the majority of samples changes in the expression of SRBs were already observed at the initial time points, and were similar between different time points (Figure 2a and Supplementary Figure 4). Fifth, we could not follow a standard case-control approach to assess the impact of treatment on sexual conversion rates, because all malaria patients must be provided immediate treatment and therefore comparing sexual conversion rates between treated and untreated patients is not possible. Six, another possible confounder could be that some of the transcripts for SRBs were derived from circulating mature gametocytes rather than from sexual rings. However, this is an unlikely confounder because SRBs are expressed at much lower levels in mature gametocytes than in sexual rings, and at all time points analysed mature gametocytes were far less abundant than rings (Supplementary Data Set 2). Lastly, the differences between cohorts in the methods to prepare RNA may be a potential source of bias, but pre- and post-treatment samples within each cohort were always prepared in parallel by the same method. Therefore, the gene expression ratio between them (FC) is expected to be independent from the preparation method (both samples would be affected in the same way).

In spite of these limitations, we can conclude that in many patients SRBs relative transcript levels clearly increased after ART-based treatment, suggesting increased sexual conversion. This is relevant in the

context of widespread use of ACTs for malaria treatment and also for mass drug administration as part of malaria elimination efforts. Our study further defines and validates a panel of markers of sexual conversion for studies aiming to investigate the impact of environmental and host factors on sexual conversion rates in natural infections, and identifies parasite age as a factor that influences the effect of ART-based treatment on sexual conversion rates. Larger studies will be needed to unambiguously establish which factors influence the effect of ART-based treatment on sexual conversion rates. These studies should use relatively homogeneous cohorts in which specific factors such as *K13* mutations occur at intermediate frequencies. Frequent sampling could be limited to the first 24 h after treatment, as this was the most informative time period.

Contributors

H.P.P. performed the majority of laboratory experiments and analysed the data. P.G., A.M.S., A.M., D.F.O. and E.R.-V. performed experiments. H.M.N., I.V., T.H., H.S. and A.R.-U. designed and performed the clinical study in Burkina Faso. N.v.H. and A.R.-U. provided the samples from the clinical study in Vietnam. A.S., R.V. and Q.B. provided the samples from the clinical study in Mozambique. A.C. and A.R.-U. provided supervision and conceived the project. H.P.P., A.C. and A.R.-U. designed the experiments, interpreted the results and wrote the manuscript. H.P.P. and A.R.-U. have verified the underlying data. All authors read and approved the final version of the manuscript.

Data sharing statement

All the data generated and used to obtain results reported here are available as supplementary information and datasets.

Declaration of interests

The authors declare no competing interests.

Acknowledgements

We are grateful to all the patients that participated in the studies. This work was supported by grants from the Spanish Ministry of Economy and Competitiveness (MINECO)/ Agencia Estatal de Investigación (AEI) (SAF2016-76190-R and PID2019-107232RB-I00 to A. C.), co-funded by the European Regional Development Fund (ERDF, European Union), and the Belgium Development Cooperation (DGD) under the Framework Agreement Program between DGD and ITM (FA3-III Vietnam, 2014–2016 & FA4 Burkina Faso, 2017-2021). The rosiglitazone adjuvant therapy trial for severe malaria was funded by the University Health Network (UHN), Toronto, Canada. ITM and ISGlobal were members of the TransGlobalHealth–Erasmus Mundus Joint Doctorate Programme, European Union (scholarship number 2016-1346 to H.P.P.). This research is part of ISGlobal's Program on the Molecular Mechanisms of Malaria, which is partially supported by the Fundación Ramón Areces. We acknowledge support from the Spanish Ministry of Science and Innovation through the "Centro de Excelencia Severo Ochoa 2019-2023" Program (CEX2018-000806-S), and support from the Generalitat de Catalunya through the CERCA Program. CISM is supported by the Government of Mozambique and the Spanish Agency for International Development (AECID).

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.104198.

References

1 WHO. *World malaria report 2021*. World Health Organization; 2021. ISBN 978-92-4-004049-6.
2 Josling GA, Williamson KC, Llinas M. Regulation of sexual commitment and gametocytogenesis in malaria parasites. *Annu Rev Microbiol*. 2018;72:501–519.
3 Ngotho P, Soares AB, Hentzschel F, Achcar F, Bertuccini L, Marti M. Revisiting gametocyte biology in malaria parasites. *FEMS Microbiol Rev*. 2019;43(4):401–414.
4 Cao P, Collins KA, Zaloumis S, et al. Modeling the dynamics of *Plasmodium falciparum* gametocytes in humans during malaria infection. *Elife*. 2019;8:e49058.
5 Eichner M, Diebner HH, Molineaux L, Collins WE, Jeffery GM, Dietz K. Genesis, sequestration and survival of *Plasmodium falciparum* gametocytes: parameter estimates from fitting a model to malariatherapy data. *Trans R Soc Trop Med Hyg*. 2001;95(5):497–501.
6 Brancucci NMB, Gerdt JP, Wang C, et al. Lysophosphatidylcholine regulates sexual stage differentiation in the human malaria parasite *Plasmodium falciparum*. *Cell*. 2017;171(7):1532–1544.
7 Neveu G, Beri D, Kafsack BF. Metabolic regulation of sexual commitment in *Plasmodium falciparum*. *Curr Opin Microbiol*. 2020;58:93–98.
8 Carter LM, Kafsack BF, Llinas M, Mideo N, Pollitt LC, Reece SE. Stress and sex in malaria parasites: why does commitment vary? *Evol Med Public Health*. 2013;2013(1):135–147.
9 Baker DA. Malaria gametocytogenesis. *Mol Biochem Parasitol*. 2010;172(2):57–65.
10 Bousema T, Drakeley C. Epidemiology and infectivity of *Plasmodium falciparum* and *Plasmodium vivax* gametocytes in relation to malaria control and elimination. *Clin Microbiol Rev*. 2011;24(2):377–410.
11 Thommen BT, Passecker A, Buser T, Hitz E, Voss TS, Brancucci NMB. Revisiting the effect of pharmaceuticals on transmission stage formation in the malaria parasite *Plasmodium falciparum*. *Front Cell Infect Microbiol*. 2022;12:802341.
12 Kafsack BF, Rovira-Graells N, Clark TG, et al. A transcriptional switch underlies commitment to sexual development in malaria parasites. *Nature*. 2014;507(7491):248–252.
13 Sinha A, Hughes KR, Modrzynska KK, et al. A cascade of DNA-binding proteins for sexual commitment and development in *Plasmodium*. *Nature*. 2014;507(7491):253–257.
14 Josling GA, Russell TJ, Venezia J, et al. Dissecting the role of PfAP2-G in malaria gametocytogenesis. *Nat Commun*. 2020;11(1):1503.
15 Llorà-Batlle O, Michel-Todó L, Witmer K, et al. Conditional expression of PfAP2-G for controlled massive sexual conversion in *Plasmodium falciparum*. *Sci Adv*. 2020;6(24):eaaz5057.

16 Poran A, Notzel C, Aly O, et al. Single-cell RNA sequencing reveals a signature of sexual commitment in malaria parasites. *Nature*. 2017;551(7678):95–99.
17 Brancucci NM, Bertschi NL, Zhu L, et al. Heterochromatin protein 1 secures survival and transmission of malaria parasites. *Cell Host Microbe*. 2014;16(2):165–176.
18 Filarsky M, Fraschka SA, Niederwieser I, et al. GDV1 induces sexual commitment of malaria parasites by antagonizing HP1-dependent gene silencing. *Science*. 2018;359(6381):1259–1263.
19 Bancells C, Llora-Batlle O, Poran A, et al. Revisiting the initial steps of sexual development in the malaria parasite *Plasmodium falciparum*. *Nat Microbiol*. 2019;4(1):144–154.
20 Farid R, Dixon MW, Tilley L, McCarthy JS. Initiation of gametocytogenesis at very low parasite density in *Plasmodium falciparum* infection. *J Infect Dis*. 2017;215(7):1167–1174.
21 Usui M, Prajapati SK, Ayanful-Torgby R, et al. *Plasmodium falciparum* sexual differentiation in malaria patients is associated with host factors and GDV1-dependent genes. *Nat Commun*. 2019;10(1):2140.
22 Pelle KG, Oh K, Buchholz K, et al. Transcriptional profiling defines dynamics of parasite tissue sequestration during malaria infection. *Genome Med*. 2015;7(1):19.
23 Prajapati SK, Ayanful-Torgby R, Pava Z, et al. The transcriptome of circulating sexually committed *Plasmodium falciparum* ring stage parasites forecasts malaria transmission potential. *Nat Commun*. 2020;11(1):6159.
24 Venugopal K, Hentzschel F, Valkiūnas G, Marti M. *Plasmodium* asexual growth and sexual development in the haematopoietic niche of the host. *Nat Rev Microbiol*. 2020;18(3):177–189.
25 Churcher TS, Bousema T, Walker M, et al. Predicting mosquito infection from *Plasmodium falciparum* gametocyte density and estimating the reservoir of infection. *Elife*. 2013;2:e00626.
26 Bousema T, Drakeley C. Determinants of malaria transmission at the population level. *Cold Spring Harb Perspect Med*. 2017;7(12):a025510.
27 Bradley J, Stone W, Da DF, et al. Predicting the likelihood and intensity of mosquito infection from sex specific *Plasmodium falciparum* gametocyte density. *Elife*. 2018;7:e34463.
28 Barry A, Bradley J, Stone W, et al. Higher gametocyte production and mosquito infectivity in chronic compared to incident *Plasmodium falciparum* infections. *Nat Commun*. 2021;12(1):2443.
29 Ahmad A, Soumare HM, Camara MM, et al. Infectivity of patent *Plasmodium falciparum* gametocyte carriers to mosquitoes: establishing capacity to investigate the infectious reservoir of malaria in a low-transmission setting in The Gambia. *Trans R Soc Trop Med Hyg*. 2021;115(12):1462–1467.
30 Portugaliza HP, Llora-Batlle O, Rosanas-Urgell A, Cortes A. Reporter lines based on the *gexpo2* promoter enable early quantification of sexual conversion rates in the malaria parasite *Plasmodium falciparum*. *Sci Rep*. 2019;9(1):14595.
31 Tiburcio M, Dixon MW, Looker O, Younis SY, Tilley L, Alano P. Specific expression and export of the *Plasmodium falciparum* gametocyte exported protein-5 marks the gametocyte ring stage. *Malar J*. 2015;14(1):334.
32 Pradel G. Proteins of the malaria parasite sexual stages: expression, function and potential for transmission blocking strategies. *Parasitology*. 2007;134(Pt.14):1911–1929.
33 Eastman RT, Fidock DA. Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria. *Nat Rev Microbiol*. 2009;7(12):864–874.
34 Nosten F, White NJ. Artemisinin-based combination treatment of falciparum malaria. *Am J Trop Med Hyg*. 2007;77(6 suppl):181–192.
35 Ariey F, Witkowski B, Amaratunga C, et al. A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature*. 2014;505(7481):50–55.
36 Birnbaum J, Scharf S, Schmidt S, et al. A Kelch13-defined endocytosis pathway mediates artemisinin resistance in malaria parasites. *Science*. 2020;367(6473):51–59.
37 Wicht KJ, Mok S, Fidock DA. Molecular mechanisms of drug resistance in *Plasmodium falciparum* malaria. *Annu Rev Microbiol*. 2020;74:431–454.
38 Dondorp AM, Nosten F, Yi P, et al. Artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med*. 2009;361(5):455–467.
39 Phyo AP, Ashley EA, Anderson TJC, et al. Declining efficacy of artemisinin combination therapy against *P. Falciparum* malaria on the Thai-Myanmar border (2003–2013): the role of parasite genetic factors. *Clin Infect Dis*. 2016;63:784–791.
40 van der Pluijm RW, Imwong M, Chau NH, et al. Determinants of dihydroartemisinin-piperaquine treatment failure in *Plasmodium falciparum* malaria in Cambodia, Thailand, and Vietnam: a prospective clinical, pharmacological, and genetic study. *Lancet Infect Dis*. 2019;19(9):952–961.
41 Saunders DL, Vanachayangkul P, Lon C. Dihydroartemisinin-piperaquine failure in Cambodia. *N Engl J Med*. 2014;371(5):484–485.
42 Woodrow CJ, White NJ. The clinical impact of artemisinin resistance in Southeast Asia and the potential for future spread. *FEMS Microbiol Rev*. 2017;41(1):34–48.
43 Siddiqui FA, Liang X, Cui L. *Plasmodium falciparum* resistance to ACTs: emergence, mechanisms, and outlook. *Int J Parasitol Drugs Drug Resist*. 2021;16:102–118.
44 Mairet-Khedim M, Leang R, Marmai C, et al. Clinical and in vitro resistance of *Plasmodium falciparum* to artesunate-amodiaquine in Cambodia. *Clin Infect Dis*. 2021;73(3):406–413.
45 Uwimana A, Legrand E, Stokes BH, et al. Emergence and clonal expansion of in vitro artemisinin-resistant *Plasmodium falciparum* kelch13 R561H mutant parasites in Rwanda. *Nat Med*. 2020;26(10):1602–1608.
46 Balikagala B, Fukuda N, Ikeda M, et al. Evidence of artemisinin-resistant malaria in Africa. *N Engl J Med*. 2021;385(13):1163–1171.
47 Galatas B, Saúte F, Martí-Soler H, et al. A multiphase program for malaria elimination in southern Mozambique (the Magude project): a before-after study. *PLoS Med*. 2020;17(8):e1003227.
48 WHO. *Mass Drug Administration for Falciparum Malaria: A Practical Field Manual*. World Health Organization; 2017.
49 von Seidlein L, Peto TJ, Landier J, et al. The impact of targeted malaria elimination with mass drug administrations on falciparum malaria in Southeast Asia: a cluster randomised trial. *PLoS Med*. 2019;16(2):e1002745.
50 Chawla J, Oberstaller J, Adams JH. Targeting gametocytes of the malaria parasite *Plasmodium falciparum* in a functional genomics era: next steps. *Pathogens*. 2021;10(3):346.
51 WWARN_Gametocyte_Study_Group. Gametocyte carriage in uncomplicated *Plasmodium falciparum* malaria following treatment with artemisinin combination therapy: a systematic review and meta-analysis of individual patient data. *BMC Med*. 2016;14:79.
52 von Seidlein L, Drakeley C, Greenwood B, Walraven G, Targett G. Risk factors for gametocyte carriage in Gambian children. *Am J Trop Med Hyg*. 2001;65(5):523–527.
53 Price RN, Nosten F, Luxemburger C, et al. Effects of artemisinin derivatives on malaria transmissibility. *Lancet*. 1996;347(9016):1654–1658.
54 Sawa P, Shekalaghe SA, Drakeley CJ, et al. Malaria transmission after artemether-lumefantrine and dihydroartemisinin-piperaquine: a randomized trial. *J Infect Dis*. 2013;207(11):1637–1645.
55 Okell LC, Drakeley CJ, Ghani AC, Bousema T, Sutherland CJ. Reduction of transmission from malaria patients by artemisinin combination therapies: a pooled analysis of six randomized trials. *Malar J*. 2008;7:125.
56 Ippolito MM, Johnson J, Mullin C, et al. The relative effects of artemether-lumefantrine and non-artemisinin antimalarials on gametocyte carriage and transmission of *Plasmodium falciparum*: a systematic review and meta-analysis. *Clin Infect Dis*. 2017;65(3):486–494.
57 Bousema JT, Schneider P, Gouagna LC, et al. Moderate effect of artemisinin-based combination therapy on transmission of *Plasmodium falciparum*. *J Infect Dis*. 2006;193(8):1151–1159.
58 Bousema T, Okell L, Shekalaghe S, et al. Revisiting the circulation time of *Plasmodium falciparum* gametocytes: molecular detection methods to estimate the duration of gametocyte carriage and the effect of gametocytocidal drugs. *Malar J*. 2010;9:136.
59 Karl S, Laman M, Moore BR, et al. Gametocyte clearance kinetics determined by quantitative magnetic fractionation in melanesian children with uncomplicated malaria treated with artemisinin combination therapy. *Antimicrob Agents Chemother*. 2015;59(8):4489–4496.
60 Targett G, Drakeley C, Jawara M, et al. Artesunate reduces but does not prevent posttreatment transmission of *Plasmodium falciparum* to *Anopheles gambiae*. *J Infect Dis*. 2001;183(8):1254–1259.
61 Koepfli C, Yan G. *Plasmodium* gametocytes in field studies: do we measure commitment to transmission or detectability? *Trends Parasitol*. 2018;34(5):378–387.
62 Babiker HA, Schneider P, Reece SE. Gametocytes: insights gained during a decade of molecular monitoring. *Trends Parasitol*. 2008;24(11):525–530.

63 Butcher GA. Antimalarial drugs and the mosquito transmission of *Plasmodium*. *Int J Parasitol*. 1997;27(9):975–987.
64 Buckling A, Ranford-Cartwright LC, Miles A, Read AF. Chloroquine increases *Plasmodium falciparum* gametocytogenesis in vitro. *Parasitology*. 1999;118(Pt 4):339–346.
65 Peatey CL, Skinner-Adams TS, Dixon MW, McCarthy JS, Gardiner DL, Trenholme KR. Effect of antimalarial drugs on *Plasmodium falciparum* gametocytes. *J Infect Dis*. 2009;200(10):1518–1521.
66 Reece SE, Ali E, Schneider P, Babiker HA. Stress, drugs and the evolution of reproductive restraint in malaria parasites. *Proc Biol Sci*. 2010;277(1697):3123–3129.
67 Brancucci NM, Goldowitz I, Buchholz K, Werling K, Marti M. An assay to probe *Plasmodium falciparum* growth, transmission stage formation and early gametocyte development. *Nat Protoc*. 2015;10(8):1131–1142.
68 Buckling A, Crooks L, Read A. *Plasmodium chabaudi*: effect of antimalarial drugs on gametocytogenesis. *Exp Parasitol*. 1999;93(1):45–54.
69 Buckling AG, Taylor LH, Carlton JM, Read AF. Adaptive changes in *Plasmodium* transmission strategies following chloroquine chemotherapy. *Proc Biol Sci*. 1997;264(1381):553–559.
70 Schneider P, Greischar MA, Birget PLG, Repton C, Mideo N, Reece SE. Adaptive plasticity in the gametocyte conversion rate of malaria parasites. *PLoS Pathog*. 2018;14(11):e1007371.
71 Portugaliza HP, Miyazaki S, Geurten FJ, et al. Artemisinin exposure at the ring or trophozoite stage impacts *Plasmodium falciparum* sexual conversion differently. *Elife*. 2020;9:e60058.
72 Rovira-Vallbona E, Van Hong N, Kattenberg JH, et al. Efficacy of dihydroartemisinin/piperaquine and artesunate monotherapy for the treatment of uncomplicated *Plasmodium falciparum* malaria in central Vietnam. *J Antimicrob Chemother*. 2020;75(8):2272–2281.
73 Varo R, Crowley VM, Sitoe A, et al. Safety and tolerability of adjunctive rosiglitazone treatment for children with uncomplicated malaria. *Malar J*. 2017;16(1):215.
74 Mira-Martínez S, van Schuppen E, Amambua-Ngwa A, et al. Expression of the *Plasmodium falciparum* clonally variant *clag3* genes in human infections. *J Infect Dis*. 2017;215(6):938–945.
75 Casas-Vila N, Pickford AK, Portugaliza HP, Tintó-Font E, Cortés A. Transcriptional analysis of tightly synchronized *Plasmodium falciparum* intraerythrocytic stages by RT-qPCR. *Methods Mol Biol*. 2021;2369:165–185.
76 Gruenberg M, Hofmann NE, Nate E, et al. qRT-PCR versus IFA-based quantification of male and female gametocytes in low-density *Plasmodium falciparum* infections and their relevance for transmission. *J Infect Dis*. 2020;221(4):598–607.
77 Hofmann N, Mwingira F, Shekalaghe S, Robinson LJ, Mueller I, Felger I. Ultra-sensitive detection of *Plasmodium falciparum* by amplification of multi-copy subtelomeric targets. *PLoS Med*. 2015;12(3):e1001788.
78 Natama HM, Ouedraogo DF, Sorgho H, et al. Diagnosing congenital malaria in a high-transmission setting: clinical relevance and usefulness of *P. falciparum* HRP2-based testing. *Sci Rep*. 2017;7(1):2080.
79 Erhart A, Ngo DT, Phan VK, et al. Epidemiology of forest malaria in central Vietnam: a large scale cross-sectional survey. *Malar J*. 2005;4:58.
80 Ménard D, Khim N, Beghain J, et al. A Worldwide Map of *Plasmodium falciparum* K13-Propeller Polymorphisms. *N Engl J Med*. 2016;374(25):2453–2464.
81 van Biljon R, van Wyk R, Painter HJ, et al. Hierarchical transcriptional control regulates *Plasmodium falciparum sexual* differentiation. *BMC Genomics*. 2019;20(1):920.
82 Zhu L, Tripathi J, Rocamora FM, et al. The origins of malaria artemisinin resistance defined by a genetic and transcriptomic background. *Nat Commun*. 2018;9(1):5158.
83 Klonis N, Xie SC, McCaw JM, et al. Altered temporal response of malaria parasites determines differential sensitivity to artemisinin. *Proc Natl Acad Sci USA*. 2013;110(13):5157–5162.
84 Witkowski B, Amaratunga C, Khim N, et al. Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: in-vitro and ex-vivo drug-response studies. *Lancet Infect Dis*. 2013;13(12):1043–1049.
85 Mok S, Ashley EA, Ferreira PE, et al. Drug resistance. Population transcriptomics of human malaria parasites reveals the mechanism of artemisinin resistance. *Science*. 2015;347(6220):431–435.
86 Khoury DS, Cao P, Zaloumis SG, Davenport MP. Artemisinin resistance and the unique selection pressure of a short-acting antimalarial. *Trends Parasitol*. 2020;36(11):884–887.
87 Pett H, Gonçalves BP, Dicko A, et al. Comparison of molecular quantification of *Plasmodium falciparum* gametocytes by Pfs25 qRT-PCR and QT-NASBA in relation to mosquito infectivity. *Malar J*. 2016;15(1):539.
88 Alkema M, Reuling IJ, de Jong GM, et al. A randomized clinical trial to compare *Plasmodium falciparum* gametocytemia and infectivity after blood-stage or mosquito bite-induced controlled malaria infection. *J Infect Dis*. 2021;224(7):1257–1265.
89 Dogovski C, Xie SC, Burgio G, et al. Targeting the cell stress response of *Plasmodium falciparum* to overcome artemisinin resistance. *PLoS Biol*. 2015;13(4):e1002132.
90 Hott A, Casandra D, Sparks KN, et al. Artemisinin-resistant *Plasmodium falciparum* parasites exhibit altered patterns of development in infected erythrocytes. *Antimicrob Agents Chemother*. 2015;59(6):3156–3167.
91 Intharabut B, Kingston HW, Srinamon K, et al. Artemisinin Resistance and Stage Dependency of Parasite Clearance in Falciparum Malaria. *J Infect Dis*. 2019;219(9):1483–1489.
92 Plouffe DM, Wree M, Du AY, et al. High-throughput assay and discovery of small molecules that interrupt malaria transmission. *Cell Host Microbe*. 2016;19(1):114–126.
93 Adjalley SH, Johnston GL, Li T, et al. Quantitative assessment of *Plasmodium falciparum* sexual development reveals potent transmission-blocking activity by methylene blue. *Proc Natl Acad Sci USA*. 2011;108(47):E1214–E1223.
94 Lee RS, Waters AP, Brewer JM. A cryptic cycle in haematopoietic niches promotes initiation of malaria transmission and evasion of chemotherapy. *Nat Commun*. 2018;9(1):1689.
95 Ouologuem DT, Kone CO, Fofana B, et al. Differential infectivity of gametocytes after artemisinin-based combination therapy of uncomplicated falciparum malaria. *Afr J Lab Med*. 2018;7(2):784.
96 Challenger JD, Gonçalves BP, Bradley J, et al. How delayed and non-adherent treatment contribute to onward transmission of malaria: a modelling study. *BMJ Glob Health*. 2019;4(6):e001856.
97 WHO. *Policy Brief on Single-Dose Primaquine as a Gametocytocide in* Plasmodium falciparum *Malaria*. World Health Organization; 2015.
98 Stepniewska K, Humphreys GS, Gonçalves BP, et al. Efficacy of single-dose primaquine with artemisinin combination therapy on *Plasmodium falciparum* gametocytes and transmission: an individual patient meta-analysis. *J Infect Dis*. 2022;225(7):1215–1226.
99 Stone W, Mahamar A, Sanogo K, et al. Pyronaridine-artesunate or dihydroartemisinin-piperaquine combined with single low-dose primaquine to prevent *Plasmodium falciparum* malaria transmission in Ouélessébougou, Mali: a four-arm, single-blind, phase 2/3, randomised trial. *Lancet Microbe*. 2022;3(1):e41–e51.