Triple antimalarial drug combinations for the treatment of falciparum malaria

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Publication date
2021
Document Version
Final published version

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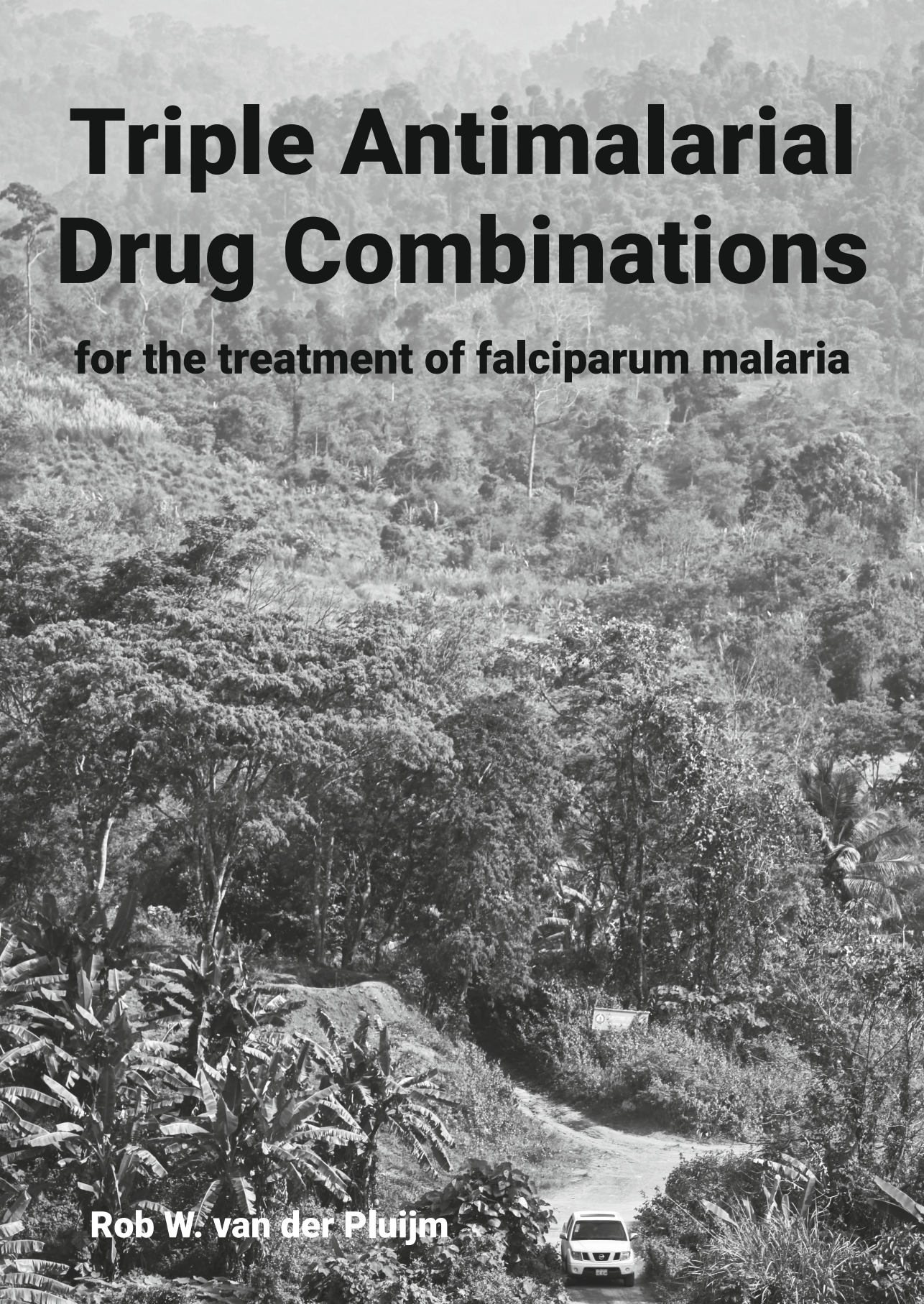

Rob W. van der Pluijm

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Cover design: Yasmin Katlich | www.persoonlijkproefschrift.nl
Design/lay-out: Danielle Balk | www.persoonlijkproefschrift.nl
Printing: Ridderprint | www.ridderprint.nl

Triple antimalarial drug combinations for the treatment of falciparum malaria

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

prof. dr. ir. K.I.J. Maex

ten overstaan van een door het College voor Promoties ingestelde commissie,

in het openbaar te verdedigen in de Agnietenkapel

op vrijdag 10 september 2021, te 16.00 uur

door Robertus Wilhelmus van der Pluijm

geboren te Tilburg

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Introduction and scope of thesis

1

INTRODUCTION

Five malaria species cause disease in humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*.[1] In 2019, malaria was endemic in 87 countries, and an estimated 229 million cases of malaria were recorded worldwide, of which about 95% occurred in sub-Saharan Africa. Over 95% of infections are caused by *Plasmodium falciparum* malaria. In the same year, 409,000 deaths, mostly in children in sub-Saharan Africa, have been recorded. Between 2000 and 2019, the yearly global malaria case incidence rate has decreased from 80 to 56.8 per 1000 persons at risk. In the same time, the malaria attributable mortality rate has dropped from 24.7 to 10.1 per 100,000 persons at risk.[2] Large-scale deployment of long-lasting insecticide treated bed nets (LLITN), indoor residual spraying, and other measures aimed at vector control have played an important role in reducing the burden of malaria. Besides measures aimed at the vector, preventive measures can be aimed at the *Plasmodium* parasite. Intermittent preventive treatment of malaria in pregnancy (IPTp) and in infants (IPTi) and seasonal malaria chemoprevention (SMC) have also contributed to a reduction in the incidence of malaria.[3]

Finally, effective antimalarial therapies against clinical stages of malaria have been a crucial factor in the recent gains in the fight against malaria. The use of an effective treatment reduces the morbidity and mortality of each symptomatic infection, while reducing the potential of the infection to be transmitted to the next host.

Out of all antimalarials available to date, the artemisinins are the most potent antimalarials. Dihydroartemisinin is the active metabolite of artemisinin, artesunate and artemether, and is available as a drug itself. The working mechanism is thought to be based on the oxidative stress that arises due to a reaction between the endoperoxide ring in the dihydroartemisinin molecule and the haem molecule in the erythrocytes, which subsequently disrupts essential processes in the intraerythrocytic stage of the malaria parasite.[4]

Artemisinin-based Combination Therapies (ACTs), in which an artemisinin component is combined with a slowly eliminated partner drug such as piperaquine, mefloquine, lumefantrine, amodiaquine or sulphadoxine-pyrimethamine, are the recommended first-line treatment for *Plasmodium falciparum* in all malaria endemic countries.[3]

Artemisinin-based combination therapies for the treatment of *Plasmodium falciparum* malaria

In 1972, artemisinins were isolated from the *Artemisia annua* plant by the Chinese chemist Tu Youyou and her team.[5] A patient suffering from uncomplicated *falciparum* malaria is likely to carry between 10^9 and 10^{11} parasites.[6] In the initial three days of treatment with an artemisinin or artemisinin-based combination therapy, the parasite load rapidly

decreases with a 10,000-fold reduction per each 48 hours asexual parasite life cycle, provided the parasite is artemisinin sensitive.[7] The rapid load reduction is related to the fast clearance of ring-stage parasites, which distinguishes artemisinins from other antimalarials such as quinine. As a result, the use of intravenous artesunate reduces the mortality of severe malaria in adults by around 35% and around 22% in children when compared to intravenous quinine.[8, 9] The active metabolite of all artemisinin compounds, dihydroartemisinin, is rapidly cleared with an elimination half-life of less than 1 hour, thereby limiting the selective window for drug-resistant parasites.[10, 11] However, even a 7-day monotherapy with an artemisinin is not completely effective, most likely due to the short half-life of artemisinin and potentially due to artemisinin induced dormancy in asexual-stage parasites.[12-14] Early in their development, it was suggested to combine artemisinins with one or more slowly eliminated 'partner' drugs.[15] Studies in the early 1990s found that a 3-day regimen of the combination of artesunate and mefloquine was safe, well tolerated and effective in treating mefloquine resistant *Plasmodium falciparum* malaria on the Thai-Burmese border.[16] Thus, combining two results in to high efficacy, even though the individual components of the treatment are not sufficient to clear an infection. An ACT is generally administered for three consecutive days. Each dose contains an artemisinin and a partner drug. In the first three days of treatment, the artemisinin component leads to a rapid reduction in parasites, while the concentration of the partner drug in the body increases with each dose. Parasites that survive the initial three days are left to be killed by the partner drugs.[17] The long half-life of the partner drug provides a post-prophylactic effect by preventing reinfections for as long as the concentrations of the partner drug remain above the minimum inhibitory concentration of the parasites that are introduced by a new mosquito bite.[18, 19] Due to their high efficacy and post-prophylactic effects, ACTs have played an important role in reducing the global burden of malaria. Combining artemisinins and partner drugs has also prolonged the longevity of each component as the chance of each invididual parasite surviving both treatments approaches the product of the chance of development of resistance against either compound.[17] However, the relative recent emergence of artemisinin and partner drug resistance pose a major threat to this recent progress.

Resistance in *Plasmodium falciparum* malaria

Chloroquine resistant *Plasmodium falciparum* strains emerged independently several times in the 1950s and 1960s. One of these chloroquine resistant lineages originated from Cambodia in Southeast Asia and spread throughout the Indian sub-continent and sub-Saharan Africa.[20] After the arrival of chloroquine resistance, several studies have reported a two to three-fold increase in cases of severe malaria and malaria-related deaths.[21] Sulfadoxine-pyrimethamine was introduced to replace chloroquine. In a similar way, sulfadoxine-pyrimethamine-resistant parasites emerged in Southeast Asia and spread throughout sub-Saharan Africa.[22]

Artemisinin resistance was first identified between 2006 and 2009 in western Cambodia.[7, 23] It can be defined *in vivo* as a prolonged parasite clearance half-life and is associated with higher *in vitro* survival of ring-stage parasites and mutations the *Plasmodium falciparum Kelch13* (*PfKelch13)* gene.[24-27] Artemisinin resistance results in a high number of parasites surviving the initial 3 days of artemisinin exposure. The number of parasites that are to be killed by the partner drugs is likely to be at least 10,000 times as high compared to infections with an artemisinin sensitive parasite strain.[28] As there is still a significant killing effect, this form of resistance has sometimes been referred to as 'partial resistance'. For ease of reading, this form of resistance will be referred to as artemisinin resistance throughout this thesis.

The partner drug is less potent compared to artemisinins and has a longer half-life. As a result, a large number of parasites is exposed to a partner drug with a limited potency for an extended time, providing ideal conditions for the selection of partner drug resistance. The selection of mefloquine resistance on the Thailand-Myanmar border and piperaquine resistance in northeastern Thailand, Cambodia and Vietnam has resulted in low efficacy of ACTs such as artesunate-mefloquine and dihydroartemisinin-piperaquine.[29-31]

The spread of antimalarial resistance

Artemisinin resistance is strongly associated with mutations in the *PfKelch13* gene, and the prevalence of these mutations can be used to track artemisinin resistance. So far, no signs of artemisinin and partner drug resistance spreading out of Southeast Asia have been identified.[32] However, the emergence of chloroquine and sulfadoxine-pyrimethamine resistance in Southeast Asia and its subsequent global spread should serve as warnings from the past.[20-22] To eliminate the risk of spread, *Plasmodium falciparum* should be eliminated in Southeast Asia as soon as possible.[33] Besides the risk of spreading of antimalarial resistance, there is the risk of independent emergence of artemisinin or partner drug resistance. Recent reports indicate the independent emergence of artemisinin resistance in Rwanda and French Guyana, both in regions with strong recent reductions in malaria transmission.[34-36] The threat of artemisinin and partner drug resistance spreading out of Southeast Asia and the threat of independent emergence in other areas underlines the need for treatments that are effective against multidrug resistant malaria and are less likely to fall to resistance.

Triple antimalarial drug combinations

Standard ACTs combine a rapidly metabolized artemisinin and a partner drug with a longer elimination phase. This difference in pharmacokinetics leaves the partner drug exposed after the first three days of treatment, therefore increasing the risk of development of partner drug resistance.[37] In addition, once either artemisinin or partner drug resistance is established, this facilitates the selection of resistance to partner drugs or artemisinin, respectively.

A potential strategy to prolong the utility of existing antimalarials is combining an artemisinin with two partner drugs in the form of a Triple Artemisinin-based Combination Therapy (Triple ACT or TACT). In the absence of antimalarial resistance, the probability of a parasite developing resistance to all three components will approach the product of the probability of development of resistance to each individual antimalarial. In the setting of established artemisinin resistance in which the partner drugs are exposed to a higher parasite biomass, adding a second partner drug could slow or prevent the development of partner drug resistance to either partner drug. Two aspects should be taken into account in selecting and matching partner drugs. Ideally, the partner drugs should be selected in a way that drug concentrations in the blood are above minimal parasiticidal concentrations during a similar time period, thereby preventing parasites being exposed to a single drug. Secondly, the mechanisms of actions and the mechanisms of resistance to the individual drugs should be different if not mutually counteractive.[38] Two combinations of partner drugs are of direct interest for the development of Triple ACTs. Both the combination of piperaquine and mefloquine and the combination lumefantrine and amodiaquine share comparable pharmacokinetics. Also, field and laboratory studies suggest counteracting mechanisms of resistance for both the combination of piperaquine and mefloquine as well as the combination of lumefantrine and amodiaquine. In other words, parasites that are resistant to piperaquine are more likely to be sensitive to mefloquine, whereas parasites that are resistant to lumefantrine are more likely to be sensitive to amodiaquine.[29, 39-43]

In areas with established artemisinin and partner drug resistance, the use of Triple ACTs could serve as a stopgap to provide effective treatment while potentially preventing further development of antimalarial resisistance. In areas where artemisinins and partner drugs are still effective, deployment of Triple ACTs could potentially delay or prevent the emergence of resistance.

Thesis scope

This thesis aims to describe the current extent of antimalarial resistance in *Plasmodium falciparum* malaria (part I) and to assess the safety, tolerability and efficacy of Triple Antimalarial Combination Therapies (part II). In addition, this thesis will explore potential barriers and outstanding questions that will need to be addressed to enable widescale implementation of Triple Antimalarial Combination Therapies (part III).

Epidemiology of antimalarial resistant malaria

Dihydroartemisin-piperaquine (DHA-piperaquine) is generally well tolerated and safe. In the absence of artemisinin and partner drug resistance, the efficacy of this ACT is high. In **Chapter 2** we report the efficacy rates of DHA-piperaquine in Cambodia, Vietnam and northeastern Thailand. We also assess the influence of host-factors such as age, sex and piperaquine drug levels on the risk of treatment failure. In addition, we describe the role of genetic markers as predictors of treatment failure. Finally, we describe the prevalence

of these genetic markers in the first TRAC trial (2011-2013) and the second TRAC study (2015-2018). In **Chapter 3** we describe how the artemisinin and piperaquine resistant co-lineage diversified into multiple subgroups and spread throughout Southeast Asia while acquiring new genetic features.

Safety, tolerability and efficacy of Triple ACTs

When combining antimalarials, safety and tolerability and pharmacokinetic and pharmacodynamic interactions should be taken into account. A characteristic shared by many antimalarials (quinolines and structurally related antimalarials) is their QTc-interval prolonging effect.[44] Combining multiple antimalarials could lead to increased individual drug levels as well as a synergistic QTc-interval prolonging effect. Therefore, we conducted a healthy volunteer study in 15 Thai adults (**Chapter 4**). They were consecutively treated with DHA-piperaquine, mefloquine and DHA-piperaquine. Major outcomes were (cardiac) safety, tolerability and pharmacokinetic interactions.

Between 2015 and 2018 we conducted a randomized controlled trial in 18 hospitals and health clinics in eight countries Cambodia (four sites), Thailand (three sites), Myanmar (four sites), India (three sites), Laos, Vietnam, Bangladesh, and the Democratic Republic of the Congo (one site each). A total of 1100 patients with uncomplicated *Plasmodium falciparum* were randomized between a standard ACT (DHA-piperaquine, artesunate-mefloquine or artemether-lumefantrine) and a Triple ACT (DHA-piperaquine plus mefloquine or artemether-lumefantrine plus amodiaquine). Major outcomes were efficacy, safety, tolerability of the Triple ACTs and pharmacokinetic interactions between the individual drugs in these combinations (**Chapter 5**). In addition, we report the current state of antimalarial resistance in *Plasmodium falciparum* malaria across Southeast Asia and South Asia. Given the high prevalence of artemisinin resistance in Southeast Asia using non-artemisinin based antimalarials could provide a benefit. Non-artemisinin based antimalarials include the synthetic ozonides, arterolane maleate (OZ277) and artefenomel (OZ439). Between March 2018 and May 2019 we conducted a randomized controlled trial in the Kilifi area in coastal Kenya (**Chapter 6**). Children younger than 12 years suffering from uncomplicated *Plasmodium falciparum* malaria were randomized between a treatment of arterolane-piperaquine, the Triple arterolane-piperaquine plus mefloquine and the first line treatment artemether-lumefantrine.

Future directions

Chapter 7 explores the aspects that should be taken into account when combining three antimalarials in a Triple ACT, including production costs, pharmacokinetic interactions and profiles, mechanisms of antimalarial resistance, drug safety and tolerability and dosing of the individual components. Furthermore, mathematical modelling can provide insights in the potential of Triple ACTs to prevent or delay antimalarial resistance, while also assessing the cost-effectiveness of this approach when compared to other antimalarial deployment strategies. In addition, the chapter explores the ethical considerations

related to deploying Triple ACTs, which are likely to result in more side effects, in areas where no resistance has yet emerged. Finally, stakeholders such as individual patients, national malaria-programs, international aid organisations, pharmaceutical companies and funders are identified.

Finally, **Chapter 8** summarizes the findings of this thesis and discusses future directions.

PART I

Epidemiology of antimalarial resistant malaria

Determinants of dihydroartemisinin-piperaquine treatment failure in *Plasmodium falciparum* malaria in Cambodia, Thailand, and Vietnam: a prospective clinical, pharmacological, and genetic study.

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Lancet Infectious Diseases. September 2019; 19(9): 952-961.
DOI: 10.1016/S1473-3099(19)30391-3

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SUMMARY

Background

The emergence and spread of resistance in *Plasmodium falciparum* malaria to artemisinin combination therapies in the Greater Mekong subregion poses a major threat to malaria control and elimination. The current study is part of a multi-country, open-label, randomised clinical trial (TRACII, 2015–18) evaluating the efficacy, safety, and tolerability of Triple artemisinin combination therapies. A very high rate of treatment failure after treatment with dihydroartemisinin-piperaquine was observed in Thailand, Cambodia, and Vietnam. The immediate public health importance of our findings prompted us to report the efficacy data on dihydroartemisinin-piperaquine and its determinants ahead of the results of the overall trial, which will be published later this year.

Methods

Patients aged between 2 and 65 years presenting with uncomplicated *P. falciparum* or mixed species malaria at seven sites in Thailand, Cambodia, and Vietnam were randomly assigned to receive dihydroartemisinin-piperaquine with or without mefloquine, as part of the TRACII trial. The primary outcome was the PCR-corrected efficacy at day 42. Next-generation sequencing was used to assess the prevalence of molecular markers associated with artemisinin resistance (*kelch13* mutations, in particular Cys580Tyr) and piperaquine resistance (*plasmepsin-2* and *plasmepsin-3* amplifications and *crt* mutations).

Findings

Between Sept 28, 2015, and Jan 18, 2018, 539 patients with acute *P. falciparum* malaria were screened for eligibility, 292 were enrolled, and 140 received dihydroartemisinin-piperaquine. The overall Kaplan-Meier estimate of PCR-corrected efficacy of dihydroartemisinin-piperaquine at day 42 was 50·0% (95% CI 41·1–58·3). PCR-corrected efficacies for individual sites were 12·7% (2·2–33·0) in northeastern Thailand, 38·2% (15·9–60·5) in western Cambodia, 73·4% (57·0–84·3) in Ratanakiri (northeastern Cambodia), and 47·1% (33·5–59·6) in Binh Phuoc (southwestern Vietnam). Treatment failure was associated independently with *plasmepsin2/3* amplification status and four mutations in the *crt* gene (Thr93Ser, His97Tyr, Phe145Ile, and Ile218Phe). Compared with the results of our previous TRACI trial in 2011–13, the prevalence of molecular markers of artemisinin resistance (*kelch13* Cys580Tyr mutations) and piperaquine resistance (*plasmepsin2/3* amplifications and *crt* mutations) has increased substantially in the Greater Mekong subregion in the past decade.

Interpretation

Dihydroartemisinin-piperaquine is not treating malaria effectively across the eastern Greater Mekong subregion. A highly drug-resistant *P. falciparum* co-lineage is evolving, acquiring new resistance mechanisms, and spreading. Accelerated elimination of *P. falciparum* malaria in this region is needed urgently, to prevent further spread and avoid a potential global health emergency.

Funding

UK Department for International Development, Wellcome Trust, Bill & Melinda Gates Foundation, Medical Research Council, and National Institutes of Health.

INTRODUCTION

In the 1990s, widespread multidrug resistance to *Plasmodium falciparum* malaria throughout southeast Asia led to the introduction of highly effective artemisinin combination therapies (ACTs)[45] These drugs have since contributed substantially to the sharp decline in the global malaria disease burden and saved millions of lives.[46] These gains are now threatened by the emergence and spread of artemisinin resistance in southeast Asia.[25, 33] Artemisinin resistance in *P. falciparum* has emerged and spread throughout the Greater Mekong subregion, and is characterised by slow in vivo parasite clearance resulting from reduced drug susceptibility of ring-stage parasites.[7, 26, 27] Loss of artemisinin efficacy in ACTs has facilitated the re-emergence of mefloquine resistance on the Thailand-Myanmar border and contributed to the emergence and spread of piperaquine resistance in *P. falciparum* in Cambodia and southern Vietnam.[29-31, 47]

Several non-synonymous mutations in the propeller domain of the *kelch13* gene have been associated with artemisinin resistance.[24, 25] Amplification of the *plasmepsin-2* and *plasmepsin-3* genes on chromosome 14 has been strongly associated with reduced in vitro susceptibility to piperaquine, and with reduced treatment efficacy of dihydroartemisinin-piperaquine for uncomplicated *P. falciparum* malaria. [39, 48] Genetic epidemiology studies[49, 50] have shown that a haplotype containing the *kelch13* Cys580Tyr (C580Y) mutation (referred to as KEL1 when identified by single nucleotide polymorphism haplotyping and *Pf*Pailin when identified by microsatellite haplotyping) and a haplotype containing a *plasmepsin2/3* amplification (referred to as PLA1) have merged to form a successful multidrug-resistant co-lineage that has spread throughout the eastern Greater Mekong subregion. Recently, mutations in the *crt* gene have been suggested as contributors to piperaquine resistance.[51, 52] The current study is part of a multi-country, open-label, randomised clinical trial (TRACII, 2015–18; registered with Clinicaltrials.gov, number NCT02453308) evaluating the efficacy, safety, and tolerability of Triple ACTs in areas with multidrug-resistant *P. falciparum* malaria. TRACII recruited patients in 18 regions, across eight countries. In Laos, Bangladesh, India, the Democratic Republic of the Congo, and Myanmar patients were randomly assigned to receive either artemether-lumefantrine and amodiaquine (the Triple ACT) or artemether-lumefantrine only. In Cambodia, Vietnam, Thailand, and Myanmar, patients were randomly assigned to receive either dihydroartemisinin-piperaquine and mefloquine (the Triple ACT) or dihydroartemisinin-piperaquine only. A very high rate of treatment failure (50·0% [95% CI 41·7–58·9%]) within the first 42 days after treatment with dihydroartemisinin-piperaquine was observed in 140 patients in Thailand, Cambodia,

and Vietnam. The immediate public health importance of our findings prompted us to report the efficacy data on dihydroartemisinin-piperaquine and its determinants ahead of the results of the overall trial. Additionally, we report on the evolution of molecular markers for antimalarial drug resistance in the period from the preceding study (TRACI, 2011–13) to the present study.[25]

RESEARCH IN CONTEXT

Evidence before this study

We searched PubMed for articles published before March 28, 2019, using the terms "artemisinin", "piperaquine", "resistance", and "PfCRT"', in combination with either "Cambodia", "Vietnam", or "Thailand". Of the 80 articles found, 31 articles documented the efficacy of dihydroartemisinin-piperaquine in Cambodia, Vietnam, or Thailand, or documented the prevalence of in vitro phenotypes or genetic markers of artemisinin or piperaquine resistance. Several earlier studies documented good efficacy of dihydroartemisinin-piperaquine throughout Cambodia; however, a study between 2008 and 2010 in western Cambodia found a PCR-corrected efficacy of 75·0% in Pailin and 89·3% in Pursat at day 42. More recent studies between 2012 and 2013 reported a PCR-corrected efficacy of 63·2% in Pursat and 98·4% in Ratanakiri, northeastern Cambodia, at day 63. In Binh Phuoc, southwestern Vietnam, the most recent PCR-corrected efficacy of dihydroartemisinin-piperaquine was reported in 2015 as 65·0% by day 42. A 2012–13 study in Ratanakiri, Pursat, and Preah Vihear in Cambodia found no correlation between treatment outcomes and day 7 piperaquine levels, and baseline parasite densities and patient age, but found that *kelch13* gene mutations and high piperaquine in vitro 50% inhibitory concentrations were predictors of treatment failure. Additionally, amplification in the genes encoding *plasmepsin-2* and *plasmepsin-3* has been associated with in vitro piperaquine resistance and in vivo treatment failure. Imwong and colleagues in 2017 and Amato and colleagues in 2018 independently showed that a parasite co-lineage containing a *kelch13* Cys580Tyr mutation and amplification of *plasmepsin2/3* emerged in western Cambodia and then spread across northeastern Thailand, northern and northeastern Cambodia, and southwestern Vietnam between 2007 and 2015. Recent in vitro gene editing experiments by Ross and colleagues in 2018 using the *Plasmodium falciparum* Dd2 strain and field strains from Cambodia have suggested that multiple mutations (His97Tyr, Phe145Ile, Met343Leu, and Gly353Val) in the *crt* gene also reduce piperaquine susceptibility. The *crt* Phe145Ile mutation has been associated with dihydroartemisinin-piperaquine treatment failure in a study by Agrawal and colleagues in 2017, also after adjusting for the *plasmepsin2/3* amplification status.

Added value of this study

This study shows that the efficacy of dihydroartemisinin-piperaquine in the treatment of *P. falciparum* malaria has declined substantially in western and northeastern Cambodia,

northeastern Thailand, and southwestern Vietnam. The *P. falciparum* co-lineage resistant to dihydroartemisinin-piperaquine can now be found throughout the Greater Mekong subregion, and it has acquired mutations in the *crt* gene which are independently associated with higher rates of treatment failure.

Implications of all the available evidence

This study reinforces the urgency for accelerated elimination of malaria in the Greater Mekong subregion. Dihydroartemisinin-piperaquine should no longer be used for the treatment of *P. falciparum* malaria in the eastern Greater Mekong subregion, since it provides ineffective treatment and thereby contributes to increased malaria transmission. Continued action to prevent the spread of this *P. falciparum* multidrug-resistant co-lineage to other parts of Asia and sub-Saharan Africa is urgently needed.

METHODS

Study design and participants

The study design for this prospective clinical, pharmacological, and genetic study was adapted from the WHO recommendations for surveillance of antimalarial drug efficacy. [53] The study took place at seven sites in Thailand, Cambodia, and Vietnam. Patients aged between 2 and 65 years presenting with uncomplicated *P. falciparum* or mixed species malaria, parasitaemia on microscopy but below 200 000 parasites per µL, and a tympanic temperature above 37·5°C or a history of fever in the previous 24 h were eligible for inclusion in the study. Written informed consent was obtained from all patients. Exclusion criteria were severe malaria or other severe illnesses necessitating treatment, haematocrit below 25%, allergy or contraindication to the study drugs, use of any artemisinin-containing drug in the previous 7 days or use of mefloquine in the previous 2 months, splenectomy, pregnancy, breast feeding, a QTc interval above 450 milliseconds, or a history of cardiac conduction problems.

Procedures

After clinical examination and a standardised symptom questionnaire, blood was taken for quantitation of parasitaemia, plasma piperaquine concentrations, and parasite genotyping. Patients were randomly assigned to receive dihydroartemisinin-piperaquine with or without mefloquine using sequentially numbered opaque envelopes, as part of the TRACII trial. Dihydroartemisinin-piperaquine (with or without mefloquine) was administered at baseline and hour 24 and 48 after enrolment. The target dose per day was 4 mg/kg (range 2–10) of dihydroartemisinin and 18 mg/kg (16–27) of piperaquine for adults and children who weighed at least 25 kg, and 4 mg/kg (2·5–10) of dihydroartemisinin and 24 mg/kg (20–32) of piperaquine for children who weighed less than 25 kg. The target dose of mefloquine was 8 mg/kg (5·7–11·4) per day. A single dose of primaquine (0.25 mg base/kg) was administered to all patients at hour 24.

Doses were chosen according to the weight-based WHO dosing recommendations.[54] All treatments were directly observed. Full doses were re-administered if patients vomited within 30 minutes of receiving the drugs, half doses were re-administered if patients vomited within 30–60 minutes of receiving the drugs. Patients were monitored in hospital for at least 3 days after treatment initiation and were followed up on day 7, and thereafter weekly until day 42. Each follow-up visit included a standardised symptom questionnaire and physical examination and measurement of the tympanic temperature. Patients were encouraged to return to the study centres if new symptoms appeared between follow-up visits. Recurrent *P. falciparum* malaria infections were treated with artesunate and atovaquone-proguanil for 3 days in Cambodia and Thailand,[55] and with quinine and doxycycline for 7 days in Vietnam.

Outcomes

The primary outcome for this study was the PCR-corrected efficacy by day 42. We expressed efficacy as recrudescence-free survival estimates using Kaplan-Meier analyses.

Laboratory analyses

Parasite densities were quantitated in Giemsa-stained blood smears at baseline and hour 4, 6, 8, and 12 after treatment initiation and every 6 h thereafter until two consecutive thick blood smears were negative (in 200 high-powered fields). Blood smears were repeated at each follow-up. In case of recurrent *P. falciparum* malaria infections, blood samples were obtained for parasite genotyping and drug concentration measurements. Venous plasma samples for the measurement of piperaquine concentrations were obtained at baseline and day 7. Plasma piperaquine concentrations were measured using liquid chromatography-tandem mass spectrometry as described previously with a lower limit of detection of 1·2 ng/mL.[56] DNA for next-generation sequencing of the parasite genotype was extracted from two types of samples collected at the time of admission: 20 µL dried blood spots on filter paper for targeted genotyping by amplicon sequencing (AmpSeq); and 2 mL leucocyte-depleted venous blood, filtered through cellulose column filters (cellulose powder type B, Advantec, Japan) for whole-genome sequencing (WGS). For AmpSeq, DNA from dried blood spots underwent selective whole genome amplification,[57] and selected primers were used to amplify parasite DNA at the desired loci before sequencing (Table S2). Sequence data for both genotyping methods were generated with Illumina short-read technology. WGS read counts were used to call genotypes with a standardised analysis pipeline (Pf6.0 release).[58, 59] WGS samples were genotyped at 1 043 334 quality-filtered coding single nucleotide polymorphisms, identified by the MalariaGEN *P. falciparum* Community Project V6.0 pipeline. Genotypes were called with a coverage of at least five reads, and alleles were disregarded when represented by fewer than two reads (or 5% of reads when coverage was >50). To genotype *kelch13*, we scanned sequencing reads from AmpSeq or WGS that aligned to amino acids distal to position 350 in this gene, identifying any nonsynonymous variants.

If no such variants were found, the sample was considered wild type. If at least 50% of positions had insufficient coverage the genotype was considered undetermined. A mutation was labelled as heterozygous if a proportion of reads for the wild-type allele were also found at the mutation site. *Plasmepsin2/3* status was assessed by scanning sequencing reads from AmpSeq or WGS for the characteristic duplication breakpoint.[50] To optimise completeness of genotypic data, we combined multiple methods when applicable. *Kelch13* was genotyped by WGS, supplemented by AmpSeq calls when WGS calls were undetermined. Amplifications of *plasmepsin2/3* and *mdr1* (a marker of mefloquine sensitivity) were quantified using Taqman real-time PCR (rtPCR) following previously described protocols, and supplemented by AmpSeq when rtPCR could not determine *plasmepsin2/3* status.[39, 60]. *crt* genotypes were assessed from WGS, except for low-coverage positions 218 and 220, which were assessed from AmpSeq data; remaining missing genotypes were supplemented by PCR assays.[52] All genotypes for TRACI samples were derived from WGS data. Recurrent infections were classified as recrudescences if all *msp1*, *msp2*, and *glurp* alleles matched those present at baseline as described previously.[61]

Statistical analysis

Estimates of recrudescence-free survival on day 28 and 42 after treatment initiation were obtained using Kaplan-Meier analyses. Contributors to treatment failure were assessed using Cox regression (unadjusted and adjusted), using previously described predictors of treatment failure. Patients were censored in the Kaplan-Meier analysis at the day of the following events: loss to follow-up, discontinuation of study drug, withdrawal of consent, discontinuation from the study due to non-compliance to the study protocol, *Plasmodium vivax* infection, PCR-confirmed *P. falciparum* reinfection, or PCR-undetermined recurrent *P. falciparum* infection. Parasite clearance parameters were estimated using the WorldWide Antimalarial Resistance Network parasite clearance estimator.[62] For comparisons between regions and other subgroups we used Wilcoxon rank-sum tests or Kruskal-Wallis tests (continuous data) or Fisher's exact test (binomial data). All analyses were done with Stata (version 15.1, Stata Corporation, USA).

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between Sept 28, 2015, and Jan 18, 2018, 539 patients with acute *P. falciparum* malaria were screened for eligibility, 292 were enrolled, and 140 received dihydroartemisinin-piperaquine (figure S1). These 140 patients were recruited in two sites in northeastern Thailand, Phusing (n=15) and Khun Han (n=four); three sites in Cambodia, Pailin (n=nine),

Pursat (n=eight), and Ratanakiri (n=44); and two sites in southwestern Vietnam, both in Binh Phuoc province (n=60). Pursat and Pailin are referred to as western Cambodia and Phusing and Khun Han are referred to as northeastern Thailand.

Two patients deteriorated to develop severe malaria. One 45-year-old woman (infected by a parasite with *kelch13* C580Y and *crt* His97Tyr [H97Y] mutations but with no *plasmepsin2/3* amplification) was started on intravenous artesunate 18 h after enrolment, resulting in a rapid recovery. One 49-year-old man (*kelch13* mutation unknown, *plasmepsin2/3* non-amplified, Ile218Phe [I218F] *crt* mutation) developed a rash after the first dose of dihydroartemisinin-piperaquine, which was interpreted as a drug allergy. As the patient was also deteriorating clinically (respiratory distress), he was started on intravenous quinine and ceftriaxone 12 h after enrolment. Despite an initial clinical recovery and a reduction of parasitaemia, the patient developed fever, hypoxia, and hypotension on day 4. The chest X-ray showed bilateral infiltration. Despite ventilatory support, the patient died the same day. The cause of death was recorded as acute respiratory distress syndrome. No autopsy was done. Another patient had a vasovagal collapse related to a venipuncture. The treating physician changed the antimalarial treatment to intravenous artesunate. Four other patients were censored from the Kaplan-Meier analysis: one because of prolongation of the QTc interval, two because of non-adherence to the study protocol, and one because of loss to follow-up. 118 (84%) of 140 patients treated with dihydroartemisinin-piperaquine were men (table 1). The median age of all patients was 27·0 years (IQR 18·5–37·6). Parasite counts were similar across sites; presence of gametocytaemia, at baseline, assessed by microscopy, ranged between 11·8% in northeastern Thailand and 41·2% in western Cambodia. Five (4%) of 140 patients presented with a *P. vivax* co-infection. Piperaquine was detected in baseline blood samples in 30 (23%) of 131 patients. Detectable piperaquine at baseline was associated with admission gametocytaemia (crude odds ratio [OR] 6·05, 95% CI 2·36–15·54; p=0·0001). Parasites were detected by microscopy in 94 (68%) of 138 of patients 72 h after treatment initiation, but before day 7 microscopic parasitaemia cleared in all patients. Parasite clearance half-life ($PC_{1/2}$) could be assessed in 133 patients: 109 (82%) of 133 had a $PC_{1/2}$ of more than 5·5 h (table 2).[63] Genotyping of the *kelch13* gene found 124 (91%) of 137 samples to contain either C580Y mutations or mixed C580Y and wild-type mutations (table 2). The KEL1 haplotype was found in 104 (88%) of 118 samples. *Plasmepsin2/3*amplification was detected in 103 (74%) of 139 samples, while *mdr1* amplification was not observed. One of the following six *crt* mutations were observed in 92 (74%) of 124 patients: Thr93Ser (T93S), H97Y, Phe145Ile (F145I), I218F, Met343Ile, and Gly353Val (G353V). The total follow-up time for the 140 patients was 4270 days (median follow-up was 35 days). The overall Kaplan-Meier estimate of PCR-corrected efficacy of dihydroartemisinin-piperaquine at day 42 was 50·0% (95% CI 41·1–58·3; table 3). PCR-corrected efficacies at day 42 for individual sites were 12·7% (2·2–33·0) in northeastern Thailand, 38·2% (15·9–60·5) in western Cambodia, 73·4% (57·0–84·3) in Ratanakiri (northeastern Cambodia), and

47·1% (33·5–59·6) in Binh Phuoc (southwestern Vietnam) (table 3; figure 1). Six (9%) of 71 recurrent infections were classified as reinfections. PCR correction was not possible for two recurrent infections. One patient presented with a *P. vivax* infection at day 35. Recrudescent *P. falciparum* infections were detected between day 9 and 42, with a median of 21 days. Gametocytes were detected by microscopy in only one of 65 recrudescent infections. There was no difference in the time to recrudescence between study sites (p=0·127; table 3).

Table 1: Baseline characteristics of the study population

	All sites (n=140)	**Northeastern Thailand (n=19)**	**Cambodia (n=61)**		**Vietnam, Binh Phuoc (n=60)**
			Western Cambodia (n=17)	Ratanakiri (n=44)	
Male	118 (84%)	19 (100%)	17 (100%)	31 (70%)	51 (85%)
Female	22 (16%)	0	0	13 (30%)	9 (15%)
Age (years)	27·0 (18·5-37·6)	39·1 (29·9-49·2)	29·0 (27·0-38·0)	23 (16·0-35·0)	22 (16·9-33·2)
Patients with fever at baseline >37·5°C	83 (59%)	15 (79%)	5 (29%)	29 (66%)	34 (57%)
Body temperature at baseline (°C)	37·9 (1·1)	38·7 (1·2)	37·3 (1·0)	37·9 (0·9)	37·8 (1·1)
Weight (kg)	52·6 (13·1)	64·2 (9·4)	59·1 (5·7)	46·6 (11·6)	51·2 (13·7)
Haematocrit (%)	41·0 (4·7)	43·5 (5·5)	40·2 (3·4)	41·8 (4·8)	40·0 (4·6)
Geometric mean parasite count per µL (range)	25732 (160-214223)	21031 (3,472-214223)	16125 (384-152604)	17345 (160-117562)	41814 (5024-198950)
Baseline gametocytaemia	27/137 (20%)	2/17 (12%)	7/17 (41%)	7/43 (16%)	11/60 (18%)
Geometric mean gametocyte count per µL (range)	59 (16-5120)	406 (224-736)	95 (16-5120)	51 (16-432)	33 (16-320)
Presence of Plasmodium vivax co-infection at baseline	5 (4%)	1 (5%)	1 (6%)	0	3 (5%)
Baseline detectable piperaquine	30/131 (23%)	2/18 (11%)	14/17 (82%)	6/44 (14%)	8/52 (15%)
Baseline piperaquine plasma concentration (ng/ml)	9·7 (3·5-16·4)	10·7 (9·7-11·6)	6·7 (3·5-12·3)	3·4 (2·1-4·9)	20·3 (11·4-56·0)

Data are n (%), median (IQR), or mean (SD) unless otherwise specified. The baseline parasitaemia of one patient was above the screening cut-off (214 223 parasites per µL) as the parasitaemia rose between screening and baseline.

Table 2: Parasite clearance parameters and molecular markers of antimalarial drug resistance

	All sites (n=140)	Northeastern Thailand (n=19)	Cambodia (n=61)		Vietnam, Binh Phuoc (n=60)
			Western Cambodia (n=17)	Ratanakiri (n=44)	
$PC_{1/2}$ (hours)	7·0 (1·9)	8·1 (1·5)	5·9 (1·7)	7·2 (2·0)	6·8 (1·8)
$PC_{1/2}$ >5.5 hours	109/133 (82%) (74·4-88·1)	18/18 (100%) (81·5-100·0)	10/17 (59%) (32·9-81·6)	36/42 (86%) (71·5-94·6)	45/56 (80%) (67·6-89·8)
Positive bloodsmear for asexual parasitemia 72 hours after treatment initiation (%) (95% CI)	91/134 (68%) (59·3-75·7)	16/18 (89%) (65·3-98·6)	11/17 (65%) (38·3-85·8)	22/43 (51%) (35·5-66·7)	42/56 (75%) (61·6-85·6)
Kelch13 Cys580Tyr mutations or mixed infections containing Cys580Tyr mutations (%) (95% CI)	124/137 (91%) (84·3-94·9)	18/18 (100%) (81·5-100·0)	15/16 (93%) (69·8-99·8)	36/43 (84%) (69·3-93·2)	55/60 (92%) (81·6-97·2)
KEL1 Cys580Tyr mutations or mixed infections containing KEL1 Cys580Tyr mutations (%) (95% CI)	104/118 (88%) (80·9-93·4)	14/14 (100%) (76·8-100·0)	14/16 (88%) (61·7-98·4)	32/38 (84%) (68·7-94·0)	44/50 (88%) (75·7-95·5)
Plasmepsin2/3 amplification (%) (95% CI)	103/139 (74%) (66·0-81·2)	15/19 (79%) (54·4-94·0)	11/17 (65%) (38·3-85·8)	31/43 (72%) (56·3-84·7)	46/60 (77%) (64·0-86·6)
MDR1 amplification (%) (95% CI)	0/139 (0%) (0-2·6)	0/19 (0%) (0-17·6)	0/17 (0%) (0-19·5)	0/43 (0%) (0-8·2)	0/60 (0%) (0-6·0)
crt Thr93Ser mutation (%) (95% CI)	31/124 (25%) (17·7-33·6)	0/18 (0%) (0-18·5)	1/13 (8%) (0·2-36·0)	6/39 (15%) (5·9-30·5)	24/54 (44%) (30·9-58·6)
crt His97Tyr mutation (%) (95% CI)	15/124 (12%) (6·9-19·2)	4/18 (22%) (6·4-47·6)	6/13 (46%) (19·2-74·9)	5/39 (13%) (4·3-27·4)	0/54 (0%) (0-6·6)
crt Phe145Ile mutation (%) (95% CI)	16/124 (13%) (7·6-20·1)	2/18 (11%) (1·4-34·7)	0/13 (0%) (0-24·7)	4/39 (10%) (2·9-24·2)	10/54 (19%) (9·3-31·4)
crt Ile218Phe mutation (%) (95% CI)	25/124 (20%) (13·5-28·3)	8/18 (44%) (21·5-69·2)	1/13 (8%) (0·2-36·0)	4/39 (10%) (2·9-24·2)	12/54 (22%) (12·0-35·6)
crt Met343Ile mutation (%) (95% CI)	2/124 (2%) (0·2-5·7)	0/18 (0%) (0-18·5)	1/13 (8%) (0·2-36·0)	0/39 (0%) (0-9·0)	0/54 (0%) (0-6·6)
crt Gly353Val mutation (%) (95% CI)	3/124 (3%) (0·5-6·9)	2/18 (11%) (1·4-34·7)	1/13 (8%) (0·2-36·0)	0/39 (0%) (0-9·0)	0/54 (0%) (0-6·6)

Data are mean (SD) or median (IQR) unless otherwise specified. PC1/2=parasite clearance half-life.

Table 3: Clinical outcomes related to the efficacy of dihydroartemisinin-piperaquine in the study population

	All sites (n=140)	**Northeastern Thailand (n=19)**	**Cambodia (n=61)**		**Vietnam, Binh Phuoc (n=60)**	**p-value**
			Western Cambodia (n=17)	Ratanakiri (n=44)		
Kaplan-Meier estimates of PCR corrected efficacy on day 42 (95% CI)	50·0% (41·1-58·3)	12·7% (2·2-33·0)	38·2% (15·9-60·5)	73·4% (57·0-84·3)	47·1% (33·5-59·6)	0·0001
Kaplan-Meier estimates of PCR corrected efficacy on day 28 (95% CI)	61·3% (52·5- 69·1)	25·4% (8·2-47·2)	63·7% (36·3-81·9)	83·5% (68·4-91·8)	54·7% (40·7- 66·7)	0·0009
Patients with fever >37·5 ˚C on day of recrudescent infection (%) (95% CI)	7/65 (11%) (4·4-20·9)	4/15 (27%) (7·8-55·1)	0/10 (0%) (0-30·8)	1/11 (9%) (0·2-41·3)	2/29 (7%) (0·8-22·8)	0·164
Median days from treatment initiation to recrudescent *P. falciparum* infection (range)	21 (9-42)	20 (12-35)	28 (20-35)	24 (13-38)	21 (9-42)	0·127
Patients with gametocytes visible through microscopy on day of recrudescent infection (%) (95% CI)	1/65 (2%) (0·0-8·3)	1/15 (7%) (0·2-31·9)	0/10 (0%) (0-30·8)	0/11 (0%) (0-21·8)	0/29 (0%) (0-11·9)	0·343
Mean parasite count on day of recrudescence (range)	366 (16-31651)	224 (16-30898)	155 (16-4736)	1,023 (368-7159)	444 (16-31651)	0·092
Patients with plasma piperaquine concentration <30 ng/ml at day 7 (%) (95% CI)	52/123 (42%) (33·4-51·5)	7/18 (39%) (17·3-64·3)	6/16 (38%) (15·2-64·6)	23/43 (54%) (37·7-68·8)	16/46 (35%) (21·4-50·2)	0·328
Plasma piperaquine concentration at day 7 (ng/mL)	33·5 (22·8-46·2)	33·0 (23·5-41·8)	34·9 (21·7-44·2)	29·0 (22·0-40·5)	37·7 (25·2-53·8)	0·258
Plasma piperaquine concentration at day of recrudescence (ng/mL)	16·3 (9·4-33·0)	21·3 (15·8-41·9)	13·5 (8·1-30·9)	7·6 (6·4-9·0)	22·1 (14·0-43·5)	0·001

Data are median (IQR) unless otherwise specified. p values are for differences between all four sites and were calculated using either Fisher's exact test (binomial data) or Kruskal-Wallis test (continuous data).

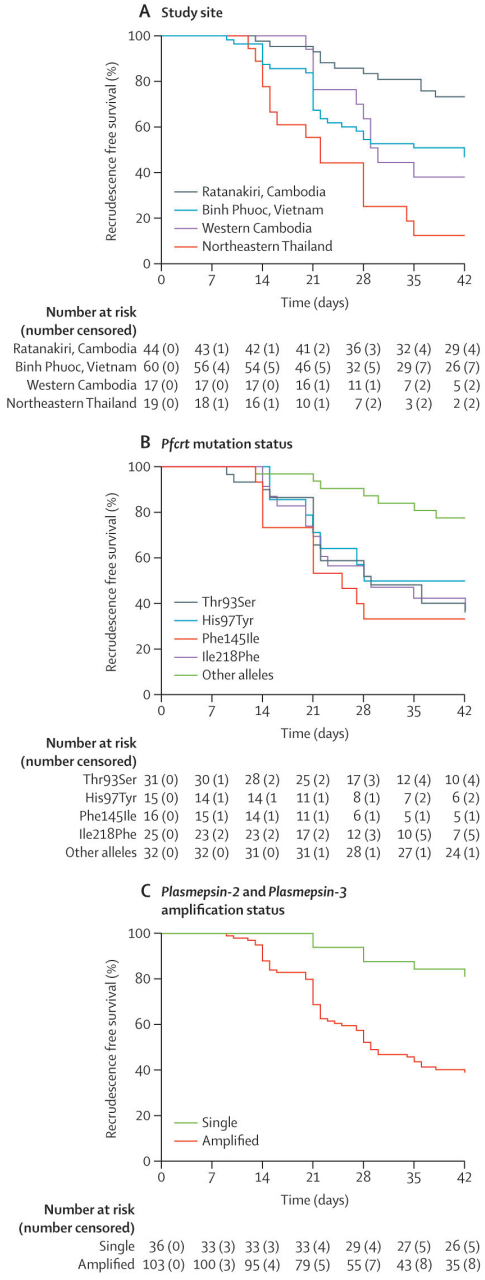


Figure 1: Kaplan-Meier survival curves of PCR-corrected efficacy of dihydroartemisin-piperaquine by (A) study site, (B) *crt* mutation status, and (C) *plasmepsin-2 and plasmepsin-3* amplification status. Other *crt* alleles indicate parasites carrying no mutations at positions 93, 97, 145, 218, 343, and 353 of the *crt* gene. Infections caused by parasites with a et343Ile or Gly353Val *crt* mutation were too scarce to be included in survival curves by *crt* mutation status.

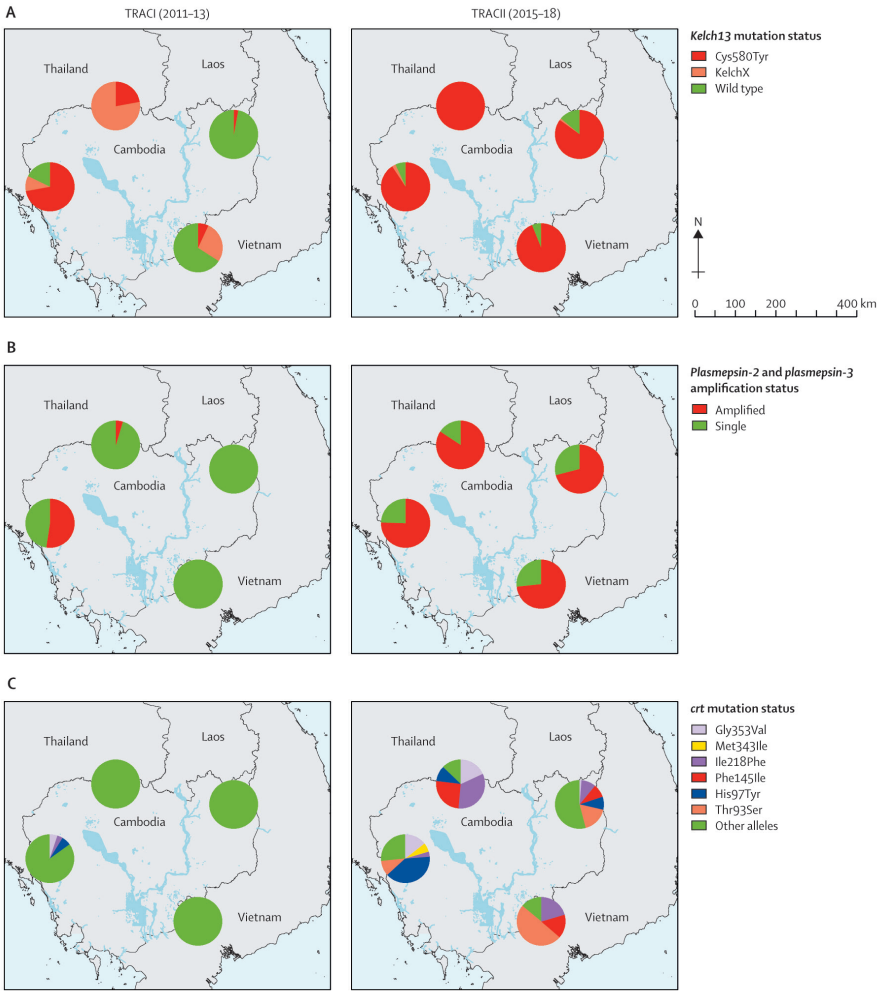


Figure 2: *kelch13* mutation status, *plasmepsin2/3* amplification status, and crt mutation status by site and country in the TRACI and TRACII trials (A) KelchX mutation status indicates parasites with a *kelch13* mutation other than Cys580Tyr. (B) Single amplification status indicates parasites without a *plasmepsin-2* and *plasmepsin*-3 amplification. (C) Other crt alleles indicate parasites carrying no mutations at positions 93, 97, 145, 218, 343, and 353 of the crt gene.

In the unadjusted Cox regression analysis, site of recruitment, *plasmepsin2/3* amplification status, $PC_{1/2}$, and the presence of the *crt* mutations T93S, H97Y, Phe145I and I218F were all associated with treatment failure (figure 1; table S1). Baseline parasitaemias and day 7 plasma piperaquine concentrations (as continuous variable or binary variable with a cut-off of 30 ng/mL)[64] were not associated with subsequent treatment failure (figure S2). We constructed a multivariable model using three genetic markers: *kelch13* mutation status, *crt* mutation status, and *plasmepsin2/3* amplification

status. In this model, *plasmepsin2/3* amplification status and the four *crt* mutations were independently associated with treatment failure. When the site of recruitment (a potential confounder) was added to the model, two *crt* mutations (T93S and F145I) and *plasmepsin2/3* amplification status remained independently associated with treatment failure. *Kelch13* mutation status was not found to be associated with treatment failure both in the unadjusted analysis (HR 3·863 [95% CI 0·943–15·816]; p=0·060) and adjusted analysis (HR 0·632 [95% CI 0·125–3·208]; p=0·580), likely related to the high overall proportion of infections carrying the C580Y *kelch13* mutation. A subgroup analysis of infections carrying both *kelch13* C580Y mutations and *plasmepsin2/3* amplifications showed that *crt* mutations at position 93, 145, and 218 were all associated with reduced clinical efficacy of dihydroartemisinin-piperaquine, suggesting a contribution in addition to the effect of the other two markers (Figure S3). Within the subgroup of *plasmepsin2/3* amplified parasites, *crt*mutations were not associated with higher plasma piperaquine concentrations at recrudescence, or with the interval to recrudescence (Figure S4) However, in patients with measurable plasma piperaquine at baseline, the *crt* F145I mutation was associated with higher piperaquine concentrations at baseline (p=0·034) and at day 7 (p=0·023) (figure S5). To report the prevalence of molecular markers of resistance, all samples obtained at the study sites during the clinical trial were used, irrespective of the treatment the patient received after enrolment (Figure S1). We compared the prevalence of molecular markers of resistance in TRACII to the results of our earlier TRACI multicentre study in 2011–13.

The marked diversity of *kelch13* mutations in the parasite population previously observed across this region,[25, 65] is now reduced to a predominance of *kelch13* C580Y mutations, present in 369 (91%) of all 404 infections (figure 2; figure S6). In TRACI the co-lineage combining the *kelch13* C580Y mutation and the *plasmepsin2/3* amplification was only found in western Cambodia in 72 (43%) of 166 infections, whereas in the current study it was found at all study sites in 296 (73%) of 404 infections. We identified six *crt* mutations that were present in low frequencies in TRACI (prevalence <5·0% in 2011–13). These mutations increased in prevalence between the two studies, appeared to be mutually exclusive, and identified specific haplotypes (table S3; figure S6). Missingness of genotyping results were mostly caused by low quantities of parasite DNA in the samples or the presence of heterozygote infections. In the current study, 272 (73%) of 375 parasites carried *crt* mutations at positions 93, 97, 145, 218, 343, or 353. In TRACI only three of these mutations (at positions 97, 218, or 353) were found in 20 (5%) of 368 infections and were present only in western Cambodia. 252 (96%) of the 263 monoclonal infections with these *crt* mutations in the current TRACII study also carried *kelch13* C580Y mutations and 207 (79%) also carried a *plasmepsin2/3* amplification (figure S7). Thus, in TRACII 201 (76%) of 263 *crt*-mutated parasites from monoclonal infections carried both a *kelch13* C580Y mutation and had *plasmepsin2/3* amplification. By comparison, during TRACI, 20 (95%) of 21 of *crt*-mutated parasites were also *kelch13* C580Y mutated, and 13 (62%) were *plasmepsin2/3* amplified.

DISCUSSION

The rapid spread of a co-lineage of multidrug-resistant *P. falciparum* across the Greater Mekong subregion has had disastrous consequences for the therapeutic efficacy of dihydroartemisinin-piperaquine in the treatment of uncomplicated *P. falciparum* malaria. Alarmingly high rates of treatment failure occurred in Cambodia, Vietnam, and Thailand, which will have contributed to increased transmission of *P. falciparum* during the study period. Given its poor efficacy, dihydroartemisinin-piperaquine should no longer be used for the treatment of *P. falciparum* malaria in the eastern Greater Mekong subregion. Fortunately, artesunate-mefloquine is known to still be highly effective in these areas,[66] and Cambodia has now changed its first-line treatment to this ACT. Recent experience[67] from the Thai-Myanmar border shows that mefloquine resistance can be selected rapidly in the presence of artemisinin resistance, which would likely limit the duration of its efficacy. Worryingly, a recent study[68] in Preah Vihear (northern Cambodia) showed that the proportion of parasites carrying all three molecular markers of resistance for artemisinins (*kelch13* mutations), piperaquine (*plasmepsin2/3* amplifications), and mefloquine (*mdr1* amplifications) increased from six (7%) of 85 parasites to ten (30%) of 33, after first-line treatment was changed to artesunate-mefloquine in February, 2016. By contrast, our study did not find any parasites carrying both amplifications of *plasmepsin2/3* and *mdr1*. The most recent ACT artesunate-pyronaridine had suboptimal efficacy in curing uncomplicated falciparum malaria in western Cambodia in 2007–08 and 2014–15,[69, 70] but more recent assessments in eastern Cambodia showed adequate cure rates.[71] Artesunate-pyronaridine has now been proposed to replace dihydroartemisinin-piperaquine in northeastern Thailand and southern Vietnam. Patients often presented with gametocytaemia and detectable plasma piperaquine at the moment of enrolment, which suggests that these patients had a recrudescent rather than a primary infection. An association between gametocytaemia and recrudescent infections was also observed when mefloquine resistance emerged in the 1990s on the western border of Thailand,[72] and is likely to promote the spread of drug resistance. In our study, gametocytes were detected by microscopy in only one of 65 patients at recrudescence, which might be explained by the early detection at low parasitaemias in the study setting, before late-stage gametocytes become visible in peripheral blood. Additionally, the use of gametocytocidal primaquine at baseline will have shortened gametocyte carriage. Over-representation of potential recrudescent infections on enrolment could have resulted in an underestimation of the efficacy of dihydroartemisinin-piperaquine in primary infections. However, the combination of high treatment coverage with the first-line treatment dihydroartemisinin-piperaquine, and high treatment failure in the same area causes an increasing number of infections to be recrudescences, which will contribute importantly to the malaria burden. Low malaria transmission in the study area precluded a larger sample size. The sample size in western Cambodia is smaller because the antimalarial drug in one study group was changed from dihydroartemisinin-piperaquine to artesunate-mefloquine. The results

reported from geographically diverse sites in the study area are representative for the eastern Greater Mekong subregion. The multidrug-resistant parasite co-lineage that originated in western Cambodia over 10 years ago has spread and evolved.[25] Day 7 piperaquine concentrations were not predictive of treatment failure in this study, whereas they are in piperaquine-sensitive infections.[64] This finding strongly suggests that in the current study parasite resistance rather than reduced exposure to piperaquine was the main determinant of treatment failure. The patient showing very early treatment failure apparent at day 9 after treatment was infected with parasites containing a *kelch13* C580Y mutation, *plasmepsin2/3* amplification, and a *crt*T93S mutation. However, the plasma piperaquine concentration at the time of recrudescense was low with 15·1 ng/mL, indicating possible underexposure to piperaquine that could have contributed to this very early treatment failure. In addition to the previously defined marker of piperaquine resistance, amplification of *plasmepsin2/3*, we found that mutations in the *crt* gene were also associated with treatment failure after dihydroartemisinin-piperaquine. Most *crt* mutations described in this study (H97Y, F145I, and G353V) have also been identified in recent gene-editing experiments by Ross and colleagues[52] to confer piperaquine resistance in vitro. They showed that all mutations except the *crt* Met343Leu (M343L) mutation resulted in a fitness loss. We did not observe clear selection of the *crt* M343L mutation, which can be related to the difference in determinants governing parasite fitness in the field setting compared with determinants obtainable in the laboratory, including the increased transmissibility of ineffectively treated infections. The findings in our study, in combination with the findings of Ross and colleagues[52] support the hypothesis that the *crt* mutations affect parasite sensitivity to piperaquine. Most *crt* mutations occurred in parasites carrying the *kelch13* C580Y mutation and *plasmepsin2/3* amplification. These *crt* mutations are distinct from the known *crt* variants conferring chloroquine resistance. A major concern is that artemisinin and partner drug resistance will continue to evolve, producing parasite strains more capable of surviving treatment, which can subsequently spread across a wider geographical area. Resistance to chloroquine and sulphadoxine-pyrimethamine has emerged in the Greater Mekong subregion in the past, and subsequently migrated from southeast Asia to the Indian subcontinent and sub-Saharan Africa, likely contributing to millions of deaths from severe malaria in African children.[20, 73] In the absence of new drug classes to replace current first-line therapies, the use of existing drugs in the form of Triple ACTs, in which an artemisinin component is combined with two partner drugs, could be a viable alternative. The results of the TRACII study are expected to be published later this year. The preliminary results indicated dihydroartemisinin-piperaquine and mefloquine to be fully efficacious in Thailand, Cambodia, and Vietnam. Nevertheless, accelerated elimination of all *P. falciparum* in the Greater Mekong subregion is needed urgently.[74] Expansion and further spread of very difficult to treat, highly resistant *P. falciparum* would cause a regional and potentially global health emergency.

ACKNOWLEDGEMENTS

This study was supported by the UK Department for International Development (201900) and the Intramural Research Program of the National Institute of Allergy and Infectious Diseases (National Institutes of Health). The Mahidol-University Oxford Tropical Medicine Research Programme is funded by the Wellcome Trust (106698/B/14/Z). Genotyping for this study was funded by the Wellcome Trust (098051 and 206194), Bill & Melinda Gates Foundation (OPP11188166), and the Medical Research Council (G0600718). We thank all patients who took part in these studies. We are especially grateful to Brian Angus, Ric Price and Christian Holm Hansen for their important guidance in their role as Data and Safety Monitoring Committee. We thank Zoë Doran, Jaruwan Tubprasert, Prayoon Yuentrakul, and Salwaluk Panapipat for site monitoring and protocol training; Thatsanun Ngernseng and Sasinun Sawaithapan for data management; Cholrawee Promnarate, Benjamas Intharabut, Thanaporn Champathai, Nitima Chanarat, and Thanawat Assawariyathipat for laboratory support; Nisarat Sorotpinya for logistical support; Patrick Hannay for financial administrative support; and Namfon Buasiri and Napaporn Piwon for patient recruitment. Genome sequencing was done by the Wellcome Sanger Institute and the Department of Molecular Tropical Medicine and Genetics of Mahidol University; we thank the staff of the Wellcome Sanger Institute Sample Logistics, Sequencing, and Informatics facilities for their contribution; Eleanor Drury, Mihir Kekre, and Katie Love for sample processing, and Victoria Simpson and the MalariaGEN Resource Centre for study coordination.

CONTRIBUTORS

RWvdP, CA, MD, DL, RH, KC, EAA, MM, NW, PYC, RJM, RMF, JT, DPK, SP, TTH, NPJD, OM, NJW, and AMD designed the study. MI, NHC, NTT-N, NTH, NVT, PJ, BH, KC, CS, RR, WK, RT, TJP, SY, SSu, SSr, SM, SO, SY, and MW recruited the study participants, collected samples, or took part in laboratory work at the study site or in the central laboratories. RWvdP, RA, RDP, SG, CGJ, and WLH generated, curated, analysed or interpreted the genetic data. RWvdP, OM, NJW, and AMD wrote the manuscript.

SUPPLEMENTARY MATERIAL

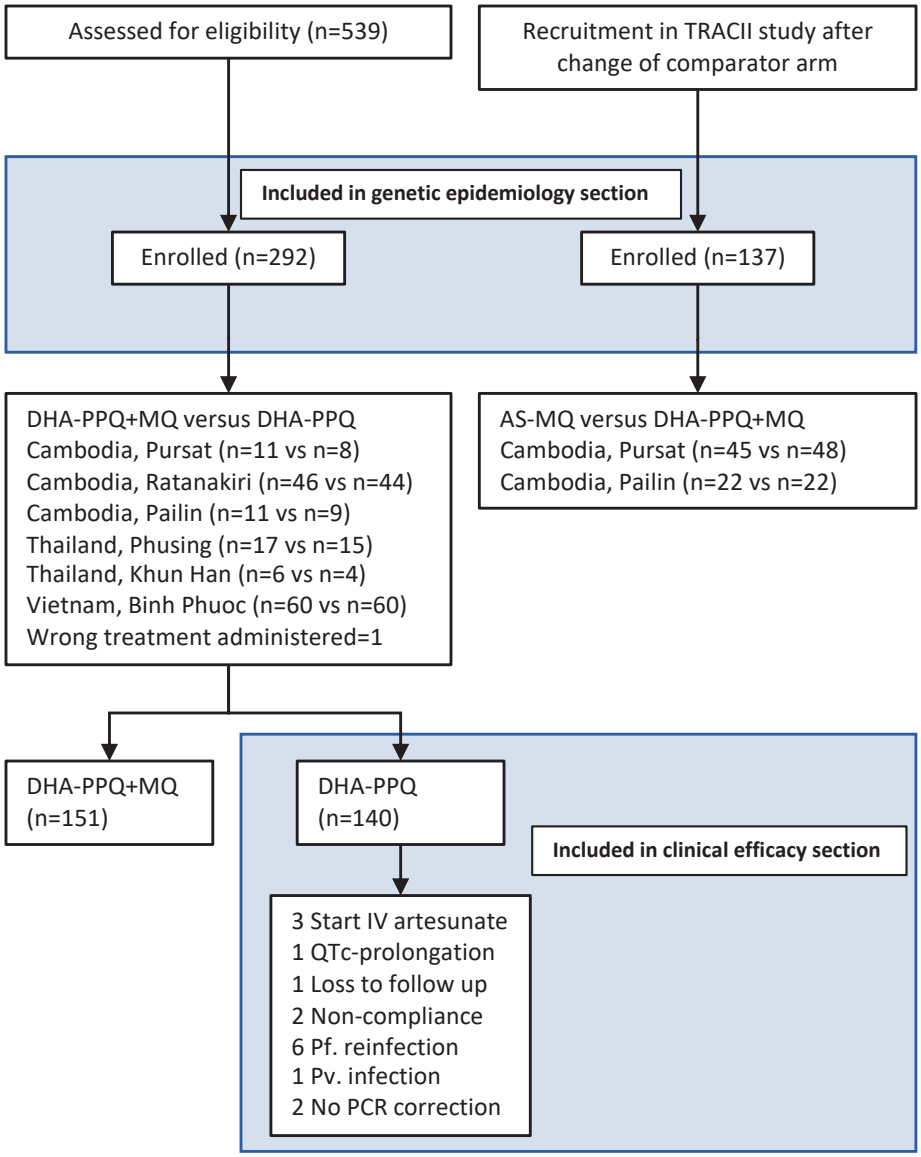
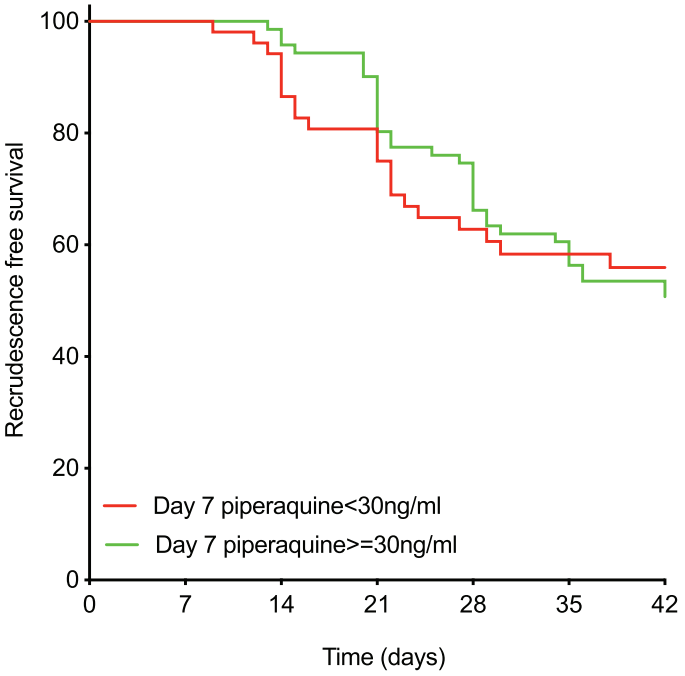


Figure S1. Simplified flow diagram of the TRACII study. The two panels indicate which patients have been included in the different sections that are presented in this manuscript.



Number at risk							
Day 7 piperaquine<30ng/ml	53	52	49	42	30	26	23
Day 7 piperaquine>=30ng/ml	72	71	70	64	52	43	38

Figure S2. Kaplan-Meier survival curves describing PCR corrected efficacy of dihydroartemisinin-piperaquine by day 7 piperaquine concentrations.

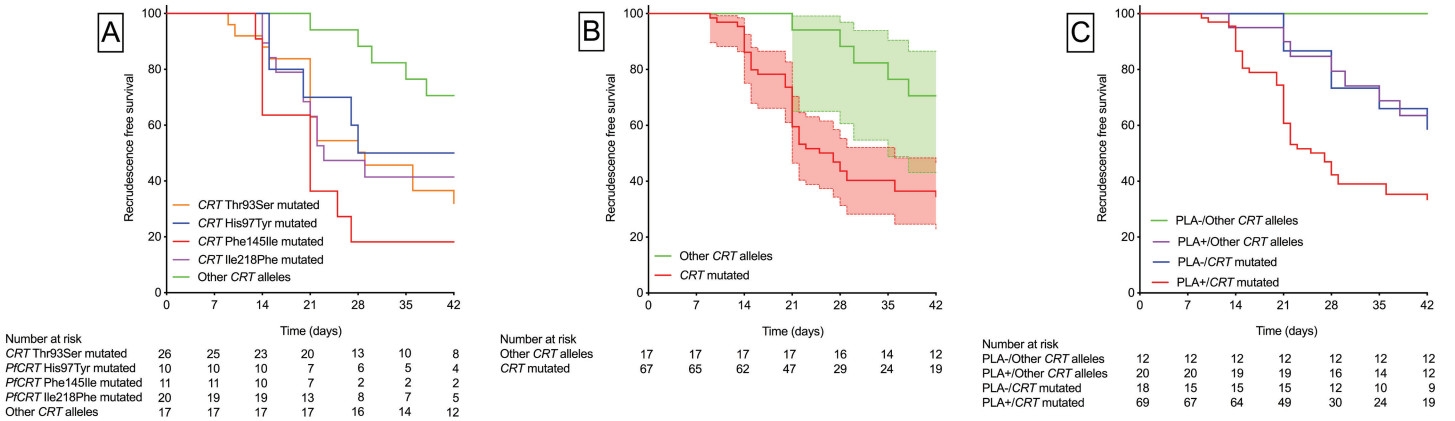


Figure S3. Kaplan-Meier survival curves describing PCR corrected efficacy of dihydroartemisin-piperaquine by *PfCRT* mutation status (for the subgroup of parasites bearing a *PfKelch13* C580Y mutation and *plasmepsin2/3* amplification) (Panel A and B) and a combination of *plasmepsin2/3* amplification and *PfCRT* mutation status (Panel C). Panel A/B/C: 'Other *PfCRT* alleles' indicates parasites carrying no mutations at position 93, 97 and 145, 218, 343 and 353 of the *PfCRT* gene. Panel B: Shaded areas indicate 95 % confidence intervals. Panel C: **PLA+** indicates parasites carrying a *plasmepsin2/3* amplification. **PLA-** indicates parasites carrying a single copy of *plasmepsin2/3*. Panel B/C: Dotted lines indicated 95 % confidence intervals. These figures are discussed in section 'Determinants of treatment failure' of the manuscript.

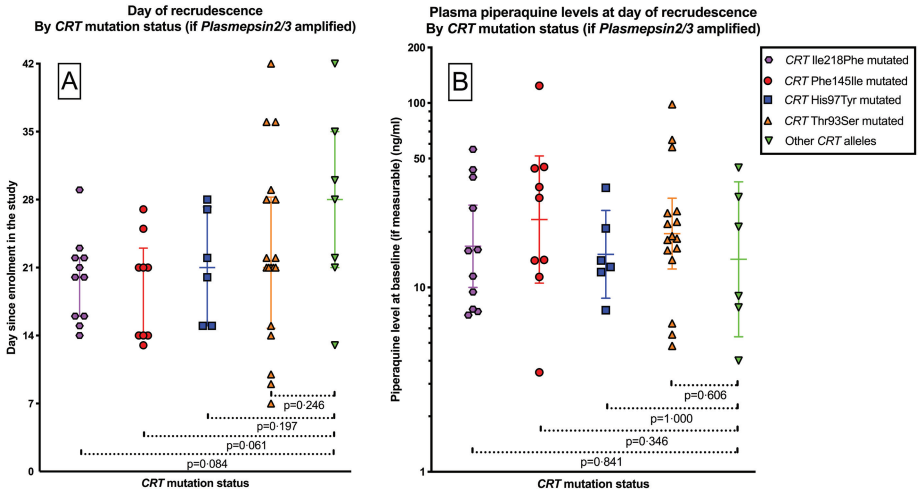


Figure S4. Day of recrudescence (Panel A) and piperaquine levels at day of recrudescence (Panel B) by crt mutation status for subgroup of parasites with a Plasmepsin2/3 amplification. Panel A: Bars indicate median and interquartile ranges. Panel B: Bars indicate geometric mean and 95% confidence intervals.

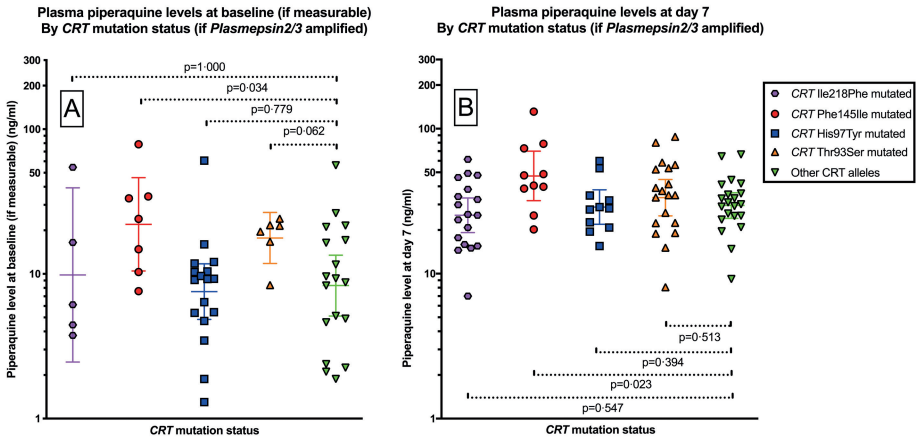


Figure S5. Baseline piperaquine levels (Panel A) and piperaquine levels at day 7 (Panel B) by crt mutation status for subgroup of parasites with a Plasmepsin2/3 amplification. Bars indicate geometric mean and 95% confidence intervals.

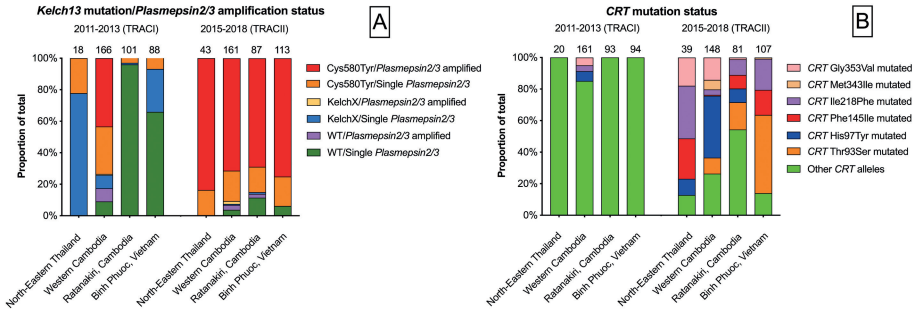


Figure S6. Frequencies of combinations for genetic markers relevant to resistance to artemisinins and piperaquine at the four sites/regions in TRACI (2011-2013) and TRACII (2015-2018). Panel A: **C580Y, KelchX and WT** indicate a *PfKelch13* C580Y mutation, a *PfKelch13* other than C580Y and *PfKelch13* wild-type, respectively. '***Plasmepsin2/3* amplified'** and **'Single *Plasmepsin2/3*'** indicate parasites with or without a *plasmepsin2/3* amplification, respectively. Panel B: Prevalence of *PfCRT* **T93S, H97Y, F145I, I218F, M343I and G353V** mutations. '**Other *PfCRT* alleles'** indicates parasites carrying no mutations at position 93, 97 and 145, 218, 343 and 353 of the *PfCRT* gene. These figures are discussed in section 'Comparison of molecular markers of resistance between 2011-2013 (TRACI) and 2015-2018 (TRACII) of the manuscript.

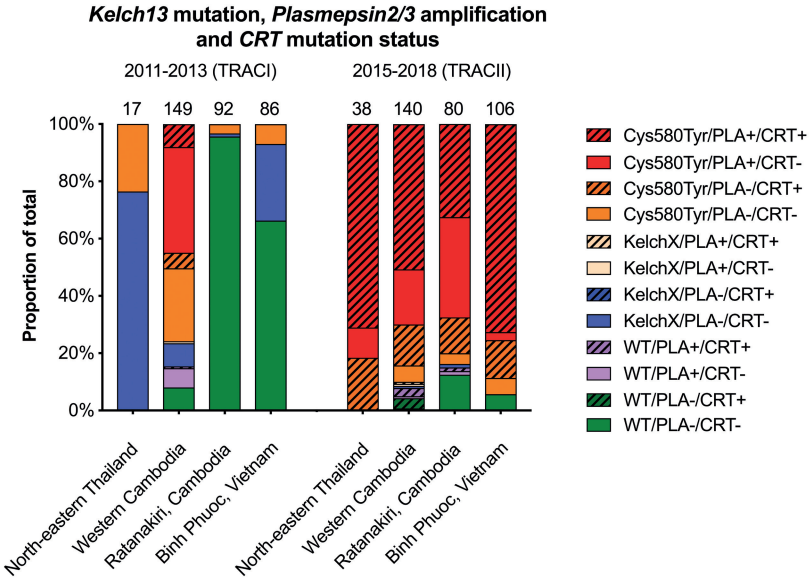


Figure S7. Frequencies of combinations for genetic markers related to resistance to artemisinins and piperaquine in TRACI (2011-2013) and TRACII (2015-2018). C580Y, KelchX and WT indicate a *PfKelch13* C580Y mutation, a *PfKelch13* other than C580Y and *PfKelch13* wild-type, respectively. **'PLA+'** and **'PLA-'** indicate parasites with or without a *plasmepsin2/3* amplification, respectively. **'CRT+'** indicate parasites with one of the *PfCRT* **T93S, H97Y, F145I, I218F, M343I and G353V** mutations whereas **'CRT-'** identifies parasites without of one of these mutations. This figure is discussed in section 'Comparison of molecular markers of resistance between 2011-2013 (TRACI) and 2015-2018 (TRACII) of the manuscript.

Table S1: Predictors of treatment failures

	HR (unadjusted) (95% CI)	p-value	HR (adjusted) (95% CI)	p-value
Site				
Ratanakiri, Cambodia (n=44)	1·000			
Binh Phuoc, Vietnam (n=60)	2·591 (1·293-5·192)	0·007		
Western Cambodia (n=17)	2·828 (1·198-6·676)	0·018		
Northeastern Thailand (n=19)	6·194 (2·821-13·600)	<0·001		
Sex				
Female (n=22)	1·000			
Male (n=118)	1·880 (0·857-4·123)	0·115		
Age (year)	0·993 (0·972-1·014)	0·492		
Baseline parasite count	1·000 (0·999-1·000)	0.064		
Parasite clearance half-life	1·166 (1·018-1·335)	0·027		
Piperaquine measurable at baseline				
No (n=101)	1·000			
Yes (n=30)	1·695 (0·978-2·937)	0·060		
***PfKelch13* status**				
WT (n=12)	1·000		1·000	
C580Y (n=119)	3·863 (0·943-15·816)	0·060	0·632 (0·125-3·208)	0·580
***Pfplasmepsin2/3* amplification status**				
No amplification (n=36)	1·000		1·000	
Amplification (n=103)	4·619 (1·991-10·717)	<0·001	3·200 (1·279-8·008)	0·013
Piperaquine levels at day 7 (continuous)	1·003 (0·998-1·009)	0·186		
Piperaquine levels at day 7>=30ng/ml (n=72)	1.000			
Piperaquine levels at day 7<30ng/ml (n=53)	0·991 (0·581-1·690)	0.974		
***PfCRT* mutation status**				
Other *PfCRT* alleles (n=32)	1·000		1·000	
Thr93Ser (n=31)	3·880 (1·617-9·313)	0·002	4·539 (1·562-13·185)	0·005
His97Tyr (n=15)	3·017 (1·057-8·613)	0·039	3·841 (1·193-12·368)	0·024
Phe145Ile (n=16)	4·942 (1·875-13·029)	0·001	7·541 (2·396-23·732)	0·001
Ile218Phe (n=25)	3·996 (1·608-9·931)	0·003	5·099 (1·715-15·161)	0·003
Met343Ile (n=2)	5·413 (1·116-26·251)	*	5·831 (1·074-31·663)	*
Gly353Val (n=3)	8·165 (2·094-31·835)	*	10·475 (1·931-56·821)	*

*Statistical significance not assessed due to small numbers. HR=Hazard Ratio. WT=Wild type.
This table is discussed in section 'Determinants of treatment failure' of the manuscript.

Table S2: Amplicon sequencing primers used to genotype variations in the crt, kelch13 and plasmepsin2/3 genes

Targets	Chromosome	Start	Stop	Length	Multiplex	Forward Primer Sequence	Reverse Primer Sequence
CRT 72, 74, 75, 76	Pf3D7_07_v3	403483	403687	204	1	ACACTCTTTCCCTACACGACGCTCTTCC GATCTTTAACAGATGGCTCACGTTmUA	TCGGCATTCCTGCTGAACCGCTCTTCCG ATCTGAGTTTCGGATGTTACAAAAmCT
CRT 93, 97	Pf3D7_07_v3	403629	403818	189	2	ACACTCTTTCCCTACACGACGCTCTTCC GATCTTTTTGCTAAAAGAACTTTAAAmCA	TCGGCATTCCTGCTGAACCGCTCTTCCG ATCTTTGGTAGGTGGAATAGATTCmUC
CRT 218, 220	Pf3D7_07_v3	404352	404600	248	1	ACACTCTTTCCCTACACGACGCTCTTCC GATCTATCTTTTGAAACACAAGAAGmAA	TCGGCATTCCTGCTGAACCGCTCTTCC GATCTATTTCCCTTGTCATGTTTGAmAA
CRT 271	Pf3D7_07_v3	404778	405026	248	2	ACACTCTTTCCCTACACGACGCTCTTCC GATCTTTTCCAATTGTTCACTTCTTmGT	TCGGCATTCCTGCTGAACCGCTCTTCCG ATCTTATTTTACCTCTACGACTGTmGT
CRT 326, 333	Pf3D7_07_v3	405189	405432	243	1	ACACTCTTTCCCTACACGACGCTCTTCC GATCTGAGCATGGGTAAGAAGCTTAmUA	TCGGCATTCCTGCTGAACCGCTCTTCCG ATCTCCTCTTGTATGTATCAACGTmUT
CRT 356	Pf3D7_07_v3	405574	405763	189	2	ACACTCTTTCCCTACACGACGCTCTTCC GATCTTGTTAGTTGTATACAAGGTCmCA	TCGGCATTCCTGCTGAACCGCTCTTCCG ATCTACGTTGTACCATCATAAACAmUT
CRT 371	Pf3D7_07_v3	405753	405965	212	1	ACACTCTTTCCCTACACGACGCTCTTCC GATCTTGGTACAACGTATCATATTTmUA	TCGGCATTCCTGCTGAACCGCTCTTCCG ATCTACGAACAAGCCATTTGATATmUA
kelch13 BTB/POZ	Pf3D7_13_v3	1724912	1725156	244	1	ACACTCTTTCCCTACACGACGCTCTTCC GATCTATGAATTTAGAACTTCGCCAmUT	TCGGCATTCCTGCTGAACCGCTCTTCCG ATCTTCCATATGCCTTATTAGAAGmCT
kelch13 BTB/POZ	Pf3D7_13_v3	1725070	1725319	249	2	ACACTCTTTCCCTACACGACGCTCTTCC GATCTTCATTATCAATACCTCCAACAmAC	TCGGCATTCCTGCTGAACCGCTCTTCCG ATCTATCGTATGAAAGCATGGGTmAG
kelch13 BTB/POZ	Pf3D7_13_v3	1725261	1725475	214	1	ACACTCTTTCCCTACACGACGCTCTTCC GATCTCATAGCTGATGATCTAGGmGG	TCGGCATTCCTGCTGAACCGCTCTTCCG ATCTCTGAGGTGTATGATCGTTTAmAG
kelch13 BTB/POZ	Pf3D7_13_v3	1725428	1725657	229	2	ACACTCTTTCCCTACACGACGCTCTTCC GATCTAATTACTTGAAACATACCATmAC	TCGGCATTCCTGCTGAACCGCTCTTCCG ATCTTATAGGTGGATTTGATGGTGmUA
kelch13 BTB/POZ	Pf3D7_13_v3	1725566	1725814	248	1	ACACTCTTTCCCTACACGACGCTCTTCC GATCTTAGACATAGGTGTACACATAmCG	TCGGCATTCCTGCTGAACCGCTCTTCCG ATCTTCTTAGATAGGGATAGTGAGmUT
kelch13 BTB/POZ	Pf3D7_13_v3	1725746	1725980	234	2	ACACTCTTTCCCTACACGACGCTCTTCC GATCTTGGGTATAGTTAACGGATTTmCT	TCGGCATTCCTGCTGAACCGCTCTTCCG ATCTAAAATTGTTGATGCAAATATmUG
Plasmepsin 2/3 breakpoint	Pf3D7_14_v3	298737	289836	var	2	ACACTCTTTCCCTACACGACGCTCTTCC GATCTCTAGGTGACCCATTTATGmAG	TCGGCATTCCTGCTGAACCGCTCTTCCG ATCTTAGCTTTAGCATCATTCAmCG

In order avoid amplicon overlapping, two different multiplexes were designed. For each amplicon, we show, from left to right: the target variants located within the amplicon, the chromosome, start and end location of the amplicon (using the Pf3D7_v3 reference); the length of the amplicon; the multiplex within which the amplicon was implemented; and the forward and reverse primer sequences.

Table S3: Prevalence of PfCRT haplotypes in TRACI (2011-2013) and TRACII (2015-2018).

PfCRT Haplotype	Counts		Allele at *PfCRT* positions															
	TRACI	TRACII	72-76	220	271	371	326	356	93	97	145	218	343	353	144	148	194	333
Wild-type (3D7-like)	9	0	CVMNK	A	Q	R	N	I	T	H	F	I	M	G	A	L	I	T
CVIET	38	0	CVIET	S	E	I	N	I	T	H	F	I	M	G	A	L	I	T
CVIET+I194T	10	0	CVIET	S	E	I	N	I	T	H	F	I	M	G	A	L	T/(-)	T
CVIET+I356T	1	0	CVIET	S	E	I	N	T	T	H	F	I	M	G	A	L	I	T
CVIET+326N+356T	191	50	CVIET	S	E	I	S	T	T	H	F	I	M	G	A	L	I/(-)	T
T93S mutation	0	82	CVIET	S	E	I	S	T	S	H	F	I	M	G	A	L	I	T
H97Y mutation	10	69	CVIET	S	E	I	S	T	T	Y	F	I	M	G	A	L	I	T
F145I mutation	0	35	CVIET	S	E	I	S	T	T	H	I	I	M	G	A	L	I	T
I218F mutation	6	48	CVIET	S	E	I	S	T	T	H	F	F	M	G	A	L	I	T
M343L mutation	2	0	CVIET	S	E	I	S	T	T	H	F	I	L	G	A	L	I	T
M343I mutation	0	10	CVIET	S	E	I	S	T	T	H	F	I	I	G	A	L	I	T
G353V mutation	8	29	CVIET	S	E	I	S	T	T	H	F	I	M	V	A	L	I	T
CVIDT+T333S	49	4	CVIDT	S	E	R	N	I	T	H	F	I	M	G	F	I	T/(-)	S
CVIET+R371I	1	0	CVIDT	S	E	I	N	I	T	H	F	I	M	G	F	I	T	T
Heterozygote infections	49	50																
Missingness at position 93, 97, 145, 218, 343 or 353	48	7																
Haplotyping not possible	6	47																

Evolution and expansion of multidrug-resistant malaria in southeast Asia: a genomic epidemiology study.

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Lancet Infectious Diseases. September 2019; 19(9): 943-951.
DOI: 10.1016/S1473-3099(19)30392-5

3

SUMMARY

Background

A multidrug-resistant co-lineage of *Plasmodium falciparum* malaria, named KEL1/PLA1, spread across Cambodia in 2008–13, causing high rates of treatment failure with the frontline combination therapy dihydroartemisinin-piperaquine. Here, we report on the evolution and spread of KEL1/PLA1 in subsequent years.

Methods

For this genomic epidemiology study, we analysed whole genome sequencing data from *P. falciparum* clinical samples collected from patients with malaria between 2007 and 2018 from Cambodia, Laos, northeastern Thailand, and Vietnam, through the MalariaGEN *P. falciparum* Community Project. Previously unpublished samples were provided by two large-scale multisite projects: the Tracking Artemisinin Resistance Collaboration II (TRAC2) and the Genetic Reconnaissance in the Greater Mekong Subregion (GenRe-Mekong) project. By investigating genome-wide relatedness between parasites, we inferred patterns of shared ancestry in the KEL1/PLA1 population.

Findings

We analysed 1673 whole genome sequences that passed quality filters, and determined KEL1/PLA1 status in 1615. Before 2009, KEL1/PLA1 was only found in western Cambodia; by 2016–17 its prevalence had risen to higher than 50% in all of the surveyed countries except for Laos. In northeastern Thailand and Vietnam, KEL1/PLA1 exceeded 80% of the most recent *P. falciparum* parasites. KEL1/PLA1 parasites maintained high genetic relatedness and low diversity, reflecting a recent common origin. Several subgroups of highly related parasites have recently emerged within this co-lineage, with diverse geographical distributions. The three largest of these subgroups (n=84, n=79, and n=47) mostly emerged since 2016 and were all present in Cambodia, Laos, and Vietnam. These expanding subgroups carried new mutations in the *crt*gene, which arose on a specific genetic background comprising multiple genomic regions. Four newly emerging *crt* mutations were rare in the early period and became more prevalent by 2016–17 (Thr93Ser, rising to 19·8%; His97Tyr to 11·2%; Phe145Ile to 5·5%; and Ile218Phe to 11·1%).

Interpretation

After emerging and circulating for several years within Cambodia, the *P. falciparum* KEL1/PLA1 co-lineage diversified into multiple subgroups and acquired new genetic features, including novel *crt* mutations. These subgroups have rapidly spread into neighbouring countries, suggesting enhanced fitness. These findings highlight the urgent need for elimination of this increasingly drug-resistant parasite co-lineage, and the importance of genetic surveillance in accelerating malaria elimination efforts.

Funding

Wellcome Trust, Bill & Melinda Gates Foundation, UK Medical Research Council, and UK Department for International Development.

INTRODUCTION

In recent years, frontline treatments for *Plasmodium falciparum* malaria have been failing in parts of southeast Asia,[29, 31, 47] a historic epicentre for the emergence and spread of antimalarial drug resistance.[75] The current treatment for *P. falciparum* consists of a fast-acting artemisinin derivative and a longer-acting partner drug, termed artemisinin combination therapy. Dihydroartemisinin with piperaquine has been the artemisinin combination therapy of choice in Cambodia, Thailand, and Vietnam for lengthy periods during the past decade. By 2008, parasites in western Cambodia began developing resistance to dihydroartemisinin-piperaquine, manifesting first through delayed clearance in response to artemisinins[7, 25, 76-78] (which might have begun several years earlier), and later with the addition of resistance to the partner drug piperaquine.[29, 47, 78] By 2013, dihydroartemisinin-piperaquine was failing to clear *P. falciparum* infections in 46% of patients treated in western Cambodia.[29] This resistance arose on a background of pre-existing resistance to multiple antimalarial drugs, leaving few treatment options and threatening plans for malaria elimination in the region. Large-scale genetic analyses have revealed the detailed epidemiology of drug resistance,[49, 58, 65, 79-81] complementing the clinical observation of increasing rates of treatment failure. Non-synonymous mutations in *kelch13*—the most prevalent of which is the Cys580Tyr (C580Y) mutation[24, 25, 58]—have proved to be valuable markers for tracking artemisinin resistance, as has amplification of *plasmepsin 2/3* [39, 41, 48, 82] in tracking piperaquine resistance. The frequency of these genetic markers increased across the eastern Greater Mekong Subregion from 2008 to 2015,[49, 50, 65, 81] corresponding with the spread of dihydroartemisinin-piperaquine treatment failure. Whole genome sequencing has provided deeper insight into the movement, demographics, and evolution of resistant parasites. Detailed analyses of a large whole genome dataset, including samples up to 2013, revealed that most parasites with the *kelch13* C580Y mutation and amplification of *plasmepsin2/3* were derived from a single parasite co-lineage, termed KEL1/PLA1, that arose in western Cambodia.[50] Such analyses raised uncertainties surrounding the future of KEL1/PLA1. Would these parasites continue their aggressive spread out from Cambodia? Would they spread clonally or heterogeneously? Could they evolve even higher levels of resistance or improved fitness? Newly emerging mutations in the *crt*gene have been reported to cause piperaquine resistance in vitro.[51, 52, 83].

These *crt* substitutions occurred on a *plasmepsin2/3* amplified background, raising the question of how mutations at multiple loci interact to produce resistant phenotypes; where and by what process the new *crt* mutations are spreading; and how these

mutations relate to the evolution and expansion of KEL1/PLA1. To address these questions, we investigated the genomic epidemiology of parasites resistant to dihydroartemisinin-piperaquine using the most recent *P. falciparum* genomic dataset currently available, including samples up to early 2018, collected across the region through the MalariaGEN *P. falciparum* Community Project.

RESEARCH IN CONTEXT

Evidence before this study

This study updates our previous work describing the emergence and spread of a multidrug-resistant *Plasmodium falciparum* co-lineage (KEL1/PLA1) within Cambodia up to 2013. A regional genetic surveillance project, Greater Mekong Subregion (GenRe-Mekong), has since reported increased frequency of dihydroartemisinin-piperaquine resistance markers in neighbouring countries. We searched PubMed using the terms "artemisinin", "piperaquine", "resistance", and "southeast Asia" for articles published since our previous study, from Oct 30, 2017, to Jan 5, 2019. Our search yielded 28 results, including reports of a recent sharp decline in the clinical efficacy of dihydroartemisinin-piperaquine in Vietnam; the spread of genetic markers of dihydroartemisinin-piperaquine resistance into neighbouring countries; and reports associating mutations in the *crt* gene with piperaquine resistance, including newly emerging *crt* variants in southeast Asia.

Added value of this study

In this genomic epidemiology study, we analysed *P. falciparum* whole genomes collected up to early 2018 from eastern southeast Asia (the geographical region comprising Cambodia, southern Laos, northeastern Thailand, and southern and central Vietnam). We describe the fine-scale epidemiology of KEL1/PLA1 genetic subgroups that have spread from Cambodia since 2015 and taken over indigenous parasite populations across eastern Southeast Asia. Several newly emerging *crt* mutations accompanied the spread and expansion of KEL1/PLA1 subgroups, suggesting a proliferation of biologically fit, multidrug-resistant parasites.

Implications of all the available evidence

The problem of *P. falciparum* multidrug resistance has substantially worsened in eastern southeast Asia since previous reports. KEL1/PLA1 has diversified and spread widely across the region since 2015, becoming the predominant parasite group in several of the endemic areas surveyed. This expansion might have been fuelled by continued exposure to dihydroartemisinin-piperaquine, resulting in sustained selection after KEL1/PLA1 became established. Continued drug pressure enabled the acquisition of further mutations, resulting in higher levels of resistance. These data show the value of genetic surveillance of pathogens and the urgent need to eliminate these dangerous parasites.

METHODS

Study design

In this genomic epidemiology study we analysed whole genome sequence data from samples in the MalariaGEN *P. falciparum* Community Project Pf6.2 data release. A large proportion of samples were collected in clinical studies, as detailed in previous publications.[25, 47, 65, 80]. Previously unpublished samples were provided by two large-scale multisite projects: the Tracking Artemisinin Resistance Collaboration II (TRAC2) and the Genetic Reconnaissance in the Greater Mekong Subregion project (GenRe-Mekong, SpotMalaria). TRAC2 did drug efficacy trials at seven sites in eastern Southeast Asia, contributing DNA from leukocyte-depleted venous blood samples taken from up to 120 symptomatic patients per site.[84] GenRe-Mekong contributed dried blood spot samples from symptomatic patients with a positive rapid diagnostic test, collected by surveillance projects at public health facilities in multiple provinces of Cambodia, Laos, and Vietnam (table S1). All patients provided informed consent through study protocols approved by the relevant local ethics authorities. Ethical approval was obtained from the National Ethics Committee for Health Research, Ministry of Health, Phnom Penh, Cambodia; the Ministry of Health National Ethics Committee For Health Research, Laos; the Ethics Committee, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; the Ethical Committee, Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam; and the Oxford Tropical Research Ethics Committee, Oxford, UK. No clinical or personal patient data were used in this analysis.

Data collection and analysis

DNA from dried blood spot samples underwent selective whole genome amplification[57] before sequencing. Sequence data were generated at the Wellcome Sanger Institute with Illumina short-read technology, and read counts at 1,043,334 quality-filtered biallelic single-nucleotide polymorphisms (SNPs) in the nuclear genome variants were called with a standardised analysis pipeline[85] (Pf6 release). Genotypes were called only with a coverage of five or more reads and alleles were disregarded when represented by fewer than two reads, or 5% of reads when coverage was higher than 50. To minimise errors and biases, we excluded from the analysis known or suspected duplicate samples, samples from time sequences and recurrences, samples sequenced with reads of fewer than 75 nucleotides, and those with insufficient coverage at more than 25% of the SNPs. After removing all SNPs that were invariant or had insufficient coverage in more than 25% of the remaining samples, we used 56 026 SNPs in our analysis. After estimating F_{WS}as previously described,[85] we removed samples with F_{WS} less than 0·95, yielding 1673 essentially monoclonal samples for analysis. Of these, 466 (28%, largely from 2016–18) were obtained from dried blood spot specimens after selective whole genome amplification, whereas the remainder were genotyped without amplification.

KEL1, PLA1, and *crt* haplotype classification

To identify *kelch13* mutations associated with artemisinin resistance, we scanned sequencing reads that align to *kelch13* amino acid positions 350 and above, identifying all non-synonymous variants. Samples without non-synonymous mutations were labelled as wild type, unless more than 25% of positions had insufficient coverage, in which case the sample was labelled as undetermined. Remaining samples were labelled according to the *kelch13* mutation found, or heterozygous if mutation sites were heterozygous. When identifying C580Y mutants, we disregarded samples that were heterozygous at that position. To assign membership to the KEL1 lineage, we tested its five characteristic SNPs.[13] Moving away from *kelch13* and ignoring missing genotypes, we counted positions carrying KEL1 characteristic alleles, until a mismatch was encountered. Samples with three or more characteristic alleles were labelled as KEL1. PLA1 parasites were identified by scanning sequencing reads for the characteristic duplication breakpoint.[50] A sample was assigned to one of the four newly emerging allele haplotypes if the corresponding position in *crt* was mutated, whereas the remaining three positions carried wild-type alleles. Remaining samples were assigned to the "no *crt*" group if all newly emerging allele positions were found to be wild type, and categorised as missing if the genotype could not be called at all the mutations.

Population genomics analysis

Analyses were done with a combination of custom software programs written in Java, R, and Python with the toolkit Scikit-Allel. To study population structure in *N* samples, we constructed an N × N pairwise distance matrix using a previously published procedure. [65] Analyses of relatedness were done with the module "cluster" from the python package "SciPy", version 0.19.

Statistical analysis

We applied the two-sided non-parametric Mann-Whitney *U* test with continuity correction to compare distributions of values, using p values less than 0·0001 as the Bonferroni-corrected significance threshold. p values less than 10^{-16} were not reported. All statistical analyses were done with the module 'stats' from the python package 'SciPy', version 0.19.

To control for spatial sampling heterogeneity, we used subsampling analyses of KEL1/PLA1 and *crt* frequency changes to balance region sample counts. We selected the earliest and latest pairs of consecutive years in which each region was represented by more than 50 samples (2010–11 and 2016–17), and estimated frequencies from 50 randomly selected samples per region. Northern Cambodia and northeastern Thailand were excluded because of insufficient samples. Median and IQR allele frequencies for the two time periods were calculated from 100 iterations.

Role of the funding source

The funders had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

We analysed a dataset of 2465 whole parasite genomes from the MalariaGEN *P. falciparum* Community Project, collected in 2007–18 from Cambodia, Laos, northeastern Thailand, and Vietnam. This geographical region is referred to as eastern Southeast Asia, the only region where KEL1/PLA1 has been found to date. After removing replicates, samples with low coverage, and highly diverse infections (F_{WS}<0·95), we analysed a dataset of 1673 samples (figure S1, S2 and table S1), in 1615 of which the KEL1/PLA1 status could be reliably identified. We identified 996 (60%) of 1673 samples as *kelch13* mutant parasites, of which 816 (82%) were C580Y. As previously reported,[58] the *kelch13* mutations were mutually exclusive (no parasites harboured more than one mutation). The next most common *kelch13* mutants were Tyr493His (Y493H; 54 [5·4%] of 996) and Arg539Thr (R539T; 53 [5·3%] of 996). Most of C580Y mutants (802 [98%] of 816) belonged to the KEL1 lineage, denoting a specific haplotype surrounding the *kelch13* locus and a single epidemiological origin in western Cambodia.[50] Of the KEL1 parasites, 551 (69%) of 802 mutants carried an amplification of the *plasmepsin2/3* genes with a shared haplotype, here named PLA1, also consistent with a single epidemiological origin at this locus (figure S3). Overall, the frequency of KEL1/PLA1 increased over the study period (figure 1).

Co-occurrence of KEL1 with PLA1 increased significantly from 2007–11 (r^2 0·28) to 2016–18 (r^2 0·41), and more than half of parasites sampled in later years were KEL1/PLA1 (354 [51%] of 695), reflecting the expansion of this co-lineage (figure 1A). Before 2009, KEL1/PLA1 was only found in western Cambodia; by 2016–18 its prevalence had risen to higher than 50% in all regions sampled except for Laos (figure 1B, table S2). This rapid rise was particularly notable in northeastern Thailand and Vietnam, where more than 80% of recent samples were KEL1/PLA1, despite their earlier absence from these areas, consistent with near-wholesale replacement of indigenous parasite populations. Increases in KEL1/PLA1 frequency in different regions and throughout eastern Southeast Asia were confirmed after correcting for uneven sampling across regions (table S3 and S4). In previous work, we showed that KEL1/PLA1 parasites from northeastern Cambodia were genetically similar to those from western Cambodia, consistent with spread from western Cambodia.[50] We extended this analysis by examining genetic similarity between parasites across the entire eastern Southeast Asia region. Overall, KEL1/PLA1 parasites had lower genetic diversity than non-KEL1/PLA1 parasites (median 0·032 *vs* 0·073; $p<10^{-16}$, Mann-Whitney *U* test; (figure 2A and figure S4). Importantly, KEL1/PLA1 parasites were genetically more similar to each other than to non-KEL1/PLA1 parasites, regardless of their geographical origins; for example,

KEL1/PLA1 parasites from Vietnam were more similar to KEL1/PLA1 parasites from other regions than to other types of parasites from Vietnam ($p<10^{-16}$ for all comparisons, Mann-Whitney *U* test; figure 2A and figure S5). This observation is consistent with KEL1/PLA1 being an invading population with origins in western Cambodia and spreading into surrounding countries. Given the high degree of genetic similarity between KEL1/PLA1 parasites, we investigated whether these parasites have spread through a single clonal expansion or as multiple independent subgroups.

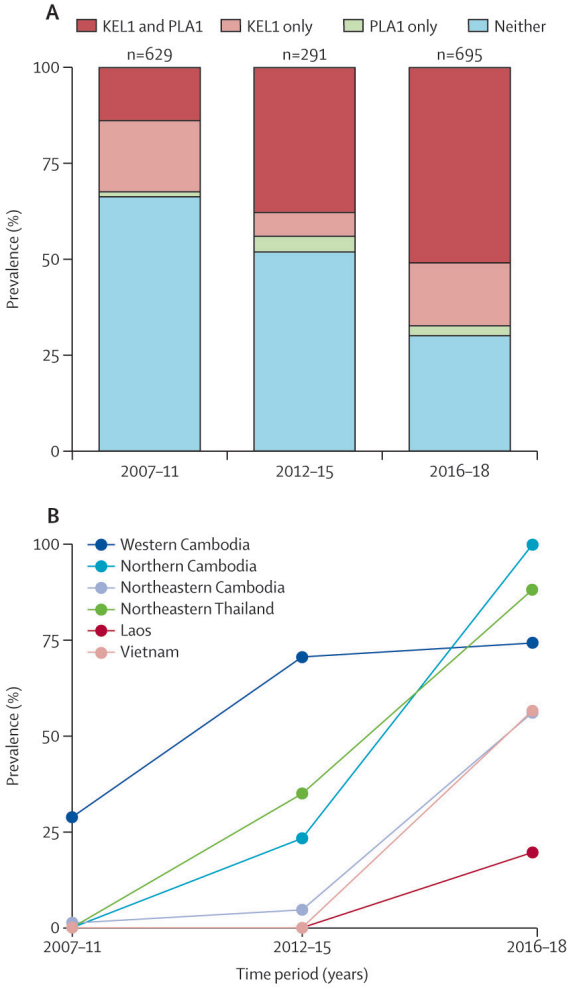


Figure 1: Rise in KEL1/PLA1 prevalence over time in eastern Southeast Asia (A) Proportions of different combinations of KEL1 and PLA1 alleles, across three time periods (2007–11, 2012–15, and 2016–18) in the eastern Southeast Asia regions surveyed in this study. (B) Change in the frequency of KEL1/PLA1 parasites during the same time periods in different geographical regions within eastern Southeast Asia.

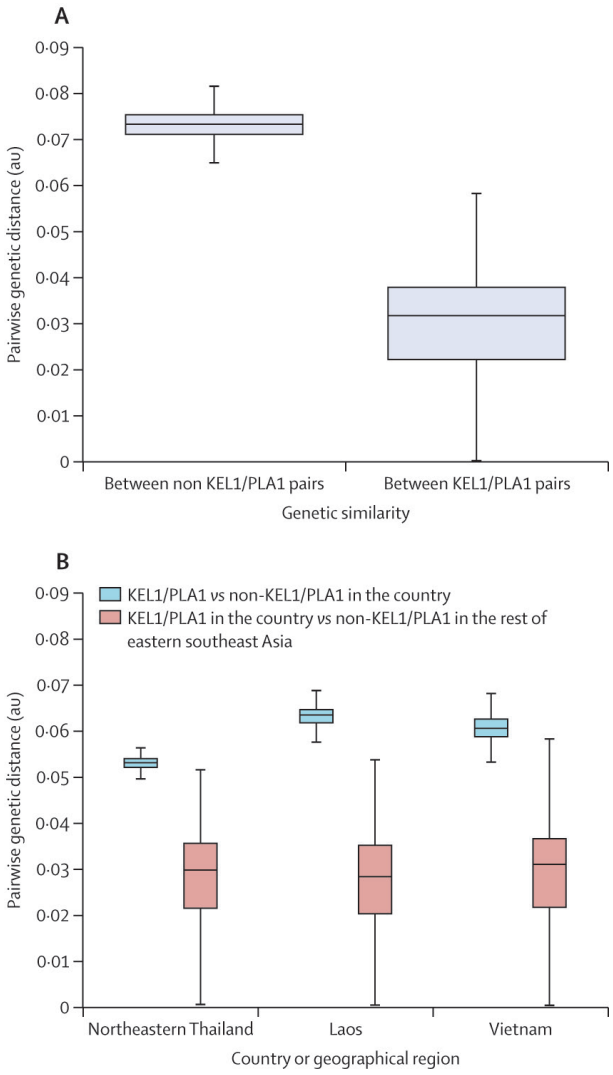


Figure 2: Genetic similarity among KEL1/PLA1 parasites across geographical regions.
(A) Boxplot comparing the distribution of pairwise genetic distance in non-KEL1/PLA1 parasites (ie, carrying neither KEL1 nor PLA1 haplotypes, n=777) with the distribution in KEL1/PLA1 parasites (n=551). (B) Boxplot comparing the distribution of pairwise distance between KEL1/PLA1 and non-KEL1/PLA1 parasites in the same geographical region (blue); and between KEL1/PLA1 parasites in the region and KEL1/PLA1 parasites outside the region (red). The number of samples analysed (in the following order: KEL1/PLA1 in the region, KEL1/PLA1 outside the region, and non-KEL1/PLA1 in the region) was 22, 529, and 14 in northeastern Thailand; 32, 519, and 193 in Laos; and 162, 389, and 207 in Vietnam. In both plots, pairwise genetic distance is expressed in an arbitrary unit, which is a function of the number of genetic differences observed among variant single-nucleotide polymorphisms (SNPs) in this dataset between pairs of samples, after correcting for linkage disequilibrium and heterozygous genotypes. Thick lines represent median values, boxes show the IQR, and whiskers represent extremes of the distribution, discounting outliers.

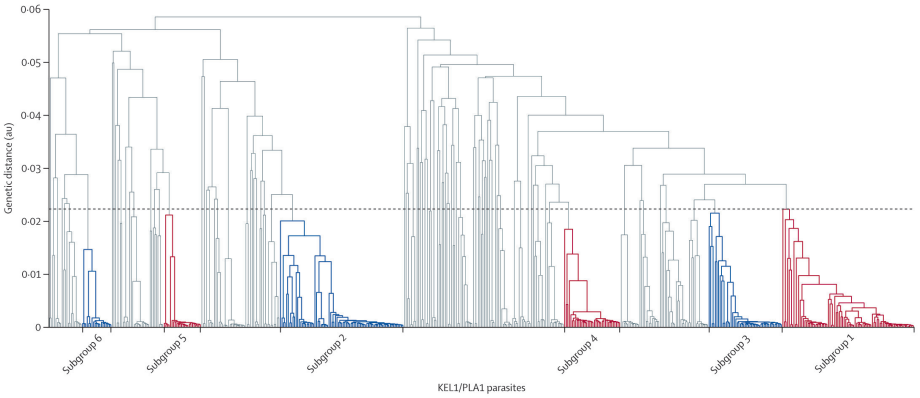


Figure 3: KEL1/PLA1 family tree. The dendrogram shows a hierarchical clustering tree of pairwise genetic distances for all 551 KEL1/PLA1 samples across eastern Southeast Asia; longer branches indicate more distant relationships. The six largest subgroups of highly related parasites are shown in red and blue, and labelled below the tree. The alternating colours highlight the different subgroups. These subgroups, numbered in order of decreasing size (subgroup 1, n=84; subgroup 2, n=79; subgroup 3, n=47; subgroup 4, n=36; subgroup 5, n=24; and subgroup 6, n=19), were identified by grouping samples with pairwise genetic distances in the lowest quartile (delimited by a dotted line). Pairwise genetic distance is expressed in an arbitrary unit, which is a function of the number of genetic differences observed among variant single-nucleotide polymorphisms (SNPs) in this dataset between pairs of samples, after correcting for linkage disequilibrium and heterozygous genotypes.

Hierarchical clustering of pairwise genetic distances was used to identify groups of closely related KEL1/PLA1 parasites (figure 3). We defined subgroups of related parasites whose pairwise genetic distance was in the lower quartile of the KEL1/PLA1 population (figure S6) and numbered these subgroups (ordered by size). The six largest subgroups together comprised more than 50% of KEL1/PLA1 samples, and broadly captured the largest expansions of near-identical parasites, with low genetic diversity within each subgroup (figure S7). The subgroups had distinct geographical, temporal, and genetic properties, reflecting separate epidemiological and evolutionary histories (figure S8). Subgroups 1 (n=84), 2 (n=79), and 3 (n=47) mostly emerged since 2016 and were all present in Cambodia, Laos, and Vietnam (figure 4A and figure 4B). These larger KEL1/PLA1 subgroups were not geographically restricted and co-existed simultaneously at the same locations across eastern Southeast Asia. This combination of high genetic similarity and broad geographical dispersal over a few years implies rapid proliferation and expansion in independent overlapping transmission waves, suggesting that these parasites possess a selective advantage. By contrast, subgroup 4 (n=36) and subgroup 6 (n=19) were largely confined to Cambodia; they were responsible for the initial KEL1/PLA1 expansion in 2007–11, but subsequently became uncommon. We also identified smaller subgroups with limited geographical and temporal distributions, such as subgroup 5 (n=24), which was almost exclusively found in northeastern Cambodia in 2016–17. The recent, rapid international expansion of some KEL1/PLA1 subgroups after years

of confinement in Cambodia raises the question of whether new genetic changes have produced advantageous phenotypic effects in these subgroups. One candidate set of driver mutations are substitutions in the *crt* gene (PF3D7_0709000) that were recently associated with piperaquine resistance.[52]

To investigate this possibility, we compared allele frequencies for all non-synonymous *crt* SNPs in an earlier sampling interval (2010–11) and a later interval (2016–17), after correcting for uneven sampling across regions (table S5). Four of these mutations (Thr93Ser [T93S], His97Tyr [H97Y], Phe145Ile [F145I], and Ile218Phe [I218F]), here referred to as newly emerging alleles, were rare in the early period (frequency ≤1%) and became more common (frequency ≥5%) by 2017–18 (T93S rising to 19·8%, H97Y to 11·2%, F145I to 5·5%, and I218F to 11·1%). These mutations were mutually exclusive: in the entire analysis dataset, we did not identify any sample carrying multiple newly emerging alleles, despite their simultaneous presence in the same geographical regions. We found that newly emerging alleles occurred on a specific constellation of other, more prevalent *crt* mutations, comprising Lys76Thr (K76T) and other mutations in common with all chloroquine-resistant parasites[86] (figure S9). Newly emerging alleles were only found on parasites possessing the most common chloroquine-resistant haplotype in eastern Southeast Asia (CVIET, named by *crt* amino acid positions 72–76), plus mutations Asn326Ser and Ile356Thr, previously associated with artemisinin-resistant *kelch13* variants.[58] Additionally, newly emerging alleles were mainly found in KEL1/PLA1 parasites (323 [78%] of 414 parasites with newly emerging alleles were also KEL1/PLA1). Consistent with the spread of KEL1/PLA1 and rising frequency of newly emerging alleles, SNPs associated with the CVIET haplotype all increased in frequency over the study period at the expense of those in other *crt* haplotypes (table S5). Three of the newly emerging alleles—T93S, F145I, and I218F—were embedded within long shared haplotypes, with reduced genetic diversity across the whole of chromosome 7 (figure S10). This denotes limited breakdown through recombination, typical of a very recent selective sweep. Consistent with a recent emergence, these newly emerging alleles were mainly found in newer KEL1/PLA1 subgroups: T93S was near fixation in subgroup 1, as was F145I in subgroup 3, whereas subgroup 2 contained a mixture of T93S and I218F parasites (figure 4C, figure S8). H97Y was distributed across multiple KEL1/PLA1 subgroups, had shorter haplotypes surrounding *crt*, and the parasites had higher levels of genetic diversity than the other newly emerging alleles, suggesting more extensive recombination. In summary, our data suggest that multiple KEL1/PLA1 subgroups were able to spread rapidly across borders in separate transmission waves, following the acquisition of one of several mutually exclusive *crt* mutations, which have emerged on a complex genetic background, including a constellation of other *crt* mutations that have accumulated over decades in eastern Southeast Asia. Northeastern Thailand provides a case study in the genomic epidemiology of these spreading multidrug-resistant parasites. In 2011, all parasites sampled from northeastern Thailand were *kelch13* R539T mutants, and possessed neither *plasmepsin 2/3* amplification nor any newly emerging alleles

in *crt*. Although they had slow parasite clearance times,[24, 25, 58] dihydroartemisinin-piperaquine remained an effective treatment because of sensitivity to piperaquine. These parasites had exceptionally low genetic diversity (figure S4), perhaps reflecting population collapse because of effective malaria control efforts. By 2017, however, KEL1/PLA1 had entirely replaced the R539T population, with a corresponding rise in dihydroartemisinin-piperaquine resistance. Although the majority of these parasites were from subgroup 3 and possessed the *crt* F145I mutation, we found other newly emerging alleles in this area, suggesting that multiple enhanced KEL1/PLA1 subgroups, possessing distinct *crt* mutations, invaded northeastern Thailand independently and replaced earlier parasite populations.

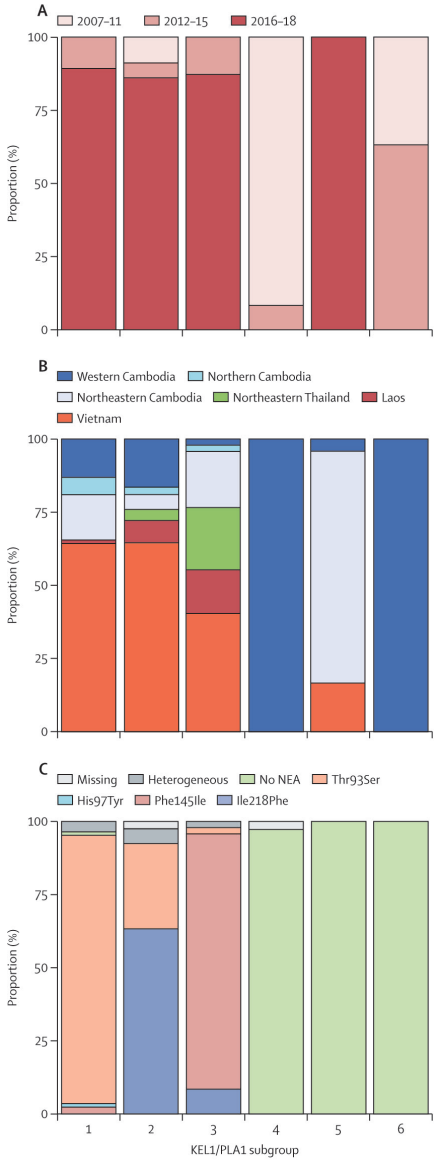


Figure 4: Distinct epidemiological and genetic properties of KEL1/PLA1 subgroups. Sample proportions by sampling time period (A) and location (B) in the six largest groups of high-similarity KEL1/PLA1 parasites. Subgroups 1–3 emerged recently and are internationally distributed, whereas subgroups 4 and 6 are older and confined to western Cambodia. Proportion of *crt* haplotypes in the same groups (C): newly emerging *crt* mutations are highly prevalent in the newer subgroups 1–3, but absent from the older geographically restricted subgroups 4 and 6, and also in subgroup 5, which has recently expanded in northeastern Cambodia. Numbers of samples are as follows: n=84 for subgroup 1, n=79 for subgroup 2, n=47 for subgroup 3, n=36 for subgroup 4, n=24 for subgroup 5, and n=19 for subgroup 6. Together, these samples comprise more than 50% of the 551 analysed KEL1/PLA1 samples.

DISCUSSION

After a decade of progress,[87] malaria incidence and mortality have been increasing since 2015, putting global malaria targets at risk.[88] Major challenges include inadequate funding, parasite drug resistance, and insecticide resistance in mosquito vectors.[89] Previous work has described a worrying situation unfolding in Southeast Asia over the 2007–13 period, with the emergence of a dominant parasite co-lineage, KEL1/PLA1, that spread across Cambodia and caused dihydroartemisinin-piperaquine treatment failure.[50] We describe the ongoing evolution and expansion of multidrug-resistant *P. falciparum*, using whole genomes sampled across eastern Southeast Asia and collected up to early 2018.

Our data clearly show that KEL1/PLA1 has continued spreading out from western Cambodia and is now highly prevalent in multiple regions of Laos, Thailand, and Vietnam, where it has frequently replaced previous indigenous populations of parasites. At all locations, KEL1/PLA1 parasites were genetically distinct from non-KEL1/PLA1 parasites, reflecting their recent shared ancestry. Genomic data show that underlying this spread is not a single lineage, but instead multiple subgroups of KEL1/PLA1 parasites, which have spread across eastern Southeast Asia in independent transmission waves. These subgroups carry newly emerging alleles in the *crt* gene, which have arisen on a specific constellation of background *crt* mutations, most frequently in KEL1/PLA1 parasites.

The rapid rise in the frequency of these *crt* alleles suggests that they are markers of an advantageous phenotype. Two newly emerging alleles (F145I and H97Y) have been shown to reduce piperaquine sensitivity in vitro,[52] and a new clinical study[84] shows that H97Y, F145I, and I218F are associated with a higher rate of dihydroartemisinin-piperaquine treatment failures. Other *crt* alleles arising on a similar genetic background might also be functionally significant—for example, Gly353Val (G353V) has been associated with reduced piperaquine sensitivity in vitro.[52] Thus, several novel *crt* variants might be capable of reducing parasite sensitivity to piperaquine, and among these the newly emerging alleles are those whose recent rise in frequency is most conspicuous in our dataset. Parasites harbouring piperaquine-resistant *crt* mutations, including F145I and G353V, were out-competed in vitro during asexual blood-stage development by lab isolates without the mutations, in the absence of drug pressure.[48] The rise in frequency of these variations, in spite of fitness cost, is further evidence that they confer an increased survival advantage under strong and sustained piperaquine pressure.

Vietnam has used dihydroartemisinin-piperaquine as first-line treatment since 2004, Cambodia during the 2008–16 period, and Thailand since 2015. Cambodia has since adopted artesunate-mefloquine as first-line treatment, whereas the other two countries are reviewing current policy and procedures. Starting around 2008, KEL1/PLA1 parasites were first detected in western Cambodia and then expanded within Cambodia. They

progressively replaced local parasite populations, such that by 2014 nearly all parasites sampled from western Cambodia were KEL1/PLA1.[50] This replacement was probably driven by resistance to dihydroartemisinin-piperaquine, consistent with the association between *plasmepsin 2/3* amplification and piperaquine resistance in parasites collected before newly emerging alleles rose in frequency.[39, 48] We propose that, after several years of continued exposure to dihydroartemisinin-piperaquine, the parasites acquired further mutations including in the *crt* gene, which conferred higher-level dihydroartemisinin-piperaquine resistance. KEL1/PLA1 subgroups possessing these *crt* mutations were able to spread rapidly across borders in the 2015–18 period. The timing of Thailand's adoption of dihydroartemisinin-piperaquine, driven by concerns about the efficacy of artesunate-mefloquine on the border with Myanmar, was particularly unfortunate as it coincided with the KEL1/PLA1 cross-border expansion. Even in Laos, where artemether-lumefantrine has been the recommended first-line drug since 2005, KEL1/PLA1 parasites have successfully colonised the southernmost province of Champasak, possibly because of cross-border importation of dihydroartemisinin-piperaquine or because of their resistance to the artemisinin component of artemether-lumefantrine.

These findings show an evolutionary process in action. Artemisinin resistance first began as delayed parasite clearance, caused by many mutually exclusive *kelch13* mutations, generally at low frequency and geographically restricted. Over time, a single *kelch13* mutation (C580Y) has become dominant in eastern Southeast Asia, in association with several other variants (eg, in *ferredoxin*, *arps10*, *mdr2*, and *crt*).[58, 65] Thus, a soft sweep (many different, independently emerging advantageous *kelch13* mutations) became a hard sweep of the *kelch13* C580Y variant as the KEL1/PLA1 co-lineage, resistant to dihydroartemisinin-piperaquine, rose in frequency and swept through Cambodia.[50] Following that harder sweep, the parasites diversified into separate evolutionary branches with emerging new properties. This perhaps reflects a general tendency for diversification after a hard sweep, as the population explores a vast evolutionary space, acquiring new mutations. The genetic background that has accumulated in eastern Southeast Asia appears to underpin the newly emerging alleles in *crt*, as multiple alleles have arisen independently within the past few years that are absent elsewhere. It remains to be seen whether one of these newly emerging alleles will become dominant and drive a new hard sweep, as *kelch13* C580Y did.

The spread of KEL1/PLA1 up to 2013 described by Amato and colleagues[50] has worsened substantially. By analogy with cancer biology, KEL1/PLA1 can be viewed as an aggressive cell line that has metastasised, invading new territories and acquiring new genetic properties. These patterns emphasise the importance of surveillance in guiding and accelerating malaria elimination. Prolonged use of dihydroartemisinin-piperaquine after resistance first emerged might have created the selective pressure for the evolution of enhanced KEL1/PLA1 subgroups. Given the spread and intensification of resistance, effective translation of genetic surveillance results is crucial to support timely decisions on first-line therapies. Many of the samples in this dataset were obtained by

amalgamating data from multiple varied studies, resulting in temporal and geographical heterogeneity that can limit the inferential power. Going forward, there is a need for systematic longitudinal surveillance, and this is now being done by malaria control programmes in Cambodia, Laos, and Vietnam, which contributed many of the recent samples included in this study. These findings highlight the importance of longitudinal genetic surveillance in guiding the elimination of multidrug-resistant *P. falciparum* from the Greater Mekong Subregion, and control and elimination efforts elsewhere.

ACKNOWLEDGMENTS

This study was funded by the Wellcome Trust (grants 098051, 206194, 203141, 090770, 204911, and 106698/B/14/Z), Bill & Melinda Gates Foundation (OPP1040463, OPP11188166), Medical Research Council (G0600718), UK Department for International Development (201900, M006212), and the Intramural Research Program of the National Institute of Allergy and Infectious Diseases. WLH is funded by Cambridge University Hospitals NHS Foundation Trust and the National Institute for Health Research. This study used data from the MalariaGEN Pf3k Project and *Plasmodium falciparum* Community Project.

Genome sequencing was done by the Wellcome Sanger Institute; sample collections were coordinated by the MalariaGEN Resource Centre. We thank the staff of Wellcome Sanger Institute Sample Logistics, Sequencing, and Informatics facilities for their contribution; Mihir Kekre and Katie Love for sample processing; Nick Harding, Alistair Miles, Chris Clarkson, and Jacob Almagro-Garcia for helpful suggestions on bioinformatics; and Victoria Simpson and Christa Henrichs for coordinating and managing MalariaGEN projects. We thank all patients and collaborators to the MalariaGEN Pf Community Project, especially investigators who submitted samples used in this study: Chris Plowe, Pascal Ringwald, Elizabeth Ashley, Xinxhuan Su, and Maciej Boni; and collaborators to the GenRe-Mekong Project: Pannapat Masingboon, Sonexay Phalivon, Nguyen Kim-Tuyen, Thanat Chookajorn, Namfon Kotanan, Zoë Doran, Salwaluk Panapipat, and Ipsita Sinha.

CONTRIBUTORS

WLH, RA, DPK, and OM designed the study. RA, CGJ, RDP, TN, SG, and OM generated and curated the data. WLH, RA, CGJ, DPK, and OM analysed and interpreted the data. RWvdP, XHSC, RJM, NJW, NPD, AMD, and OM oversaw the field projects. NTT-N, HHQ, TTH, BHo, KC, MM, RH, RL, CH, LD, CA, SS, RMF, RT, TJP, YS, PJ, BHa, SP, and NHC collected the samples. NTT-N, MI, MD, RV, KR, and ED did laboratory work to sequence the samples. WLH, RA, DPK, and OM wrote the manuscript. All authors critically reviewed the manuscript.

SUPPLEMENTARY MATERIAL

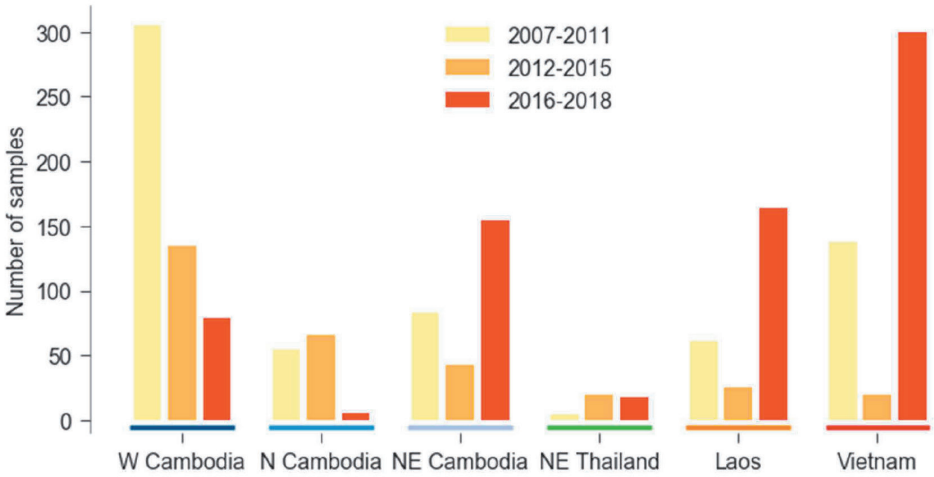
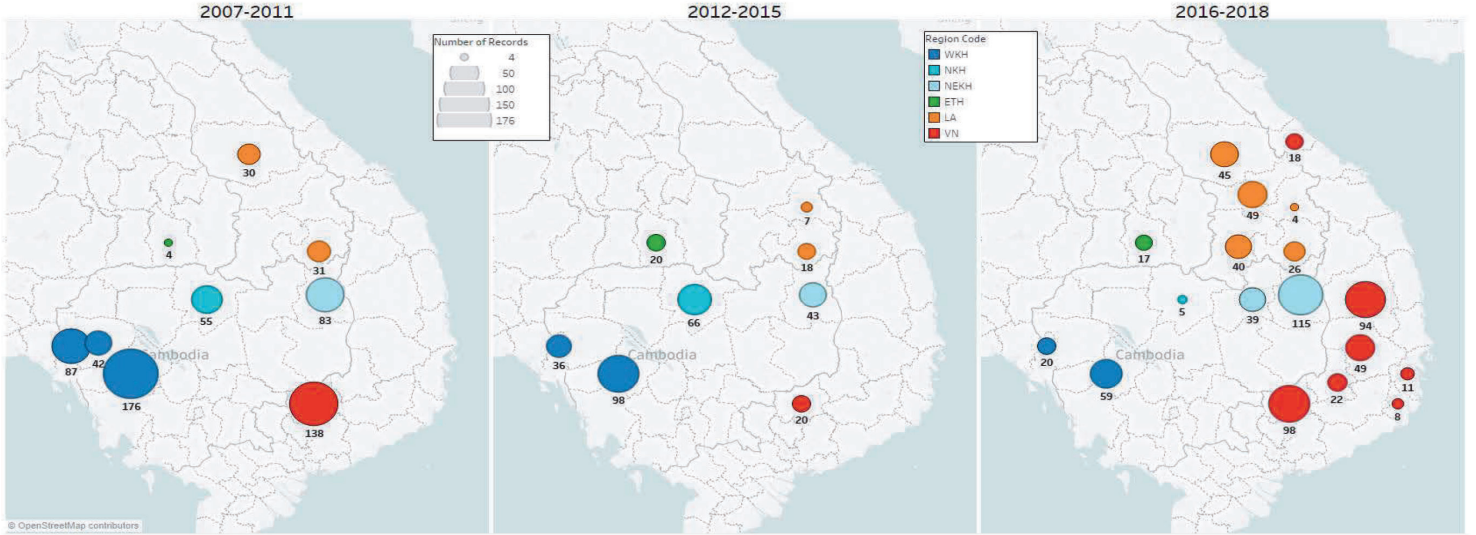


Figure S1. Sample numbers included in main analysis, broken down by country and year.



Figures S2. Distribution by region of 1,673 samples in the three analysed periods.
Markers are coloured by region, and their size reflects the number of samples (shown in the labels below each marker).

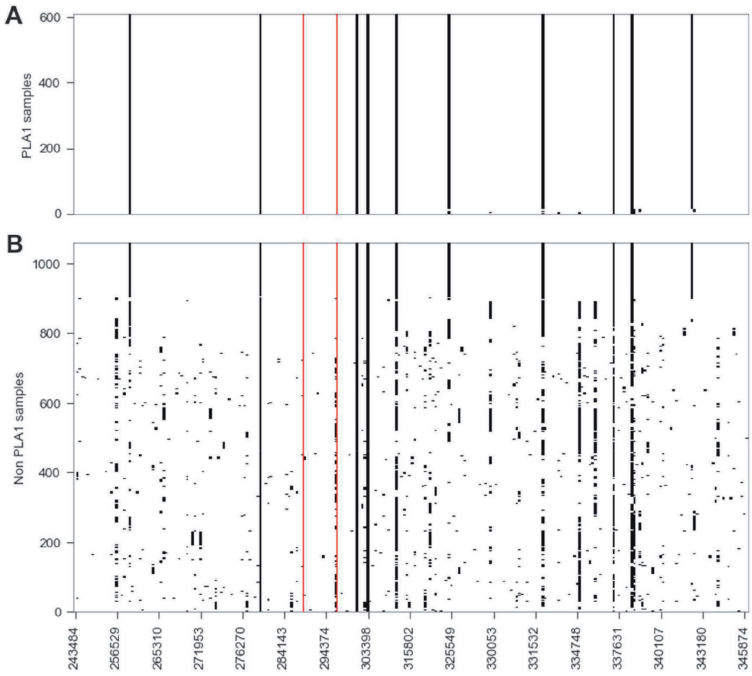


Figure S3. Haplotypes surrounding the *plasmepsin-2* and *plasmepsin-3* loci for PLA1 samples (A) and non-PLA1 samples (B). Each row represents a sample, and each column represents a single SNP variant. Cells are coloured white for the reference allele (same as 3D7 reference sequence) and black for non-reference allele. The red lines enclose SNPs in the two *plasmepsin* genes. The presence of a single shared haplotype surrounding these loci is consistent with a single epidemiological origin of the genetic background on which the amplification arose.

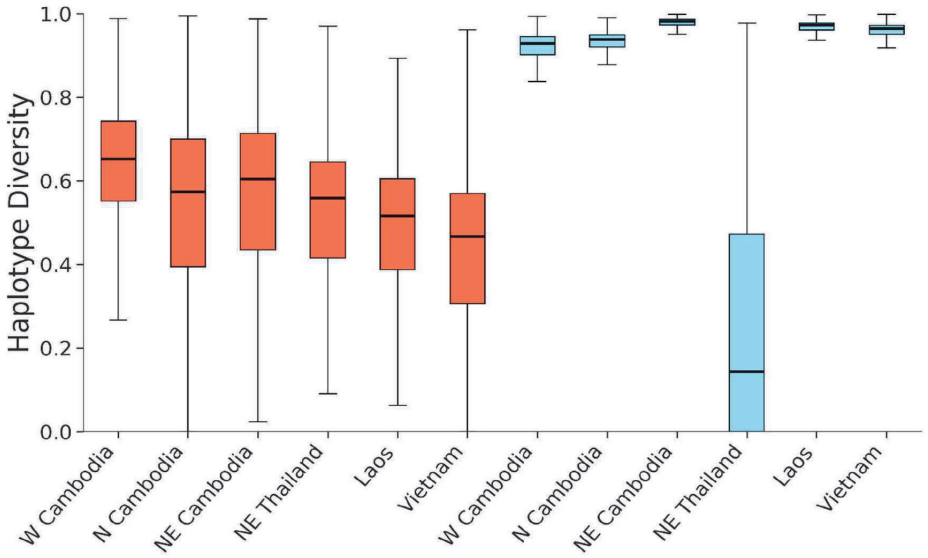


Figure S4. Low haplotype diversity in KEL1/PLA1 parasites. Boxplots show the distribution of haplotype diversity measures in rolling 100-SNP windows with 50-SNP overlaps along the whole genome for populations of KEL1/PLA1 (red, left) and non-KEL1/PLA1 (blue, right) parasites in the different regions studied. Haplotype diversity is the proportion of SNP windows that have at least 1 SNP difference between samples, where zero means all SNP-windows are identical and 1 means all have at least 1 SNP difference. Thick lines represent median values, boxes show the interquartile range, whiskers represent extremes of the distribution discounting outliers. Pairwise comparisons between the KEL1/PLA1 and non-KEL1/PLA1 groups for each region were all statistically significant with Bonferroni correction for multiple comparisons ($P<10^{-16}$, Mann-Whitney U test).

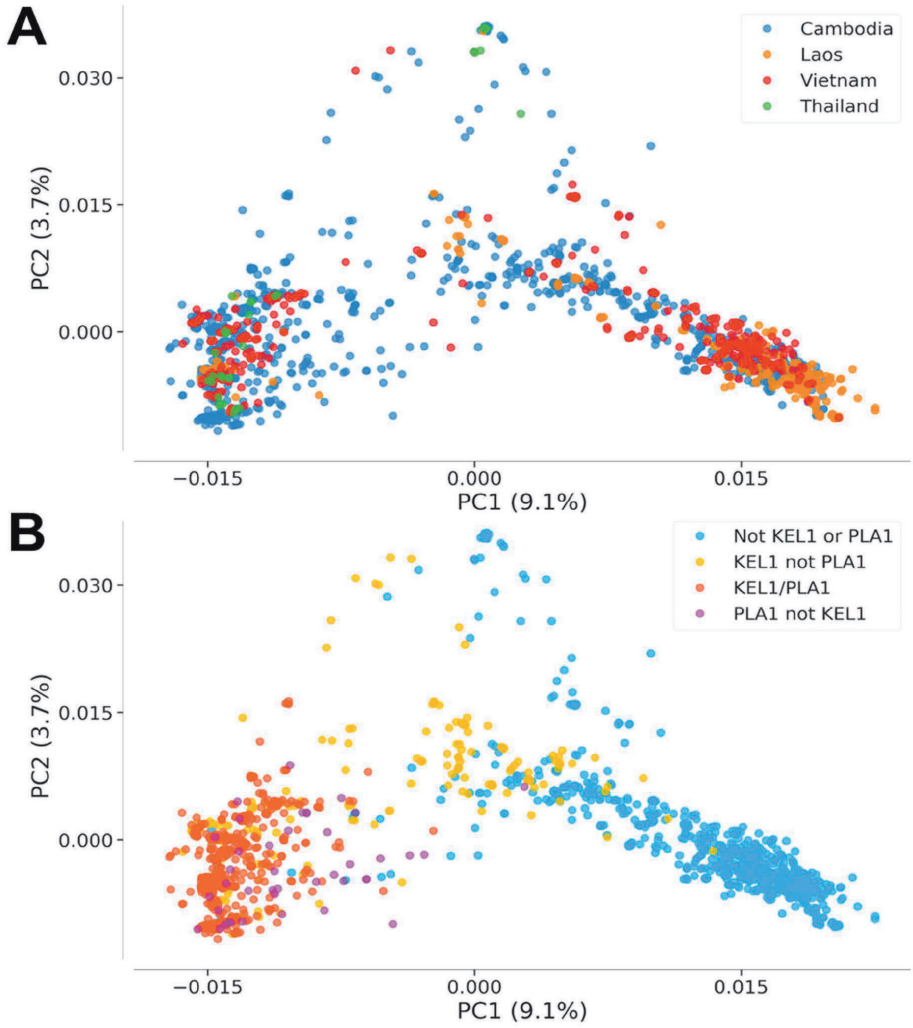


Figure S5. High degree of similarity among KEL1/PLA1 parasites, regardless of geographical origin. Principal Component Analysis (PCoA) based on genetic distance, coloured by country (A) and KEL1/PLA1 status (B). Genetic distances were calculated with correction for linkage disequilibrium and minor allele frequency cutoff of 1%.

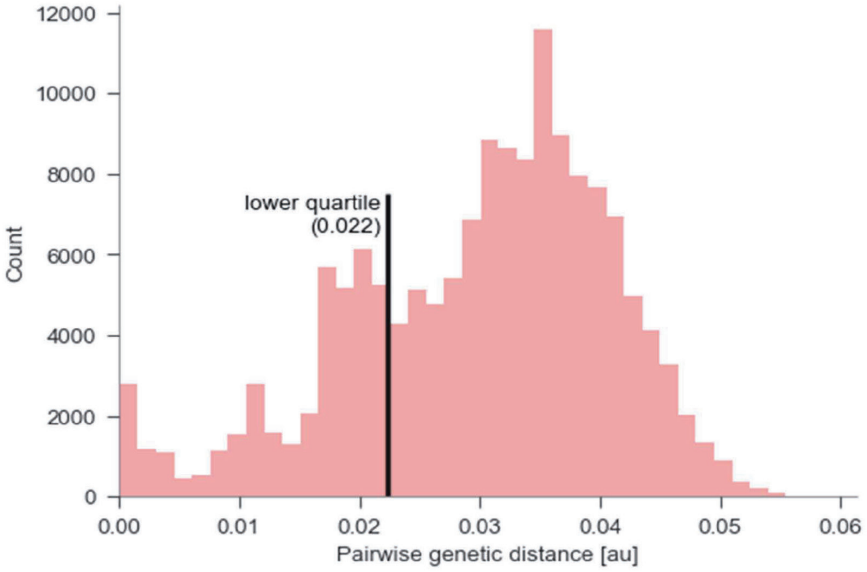


Figure S6. Histogram of pairwise genetic distances for KEL1/PLA1 samples, with lower quartile (used to define related subgroups) marked.

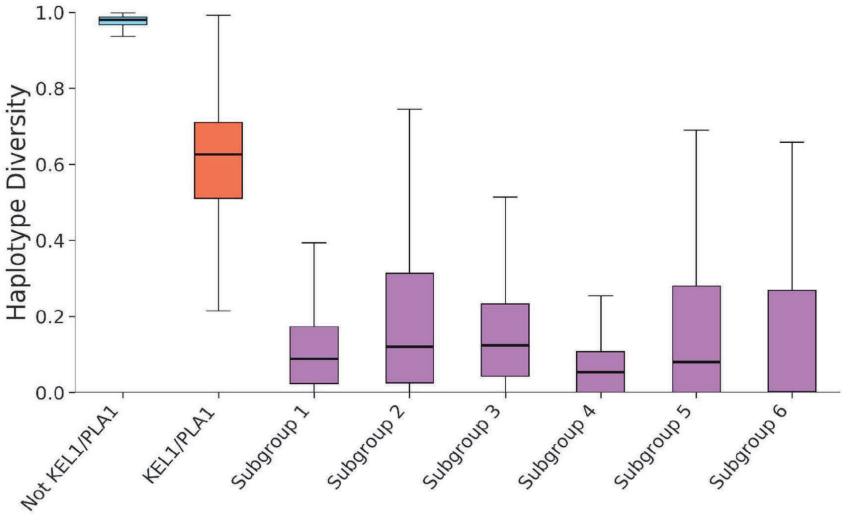


Figure S7. Haplotype diversity in KEL1/PLA1 subgroups. Boxplots show the distribution of haplotype diversity in different parasite groups, measured in rolling 100-SNP windows with 50-SNP overlaps along the whole genome. Haplotype diversity is the proportion of SNP windows that have at least 1 SNP difference between samples, where zero means all SNP-windows are identical and 1 means all have at least 1 SNP difference. Thick lines represent median values, boxes show the interquartile range, whiskers represent extremes of the distribution discounting outliers.

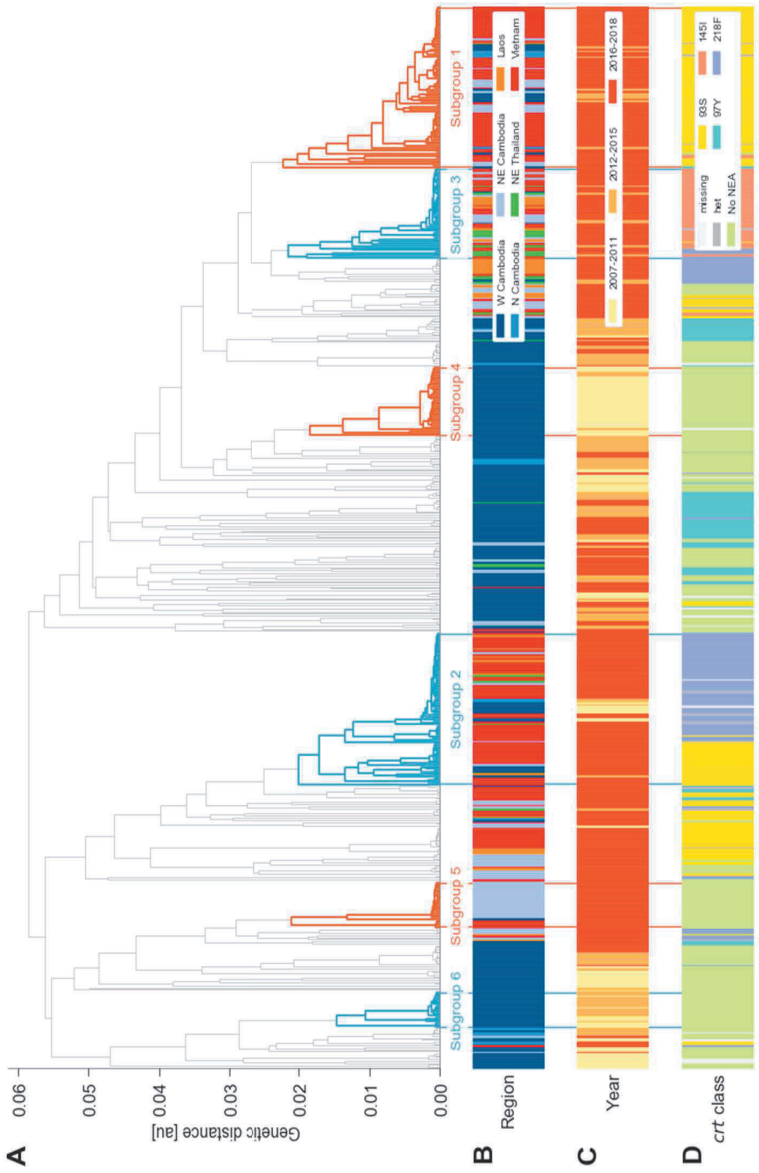


Figure S8. KEL1/PLA1 family tree, geo-temporal distribution and *crt* alleles. (A) Dendrogram of genetic distances within all 551 analysed KEL1/PLA1 samples across ESEA, identical to that shown in Figure 3. The six largest subgroups are highlighted and labelled. Colour bars indicate sampling region (B), year (C) and the presence of any Newly Emerging Alleles in *crt*, as defined in main text (D). Pairwise genetic distance is expressed in an arbitrary unit which is a function of the number of genetic differences observed among variant SNPs in this dataset between pairs of samples, after correcting for linkage disequilibrium and heterozygous genotypes.

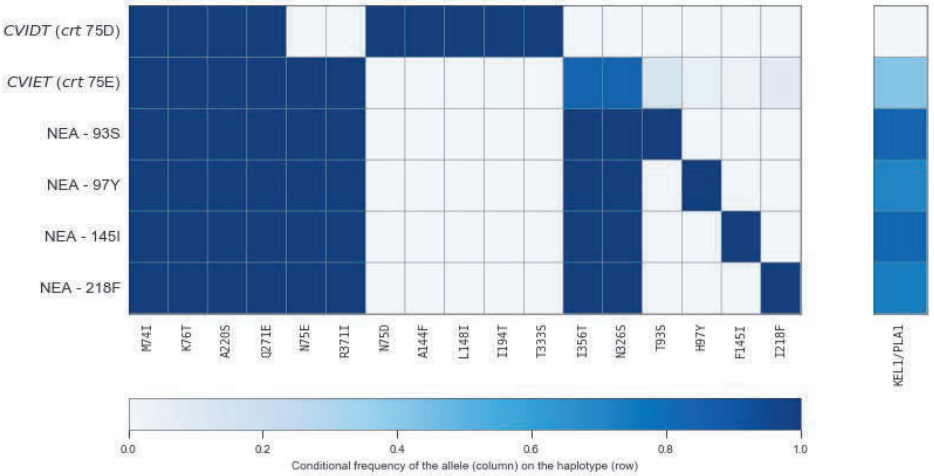


Figure S9. Association of *crt* alleles with specific genetic backgrounds. Each column represents a circulating mutation in the *crt* gene, and each row represents a genetic background, as mentioned in the main text. Each cell is coloured according to the frequency of the mutation (column) in parasites that carry the specified haplotype (row). NEAs arise on a genetic background comprising the chloroquine resistant CVIET haplotype, the additional mutations at positions 326 and 356, and the KEL1/PLA1 haplotype. Three other mutually exclusive *crt* alleles found at lower frequencies than the NEAs (H97L, M343I and G353V) were also associated with the same genetic background as the NEAs, though H97L only occurred in non-PLA1 parasites.

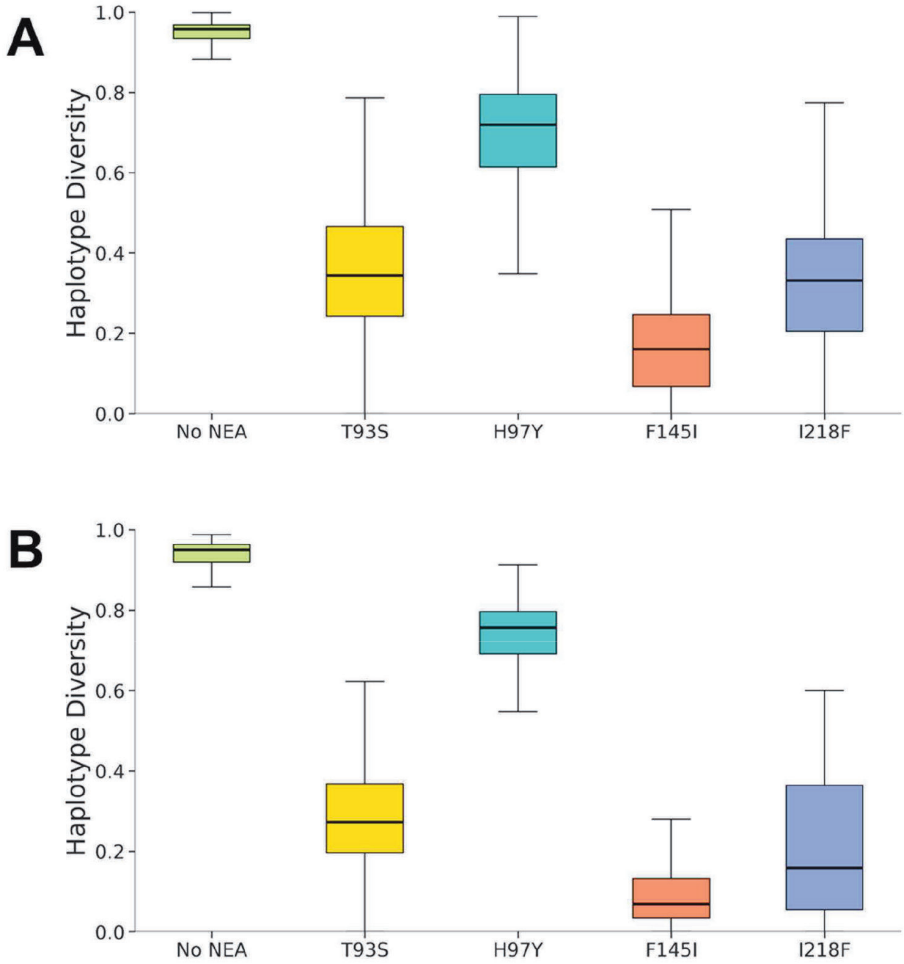


Figure S10. Haplotype diversity in parasites with *crt* Newly Emerging Alleles. Boxplots show the distribution of haplotype diversity in parasites possessing different *crt*. Newly Emerging Alleles, and parasites without NEAs, measured in rolling 100-SNP windows with 50-SNP overlaps along the whole genome (A) and just chromosome 7 (B), where *crt* is situated. Haplotype diversity is the proportion of SNP windows that have at least 1 SNP difference between samples, where zero means all SNP-windows are identical and 1 means all have at least 1 SNP difference. Thick lines represent median values, boxes show the interquartile range, whiskers represent extremes of the distribution discounting outliers. Pairwise comparisons between "no NEA" and each NEA groups were all statistically significant with Bonferroni correction for multiple comparisons ($P<10^{-16}$, Mann-Whitney U test).

Table S1: Counts of samples analysed for the different ESEA regions surveyed.

Region			
Symbol	Name	Provinces	Sample count
WKH	W Cambodia	Pailin, Pursat, Battambang	518
NKH	N Cambodia	Preah Vihear	126
NEKH	NE Cambodia	Stung Treng, Ratanakiri	280
NETH	NE Thailand	Sisaket	41
LA	Lao PDR	Savannakhet, Salavan, Sekong, Attapeu, Champasak	250
VN	Vietnam	Binh Phuoc, Dak Lak, Dak Nong, Gia Lai, Quang Tri, Ninh Thuan, Khanh Hoa	458
			1673

Table S2: Proportion of KEL1/PLA1 parasites per year in each ESEA region. Absolute numbers of samples are shown in parenthesis.

Year	W Cambodia	N Cambodia	NE Cambodia	NE Thailand	Lao PDR	Vietnam
2007	0% (0/6)	-	100% (1/1)	-	-	-
2008	15% (11/74)	-	-	-	-	-
2009	41% (9/22)	-	-	-	-	0% (0/9)
2010	23% (19/82)	-	0% (0/33)	-	0% (0/30)	0% (0/54)
2011	39% (47/121)	0% (0/55)	0% (0/49)	0% (0/4)	0% (0/31)	0% (0/75)
2012	58% (24/48)	8% (3/38)	0% (0/18)	0% (0/11)	0% (0/18)	0% (0/20)
2013	56% (20/36)	27% (6/22)	14% (1/7)	0% (0/2)	-	-
2014	72% (18/25)	80% (4/5)	6% (1/18)	-	-	-
2015	84% (21/25)	100% (1/1)	-	100% (7/7)	0% (0/7)	-
2016	78% (28/36)	100% (5/5)	47% (39/83)	100% (1/1)	0% (0/3)	69% (34/49)
2017	68% (28/41)	-	60% 41/68)	88% (14/16)	20% (32/161)	51% (128/251)
2018	100% (2/2)	-	67% (2/3)	-	-	-

Dashes indicate that no samples were available in our dataset for that region/year combination.

Table S3: Frequency of parasites with KEL1 and PLA1 haplotypes in the periods 2010-2011 and 2016-2017, corrected for sampling heterogeneity.

Haplotype status	2010-2011	2016-2017
Neither KEL1 nor PLA1	80.62% (79.46%-81.79%)	29.02% (26.91%-30.97%)
PLA1 only	1.03% (0.52%-1.55%)	4.18% (3.63%-4.67%)
KEL1 only	9.57% (8.29%-10.42%)	14.95% (13.92%-16.21%)
KEL1/PLA1	8.38% (7.73%-9.38%)	51.66% (50.00%-53.42%)

For each time period, we show median haplotype frequency (with interquartile range, IQR) estimated from 50 randomly selected samples from each region except northeastern Thailand and northern Cambodia, for which sample size was insufficient), repeated for 100 iteration.

Table S4: Frequency of KEL1/PLA1 parasites in the periods 2010-2011 and 2016-2017 for different ESEA regions, corrected for sampling heterogeneity.

Region	2010-2011	2016-2017
Western Cambodia	34.00% (31.25% - 37.62%)	74.00% (71.43% - 75.63%)
Northeastern Cambodia	0.00% (0.00% - 0.00%)	56.25% (52.15% - 61.34%)
Laos	0.00% (0.00% - 0.00%)	20.00% (18.00% - 22.45%)
Vietnam	0.00% (0.00% - 0.00%)	56.83% (52.84% - 60.96%)

For each time period, we show median haplotype frequency (with interquartile range, IQR) estimated from 50 randomly selected samples from each region (except northeastern Thailand and northern Cambodia, for which sample size was insufficient), repeated for 100 iteration.

Table S5. Variations in frequency of crt mutations between the periods 2010-2011 and 2016-2017, corrected for sampling heterogeneity.

crt variant	Frequency in 2010-2011	Frequency in 2016-2017	Frequency change
M74I	91.96% (91.00% - 92.50%)	97.24% (96.50% - 97.99%)	5.49% (4.48% - 6.27%)
N75D	75.91% (73.85% - 77.61%)	79.67% (75.49% - 84.80%)	4.40% (-0.93% - 9.36%)
N75E	89.06% (87.92% - 90.01%)	96.95% (96.15% - 97.73%)	7.86% (6.67% - 9.02%)
K76T	91.96% (91.00% - 92.50%)	97.24% (96.50% - 97.99%)	5.49% (4.48% - 6.27%)
T93S	**0.00% (0.00% - 0.00%)**	**19.75% (18.00% - 21.39%)**	**19.75% (18.00% - 21.39%)**
H97Y	**1.00% (0.50% - 1.50%)**	**11.20% (10.41% - 11.87%)**	**10.25% (9.16% - 11.23%)**
H97L	0.00% (0.00% - 0.00%)	1.71% (1.13% - 2.27%)	1.70% (1.12% - 2.27%)
A144F	25.50% (24.50% - 26.80%)	10.83% (9.60% - 12.31%)	-15.02% (-16.34% - -13.29%)
F145I	**0.00% (0.00% - 0.00%)**	**5.54% (4.54% - 6.57%)**	**5.54% (4.54% - 6.57%)**
L148I	25.51% (24.37% - 26.78%)	10.86% (9.71% - 12.31%)	-14.89% (-16.27% - -13.13%)
I194T	27.23% (25.87% - 28.27%)	15.31% (13.73% - 17.64%)	-11.80% (-13.73% - -9.15%)
I218F	**0.77% (0.51% - 1.04%)**	**11.14% (9.99% - 12.20%)**	**10.28% (9.15% - 11.35%)**
A220S	91.69% (90.76% - 92.31%)	97.34% (96.68% - 97.98%)	5.74% (4.72% - 6.48%)
Q271E	91.96% (91.00% - 92.50%)	97.27% (96.57% - 97.93%)	5.44% (4.38% - 6.22%)
H273N	1.01% (0.50% - 1.51%)	0.51% (0.00% - 1.02%)	-0.50% (-1.00% - 0.00%)
N326S	38.61% (37.41% - 39.45%)	76.49% (74.35% - 77.96%)	38.03% (35.96% - 40.01%)
T333S	25.38% (24.37% - 26.67%)	11.08% (9.88% - 12.53%)	-14.75% (-15.89% - -12.82%)
M343I	0.00% (0.00% - 0.00%)	2.26% (2.00% - 2.75%)	2.26% (2.00% - 2.75%)
G353V	1.01% (0.50% - 1.50%)	4.52% (4.50% - 5.03%)	3.99% (3.02% - 4.50%)
I356T	39.45% (38.39% - 40.42%)	77.27% (75.00% - 78.50%)	37.66% (35.85% - 39.53%)
R371I	66.50% (65.25% - 68.25%)	86.36% (84.91% - 87.94%)	19.46% (17.83% - 21.94%)

For each time period, we show median mutation frequency (with interquartile range, IQR) estimated from 50 randomly selected samples from each region (except northeastern Thailand and northern Cambodia, for which sample size was insufficient), repeated for 100 iteration. Mutations printed in **bold** are those absent in the first period (frequency ≤ 1%) and increased by >5% in the second period. Mutations below 1% frequency in at least one period were disregarded.

PART II

Safety, tolerability and efficacy of Triple Antimalarial Combination Therapies

Sequential open-label study of the safety, tolerability, and pharmacokinetic interactions between dihydroartemisinin-piperaquine and mefloquine in healthy Thai adults.

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[*] These authors contributed equally to this work.

Antimicrobial Agents and Chemotherapy. July 2019; 25;63(8):e00060-19
DOI: 10.1128/AAC.00060-19

4

ABSTRACT

Background

Artemisinin-based combination therapies (ACTs) have contributed substantially to the global decline in *Plasmodium falciparum* morbidity and mortality, but resistance to artemisinins and their partner drugs is increasing in Southeast Asia, threatening malaria control. New antimalarial compounds will not be generally available soon. Combining three existing antimalarials in the form of Triple ACTs, including dihydroartemisinin (DHA)-piperaquine plus mefloquine, is a potential treatment option for multidrug-resistant *Plasmodium falciparum* malaria.

Methods

In a sequential open-label study, healthy Thai volunteers were treated with DHA-piperaquine (120 to 960 mg), mefloquine (500 mg), and DHA-piperaquine plus mefloquine (120 to 960 mg plus 500 mg), and serial symptom questionnaires, biochemistry, full blood counts, pharmacokinetic profiles, and electrocardiographic measurements were performed.

Findings

Fifteen healthy subjects were enrolled. There was no difference in the incidence or severity of adverse events between the three treatment arms. The slight prolongation in QTc (QT interval corrected for heart rate) associated with DHA-piperaquine administration did not increase after administration of DHA-piperaquine plus mefloquine. The addition of mefloquine had no significant effect on the pharmacokinetic properties of piperaquine. However, coadministration of mefloquine significantly reduced the exposures to dihydroartemisinin for area under the concentration-time curve (−22.6%; 90% confidence interval [CI], −33.1, −10.4; $P = 0.0039$) and maximum concentration of drug in serum (−29.0%; 90% CI, −40.6, −15.1; $P = 0.0079$).

Interpretation

Mefloquine can be added safely to dihydroartemisinin-piperaquine in malaria treatment. (This study has been registered at ClinicalTrials.gov under identifier NCT02324738.)

INTRODUCTION

Artemisinin-based combination therapies (ACTs), which combine a potent and rapidly eliminated artemisinin derivative and a more slowly eliminated partner drug, have contributed significantly to the large global decline in *Plasmodium falciparum* morbidity and mortality.[2, 45, 90] However, artemisinin resistance, which manifests as slow parasite clearance resulting from reduced susceptibility of ring-stage parasites to artemisinins, has emerged and spread in Southeast Asia.[7, 23-26] Artemisinin resistance is associated with mutations in the *P. falciparum* Kelch gene on chromosome 13 (*Kelch13*). The reduction in artemisinin sensitivity has left partner drugs within the ACTs exposed to larger numbers of parasites after the initial 3 days of treatment, and this has facilitated the selection and spread of artemisinin and partner drug resistance.[33]

On the Myanmar-Thailand border the combination of artemisinin resistance and the reemergence of mefloquine resistance led to high failure rates following treatment of *Plasmodium falciparum* malaria with artesunate-mefloquine, forcing a change in policy.[30] In Cambodia and southern Vietnam, artemisinin and piperaquine resistance have led to high rates of treatment failures after dihydroartemisinin (DHA)-piperaquine.[29, 31, 47, 78, 91] The incidence of malaria has risen subsequently. New potent antimalarials, such as spiroindolones, imidazolopiperazines, and synthetic endoperoxides, are currently being tested in clinical trials, but their large-scale deployment is not expected to occur within the next 5 years.[92, 93] There is therefore an urgent need to use existing antimalarials in novel ways to counter the threat of multidrug resistance in *P. falciparum* in the Greater Mekong Subregion (GMS). Recombining three existing antimalarials in the form of Triple artemisinin-based combination therapies (Triple ACTs) could be an important option for the treatment of multidrug-resistant *Plasmodium falciparum* malaria.

The matching pharmacokinetic profiles of piperaquine and mefloquine should ensure antimalarial activity and mutual protection from both partner drugs throughout a large part of the elimination phase of the partner drugs. In addition, the Triple ACT DHA-piperaquine plus mefloquine takes advantage of an inverse relationship of piperaquine and mefloquine resistance observed in field and laboratory studies.[29, 31, 39, 40, 48, 78, 91, 94]

DHA-piperaquine is generally well tolerated, with most reported side effects being similar to and/or indistinguishable from malaria symptoms such as headache, gastrointestinal symptoms, and fatigue.[95] Mefloquine has neuropsychiatric side effects, including headache, dizziness, and sleeping disturbances, and gastrointestinal side effects, including nausea, vomiting, abdominal pain, and diarrhoea.[96] Nevertheless, it is generally well tolerated in the treatment of malaria. Piperaquine is known to cause dose-dependent electrocardiographic QT interval prolongation.[97] This has led to concerns about its proarrhythmic potential, but a recent large systematic review indicated that

the use of DHA-piperaquine does not increase the risk of sudden unexplained death.[98] QTc-interval prolongation has been reported after the treatment of *P. falciparum* with artesunate-mefloquine, but this does not correlate with mefloquine drug levels; thus, it may be attributable to a malarial rather than a drug effect.[99, 100]

In preparation for a multinational field trial on the safety, tolerability, and efficacy of the Triple ACT DHA-piperaquine plus mefloquine, we conducted a single-dose, sequential, open-label study in healthy volunteers to characterize the tolerability and the potential pharmacokinetic and pharmacodynamic interactions between dihydroartemisinin, piperaquine, and mefloquine in healthy Thai subjects.

MATERIAL AND METHODS

Sample size calculation

The primary focus was potential cardiotoxicity. DHA-piperaquine prolongs the QT interval corrected by Fridericia's formula (QTcF interval) by a mean of 10 ms (SD, 13 ms) (reanalysis of published data).[101] A sample size of 13 subjects would allow detection of a further increase of 15 ms with a power of 90% and α of 5%. The sample size was adjusted to a total of 16 subjects to allow for loss to follow-up and other unforeseen circumstances. A sample size of 11 (n = 11) volunteers would allow a paired t-test, applied to continuous pharmacokinetic variables (e.g., AUC_{LAST}, C_{max}, and T_{max}), to demonstrate a significant difference (power of 80% and α of 5%) when the standard deviation of the paired differences is not larger than the actual paired differences.

Study overview

Healthy male and female Thai subjects were enrolled in an open-label, sequential, single-dose study of orally administered DHA-piperaquine, DHA-piperaquine plus mefloquine, and mefloquine. The study was conducted in the healthy volunteer research ward at the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, and was approved by the Ethics Committee of the Faculty of Tropical Medicine (Mahidol University, Bangkok, Thailand) (TMEC 14-069) and the Oxford Tropical Research Ethics Committee (University of Oxford, Oxford, United Kingdom). The trial was registered at clinicaltrials.gov (NCT02324738).

Study subjects

Clinically healthy males and females, aged between 18 and 60 years, weighing between 36 and 75 kg, and willing to comply with the study protocol for the duration of the trial, were eligible for this study. All gave fully informed written consent. Exclusion criteria were a QTcF interval of ≥450 ms or a history of any cardiac disease or a family history of sudden cardiac death; positive hepatitis B, hepatitis C, or HIV serology; a creatinine clearance of <70 ml/min as determined by the Cockcroft-Gault equation; history of

alcohol or illicit substance abuse or dependence within 6 months of the study; use of prescription or non-prescription drugs (excluding paracetamol up to 2 g/day), vitamins, and herbal and dietary supplements within 7 days or 14 days for drugs known to have enzyme-inducing characteristics; participation in a clinical trial and receiving a new chemical entity within 30 days or twice the duration of the biological effect (whichever is longer); unwillingness to abstain from alcohol 48 h before and throughout the study; blood donation in the previous 30 days; a history of allergy to the study drugs; inability to comply with the study protocol; alanine aminotransferase (ALT) or aspartate aminotransferase (AST) levels of >1.5× the upper limit of normal; any history of renal disease, hepatic disease, and/or status after cholecystectomy; and antimalarial use in the previous 3 months. Also excluded were female subjects of child-bearing potential who could not comply with the use of effective methods of contraception during the study period until the end of the follow-up period, those who had a positive urine pregnancy test, and those who were lactating.

Study drug administration and study procedures

All subjects had participated in previous healthy volunteer studies in which they took 3 tablets of DHA-piperaquine (40/320 mg/tablet; Sigma-Tau) without any other medication (ClinicalTrials registration no. NCT01525511 and NCT02192944).[97, 101] The data obtained from that treatment round were used as a comparator for this study. The subjects were treated in 2 sequential rounds with the combination of DHA-piperaquine (3 tablets; 40/320 mg/tablet; Sigma Tau) and mefloquine (2 tablets; 250 mg base/tablet; Mequine; Thai Government Pharmaceutical Organization, Bangkok, Thailand), followed by mefloquine alone (2 tablets; 250 mg/tablet; Mequine). The two dosing rounds were separated by a wash-out period of at least 6 weeks. For every treatment round, the study drugs were administered under direct observation as a single oral dose after a standard light meal (a small cup of Thai-style porridge with chicken breast, around 200 calories, with less than 50% of the calories from fat) followed by 4 h of fasting. Fluids were restricted to 3 liters/day during the 24 h after the drug dosing. The use of illicit drugs and the intake of grapefruit or grapefruit juice was not allowed throughout the study periods. Alcohol and caffeine drinks were not allowed within 48 h prior to the study drug administration and during the admission. All subjects were admitted to the healthy volunteer research ward for a total of 2 nights and 1 day during each treatment round of the study for the clinical and pharmacokinetic evaluations. Medical history was documented, and a physical examination was performed by the study physicians before, during, and after the study. Blood tests, consisting of a complete blood count, and measurements of fasting blood sugar (FBS), serum creatinine, blood urea nitrogen, serum alkaline phosphatase, ALT, AST, total and direct bilirubin, creatine kinase, potassium, and sodium were performed at screening, at baseline, and 24 h after each drug dose. For women, a serum or urine pregnancy test was performed before each drug administration, and use of contraception was advised throughout the study period and for 4 weeks after the last dose of drugs to prevent pregnancy while using this new drug combination. An

electrocardiogram was performed at baseline and at 1, 2, 4, 8, 12, and 24 h after drug dosing (ECG-1250 Cardiofax S; Nihon Kohden, Tokyo, Japan). The QTc (QT interval corrected for heart rate) is calculate by using Fridericia's correction [QTcF = QT interval/3√(60/heart rate)] and Bazett's correction [QTcB = QT interval/√(60/heart rate)]. Symptom questionnaires, assessing the presence and severity (mild, moderate, severe, or life-threatening) of symptoms, were taken at baseline and 1, 4, and 24 h and 3 and 7 days after administration of the treatments with DHA-piperaquine plus mefloquine and mefloquine. This standard questionnaire was not used in the earlier studies. Adverse events were captured and graded according to the Division of AIDS table for grading the severity of adult and pediatric adverse events, version 1.0, December 2004, with clarification in August 2009.[102]

Pharmacokinetic study sample collection

Blood samples for drug plasma concentration measurements were taken at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, and 24 h and 3, 4, 7, 11, 15, 22, and 36 days after administration of the study drugs. All blood samples were obtained through an indwelling venous catheter during the first 24 h and by venipunctures at later time points. Blood samples were collected in fluoride-oxalate tubes (2 ml). Whole-blood samples were centrifuged for 7 min at 2,000 × *g* at 4°C to obtain plasma for DHA, mefloquine, and piperaquine concentration measurements. Plasma samples were stored immediately at −70°C or lower in a non-self-defrosting freezer until analyzed. All samples were transferred to the Department of Clinical Pharmacology, Mahidol-Oxford Tropical Medicine Research Unit, Bangkok, Thailand, for drug measurements.

Safety analysis

All subjects who received at least 1 dose of the study drug were included in the safety analysis. The safety and tolerability of DHA-piperaquine, mefloquine, and the combination of these 3 drugs were assessed by reporting the incidence of adverse events (AEs) and serious adverse events (SAEs) and comparison of the prolongation of QTcF and QTcB intervals for all study arms. QTcF and QTcB intervals were calculated using the QT interval and heart rate, as measured by the electrocardiogram machine. Statistical analysis of safety-related endpoints was performed using Stata v15.0 (StataCorp, College Station, TX, USA).

Drug analysis

Concentrations of piperaquine, dihydroartemisinin, and mefloquine were measured using methods validated according to U.S. FDA guidelines.[56, 103] Drug quantification was performed in a quality-controlled setting using liquid chromatography coupled with tandem mass spectrometry. In brief, plasma sample preparation was performed in a 96-well format on a Freedom EVO liquid handler system (Tecan, Männedorf, Switzerland) using an MPC-SD SPE plate (3M, Eagan, MN, USA) for piperaquine, Oasis HLB µElution SPE plate (Waters, Milford, MA, USA) for DHA, and a Phree phospholipids removal

plate (Phenomenex, Torrance, CA, USA) for mefloquine. Stable isotope-labeled internal standards were used to compensate for recovery and matrix effects. The extracted drugs were separated using a Dionex Ultimate 3000 UHPLC (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Gemini C_{18} column (Phenomenex) for piperaquine, a Hypersil Gold C_{18} column (Thermo Fisher Scientific) for DHA, and a Zorbax SB-CN (Agilent, Santa Clara, CA, USA) for mefloquine. An API500 Triple-quadrupole mass spectrometer and Analyst 1.7 software (ABSciex, Framingham, MA, USA) were used for drug detection and quantification. The coefficient of variation for the quality control (QC) samples was within the set limits and did not exceed 10% for any QC level. The lower limits of quantification were 1.96 ng/ml for DHA, 9.55 ng/ml for mefloquine, and 1.5 ng/ml for piperaquine.

Pharmacokinetic and pharmacodynamic analysis

A noncompartmental analysis, as implemented in the software Phoenix 64 (Certara, Princeton, NJ, USA), was performed to evaluate potential pharmacokinetic interactions of dihydroartemisinin, piperaquine, and mefloquine. Noncompartmental analyses were performed for all arms in the study. The observed concentrations were used to derive the maximum concentration (C_{max}) and the time to reach the maximum concentration (T_{max}). The drug exposure, measured as area under the concentration-time curve from administration of the drug until the last detectable drug concentration (AUC_{LAST}), was derived using the trapezoid method. Linear interpolation was used for ascending concentrations and log-linear interpolation for descending concentrations. The terminal elimination half-life ($t_{1/2}$) was estimated by $\ln 2/\lambda$, where λ is the terminal elimination rate constant, estimated from the log-linear best-fit regression of observed concentrations in the elimination phase. The terminal elimination rate constant was used to extrapolate AUC_{LAST} from the last observed concentration to infinity ($AUC_{inf} = C_{LAST}/\lambda$). The apparent elimination clearance (CL/F) and apparent volume of distribution (V/F) were calculated according to standard equations. Curve stripping was applied in the mefloquine-alone arm, as the wash-out period was not long enough to eliminate completely the mefloquine administered in the previous arm (mefloquine given together with DHA-piperaquine). Four individuals had samples collected for 24 h only after administration of DHA-piperaquine. Exposure to piperaquine for up to 24 h after dose (AUC_{24}) was estimated for these volunteers, and corresponding piperaquine data were truncated at 24 h in these volunteers when receiving DHA-piperaquine plus mefloquine. The individual AUC_{24} values were compared between treatment arms and included in the overall statistical evaluation of drug-drug interactions for piperaquine. Wilcoxon matched-pairs signed-rank tests were performed in GraphPad Prism to compare pharmacokinetic parameters when given alone and in combination with concomitant treatment. The geometric means (with 90% confidence interval) of the ratios of T_{max}, C_{max}, and AUC_{LAST} when given alone and in combination with other drugs were evaluated using the bioequivalence function in Phoenix 64 and were plotted in GraphPad Prism, v 8.1 (GraphPad Software Inc., CA, USA). Ordinary linear regression analysis (GraphPad

Prism, v 8.1) was used to quantify the relationship between drug concentrations and prolongation of QTcF interval, and the mean value and 95% confidence interval of the slope of the regression were compared between arms.

RESULTS

Study participants

Fifteen subjects (six males) was screened and subsequently enrolled in this study. The weight and body mass index (BMI) before the first dose of DHA-piperaquine ranged from 49.8 to 73.0 kg and 20.0 to 27.9 kg/m^2, respectively (table 1).

Table 1: Subject baseline demographics before drug administration

Variable	Result
Age (year)	41.6 (24.3–51.3)
Male (n/N (%))	6/15 (40)
Height (cm)	165 (144–178)
Body weight (kg)	61.6 (49.8–73.0)
QTcF interval (ms)	423 (407–439)
QTcB interval (ms)	429 (407–458)
Plasma aspartate aminotransferase (U/liter)	17 (13–24)
Plasma alanine aminotransferase (U/liter)	13 (9–32)
Plasma alkaline phosphatase (U/liter)	51 (35–70)
Plasma total bilirubin (micromol/liter)	6.8 (1.7–11.5)
Plasma creatinine (mol/liter)	70.7 (44.2–88.4)

QTcF is Fridericia-corrected QT intervals and QTcB is Bazett-corrected QT intervals.
Data are presented as medians (ranges) unless otherwise specified.

Safety

All subjects were included in the safety analyses. A total of 97 adverse events related to clinical symptoms were reported (table 2). Nearly all were graded as mild (95/97, 97.9%), and two were moderate in severity. A mild rise in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was documented in one subject after DHA-piperaquine plus mefloquine. Adverse events were attributed as possibly related to the study drugs in 72 out of 97 cases (74.2%), whereas the other events were judged as not related to the study drugs. In general, there was no difference in the incidence or severity of the study drug-related AEs when subjects were treated with DHA-piperaquine plus mefloquine or mefloquine. The most common adverse events after treatment with DHA-piperaquine plus mefloquine and mefloquine were mild to moderate dizziness, nausea, abdominal pain/discomfort, and disturbance of sleeping, which are all known side effects of mefloquine. One volunteer suffered from a moderate-severity neuropsychiatric reaction, as she reported anxiety, nausea, dizziness, and palpitations within 24 h after

the administration of DHA-piperaquine and mefloquine. These complaints resolved completely by day 3. There were 2 serious adverse events (SAEs) during the previous studies in which DHA-piperaquine was administered. One subject had a rickettsial infection at day 24 after drug administration causing hospitalization. Another subject was hospitalized at day 12 after drug administration due to unstable angina pectoris. Both SAEs were assessed as not related to the study interventions.

Cardiac effects

In assessment of the QT interval (see Fig. S1 in the supplemental material), Bazett's correction resulted in a significant overcorrection of QT intervals ($P < 0.0001$), whereas Fridericia's correction generated no residual trend in the corrected QT intervals versus heart rates. Baseline heart rates, QT interval corrected by Fridericia's formula (QTcF interval), and Bazett-corrected QT interval (QTcB) were similar before each of the interventions. A maximum increase of the QTcF and QTcB was seen 4 h after administration of both DHA-piperaquine and DHA-piperaquine plus mefloquine (table 3 and figure 1). No significant prolongation of either QTc interval was found after administration of mefloquine alone. The mean (standard deviation [SD]) increase of QTcF and QTcB intervals between baseline and hour 4 was not significantly different after DHA-piperaquine and DHA-piperaquine plus mefloquine: 4.2 (10.3) ms versus 3.5 (9.2) ms and 1.8 (11.4) ms versus 5.6 (10.3) ms, respectively. QTcF prolongations correlated positively with plasma piperaquine concentrations (figure 2), and this relationship was unaffected by the coadministration of mefloquine (figure 2A and figure 2B). Pooling all piperaquine data resulted in 1.08-ms QTcF prolongation per 100-ng/ml increase in piperaquine concentrations (figure 2C). QTcF prolongation did not correlate with mefloquine plasma concentrations (figure 2D).

Table 2: Clinical adverse events and serious adverse events

	DHA-piperaquine		DHA-piperaquine plus mefloquine		Mefloquine	
	Possibly related to study drugs	Not related to study drug	Possibly related to study drugs	Not related to study drug	Possibly related to study drugs	Not related to study drug
Central nervous system			9[c]	3	9	2
Cardiovascular		1[a] (1 SAE)	3		1	
Gastrointestinal			20		14	2
Musculoskeletal			1	4	3	1
Dermatology			0	2[d]		
Infections		5[b] (1 SAE)				
Sleeping disturbance			4	4	8	1
Total	0	6	37	13	35	6

Subjects treated with DHA-piperaquine alone did not undergo a systematic symptom questionnaire. a. Unstable angina pectoris at day 12.
b. One case of acute bronchitis, 2 of acute pharyngitis, 1 rickettsial infection, 1 viral infection of unknown origin. c. One neuropsychiatric reaction (anxiety, nausea, dizziness, and palpitations).d. One case of urticaria in subject with preexisting allergy to an unknown allergen.

Table 3: Mean changes compared to baseline in heart rate and QTc interval, stratified by drug regimen

	DHA-piperaquine			DHA+piperaquine plus mefloquine			Mefloquine		
Time point	ΔHR (bpm)	ΔQTcF (ms)	ΔQTcB (ms)	ΔHR (bpm)	ΔQTcF (ms)	ΔQTcB (ms)	ΔHR (bpm)	ΔQTcF (ms)	ΔQTcB (ms)
H1	2.2 (4.2)	-2.4 (8.2)	-0.3 (9.9)	**3.1 (3.7)**	**-8.2 (7.8)**	**-4.9 (8.3)**	2.1 (3.4)	-4.8 (12.2)	-2.6 (12.2)
H2	0.1 (4.5)	-4.0 (9.1)	-4.0 (10.9)	**2.9 (4.0)**	**-4.7 (5.8)**	-1.5 (7.0)	0.8 (3.3)	**-6.0 (6.0)**	**-5.1 (7.7)**
H4	-2.0 (4.6)	4.2 (10.3)	1.8 (11.4)	1.7 (3.7)	3.5 (9.2)	5.6 (10.3)	-0.1 (3.1)	-1.6 (7.0)	-1.7 (8.3)
H8	**2.9 (4.8)**	-4.0 (21.6)	-1.1 (22.3)	**4.2 (3.3)**	-1.4(8.2)	3.1 (9.5)	**6.0 (4.8)**	**-10.8 (10.4)**	-4.7 (12.7)
H12	2.6 (6.4)	-5.0 (16.5)	-2.3 (19.8)	**5.7 (3.1)**	-1.3 (9.7)	4.8 (9.9)	**6.9 (5.3)**	**-7.6 (9.0)**	-0.6 (12.4)

Changes in heart rate (ΔHR), ΔQTcF, and ΔQTcB between baseline and up to 12 h after administration of DHA-piperaquine alone, DHA-piperaquine plus mefloquine, and mefloquine alone. Data are presented as mean (SD). Significant changes (by paired t test) compared to baseline are indicated in bold.

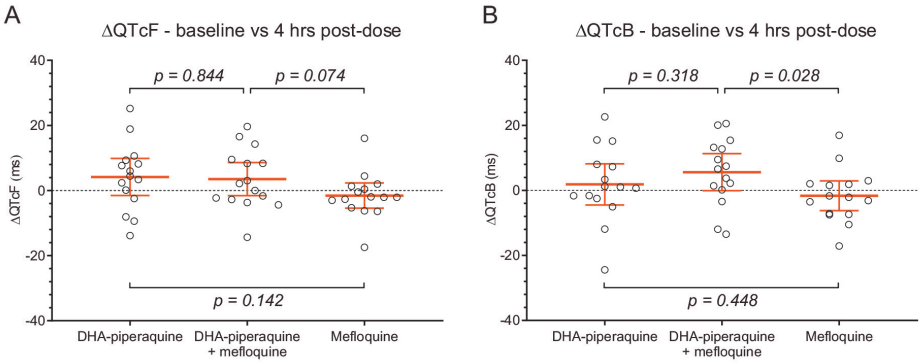

Figure 1. Changes in the electrocardiogram QTcF (A) and QTcB (B) between baseline and 4 h after administration of DHA-piperaquine alone, DHA-piperaquine plus mefloquine, and mefloquine alone. Open circles are observed changes in QTc intervals, and solid red lines are mean values ± 95% confidence intervals

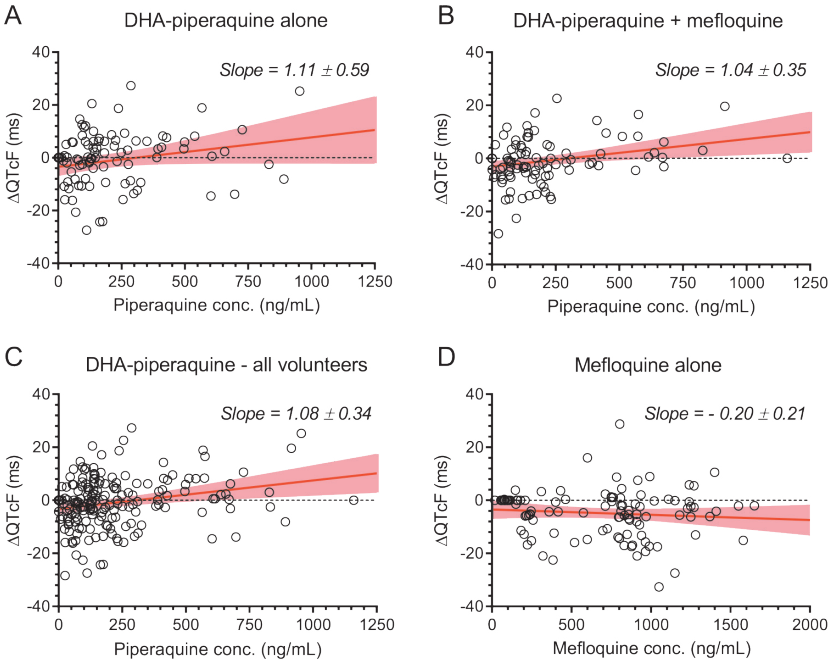

Figure 2. Ordinary linear regression of QTcF interval prolongations (ΔQTcF) versus piperaquine drug concentrations (A, B, and C) and versus mefloquine drug concentrations (D). (A and B) The relationship between piperaquine drug concentrations and ΔQTcF in healthy volunteers receiving DHA-piperaquine alone (A) and DHA-piperaquine plus mefloquine (B). (C)Relationship between piperaquine drug concentrations and ΔQTcF for all volunteers receiving DHA-piperaquine (all arms). (D) Relationship between mefloquine drug concentrations and ΔQTcF for volunteers receiving mefloquine alone. Open circles are observed ΔQTcF at specific drug concentrations. Slopes are displayed as mean regression lines (solid red lines) and 95% confidence intervals (shaded area) and as mean values ± standard errors.

Pharmacokinetic analysis

Pharmacokinetic parameter estimates for dihydroartemisinin, piperaquine, and mefloquine when given alone and in combination are presented in table 4. Coadministration of mefloquine did not significantly impact the pharmacokinetic properties of piperaquine (maximum concentration of drug in serum [C_{max}], −0.82% [90% confidence interval, or CI, −22.9, 27.6; $P = 0.888$]; area under the concentration-time curve from administration of the drug until the last detectable drug concentration [AUC_{LAST}], −10.3% [90% CI, −25.1, 7.30; $P = 0.239$]; time to maximum concentration of drug in serum [T_{max}], −8.83% [90% CI, −21.4, 5.77; $P = 0.301$]) (figure 3 and figure 4). One individual in the mefloquine-alone arm was removed, as he had much lower plasma concentration than the rest of the patients (C_{max}, after curve stripping, for this individual was 95.0 ng/ml compared to a median of 1,040 ng/ml for the other individuals in this arm). The reason for the very low concentrations are unknown, but this was in the mefloquine-alone arm, so a drug-drug interaction can be excluded. The same participant had normal mefloquine concentrations when given mefloquine with DHA-piperaquine, suggesting normal distribution and elimination properties. Except for a significantly shorter time to peak levels of mefloquine (T_{max}, −30.8% [90% CI, −45.9, −11.6; $P = 0.0475$]), there was also no significant impact on the pharmacokinetic properties of mefloquine when given with DHA-piperaquine (C_{max}, 9.44% [90% CI, −3.97, 24.7; $P = 0.348$]; AUC_{LAST}, −6.17% [90% CI, −18.8, 8.39; $P = 0.350$]). However, coadministration of DHA-piperaquine and mefloquine did result in a significantly lower exposure to dihydroartemisinin and a longer time to peak levels of dihydroartemisinin (C_{max}, −29.0% [90% CI, −40.6, −15.1; $P = 0.0022$]; AUC_{LAST}, −22.6% [90% CI, −33.1, −10.4; $P = 0.0039$]; T_{max}, 34.0% [90% CI, 9.87, 63.5; $P = 0.0079$]).

Table 4: Pharmacokinetic parameter estimates for 15 healthy volunteers stratified by drug regimen

Parameter and regimen component	DHA-piperaquine alone	Mefloquine alone	Co-administered	P value
Dihydroartemisinin				
Tmax (h)	1.00 (1.00-2.00)		1.50 (0.500-3.00)	**0.0112**
Cmax (ng/ml)	387 (184-792)		275 (124-510)	**0.0026**
AUClast (h*mg/ml)	901 (394-2000)		673 (360-1550)	**0.0151**
AUCinf (h*mg/ml)	908 (398-2030)		684 (365-1580)	**0.0151**
t1/2 (h)	2.03 (1.13-2.60)		1.94 (1.06-2.26)	0.0637
CL/F (liters/h)	132 (59-302)		176 (75.8-329)	0.0413
V/F (liters)	337 (164-678)		436 (197-846)	0.0946
Piperaquine				
Tmax (h)	4.00 (2.00-4.00)		3.00 (2.00-4.00)	0.438
Cmax (ng/ml)	539 (240-1040)		631 (229-1160)	0.668
AUClast (h*mg/ml)	17.1[a] (8.11-36.8)		16.8 (7.36-27.7)	0.277
AUCinf (h*mg/ml)	19.8[a] (12.2-58.5)		22.4 (8.81-32.5)	0.966
t1/2 (h)	12.7[a] (7.35-36.6)		13.7 (6.97-53.0)	0.7
CL/F (liters/h)	25.9[a] (8.79-42.7)		24.7 (17.1-62.9)	0.365
V/F (liters)	9600[a] (7500-33900)		16100 (4660-34300)	0.32
Mefloquine				
Tmax (h)		6.00 (4.00-10.0)	4.00 (2.00-10.0)	0.0679
Cmax (ng/ml)		1040 (711-1580)	1110 (854-1450)	0.358
AUClast (h*mg/ml)		334 (261-596)	295 (232-518)	0.194
AUCinf (h*mg/ml)		431 (342-1050)	386 (330-1160)	0.241
t1/2 (h)		16.4 (12.7-28.6)	19.4 (13.0-32.9)	0.357
CL/F (liters/h)		1.16 (0.475-1.46)	1.29 (0.432-1.51)	0.173
V/F (liters)		638 (48-965)	721 (484-1160)	0.194

Values are presented as median (min-max). Tmax is the time to reach maximum concentration, Cmax is the maximum concentration, AUCLAST is total exposure up to the last observation, AUCinf is the total exposure extrapolated to infinity, t1/2 is the terminal elimination half-life, CL/F is the apparent elimination clearance, and V/F is the apparent volume of distribution. The P value was obtained from the Wilcoxon matched-pairs signed-rank test. a. Based on 15 volunteers. Four volunteers were sampled for 24 h after dose administration and therefore were excluded from this parameter summary but included in the statistical analysis.

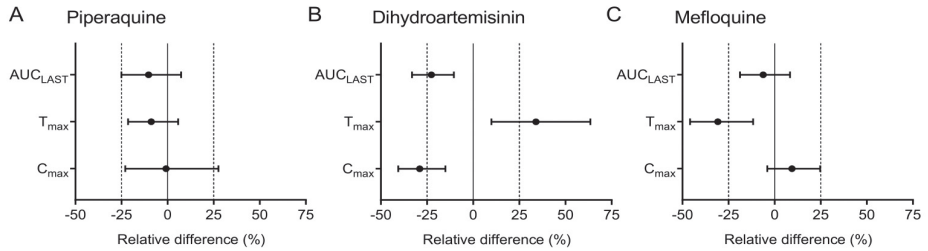

Figure 3. Forest plots showing the geometric mean pharmacokinetic parameter ratios based on 15 individuals (14 for mefloquine) and the corresponding 90% confidence interval for the drugs given alone or in combination with other drugs. AUCLAST represents the area under the concentration-time curve from time zero to the last measurable concentration. Cmax is the maximum concentration, and Tmax is the time to reach the maximum concentration. Solid vertical lines represent no interaction (zero difference), while vertical dashed lines represent a clinically relevant effect of ± 25% relative difference. (A) Piperaquine pharmacokinetic parameter ratios when DHA-piperaquine is given alone and in combination with mefloquine. (B) DHA pharmacokinetic parameters when DHA-piperaquine is given alone and in combination with mefloquine. (C) Mefloquine pharmacokinetic parameters when mefloquine is given alone and in combination with DHA-piperaquine.

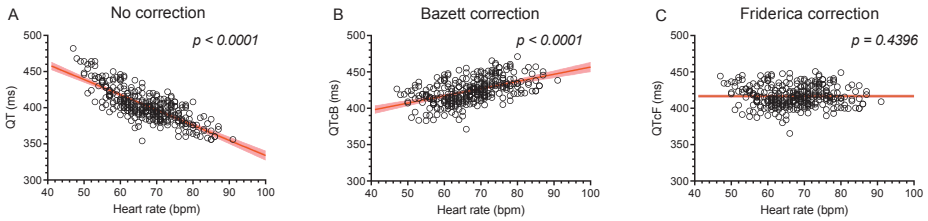

Figure 4. Ordinary linear regression of observed QT intervals and heart rates, when applying (A) no correction, (B) Bazett's correction, and (C) Fridericia's correction. Open circles are observed QT(c) intervals at specific heart rates. Slopes are displayed as mean regression lines (solid red lines) and 95% confidence intervals (shaded areas), and p-values demonstrate if the regression line deviates significantly from zero.

DISCUSSION

In extensive clinical use, DHA-piperaquine has proved a well-tolerated and highly effective antimalarial drug. The main clinical concern has been the effect of piperaquine on ventricular repolarization (manifest by electrocardiographic QT prolongation), although recent large studies suggest that this does not translate into an increased risk of lethal ventricular tachyarrhythmias.[97] Halofantrine, an antimalarial drug that has now been withdrawn, caused marked QT prolongation and did cause lethal ventricular tachyarrhythmias.[104] This effect was accentuated by concomitant exposure to mefloquine. Naturally, there was concern that adding mefloquine to piperaquine could be dangerous, but fortunately there was no evidence of additional QT prolongation. As expected, DHA-piperaquine prolonged the QTcF and QTcB intervals, with a maximum effect at peak levels around 4 h postdose, but the addition of mefloquine did not lead to an increase of QTc (QT interval corrected for heart rate) prolongation. One subject suffered from a moderate-severity neuropsychiatric reaction after DHA-piperaquine plus mefloquine, reflecting the known, neuropsychiatric side effects of mefloquine. A recent pooled analysis found the risk of neuropsychiatric events to be 7.6/10,000 treatments (95% CI, 4, 12).[96]

There is a potential for drug-drug interactions between mefloquine and piperaquine, since both drugs are metabolized by cytochrome P450 (CYP) 3A4, which can be inhibited by piperaquine according to *in vitro* studies.[105, 106] However, this study showed no clinically relevant interactions. Only the time to reach the maximum concentration showed a significant difference. However, the pharmacokinetic properties of dihydroartemisinin were affected by co-administration of mefloquine, a drug-drug interaction that could be of clinical significance. The total exposure to dihydroartemisinin was 23% lower (90% CI, −33.1, −10.4) when combined with mefloquine. The reason for this is unknown, but it could be a consequence of altered absorption or metabolism/elimination of the drug. Data generated here do not provide any insight into this effect, and further studies are needed to understand the underlying mechanism of this interaction. However, previous studies in healthy volunteers (n = 10) and patients (n = 207) with uncomplicated falciparum malaria in Thailand demonstrated no pharmacokinetic drug-drug interactions when administering dihydroartemisinin and mefloquine alone or in combination.[107, 108] The dose of dihydroartemisinin in the DHA-piperaquine combination is already relatively low (2.25 to 2.50 mg/kg of body weight) compared to that of other ACTs (4 mg/kg), so it will be important to assess if this interaction has clinical relevance in the larger series of patients.

The power of multidrug approaches for the management of infections is evident from high efficacy and the relatively slow emergence and spread of resistance to therapies for HIV and tuberculosis. The chance of a malaria parasite developing resistance to two antimalarials *de novo* is small, provided that the mechanisms are unrelated and not conferred by the same mutation. Combining three antimalarials reduces the risk even

further. In the case of Triple ACTs, the artemisinins kill a large amount of parasites in the first 3 days of treatment. Any remaining parasites that normally would face only one partner drug would now be exposed to two partner drugs, reducing the chance of treatment failures and providing mutual protection against resistance. In the short term, the combination of DHA-piperaquine plus mefloquine could lead to a restored efficacy in areas affected by resistance to piperaquine (eastern GMS) or mefloquine (eastern Myanmar). In the longer term, deployment of Triple ACTs could slow down or prevent the emergence of artemisinin and partner drug resistance.

It is likely that artemisinin resistance will compromise the efficacy of ACTs containing novel partner drugs and will contribute to selection of resistance against partner drugs that are still under development or have only been deployed on a small scale. For example, the artesunate and pyronaridine (Pyramax) combination had failure rates of up to 16.0% and 10.2% in two sites in western Cambodia, an area with a high prevalence of artemisinin resistance, despite high efficacy in other African and Asian countries.[70, 109] Therefore, combining any novel antimalarial with two other antimalarials in order to prolong the longevity of novel and existing compounds warrants further consideration. The wash-out period between each regimen was 6 weeks only and was therefore too short to eliminate the drugs completely. The median (range) mefloquine concentration was 87.9 (17.9 to 179) ng/ml at the end of the 6-week wash-out period. The median (range) maximum concentration when giving mefloquine alone was 1,095 ng/ml (866 to 1,650), without curve stripping, indicating that the predose concentrations contributed approximately 8% to the total peak concentration (median [range] of 7.53% [1.67% to 18.0%]). However, the application of curve stripping corrected for this bias and allowed the pharmacokinetic parameters to be compared when mefloquine was given alone and in combination with DHA-piperaquine. Another limitation was that all subjects were recruited from separate earlier studies, making use of existing DHA-piperaquine-alone arms. Although this design did not allow us to perform a complete study arm randomization, we acknowledge that randomization of the treatment sequence of DHA-piperaquine plus mefloquine and mefloquine alone could have helped to minimize the potential confounding carryover effect. However, this design permitted us to reuse available data and, therefore, reduce the duration and cost of the study as well as potential discomfort from drug administrations and sampling in healthy volunteers.

This pharmacokinetic/pharmacodynamic study was conducted according to current European Medicines Agency and U.S. FDA guidance, and we believe that the data generated are important and increase insights into safety of the proposed Triple ACT. However, malaria has been shown to have a substantial impact on the QT interval and may lead to false conclusions on QT prolongation properties of antimalarial drugs. Potential drug-drug interactions might also be somewhat different in malaria patients than healthy volunteers due to the acute disease effect associated with malaria during the first days of treatment. Thus, the findings in this study need to be confirmed in patients with malaria.

CONTRIBUTORS

RWvdP, BH, RH, SP, MM, NW, KC, PS, NJW, AMD, JT and PJ designed the study or were involved in the organisation of the trial, or both. RWvdP, BH and PJ recruited the study participants and collected samples. or took part in laboratory work at the study site or in the central laboratories. RMH, MW, JT generated or analysed the pharmacological data, or both. RWvdP, BH, SP, MM, NJW, AMD and PJ wrote the manuscript.

ACKNOWLEDGMENTS

We are grateful to the volunteers and the nurses of the healthy volunteer research ward, Hospital for Tropical Diseases, Bangkok, Thailand. We are grateful to Thatsanun Ngernseng for data management support. The study was funded by the Department for International Development (DFID). The Mahidol Oxford Tropical Medicine Research group is funded by the Wellcome Trust. The funders had no part in the study design, implementation, or analysis of the study or in the decision to publish the results. The investigators/authors from the Faculty of Tropical Medicine and the Mahidol-Oxford Tropical Medicine Research Unit declare no conflicts of interest. The pharmacokinetic analysis was funded through a grant from the Bill & Melinda Gates Foundation to Joel Tarning. The study was funded by the Department for International Development (DFID) and the Bill & Melinda Gates Foundation and registered on ClinicalTrials.gov (NCT02324738). The MORU research program is supported by the Wellcome Trust.

Triple artemisinin-based combination therapies versus artemisinin-based combination therapies for uncomplicated Plasmodium falciparum malaria: a multicentre, open-label, randomised clinical trial

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Lancet. April 2020; 25;395(10233): 1345-1360.
DOI: 10.1016/S0140-6736(20)30552-3

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SUMMARY

Background

Artemisinin and partner-drug resistance in *Plasmodium falciparum* are major threats to malaria control and elimination. Triple artemisinin-based combination therapies (Triple ACTs), which combine existing co-formulated ACTs with a second partner drug that is slowly eliminated, might provide effective treatment and delay emergence of antimalarial drug resistance.

Methods

In this multicentre, open-label, randomised trial, we recruited patients with uncomplicated *P. falciparum* malaria at 18 hospitals and health clinics in eight countries. Eligible patients were aged 2–65 years, with acute, uncomplicated *P. falciparum* malaria alone or mixed with non-*falciparum* species, and a temperature of 37·5°C or higher, or a history of fever in the past 24 h. Patients were randomly assigned (1:1) to one of two treatments using block randomisation, depending on their location: in Thailand, Cambodia, Vietnam, and Myanmar patients were assigned to either dihydroartemisinin–piperaquine or dihydroartemisinin–piperaquine plus mefloquine; at three sites in Cambodia they were assigned to either artesunate–mefloquine or dihydroartemisinin–piperaquine plus mefloquine; and in Laos, Myanmar, Bangladesh, India, and the Democratic Republic of the Congo they were assigned to either artemether–lumefantrine or artemether–lumefantrine plus amodiaquine. All drugs were administered orally and doses varied by drug combination and site. Patients were followed-up weekly for 42 days. The primary endpoint was efficacy, defined by 42-day PCR-corrected adequate clinical and parasitological response. Primary analysis was by intention to treat. A detailed assessment of safety and tolerability of the study drugs was done in all patients randomly assigned to treatment. This study is registered at ClinicalTrials.gov, NCT02453308, and is complete.

Findings

Between Aug 7, 2015, and Feb 8, 2018, 1100 patients were given either dihydroartemisinin–piperaquine (183 [17%]), dihydroartemisinin–piperaquine plus mefloquine (269 [24%]), artesunate–mefloquine (73 [7%]), artemether–lumefantrine (289 [26%]), or artemether–lumefantrine plus amodiaquine (286 [26%]). The median age was 23 years (IQR 13 to 34) and 854 (78%) of 1100 patients were male. In Cambodia, Thailand, and Vietnam the 42-day PCR-corrected efficacy after dihydroartemisinin–piperaquine plus mefloquine was 98% (149 of 152; 95% CI 94 to 100) and after dihydroartemisinin–piperaquine was 48% (67 of 141; 95% CI 39 to 56; risk difference 51%, 95% CI 42 to 59; p<0·0001). Efficacy of dihydroartemisinin–piperaquine plus mefloquine in the three sites in Myanmar was 91% (42 of 46; 95% CI 79 to 98) versus 100% (42 of 42; 95% CI 92 to 100) after dihydroartemisinin–piperaquine (risk difference 9%, 95% CI 1 to 17; p=0·12). The 42-day PCR corrected efficacy of dihydroartemisinin–piperaquine plus mefloquine

(96% [68 of 71; 95% CI 88 to 99]) was non-inferior to that of artesunate–mefloquine (95% [69 of 73; 95% CI 87 to 99]) in three sites in Cambodia (risk difference 1%; 95% CI −6 to 8; p=1·00). The overall 42-day PCR-corrected efficacy of artemether–lumefantrine plus amodiaquine (98% [281 of 286; 95% CI 97 to 99]) was similar to that of artemether–lumefantrine (97% [279 of 289; 95% CI 94 to 98]; risk difference 2%, 95% CI −1 to 4; p=0·30). Both Triple ACTs were well tolerated, although early vomiting (within 1 h) was more frequent after dihydroartemisinin–piperaquine plus mefloquine (30 [3·8%] of 794) than after dihydroartemisinin–piperaquine (eight [1·5%] of 543; p=0·012). Vomiting after artemether–lumefantrine plus amodiaquine (22 [1·3%] of 1703) and artemether–lumefantrine (11 [0·6%] of 1721) was infrequent. Adding amodiaquine to artemether–lumefantrine extended the electrocardiogram corrected QT interval (mean increase at 52 h compared with baseline of 8·8 ms [SD 18·6] *vs* 0·9 ms [16·1]; p<0·01) but adding mefloquine to dihydroartemisinin–piperaquine did not (mean increase of 22·1 ms [SD 19·2] for dihydroartemisinin–piperaquine *vs* 20·8 ms [SD 17·8] for dihydroartemisinin–piperaquine plus mefloquine; p=0·50).

Interpretation

Dihydroartemisinin–piperaquine plus mefloquine and artemether–lumefantrine plus amodiaquine Triple ACTs are efficacious, well tolerated, and safe treatments of uncomplicated *P. falciparum* malaria, including in areas with artemisinin and ACT partner-drug resistance.

Funding

UK Department for International Development, Wellcome Trust, Bill & Melinda Gates Foundation, UK Medical Research Council, and US National Institutes of Health.

INTRODUCTION

Artemisinin-based combination therapies (ACTs) have contributed substantially to the reduction in the global burden of malaria.[110] However, progress is now threatened by the emergence and spread of artemisinin and ACT partner-drug resistance in Southeast Asia.[25, 67] The combination of resistance first to artemisinins and then to the ACT partner drugs, including piperaquine, mefloquine, amodiaquine, and sulfadoxine–pyrimethamine, has led to unacceptably high rates of treatment failure (ie, recrudescent infections) with artesunate–mefloquine on the Thailand–Myanmar border and dihydroartemisin-piperaquine in Cambodia, eastern Thailand, and southern Vietnam. [30, 84] Molecular epidemiology studies show that the failure of dihydroartemisinin–piperaquine is caused by a single lineage of a multidrug-resistant parasite that has spread across Cambodia, northeastern Thailand, southern Laos, and southern Vietnam.[49] The emergence of multidrug resistance has forced a series of treatment policy changes but progressively fewer treatment options for *Plasmodium falciparum* malaria are available in

the Greater Mekong subregion. Yet, new compounds will not become generally available within the next few years.[111] A major concern is the potential spread of ACT resistance to the Indian subcontinent and sub-Saharan Africa. In the past, chloroquine resistance and sulfadoxine-pyrimethamine resistance that emerged in Southeast Asia spread to sub-Saharan Africa and contributed to millions of childhood deaths.[20, 21, 73] Combining drugs with different mechanisms of action to prevent the emergence and spread of antimicrobial resistance is a widely accepted approach, for instance, for the treatment of tuberculosis and infections caused by HIV, *Helicobacter pylori*, and multidrug-resistant bacteria.[112] This principle of combining drugs also underlies ACTs, but the slowly eliminated component is unprotected by the rapidly eliminated artemisinin component after the third day of treatment. Triple artemisinin-based combination therapies (Triple ACTs), which combine a conventional ACT with a second slowly eliminated partner drug, add additional antimalarial activity and provide mutual protection for the partner drugs.[113] Furthermore, combining piperaquine with mefloquine and lumefantrine with amodiaquine potentially exploits counterbalancing resistance mechanisms.[29, 39-43] We did a multicentre randomised controlled trial comparing the efficacy, safety, and tolerability of two Triple ACTs, dihydroartemisinin–piperaquine plus mefloquine and artemether–lumefantrine plus amodiaquine, compared with their corresponding standard ACTs.

RESEARCH IN CONTEXT

Evidence before this study

We searched PubMed for articles published from database inception until Jan 30, 2020, using the terms “resistance” AND “malaria” AND “Triple”, which resulted in 265 articles. One study in healthy adult volunteers from Thailand reported no difference in the extension of electrocardiogram corrected QT interval after treatment with dihydroartemisinin–piperaquine compared with dihydroartemisinin–piperaquine plus mefloquine. In that study, coadministration of mefloquine with dihydroartemisinin–piperaquine reduced exposure to dihydroartemisinin.

A modelling study published in 2018 modelled the potential for dihydroartemisinin–piperaquine plus mefloquine (with mefloquine given as three doses of 6·7 mg/kg) to achieve parasitological efficacy, even in the scenario in which resistance to dihydroartemisinin and piperaquine had emerged. To date, no studies reporting the safety, tolerability, and efficacy of Triple artemisinin-based combination therapies (Triple ACTs) in the clinical setting have been reported.

Added value of this study

To our knowledge, this is the first clinical study to assess the safety, tolerability, and efficacy of two Triple ACTs that combine three existing antimalalarial drugs—

dihydroartemisinin– piperaquine plus mefloquine and artemether–lumefantrine plus amodiaquine—compared with currently used ACTs for the treatment of uncomplicated *Plasmodium falciparum* malaria. Incidence of vomiting within the first hour of treatment with both Triple ACTs were low, but adding mefloquine or amodiaquine to the existing ACTs was associated with a slight increase in the incidence of vomiting. Amodiaquine extended the QT interval, but not to the extent associated with cardiac arrhythmias, and overall the two Triple ACTs were safe and well tolerated. Dihydroartemisinin–piperaquine plus mefloquine and artesunate–mefloquine were also very effective in Cambodia, Thailand, and Vietnam, areas where dihydroartemisinin– piperaquine is no longer effective because of high prevalence of both artemisinin and piperaquine resistance. Although adding amodiaquine reduced exposures to lumefantrine, artemether, and its active metabolite dihydroartemisinin, the artemether–lumefantrine plus amodiaquine Triple ACT was highly effective.

Implications of all the available evidence

Triple ACTs are a safe, well tolerated, efficacious, and a readily available new option for the treatment of uncomplicated *P. falciparum* malaria and could improve treatment outcomes in areas with increasing artemisinin and partner-drug resistance in the Greater Mekong subregion. In areas where such resistance has not yet emerged, deployment of Triple ACTs might delay the emergence and spread of resistance.

METHODS

Study design and participants

In this multicentre, open-label, randomised controlled trial, we recruited patients from 18 hospitals and health clinics in eight countries: Cambodia (four sites), Thailand (three sites), Myanmar (four sites), India (three sites), Laos, Vietnam, Bangladesh, and the Democratic Republic of the Congo (one site each). Patients presented directly to the study sites with fever or were referred by malaria field workers after pre-screening with a malaria rapid diagnostic test. Eligible patients were aged 2–65 years, with acute, uncomplicated *P. falciparum* malaria alone or mixed with non-*falciparum* species, and a temperature of 37·5°C or higher, or a history of fever in the past 24 h. In Democratic Republic of the Congo, only children younger than 12 years were eligible for inclusion. A further inclusion criterion was a parasitaemia of 5000–200 000 parasites per µL of blood, except in the Democratic Republic of the Congo where the range was 10 000–250 000 parasites per µL of blood, and in Cambodia where any parasitaemia of <200 000 parasites per µL of blood was allowed. Exclusion criteria were a contraindication to any study drug, the use of artemisinins in the previous 7 days, a previous splenectomy, pregnancy or breastfeeding, an electrocardiogram (ECG) corrected QT (QTc) interval of more than 450 ms, a documented or self-reported history of cardiac conduction problems, a low haematocrit (<25% in Asian sites and <15% in the Democratic Republic of the Congo),

and participation in clinical trials in the previous 3 months. Written informed consent was obtained from all participants before any study procedures were done. The protocol was approved by the Oxford Tropical Research Ethics Committee and for each site by the relevant institutional review board, national ethics committee, or both. The trial was monitored by the Mahidol-Oxford Tropical Medicine Research Unit Clinical Trials Support Group.

Randomisation and masking

Patients were randomly assigned (1:1) to one of two treatments at all study sites. The randomisation sequence was generated by an independent statistician in blocks of 8, 10, and 12 for all sites. Study number and treatment allocation codes were provided in sequentially numbered opaque envelopes. The comparator treatment was the first-line ACT in that area at the time of the start of the trial. Patients in Thailand, three sites in Cambodia (Pursat, Pailin, and Ratanakiri), Vietnam, and three sites in Myanmar (Thabeikkyin, Pyay, and Ann) were randomly assigned to either dihydroartemisinin–piperaquine or dihydroartemisinin–piperaquine plus mefloquine. In two Cambodian sites (Pursat and Pailin) the comparator treatment was changed from dihydroartemisinin–piperaquine to artesunate–mefloquine after recruitment of 19 patients at the Pursat site and 20 at the Pailin site because of very high failure rates with dihydroartemisinin–piperaquine. In Preah Vihear, the final site in Cambodia, which started later than the other three sites, artesunate–mefloquine was used as the comparator treatment throughout the study. In Laos, Bangladesh, India, Democratic Republic of the Congo, and one site in Myanmar (Pyin Oo Lwin), patients were randomly assigned to either artemether–lumefantrine or artemether–lumefantrine plus amodiaquine. Study staff, patients, and investigators were unmasked to treatment assignment, while laboratory staff were masked.

Procedures

All drugs were administered orally, directly observed by a member of the study team. Artemether–lumefantrine containing study drugs were given with a fatty snack to improve absorption of lumefantrine. The ACTs artemether–lumefantrine and dihydroartemisinin–piperaquine were dosed according to WHO guidelines.[54] All treatments were given as three (for piperaquine and mefloquine containing treatments) or six (for lumefantrine and amodiaquine containing treatments) doses. The target dose of amodiaquine was 10 mg base per kg per day, administered as a split-dose twice daily (together with artemether–lumefantrine). The target dose of mefloquine was 8 mg/kg per day, administered as a once daily dose (together with dihydroartemisinin–piperaquine). Mefloquine doses in the artesunate–mefloquine and dihydroartemisinin–piperaquine plus mefloquine study groups were similar. A single low dose of the gametocytocidal drug primaquine was given after 24 h (0·25 mg base per kg, except for age-based dosing in India, in accordance with national policies). Dosing schedules are detailed in the appendix of the published manuscript.

A full dose of study drug was readministered in case of vomiting within 30 min, or a half dose if vomiting occurred 30–60 min after administration. All patients were admitted to the study site for at least 3 consecutive nights and followed up on day 7, and thereafter weekly up to day 42. A standardised symptom questionnaire and physical examination, including heart rate, blood pressure, respiratory rate, and tympanic temperature, were done and recorded on each day of admission and each day of follow-up. In case of the development of signs indicating severe malaria or an increase in parasitaemia 12 h after start of antimalarial therapy, rescue treatment with intravenous artesunate was started. Adverse events were graded according to the Division of AIDS table for Grading the Severity of Adult and Paediatric Adverse Events (version 2.0).[102] Laboratory abnormalities were graded according to preprepared tables. A 12-lead ECG was done at screening, baseline, and 4 h, 48 h, and 52 h after drug administration. In patients treated with dihydroartemisinin–piperaquine, ECGs were also done 24 h and 28 h after drug administration, and at the Indian sites also at 60 h and 64 h after drug administration. Recurrent infections were treated with an alternative ACT or a different drug combination.

Asexual *P. falciparum* densities were counted with microscopy at screening, baseline, and at 4 h, 6 h, 8 h, and 12 h after drug administration, and thereafter every 6 h until two Giemsa-stained consecutive blood films were negative. Asexual parasite clearance half-lives were determined on site with the WorldWide Antimalarial Resistance Network parasite clearance estimator,[62] with a cutoff of 5 h defining an extended parasite clearance half-life. Biochemistry measurements (serum alanine aminotransferase, aspartate aminotransferase, total bilirubin, alkaline phosphatase, and creatinine) and full blood count or haemoglobin measurements, or both, were done at baseline, and at days 3, 7, and 28 after drug administration. Blood samples were taken at baseline, day 7, and at any recurrent infection in all patients for measurement of antimalarial drug concentration (piperaquine, lumefantrine and its active metabolite desbutyl-lumefantrine, and artemether and its active metabolite dihydroartemisinin). Additional dense pharmacokinetic sampling was done in the first 20 patients given ACT and the first 20 patients given Triple ACT at one site using artemether–lumefantrine with and without amodiaquine (Ramu, Bangladesh) and at one site using dihydroartemisinin–piperaquine with and without mefloquine (Binh Phuoc, Vietnam) at 1, 2, 4, 6, 8, 12, 24, 52 h, and day 4, 7, and 28 (and day 42 for patients given dihydroartemisinin–piperaquine plus mefloquine) after administration of the first dose of the study drug. Plasma samples were shipped on dry ice to the Department of Clinical Pharmacology, Mahidol-Oxford Tropical Medicine Unit at Mahidol University (Bangkok, Thailand) for measurement of antimalarial drug concentration. Concentrations of artemether, dihydroartemisinin, piperaquine, lumefantrine, and desbutyl-lumefantrine were measured using validated liquid chromatography-mass spectroscopy methods and standard procedures.[56, 103, 114] Molecular markers of resistance to artemisinins (*P. falciparum Kelch 13* mutations [*kelch13*]), piperaquine (*plasmepsin2/3* gene amplification) and mefloquine (*mdr1* gene

amplification) were assessed as described previously.[59, 77, 84] *Kelch13* mutations Y493H, R539T, R561H, and C580Y have previously been associated with slow parasite clearance. *Kelch13* A578S has been shown not to be associated with slow parasite clearance.[63] Recurrent infections were classified as recrudescent infections if all *msp1*, *msp2*, and *glurp*alleles matched those that were present at baseline.[61]

Outcomes

The primary outcome was efficacy, defined by the 42-day PCR-corrected adequate clinical and parasitological response (ACPR) of Triple ACTs and ACTs within each site.[61] Day-42 PCR corrected ACPR was analysed by intention-to-treat (ITT) analysis, and supported by per-protocol and Kaplan-Meier survival analyses. Secondary outcomes were the differences in parasite clearance half-lives stratified by *kelch13* mutation status, PCR uncorrected 42-day ACPR (including patients with *P. falciparum* reinfection), incidence of vomiting within 1 h after study drug administration, fever clearance time (time until a temperature of <37·5°C), incidence of adverse events and serious adverse events, extension of the Bazett's QTc-interval (QTcB-interval), extension of the QTc-interval of more than 500 ms or of more than 60 ms compared with baseline, and changes in heart rate. A further secondary endpoint was assessment of pharmacokinetic interactions between Triple ACT components. Secondary endpoints not included in this report include the detailed parasite genome-wide and transcriptomic analyses, including gametocyte dynamics, and in-vitro parasite drug sensitivity analyses, which will all be reported separately.

Statistical analysis

We determined sample sizes per site in three different ways. For the sites in Thailand, Cambodia, and Vietnam that were comparing dihydroartemisinin–piperaquine with dihydroartemisinin–piperaquine plus mefloquine, we anticipated a PCR-corrected failure rate of dihydroartemisinin–piperaquine of 30–35%. Recruiting 50 patients in each treatment group would provide 80% power to detect a decrease in PCR-adjusted failure rates at day 42 to 10% or less (α=0·05, two-sided z test). In the sites where the efficacy of dihydroartemisinin–piperaquine plus mefloquine was compared with that of artesunate–mefloquine, the sample size was calculated to assess non-inferiority of dihydroartemisinin–piperaquine plus mefloquine. Assuming an ACT efficacy of 98% and a non-inferiority margin of 8%, a sample size of 49 participants per group was needed to declare non-inferiority with a one-sided α=0·025 and 80% power. Allowing for 20% loss to follow-up, this resulted in a sample size of 60 patients per study group In sites where artemisinin resistance was not established at the start of the study, a sample size of 120 would allow the detection of a prevalence of extended parasite clearance half-lives above 10% compared with a background prevalence of 3·5% with a power of 80%. We assessed the superiority of the efficacy of ACTs and Triple ACTs at each site using Fisher's exact test. Effect sizes are given as absolute differences with 95% CIs. In the ITT analysis, patients needing intravenous artesunate treatment, patients

who discontinued the trial or study drugs before completion, had a PCR result that did not allow the distinction between reinfection and recrudescence, and with *Plasmodium vivax* infection during follow-up were imputed as treatment failures. Patients who re-presented with a PCR-confirmed *P. falciparum* reinfection or who were lost to follow-up were imputed as a treatment success. In the per-protocol analysis, patients with any of these events were excluded from the analysis. We obtained day 42 recrudescence-free survival estimates using Kaplan-Meier analysis. We compared changes in heart rate and QTc-interval and parasite clearance half-lives between treatment groups using the unpaired *t* test and incidence of vomiting within the first hour after drug administration using the χ^2 test.

Our analyses for assessing non-inferiority were based on the one-sided CI for the difference in efficacy between treatments. The 42-day PCR-corrected efficacy of dihydroartemisinin–piperaquine plus mefloquine was declared non-inferior to that of artesunate–mefloquine if the lower end of the 95% CI for the difference in efficacy was greater the non-inferiority margin of −8%. We compared PCR uncorrected efficacy using a Fisher's exact test. We normalised the relevant tolerability and safety results as incidence per 100 patients and also calculated incidences using a Poisson distribution and assessed their differences using Fisher's exact test. We also calculated incidence rate ratios comparing treatment groups and the incidences were normalised per 100 patients. Drug exposure (ie, area under the curve [AUC]) was the primary pharmacometric parameter to evaluate drug–drug interactions and we analysed it after the first and last dose separately. Additionally, we calculated C_{max} and T_{max} values. Exposure between first dose and second dose (AUC_T) was expressed as AUC_{0-8} for artemether–lumefantrine with or without amodiaquine and as AUC_{0-24} for dihydroartemisinin–piperaquine with or without mefloquine. We report drug concentrations with 90% CIs. Exposure after the last dose ($AUC_{T\_lastdose}$) was defined as the AUC after the last dose (52 h for dihydroartemisinin–piperaquine with or without mefloquine and 96 h for artemether–lumefantrine with or without amodiaquine) to the last collected sample (day 28 for lumefantrine and desbutyl-lumefantrine and day 42 for piperaquine). All tests were done at a 5% significance level. A Data and Safety Monitoring Board (DSMB) met before the start of the trial and after recruitment of 20, 100, 200, 500, and 800 patients. We did most analyses using Stata version 15.1. This study was registered on ClinicalTrials.gov, NCT02453308.

Role of the funding sources

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

RESULTS

Between Aug 7, 2015, and Feb 8, 2018, of 2790 individuals screened, 1100 patients with acute uncomplicated *P. falciparum* malaria were enrolled and randomly assigned to dihydroartemisinin–piperaquine (183 [16·6%]), dihydroartemisinin–piperaquine plus mefloquine (269 [24·5%]), artesunate–mefloquine (73 [6·6%]), artemether–lumefantrine (289 [26·3%]), or artemether–lumefantrine plus amodiaquine (286 [26·0%]; figure 1). Baseline characteristics were similar between study groups (table 1) and 29 (3·0%) patients had mixed *P. falciparum* and *P. vivax* infections. In 15 sites, the study was stopped before target recruitment was reached because of decreasing malaria transmission based on a DSMB recommendation which was discussed and agreed by the investigators. 113 patients were excluded from the per-protocol analysis, of whom ten patients required intravenous artesunate treatment, as determined by the investigator on site (figure 1). Six patients discontinued the study drug and started standard antimalarial treatment due to abnormal baseline laboratory results or extension of the QTc-interval, as prespecified in the protocol.

Overall, the 42-day PCR corrected efficacy of dihydroartemisinin–piperaquine plus mefloquine (97%; 95% CI 93 to 99) was higher than for dihydroartemisinin–piperaquine (60%; 52 to 67), with a risk difference of 37% (29 to 45; p<0·0001. The 42-day PCR corrected efficacy of dihydroartemisinin–piperaquine plus mefloquine was superior to dihydroartemisinin–piperaquine in Binh Phuoc, Vietnam (p<0·0001); Phusing (p<0·0001) and Khun Han (p=0·015), Thailand; Ratanakiri (p<0·0001) and Pursat (p=0·0002), Cambodia (table 2). Efficacy of dihydroartemisinin–piperaquine plus mefloquine was not superior to dihydroartemisinin–piperaquine in Pailin, Cambodia (p=0·13; table 2). Because of the high failure rates after dihydroartemisinin–piperaquine and the switch of first-line treatment from dihydroartemisinin–piperaquine to artesunate–mefloquine, the DSMB advised changing the comparator treatment to artesunate–mefloquine in Pailin, Pursat, and Preah Vihear, all in Cambodia. By that timepoint, 19 patients had been recruited in Pursat and 20 patients had been recruited in Pailin. The comparator was not changed in Vietnam and Thailand because national first-line treatment was dihydroartemisinin–piperaquine throughout the duration of the trial. Overall, the 42-day PCR corrected efficacy of dihydroartemisinin–piperaquine plus mefloquine (96%; 88 to 99) was non-inferior to that of artesunate–mefloquine (95%; 87 to 99), risk difference 1% (−6 to 8; p=1·00). The 42-day PCR corrected efficacy of dihydroartemisinin–piperaquine plus mefloquine was non-inferior to that of artesunate–mefloquine in Pursat (p=0·36), Pailin (p=0·50), and Preah Vihear, Cambodia (table 2). In Myanmar, 42-day PCR-corrected efficacy of dihydroartemisinin–piperaquine was 100% (42 of 42; 95% CI 92 to 100) in the three sites combined, and efficacy of dihydroartemisinin–piperaquine plus mefloquine in these sites in Myanmar was 91% (42 of 46; 95% CI 79 to 98; p=0·12).

Figure 1. Study profile. ITT=intention-to-treat. QTc-interval=corrected QT interval. *Reasons are non-exclusive.

Table 1: Baseline characteristics of study patients, by randomly assigned treatment. Data are n (%), median (IQR), or mean (SD), unless otherwise stated. QTcB-interval=corrected QT interval using Bazett's formula.

	Dihydroartemisinin-piperaquine (n=183)	**Dihydroartemisinin-piperaquine plus mefloquine (n=269)**	**Artesunate-mefloquine (n=73)**	**Artemether-lumefantrine (n=289)**	**Artemether-lumefantrine plus amodiaquine (n=286)**	**Total population (n=1100)**
Median age (years) (IQR)	26·0 (18·0-36·0)	28 (20·0-37·0)	32·0 (24·0-43·0)	18·0 (7·0-28·0)	17·0 (7·0-29·0)	23·0 (13·0-34·0)
Male sex	151 (83%)	231 (86%)	72 (99%)	202 (70%)	198 (69%)	854 (78%)
Female sex	32 (18%)	38 (14%)	1 (1%)	87 (30%)	88 (31%)	246 (22%)
Number of patients baseline tympanic temperature >37·5°C	102 (56%)	158 (59%)	38 (52%)	142 (49%)	134 (47%)	574 (52%)
Weight, kilograms	51·4 (13·4)	52·5 (11·6)	56·5 (8·2)	38·2 (17·7)	37·6 (17·7)	45·0 (16·9)
Height, centimetres	157·4 (14·7)	159·6 (11·3)	164·1 (5·7)	140·8 (25·1)	139·7 (24·9)	149·4 (22·1)
QTcB-interval, milliseconds	411·9 (17·2)	412·8 (17·8)	411·5 (16·0)	414·6 (18·8)	415·0 (17·9)	413·6 (17·9)
Haematocrit, %	40·6 (5·3)	40·3 (5·3)	40·1 (5·3)	36·3 (5·6)	37·1 (6·2)	38·5 (5·9)
Parasite count per µL, geometric mean (range)*	26035 (160-214223)	18776 (48-565200)	14179 (96-217602)	40278 (384-449711)	45396 (1520-379814)	29865 (48-565200)
Mixed infection (*Plasmodium falciparum* and *Plasmodium vivax*) present at baseline	5 (3%)	9 (3%)	7 (10%)	6 (2%)	2 (1%)	29 (3%)

*The baseline parasitaemia in some patients was above the screening cutoff as the parasitaemia increased between screening and baseline.

Table 2: Day-42 PCR-corrected efficacy after dihydroartemisinin–piperaquine, dihydroartemisinin–piperaquine plus mefloquine, and artesunate–mefloquine treatment, by site (ITT analysis).

		Dihydroartemisinin-piperaquine	Dihydroartemisinin-piperaquine+ mefloquine	Artesunate-mefloquine	Risk difference	P-value
Vietnam	Binh Phuoc (n=120)	26/60 (43%; 31 to 57)	58/60 (97%; 89 to 100)		53% (40 to 67)	<0·001
Thailand	Phusing (n=32)	2/15 (13%; 2 to 41)	17/17 (100%; 81 to 100)		87% (70 to 100)	<0·001
	Khun Han (n=11)	1/5 (20%; 1 to 72)	6/6 (100%; 54 to 100)		80·0 (45 to 100)	0·015
	Tha Song Yang (n=1)		1/1 (100%; 3 to 100)		NA	NA
Cambodia	Ratanakiri (n=90)	32/44 (73%; 57 to-85)	46/46 (100%; 92 to 100)		27% (14 to 41)	<0·001
	Pursat					
	Dihydroartemisinin-piperaquine comparator treatment (n=19)	1/8 (13%; 0 to 53)	11/11 (100%; 72 to 100)		88% (65 to 100)	0·0002
	Artesunate–mefloquine comparator treatment (n=93)		44/45 (98%; 88 to 100)	44/48 (92%; 80 to 98)	6% (–3 to 15)	0·36
	Pailin					
	Dihydroartemisinin–piperaquine comparator treatment (n=20)	5/9 (56%; 21 to 86)	10/11 (91%; 59 to 100)		35% (–1 to 72)	0·13
	Artesunate–mefloquine comparator treatment (n=44)		20/22 (91%; 71 to 99)	22/22 (100%; 85 to 100)	9% (–3 to 21)	0·50
	Preah Vihear (n=7)		4/4 (100%; 40 to 100)	3/3 (100%; 29 to 100)	0·0	NA

Table 2: Continued.

		Dihydroartemisinin-piperaquine	Dihydroartemisinin-piperaquine+ mefloquine	Artesunate-mefloquine	Risk difference	P-value
Myanmar	Thabeikkyin (n=27)	13/13 (100%; 75 to 100)	13/14 (93%; 66 to 100)		–7% (–21 to 6)	1·00
	Pyay (n=32)	15/15 (100%; 78 to 100)	15/17 (88%; 64 to 99)		–12% (–27 to 4)	0·50
	Ann (n=29)	14/14 (100%; 77 to 100)	14/15 (93%; 68 to 100)		7% (–6 to 19)	1·00
Total	Dihydroartemisinin–piperaquine vs dihydroartemisinin–piperaquine plus mefloquine					
	Overall (n=381)	109/183 (60%; 52 to 67)	191/198 (97%; 93 to 99)		37% (29 to 45)	<0·0001
	In Vietnam, Thailand and Cambodia (n=293)	67/141 (48%; 39 to 56)	149/152 (98%; 94 to 100)		51% (42 to 59)	<0·0001
	In Myanmar (n=88)	42/42 (100%; 92 to 100)	42/46 (91%; 79 to 98)		9% (1 to 17)	0·12
	Artesunate–mefloquine vs dihydroartemisinin–piperaquine plus mefloquine (n=144)					
	Overall (n=144)		68/71 (96%; 88 to 99)	69/73 (95%; 87 to 99)	1% (–6 to 8)	1·00

Data are n/N (%; 95% CI) or risk difference. p values are from two-sided Fisher's exact tests. NA=not applicable. ITT=intention-to-treat.

Table 3: Day-42 PCR corrected efficacy after artemether–lumefantrine and artemether–lumefantrine plus amodiaquine treatment, by site (ITT analysis).

		artemether-lumefantrine	artemether-lumefantrine plus amodiaquine	Risk difference	P value
Myanmar	Pyin Oo Lwin (n=27)	12/12 (100%; 74 to 100)	15/15 (100%; 78 to 100)	0 (NA)	NA
Laos	Sekong (n=11)	5/6 (83%; 36 to 100)	5/5 (100%; 48 to 100)	17% (–13 to 47)	1·00
Bangladesh	Ramu (n=120)	57/60 (95%; 86 to 99)	58/60 (97%; 89 to 100)	2% (–5 to 9)	1·00
India	Agartala (n=83)	44/44 (100%; 92 to 100)	38/39 (97%; 87 to 100)	3% (–2 to 8)	0·47
	Rourkela (n=112)	52/56 (93%; 83 to 98)	54/56 (96%; 88 to 100)	4% (–5 to 12)	0·68
	Midnapur (n=102)	50/51 (98%; 90 to 100)	51/51 (100%; 93 to 100)	2% (–2 to 6)	1·00
DR Congo	Kinshasa (n=120)	59/60 (98%; 91 to 100)	60/60 (100%; 94 to 100)	2% (–2 to 5)	1·00
Total	Artemether–lumefantrine vs artemether–lumefantrine plus amodiaquine (n=575)	279/289 (97%; 94 to 98)	281/286 (98%; 96 to 99)	2% (–1 to 4)	0·30

Data are n/N (%; 95% CI) or risk difference. p values are from two-sided Fisher's exact tests. NA=not applicable. ITT=intention-to-treat.

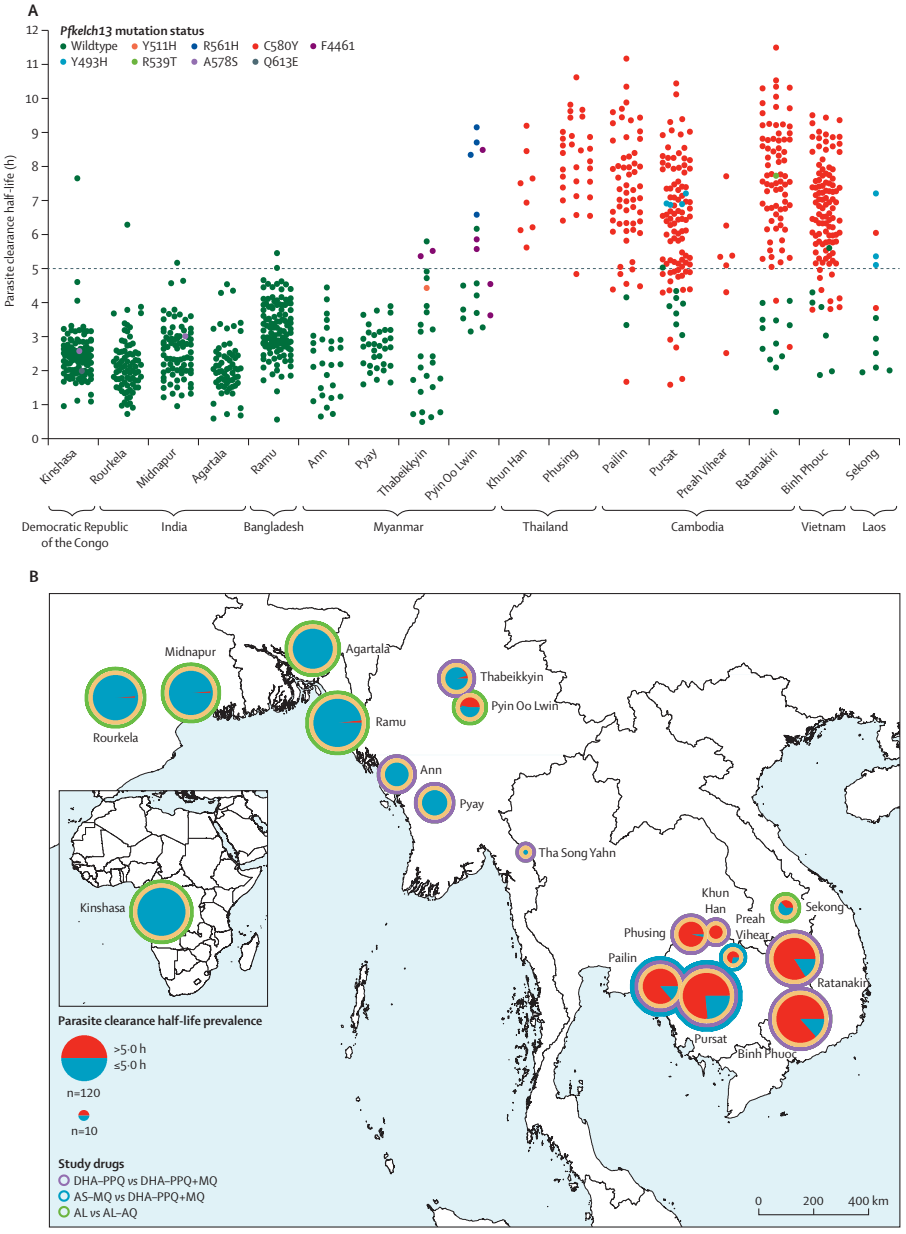


Figure 2: Parasite clearance half-lives and the presence of the *Pfkelch13* mutations by study site. (A) Parasite clearance half-lives for each individual participant by study site, with the dotted line showing the 5 h cutoff point; participants with polyclonal infections were excluded from this graph. (B) Location of the study sites and pie charts show the proportions of participants with a parasite clearance half-life of more than 5 h and less than 5 h and which drugs were trialled at each site. AL=artemether–lumefantrine. AQ=amodiaquine. AS-MQ=artesunate–mefloquine. DHA-PPQ=dihydroartemisinin–piperaquine. MQ=mefloquine

The overall 42-day corrected efficacy of artemether–lumefantrine (97%, 95% CI 94 to 98) was comparable to that of artemether–lumefantrine plus amodiaquine (98% [96 to 99]; risk difference 2%, [–1 to 4, p=0·30]; table 3). The 42-day PCR corrected efficacy of artemether–lumefantrine was above 90% in all seven sites except for Sekong, Laos, where one of six enrolled patients had a recrudescent infection (table 3). The efficacy of artemether–lumefantrine plus amodiaquine ranged between 96% and 100% in all sites where it was tested. No differences in efficacy between artemether–lumefantrine and artemether–lumefantrine plus amodiaquine were observed at any site). The results of the per-protocol analysis and Kaplan-Meier survival analysis showed similar results (figure S1 and S2).

Most patients in Cambodia, Vietnam, and Thailand had extended parasite clearance half-lives of longer than 5 h (figure 2; table S1). Genotyping of the *kelch13* gene, a marker of artemisinin resistance, was possible in 1036 of 1100 patients (table S2a and S2b). C580Y was the dominant *kelch13* mutation in Cambodia, eastern Thailand, Vietnam, and Laos, but was not observed elsewhere. In Pyin Oo Lwin, Myanmar, ten (37%) of 27 infections were caused by parasites carrying *kelch13* mutations (F446I n=5; R561H n=5; table S2a). In the other sites in Myanmar, and in the sites in Bangladesh, India, and the Democratic Republic of the Congo, almost all parasite clearance half-lives were shorter than 5 h. In Midnapur, India, ten (11%) of 89 infections were caused by *kelch13* mutated parasites including mutations at positions 364, 464, 496, 545, 548, 567, 578, 637, 662, and 704. However, nine of these ten *kelch13* mutated infections were mixed strain infections also containing *kelch13* wildtype parasites (table S2b). Similarly, all *kelch13* mutant infections in the other two sites in India, Rourkela (three [3%] of 87) and Agartala (six [9%] of 69) were mixed with wildtype infections. In the sites in Myanmar (other than Pyin Oo Lwin), in Bangladesh, and in the other sites in India and the Democratic Republic of the Congo, *kelch13* mutations were rare. *Plasmepsin2/3* gene amplifications, a marker of piperaquine resistance, were present in high frequencies in Cambodia, Thailand, and Vietnam (table S2b), but were absent in all the other countries. *Mdr1* gene amplifications, a marker for mefloquine resistance, were not observed anywhere. Parasite half-lives in *kelch13* C580Y mutated infections were shorter in patients treated with dihydroartemisinin–piperaquine plus mefloquine (mean 6·93 h [SD 1·77]) than among those treated with dihydroartemisinin–piperaquine (7·39 h [1·46]; p=0·019) and were similar to the half-lives in those treated with artesunate–mefloquine (7·02 h [1·81]; p=0·752; figure S3). In patients with a *kelch13* wildtype infection, parasite clearance half-lives were longer with dihydroartemisinin–piperaquine plus mefloquine (2·90 h [1·15]) than with dihydroartemisinin–piperaquine alone (2·40 h [SD 0·98]; p=0·020). We found no difference in parasite clearance half-lives in *kelch13* wildtype infections after treatment with artemether–lumefantrine (2·65 h [1·39]) compared with after artemether–lumefantrine plus amodiaquine (2·69 h [1·17]; p=0·702). PCR-uncorrected day-42 ACPR were similar to the PCR-corrected ACPR outcome data, except for the high-transmission site in the Democratic Republic of the Congo, where reinfection is

common and the uncorrected ACPR was 75% (45 of 60; 95% CI 62–85) with artemether-lumefantrine and 78% (47 of 60; 66–88) with artemether-lumefantrine plus amodiaquine. Fever clearance times were not different between the ACTs and corresponding Triple ACTs (data not shown).

Overall all drug regimens were well tolerated and most reported adverse clinical symptoms were mild or moderate in severity (table 4). Most clinical adverse events occurred in the first week after enrolment (tables S4). The incidence of clinical adverse events in patients treated with dihydroartemisinin–piperaquine plus mefloquine (277 adverse events in 269 patients) were not different from dihydroartemisinin–piperaquine (171 adverse events in 183 patients; incidence rate ratio 1·1, 95% CI 0·9–1·3; p=0·32), whereas patients treated with artesunate–mefloquine (94 adverse events in 73 patients) had more clinical adverse events than did those treated with dihydroartemisinin–piperaquine (incidence rate ratio 1·4, 95% CI 1·1–1·8; p=0·014), including more abdominal complaints, dizziness, blurred vision, and sleeping disturbances. The incidence of clinical adverse events was also higher with artemether–lumefantrine plus amodiaquine (153 adverse events in 286 patients) than with artemether–lumefantrine (121 adverse events in 289 patients; incidence rate ratio 1·3, 95% CI 1·0–1·6; p=0·0436), including more abdominal symptoms—eg, loss of appetite, nausea, and vomiting.

Vomiting within the first hour after administration of study drug was infrequent but occurred more after dihydroartemisinin–piperaquine plus mefloquine (30 [3·8%] of 794) than after dihydroartemisinin–piperaquine (eight [1·5%] of 543; p=0·012; table 4; table S5). A similar proportion of patients had vomiting within the first hour after artemether–lumefantrine plus amodiaquine (22 [1·3%] of 1703) versus artemether–lumefantrine (11 [0·6%] of 1721; p=0·055).

Table 4: Incidence of adverse events and other indicators of study drug toxicity, by study treatment group

	Dihydroartemisin-piperaquine		Dihydroartemisinin-piperaquine plus mefloquine		Artesunate-mefloquine		Artemether-lumefantrine		Artemether-lumefantrine plus amodiaquine	
Number of patients	183		269		73		289		286	
Vomiting within one hour after treatment/number of treatments	8/543 (1·5)		30/794 (3·8)		3/219 (1·4)		11/1721 (0·6)		22/1703 (1·3)	
Serious adverse events (SAEs)	6/183 (3·3)		10/269 (3·7)		2/73 (2·7)		4/289 (1·4)		2/286 (0·7)	
Drug related SAEs	4/183 (2·2)		4/269 (1·5)		1/73 (1·4)		0/289 (0)		1/286 (0·3)	
QTcB >60ms above baseline	5/183 (2·7)		6/269 (2·2)		0/73 (0·0)		1/289 (0·3)		1/286 (0·3)	
QTcB >500ms	0/183 (0·0)		1/269 (0·4)		0/73 (0·0)		0/289 (0·0)		0/286 (0·0)	
Bradycardia	24/183 (13·1)		44/269 (16·4)		9/73 (12·3)		18/289 (6·2)		52/286 (18·2)	
Grading of adverse events	1-2	3-4	1-2	3-4	1-2	3-4	1-2	3-4	1-2	3-4
Symptoms										
Headache	43 (23·5)	0 (0·0)	40 (14·9)	1 (0·4)	7 (9·6)	0 (0·0)	25 (8·7)	1 (0·3)	13 (4·5)	0 (0·0)
Fatigue	26 (14·2)	0 (0·0)	29 (10·8)	1 (0·4)	3 (4·1)	1 (1·4)	14 (4·8)	0 (0·0)	21 (7·3)	0 (0·0)
Abdominal pain	9 (4·9)	0 (0·0)	17 (6·3)	0 (0·0)	6 (8·2)	0 (0·0)	9 (3·1)	0 (0·0)	13 (4·5)	0 (0·0)
Loss of appetite	19 (10·4)	0 (0·0)	19 (7·1)	0 (0·0)	8 (11·0)	0 (0·0)	25 (8·7)	0 (0·0)	31 (10·8)	0 (0·0)
Nausea	17 (9·3)	0 (0·0)	39 (14·5)	0 (0·0)	5 (6·8)	0 (0·0)	3 (1·0)	0 (0·0)	14 (4·9)	0 (0·0)
Vomiting	15 (8·2)	0 (0·0)	28 (10·4)	0 (0·0)	6 (8·2)	0 (0·0)	10 (3·5)	0 (0·0)	22 (7·7)	0 (0·0)
Diarrhoea	9 (4·9)	0 (0·0)	25 (9·3)	0 (0·0)	8 (11·0)	0 (0·0)	7 (2·4)	0 (0·0)	5 (1·7)	0 (0·0)
Itching	3 (1·6)	0 (0·0)	3 (1·1)	0 (0·0)	2 (2·7)	0 (0·0)	4 (1·4)	0 (0·0)	4 (1·4)	0 (0·0)
Dizziness	21 (11·5)	1 (0·5)	38 (14·1)	2 (0·7)	16 (21·9)	2 (2·7)	18 (6·2)	0 (0·0)	25 (8·7)	0 (0·0)
Blurred vision	1 (0·5)	0 (0·0)	9 (3·3)	0 (0·0)	11 (15·1)	0 (0·0)	3 (1·0)	0 (0·0)	2 (0·7)	0 (0·0)
Sleep disturbance	8 (4·4)	0 (0·0)	25 (9·3)	1 (0·4)	16 (21·9)	3 (4·1)	2 (0·7)	0 (0·0)	3 (1·0)	0 (0·0)
Total	171/183 (93·4)	1/183 (0·5)	272/269 (101·1)	5/269 (1·9)	88/73 (120·5)	6/73 (8·2)	120/289 (41·5)	1/289 (0·3)	153/286 (53·5)	0/286 (0·0)
Laboratory abnormalities										
Creatinine*	17/148 (11·5)	0/148 (0·0)	17/229 (7·4)	1/229 (0·4)	2/73 (2·7)	0/73 (0·0)	23/232 (9·9)	2/232 (0·8)	38/230 (16·5)	6/230 (2·6)
Total bilirubin	22/183 (12·0)	2/183 (1·1)	21/269 (7·8)	3/269 (1·1)	2/73 (2·7)	0/73 (0·0)	11/289 (3·8)	1/289 (0·3)	9/286 (3·1)	0/286 (0·0)
Alkaline phosphatase	5/183 (2·7)	0/183 (0·0)	7/269 (2·6)	0/269 (0·0)	3/73 (4·1)	0/73 (0·0)	17/289 (5·9)	0/289 (0·0)	21/286 (7·3)	0/286 (0·0)
Alanyl transferase (ALT)**	31/163 (19·0)	3/163 (1·8)	43/246 (17·5)	2/246 (0·8)	23/73 (31·5)	2/73 (2·7)	43/289 (14·9)	1/289 (0·3)	32/286 (11·1)	0/286 (0·0)
Aspartate transferase (AST)***	35/183 (19·1)	1/183 (0·5)	33/269 (12·2)	1/269 (0·4)	18/73 (24·7)	1/73 (1·3)	63/283 (22·2)	2/283 (0·7)	47/281 (16·7)	1/281 (0·3)
Anaemia (haemoglobin)	40/159 (25·2)	5/159 (1·4)	39/244 (16·0)	3/244 (1·2)	11/73 (15·1)	1/73 (1·4)	66/235 (28·1)	25/235 (10·6)	71/227 (31·3)	16/227(7·0)
Leukocytopenia	0/114 (0·0)	0/114 (0·0)	0/149 (0·0)	1/149 (0·7)	1/22 (4·5)	1/22 (4·5)	0/165 (0·0)	1/165 (0·6)	1/160 (0·6)	0/160 (0·6)
Neutropenia	2/113 (1·8)	0/113 (0·0)	4/149 (2·7)	0/149 0·0)	1/22 (4·5)	0/22 (0·0)	6/161 (3·7)	6/161 (3·7)	6/156 (3·8)	1/156 (0·6)
Thrombocytopenia	9/115 (7·8)	2/115 (1·7)	16/149 (10·7)	2/149 (1·3)	2/22 (9·1)	0/22 (0·0)	26/162 (16·0)	2/162 (1·2)	23/157 (14·6)	1/157 (0·6)

Data are n/N (%), where n is number of events and N is number of patients, with a normalised incidence per 100 patients in parentheses, unless otherwise indicated. Incidence of QTcB increases of >60 ms above baseline or bradycardia (defined as ≤54 heartbeats per min) are defined as a patient encountering these abnormalities at one or more timepoints at 4 h, 48 h, or 52 h after treatment. QTcB=Bazett's corrected QT-interval. Any worsening of self-reported vomiting was recorded as an adverse event. *Results of creatinine measurements from sites in Midnapur (India), Pyay (Myanmar), Phusing and Khun Han (Thailand), and Sekong (Laos) were not available, and the denominator number of patients is amended to reflect this fact. **Results from Phusing and Khun Han (Thailand) were not available, and the denominator number of patients is amended to reflect this fact. ***Results from Sekong, Laos were not available, and the denominator number of patients is amended to reflect this fact.

No difference in extension of the ECG QTcB-interval was seen at 52 h compared with baseline after treatment with dihydroartemisinin–piperaquine (mean increase in QTcB 22·1 ms [SD 19·2]) compared with dihydroartemisinin–piperaquine plus mefloquine (20·8 ms [SD 17·8]; p=0·50; figure S4, table S6a and table S6b). Frequency of QTcB-interval extensions of more than 60 ms compared with baseline was similar between dihydroartemisinin–piperaquine (five [2·7%] of 183), dihydroartemisinin–piperaquine plus mefloquine (six [2·2%] of 269), and artesunate–mefloquine (0 of 73). One patient developed a QTcB-interval of more than 500 ms at 52 h after dihydroartemisinin–piperaquine. The decrease in heart rate after dihydroartemisinin–piperaquine plus mefloquine (mean decrease of 21·8 beats per min [bpm; SD 13·7]) and artesunate–mefloquine (14·5 bpm [13·7]) was less than that after dihydroartemisinin–piperaquine alone (25·8 bpm [SD 15·0]; figure S4 and table S6c). The incidence of bradycardia (ie, ≤54 bpm) at 4 h, 48 h, or 52 h after treatment was similar with dihydroartemisinin–piperaquine and with dihydroartemisinin–piperaquine plus mefloquine (p=0·42; table 4). The QTcB-interval was more extended at 52 h compared with baseline with artemether–lumefantrine plus amodiaquine than with artemether–lumefantrine alone (mean increase of 8·8 ms [SD 18·6] *vs*0·9 ms [16·1]; p=0·01), and the decrease in heart rate was more pronounced with artemether–lumefantrine plus amodiaquine than with artemether–lumefantrine alone (mean decrease of 29·6 bpm [SD 16·3] *vs* 20·9 bpm [SD 16·9]; p=0·01; figure S4 and table S6c). Overall, bradycardia was more frequent in patients treated with artemether–lumefantrine plus amodiaquine than with artemether–lumefantrine alone (p=0·01).

We saw no haematological differences between any of the treatment groups (table 4). The incidence of mild-to-moderate increases in liver enzymes was similar with all treatments. 20 patients developed a hepatotoxic adverse event that was graded as severe or higher (defined as an alanine aminotransferase, aspartate aminotransferase, or alkaline phosphatase concentration of >5·0 × the upper limit of normal [ULN] or total bilirubin >2·5 × ULN), with no difference between treatment groups. None of the patients fulfilled Hy's law criteria for liver toxicity (alanine aminotransferase or aspartate aminotransferase >3 × ULN and total bilirubin >2 × ULN). We found no evidence of nephrotoxicity of the two Triple ACTs, although small increases in serum creatinine were more frequent after amodiaquine-containing Triple ACTs than with the other treatments (table 4).

24 serious adverse events were reported in 1100 patients, of which 11 were judged to be possibly (n=10) or probably (n=1) drug related (table S7). The incidence of serious adverse events was similar after treatment with ACTs or Triple ACTs. In northeastern Thailand (Khun Han), one patient died of severe malaria after treatment with dihydroartemisinin–piperaquine; this incident has been reported in detail elsewhere. [84] Two male patients in Myanmar treated with dihydroartemisinin-piperaquine plus mefloquine, progressed to severe malaria in the first 12 h of treatment, and fortunately

intravenous artesunate resulted in a rapid clinical recovery. In two patients (one in Myanmar given dihydroartemisinin–piperaquine plus mefloquine and one in Vietnam given dihydroartemisinin–piperaquine) an initial decrease in parasitaemia in the first 12 h after treatment was followed by an increase in parasitaemia, after which intravenous artesunate was started, resulting in rapid parasite clearance. None of these four patients had early vomiting after the study drug. Two young previously healthy males (aged 14 and 17 years), one treated with dihydroartemisinin–piperaquine and one with dihydroartemisinin–piperaquine plus mefloquine, developed sinus bradycardia (<54 beats per min) on the first day of treatment, both interpreted as physiological or possibly related to study drug. One male patient, aged 23 years, who was treated with dihydroartemisinin–piperaquine plus mefloquine developed convulsions at day 2. This event was interpreted as a post-malaria neurological syndrome, which can be associated with use of mefloquine, but generally occurs later in the course after severe malaria. [115] One male child aged 11 years who was given dihydroartemisinin–piperaquine plus mefloquine developed a QTcB interval extension to 503 ms at 52 h, the time of the expected peak level of piperaquine. One male child aged 5 years who was given artemether–lumefantrine plus amodiaquine developed general weakness and a relative bradycardia (45–55 beats per min) at day 2 of enrolment; investigators deemed this event to probably be due to a pre-existing hypokalaemia and malnourished state. The patient recovered after intravenous replacements of electrolytes and fluids.

Assessing the pharmacokinetics of the addition of mefloquine to dihydroartemisinin–piperaquine, the only observed significant drug–drug interaction was a shorter absorption time for piperaquine (T_{max} −28·4%, 90% CI −47·6 to −2·07) when administered with mefloquine (figure 3; table S3). We found a non-significant decrease in the exposure to dihydroartemisinin (−18·8%, −35·1 to 1·53) and piperaquine (−25·1%, −45·5 to 2·93) after adding mefloquine to the first dose of dihydroartemisinin–piperaquine. Exposure to piperaquine after the last dose ($AUC_{T\_lastdose}$) or the piperaquine day 7 concentrations were unaffected by adding mefloquine (figure 3). Adding amodiaquine to artemether–lumefantrine resulted in lower peak concentrations of both artemether (C_{max} −24·9%, 90% CI −42·9 to −1·31) and its active metabolite dihydroartemisinin (C_{max} −32·0%, −51·1 to −5·39), and a non-significant decrease in the exposure to artemether (AUC_T −15·9%, 90% CI −33·1 to 5·77) and dihydroartemisinin (−24·6%, −45·0 to 3·38). We also saw a non-significant decrease in exposure to both lumefantrine (AUC_T −32·0%, 90% CI −72·3 to 67·5) and desbutyl-lumefantrine (−20·0%, −58·3 to −53·4) after the first dose. After the last dose, exposure to both lumefantrine ($AUC_{T\_lastdose}$ −48·4%, 90% CI −64·5 to −25·0%) and desbutyl-lumefantrine (−45·7%, −63·8 to −18·5) were decreased and day-7 plasma lumefantrine concentrations were lower after artemether–lumefantrine plus amodiaquine (n=148; median 508·5 ng/mL [IQR 305·8–727·8]) than after artemether–lumefantrine (n=152; median 614·5 ng/mL [355·3–1008]; figure 3). A more detailed description on the pharmacokinetic profiles of the study drugs will be reported separately.

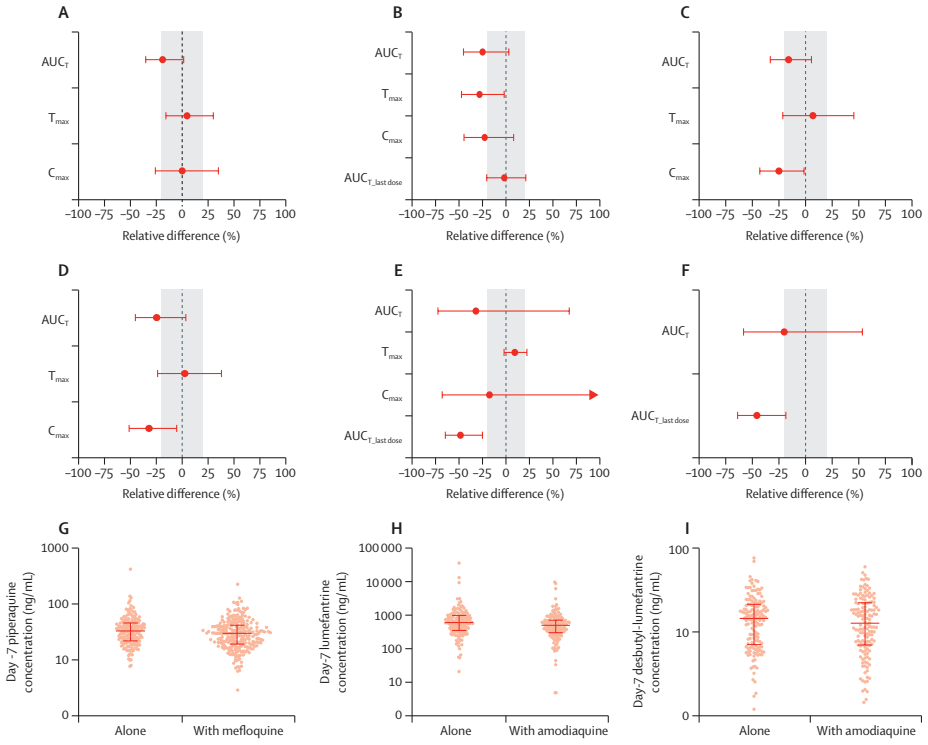


Figure 3. Pharmacokinetic drug–drug interactions Effect of mefloquine on dihydroartemisinin (A) and on piperaquine (B) when treatment is dihydroartemisinin–piperaquine with or without mefloquine. Effect of amodiaquine on artemether (C), active metabolite dihydroartemisinin (D), lumefantrine (E), and desbutyl-lumefantrine when treatment is artemether–lumefantrine with or without amodiaquine. (G) Effect of mefloquine on day-7 piperaquine plasma concentrations when the treatment is dihydroartemisinin–piperaquine with or without mefloquine. Effect of amodiaquine on day 7 lumefantrine (H) and desbutyl-lumefantrine (I) plasma concentrations when the treatment is artemether–lumefantrine with or without amodiaquine. The plots in panels A–F show the geometric mean ratios and 90% CIs of drug–drug interactions related to the specific pharmacokinetic parameters. The dashed line represents zero effect, and the dotted lines show plus or minus 20% effect. In the scatter plots in panels G–I, the red bars show the median and IQR of day 7 plasma concentrations. C_{max}=maximum plasma concentration divided with mg/kg dose. T_{max}=time to reach maximum concentration. AUCT=area under the concentration-time curve to time T after administration of the first dose, divided by the mg/kg dose. $AUC_{T–lastdose}$=area under the concentration-time curve to time T after administration of the last dose, divided by the mg/kg dose.

DISCUSSION

To our knowledge, this is the first clinical study of the two Triple ACTs, dihydroartemisinin–piperaquine plus mefloquine and artemether–lumefantrine plus amodiaquine. We found that both combinations were highly efficacious in the treatment of uncomplicated falciparum malaria, and were safe and well tolerated. Except for a slight increase in incidence of vomiting within 1 h of treatment, neither combination was associated with more adverse effects than those known for the individual components. The addition of mefloquine to dihydroartemisinin–piperaquine did not further extend the QTc-interval and the addition of amodiaquine to artemether–lumefantrine resulted in small increases in QTc-interval and decreases in heart rate, which do not have clinical importance.

Dihydroartemisinin–piperaquine plus mefloquine was highly efficacious even in areas in Cambodia, Thailand, and Vietnam where dihydroartemisinin–piperaquine alone gave unacceptably high rates of recrudescent infections. Artesunate–mefloquine was also an effective treatment in Cambodia, but this combination is known to be vulnerable to the emergence of mefloquine resistance in artemisinin-resistant parasite populations.[30]

In Cambodia, *P. falciparum* isolates did not show *Mdr1* amplification, the molecular marker of mefloquine resistance, presumably as a consequence of the cessation of drug pressure 5–8 years previously when increasing rates of treatment failure led to artesunate–mefloquine to being abandoned as first-line therapy (unpublished, Dondorp AM). On the Thailand–Myanmar border, artesunate–mefloquine was highly efficacious for over a decade, but mefloquine resistance was rapidly acquired after the arrival of artemisinin-resistant *P. falciparum*, a scenario likely to repeat in Cambodia and southern Vietnam. However, the current high efficacy of dihydroartemisinin–piperaquine plus mefloquine in areas with high rates of dihydroartemisinin–piperaquine failure is threatened by worsening piperaquine resistance. Although initial observations suggested that concomitant amplification of *mdr1* and *plasmepsin2/3* was rare, implying the presence of counter-balancing resistance mechanisms,[39, 48] in recent years parasites carrying both amplifications have been observed more frequently in Cambodia.[68] In a previous study in healthy volunteers, dihydroartemisinin exposure was reduced by 23% with the addition of mefloquine to dihydroartemisinin–piperaquine.[116] This finding is of concern because of the relatively low dose of dihydroartemisinin in the fixed dose dihydroartemisinin–piperaquine regimen. The reduction in exposure to dihydroartemisinin was not observed in the current study, although parasite clearance half-life was extended in wildtype parasite infections treated with dihydroartemisinin–piperaquine plus mefloquine compared with such infections treated with dihydroartemisinin–piperaquine. In artemisinin-resistant infections, parasite clearance was more rapid with dihydroartemisinin–piperaquine plus mefloquine.

Artemether–lumefantrine plus amodiaquine was well tolerated, with only 1% of patients given this treatment vomiting within 1 h. This rate is lower than the approximately 5% reported in previous studies in which artesunate-amodiaquine was given as a once daily dose.[117] This improved tolerability might be explained by lower peak concentrations of amodiaquine and its active metabolite desethyl-amodiaquine, resulting from splitting the daily dose, which will not affect overall drug exposure.

Amodiaquine and mefloquine both have bitter tastes, which could compromise acceptability in young children. In future pharmaceutical development, masking the taste of both amodiaquine and mefloquine in paediatric formulations might be necessary to optimise treatments in this important age group. Adding amodiaquine resulted in reduced exposures to artemether and the active metabolite dihydroartemisinin and almost 50% lower exposure to lumefantrine after the last dose. The mechanism underlying these interactions is unknown. Nevertheless, clinical efficacy of this Triple ACT was excellent and observed drug concentrations in plasma remained adequate for parasite clearance. Whether higher doses of artemether–lumefantrine should be used is currently uncertain. In Myanmar, Bangladesh, India, and the Democratic Republic of the Congo both artemether–lumefantrine and artemether–lumefantrine plus amodiaquine were highly efficacious with cure rates over 98%, which is in accordance with the observed low prevalence of *kelch13* mutations in these study locations. A trial comparing artemether–lumefantrine with artemether–lumefantrine plus amodiaquine in areas with high levels of multidrug-resistant falciparum malaria in Cambodia and Vietnam is ongoing (NCT03355664). This Triple ACT might be the preferred choice for countries in the eastern Greater Mekong subregion where ACTs are increasingly unsuccessful, and where deployment of artesunate–mefloquine plus piperaquine is suboptimal because of potential resistance to all three components. In the Indian study sites, including in Midnapur, west Bengal, a variety of *kelch13* mutant *P. falciparum* strains were observed. These mutations are not in the current list of *kelch13* mutations associated with delayed parasite clearance and were observed at very low frequencies.[63] Parasite clearance half-lives were not extended in these infections, but nearly all were multiclonal admixed with wildtype genotypes, confounding the parasite clearance assessment. The mutations were also different from the *kelch13* mutations reported previously from west Bengal,[118] and might represent low frequencies of background *Pfkelch* mutations that are not under selection, as also observed in African parasite populations in higher transmission settings.[58]

Our study had several limitations. For instance, it was unblinded. Although this factor could have affected assessment of subjective outcomes, such as symptom severity and attribution of causality of adverse events to study drugs, it is unlikely to have affected objective endpoints, such as treatment efficacy, and measures of cardiac, renal, and hepatic toxicity. The study might have been underpowered to declare non-inferiority between study groups in sites without ACT failure, because the sample size in those areas was based on the detection of changes in parasite clearance half-lives. Another limitation

is that children, who carry most of the malaria burden in sub-Saharan Africa, were under-represented with only the site in the Democratic Republic of the Congo explicitly studying this patient group. Our focus initially was on areas where ACT resistance is established, or that are threatened by such resistance due to geographical proximity. A large follow-up project testing artesunate–mefloquine plus piperaquine and artemether–lumefantrine plus amodiaquine with greater focus on sub-Saharan Africa is currently in preparation (NCT03923725 and NCT03939104).

With the increasing failure of conventional ACTs, use of Triple ACTs might become essential for treatment of uncomplicated *falciparum* malaria in the Greater Mekong subregion in the near future. This region is aiming for accelerated malaria elimination before increasing antimalarial drug resistance renders *P. falciparum* malaria close to untreatable. The Triple ACTs we studied here could prevent a resurgence of malaria that often accompanies spreading antimalarial drug resistance. Because two well-matched partner drugs provide mutual protection against resistance, deployment of Triple ACTs is expected to extend the useful life of the few effective available and affordable antimalarial drugs. This approach would break the well known repeated historical inefficient sequence in malaria chemotherapy—waiting for resistance to emerge and spread before changing therapy. Fortunately, to date, artemisinin resistance-related delayed parasite clearance has not worsened in Southeast Asia and has not spread to or emerged in sub-Saharan Africa, so this class of drugs still provides useful antimalarial activity in combinations. The presented Triple ACTs, combine existing antimalaria drugs and could be made available in the near future and might buy important time before new antimalarial compounds become available. In areas not yet affected by antimalarial resistance, Triple ACTs might have the potential to delay the emergence and spread of antimalarial resistance and could help prevent importation of drug resistance from the Greater Mekong subregion. This study shows that artemether–lumefantrine plus amodiaquine and dihydroartemisinin–piperaquine plus mefloquine are well tolerated, safe, and efficacious Triple ACTs.

CONTRIBUTORS

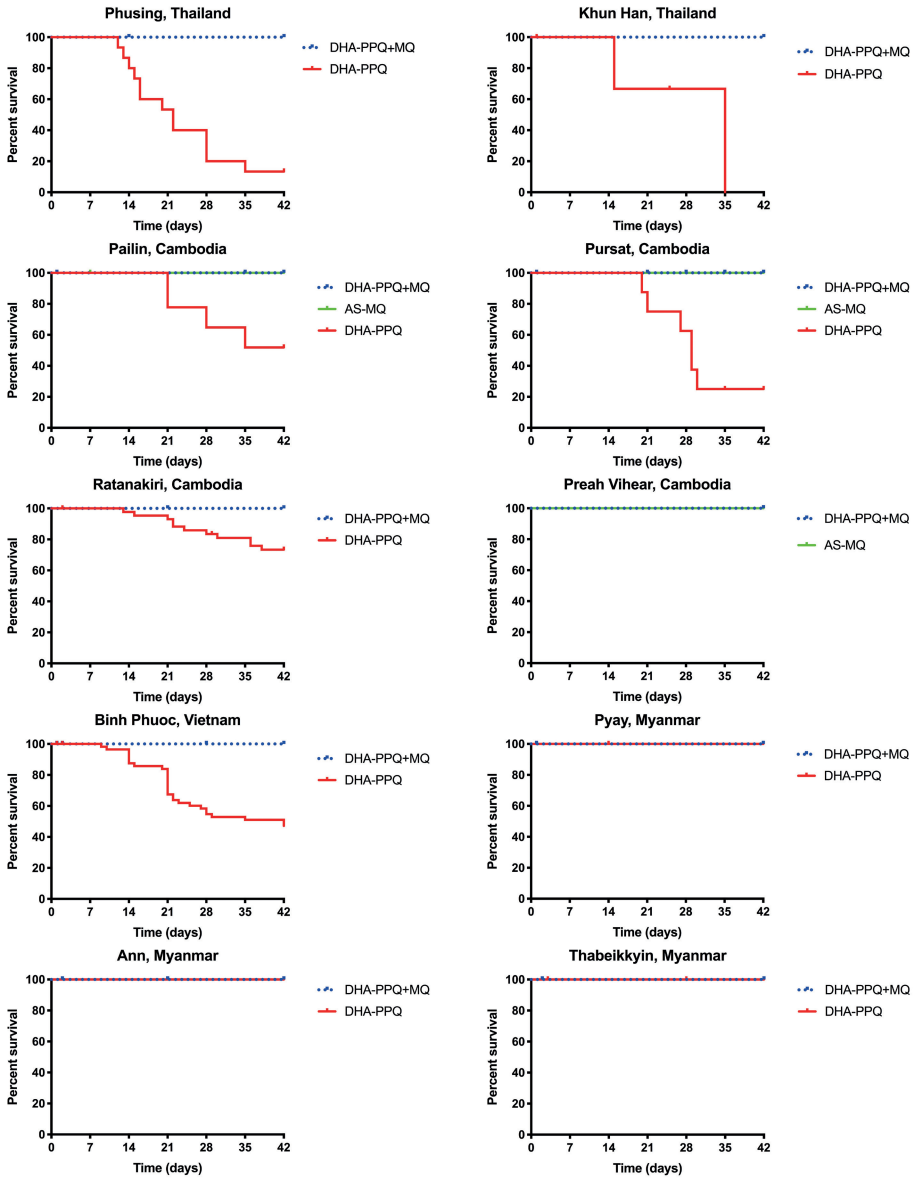
RWvdP, RMH, DL, ARA, CA, MMu, NW, CJW, MPG, MvV, RMF, PYC, LvS, MD, RJM, DPK, MI, PJ, KL, TMH, KC, RH, CF, EA, MMa, PNN, TTH, NV, FS, SP, AF, OM, JT, NPJD, NJW, and AMD designed the study or were involved in the organisation of the trial, or both, and training of the study teams or data management or both. RT, APP, AuI, PS, SSa, PKB, AT, SB, MO, NHC, YS, SSu, SSr, SM, SO, SY, KC, CS, RR, WK, NTH, NVT, BH, JJC, AKM, JH, MT, KG, TG, TJP, and NTT-N recruited the study participants, collected samples, or took part in laboratory work at the study site or in the central laboratories. RWvdP, RMH, MW, JT, NJW, and AMD generated or analysed the pharmacological data,

or both. RA, RDP, CGJ, SG, OM, and MI generated and analysed the parasite genetic data. RWvdP, MMu, NJW, and AMD wrote the manuscript.

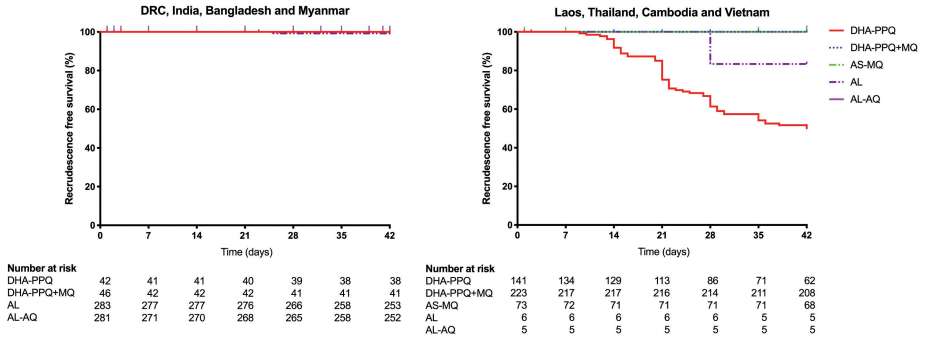
ACKNOWLEDGMENTS

This study was supported by the UK Department for International Development (201900) and the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, US National Institutes of Health. The Mahidol-University Oxford Tropical Medicine Research Programme is funded by the Wellcome Trust (106698/B/14/Z). Genotypes for this study were generated through the MalariaGEN SpotMalaria Project and GenRe Mekong project, which are projects coordinated by the MalariaGEN Resource Centre with funding from Wellcome (206194, 090770) and the Bill & Melinda Gates Foundation (OPP1118166, OPP1204268). The pharmacokinetic analysis was partially funded by the Bill & Melinda Gates Foundation (OPP1134284). We thank all patients who took part in these studies. We thank Prof Abul Khair Mohammad Shamsuzzaman, Prof Amir Hossain, Prof Ridwanur Rahman, Prof Rasheda Samad, Prof Aniruddha Ghose, Prof Abdus Sattar, Mohammad Jahirul Karim, Abdullah Abu Sayeed, Rafiqul Hasan, and Ma-Yin-Nu for organising, facilitating, and supervising the study in the site in Ramu, Bangladesh. We also thank Brian Angus, Ric Price, and Christian Holm Hansen for their important guidance in their role as Data and Safety Monitoring Committee. We thank Zoë Doran, Jaruwan Tubprasert, Prayoon Yuentrakul, and Salwaluk Panapipat for site monitoring and protocol training; Thatsanun Ngernseng and Sasinun Sawaithapan for data management; Cholrawee Promnarate, Benjamas Intharabut, Thanaporn Champathai, Nitima Chanarat, Ranitha Vongpromek, Teeradet Khomvarn, Nakararin Aud-ai, and Thanawat Assawariyathipat for laboratory support provided by the WorldWide Antimalarial Resistance Network; Nisarat Sorotpinya for logistical support; Patrick Hannay for financial administrative support; and Namfon Buasiri and Napaporn Piwon for patient recruitment. We thank the staff of Department of Molecular Tropical Medicine and the Wellcome Sanger Institute Sample Logistics, Sequencing, and Informatics facilities for their contribution; Eleanor Drury, Mihir Kekre, Mozam Ali, and Katie Love for sample processing, and Victoria Simpson and the MalariaGEN Resource Centre for coordination of the genotyping.

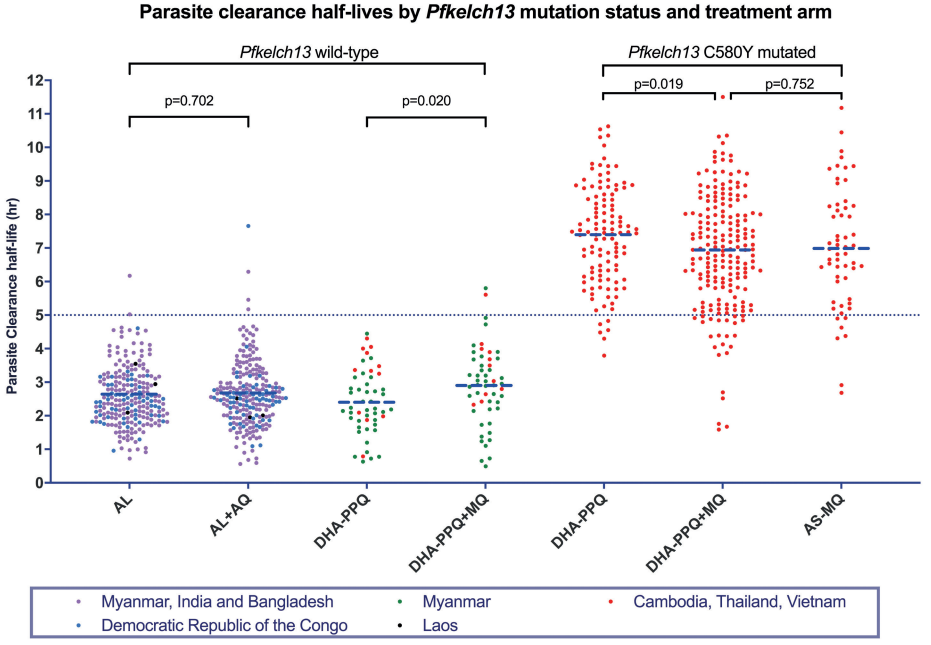
SUPPLEMENTARY MATERIAL



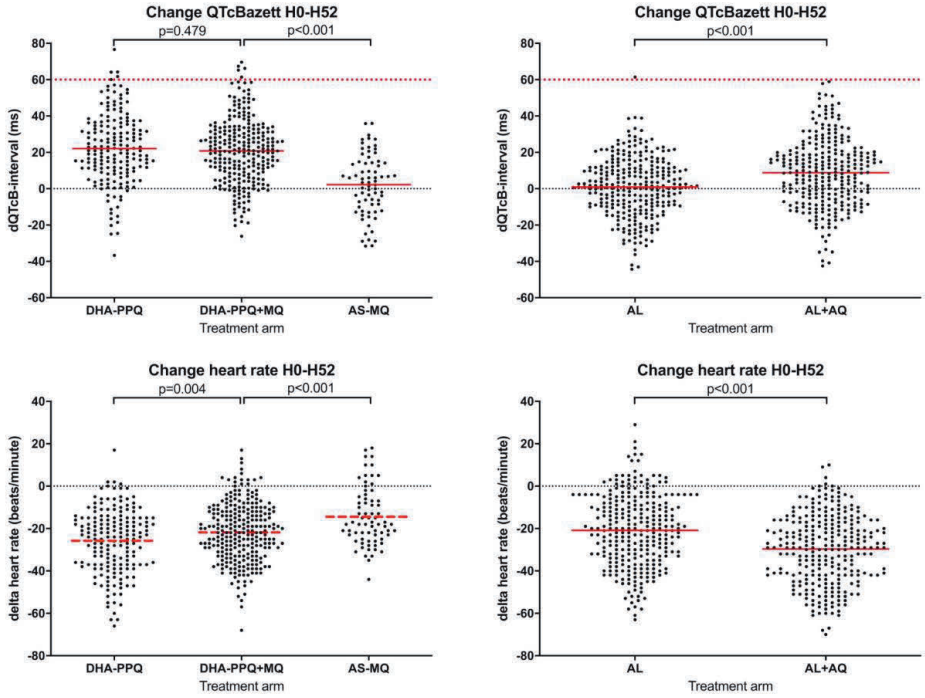
Supplementary figure S1. Kaplan-Meier survival curves by site and arm.
Kaplan-Meier estimates are shown for the time to *Plasmodium falciparum* recrudescent infections following treatment with DHA-piperaquine versus DHA-piperaquine plus mefloquine versus artesunate-mefloquine. DHA-PPQ: dihydroartemisinin-piperaquine; MQ: mefloquine; AS-MQ: artesunate-mefloquine.



Supplementary figure S2 Kaplan-Meier survival curves by arm in study sites in the Democratic Republic of the Congo (DRC), India, Bangladesh and Myanmar (left) and Laos, Thailand, Cambodia and Vietnam (right). DHA-PPQ: dihydroartemisinin-piperaquine; MQ: mefloquine; AS-MQ: artesunate-mefloquine; AL: artemether-lumefantrine; AQ: amodiaquine.



Supplementary figure S3 Parasite clearance half-lives by study arm, stratified by *kelch13* mutation status (either C580Y mutated or wild-type).
The colour of each individual dot indicates the country in which each individual was recruited. Grouping of the countries is based on treatment arms and the presence or absence of preceding high levels of artemisinin resistance. Only subjects infected by either a homozygote *kelch13* Wild-type or C580Y mutated infection were included in this figure. All other *kelch13* mutations were excluded because of low numbers. DHA-PPQ: dihydroartemisinin-piperaquine; MQ: mefloquine; AS-MQ: artesunate-mefloquine; AL: artemether-lumefantrine; AQ: amodiaquine. Reference bars indicate mean changes. Comparisons were performed using an unpaired t-test.



Supplementary figure S4 Changes in ECG QTc intervals and heart rates over time.
Each individual dot represents the change (dQTcB) within each subject of the QTc (Bazett) interval and heart rate between baseline and hour 52 after DHA-PPQ, DHA-PPQ+MQ or AS-MQ (panel A/B) and after AL or AL+AQ (panel C/D). Reference bars indicate mean changes. The black dotted line indicates a change in QTcBazett of 60 miliseconds, which was used as a safety cut-off in this trial. DHA-PPQ: dihydroartemisinin-piperaquine; MQ: mefloquine; AS-MQ: artesunate-mefloquine; AL: artemether-lumefantrine; AQ: amodiaquine. Comparisons were performed using an unpaired t-test.

Supplementary table S1: Parasite clearance characteristics and day 3 positivity by site

Country	Site	Patients with PC1/2 result (n)*	PC1/2 (h)*	PC1/2 >5 h (%; 95% CI)*	Time to 50% parasite clearance (h)*	Positive blood smear for asexual parasitaemia 72 h after treatment initiation (%; 95% CI)
Vietnam	Binh Phuoc	111	6·6 (1·6)	96/111 (86·5) (78·7-92·2)	11·0 (5·7)	86/115 (74·8) (65·8-82·4)
Cambodia	Ratanakiri	87	7·0 (2·3)	73/87 (83·9) (74·5-90·9)	10·2 (5·4)	47/90 (52·2) (41·4-62·9)
	Preah Vihear	7	5·2 (1·6)	5/7 (71·4) (29·0-96·3)	7·1 (3·9)	1/7 (14·3) (0·4-57·9)
	Pursat	103	6·3 (1·8)	79/103 (76·7) (67·3-84·5)	10·9 (4·9)	50/110 (45·5) (35·9-55·2)
	Pailin	59	7·2 (1·8)	51/59 (86·4) (75·0-94·0)	12·2 (5·6)	46/64 (71·9) (59·2-82·4)
Thailand	Phusing	30	8·2 (1·2)	29/30 (96·7) (82·8-99·9)	12·2 (3·4)	30/32 (93·8) (79·2-99·2)
	Khun Han	9	8·0 (2·6)	9/9 (100·0) (66·4-100·0)	13·5 (5·8)	8/10 (80·0) (44·4-97·5)
	Tha Song Yahn	1	4·7 (NA)	0/1 (0·0) (0·0-97·5)	3·9 (NA)	0/1 (0·0) (0·0-97·5)
Myanmar	Thabeikkyin	23	2·5 (1·5)	1/23 (4·3) (0·1-21·9)	7·8 (7·2)	2/27 (7·4) (0·9-24·3)
	Pyay	32	2·7 (0·6)	0/32 (0·0) (0·0-10·9)	6·5 (4·2)	0/32 (0·0) (0·0-10·9)
	Ann	27	2·3 (1·0)	0/27 (0·0) (0·0-12·8)	7·2 (4·2)	0/29 (0·0) (0·0-11·9)
	Pyin Oo Lwin	15	6·8 (4·5)	7/15 (46·7) (22·3-73·4)	10·5 (4·6)	4/24 (16·7) (4·7-37·4)
Laos	Sekong	11	3·9 (1·8)	4/11 (36·4) (10·9-69·2)	7·1 (3·9)	1/11 (9·1) (0·2-41·3)

Supplementary table S1: Continued.

Country	Site	Patients with PC1/2 result (n)*	PC1/2 (h)*	PC1/2 >5 h (%; 95% CI)*	Time to 50% parasite clearance (h)*	Positive blood smear for asexual parasitaemia 72 h after treatment initiation (%; 95% CI)
Bangladesh	Ramu	117	3·2 (0·8)	2/117 (1·7) (0·2-6·0)	6·5 (3·6)	0/120 (0·0) (0·0-3·0)
India	Agartala	63	2·1 (0·8)	0/63 (0·0) (0·0-5·7)	6·8 (4·2)	0/83 (0·0) (0·0-4·3)
	Midnapur	80	2·6 (0·8)	1/80 (1·3) (0·0-6·8)	7·7 (3·6)	0/102 (0·0) (0·0-3·6)
	Rourkela	79	2·2 (0·8)	1/79 (1·3) (0·0-6·9)	8·1 (4·9)	0/110 (0·0) (0·0-3·3)
DRC	Kinshasa	117	2·5 (0·7)	1/117 (0·8) (0·0-4·7)	8·6 (5·5)	2/120 (1·7) (0·2-5·9)

*Patients with mixed infections are excluded. Data are mean (SD) unless otherwise specified. PC1/2=parasite clearance half-life.

Supplementary table S2a: Molecular markers of resistance by site

Country	Site	n	Wildtype *kelch13* (n/N, %, CI 95%)	C580Y *kelch13* mutation (or mixed C580Y/other) (n/N , %,CI 95%)	Other *kelch13* mutation (or mixed mutation/other) (n/N, %, CI 95%)	Plasmepsin 2/3 amplification (n/N,%,CI 95%)	Mdr1 amplification (n/N,%,CI 95%)
Vietnam	Binh Phuoc	120	7/120 (5·8) (2·4-11·6)	113/120 (94·2) (88·4-97·6)	NA	88/120 (73·3) (64·5-81·0)	0/120 (0·0) (0-3·0)
Cambodia	Ratanakiri	90	12/89 (13·5) (7·2-22·4)	76/89 (85·4) (76·3-92·0)	1/89 (1·1) (0-6·1)	63/89 (70·8) (60·2-79·9)	0/89 (0·0) (0-4·1)
	Preah Vihear	7	NA	7/7 (100·0) (59·0-100·0)	NA	7/7 (100·0) (59·0-100·0)	0/7 (0·0) (0-41·0)
	Pursat	112	8/107 (7·5) (3·3-14·2)	95/107 (88·8) (81·2-94·1)	4/107 (3·7) (1·0-9·3)	87/110 (79·1) (70·3-86·3)	0/110 (0·0) (0-3·3)
	Pailin	64	2/64 (3·1) (0·4-10·8)	62/64 (96·9) (89·2-99·6)	NA	43/62 (69·4) (56·3-80·4)	0/64 (0·0) (0-5·6)
Thailand	Phusing	32	NA	32/32 (100·0) (89·1-100·0)	NA	26/32 (81·3) (63·6-92·8)	0/32 (0·0) (0-10·9)
	Khun Han	11	NA	11/11 (100·0) (71·5-100·0)	NA	10/11 (90·9) (58·7-99·8)	0/11 (0·0) (0-28·5)
Myanmar	Thabeikkyin	27	23/27 (85·2) (66·3-95·8)	NA	4/27 (14·8) (4·2-33·7)	0/27 (0·0) (0-12·8)	0/27 (0·0) (0-12·8)
	Pyay	32	32/32 (100·0) (89·1-100·0)	NA	NA	0/32 (0·0) (0-10·9)	0/32 (0·0) (0-10·9)
	Ann	29	27/28 (96·4) (81·7-99·9)	NA	1/28 (3·6) (0-18·3)	0/28 (0·0) (0-12·3)	0/28 (0·0) (0-12·3)
	Pyin Oo Lwin	27	17/27 (63·0) (42·4-80·6)	NA	10/27 (37·0) (19·4-57·6)	0/25 (0·0) (0-13·7)	0/26 (0·0) (0-13·2)
Laos	Sekong	11	6/11 (54·5) (23·4-83·3)	2/11 (18·2) (2·3-51·8)	3/11 (27·3) (6·0-61·0)	0/11 (0·0) (0-28·5)	0/11 (0·0) (0-28·5)
Bangladesh	Ramu	120	117/117 (100·0) (96·9-100·0)	NA	NA	0/119 (0·0) (0-3·1)	0/119 (0·0) (0-3·1)
India	Agartala	83	63/69 (91·3) (82·0-96·7)	NA	6/69 (8·7) (3·3-18·0)	0/11 (0·0) (0-28·5)	0/11 (0·0) (0-28·5)
	Midnapur	102	79/89 (88·8) (80·3-94·5)	NA	10/89 (11·2) (5·5-19·7)	0/11 (0·0) (0-28·5)	0/11 (0·0) (0-28·5)
	Rourkela	112	84/87 (96·6) (90·3-99·3)	NA	3/87 (3·4) (0·7-9·7)	0/14 (0·0) (0-23·2)	0/14 (0·0) (0-23·2)
DRC	Kinshasa	120	115/119 (96·6) (91·6-99·1)	NA	4/119 (3·4) (0·1-8·4)	0/103 (0·0) (0-3·5)	0/103 (0·0) (0-3·5)

Supplementary table S2b: Molecular markers of resistance by site (*kelch13* mutation status)

	Bangladesh	DRC	India			Cambodia				Laos	Myanmar				Thailand		Vietnam	
Kelch13-status	Ramu	Kinshasa	Rourkela	Midnapur	Agartala	Pursat	Preah Vihear	Ratanakiri	Pailin	Sekong	Pyin Oo Lwin	Thabbeikyin	Pyay	Ann	Phusing	Khun Han	Binh Phuoc	Total
WT	117	115	84	79	63	8		12	2	6	17	23	32	27			7	592
D353N,WT			1															1
R365K,WT				1														1
D399N,WT			1															1
F446I											5	2						7
F446I,WT												1						1
D464N,WT				1														1
G453S,WT					1													1
Y493H						4				3								7
Y493H,C580Y,WT						1			1									2
G496D,WT				1														1
Y511H												1						1
R539T								1										1
G545R,WT				1														1
G548V,WT				1														1
R561H											5							5
R561H,C580Y,WT																	1	1
E567K,WT				1														1
A578S		1		1														2
A578S,WT		1																1
C580Y						91	7	74	57	2					30	11	106	378
C580Y,WT						3		2	4					1	2		5	17
Q613E		1																1
G625E,WT					1													1
V637I,WT				1														1
F662Y,WT				1														1
K658R,WT			1															1
E668K,WT					1													1
G690D,WT					1													1
S695T,WT		1																1
H697N,C580Y																	1	1
N704T,WT				1														1
G709D,WT					1													1
G718S,WT					1													1
Total	117	119	87	89	69	107	7	89	64	11	27	27	32	28	32	11	120	1,036

Supplementary Table S3: Relative differences in C_{max}, T_{max} and AUC after Triple ACTs versus ACTs

	Relative difference (90% confidence interval)			
	C_{max}	T_{max}	AUC_T	AUC_{T-}last dose
Dihydroartemisinin DHA-PPQ+MQ (n=19) versus DHA-PPQ (n=21)	0·080 (-25·8, 35·0)	4·75 (-15·7, 30·1)	-18·8 (-35·1, 1·53)	-
Piperaquine DHA-PPQ+MQ (n=19) versus DHA-PPQ (n=21)	-22·9 (-45·0, 8·03)	-28·4 (-47·6, -2·07)	-25·1 (-45·5, 2·93)	-2·03 (-20·7, 21,0)
Artemether AL+AQ (n=21) versus AL (n=20)	-24·9% (-42·9, -1·31)	7·07% (-21·2, 45·6)	-15·9% (-33·1, 5·77)	-
Dihydroartemisinin AL+AQ (n=21) versus AL (n=20)	-32·0% (-51·1, -5·39)	2·44% (-23·7, 37·6)	-24·6% (-45·0, 3·38)	-
Lumefantrine AL+AQ (n=21) versus AL (n=20)	-17·5% (-67·8, 111)	9·55% (-1·96, 22·4)	-32·0% (-72·3, 67·5)	-48·4 (-64·5, -25·0)
Desbutyl-lumefantrine AL+AQ (n=21) versus AL (n=20)	-	-	-20·0% (-58·3, -53·4)	-45·7 (-63·8, -18·5)

Relative differences in C_{max}, T_{max}, AUC_T and AUC_T last dose after treatment with the DHA-PPQ+MQ and AL+AQ in comparison with the corresponding ACTs DHA-PPQ and AL, respectively. DHA-PPQ: dihydroartemisinin-piperaquine, MQ: mefloquine, AL: artemether-lumefantrine, AQ: amodiaquine, C_{max} is the maximum concentration divided by mg/kg dose, T_{max} is the time to reach the maximum concentration, AUC_T is the area under the concentration-time curve to time T divided by mg/kg dose, and AUC_{T-}last dose is the area under the concentration-time curve to time T divided by mg/kg dose after administration of the last dose.

Supplementary table S4: Adverse events related to reported symptoms in the first 7 days of treatment by study arm

	DHA-piperaquine		DHA-piperaquine +mefloquine		Artesunate-mefloquine		Artemether-lumefantrine		Artemether-lumefantrine +amodiaquine	
	183		269		73		289		286	
Grading of adverse events	1-2	3-4	1-2	3-4	1-2	3-4	1-2	3-4	1-2	3-4
Headache	11 (6·0)	0 (0·0)	26 (9·7)	1 (0·4)	4 (5·5)	0 (0·0)	10 (3·5)	1 (0·3)	9 (3·1)	0 (0·0)
Fatigue	11 (6·0)	0 (0·0)	23 (8·6)	0 (0·0)	3 (4·1)	1 (1·4)	6 (2·1)	0 (0·0)	9 (3·1)	0 (0·0)
Abdominal pain	6 (3·3)	0 (0·0)	11 (4·1)	0 (0·0)	4 (5·5)	0 (0·0)	6 (2·1)	0 (0·0)	8 (2·8)	0 (0·0)
Loss of appetite	8 (4·4)	0 (0·0)	14 (5·2)	0 (0·0)	8 (11·0)	0 (0·0)	14 (4·8)	0 (0·0)	21 (7·3)	0 (0·0)
Nausea	10 (5·5)	0 (0·0)	36 (13·4)	0 (0·0)	5 (6·8)	0 (0·0)	3 (1·0)	0 (0·0)	12 (4·2)	0 (0·0)
Vomiting	11 (6·0)	0 (0·0)	27 (10·0)	0 (0·0)	6 (8·2)	0 (0·0)	7 (2·4)	0 (0·0)	19 (6·6)	0 (0·0)
Diarrhoea	5 (2·7)	0 (0·0)	23 (8·6)	0 (0·0)	6 (8·2)	0 (0·0)	3 (1·0)	0 (0·0)	4 (1·4)	0 (0·0)
Itching	3 (1·6)	0 (0·0)	3 (1·1)	0 (0·0)	1 (1·4)	0 (0·0)	2 (0·7)	0 (0·0)	2 (0·7)	0 (0·0)
Dizziness	11 (6·0)	1 (0·5)	36 (13·4)	2 (0·7)	16 (21·9)	2 (2·7)	9 (3·1)	0 (0·0)	15 (5·2)	0 (0·0)
Blurred vision	1 (0·5)	0 (0·0)	7 (2·6)	0 (0·0)	10 (13·7)	0 (0·0)	2 (0·7)	0 (0·0)	2 (0·7)	0 (0·0)
Sleeping disturbance	7 (3·8)	0 (0·0)	20 (7·4)	1 (0·4)	13 (17·8)	3 (4·1)	1 (0·3)	0 (0·0)	1 (0·3)	0 (0·0)
Total	84 / 183 (45·9)	1/183 (0·5)	226 / 269 (84·0)	4/269 (1·5)	76 / 73 (104·1)	6/73 (8·2)	63 / 289 (21·8)	1 / 289 (0·3)	102 / 286 (35·7)	0/286 (0·0)

Supplementary table S5: Vomiting rates within 1 hour after study drug administration.

Vomiting rates per timepoint by study arm and age category						
Time point	**DHA-PPQ ≥12 years**	**DHA-PPQ+MQ ≥12 years**	**ART-MQ ≥12 years**	**DHA-PPQ <12 years**	**DHA-PPQ+MQ <12 years**	**ART-MQ <12 years**
	n=171	n=258	n=73	n=12	n=11	n=0
H0	2/171	5/258	1/73	0/12	3/11	NA
H24	1/168	8/252	1/73	0/12	0/11	NA
H48	4/168	14/251	1/73	1/12	0/11	NA
Total	7/507	27/761	3/219	1/36	3/33	NA
	1·4%	3·5%	1·4%	2·8%	9·1%	NA
Time point	**AL Asia ≥12 years**	**AL+AQ Asia ≥12 years**	**AL Asia <12 years**	**AL+AQ Asia <12 years**	**AL Children DRC**	**AL+AQ Children DRC**
	n=187	n=174	n=42	n=52	n=60	n=60
H0	0/187	4/174	2/42	0/52	5/60	1/60
H8	0/187	2/174	0/40	0/52	1/60	1/60
H24	0/187	2/171	0/40	3/52	0/60	2/60
H36	0/187	2/171	0/40	0/52	3/60	0/60
H48	0/186	1/171	0/40	0/52	0/60	2/60
H60	0/185	1/171	0/40	0/51	0/60	1/60
Total	0/1119	12/1032	2/242	3/311	9/360	7/360
	0·0%	1·2%	0·8%	1·0%	2·5%	1·9%

Supplementary table S6a: QTcBazett-intervals and changes in QTcBazett-intervals over time

Time-point	DHA-PPQ		DHA-PPQ+MQ		AS-MQ		AL		AL+AQ	
	QTcB	Δ-QTcB	QTcB	Δ-QTcB	QTcB	Δ-QTcB	QTcB	Δ-QTcB	QTcB	Δ-QTcB
H0	411·9 (17·2)	NA	412·8 (17·8)	NA	411·5 (16·0)	NA	414·6 (18·8)	NA	415·0 (17·9)	NA
H4	420·3 (19·7)	8·4 (15·2)	419·3 (19·2)	6·4 (15·0)	411·8 (15·7)	0·2 (14·2)	415·8 (20·2)	1·1 (14·5)	416·7 (19·1)	1·6 (14·7)
H24	417·9 (18·2)	6·1 (13·6)	418·5 (17·7)	5·4 (12·9)	411·8 (14·8)	0·3 (14·3)	NA	NA	NA	NA
H28	427·5 (19·4)	15·5 (15·5)	427·9 (19·4)	14·7 (16·7)	412·4 (12·8)	0·9 (15·2)	NA	NA	NA	NA
H48	423·3 (19·2)	11·6 (15·2)	424·2 (18·6)	11·3 (17·3)	413·2 (12·9)	1·6 (16·1)	415·1 (19·0)	0·5 (16·3)	420·6 (19·1)	5·6 (17·6)
H52	433·7 (22·9)	22·1 (19·2)	433·6 (19·9)	20·8 (17·8)	413·8 (15·2)	2·2 (16·7)	415·5 (19·6)	0·9 (16·1)	423·6 (20·3)	8·8 (18·6)
H60	NA	NA	NA	NA	NA	NA	415·3 (19·9)	0·5 (17·9)	423·8 (21·0)	6·2 (17·3)
H64	NA	NA	NA	NA	NA	NA	414·1 (20·3)	-0·7 (18·3)	424·4 (20·4)	6·8 (18·8)

ECGs at hour 60 and hour 64 were only performed in subjects in the three Indian sites (artemether-lumefantrine: n=100; artemether-lumefantrine plus amodiaquine: n=99)

Supplementary table S6b: QTcFridericia-intervals and changes in QTcFridericia-intervals over time

Time-point	DHA-PPQ		DHA-PPQ+MQ		AS-MQ		AL		AL+AQ	
	QTcF	dQTcF	QTcF	dQTcF	QTcF	dQTcF	QTcF	dQTcF	QTcF	dQTcF
H0	385·1 (18·7)	NA	388·4 (19·2)	NA	391·0 (18·9)	NA	383·1 (20·0)	NA	383·3 (21·0)	NA
H4	397·7 (21·6)	12·6 (17·8)	397·7 (21·4)	9·2 (18·4)	394·8 (16·9)	3·9 (14·6)	387·2 (20·7)	4·0 (17·0)	387·6 (22·1)	4·2 (17·0)
H24	402·4 (18·1)	17·2 (16·0)	404·9 (19·1)	15·9 (17·6)	399·3 (15·8)	8·3 (16·0)	NA	NA	NA	NA
H28	415·4 (20·4)	30·3 (19·4)	416·1 (21·5)	27·1 (20·2)	398·9 (15·0)	7·9 (15·9)	NA	NA	NA	NA
H48	415·0 (17·8)	30·0 (18·4)	417·6 (18·5)	29·1 (19·3)	406·8 (11·8)	15·6 (17·6)	396·5 (17·8)	13·4 (18·1)	411·4 (20·6)	28·2 (21·4)
H52	428·3 (21·6)	43·3 (22·3)	427·5 (20·5)	39·1 (21·1)	405·6 (15·6)	14·3 (17·0)	398·7 (17·9)	15·8 (18·3)	414·7 (21·6)	31·7 (22·4)
H60	NA	NA	NA	NA	NA	NA	401·3 (20·8)	17·4 (22·9)	420·9 (21·9)	31·9 (22·0)
H64	NA	NA	NA	NA	NA	NA	399·3 (18·6)	15·1 (21·1)	422·1 (21·5)	33·0 (23·4)

ECGs at hour 60 and hour 64 were only performed in subjects in the three Indian sites (artemether-lumefantrine: n=100; artemether-lumefantrine plus amodiaquine: n=99)

Supplementary table S6c: Heart rates and changes in heart rates over time

Time-point	DHA-PPQ		DHA-PPQ+MQ		AS-MQ		AL		AL+AQ	
	Heart rate	Δ-Heart rate	Heart rate	Δ-Heart rate	Heart rate	Δ-Heart rate	Heart rate	Δ-Heart rate	Heart rate	Δ-Heart rate
H0	91·5 (16·9)	NA	88·1 (15·9)	NA	83·2 (15·0)	NA	99·8 (24·6)	NA	99·5 (22·6)	NA
H4	85·5 (16·9)	-6·1 (12·1)	84·1 (16·3)	-4·1 (12·7)	78·6 (14·3)	-4·6 (9·9)	95·3 (24·2)	-4·4 (14·0)	95·7 (22·8)	-3·7 (14·5)
H24	76·5 (13·5)	-15·0 (13·6)	74·5 (14·4)	-13·1 (13·4)	73·0 (10·8)	-10·2 (10·5)	NA	NA	NA	NA
H28	72·4 (12·4)	-19·3 (13·0)	72·2 (13·0)	-15·5 (12·9)	74·1 (10·7)	-9·1 (11·5)	NA	NA	NA	NA
H48	68·3 (11·3)	-23·1 (13·7)	66·6 (10·0)	-21·4 (13·4)	66·5 (9·6)	-16·4 (14·0)	81·2 (19·4)	-18·6 (16·8)	70·3 (16·1)	-29·3 (16·2)
H52	65·5 (10·9)	-25·8 (15·0)	66·2 (10·9)	-21·8 (13·7)	68·4 (10·1)	-14·5 (13·7)	78·9 (19·1)	-20·9 (16·9)	70·0 (16·4)	-29·6 (16·3)
H60	NA	NA	NA	NA	NA	NA	75·4 (16·0)	-22·6 (20·3)	63·4 (10·4)	-30·2 (15·8)
H64	NA	NA	NA	NA	NA	NA	76·0 (15·0)	-21·7 (18·2)	62·8 (9·4)	-30·8 (16·4)

ECGs at hour 60 and hour 64 were only performed in subjects in the three Indian sites (artemether-lumefantrine: n=100; artemether-lumefantrine plus amodiaquine: n=99)

Supplementary Table S7: Listing of Serious Adverse Events

Description SAE	Start day SAE	Last day SAE	Age (years)	Sex	Study arm	Relationship to study drug	Maximum severity	Outcome event	Action taken
Sinus bradycardia with sinus arrhythmia without clinical consequences in young male	1	6	14	M	DHA-PPQ	Possibly related	Moderate	Resolved	Discontinuation of study drug Treatment with artesunate-mefloquine
Vasovagal collapse after venous puncture and before toilet visit	1	1	39	M	DHA-PPQ	Possibly related	Severe	Resolved	Discontinuation of study drug Treatment with artesunate-mefloquine
Pneumonia	16	21	45	M	DHA-PPQ	Not related	Severe	Resolved	Treatment for pneumonia
Admission for treatment of recurrent Plasmodium falciparum infection	28	32	54	M	DHA-PPQ	Probably related	Moderate	Resolved	Treatment for recurrent infection
Severe malaria	0	6	45	F	DHA-PPQ	Not related	Severe	Resolved	Discontinuation of study drug. Treatment with artesunate intravenously
Severe malaria with ARDS	1	4	49	M	DHA-PPQ	Possibly related	Potentially life-threatening	Fatal	Discontinuation of study drug Treatment with artesunate intravenously
Severe malaria	0	6	20	M	DHA-PPQ+MQ	Not related	Severe	Resolved	Discontinuation of study drugs Treatment for severe malaria

Supplementary Table S7: Continued.

Description SAE	Start day SAE	Last day SAE	Age (years)	Sex	Study arm	Relationship to study drug	Maximum severity	Outcome event	Action taken
Severe malaria	1	7	52	M	DHA-PPQ+MQ	Not related	Severe	Resolved	Discontinuation of study drugs Treatment for severe malaria
Severe malaria	0	7	31	M	DHA-PPQ+MQ	Not related	Potentially life-threatening	Resolved	Discontinuation of study drug. Treatment with artesunate intravenously
Post malaria neurological syndrome	2	4	23	M	DHA-PPQ+MQ	Possibly related	Moderate	Resolved	No action taken
Acute disorientation, potentially due to hyponatraemia	1	7	38	M	DHA-PPQ+MQ	Possibly related	Severe	Resolved	Discontinuation of study drug Treatment with artesunate intravenously
Shortness of breath most likely due to malaria/fever	0	1	33	M	DHA-PPQ+MQ	Not related	Severe	Resolved	Discontinuation of study drug Treatment with artesunate intravenously
Junctional rhythm and/or sinus bradycardia without clinical consequences in young male	1	9	17	M	DHA-PPQ+MQ	Possibly related	Moderate	Resolved	Discontinuation of study drug Treatment with artesunate intravenously
Cellulitis leg after mosquito bite	33	38	46	M	DHA-PPQ+MQ	Not related	Severe	Resolved	Antibiotic treatment for cellulitis

Supplementary Table S7: Continued.

Description SAE	Start day SAE	Last day SAE	Age (years)	Sex	Study arm	Relationship to study drug	Maximum severity	Outcome event	Action taken
Persistent fever and increased AST and ALT without an identified cause	3	7	27	M	DHA-PPQ+MQ	Not related	Moderate	Resolved	Antibiotics for fever without an identified cause
QTcBazett-interval>500 milliseconds at hour 52	2	3	11	M	DHA-PPQ+MQ	Possibly related	Severe	Resolved	No action taken
Anaemia, most likely due to malaria infection and hemolysis	5	7	30	M	AS-MQ	Not related	Severe	Resolved	No action taken
AST and ALT increase to grade 4 (Chronic hepatitis C infection)	3	7	27	M	AS-MQ	Possibly related	Potentially life-threatening	Resolved	No action taken
Admission for treatment of recurrent Plasmodium falciparum infection	25	29	5	F	AL	Possibly related	Mild	Resolved	Treatment for recurrent infection
Febrile convulsion	0	5	6	M	AL	Not related	Potentially life-threatening	Resolved	Discontinuation of study drug Treatment with artesunate intravenously
Anaemia, most likely due to malaria infection and haemolysis	1	7	8	M	AL	Not related	Potentially life-threatening	Resolved	No action taken
Creatinine grade 4 and oliguria and fever of unknown cause	6	11	27	M	AL	Not related	Potentially life-threatening	Resolved	Antibiotics for fever without cause
Weakness and relative bradycardia (hypokalaemia and malnutrition)	2	7	5	M	AL+AQ	Possibly related	Potentially life-threatening	Resolved	Discontinuation of study drugs Potassium intravenously
Creatinine increase to grade 4 of unknown cause	28	54	36	M	AL+AQ	Not related	Potentially life-threatening	Resolved	No action taken

Arterolane–piperaquine–mefloquine versus arterolane– piperaquine and artemether–lumefantrine in the treatment of uncomplicated Plasmodium falciparum malaria in Kenyan children: a single-centre, open-label, randomised, non-inferiority trial

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The Lancet Infectious Diseases. 2021 Jun 7;S1473-3099(20)30929-4

6

SUMMARY

Background

Triple antimalarial combination therapies combine potent and rapidly cleared artemisinins or related synthetic ozonides, such as arterolane, with two more slowly eliminated partner drugs to reduce the risk of resistance. We aimed to assess safety, tolerability, and efficacy of arterolane-piperaquine-mefloquine versus arterolane-piperaquine and artemether-lumefantrine for the treatment of uncomplicated falciparum malaria in Kenyan children.

Methods

In this single-centre, open-label, randomised, non-inferiority trial done in Kilifi County Hospital, Kilifi, coastal Kenya, children with uncomplicated *Plasmodium falciparum* malaria were recruited. Eligible patients were aged 2–12 years and had an asexual parasitaemia of 5000–250 000 parasites per µL. The exclusion criteria included the presence of an acute illness other than malaria, the inability to tolerate oral medications, treatment with an artemisinin derivative in the previous 7 days, a known hypersensitivity or contraindication to any of the study drugs, and a QT interval corrected for heart rate (QTc interval) longer than 450 ms. Patients were randomly assigned (1:1:1), by use of blocks of six, nine, and 12, and opaque, sealed, and sequentially numbered envelopes, to receive either arterolane– piperaquine, arterolane–piperaquine–mefloquine, or artemether–lumefantrine. Laboratory staff, but not the patients, the patients' parents or caregivers, clinical or medical officers, nurses, or trial statistician, were masked to the intervention groups. For 3 days, oral artemether–lumefantrine was administered twice daily (target dose 5–24 mg/kg of bodyweight of artemether and 29–144 mg/kg of bodyweight of lumefantrine), and oral arterolane–piperaquine (arterolane dose 4 mg/kg of bodyweight; piperaquine dose 20 mg/kg of bodyweight) and oral arterolane–piperaquine– mefloquine (mefloquine dose 8 mg/kg of bodyweight) were administered once daily. All patients received 0·25 mg/kg of bodyweight of oral primaquine at hour 24. All patients were admitted to Kilifi County Hospital for at least 3 consecutive days and followed up at day 7 and, thereafter, weekly for up to 42 days. The primary endpoint was 42-day PCR-corrected efficacy, defined as the absence of treatment failure in the first 42 days post-treatment, of arterolane– piperaquine–mefloquine versus artemether–lumefantrine, and, along with safety, was analysed in the intention-to-treat population, which comprised all patients who received at least one dose of a study drug. The non-inferiority margin for the risk difference between treatments was –7%. The study is registered in ClinicalTrials.gov, NCT03452475, and is completed.

Findings

Between March 7, 2018, and May 2, 2019, 533 children with *P falciparum* were screened, of whom 217 were randomly assigned to receive either arterolane–piperaquine (n=73), arterolane–piperaquine–mefloquine (n=72), or artemether–lumefantrine (n=72) and

comprised the intention-to-treat population. The 42-day PCR-corrected efficacy after treatment with arterolane–piperaquine–mefloquine (100%, 95% CI 95–100; 72/72) was non-inferior to that after treatment with artemether–lumefantrine (96%, 95% CI 88–99; 69/72; risk difference 4%, 95% CI 0–9; p=0·25). Vomiting rates in the first hour post-drug administration were significantly higher in patients treated with arterolane– piperaquine (5%, 95% CI 2–9; ten of 203 drug administrations; p=0·0013) or arterolane–piperaquine–mefloquine (5%, 3–9; 11 of 209 drug administrations; p=0·0006) than in patients treated with artemether–lumefantrine (1%, 0–2; three of 415 drug administrations). Upper respiratory tract complaints (n=26 for artemether–lumefantrine; n=19 for arterolane–piperaquine–mefloquine; n=23 for arterolane–piperaquine), headache (n=13; n=4; n=5), and abdominal pain (n=7; n=5; n=5) were the most frequently reported adverse events. There were no deaths.

Interpretation

This study shows that arterolane–piperaquine–mefloquine is an efficacious and safe treatment for uncomplicated falciparum malaria in children and could potentially be used to prevent or delay the emergence of antimalarial resistance.

Funding

UK Department for International Development, The Wellcome Trust, The Bill & Melinda Gates Foundation, Sun Pharmaceutical Industries

INTRODUCTION

Artemisinin-based combination therapies (ACTs) are first-line drugs for the treatment of uncomplicated *falciparum* malaria in all malaria endemic countries. ACTs have contributed importantly to the decrease in malaria transmission in the current millennium[87].

However, artemisinin resistance has emerged and spread in the Greater Mekong Subregion in Southeast Asia [25], followed by rapidly increasing resistance to the ACT partner drugs mefloquine and piperaquine. This resistance has caused high treatment failure in patients with uncomplicated *falciparum* malaria treated with artesunate-mefloquine (on Thailand-Burma border) and dihydroartemisinin-piperaquine (in Cambodia, eastern Thailand and Vietnam) [30, 84]. Fit multidrug-resistant malaria parasites could spread to the Indian subcontinent and to sub-Saharan Africa, as has happened in the past with chloroquine and sulphadoxine-pyrimethamine [20, 73]. In addition, artemisinin resistant *P. falciparum* can emerge independently in Africa, as recently shown in Rwanda [34]. Because novel antimalarial compounds are not likely to be registered within the next 5 years [111], strategies that use currently available drugs have to be developed to treat multidrug resistant falciparum malaria and to delay its spread. These strategies include the use of triple Triple ACTs and the use of non-

artemisinin-based triple drug combination therapies. Triple ACTs combine an artemisinin with two partner drugs that are slowly eliminated and have similar pharmacokinetic profiles to each other. Ideally, the two partner drugs in Triple ACTs have counteracting resistance mechanisms, like those between lumefantrine and amodiaquine and between piperaquine and mefloquine [39, 41-43]. The Triple ACTs artemether-lumefantrine plus amodiaquine and dihydroartemisinin-piperaquine plus mefloquine were recently shown to be efficacious, safe, and well tolerated for the treatment of uncomplicated falciparum malaria, including in regions with high ACT failure rates [119]. Non-artemisinin based antimalarials include the synthetic ozonides, arterolane maleate (OZ277) and artefenomel (OZ439). The fixed dose combination arterolane-piperaquine was shown to be an effective and safe treatment for *Plasmodium falciparum* malaria in both children and adults across India and Africa [120, 121] and for *Plasmodium vivax* malaria [122]. The current dosing scheme for arterolane-piperaquine is age-based rather than weight based, resulting in variable dosing per patient weight band. Retrospective analyses of dose finding studies in Africa and Asia indicated that an arterolane dose of 4 mg/kg or higher is needed for optimal parasite clearance (Joel Tarning, unpublished). We aimed to assess the safety, tolerability and efficacy of arterolane-piperaquine plus mefloquine versus arterolane-piperaquine and artemether-lumefantrine, the current first-line treatment in the study area. In the groups containing arterolane-piperaquine, we used novel dosing schedules aimed at an arterolane dose of 4 mg/kg, while maintaining the same arterolane:piperaquine ratio as in the original formulation.

RESEARCH IN CONTEXT

Evidence before this study

We searched PubMed for articles published between database inception and August 31, 2020, using the terms ‘‘arterolane” AND “malaria” which resulted in 34 articles. In addition, we searched PubMed on the same data using terms “malaria” AND (“Triple ACT” OR “TACT”) which resulted in 35 articles. Arterolane maleate is a synthetic, rapidly acting, potent ozonide antimalarial. The fixed dose combination of arterolane-piperaquine has been shown to be effective and safe for the treatment of *Plasmodium falciparum* and *Plasmodium vivax* malaria in both children and adults across India. There were no studies on triple antimalarial combinations including arterolane-piperaquine. A study in healthy Thai adult volunteers found that exposure to the artemisinin dihydroartemisinin (DHA) was decreased when DHA-piperaquine was combined with mefloquine. The TRACII trial showed that the DHA-piperaquine plus mefloquine and artemether-lumefantrine plus amodiaquine were highly efficacious, safe and well tolerated in patients with *Plasmodium falciparum* malaria.

Added value of this study

In this study we show that both arterolane-piperaquine and arterolane-piperaquine plus mefloquine are highly efficacious, safe and well tolerated treatments for uncomplicated

falciparum malaria in Kenyan children. The pharmacokinetic profile of arterolane was not affected by the addition of mefloquine to arterolane-piperaquine.

Implications of all the available evidence

Deploying arterolane-based triple-combination therapies could delay the development of antimalarial drug resistance against arterolane and its partner drugs, because the chance of parasites developing resistance to all three drugs is the product of the chance of developing resistance to each individual drug.

METHODS

Study design and participants

We did a single-centre, open label, non-inferiority, randomized trial in children aged 2 up to 12 years of age in the Kilifi County Hospital in Coastal Kenya (Figure S1). Participants were recruited from Kilifi County Hospital and the Pingilikani dispensary, a dispensary in Banda ra Salama, Pingilikani Sub-Location, with intermediate-to-high malaria transmission that is about 30 km from Kilifi County Hospital (Figure S1). Febrile patients presenting at the Pingilikani dispensary were screened by use of a rapid diagnostic test (SD BIOLINE Malaria Ag P.f/Pan; Abbott Diagnostics Korea; Seoul, South Korea) for *Plasmodium falciparum* malaria. For patients with a positive rapid diagnostic test but no signs of severe or complicated malaria or other disease, written informed consent was obtained from their parent or guardian, after which patients were admitted to Kilifi County Hospital. Patients directly presenting with fever at Kilifi County Hospital were screened for *P falciparum* malaria by use of a rapid diagnostic test and a blood film.

Eligible participants were aged 2–12 years and had uncomplicated *P falciparum* infection, defined as a positive blood smear with asexual forms of *P falciparum* (that might be mixed with non-*falciparum* species), an asexual parasitaemia of 5000–250 000 parasites per µL, and a fever (a tympanic temperature >37·5°C or a history of fever within the past 48 h before enrolment). They were also able to take oral medication, were willing and able to comply with the study protocol for the duration of the study, and had a parent or guardian provide written informed consent. An independent witness was sought in case of an illiterate parent. The exclusion criteria were: signs of severe or complicated malaria according to WHO guidelines, the need for immediate treatment with a parenteral antimalarial, as judged by the treating clinician; an acute illness other than malaria requiring urgent systemic treatment, such as antibiotics; a previous splenectomy; treatment with an artemisinin or an ACT in the previous 7 days; treatment with mefloquine in the previous 2 months; a known hypersensitivity or contraindication to any of the study drugs; a QT interval corrected for heart rate (QTc interval) using Bazett's correction method (QTcB interval) of more than 450 ms; a known personal or family history of cardiac conduction problems; or participation in another clinical trial in the previous 3 months.

The protocol was approved by the Oxford Tropical Research Ethics Committee in the UK and the Kenya Medical Research Institute Scientific and Ethics Review Unit in Kenya. The trial was monitored collaboratively by the Mahidol-Oxford Tropical Medicine Research Unit (MORU) and Kenya Medical Research Institute Wellcome Trust Research Programme clinical trials support groups.

Randomisation and masking

The admitting clinician enrolled all participants, assigned them to the trial groups, and was involved with subsequent patient care and reviews. By use of block sizes of six, nine, and 12, and opaque, sealed, sequentially numbered envelopes, patients were randomly assigned (1:1:1) to receive either arterolane–piperaquine, arterolane–piperaquine–mefloquine, or artemether–lumefantrine. Randomisation sequences were prepared by use of a computer code, with a computer seed included in the program to allow for reproducibility, before the start of the trial by the trial statistician (MM), who conducted the final analysis. Using the randomisation sequences, the envelopes containing treatment allocation information were prepared by the MORU clinical trial support group. All samples were deidentified and the laboratory staff were masked to group assignment. The patients, the patients' parents or caregivers, clinical or medical officers, nurses, and the trial statistician were not masked to the intervention groups. All treatments were directly observed.

Procedures

For 3 consecutive days, oral artemether–lumefantrine was administered twice daily with a fatty snack or drink (containing at least 2 g of fat) to maximise absorption, whereas oral arterolane–piperaquine and oral arterolane– piperaquine–mefloquine were administered once daily with a non-fatty snack or water.[123] Artemether–lumefantrine (Coartem; 20 mg of artemether and 120 mg of lumefantrine per tablet; Novartis, Basel, Switzerland) was dosed by bodyweight according to WHO's guidelines for the treatment of malaria (target dose 5–24 mg/kg of bodyweight of artemether and 29–144 mg/kg of bodyweight of lumefantrine) and was administered at hours 0, 8, 24, 36, 48, and 60.[54] The weight-based dosing schedule of arterolane-piperaquine (Synriam; 37.5 mg arterolane maleate and 187.5 mg of piperaquine phosphate per tablet; Sun Pharmaceuticals, Gurugram, India; administered at hours 0, 24, and 48) aimed for an arterolane dose of 4 mg/kg of bodyweight and a piperaquine dose of 20 mg/kg of bodyweight, matching WHO's recommendations for the dosing of dihydroartemisinin-piperaquine.[54] Mefloquine (Lariam; 250 mg per tablet; Roche, Basel, Switzerland) was administered at hours 0, 24, and 48, together with arterolane–piperaquine, aiming for a dose of 8 mg/kg of bodyweight per day. A single low dose of oral primaquine (Centurion Laboratories; Vadodara, India) was administered 24 h after the start of treatment, according to patient age (target dose 0·25 mg/kg of bodyweight.[124] Dosing schedules are summarised in the appendix. Intravenous artesunate (2·4 mg/kg of bodyweight) was administered as a rescue treatment if uncomplicated malaria progressed to severe malaria. The dosing

of artesunate was scheduled according to WHO guidelines for severe malaria. In case of vomiting within the first 30 min after study drug administration, a full dose of the study drug was readministered. in case of vomiting between 30 min and 60 min post-administration, a half dose of the study drug was re-administrated. All patients were admitted to the Kilifi County Hospital for at least 3 consecutive days and followed up at day 7 and, thereafter, weekly up to 42 days. During each day of admission and at days of follow up, a standardised symptom questionnaire, physical examination, and a measurement of the vital signs were obtained.

A 12-lead electrocardiograph was done at screening, baseline, hour 4, hour 24, hour 28, hour 48 and hour 52. For this study the QTc-interval was calculated by use of both Bazett's (QTcB) as well as Fridericia's correction method (QTcF). Biochemistry and full blood count measurements were done at baseline, day 3, day 7 and day 28. If baseline biochemistry values were abnormal (grade 3 or 4) or the QTc interval prolonged by more than 60 milliseconds at any timepoint compared with baseline, arterolane-piperaquine-mefloquine or arterolane-piperaquine were discontinued and replaced by artemether-lumefantrine. Asexual *Plasmodium falciparum* parasite densities were assessed microscopically at screening, baseline, hour 4, 6, 8, 12 and thereafter every 6 hours until two consecutive blood films were negative. In every participant, parasite densities were also assess at hours 24, 48 and hour 72 (even if two consecutive negative blood films were seen before these timepoints). A venous or capillary blood film was examined at each weekly follow up until day 42 to detect a recurrent infection. A recurrent infection was defined as a blood smear positivity for asexual *Plasmodium falciparum*. According to local guidelines, all recurrent infections were treated with artemether-lumefantrine. Whole blood was collected on dried blood spots at baseline and the day of recurrent infections. Genetic markers of *P falciparum* resistance to artemisinin (*kelch13* non-synonymous mutations) and piperaquine (*plasmepsin2/3* gene amplification) were identified by use of the SPOTMalaria V2 platform, which uses a multiplexed amplicon sequencing method, implemented on Illumina sequencers.

For PCR correction, DNA extraction and purification were done using the standardised kit (QIAamp DNA Mini Kit, Qiagen, Dusseldorf, Germany). Primer sequences, PCR amplification, and analyses were based on methods described previously.[125] Recurrent infections were classified as a recrudescence if all *msp1*, *msp2*, and *glurp* alleles matched those that were present at baseline and as a reinfection if there were one or more allelic differences.[61] Blood samples for pharmacokinetic measurements were obtained 0·5 h, 2·0 h, 6·0 h, 18·0 h, and 48·0 h after baseline in half the patients and 1·0 h, 3·0 h, 12·0 h, 24·0 h, and 72·0 h after baseline in the other half of patients. Plasma samples were shipped on dry ice to Sun Pharmaceutical Industries (the Clinical Pharmacology and Pharmacokinetics Unit) in Gurugram, India, for the assessment of plasma arterolane concentrations using a validated liquid chromatography with tandem mass spectrometry method.

Outcomes

The primary endpoint was the 42-day PCR-corrected efficacy, defined as the absence of treatment failure in the first 42 days after treatment, of arterolane–piperaquine–mefloquine versus artemether–lumefantrine. The PCR-corrected efficacy denotes the absence of recrudescent infections during follow-up. The PCR-uncorrected efficacy denotes the absence of recrudescence and reinfections during follow-up. The 42-day PCR-corrected efficacy of arterolane–piperaquine–mefloquine versus arterolane–piperaquine was an important secondary endpoint.

Other prespecified secondary endpoints were parasite clearance half-lives, slide-positive parasitaemia at day 3, fever clearance times, 28-day PCR-corrected efficacy and 28-day and 42-day PCR-uncorrected efficacy, the proportion of patients completing a full treatment course, vomiting rates within 1 h of study drug administration, the prevalence of adverse events and serious adverse events, changes in heart rate at any timepoint, prolongation of the QTc interval (>60 ms or >500 ms) at hours 4, 24, 28, 48, and 52, and the pharmacokinetic profile of arterolane. Detailed results from the genomics and transcriptomics analyses will be reported separately.

Serious adverse events were reported to the sponsor, the appropriate ethics committees, the regulator, and an independent data and safety monitoring board within 24 h of awareness by the study team. Serious adverse events were defined as per the International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use guidelines for good clinical practice. The data and safety monitoring board met before the start of the trial and evaluated unblinded safety data after recruitment of 30 patients and then 100 patients. All adverse events were graded according to the Division of Acquired Immune Deficiency Syndrome Table for Grading the Severity of Adult and Paediatric Adverse Events (version 2.1; March, 2017), in which grade 1 is mild, grade 2 is moderate, grade 3 is severe, and grade 4 is potentially life-threatening. [102]

Statistical analysis

We hypothesized that the 42-day PCR-corrected efficacy of arterolane-piperaquine plus mefloquine would be non-inferior to artemether-lumefantrine. Based on the experience elsewhere in Africa, we assumed the efficacy of artemether-lumefantrine in Kenya was 98% [120, 126]. WHO guidelines state that a change of first-line treatment should be considered if the efficacy of the first-line treatment is 90% or less. Therefore, we chose a –7% non-inferiority margin for arterolane–piperaquine–mefloquine versus artemether–lumefantrine. With this non-inferiority margin, a power of 80%, and a one-sided significance level of 0·025, a sample size of 63 patients per group was needed. Enrolling 73 patients per group allowed for a 15% loss to follow-up. In a secondary analysis, the efficacy of arterolane–piperaquine– mefloquine was compared with that of arterolane–piperaquine. Efficacy is reported as proportions. In addition, efficacy is reported as

recrudescent and recurrent infection free survival rates using Kaplan-Meier survival methods. We compared efficacy of the study arms using Fisher's exact test. Effect sizes are given as absolute differences or hazard ratios (HRs) with 95% CIs. Non-inferiority was assessed by constructing a two-sided 95% CI on the difference between arterolane–piperaquine–mefloquine and either of the non-triple combinations. Non-inferiority was concluded if the lower bound of the 95% CI did not exceed the non-inferiority margin of –7% of the risk difference. We analysed our primary outcome, safety, and secondary outcomes in the intention-to-treat population, which comprised all patients who received at least one dose of a study drug. Patients requiring rescue treatment with intravenous artesunate or who had a PCR-unclassified recurrent *P falciparum* infection were included in the treatment failure group in the intention-to-treat analysis. Patients who presented with a malaria reinfection, withdrew consent, or were lost to follow-up were included in the treatment success group in the intention-to-treat analysis.

Patients with any of these events or in whom the study drug was replaced by artemether–lumefantrine (eg, because of a prolonged QTc interval) were excluded from the per-protocol analysis and were censored from the Kaplan-Meier survival analysis. We repeated our analyses of the primary outcome and secondary efficacy outcomes in the per-protocol population.

Parasite clearance half-lives were estimated by use of the Worldwide Antimalarial Resistance Network's parasite clearance estimator.[62] The prevalence of adverse events related to symptoms, physical examination, and laboratory abnormalities were compared by use of descriptive statistics. Changes in heart rate and QTc intervals were compared by use of the unpaired *t* test. The incidences of vomiting within the first hour after drug administration were compared between study groups by use of a χ^2 test. p-values are given and statistical significance was declared at 5%. All aforementioned analyses were done in Stata, version 15.

Collected pharmacokinetic data were analysed by use of a non-compartmental approach in Pkanalix, version 2019R2, to assess differences in the pharmacokinetic profile of arterolane with or without mefloquine coadministration. We assumed that arterolane, which is not locally available, was undetectable at baseline (at 0 h) and concentrations less than the lower limit of quantification were replaced with a value equal to half the lower limit of quantification. Because of the sparse sampling design, a non-compartmental analysis was done on the median concentration at each sampling timepoint (naive pooled analysis) comparing the children receiving arterolane– piperaquine with the children receiving arterolane– piperaquine–mefloquine. The analysis was done only after the first dose. The maximum concentration (Cmax) and the time to reach the maximum concentration (Tmax) were derived from the observed data. The terminal elimination rate constant (λ) was estimated by use of the software's best fit functionality (based on an adjusted R^2 value and a uniform weighing). The elimination half-life was calculated as

ln(2/λ). Exposure (area under the concentration time curve [AUC]) was calculated with the trapezoidal method by use of the linear method for ascending concentrations and the log-linear method for descending concentrations. Exposure was calculated to the last timepoint (AUClast) and, by use of λ to extrapolate from the last observed concentration, to infinity (AUC∞). Standard equations were used to calculate apparent elimination clearance and apparent volume of distribution. To evaluate the potential pharmacokinetic differences between arterolane, piperaquine, and mefloquine, concentrations at each sampling timepoint (including the 48 h and 72 h samples) were compared between groups by use of a Mann-Whitney *U*-test in GraphPad Prism, version 8.2.1. A data and safety monitoring board evaluated unblinded safety data after recruitment of 30 patients and then 100 patients. The study is registered in ClinicalTrials. gov, NCT03452475.

Role of the funding source

Arterolane concentrations were measured and financed by Sun Pharmaceutical Industries (Gurugram, India), masked to the treatment group. Arterolane–piperaquine (Synriam) was provided for the study by Sun Pharmaceutical Industries. Other study drugs were purchased against their commercial value. The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Between March 7, 2018, and May 2, 2019, 533 children with an initial rapid diagnostic test positive for *P falciparum* were screened (figure 1). Of these, 217 patients were enrolled in the trial and randomly assigned to receive either arterolane–piperaquine (n=73), arterolane–piperaquine–mefloquine (n=72), or artemether–lumefantrine (n=72). All 217 patients were included in the intention-to-treat and safety analyses. 56 patients were excluded from the per-protocol analysis (figure 1). The median age of all 217 patients was 7·1 years (IQR 4·6–9·6) and just over half were male (table 1). Baseline characteristics were similar between the three study groups (table 1). Throughout the trial, 59 recurrent infections were identified, of which 56 were reinfections, two were unclassified (PCR correction was not possible because of a low DNA sample concentration at the day of recurrence), and one was a recrudescent infection. Of the 56 patients with reinfections, 38 were reinfected before day 42 and were excluded from the per-protocol analysis, 12 were reinfected on day 42, and six were reinfected after having stopped their study drug (figure 1).

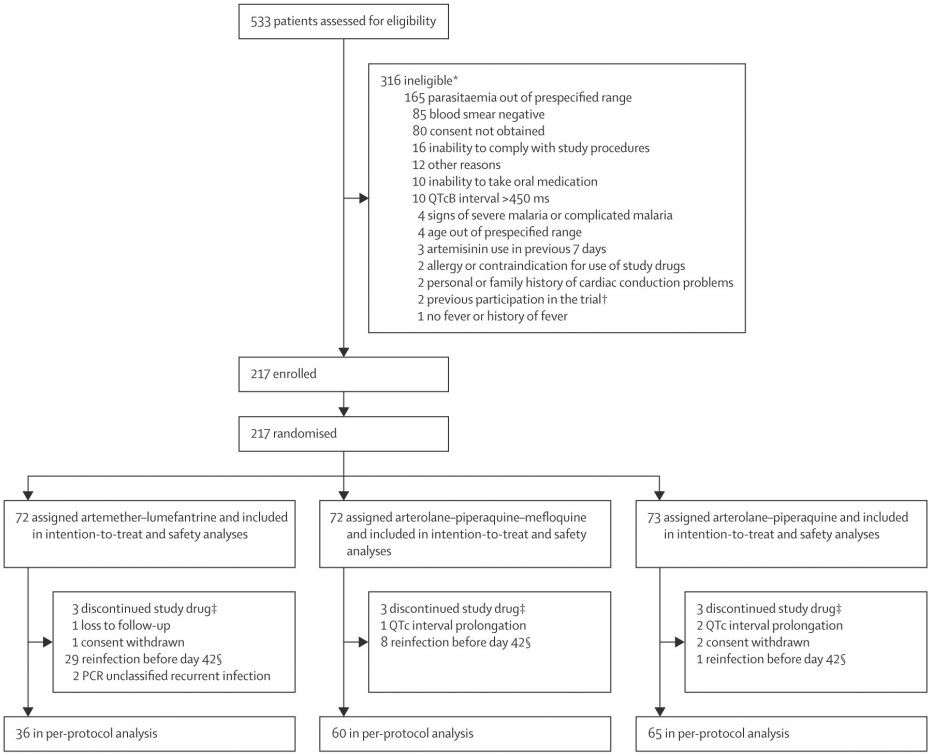

Figure 1. Trial profile

QTcB interval=QT interval corrected for heart rate by use of Bazett's formula. *Reasons for exclusion are not exclusive. Some patients fulfilled more than one exclusion criterion. †After enrolment, randomisation, and administration of the first study drug dose (artemether–lumefantrine), it was found that one patient had been enrolled for a second time (one patient was directly excluded at screening as they had participated in the trial earlier). This patient was not included in the analysis. ‡The study drugs were discontinued in nine patients because of laboratory abnormalities at baseline, as per protocol. These nine patients were included in the intention-to-treat analysis and were excluded from the per-protocol analysis. §Patients with a reinfection at day 42 were included in the per-protocol analysis as a treatment success. A total of six reinfections (one in the artemether–lumefantrine group; two in the arterolane–piperaquine–mefloquine group; three in the arterolane–piperaquine group) occurred in patients that were excluded from the per-protocol analysis because their drugs were discontinued due to QTc interval prolongation (n=2) or baseline laboratory abnormalities (n=4).

Table 1: Baseline characteristics.

	Artemether-lumefantrine (n=72)	Arterolane-piperaquine-mefloquine (n=72)	Arterolane-piperaquine (n=73)	Total (n=217)
Sex				
Female sex	29 (40%)	34 (47%)	42 (58%)	105 (48%)
Male sex, n (%)	43 (60%)	38 (53%)	31 (42%)	112 (52%)
Female sex, n (%)	29/72 (40·3)	34/72 (47·2)	42/73 (57·5)	105/217 (48·4)
Median age, years	7·6 (4·9-9·1)	7·6 (4·3-9·8)	6·7 (4·1-9·5)	7·1 (4·6-9·6)
Mean tympanic temperature, °C	37·5 (1·2)	37·5 (1·2)	37·4 (1·1)	37·5 (1·2)
Weight, kg	18·8 (4·9)	19·4 (6·3)	18·6 (5·9)	18·9 (5·7)
Height, cm	114·7 (13·5)	114·4 (18·2)	112·9 (17·0)	114·0 (16·3)
Median heart rate, beats per minute	118 (103-131)	118 (104-131)	119 (110-130)	119 (105-131)
Median respiratory rate, breaths per minute	29 (26-32)	28 (26-34)	29 (25-32)	29 (26-32)
Median systolic blood pressure, mmHg	107 (100-117)	111 (103-117)	109 (101-118)	109 (101-117)
Median diastolic blood pressure, mmHg	66 (62-74)	70 (64-77)	69 (64-77)	69 (63-77)
QTcB interval ms	420·1 (15·3)	418·9 (16·3)	419·2 (14·3)	419·4 (15·2)
QTcF interval, ms	376·8 (16·9)	376·5 (19·4)	374·0 (17·2)	375·8 (17·8)
Haematocrit, %	30·8 (6·0)	31·0 (3·7)	31·3 (4·2)	31·0 (4·7)
Geometric mean arasite count per *ul**	61,683 (848-358,726)	34,305 (384-326,020)	52,508 (80-571,530)	47,999 (80-571,530)
Gametocytaemia	3 (4%)	1 (1%)	2 (3%)	6 (2%)
Bed net use in night before enrolment	43 (59%)	49 (68%)	51 (70%)	143 (66%)

Data are n (%), median (IQR), mean (SD), or mean (SD; range). QTcB interval=QT interval corrected for heart rate by use of Bazett's formula. QTcF interval=QT interval corrected for heart rate by use of Fridericia's formula. *In some cases, the baseline parasitaemia concentration is outside the screening cutoff range because the parasitaemia decreased or increased between screening and baseline.

In the ITT, the 42-day PCR-corrected efficacy was 100% (95% CI 95–100; 73/73) for patients treated with arterolane–piperaquine, 100% (95–100; 72/72) for patients treated with arterolane– piperaquine–mefloquine, and 96% (88–99; 69/72) for patients treated with artemether–lumefantrine (table 2; figure S2). The 42-day PCR-corrected efficacy for arterolane–piperaquine–mefloquine was non-inferior to that of artemether–lumefantrine as the lower limit of the 95% CI of the risk difference did not cross the –7% non-inferiority margin (risk difference 4%, 95% CI 0–9; p=0·25). Kaplan-Meier survival analyses showed similar results (figure 2; table S1). Furthermore, the 42-day PCR-corrected efficacy of arterolane–piperaquine– mefloquine was non-inferior to that of arterolane–piperaquine (table 2). The two patients with recurrent infections for which PCR correction was not possible were treated with artemether–lumefantrine.

When reconsidering and imputing these two patients as having reinfections, the 42-day PCR-corrected efficacy of artemether–lumefantrine increases to 99% (95% CI 93–100; 71/72). In the ITT population, the 42-day PCR-uncorrected efficacy was 90% for arterolane–piperaquine, 78% for arterolane–piperaquine–mefloquine, and 50% for artemether-lumefantrine (table 2; figure S2), reflecting a shorter post-treatment prophylactic effect conferred by lumefantrine. The 42-day PCR-uncorrected efficacy of arterolane–piperaquine–mefloquine was non-inferior to that of artemether–lumefantrine (table 2), whereas the 42-day PCR-uncorrected efficacy of arterolane–piperaquine–mefloquine was inferior to that of arterolane–piperaquine (table 2). However, in the Kaplan-Meier analysis, the CIs of the efficacy of these two groups overlap (figure 2B). The 28-day PCR-corrected efficacy of arterolane–piperaquine–mefloquine was non-inferior to that of artemether–lumefantrine and that of arterolane–piperaquine (table 2; figure S3). The results from the per-protocol analysis confirmed the results from the intention-to-treat analysis (table 2).

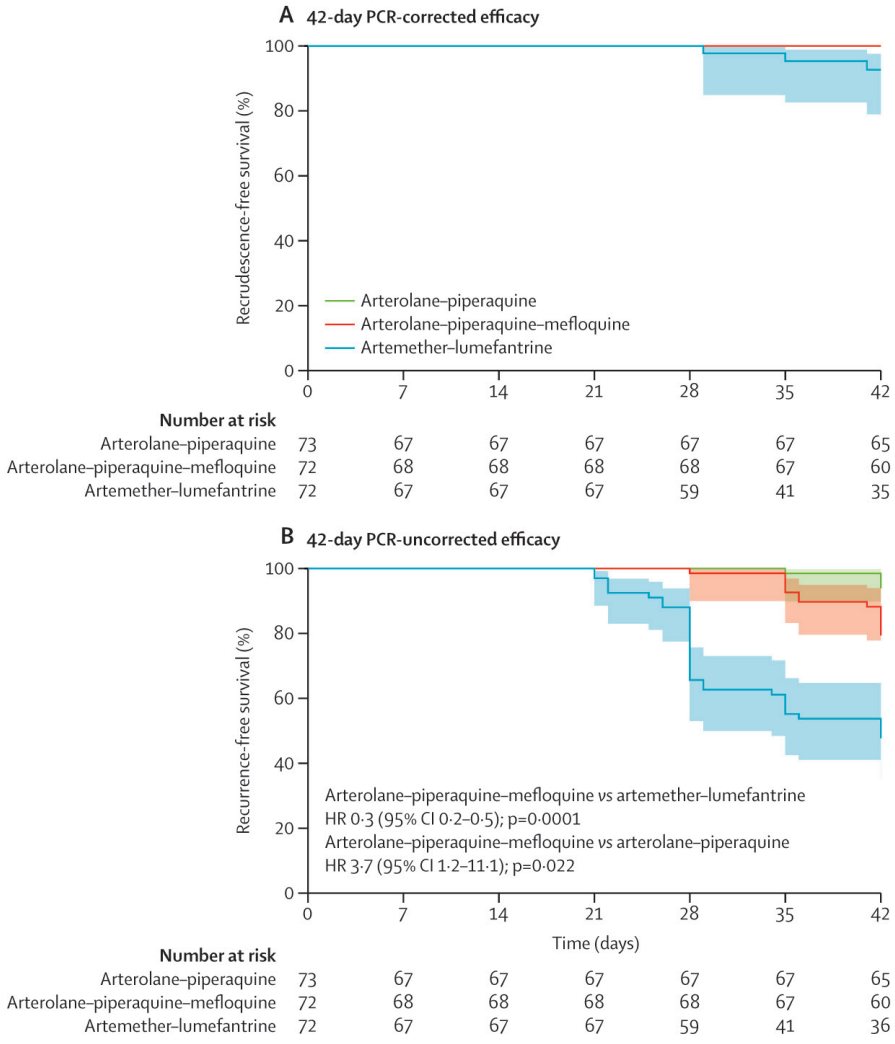

Figure 2. Kaplan-Meier survival curves by treatment group.
42-day Kaplan-Meier survival estimates are shown for the time to *Plasmodium falciparum* recrudescent (A) and recurrent (B) infections following treatment with artemether–lumefantrine, arterolane–piperaquine–mefloquine, and arterolane–piperaquine. No meaningful HR was obtained for42-day PCR-corrected efficacy because one group had a number of participants with the event, but the other two groups had no or only one event. HR=hazard ratio.

Table 2: 42-day and 28-day PCR-corrected and PCR-uncorrected efficacy according to antimalarial treatment in the intention-to-treat and per-protocol populations.

	Artemether-lumefantrine	**Arterolane-piperaquine-mefloquine**	**Arterolane-piperaquine**	**Arterolane-piperaquine-mefloquine versus artemether-lumefantrine**	**Arterolane-piperaquine-mefloquine versus arterolane-piperaquine**
Efficacy at day 42				Risk difference (95%CI) (p-value)	
PCR corrected in the intention-to-treat population	69/72 (96%) (88-99)	72/72 (100%) (95-100)	73/73 (100%) (95-100)	4 (0 to 9) 0·245	0
PCR uncorrected in the intention-to-treat population	36/72 (50%) (38-62)	56/72 (78%) (66-86)	66/7 (90%) (81-96)	28 (13 to 43) 0·0009	-12 (-24 to -1) 0·043
PCR corrected in the per-protocol population	35/36 (97%) (86-100)	60/60 (100%) (94-100)	65/65 (100%) (95-100)	3 (-3 to 8) 0·375	0
PCR uncorrected in the per-protocol population	32/65 (49%) 37-62)	54/68 (79%) (68-88)	62/66 (94%) (85-98)	30 (15 to 46) 0·0003	-15 (-26 to -3) 0·0021
Efficacy at day 28					
PCR corrected in the intention-to-treat population	72/72 (100%) (95-100)	72/72 (100%) (95-100)	73/73 (100%) (95-100)	0	0
PCR uncorrected in the intention-to-treat population	48/72 (67%) (55-77)	69/72 (96%) (88-99)	70/73 (96%) (89-99)	29 (17 to 41) <0·0001	0 (-7 to 6) 1·00
PCR corrected in the per-protocol population	44/44 (100%) (92-100)	67/67 (100%) (95-100)	67/67 (100%) (95-100)	0 (NA) NA	0 (NA) NA
PCR uncorrected in the per-protocol population	42/65 (65%) (52-76)	67/68 (99%) (92-100)	66/66 (100%) (95-100)	34 (22 to 46) <0·0001	-2 (-4 to 1) 1·00

Data are n/N (%) (95% CI), unless otherwise specified. p values were calculated by use of two-sided Fisher's exact tests.

Parasite clearance half-lives could be calculated in 208 of 217 patients (figure 3A; Table S2). Half-lives were longer than 5 h in seven of 208 patients (3%, 95% CI 1–7). There was no significant difference between the mean parasite clearance half-lives in patients treated with arterolane–piperaquine (2·6 h, 95% CI 1·4–4·0), arterolane–piperaquine–mefloquine (2·7 h, 1·6–4·5), and artemether–lumefantrine (2·9, 1·6–4·7; figure 3A; table S2). Slide-positive parasitaemia at day 3 was rare (n=2) and fever clearance times were similar between the three groups (Table S2).

We obtained *Pfkelch13* genotypes for 211 (97%) of 217 baseline samples. Of these, 203 (96%) did not carry any non-synonymous mutations. The Ala578Ser mutation, which is present throughout Africa and not associated with artemisinin resistance, was found in four samples. The other four samples were from mixed infections and contained rare, non-synonymous mutations (Asp399Asn, Ala486Ser, Cys542Arg, and Gly665Ser), whose effect on artemisinin sensitivity is unknown. Amplification of the *Pfplasmepsin*2/3 gene was found in none of the 103 samples in which amplification status could be determined.

The proportions of patients that completed a full treatment course were similar between treatments (table S3). Reasons for discontinuation included baseline abnormalities in biochemistry results (n=9) and prolongation of the QTc interval (n=3).

Vomiting rates were significantly higher in patients treated with arterolane–piperaquine (p=0·0013) or arterolane–piperaquine–mefloquine (p=0·0006) than in patients treated with artemether–lumefantrine (table 3; table S4). All patients were retreated successfully with an oral dose of the same drug combination. Vomiting rates were not significantly different between patients treated with arterolane-piperaquine or arterolane-piperaquine plus mefloquine (p=1·00, table 3, table S4). The numbers of patients that vomited within the first hour after treatment at least once after enrolment in the trial were similar comparable after arterolane-piperaquine (9/73 (12·3%)) and arterolane-piperaquine plus mefloquine (8/72 (11·1%)) (p=1·00, table 3). Prolongation of the QTcB interval at hour 52, the time of expected highest concentrations of piperaquine, was greater after treatment with arterolane-piperaquine (mean increase 18·9 ms (SD 19·9);p<0·0001) or arterolane-piperaquine plus mefloquine (mean increase 15·7 ms (SD 23·4); p=0·0007) than after treatment with artemether-lumefantrine (mean increase 3·6 ms (SD 16·3)(Figure S4, table S5).

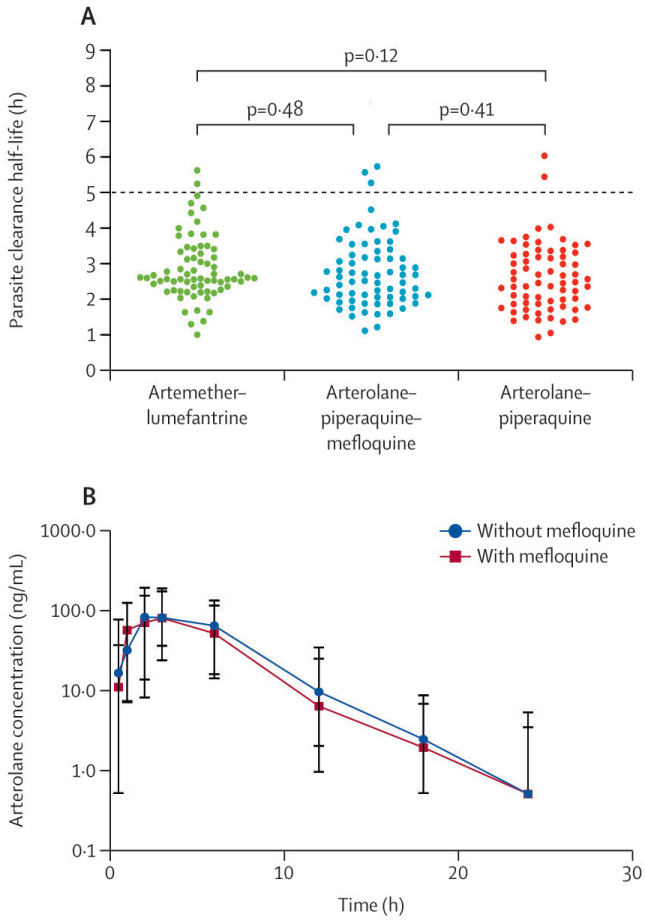

Figure 3: Parasite clearance half-lives and arterolane pharmacokinetics (A) Parasite clearance half-lives by study group. Each individual dot represents an individual patient's parasite clearance half-life after treatment with artemether-lumefantrine, arterolane-piperaquine-mefloquine, or arterolane-piperaquine. Reference bars indicate the mean value for each study group. The red dashed line indicates a half-life of 5 hours, a common cutoff value for the delayed parasite clearance phenotype. Comparasions were done by us of an unpaired t-test. (B) Pharmacokinetic concentration-time profiles of arterolane at an oral dose of 4 mg/kg, given in combination with piperaquine-mefloquine or piperaquine alone. The markers represent the median concentrations, and the bars represent the 5-95th percentiles within each sample collection timepoint.

Prolongation of the QTcF interval at hour 52 was greater after treatment with arterolane-piperaquine (mean increase 35·9 ms (SD 20·4)) or arterolane-piperaquine plus mefloquine (mean increase 31·9 ms (SD 25·8)) than after treatment with artemether-lumefantrine (mean increase 15·5 ms (SD 16·9 ; p<0·0001 for both)). There was no significant difference in QTcB interval prolongation (p=0·29) or QTcF interval prolongation (p=0·68) at hour 52 between treatment with arterolane–piperaquine and treatment with arterolane–piperaquine–mefloquine (figure S4). QTc intervals for the other prespecified timepoints can be found in the appendix (table S5)**.** QTcF and QTcB intervals longer than 500 ms were not observed in any patient (table 3). The prevalence of a QTcB interval prolongation more than 60 ms than that at baseline was similar in patients treated with arterolane–piperaquine, arterolane– piperaquine–mefloquine, and artemether–lumefantrine (table 3). Changes in heart rate from baseline to hour 52 were similar between the arterolane–piperaquine group and the arterolane–piperaquine–mefloquine group (figure S4, table S6). Heart rate decreased more in the arterolane–piperaquine group (p=0·0007) and the arterolane–piperaquine–mefloquine group (p=0·024) than in the artemether–lumefantrine group (figure S4, table S6). The prevalence of bradycardia (heart rate ≤54 beats per min) was not different after treatment with arterolane– piperaquine, arterolane–piperaquine–mefloquine, or artemether–lumefantrine (table 3).

Table 3: Safety outcomes in the intention-to-treat population.

	Artemether-lumefantrine (n=72)	**Arterolane-piperaquine-mefloquine (n=72)**	**Arterolane-piperaquine (n=73)**
Vomiting per number of treatments*	3/415 (1%, 0-2)	11/209 (5%, 3-9)	10/203 (5%, 2-9)
Vomiting at least once during the first h after treatment	3 (4%)	8 (11%)	9 (12%)
QTcB interval >60 ms more than baseline	0	1 (1%)	2 (3%)
QTcF interval >60 ms more than baseline	1 (1%)	13 (18%)	14 (19%)
QTcB interval >500 ms	0	0	0
QTcF interval >500 ms	0	0	0
Bradycardia (≤54 beats per min)	0	2 (3%)	1 (1%)

Data are n/N (%, 95% CI) or n (%). QTcB interval=QT interval corrected for heart rate by use of Bazett's formula. QTcF interval=QT interval corrected for heart rate by use of Fridericia's formula. *Vomiting per number of treatments relates to observed vomiting within 1 h after drug administration. χ^2 tests were used to compare vomiting rates (p=0·0006 for arterolane–piperaquine–mefloquine *vs* artemether–lumefantrine; p=0·0013 for arterolane–piperaquine *vs* artemether– lumefantrine; p=0·88 for arterolane–piperaquine–mefloquine *vs* arterolane– piperaquine).

Headache, abdominal pain, and symptoms of upper respiratory tract infection were the most frequently reported adverse events (table 4). Overall, the prevalence of clinical adverse events (excluding upper respiratory tract complaints) were similar for patients treated with arterolane–piperaquine and patients treated with arterolane–piperaquine–mefloquine (table 4). Mild-to-moderate headache was reported by patients treated with artemether–lumefantrine at a higher frequency than by patients in the other treatment groups, which resulted in a higher total of adverse events for patients treated with artemether–lumefantrine than for patients treated with arterolane–piperaquine or arterolane–piperaquine– mefloquine (table 4). The most common biochemical adverse event was an abnormality in plasma creatinine concentrations (table 4). Mild-to-moderate increases in creatinine concentrations (not exceeding 0·94 mg/dL or 83 µmol/L) were found in 134 (62%) of 217 patients (table 4). This high proportion is probably related to the predefined, relatively low normal values for creatinine concentrations, which were not calibrated specifically for our study population. No difference in the prevalence of adverse events related to liver and renal toxicity was found between the study groups (table 4). None of the patients in this trial fulfilled Hy's criteria for liver toxicity (alanine aminotransferase or aspartate transferase concentrations >3 × the upper limit of normal and total bilirubin concentration >2 × the upper limit of normal). There were no differences between study groups in the prevalence of haematological adverse events and in the change in haemoglobin concentration (table 4). A total of six serious adverse events fulfilling predefined criteria were reported, of which four were in patients treated with arterolane–piperaquine, one was in a patient treated with arterolane–piperaquine–mefloquine, and one was in a patient treated with artemether– lumefantrine (appendix p 9). Grade 4 thrombocytopenia occurred at day 3 (thrombocyte count 19 000 platelets per µL, which recovered to 241 000 platelets per µL by day 7) in a 4-year-old boy and was determined to be disease-related and unrelated to the study drug, arterolane–piperaquine. In three patients, two treated with arterolane–piperaquine and one treated with arterolane– piperaquine–mefloquine, QTcB interval prolongation compared with baseline exceeded 60 ms, resolved within 1 day, and was classified as definitely related to the study drugs. One patient treated with arterolane–piperaquine had a delayed discharge (day 4 instead of day 3; classified as possibly related) as parasite clearance was slow, although clinical recovery was rapid. One 4-year-old girl treated with artemether–lumefantrine was hospitalised for 2 days longer because of a urinary tract infection, which was classified as being unrelated to the study drug. A single dose of primaquine given at hour 24 was well tolerated.

The median concentration–time profiles for the two groups given arterolane are shown in figure 3B and the results from the non-compartmental analysis (ie values for AUC ∞, AUC_{last}, C_{max}, T_{max}, the apparent elimination clearance, the apparent volume of distribution,and the eliminination half-life are shown in table S8. The results indicated no substantial differences in overall arterolane exposures between patient receiving arterolane-piperaquine compared with those receiving arterolane-piperaquine plus

mefloquine (figure 3B). Comparing concentrations at each protocol timepoint, by treatment group, showed no significant difference in arterolane concentrations, except lower arterolane concentrations at hour 72 after arterolane-piperaquine plus mefloquine (p=0·0091; table S9).

Table 4: Adverse events and safety outcomes according to antimalarial treatment in the intention-to-treat population.

	Artemether-lumefantrine (n=72)		**Arterolane-piperaquine-mefloquine (n=72)**		**Arterolane-piperaquine (n=73)**	
	Grades 1-2	Grades 3-4	Grades 1-2	Grades 3-4	Grades 1-2	Grades 3-4
Symptoms						
Upper respiratory complaints*	26 (36%)	0	19 (26%)	0	23 (32%)	0
Headache	13 (18%)	0	4 (6%)	0	5 (7%)	0
Fatigue	1 (1%)	0	0	0	0	0
Abdominal pain	7 (10%)	0	5 (7%)	0	5 (7%)	0
Loss of appetite	2 (3%)	0	1 (1%)	0	1 (1%)	0
Nausea	0	0	0	0	1 (1%)	0
Vomiting†	3 (4%)	0	1 (1%)	0	2 (3%)	0
Diarrhoea	2 (3%)	0	2 (3%)	0	2 (3%)	0
Itching	2 (3%)	0	4 (6%)	0	3 (4%)	0
Dizziness	1 (1%)	0	0	0	0	0
Blurred vision	0	0	0	0	0	0
Sleep disturbance	1 (1%)	0	0	0	0	0
Total‡	33 (46%)	0	17 (24%)	0	19 (26%)	0
Laboratory abnormalities						
Creatinine	47 (65%)	0	42 (58%)	1 (1%)	45 (62%)	0
Total bilirubin	3 (4%)	0	0	1 (1%)	1 (1%)	0
Alkaline phosphatase	0	0	0	0	3 (4%)	0
Alanine aminotransferase	3 (4%)	0	3 (4%)	0	3 (4%)	1 (1%)
Aspartate aminotransferase	2 (3%)	0	5 (7%)	0	3 (4%)	0
γ-glutamyl transferase	6 (8%)	1 (1%)	5 (7%)	0	3 (4%)	0
Heamoglobin decrease ¶	12 (17%)	1 (1%)	9 (13%)	1 (1%)	11 (15%)	1 (1%)
Leukopenia	1 (1%)	0	0	0	0	0
Neutropenia	6 (8%)	0	3 (4%)	0	1 (1%)	1 (1%)
Lymphopenia	0	0	0	0	0	0
Thrombopenia	3 (4%)	1 (1%)	4 (6%)	0	5 (7%)	2 (3%)

Data are n (%), n/N, or n/N (%). *Upper respiratory tract complaints that were described as cough (if mild), conjunctivitis, nasopharyngitis, otitis media, rhinitis, rhinorrhoea, tonsillitis, and upper respiratory tract infections. †The adverse event named vomiting relies on self-reporting, which could explain the observed discrepancy in the prevalence of vomiting and vomiting per number of treatments. ‡Does not include upper respiratory tract complaints. ¶The decrease is compared to the previous timepoint.

DISCUSSION

To our knowledge, this clinical trial is the first to compare the efficacy and safety of the triple antimalarial combination therapy, arterolane–piperaquine–mefloquine, with arterolane–piperaquine and artemether–lumefantrine for the treatment of uncomplicated falciparum malaria in Kenyan children. In addition, the study used a new weight-based dosing schedule for arterolane–piperaquine. The efficacy of arterolane–piperaquine–mefloquine was non-inferior to that of artemether–lumefantrine and arterolane–piperaquine, and all three treatment combinations were well tolerated.

Related to the much shorter plasma half-life of lumefantrine (half-life 3–4 days) compared with mefloquine (half-life 10–20 days) and piperaquine (terminal plasma half-life 20–30 days), the 42-day PCR-uncorrected efficacy, which includes reinfections with *P falciparum*, was low for treatment with artemether–lumefantrine, as result of a short post-treatment prophylactic effect, as has been described previously.[127]. Arterolane exposure was similar between patients treated with arterolane–piperaquine and patients treated with arterolane–piperaquine–mefloquine. This result is reassuring, because a drug–drug interaction study in healthy volunteers observed a 25% decrease in exposure of the artemisinin derivative dihydroartemisinin after the addition of mefloquine to dihydroartemisinin– piperaquine.[116]

In both the arterolane–piperaquine and arterolane–piperaquine–mefloquine groups, vomiting rates were low, although higher than those observed after treatment with artemether–lumefantrine. For the individual patient, the slightly worse tolerability of arterolane–piperaquine and arterolane–piperaquine– mefloquine might be outweighed by their longer post-prophylactic effect compared with artemether–lumefantrine, which could result in fewer malaria episodes, especially in areas of high malaria transmission.

Prolongation of the QTc interval at hour 52 was significantly greater with arterolane–piperaquine or arterolane–piperaquine–mefloquine than with artemether–lumefantrine. Importantly, the addition of mefloquine to arterolane–piperaquine did not further increase the QTc interval, which supports similar observations comparing QTc intervals after the addition of mefloquine to DHA-piperaquine.[119]

The prolongation of the QTc-interval after other piperaquine containing antimalarials, has been shown not to be associated with an increased risk for sudden death [98]. The total prevalence of clinical adverse events was similar for patients treated with arterolane–piperaquine and patients treated with arterolane–piperaquine–mefloquine but was higher for patients treated with artemether–lumefantrine. The prevalence of laboratory abnormalities was similar between the treatment groups.

Combining arterolane with two partner drugs in a triple therapy could preserve the efficacy of each individual drug, as the chance of parasites developing resistance to all

three drugs is the product of the chance of developing resistance to each individual drug, assuming there are no interactions between resistance mechanisms. The cost of a triple antimalarial therapy will be slightly higher than the cost of a standard ACT. However, these increases in costs should be considered against the costs associated with the emergence of multidrug-resistant malaria, which would probably increase the morbidity and mortality of malaria and could set back successes in malaria control and elimination.

Like the artemisinins, synthetic ozonides contain an endoperoxide bridge considered necessary for their parasiticidal potency, and in-vitro studies have shown reduced parasiticidal potency of arterolane (OZ277) in *Pfkelch13*-mutated (artemisinin-resistant) strains, suggesting cross-resistance.[128, 129] However, the slightly longer plasma half-life of arterolane (around 3 h) compared with dihydroartemisinin (<1·5 h) might prolong and thus increase its parasiticidal activity in artemisinin-resistant *P falciparum* infections.[130] Trials evaluating triple arterolane-based combinations in the setting of artemisinin and partner drug resistance are pending. Arterolane– piperaquine is not yet recommended, nor prequalified, by WHO for the treatment of malaria.

Our study had several limitations. The unblinded design could have affected the assessment of adverse events and the attribution of relatedness to the study drugs. However, objective measures, such as parasite clearance half-lives, treatment efficacy, electrocardiograph readings, and laboratory outcomes, are very unlikely to have been affected by the unblinded design of our study. Because our study relied on self-reporting of symptoms and all participants were young children, it is probable that the prevalence of complaints is an underestimation. The number of patients recruited to our study was small, and it is possible that less frequent side-effects were not identified. Furthermore, the study was done in a hospital setting, and each drug dose was administered and observed by a staff member, which might have resulted in a higher study drug adherence than that expected in a non-supervised setting. The most common reasons for study exclusion were having a parasitaemia out of our prespecified range or a negative blood smear. Future studies could evaluate the use of these drugs in clinical settings where diagnosis relies on rapid diagnostic tests because of the unavailability of microscopy. Further studies assessing treatment adherence in a non-clinical, non-supervised setting are needed. In addition, the relevance of the increased rates of vomiting we observed with triple ACTs compared with artemether–lumefantrine, with respect to treatment efficacy, should be assessed. The addition of primaquine is unlikely to have affected the comparison of efficacy and safety between the three study groups because primaquine does not affect asexual-stage *P falciparum*.

In conclusion, dosed according to weight, the efficacy of arterolane–piperaquine–mefloquine was non-inferior to that of artemether–lumefantrine and arterolane–piperaquine, and all combinations were safe and well tolerated, in the treatment of uncomplicated falciparum malaria in Kenyan children.

CONTRIBUTORS

RWvdP, PN, WRJT, MD, MM, NW, MN, CN, JW, MB, JT, NPJD, NJW, PB, and AMD designed the study. MH, RWvdP, PN, and MD were involved in the organisation of the trial. MH, RWvdP, PN, MN, NM, MB, MD, and NW were involved in the training of the study teams. BM and NW were involved in data management. JW, MN, NM, and MB recruited the study participants. JW and MN collected the study samples. CN, GM, and PK did the laboratory work. AAL and AK coordinated the measurement of arterolane concentrations in the plasma samples. RMH and JT analysed the pharmacological data. MI, SG, and OM generated and analysed the parasite genetic data. MH, RWvdP, MM, NW, and AMD accessed and verified the data. MH, RWvdP, MM, NJW, PB, and AMD wrote the first draft of the manuscript. All authors read and contributed to the final version of the manuscript. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

DECLARATION OF INTERESTS

We declare no competing interests.

ACKNOWLEDGMENTS

This study was supported by the UK Department for International Development (201900). The Mahidol Oxford Tropical Medicine Research Programme and the Kenya Medical Research Institute Wellcome Trust Research Programme are funded by the Wellcome Trust of the UK. The pharmacokinetic drug measurements were funded and done by Sun Pharmaceutical Industries, Gurugram, India. The pharmacokinetic data analysis was funded by the Bill & Melinda Gates Foundation. We thank all patients who took part in our study. We also thank Brian Angus, Ric Price, and Christian Holm Hansen for their important guidance in their roles on the data and safety monitoring board. We thank Prayoon Yuentrakul for site monitoring and protocol training; Clinton Mwatata and Stephen Mwangala for data entry; Cholrawee Promnarate, Thanaporn Champathai, Raniṭha Vongpromek, and Thanawat Assawariyathipat for laboratory support provided by the WorldWide Antimalarial Resistance Network; and Patrick Hannay for financial administrative support. We thank the staff of the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, for their contribution to the PCR correction of recurrent infections. This publication uses data from the MalariaGEN SPOTMalaria Project; the project is coordinated by the MalariaGEN Resource Centre, with funding from the Wellcome Trust (206194, 090770). We thank the staff in the sample management, genotyping, sequencing, and informatics teams of the Wellcome Sanger Institute for their contribution.

SUPPLEMENTARY MATERIAL

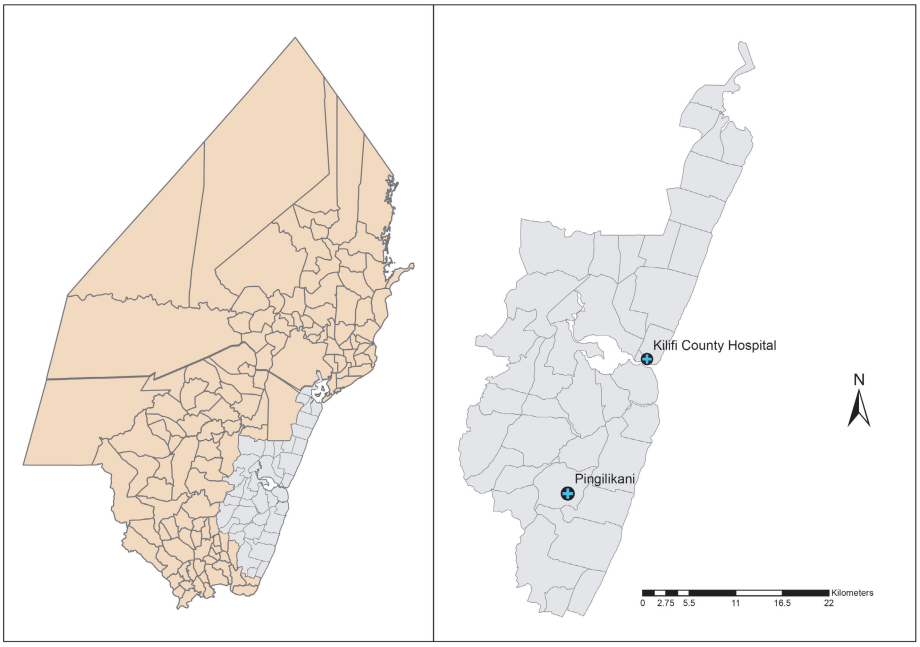

Figure S1. Location of Pingilikani dispensary and Kilifi County Hospital in Kenya

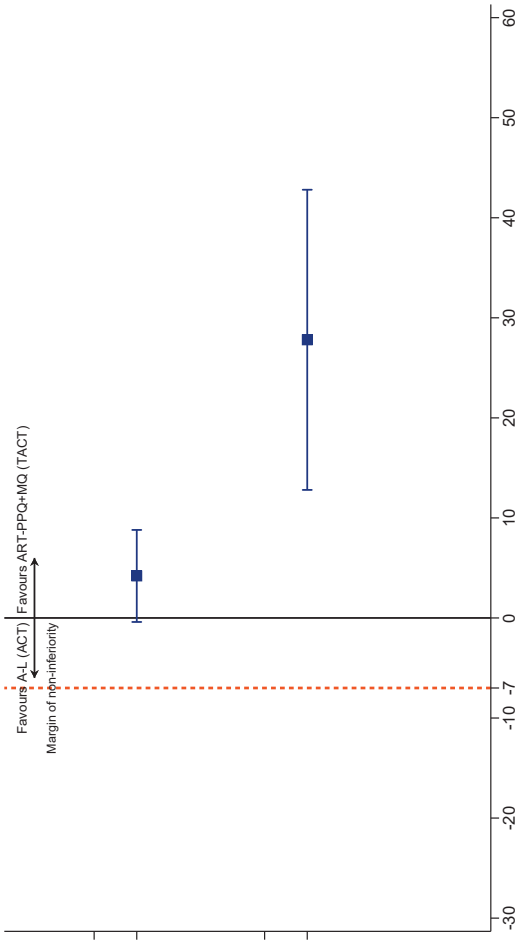

Figure S2. Comparison of day 42 PCR corrected and uncorrected efficacy by study arm
Comparison of 42 day efficacy (risk difference with 95% CI) after receiving artemether-lumefantrine (AL) or arterolane-piperaquine+mefloquine (ART-PPQ+MQ). The 42 day PCR corrected efficacy denotes the absence of recrudescent infections during the 42 days of follow-up. The 42 day PCR uncorrected efficacy denotes the absence of both recrudescence and reinfections with *P. falciparum* during the follow-up period.

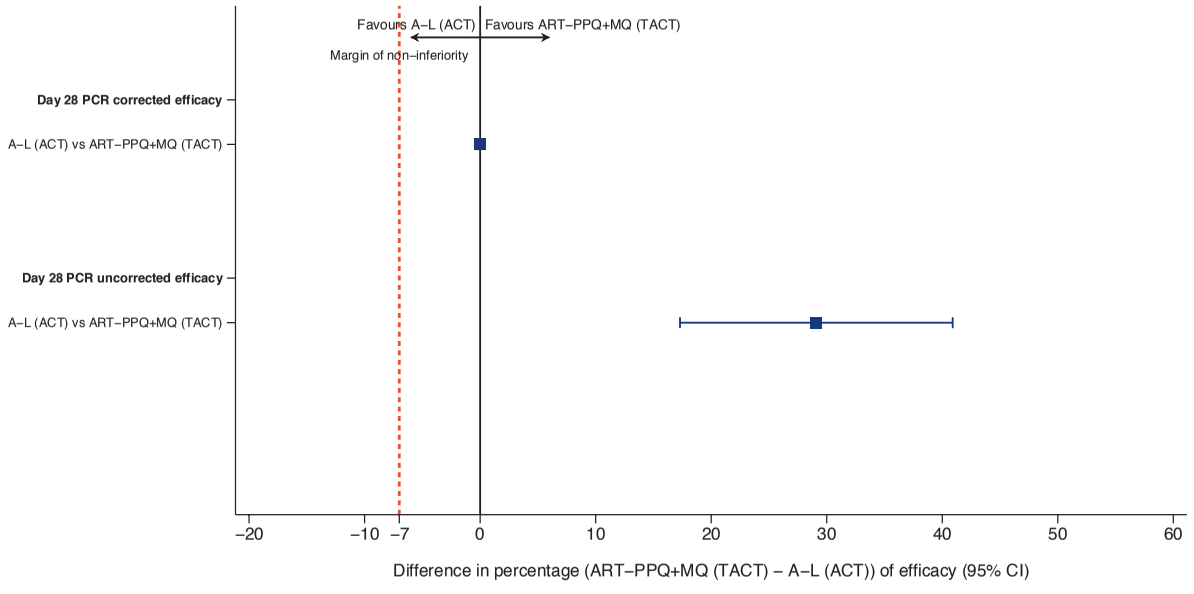

Figure S3. Comparison of day 28 PCR corrected and uncorrected efficacy by study arm

Comparison of 28 day efficacy (risk difference with 95% CI) after receiving artemether-lumefantrine (AL) or arterolane-piperaquine+mefloquine (ART-PPQ+MQ). The 28 day PCR corrected efficacy denotes the absence of recrudescent infections during the first 28 days of follow-up. The 28 day PCR uncorrected efficacy denotes the absence of both recrudescence and reinfections with *P. falciparum* during the first 28 days of follow-up.

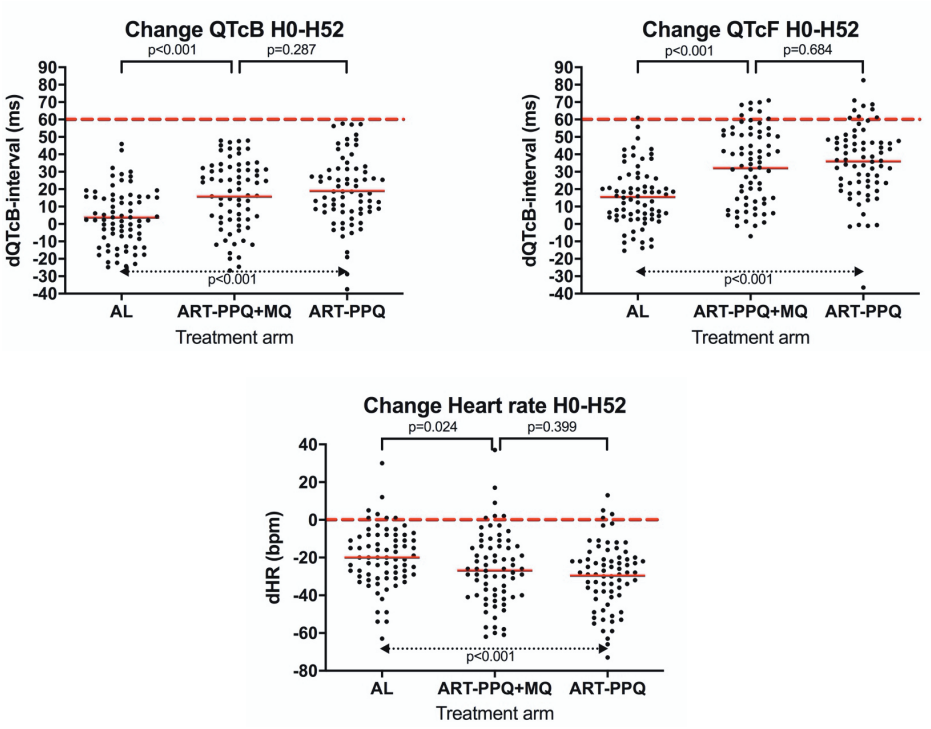

Figure S4. Changes in QTcBazett-interval, QTc-Fridericia-interval and heart rates

Each individual dot represents the change within each subject of the QTcBazett-interval (QTcB), QTcFridericia-interval (QTcF) and heart rate between baseline and hour 52 after arterolane-piperaquine (ART-PPQ), arterolane-piperaquine+mefloquine (ART-PPQ+MQ) and artemether-lumefantrine (AL). Hour 52 after baseline represents the timing of the expected peak values of piperaquine. Reference bars indicate mean changes. The red dashed line indicates a change in QTc-interval of 60 miliseconds, which was used as a safety cut-off in this trial. Comparisons were performed using an unpaired t-test.

Table S1: 42 day and 28 day efficacy (PCR corrected and uncorrected) using Kaplan Meier survival analysis

	Artemether-lumefantrine	**Arterolane-piperaquine +mefloquine**	**Arterolane-piperaquine**	**Arterolane-piperaquine+mefloquine versus artemether-lumefantrine**	**Arterolane-piperaquine+mefloquine versus Arterolane-piperaquine**
	% (95% CI)	% (95% CI)	% (95% CI)	Hazard ratio (95%CI) (p-value)	
PCR corrected efficacy at day 42	93 (79 to 98)	100 (NA)	100 (NA)	NA*	1 (NA) NA
PCR uncorrected efficacy at day 42	48 (36 to 59)	79 (68 to 87)	94 (85 to 98)	0.3(0.2 to 0.5) (0·0001)	3.7(1.2 to 11.1) (0·022)
PCR corrected efficacy at day 28	100·0 (NA)	100·0 (NA)	100 (NA)	1 (NA) NA	1 (NA) NA
PCR uncorrected efficacy at day 28	66 (53 to 76)	99 (90 to 100)	100 (NA)	0.04(0.005 to 0.3) (0·0015)	NA*

NA* no meaningful Hazard ratio is obtained because one arm has a number of participants with an event, but the other two arms have, no/or only one event. This phenomenon creates complexities for the Maximum Likelihood Estimation (MLE) algorithms and the estimated hazard ratios become infinite (too large).

Table S2: Parasite clearance characteristics, parasite genotypes and fever clearance time by arm

	Artemether-lumefantrine	Arterolane-piperaquine+mefloquine	Arterolane-piperaquine
Mean parasite clearance half-life in hours (CI 95%)*	2·9 (1·6-4·7)	2·7 (1·6-4·5)	2·6 (1·4-4·0)
Median parasite clearance half-life in hours (25^{th}-75^{th} percentile)	2·6 (2·4-3·3)	2·6 (2·1-3·2)	2·5 (1·9-3·3)
Parasite clearance half-life >5 hours (n/N, %, CI 95%)	2/70 (2·9) (0·3-9·9)	3/70 (4·3) (0·9-12·0)	2/68 (2·9) (0·4-10·2)
Median time to 50% parasite clearance in hours (25^{th}-75^{th} percentile)	5·9 (4·1-7·3)	6·3 (4·4-7·6)	5·5 (4·0-7·0)
Median time to 90% parasite clearance in hours (25^{th}-75^{th} percentile)	12·6 (9·8-14·5)	12·0 (10·0-13·5)	10·8 (9·2-13·2)
Day 3 positivity) (n/N, %, CI 95%)	1/73 (1·4) (0-7·4)	0/70 (0) (0-5·1)	1/73 (1·4) (0-7·4)
Pfkelch13 genotype: wild-type	66/67 (98·5) (92·0-99·9)	71/71 (0) (94·9-100)	66/73 (90·4) (81·2-96·1)
Pfkelch13 genotype: A578S or mixed infection wild-type/A578S	1/67 (0) (0-5·4)	0/71 (0) (0-5·1)	3/73 (4·1) (0·9-11·5)
Pfkelch13 genotype: Other non-synonymous mutations	0/67 (0) (0-5·4)	0/71 (0) (0-5·1)	4/73 (5·5) (1-5·13·4)
Pfplasmepsin2/3 gene amplification	0/31 (0) (0-11·2)	0/31 (0) (0-11·2)	0/41 (0) (0-8·6)
Median fever clearance time in hours (25^{th}-75^{th} percentile)	36 (24-48)	36 (24-48)	36 (24-48)

*Parasite clearance half-lives were not obtained in 9 patients.

Table S3: Proportions of patients that completed a full course of treatment

	Artemether-lumefantrine	Arterolane-piperaquine +mefloquine	Arterolane-piperaquine	Arterolane-piperaquine +mefloquine versus Artemether-lumefantrine	Arterolane-piperaquine versus Artemether-lumefantrine	arterolane-piperaquine +mefloquine versus Arterolane-piperaquine
	n/N (%) (95% CI)	n/N (%) (95% CI)	n/N (%) (95% CI)	Difference (95%CI) (p-value)		
Completing full course of study drug	66/72 (91·7) (82·7 to 96·9)	68/72 (94·4) (86·4 to 98·5)	68/73 (93·2) (84·7 to 97·7)	2·7 (-5·6 to 11·0) 0·745	1·5 (-7·1 to 10·0) 0·765	1·2 (-6·6 to 9·0) 1·00

Table S4: Vomiting rates per timepoint within 1 hour study drug administration

	Artemether-lumefantrine	**Arterolane-piperaquine+ mefloquine**	**Arterolane-piperaquine**
	n=72	n=72	n=73
First hour after H0	1/72 (1·4)	7/72 (9·7)	5/73 (6·8)
First hour after H8	2/69 (2·9)	NA	NA
First hour after H24	0/69 (0)	4/69 (5·8)	3/70 (4·3)
First hour after H36	0/69 (0)	NA	NA
First hour after H48	0/69 (0)	0/68 (0)	2/66 (3·0)
First hour after H60	0/67 (0)	NA	NA
Total	3/415 (0·7)	11/209 (5·3)	10/203 (4·9)

Results are presented as vomiting number of administrations (n/N, %)**,** Comparison of incidence of vomiting within 1 hour after drug administration, Artemether-lumefantrine versus arterolane-piperaquine+mefloquine=0·0006, Artemether-lumefantrine versus arterolane-piperaquine=0·0013, Arterolane-piperaquine versus arterolane-piperaquine+mefloquine=1·000

Table S5: QTc-intervals and changes in QTc-intervals over time

Time-point	**Artemether-lumefantrine**				**Arterolane-piperaquine+mefloquine**				**Arterolane-piperaquine**			
	QTcB	Δ-QTcB	QTcF	Δ-QTcF	QTcB	Δ-QTcB	QTcF	Δ-QTcF	QTcB	Δ-QTcB	QTcF	Δ-QTcF
H0	420·1 (15·3)	NA	376·8 (16·9)	NA	418·9 (16·3)	NA	376·5 (19·4)	NA	419·2 (14·3)	NA	374·0 (17·2)	NA
H4	421·1 (17·9)	1·0 (15·4)	381·5 (18·4)	4·8 (15·6)	431·2 (20·3)	12·2 (17·6)	394·6 (23·0)	18·0 (19·5)	435·8 (19·6)	16·6 (15·8)	396·3 (22·3)	22·3 (16·5)
H24	419·8 (14·7)	-0·2 (15·4)	384·1 (16·1)	7·4 (17·9)	426·1 (16·7)	7·2 (15·9)	395·0 (16·7)	18·5 (17·2)	425·4 (15·1)	6·2 (13·9)	391·2 (16·1)	17·2 (14·1)
H28	421·7 (15·7)	1·6 (16·7)	389·1 (16·7)	12·3 (17·5)	438·7 (28·8)	19·8 (28·5)	409·1 (25·7)	32·6 (24·7)	442·9 (19·6)	23·7 (19·4)	411·8 (18·9)	37·8 (18·9)
H48	420·6 (15·3)	0·5 (16·8)	388·8 (15·8)	12·1 (17·0)	424·0 (16·9)	5·1 (17·5)	396·4 (17·9)	19·9 (17·7)	424·2 (16·2)	5·0 (16·6)	394·5 (15·1)	20·5 (15·5)
H52	423·7 (14·6)	3·6 (16·3)	392·2 (16·6)	15·5 (16·9)	434·6 (22·6)	15·7 (23·4)	408·4 (26·4)	31·9 (25·8)	438·2 (18·1)	18·9 (19·9)	410·1 (20·1)	35·9 (20·4)

Data are provided as mean and standard deviation

Table S6: Heart rates and changes in heart rates over time

Time-point	Artemether-lumefantrine		Arterolane-piperaquine+mefloquine		Arterolane-piperaquine	
	Heart rate	Δ-Heart rate	Heart rate	Δ-Heart rate	Heart rate	Δ-Heart rate
H0	117 (20)	NA	116 (21)	NA	121 (20)	NA
H4	110 (19)	-7 (14)	104 (18)	-12 (15)	108 (16)	-13 (14)
H24	104 (19)	-13 (15)	96 (18)	-20 (18)	101 (16)	-20 (14)
H28	99 (16)	-19 (15)	93 (17)	-24 (21)	94 (16)	-27 (16)
H48	98 (18)	-19 (17)	92 (20)	-24 (20)	94 (16)	-27 (17)
H52	97 (19)	-20 (16)	89 (19)	-27 (20)	91 (18)	-30 (17)

Data are provided as mean and standard deviation

Table S7: Listing of Serious Adverse Events

SAE number	SAE description	Study arm	Age	Sex	Relatedness SAE to study drugs	Severity of SAE
1	Thrombocytopenia	Arterolane-piperaquine	4	Male	Not Related	Life-threatening
2	Prolonged QTc interval	Arterolane-piperaquine	4	Male	Definitely related	Severe
3	Prolonged QTc interval	Arterolane-piperaquine	6·7	Male	Definitely related	Severe
4	Prolonged parasite clearance	Arterolane-piperaquine	5·1	Female	Possibly related	Mild
5	Presumed urinary tract infection	Artemether-lumefantrine	4·2	Female	Not related	Moderate
6	Prolonged QTc interval	Arterolane-piperaquine+mefloquine	2·1	Female	Definitely related	Moderate

Table S8: Pharmacokinetic parameters of arterolane, using a naïve-pooled non-compartmental analysis

Parameter	Arterolane-piperaquine	Arterolane-piperaquine+mefloquine
AUC_{∞} (h×ng/mL)	592	518
AUC_{last} (h×ng/mL)	590	516
C_{max} (ng/mL)	83·3	80·9
T_{max} (h)	2·00	3·00
CL/F (L/h)	122	140
V/F (L)	499	663
$t_{1/2}$ (h)	2·83	3·29

AUC is the area under the concentration time curve, C_{max} is the maximum concentration, T_{max} is the time to reach the maximum concentration, CL/F is the apparent elimination clearance, V/F is the apparent volume of distribution, $t_{1/2}$ is the terminal elimination half-life.

Table S9: Comparison of arterolane drug concentrations at individual timepoints

Protocol time (h)	Arterolane drug concentrations (ng/mL)[a]		
	Arterolane given alone, n	Arterolane given together with mefloquine, n	p-value[b]
0.5	16.7 (7.84-35.5), n=36	11.1 (3.17-22.4), n=36	0.072
1	32.1 (20.2-75.2), n=37	57.2 (24.8-75.2), n=36	0.27
2	83.3 (55.2-149), n=36	70.7 (44.1-97.0), n=36	0.11
3	81.8 (53.6-113), n=37	80.9 (63.2-116), n=36	0.73
6	65.0 (34.9-91.8), n=36	52.0 (34.8-76.5), n=36	0.51
12	9.72 (5.11-17.3), n=37	6.44 (2.57-14.1), n=36	0.084
18	2.48 (0.954-3.95), n=36	1.96 (0.946-4.43), n=36	0.85
24	0.515 (0.515-1.35), n=37	0.515 (0.515-0.515), n=35	0.14
48	2.07 (0.696-3.34), n=34[c]	1.33 (0.515-2.34), n=35[c]	0.083
72	2.23 (1.49-5.82), n=35[d]	1.19 (0.515-2.47), n=33[d]	0.0091

Data below the lower limit of quantification (1.03 ng/mL) were replaced with half the limit of quantification concentration (0.515 ng/mL). n is the number of subjects included in the analysis.

[a] Presented as median (25^{th}-75^{th} percentiles)

[b] p-value calculated with Mann-Whitney test

[c] Patients who received only one dose were excluded from the analysis at this timepoint.

[d] Patients who received only one or two doses were excluded from the analysis at this timepoint.

Study drug dosing schedules

Arterolane maleate-piperaquine phosphate dosing schedule (administered at H0, H24 and H48)		
Body weight (kg)	Arterolane-piperaquine (37·5/187·5 mg)	Arterolane-piperaquine (150/750 mg)
5-7·9	0·75	0
8-10·9	1·25	0
11-16·9	1·75	0
17-24·9	2·5	0
25-35·9	3·5	0
36-59·9	1	1
60-79·9	3	1
80-100	1	2

Mefloquine dosing schedule (administered at H0, H24 and H48)	
Body weight (kg)	Milliliter (50 mg/ml)
5-5·9	0·8 milliliter
6-6·9	1 milliliter
7-7·9	1·2 milliliter
8-8·9	1·3 milliliter
9-9·9	1·5 milliliter
10-10·9	1·7 milliliter
11-11·9	1·8 milliliter
	Tablets (250mg/tablet)
12-16·9	0·5 tablet
17-23·9	0·75 tablet
24-33·9	1 tablet
34-43·9	1·25 tablets
44-48·9	1·5 tablets
49-53·9	1·75 tablets
54-63·9	2 tablets
64-71·9	2·25 tablets
72-77·9	2·5 tablets
78-100	2·75 tablets

Artemether-lumefantrine dosing schedule (administered at H0, H8, H24, H36, H48 and H60)	
Body weight (kg)	Artemether-lumefantrine tablet 20mg/120mg
5-14·9	1 tablet
15-24·9	2 tablets
25-34·9	3 tablets
≥35	4 tablets

Primaquine dosing schedule (administered at H24)	
Age (months)	Primaquine dose base in mg
6-<12	1·25 mg
12-<72	2·5 mg
72-<120	5 mg
120-<180	7·5 mg

PART III

Summary, general discussion and future perspectives

Triple Artemisinin-Based Combination Therapies for Malaria – A New Paradigm?

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Trends in Parasitology. October 2020; 12;S1471-4922(20)30254-3
DOI: 10.1016/j.pt.2020.09.011

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SUMMARY

Recent gains in the fight against malaria are threatened by the emergence and spread of artemisinin and partner drug resistance in *Plasmodium falciparum* in the Greater Mekong Subregion (GMS). When artemisinins are combined with a single partner drug, all recommended artemisinin-based combination therapies have shown reduced efficacy in some countries in the GMS at some point. Novel drugs are not available for the near future. Triple artemisinin-based combi- nation therapies, combining artemisinins with two currently available partner drugs, will provide one of the last remaining safe and effective treatments for falciparum malaria that can be deployed rapidly in the GMS, whereas their deployment beyond the GMS could delay or prevent the global emergence and spread of resistance to currently available drugs.

Highlights

Artemisinin and partner drug resistance have resulted in high failure rates of artemisinin-based combination therapies (ACTs) in the GMS. Spread or emergence of resistance beyond the GMS are threats to malaria control. Triple ACTs, combining an artemisinin and two existing partner drugs, could be a stop-gap therapy for treating multidrug-resistant malaria until new antimalarials are available. Where resistance is not established, deployment of Triple ACTs could delay or prevent emergence of resistance and could prolong the longevity of antimalarial compounds used in any Triple-drug combination.

Triple ACTs must be safe, well-tolerated, effective, and affordable. Fixed-dose combinations of three drugs in the same tablet will likely improve adherence. Barriers that hinder deployment and adherence must be identified and addressed early in the development of Triple ACTs.

Artemisinin-Based Combination Therapies Are First-line Treatments for Malaria

Artemisinins are the most potent antimalarial drugs available to date, with a 10 000-fold reduction in *Plasmodium falciparum* parasite burden per 48 h asexual parasite life cycle period in infections caused by artemisinin-sensitive parasites.[7] Artemisinins have a short elimination half-life of ~1 h, which then requires a longer regimen for complete elimination of the infection, but also shortens the window of selection in which drug-resistant parasites outgrow drug-sensitive parasites.[131, 132] As early as 1984, Li *et al*. recognised that, even with 3 days of high-dose artemisinin treatment, ~40% of patients will come back with recrudescent infections.[15] [Due to the short half-life of artemisinin, and probably also due to artemisinin-induced dormancy in asexual-stage parasites, even 7 days of monotherapy is not completely effective.[12-14] Early in its development, Chinese investigators already suggested that artemisinin should be used as combination therapy with one or more partner drugs.[15] Artesunate combined with mefloquine was one of the first artemisinin-based combination therapies (ACTs)

evaluated, showing that a 3-day regimen was safe, well tolerated, and highly effective in treating uncomplicated multidrug-resistant *P. falciparum* malaria in western Thailand.[16] Combining two existing drugs led to high efficacy rates despite the drugs not being fully effective on their own because of their pharmacokinetic properties (artemisinins) or parasite resistance (mefloquine). In the first 3 days of treatment the total parasite load is rapidly reduced by the highly potent yet quickly eliminated artemisinin component, and the remaining parasites are subsequently killed by the less potent yet long-lasting partner drug.[37, 133] In 2006 the World Health Organization (WHO) recommended ACTs as global first-line treatments for *P. falciparum* malaria.[54] Five ACTs are recommended by the WHO: artemether–lumefantrine, artesunate–amodiaquine, artesunate–mefloquine, artesunate–sulfadoxine–pyrimethamine, and dihydroartemisinin–piperaquine.[54] A sixth ACT, artesunate–pyronaridine, has recently been added.[134]

Along with large-scale distribution of insecticide-treated bed nets and chemoprevention in pregnant women and children, the rollout of ACTs has been key in reducing the incidence of malaria and related deaths over the past decade, particularly in sub-Saharan Africa, which bears the brunt of the global malaria burden.[87, 90, 110]

Antimalarial Drug Resistance: Past and Present

Chloroquine-resistant *P. falciparum* parasites appear to have evolved independently in the 1950s and 1960s on at least four separate occasions, but the lineage originating from Cambodia in Southeast Asia migrated westward, and by the late 1980s chloroquine-resistant parasites had spread throughout the African continent [20] with a direct and severe impact. A two- to three-fold increase in malaria-related deaths was observed in multiple countries and different epidemiological contexts across the African continent, with up to a sixfold increase in malaria-attributable mortality reported in some locations.[21] Sulfadoxine- and pyrimethamine-resistant parasites again emerged and spread from Southeast Asia to Africa very soon after the combination was introduced to replace chloroquine, rendering these safe, cheap, and widely available drugs ineffective for the treatment of *P. falciparum* malaria.[22] Artemisinin-resistant *P. falciparum* emerged in western Cambodia probably already in the early 2000s, even before artemisinin-based combination therapies (ACTs) were recommended by the World Health Organization as first-line treatments. It is again likely to have resulted from their widespread inappropriate use as monotherapies at suboptimal dosing in nonimmune populations with higher parasite densities, exacerbated by substandard drugs.[135] Molecular epidemiology has facilitated the tracking of artemisinin-resistant *P. falciparum* across Southeast Asia.[59, 136] Its spread through the GMS triggered an emergency response to eliminate malaria from the region to prevent further spread to South Asia and onwards to Africa. There have nonetheless been observations of artemisinin-resistant *P. falciparum* in India, and more recently independent emergence of artemisinin resistance has been confirmed in Rwanda.[34, 118, 137] Both settings provide a context for the emergence of artemisinin resistance. In India, artesunate–sulfadoxine–pyrimethamine was until recently the first-

line ACT in the presence of widespread sulfadoxine–pyrimethamine resistance, providing an environment for the emergence and spread of artemisinin resistance. In Rwanda, ACT coverage has risen to very high levels, which has contributed to an impressive reduction in *P. falciparum* transmission. However, this has left an intense selective drug pressure on the remaining parasites, infecting a population with low immunity. This implies a worrying scenario of artemisinin-resistant *P. falciparum* spreading across South Asia simultaneously with its *de novo* emergence in Africa. They underline the immediacy and importance of the need for globally deployable novel treatments for multidrug-resistant *P. falciparum*.

Artemisinin and Partner Drug Resistance Cause ACT Failure

Fast clearance of ring-stage parasites distinguishes artemisinins from other antimalarials. Artemisinin resistance in *P. falciparum* is characterised by much slower ring-stage parasite clearance following treatment with an ACT,[62] and was first described in 2009 in western Cambodia.[7] Since then, artemisinin resistance, defined clinically in the GMS as a parasite clearance half-life of >5.5 h, has spread through the GMS and is now present in >80% of infections in northeastern Thailand, Cambodia, and Vietnam.[84] The *in vivo* artemisinin-resistant phenotype associates with *in vitro* higher survival in a purposely designed ring-stage survival assay. It also has a strong association with point mutations in the propeller domain of the *P. falciparum Kelch* gene (*K13*),[24, 138, 139] which indicated the presence of artemisinin resistance at a lower frequency in southern Laos.[140] Reduced sensitivity to artemisinin leads to a larger proportion of parasites surviving the initial 3 days of treatment, which results in an increase in parasite biomass from ~10^5–10^6 to 10^8–10^9 parasites exposed to only the ACT partner drug.[33] The residual partner drug levels will suppress sensitive parasites but allow selection of resistant parasites. Artemisinin resistance followed by selection for partner drug resistance has been observed recently for mefloquine (Thai–Myanmar border) and for piperaquine (northeastern Thailand, Cambodia, and Vietnam), and maybe also amodiaquine (Cambodia), and has resulted in unacceptably high failure rates of these ACTs.[29-31, 84, 141] Therapeutic efficacy studies (TESs), following the WHO protocol, show that artesunate–mefloquine, artesunate–pyronaridine, and artemether–lumefantrine are currently efficacious in Cambodia,[134] although all of these have shown compromised efficacy in past studies.[70, 142, 143] The three ACTs in Cambodia that now show treatment efficacies ≤90%, the WHO-defined threshold for minimal efficacy acceptable for a first-line antimalarial drug, are dihydroartemisinin–piperaquine, artesunate–sulfadoxine-pyrimethamine, and artesunate–amodiaquine.[134] A major concern is the potential for artemisinin and partner drug resistance to spread to Myanmar, Bangladesh, India, and sub-Saharan Africa, similar to the previous spread of resistance to chloroquine and sulfadoxine–pyrimethamine that contributed to millions of malaria-related deaths in African children (Box 1).[20, 21, 73] Recent reports from India of decreased *in vitro* and *in vivo* sensitivity of *P. falciparum* to artemisinins, although in only a few cases or isolates, will have to be monitored closely.[118, 137] Mutations in the *K13* gene have been reported from Africa, but in low frequencies consistent with

background mutation rates rather than gene selection, or in *K13* positions that are not associated with artemisinin resistance, such as the *K13* A578S mutation.[32, 110, 144, 145] However, recent reports from South America and Africa of what appears to be independent emergence of artemisinin-resistant parasites with *K13* mutations are a cause for alarm. Parasites carrying a *K13* C580Y mutation of independent origin and showing an artemisinin-resistant phenotype *in vitro* have been isolated from patients in French Guyana.[35] Studies conducted at three sites in Rwanda showed a 1–20% prevalence of parasites with a single origin carrying a validated artemisinin-resistant marker, *K13* R561H. Infections with these parasites were associated with delayed parasite clearance and constitute strong first evidence of local emergence and spread of artemisinin resistance in Africa.[34] New treatments are urgently needed in areas where artemisinin and partner drug resistance are established. They must be effective, well tolerated, safe, affordable, and less likely to fall to resistance. Although antimalarial drugs in the development pipeline look promising, it is difficult to predict if and when these will reach the market. Most estimates are that new compounds will not be available for wide-scale deployment within the next 4 years.[111, 146] Meanwhile, the window of opportunity to prevent widespread resurgence of multidrug-resistant malaria followed by a likely increase of difficult-to-treat multidrug-resistant falciparum malaria infections is narrowing. Occasional case reports of patients with severe falciparum malaria not responding to intravenous artesunate therapy is particularly concerning.[147] In the recent past, high treatment failure with the then first-line antimalarial dihydroartemisinin-piperaquine in eastern Cambodia and southwestern Vietnam was anecdotally associated with an increase in falciparum malaria cases.

Strategies to Overcome ACT Failure Using Existing Antimalarials

Using existing antimalarials in novel ways could sustain their efficacy.[4, 33] One option is to prolong the duration of therapy. A 3-day course of oral artesunate followed by a 3-day course of ACTs was fully effective, even in areas with clearly established artemisinin resistance.[25] However, this could likely result in lower adherence to the therapy since patients generally feel better quickly, which makes them less likely to complete a 6-day treatment course. Alternatively, different ACTs could be used as first-line treatment, either sequentially or at the same time.[148] If deployed sequentially, the first-line treatment policy would be changed at fixed intervals or when resistance to one of the partner drugs reaches a predefined threshold. In theory, using multiple ACTs at the same time could be the better approach as it would reduce the selective pressure on individual partner drugs – the chance for a parasite to develop resistance to the partner drugs in circulation approaches the product of the probabilities of developing resistance to each partner drug, provided there is no cross-resistance between the drugs in use.[17, 149] Comprehensive and consistent implementation of such strategies, however, is complex, costly, and challenging in under-resourced countries. In the GMS, rapid change to a new ACT has proven to be difficult – in two countries, it could not be achieved before multidrug-resistant parasites had already spread widely.[84]

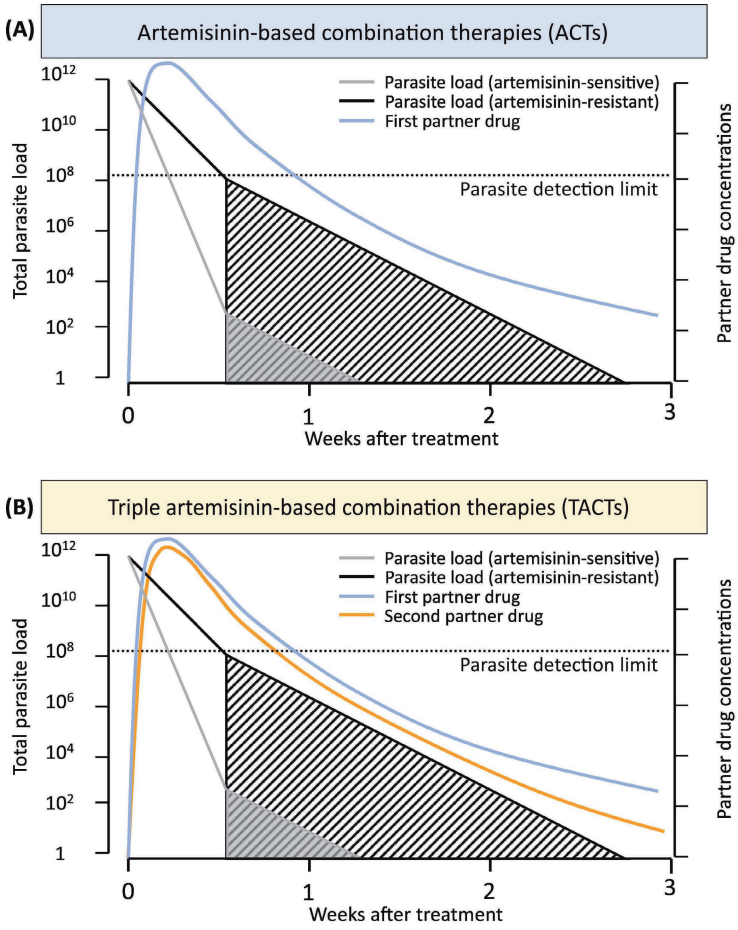

Figure 1. Parasite Clearance Dynamics and Partner Drug Elimination Dynamics in Artemisinin-Based Combination Therapies (ACTs) and Triple Artemisinin-Based Combination Therapies (Triple ACTs). (A) Following 3 days of treatment, parasites are rapidly cleared predominantly by the highly potent yet rapidly eliminated artemisinins. With effective ACTs, any remaining parasites that have not been cleared by artemisinins will be cleared by the partner drug. In the case of artemisinin resistance, parasite reduction over one 48 h parasite life cycle is 100-fold compared to 10 000-fold in sensitive strains, leaving a much higher parasite load after 3 days of ACT treatment for the partner drug to clear (lined area) compared to the case of artemisinin-sensitive parasites (grey area). Thereby artemisinin resistance leads to a larger parasite biomass exposed to a single partner drug. This higher parasite burden, facing declining partner-drug concentrations during an extended period, enables the selection of partner-drug-resistant parasites. Treatment failure occurs when parasites survive both the artemisinin and partner drug. (B) Triple ACTs involve addition of a second partner drug, carefully chosen on the basis of having a matching pharmacokinetic profile, different mode of action, and no cross-resistance, compared to the other partner drug. This approach could increase the efficacy of Triple ACTs compared to ACTs, when resistance already exists, and provide a stronger defence against resistance when resistance has not yet emerged, thereby prolonging the longevity of artemisinins and partner drugs.

Triple Artemisinin-Based Combination Therapies as First-Line Treatment for Malaria

Another strategy to prolong the utility of existing antimalarials is to combine two partner drugs with artemisinins in Triple artemisinin-based combination therapies (Triple ACTs). Such Triple-drug combinations are now standard for treatment of tuberculosis and HIV infections.[150, 151] Compared to ACTs, this could sustain efficacy of the drugs over longer periods, even in the context of artemisinin resistance, wherein partner drugs are exposed to a higher parasite biomass, are unprotected, and are thus more prone to the development of resistance. Partner drugs for Triple ACTs would need to be carefully chosen to provide mutual protection. Their elimination half-lives, or more precisely the time periods of blood concentrations above the minimal parasiticidal concentrations, must be similar to avoid in effect exposing parasites to a single drug (Figure 1). Further, the mechanisms of resistance to either drug must at least be distinct if not mutually counteractive. As with multiple first-line treatments, this approach would reduce the chance of resistance emerging to either of the partner drugs while delivering highly effective treatment to the individual patient. The recent Tracking Resistance to Artemisinin Collaboration II (TRACII) trial assessed the safety, tolerability, and efficacy of two such Triple ACTs.[119] Based on their pharmacokinetic profiles, mefloquine was added to the ACT dihydroartemisinin–piperaquine (DHA–PPQ) and amodiaquine was added to artemether–lumefantrine (AL) respectively to constitute the Triple ACTs. These combinations also took advantage of counterbalancing resistance mechanisms observed in field and laboratory studies between piperaquine versus mefloquine and lumefantrine versus amodiaquine.[29, 42, 43, 60, 152-154] Both Triple ACTs were highly efficacious throughout Asia. In Thailand, Cambodia, and Vietnam, where DHA–PPQ failed in ~50% of patients, DHA–PPQ+MQ was 98% effective, whereas efficacy data of AL+AQ in the same areas will be published shortly. Triple ACTs were safe and generally well tolerated, although loss of appetite, nausea and vomiting occurred with slightly higher frequency. This indicates that Triple ACTs could present an effective option to treat drug-resistant malaria and could be made available quickly. Atovaquone and pyronaridine have very different mechanisms of action, which makes this also a potentially attractive combination.[155, 156] A trial comparing the Triple ACT artesunate–pyronaridine with atovaquone–proguanil and the Triple ACT artesunate–mefloquine with atovaquone–proguanil versus the ACT artesunate–pyronaridine is currently underway in Cambodia (ClinicalTrials.gov identifier: NCT03726593).

Global Deployment of Triple ACTs: Potential Strategies and Barriers

Multiple reasons prevented rapid uptake of ACTs throughout the world, including costs and availability, delays in decision making and implementation of policy, and concerns from policymakers, healthcare providers, and patients. Similar and additional issues will likely surface with the potential future roll-out of Triple ACTs.

Dosing, Formulation, and Production of Triple ACTs

Optimizing the composition of Triple ACTs and dosing regimens has to consider age-stratified pharmacokinetic drug profiles, dose–effect relationships, and dose-related toxicity and tolerability.[97, 157, 158] Suboptimal dosing of any of the components of the Triple ACTs facilitates incomplete parasite clearance and subsequent recrudescences, and thus selection of drug-resistant parasites.[158-161] Coformulation enables accurate dosing, but pharmaceutical issues sometimes affect drug stability. Additionally, *in vivo* interactions between the drugs in the combination need to be assessed.[116] A multitude of factors will determine the additional costs associated with the development of the Triple ACTs. However, most antimalarials currently in use are off-patent, thereby limiting the costs of individual drugs. Although coformulation of Triple ACTs would be preferable regarding patient adherence, co-blistering of an ACT and a second partner drug should be considered as a more rapidly available option in case Triple ACTs are needed urgently in the coming years.

Efficacy, Safety, and Tolerability of Triple ACTs in Adults and Children in Asia and Africa

The safety and tolerability of all currently available ACTs and individual antimalarial components are well established. Common side effects of antimalarial drugs are fatigue, headache, dizziness, nausea, vomiting, and abdominal pain. Most of these symptoms are also common in malaria infections and therefore often result in an overestimation of the side effects of antimalarials. Combining an ACT with another drug will likely result in additional side effects or increase the intensity of side effects attributable to the antimalarial treatment. It is important to quantify this increased risk of adverse events and to assess whether they are dose- or concentration-dependent. Many current antimalarials, including quinine, chloroquine, amodiaquine, mefloquine, and piperaquine have cardiovascular side effects such as exacerbation of malaria-related orthostatic hypotension, sinus bradycardia, and QTc-interval prolongation, but all well within acceptable safety limits when used in therapeutic doses.[162, 163] The QTc-interval is often used as a surrogate marker for the arrhythmogenic risk of antimalarials. The TRACII trial (1100 patients randomised to receive DHA–PPQ (n = 183), DHA–PPQ+MQ (n = 269), artesunate–mefloquine (n = 73), AL (n = 289) and AL+AQ (n = 286)) showed that the addition of mefloquine to DHA–PPQ did not further increase the prolongation of the QTc-interval that is seen over time after administration of DHA–PPQ [51]. Addition of amodiaquine to AL slightly increased the prolongation of the QTc-interval, comparable to administration of amodiaquine alone, and did not raise safety concerns. More extensive data on the safety, tolerability, and efficacy of Triple ACTs, in particular in children from sub-Saharan Africa, will become available in the near future (ClinicalTrials.gov Identifiers: NCT03923725 and NCT03939104).

The (Cost-)effectiveness of Triple ACTs to Prevent or Delay the Emergence of Resistance

Clinical trials on Triple ACTs will not be able to address the potential long-term benefits of the deployment of Triple ACTs to prevent or delay the development of antimalarial drug resistance, and modelling could provide valuable insights here. In the past, mathematical modelling identified the role of mismatched pharmacokinetic and pharmacodynamic (PK/PD) profiles in promoting the evolution of drug-resistant malaria.[164] In recent times there has been a rapid growth in the development and application of models to guide antimalarial drug development, including PK/PD modelling to identify appropriate candidates for development, incorporating laboratory measures of drug resistance and the epidemiological spread of resistance, and transmission models to guide drug deployment and use scenarios.[165, 166] Mathematical modelling has been used to estimate economic losses that would follow ACT failure; direct medical costs for malaria treatment would be 28% higher (US$ 146 million) and the cost of policy change if switching between ACTs would be US$ 130 million.[167] Extensions of such models may be helpful in forecasting the future impact of deploying novel antimalarial therapies, although there are limitations and assumptions inherent to each model. Mathematical modelling could assess the likely epidemiological impact of Triple ACTs as first-line antimalarial treatments in different malaria-endemic settings. This will quantify the potential of Triple ACTs to delay the emergence and spread of artemisinin- and partner-drug-resistant falciparum malaria in the context of different transmission intensities, population mobility, and fitness costs of mutations conferring drug resistance. Pharmacokinetic modelling for partner drugs could determine the dose of the partner drugs at which toxicity is minimised while drug levels are sufficiently high to kill parasites that were not eliminated in the first 3 days of treatment, while shortening the window of selection, defined as the time that parasites are exposed to suboptimal drug levels.[113] In addition, economic modelling could assess the cost-effectiveness of deploying Triple ACTs in different endemic settings, incorporating the potential additional costs of Triple ACTs versus conventional ACTs versus the savings made through reducing and preventing malaria morbidity and mortality resulting from antimalarial drug resistance.

Will Triple ACTs Be Accepted in the Setting of ACTs That Are Still Efficacious?

Ethical aspects of malaria control and elimination have received limited attention but need to be addressed to minimise potential injustice to the autonomy of individuals while ensuring an advantageous risk–benefit balance.[168] Deploying Triple ACTs as first-line treatment in Africa in the absence of widespread ACT failure raises an ethical question: why should a patient in Africa, often a child, take three drugs for malaria instead of two when two drugs work, and when the addition of a drug may result in increased side effects? This question shares some similarities with the ethical considerations of mass antimalarial drug administration, where the long-term benefit for the community will have to be taken partly on trust and cannot be guaranteed.[169] Social and cultural issues delayed the adoption of ACTs over inefficacious conventional antimalarials in Africa.

[170] Past experience suggests that Triple ACTs may not be accepted and prescribed without prior engagement with communities to devise culturally sensitive methods of increasing acceptance and adherence. Critical examination of the views of stakeholders, ranging from patients and parents to regulatory authorities and national malaria control programmes, will contribute to developing recommendations to guide deployment of Triple ACTs in Africa. Without such engagement, acceptance of Triple ACTs is likely to be poor which, in turn, would reduce the impact of deploying Triple ACTs to prevent or delay emergent resistance. The recent emergence of artemisinin resistance in Rwanda, however, might change the risk–benefit and ethical analyses on the deployment of Triple ACTs in Africa, making Triple ACTs more urgently needed.

Market Positioning and Large-Scale Production and Deployment of Triple ACTs

The transition to ACTs for the treatment of malaria has been described as one of the major challenges faced by malaria control in the recent past.[171] Indeed, cost has been cited as the main factor that prevented governments from switching to ACTs.[172] In addition to the social and cultural aspects described previously, market instability had a major influence in deterring widespread adoption and deployment of ACTs, and barriers were not restricted to supply and demand dynamics.[173, 174] Lack of coordination in distribution chains and of private sector compliance have also posed additional challenges. Many stakeholders are involved in the decision to deploy new antimalarials and their views, attitudes, and concerns need to be assessed at an early stage in order to devise strategies for appropriate market positioning of Triple ACTs. Manufacturers will have to be convinced that investments in developing and producing Triple ACTs are warranted. There is a large market; over the period 2010–2018, ~3 billion treatment courses of ACTs were procured for the treatment of malaria, worldwide.[110]

However, malaria is mainly a disease of underprivileged and poor populations and therefore antimalarial drug prices need to be kept very affordable for both the private and public sector. A dialogue between manufacturers and WHO, national malaria-control programmes, academic groups, civil society representation, Medicines for Malaria Venture (MMV), funding mechanisms such as Global Fund, Wellcome, Bill and Melinda Gates Foundation, and other stakeholders will be important to support manufacturers in this decision making.

Concluding Remarks

The underlying scientific rationale for Triple ACTs is easily understood: if drugs with different targets and/or resistance mechanisms are combined, the probability that resistance will emerge to any of its components will be reduced. Effective antimalarials are the cornerstone of national malaria-control programmes, and prevention or delay of antimalarial resistance is essential for the longer-term goal of malaria elimination. In low-transmission zones with high ACT failure rates, such as Cambodia, effective treatment with Triple ACTs will reduce transmission, which is amplified by the prevention of recrudescent infections that produce more gametocytes than primary infections and

are more transmissible.[175, 176] Some key questions should be addressed to facilitate the development, deployment, and uptake of Triple ACTs. Choosing the right combinations of drugs and the optimum dose of each individual component is important. Co-formulating drugs in Triple combinations requires investments by manufacturers. Early provision of a business case analysis for pharmaceutical companies on the economic viability of Triple ACTs, which includes current funding structures for malaria drugs, such as the Global Fund, will likely help engage manufacturers. From the start of the development process it will be important to have insight in the regulatory requirements for licensing of Triple ACTs through WHO prequalification or stringent regulatory authorities. For the potential adaptation of Triple ACTs in treatment guidelines, it will be prudent that study plans, and results, are shared between academia and nonacademic stakeholders, and early involvement of policy makers and the WHO is important. More information on the safety, efficacy, and tolerability of Triple ACTs is needed, especially in children in sub-Saharan Africa. Additionally, the safety of repeated treatments with Triple ACTs should be assessed to support their deployment in high-transmission areas. In areas like the GMS, where artemisinin and partner drug resistance has emerged, Triple ACTs outperforming the efficacy of standard ACTs will be a strong argument for their deployment. For areas not yet affected by these resistance problems, a continued discussion will be needed on the pros and cons of Triple ACT as a new paradigm to break the ever-returning chain of events associated with drug resistance that severely reduces the useful lifetime of valuable antimalarial drugs.[38, 177] With reduced *P. falciparum* transmission in many parts of South America and sub-Saharan Africa the chance of artemisinin and partner drug emergence and spread increases for a variety of reasons. Reduced transmission, which involves better treatment coverage, will decrease population level immunity, which will increase the proportion of symptomatic patients seeking treatment, and will increase further antimalarial drug pressure on the parasite population. Higher drug pressure increases the chance for antimalarial drug resistance to emerge, and reduced immunity increases the probability of survival of low-grade resistant parasites. With reduced transmission, there will be fewer multiclonal infections with subsequent recombination events during the sexual stage of the parasite in the *Anopheles* mosquito, increasing the chance that resistant haplotypes remain intact. When transmission further decreases, genetic diversity will decline, also translating to fewer recombination events between artemisinin- (or partner drug-) sensitive and resistant strains. The increased ACT coverage, and the reduction in *P. falciparu*m malaria transmission, might thus have been drivers for the recently described emergence and local spread of artemisinin resistance in Rwanda. [34] To prevent the further spread or emergence of multidrug-resistant falciparum malaria the deployment of Triple ACTs, worldwide, deserves serious consideration.

As described, several important issues around deployment of Triple ACTs need to be addressed; this will require a multifaceted approach to identify and overcome barriers and provide convincing evidence of the potential benefit of Triple ACTs in order to gain support and endorsement by policy makers. This is being addressed by the Development

of Triple Artemisinin-based Combination Therapies (DeTACT) project, which takes a holistic approach by partnering with pharmaceutical companies, MMV, collaborators in Asia and Africa to conduct clinical trials, and experts in the fields of mathematical modelling, market positioning, bioethics, and engagement. Realizing the vision of a world free of malaria might become a reality if we act now.[178, 179]

CONTRIBUTORS

RWvdP, CA, MD and AMD wrote the manuscript.

ACKNOWLEDGEMENTS

We are grateful to UK aid and the UK Government's Foreign, Commonwealth & Development Office for financial support. We are grateful to Professor Sir Nicholas J. White for providing the original version of figure 1.

Summary, general discussion and future perspectives

8

SUMMARY OF FINDINGS

INTRODUCTION

Chapter 1 sets the stage for the work that is included in this thesis. The relative recent emergence and spread of artemisinin and partner drug resistance in Southeast Asia pose a major threat to the worldwide fight against malaria. If artemisinin and partner drug resistance were to spread to sub-Saharan Africa or emerge there independently, the malaria related mortality would likely increase dramatically. Potentially, Triple ACTs prevent or at least slow down the future emergence and spread of antimalarial resistance. This thesis focusses on the current extent of artemisinin and partner drug resistance and the safety, tolerability and efficacy of Triple ACTs.

EPIDEMIOLOGY OF ANTIMALARIAL RESISTANT MALARIA

Chapter 2 describes the alarmingly low day 42 PCR-corrected efficacy rates of dihydroartemisinin(DHA)-piperaquine in northeastern Thailand (12·7%), western Cambodia (38·2%), northeastern Cambodia (73·4%) and southwestern Vietnam (47·1%). Treatment failure was associated with amplifications of the *plasmepsin2/3* gene and 4 mutations in the chloroquine resistance transporter (*crt*) gene (T93S, H97Y, Phe145I and I218F). *Kelch13* gene mutation status was not found to be associated with treatment failure most likely due to the high overall proportion of infections carrying the C580Y *kelch13* gene mutation (369/404 (91%)). Most of these C580Y *kelch13* gene mutations were of the KEL1 haplotype, suggesting the spread of one strain throughout the region. *Plasmepsin2/3* gene amplification was detected in three-quarter of the samples, while *mdr1* gene amplification was not observed. We identified six mutations in the *crt* gene that increased in prevalence between the two periods in which samples were obtained (2011-2013 and 2015-2018), These mutations appeared to be mutually exclusive, and identified specific haplotypes. The increase of the prevalence of *kelch13* C580Y mutations (KEL1), *plasmepsin2/3* amplifications and the six *crt* mutations support the hypothesis that these genetic markers have selective advantages as they will increase the chance of a treatment failure and subsequent transmission.

In **Chapter 3,** we describe the results of an analysis of 2465 whole parasites genomes, collected between 2007 and 2018 from Cambodia, Laos, northeastern Thailand, and Vietnam. This analysis showed that the KEL1 haplotype, bearing the C580Y *kelch13* mutation combined with a specific *plasmepsin2/3* (PLA1) haplotype. After emerging most likely within western Cambodia and under the continued pressure of region wide DHA-piperaquine use, this KEL1/PLA1 co-lineage diversified into multiple subgroups and acquired new genetic features, including the aforementioned *crt* mutations. Again, the rapid emergence of *kelch13* C580Y mutations (KEL1 haplotype), *plasmepsin2/3*

amplification (PLA1 haplotype) and *crt* mutations suggest them to be markers of an advantageous phenotype. In addition, it is a strong example of the evolutionary processes that occur sequentially when failing drugs are continued. Artemisinin resistance facilitated the selection of piperaquine resistance in the form of *plasmepsin2/3* amplified and *crt* mutated parasites. Finally, **Chapter 2 and 3** show the potential of a multidrug resistant lineage to spread throughout a region rapidly.

SAFETY, TOLERABILITY AND EFFICACY OF TRIPLE ANTIMALARIAL COMBINATION THERAPIES

In **Chapter 4** we describe the results of a healthy volunteer study in 15 Thai adults in which patients were treated with a single dose of the ACT DHA-piperaquine, the Triple ACT DHA-piperaquine plus mefloquine or mefloquine alone. As expected, DHA-piperaquine prolonged the QTc interval slightly. The addition of mefloquine did not further prolong the QTc interval.

These reassuring results are important as the potential synergistic effect of piperaquine and mefloquine on the prolongation of the QTc-interval was a major concern prior to this study.

The most common adverse events after DHA-piperaquine plus mefloquine were moderate dizziness, nausea, abdominal discomfort and disturbance of sleeping, all known side effects of mefloquine. The addition of mefloquine to DHA-piperaquine did not affect the pharmacokinetics of piperaquine. However, the addition of mefloquine did result in a 22.6% lower exposure (AUC) to DHA and a 29.0% lower peak value (C_{MAX}) DHA levels.

These results are of importance as inadequate exposure to artemisinins is a risk factor for the development of artemisinin resistance.

The results of the clinical trial aimed at comparing the safety, tolerability and efficacy of Triple ACTs and ACTs are presented in **Chapter 5**. In this multicentre, open-label, randomised trial, 1100 patients with uncomplicated *P. falciparum* malaria were recruited at eighteen hospitals and health clinics in eight countries. Patients were randomized between DHA–piperaquine or DHA–piperaquine plus mefloquine; between artesunate-mefloquine or DHA-piperaquine plus mefloquine; or between artemether-lumefantrine or artemether-lumefantrine plus amodiaquine. We found unacceptable low efficacy rates of DHA-piperaquine in northeastern Thailand, Cambodia and Vietnam. The efficacy of DHA-piperaquine in Myanmar was high. The efficacy of the Triple ACT DHA-piperaquine plus mefloquine was above 95% throughout Thailand, Cambodia, Vietnam and Myanmar. The efficacy of the ACT artesunate-mefloquine was also above 95% throughout Cambodia. We found high efficacy rates of artemether-lumefantrine

in Laos, Myanmar, Bangladesh, India and the Democratic Republic of the Congo. The addition of amodiaquine to artemether-lumefantrine did not reduce the incidence of reinfections in the Democratic Republic of the Congo.

The C580Y *kelch13* mutation was found in high frequencies in Cambodia, northeastern Thailand and Vietnam and was not found West of Thailand. About half of the infections in Pyin Oo Lwin, Myanmar were caused by parasites carrying *kelch13* mutations, which were not C580Y mutations. In all other sites in Myanmar, Bangladesh, India and the DRC *kelch13* mutations were rare. As expected, there was an association between parasite clearance half-lives and the presence of *kelch13* mutations. Accordingly, parasite clearance half-lives were found to be prolonged in Cambodia, Thailand and Vietnam and to a lesser extent in Laos and Pyin Oo Lwin, Myanmar. In the other sides, prolonged parasite clearance half-lives were rarely found. Both Triple ACTs were generally well tolerated and incidence rates of adverse events after Triple ACTs and ACTs were comparable. However, the addition of mefloquine to DHA-piperaquine did increase vomiting rates within the first hour after administration of the medication. The incidence of serious adverse events was similar after treatment with ACTs or Triple ACTs. As in the healthy volunteer trial described in **Chapter 4** the addition of mefloquine to DHA-piperaquine did not further prolong the QTc-interval. The addition of amodiaquine to artemether-lumefantrine prolonged the QTc-interval in an amount comparable to the prolongation which has been reported after amodiaquine alone. Although the decrease was not significant, we did observe an important decrease of exposure to DHA and piperaquine (AUC) after the addition of mefloquine to DHA-piperaquine. The addition of amodiaquine to artemeter-lumefantrine resulted in lower peak concentrations (C_{MAX}) of both artemether and its active metabolite DHA. Furthermore, we observed a non-significant yet concerning decrease in exposure (AUC) to both lumefantrine and its metabolite desbutyllumefantrine.

In **Chapter 6** we describe the results of a single centre, open-label, randomised controlled trial in the Kilifi County Hospital in coastal Kenya. 217 children under the age of 12 suffering from uncomplicated *Plasmodium falciparum* malaria were included. The efficacies of arterolane-piperaquine and arterolane-piperaquine plus mefloquine were found to be non-inferior to the current first line treatment artemether-lumefantrine. Reinfections occurred less frequently in patients treated with arterolane-piperaquine and arterolane-piperaquine plus mefloquine, most likely due to the longer post-prophylactic effect of piperaquine and mefloquine compared to that of lumefantrine. No indications of artemisinin resistance, either defined phenotypically (prolonged parasite clearance half-life) or genotypically (*kelch13* mutations) were observed. Arterolane-piperaquine and the Triple arterolane-piperaquine plus mefloquine were found to be safe and well tolerated. Vomiting rates were comparable after treatment with arterolane-piperaquine and arterolane-piperaquine plus mefloquine however both of these combinations showed higher vomiting rates compared to after artemether-lumefantrine. In accordance with

the results in **Chapter 5** the addition of mefloquine to arterolane-piperaquine did not further the prolong the QTc-interval, suggesting that the combination of arterolane-piperaquine plus mefloquine is safe for young children with respect to cardiotoxicity. We found no difference in overall arterolane exposure between arterolane-piperaquine and arterolane-piperaquine plus mefloquine treated patients.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Southeast Asia as the cradle of antimalarial resistance

Chloroquine and sulphadoxine-pyrimethamine resistance both emerged in Southeast Asia and spread from there throughout the world.[20, 22, 73] With the emergence of artemisinin and partner drug resistance, history seems to repeat itself. Several factors explaining why this region is the cradle of antimalarial resistance can be proposed: widespread inappropriate use as monotherapies, substandard dosing regime and the use of substandard or fake antimalarials.[135, 180]

Another factor could be the relative low levels of transmission, resulting in low levels of immunity in the population at risk. Reduced immunity will lead to increases health care seeking behaviour as the immune system is less able to control each new malaria infection. Increased healthcare seeking behaviour involves increased drug pressure for the parasite population in the region. On a population level, this increased drug pressure can accelerate the selection of antimalarial resistance.[181-183] In a region of high-level immunity, parasites with a higher level of resistance are likely to be cleared by the combination of antimalarial treatment and the host's immune system. In regions with low levels of immunity these resistant parasites are more likely to survive and be passed on to a next person.[184] Also, lower levels of immunity is associated with higher parasitaemias at the moment of treatment, which in itself is a risk factor for *de novo* emergence of resistance[160] and ACT treatment failure.[185] Finally, there are strong indications that artemisinin resistance arose on a complex genetic background favourable to the resistance conferring mutations such as the *kelch13* mutations.[58] Potentially, this genetic background might compensate the fitness loss often associated with resistance mutations. Alternatively, the background could enhance the phenotypic effect of *kelch13* mutations on artemisinin resistance. As genetic diversity declines due to the combination of reduced transmission and the continued high levels selection pressure due to extensive drug use, the genetic background and for instance *kelch13* mutations are less likely to be broken up by recombinations with wild-type parasites.

The current extent of artemisinin and partner drug resistance in the Southeast Asia

Reduced sensitivity to artemisinins results in a larger number of parasites surviving the initial three days of ACT treatment, which results in these parasites being exposed to only the ACT partner drug, resulting in an increased chance of selection of partner drug resistance.[186]

In **Chapter 2** and **Chapter 3** we indeed observed the rapid spread of the C580Y *kelch13* mutation on the KEL1 haplotype, most likely originating from western Cambodia, throughout northeastern Thailand, the whole of Cambodia and the South of Vietnam, while replacing all other lineages carrying other *kelch13* mutations that could be found in the region. At the same time piperaquine resistance spread from Western Cambodia throughout the region, resulting in unacceptable low efficacy of DHA-piperaquine. Similarly, low efficacy rates have been reported for artesunate-mefloquine on the Thai-Burmese border, artesunate-amodiaquine and artesunate-pyronaridine in Cambodia.[30, 70, 141] One of the goals of the study described in **Chapter 5** aimed to map the current extent of artemisinin resistance throughout Southeast Asia and the Indian Subcontinent. We found that lineages carrying the *kelch13* C580Y mutations have almost completely replaced the lineages carrying wild-type *kelch13* and non-C580Y *kelch13* mutations in Cambodia, northeastern Thailand and southern Vietnam. However, the spread of the C580Y mutated lineage was not found to have occurred westwards into Myanmar, Bangladesh and India. As is evident from the current COVID-19 pandemic our world is becoming more and more connected. Malaria endemic areas in Southeast Asia are strongly connected with malaria endemic areas in Myanmar, Bangladesh, India and sub-Saharan Africa, providing ample opportunities for resistance to spread by land, water and air.[187]

Independent emergence of antimalarial resistance

The independent emergence of antimalarial resistance is another threat to the longevity of the currently used antimalarials. Between 2016 and 2017, around 9% of parasites genotyped in the north of French Guiana, were found to carry *kelch13* C580Y mutations while showing an artemisinin-resistant phenotype *in vitro*. [35] *kelch13* mutations have been reported in sub-Saharan Africa, but generally at levels suggesting background mutation rates instead of clear selection of the mutations.[32] However, recent reports from Rwanda suggest the local emergence of the *kelch13* R561H mutations, a validated marker of artemisinin resistance.[34, 36] The high ACT coverage in French Guiana and Rwanda has contributed to a strong reduction in *Plasmodium falciparum* transmission. As we argue in **Chapter 7** and above the reduction in transmission will have led to lower levels of immunity on a population level and a reduction in genetic diversity. These, combined with the continued high drug pressure, could have been important factors in the local emergence of artemisinin resistance. As malaria transmission will reduce further under the influence of socio-economic processes, roll-out of preventive measures such as LLITN, indoor residual spraying and chemoprophylaxis and the recent highly promising malaria vaccines, the risk of independent emergence of antimalarial resistance becomes larger every year. Until malaria is fully eradicated it is crucial for antimalarial treatments to remain effective, safe, well tolerated and affordable. Although initial reports on efficacy of novel compounds look promising novel compounds might not be available for wide-scale use in the next few years.[111, 146]

Retaining efficacy of existing antimalarials

Potentially, using existing antimalarials in novel ways could protect the individual components from falling victim to antimalarial resistance. As we discuss in **Chapter 7** one option would be to prolong the administration of an ACT or administer two different ACTs consecutively.[148] However, adherence to these 5-to-6-day regimens might be suboptimal as patients generally will feel better within one or two days after start of the antimalarial treatment. An alternative approach aimed at reducing the selective pressure on individual drugs is to deploy multiple first line treatments in one population. Alternatively, a country could cycle through different ACTs as first line treatments, while changing first line treatments at pre-set moments or when levels of resistance to individual partner drugs increase. However, both approaches could be more costly and more challenging in a country with limited resources. In addition, an approach of cycling through ACT would require international coordination as poor alignment of first line policies in two neighbouring countries could facilitate the spread of resistance to specific partner drugs to neighbouring countries. A good example of this phenomena is the recent history of northeastern Thailand and Vietnam, where the first line treatment was changed to DHA-piperaquine in 2015 and 2016, respectively.[188] At the same time piperaquine resistant parasites were spreading throughout Cambodia to Vietnam and northeastern Thailand (**Chapter 3**). As a result, within years after implementation the efficacy was only 12.7% in northeastern Thailand and only 47.1% in southwestern Vietnam, as we report in **Chapter 2**.

Combining three antimalarials in the form of a Triple Antimalarial Combination Therapy could be a potentially way of retaining the efficacy of each individual compound, while limiting the costs and organizational difficulties of a national antimalarial policy.

Triple Antimalarial Combination Therapies, the new paradigm for malaria treatment?

Artemisinin-based combination therapies have proven to be safe, well tolerated and highly effective treatments for uncomplicated malaria. As long as malaria parasites are sensitive to both components, the efficacy of ACTs is generally in the range of 98% and higher. However, a decrease in artemisinin sensitivity would lead to a significant increase in parasites surviving the initial three days of treatment, resulting in the partner drug facing a larger number of parasites alone, which in turn favours the selection of partner drug resistance.

A potential solution to this problem could be to combine an existing ACT with an additional partner drug in the form of Triple ACT. Adding a second partner drug to an existing ACT could have a synergistic effect on parasite killing. In addition, both partner drugs could provide mutual protection to each other as the chance of a parasite being resistant to both partner drugs would be in the range of the product of the chance of being resistant to each individual partner drug, provided there is no cross resistance.

Several aspects should be taken into account when combining partner drugs. Firstly, the partner drugs should have different modes of action and no cross-resistance should exist between the individual compounds. Where possible, it would be preferable to choose combinations in which mechanisms of resistance are mutually counteractive. In other words, mechanisms that lead to resistance to one partner drug should make parasites more sensitive to the other partner drug. Secondly, the time the concentrations of the two partner drugs are above minimal parasiticidal concentrations should be similar to ensure mutual protection throughout their elimination phase. Thirdly, combining the antimalarials should have no detrimental effects on the bioavailability of the individual compounds. Finally, the resulting Triple ACTs should be highly effective, well tolerated and safe, while remaining affordable.

As discussed in **Chapter 4, 5, 6 and 7** the Triple combinations dihydroartemisinin-piperaquine plus mefloquine, artemether-lumefantrine plus amodiaquine and arterolane-piperaquine plus mefloquine full-fill most of these criteria. The combination of DHA-piperaquine plus mefloquine was generally well tolerated, did not result in cardiotoxicity and was highly effective. However, the addition of mefloquine resulted in a reduced exposure to dihydroartemisinin and piperaquine (**Chapter 4 and 5**). This is an important finding and needs to be taken into account when developing this Triple ACT further. For instance, dihydroartemisinin and piperaquine dosing could be increased to account for this reduced exposure. An important finding in **Chapter 5** was the high efficacy of the ACT artesunate-mefloquine in Cambodia. We cannot rule out that the high efficacy of the Triple ACT dihydroartemisinin-piperaquine plus mefloquine was the result of high mefloquine sensitivity alone instead of a potential synergistic effect of piperaquine and mefloquine. To answer this question, future studies should assess whether the Triple ACT dihydroartemisinin-piperaquine plus mefloquine remains effective if and when the combination of artesunate-mefloquine starts to fail in this region. The high prevalence of artemisinin and piperaquine resistance raises the question whether deployment of Triple ACTs could result in the selection of 'Triple-resistant' parasites. In the case of the Triple DHA-piperaquine plus mefloquine the continued drug pressure of DHA-piperaquine would lead to the continued selective advantage of mechanisms conferring artemisinin and piperaquine resistance whereas the use of mefloquine will increase the risk of the selection of mefloquine resistance. Given the current extent of piperaquine resistance the Triple DHA-piperaquine plus mefloquine would not be the first Triple ACT of choice to be deployed in Southeast Asia. On the other hand, a recent study found that the prevalence of 'Triple mutants' carrying the C580Y *kelch13* mutation, and amplification of both the *plasmepsin2/3* and *mdr1* genes was very low. Only 4 out of 6722 samples obtained in Thailand, Cambodia, Laos, Myanmar and Vietnam between 2007 and 2019 were found to carry all of these three markers of resistance, even though DHA-piperaquine and mefloquine were used in the region at large scale sequentially or at the same time (Mallika Imwong et al., unpublished data). This observation could support both the hypothesis of counteracting resistance mechanisms between piperaquine and

mefloquine or the hypothesis of a relative fitness disadvantage of these 'Triple mutants'. As an alternative to deploying DHA-piperaquine plus mefloquine in Southeast-Asia, the Triple ACT artemether-lumefantrine plus amodiaquine could be deployed.

The Triple ACT artemether-lumefantrine plus amodiaquine was found to be just as highly effective as the comparator artemether-lumefantrine in Laos, Myanmar, Bangladesh, India and the Democratic Republic of the Congo (DRC). In the DRC, the incidence and timing of reinfections was not affected by the addition of amodiaquine, most likely due to similar elimination times of lumefantrine, amodiaquine and their metabolites.

The reduced peak concentrations of artemether and its active metabolite dihydroartemisinin as well as a non-significant yet concerning decreased AUC of both lumefantrine and its metabolite desbutyllumefantrine should be taken into account when developing the Triple ACT artemether-lumefantrine plus amodiaquine further.

This Triple ACT was only trialled in areas without established artemisinin resistance. The recently completed 'TACT-CV' study (ClinicalTrials.gov: NCT03355664), which was conducted in Cambodia and Vietnam will provide insight in the efficacy of the Triple ACT artemether-lumefantrine plus amodiaquine compared to that of artemether-lumefantrine in the setting of clearly established artemisinin resistance.

In the TRACII trial (**Chapter 5**) children were underrepresented, whereas most malaria infections globally occur in young children, especially in sub-Saharan Africa.[2] The 'DeTACT' study, which is a direct follow up study of the TRACII study, will focus on the safety, efficacy and tolerability of Triple ACTs in children in sub-Saharan Africa. In addition, this trial will also continue to assess the efficacy of the Triple ACTs artemether-lumefantrine plus amodiaquine and artesunate-mefloquine plus piperaquine in the Southeast Asia (ClinicalTrials.gov Identifier: NCT03923725).

In **Chapter 6** we trialled a non-artemisinin based Triple Antimalarial Combination Therapy, consisting out of the synthetic ozonide arterolane, piperaquine and mefloquine. As expected, the efficacy of this Triple was equal to that of arterolane-piperaquine and the efficacy of both combinations was non-inferior to that of the artemether-lumefantrine. As all patients were children under the age of 12 this study provides important insights in the tolerability and safety of the piperaquine-mefloquine based triple therapies in children in sub-Saharan Africa.

FUTURE PERSPECTIVES

DHA-piperaquine and artemether-lumefantrine are already extensively used globally. If needed, co-blistered Triple ACTs based on DHA-piperaquine and artemether-lumefantrine could be produced and distributed rapidly. However, co-formulation of a Triple into one tablet will simplify and ensure the adequate intake of all components of the Triple ACT, comparable to the beneficial effects of coformulating antiretroviral therapies for HIV.

The concept of a Triple Antimalarial Combination Therapy can also be applied to novel non-artemisinin compounds that are currently under development, such as the highly active and promising spiroindolone Cipargamin.[92] Most novel compounds that are under development are combined with one other partner drug. As with artemisinin-based combination therapies this leaves the individual compounds at a higher risk of being lost to resistance. Although combining novel compounds with two other antimalarials will likely increase development costs significantly and could pose some difficulties in drug development due to stability issues, it might be cost-effective in the long term as the novel compounds are more likely to remain effective.

A synergistic killing effect of two partner drugs would make the combination more suitable to be combined in a Triple ACT. The *in vivo* assessment of synergism would be extremely complicated and costly as it would most likely need large clinical trials with at least 2 or 3 study arms. An alternative is the *in vitro* identification of potential synergistic mechanisms.[189] *In vitro* screening for synergistic could prove to be a cost-effective and relatively easily scalable method to identify suitable candidate combination of antimalarial drugs. The combinations of antimalarial drugs identified through these methods could be trialled in healthy volunteer studies and clinical trials subsequently.

In theory, Triple ACTs could serve as a stopgap in areas with widespread resistance and could be used to prevent or delay the emergence of resistance in areas with no resistance yet. In practice, it is difficult to predict the real effect of deployment of Triple ACTs on the emergence and spread of antimalarial resistance. Although there are limitations to each model, mathematical modelling could provide insights in the potential benefits of deploying Triple ACTs, both in terms of preventing or delaying resistance and cost-effectiveness of this strategy.[113]

An alternative way to assess the potential of Triple ACTs is to conduct large scale randomized trials in which inhabitants of different regions are, for an extended period, either treated with either ACT-1, ACT-2 or a Triple ACT which contains both partner drugs included in either ACT-1 and ACT-2. Assuming counteracting resistance mechanisms as is suspected for lumefantrine and amodiaquine, over time the continued drug pressure by ACT-1 could push the parasite population towards a genotype and phenotype that is less sensitive to the partner drug in ACT-1 and more sensitive to the

partner drug in ACT-2. The population treated with ACT-2 would develop in the opposite direction. We hypothesize that no directional shift will be seen in the population treated with the Triple ACT containing both partner drugs.

In the past, the transition from monotherapies to ACTs has been slow and a shift towards Triple ACTs might be hindered in a similar way.[171]

Conducting laboratory and field studies in the developing world is complicated and costly. Although malaria affects hundreds of millions of people every year, it is difficult to obtain research funds. Adequate funding of future studies will be crucial to detect the emergence and spread of antimalarial resistance and the critically assess the advantages and disadvantages of Triple Antimalarial Combination Therapies. In addition, funds should be made available for community engagement and drug development.

One important question is whether it is ethical to treat the current generation with a combination that will most likely have more side effects, to prevent the emergence of resistance in the future, whereas the standard ACTs are still effective.[190] As with ACTs in the past, the uptake of Triple ACTs could also be hindered by concerns about the safety and efficacy of 'western' medicines and the reliance on traditional remedies, but also by logistical obstacles.[170] Early engagement with community representatives will be crucial to ensure the rapid uptake of Triple ACTs.

Drug manufacturers should have confidence that investments in the development of Triple ACTs will be merited. However, as malaria is a disease affecting the poor the prices of Triple ACTs should be comparable to ACTs. One potential solution to this problem is a scheme in which drug manufacturers are rewarded by a substantial financial reimbursement for each new triple combination that reaches the market. The Global Fund, which functions as a financing mechanism aimed at elimination of malaria, HIV and tuberculosis, has WHO prequalification as a requisite for funding of antimalarial drugs that are used in national policies. For this reason drug manufacturers and academical research groups should focus on the prerequisites for WHO prequalification when developing and trialling new triple antimalarial drug combinations and should communicate and collaborate with the WHO at an early stage. Finally, national malaria programmes tend to follow the advice of the World Health Organization (WHO) when deciding on a national malaria policy. For this reason, once co-formulated Triple ACTs have been developed and have been proven to be effective, safe and well tolerated and modelling studies and/or field studies show favourable results a decision on deploying Triple ACTs on a global scale should be made by the WHO. This decision should be made without delay once sufficient evidence is available. Once a decision is made in favour of Triple ACTs the successful deployment will depend on the strong collaboration between the WHO, national malaria programmes, representatives of civil society, academic research groups, international funding mechanisms and other stakeholders.

Appendices

NEDERLANDSE SAMENVATTING

INTRODUCTIE

Hoofdstuk 1 beschrijft de achtergrond van het werk in dit proefschrift. De relatief recente opkomst en verspreiding van resistentie tegen artemisinine en partner medicatie in Zuidoost-Azië vormt een groot gevaar voor de wereldwijde strijd tegen malaria.

Indien resistentie tegen artemisinine en partner medicatie zich zou verspreiden naar of onafhankelijk zou opkomen in Sub-Sahara-Afrika zal de aan malaria gerelateerde mortaliteit sterk stijgen. Mogelijk kunnen Triple ACTs het opkomen en het zich verspreiden van resistentie voorkomen of vertragen. Dit proefschrift richt zich op het in kaart brengen van de huidige prevalentie van resistentie tegen artemisinine en partner medicatie. Daarnaast richt dit proefschrift zich op het beoordelen van de veiligheid, verdraagbaarheid en effectiviteit van Triple Antimalaria Combinatie Therapieën.

EPIDEMIOLOGIE VAN BEHANDELING RESISTENTE MALARIA

Hoofdstuk 2 beschrijft de alarmerend lage effectiviteit (PCR-gecorrigeerd) op dag 42 van dihydroartemisinine(DHA)-piperaquine in noordoost-Thailand (12·7%), west-Cambodja (38·2%) en noordoost Cambodja (73·4%) en zuidwest Vietnam (47·1%). Therapiefalen was geassocieerd met amplificaties van het *plasmepsin2/3* gen en 4 mutaties in het 'chloroquine resistance transporter' (*crt*) gen (T93S, H97Y, Phe145I and I218F). Mutaties in het *kelch13* gen bleken niet geassocieerd met therapie falen, meest waarschijnlijk vanwege het feit dat een groot deel van de infecties een gebaseerd waren op parasieten met een C580Y *kelch13* mutatie (369/404 (91%)). De meest van deze C580Y *kelch13* mutaties werden gevonden op het KEL1 haplotype. Dit suggereert de verspreiding van 1 stam door de regio.

Amplificatie van het *plasmepsin2/3* gen werd gevonden in drie-vierde van de monsters, terwijl er geen amplificaties van het *mdr1* gen werden geobserveerd. Wij identificeerden 6 mutaties in het *crt* gen die in prevalentie waren toegenomen tussen de twee periodes waarin monsters werden verzameld (2011-2013 en 2015-2018). Deze mutaties bleken elkaar uit te sluiten en konden gebruikt worden om specifieke haplotypes te identificeren.

De toename in de prevalentie van *Kelch13* C580Y mutaties (KEL1), *plasmepsin2/3* amplificaties en de 6 *crt* mutaties ondersteunen de hypothese dat deze genetisch 'markers' een selectievoordeel hebben doordat zij de kans op therapiefalen en daarop volgende transmissie vergroten.

In **hoofdstuk 3** beschrijven wij de resultaten van een analyse van het volledige genoom van 2465 parasieten die werden verzameld tussen 2007 en 2018 in Cambodja, Laos,

noordoost Thailand en Vietnam. Deze analyse toonde dat het KEL1 haplotype met daarop de C580Y *kelch13* mutatie verbonden is geraakt met het PLA1 haplotype met daarop een specifiiek *plasmepsin2/3* (PLA1) haplotype. Parasieten met deze KEL1/PLA1 combinatie zijn meest waarschijnlijk opgekomen in west-Cambodja en onder de druk van het voortdurende gebruik van DHA-piperaquine gediversifieerd in verschillende subgroepen, waarbij zij nieuwe genetische eigenschappen verkregen, zoals de bovengenomende mutaties in het *crt* gen. Ook hier suggereert het snel opkomen van deze *kelch13* C580Y mutaties (KEL1 haplotype) en *plasmepsin2/3* amplificaties (PLA1 haplotype) en de *crt* mutaties dat deze genetische 'markers' leiden tot een selectievoordeel.

Daarnaast zijn deze resultaten een duidelijk voorbeeld van de evolutionaire processen die plaatsvinden wanneer niet effectieve behandelingen worden gecontinueerd. Artemisinine resistentie faciliteerde de selectie van resistentie tegen piperaquine in de vorm van *plasmepsin2/3* geamplificeerde en *crt* gemuteerde parasieten. Tenslotte tonen **hoofdstuk 2 en 3** het potentieel van een 'multidrug' resistente stam om zich snel te verspreiden door een regio.

VEILIGHEID, VERDRAAGBAARHEID EN EFFECTIVITEIT VAN TRIPLE ANTIMALARIA COMBINATIE THERAPIEEN

In **hoofdstuk 4** beschrijven wij de resultaten van een studie met 15 Thaise gezonde volwassen vrijwilligers waarbij patiënten werden behandeld met de ACT DHA-piperaquine, de Triple ACT DHA-piperaquine plus mefloquine of mefloquine alleen. Zoals verwacht verlengde de toediening van DHA-piperaquine het QTc-interval in geringe mate. Het toevoegen van mefloquine verlengde de het QTc-interval niet verder. Deze geruststellende resultaten zijn van belang aangezien de potentiele synergistische effecten van piperaquine en mefloquine op de verlenging van het QTc-interval een punt van grote zorg waren voorafgaande aan deze studie.

De meeste gerapporteerde 'adverse events' na DHA-piperaquine plus mefloquine waren matige duizeligheid, misselijkheid, buikklachten en verstoringen van de slaap. Dit zijn allemaal bekende bijwerkingen van mefloquine. Het toevoegen van mefloquine aan DHA-piperaquine had geen effect op de farmacokinetiek van piperaquine. Echter, het toevoegen van mefloquine resulteerde in een 22.6% lagere blootstelling (AUC) aan dihydroartemisinine en een 29.0% lagere piekspiegel (C_{MAX}) van dihydroartemisinine. Deze resultaten zijn van belang aangezien onvoldoende blootstelling aan artemisinines een risicofactor is voor het ontwikkelen van artemisinine resistentie.

De resultaten van de grote klinische trial gericht op het vergelijken van de veiligheid, verdraagbaarheid en effectiviteit van Triple ACTs en ACTs worden beschreven in **hoofdstuk 5**. In de multicenter, open-label, gerandomiseerde studie werden 1100

patiënten met ongecompliceerde *P. falciparum* malaria geïncludeerd in achttien ziekenhuizen en gezondheidsklinieken in acht landen. Patienten werden gerandomiseerd tussen DHA-piperaquine of DHA-piperaquine plus mefloquine; tussen artesunaat-mefloquine of DHA-piperaquine plus mefloquine; of tussen artemether-lumefantrine of artemether-lumefantrine plus amodiaquine.

Wij vonden een onacceptabel lage effectiviteit van DHA-piperaquine in noordoost Thailand, Cambodja en Vietnam. De effectiviteit van DHA-piperaquine in Myanmar was hoog. De effectiviteit van de Triple ACT DHA-piperaquine plus mefloquine was hoger dan 95% in Thailand, Cambodja, Vietnam en Myanmar. De effectiviteit van artesunaat-mefloquine was ook hoger dan 95% in Cambodja. Wij vonden een hoge effectiviteit van artemether-lumefantrine in Laos, Myanmar, Bangladesh, India en de Democratische Republiek Congo (DRC). Het toevoegen van amodiaquine aan artemether-lumefantrine verlaagde de incidentie van herinfecties in de DRC niet.

De C580Y *kelch13* mutatie werd in hoge frequentie gevonden in Cambodja, noordoost-Thailand en Vietnam maar werd niet gevonden ten westen van Thailand. Ongeveer de helft van de infecties in Pyin Oo Lwin (Myanmar) werden veroorzaakt door parasieten met een *kelch13* mutatie anders dan de C580Y mutatie.

In alle andere locaties in Myanmar, Bangladesh, India en de DRC waren *kelch13* mutaties zeldzaam. Zoals verwacht was er een associatie tussen de 'parasite-clearance' halfwaardetijd en de aanwezigheid van *kelch13* mutaties. Daarbij passend zijn de 'parasite clearance' halfwaardetijden verlengd in Cambodja, Thailand en Vietnam en in mindere mate in Laos en in Pyin Oo Lwin in Myanmar. In de andere gebieden werd een verlengde halfwaardetijd slechts sporadisch gevonden. Beide Triple ACTs werden over het algemeen goed verdragen en de incidentie van 'adverse events' was vergelijkbaar na Triple ACTs en ACTs. Echter, het toevoegen van mefloquine aan DHA-piperaquine verhoogde de frequentie van braken binnen een uur na inname van de medicatie. De incidentie van 'Serious Adverse Events' was vergelijkbaar na ACTs en Triple ACTs.

Zoals in de gezonde vrijwilligersstudie in **hoofdstuk 4** leidde het toevoegen van mefloquine aan DHA-piperaquine niet tot een verdere verlenging van het QTc-interval. Het toevoegen van amodiaquine aan artemether-lumefantrine verlengde het QTc-interval in een mate vergelijkbaar met wat is beschreven voor amodiaquine alleen. Hoewel het effect niet statistisch significant is observeerden wij een belangrijke verlaging in de blootstelling aan DHA en piperaquine (AUC) na het toevoegen van mefloquine aan DHA-piperaquine.

Het toevoegen van amodiaquine aan artemeter-lumefantrine leidde tot een lager topspiegel (C_{MAX}) van zowel artemether en haar actieve metaboliet DHA. Daarnaast

vonden wij een niet statistisch significante maar wel zorgwekkende verlaagde blootstelling (AUC) aan zowel lumefantrine en haar metaboliet desbutyllumefantrine.

In **hoofdstuk 6** beschrijven wij de resultaten van een open label, gerandomiseerde studie in het 'Kilifi County Hospital' aan de kust van Kenia. 217 kinderen jonger dan 12 jaar met ongecompliceerde *Plasmodium falciparum* malaria werden geïncludeerd. De effectiviteit van arterolane-piperaquine en arterolane-piperaquine plus mefloquine was niet-inferieur aan die van de eerstelijnsbehandeling artemether-lumefantrine. herinfecties vonden minder frequent plaats in patiënten die werden behandeld met arterolane-piperaquine en arterolane-piperaquine plus mefloquine, meest waarschijnlijk ten gevolge van een langer post-profylactisch effect van piperaquine en mefloquine in vergelijking met dat van lumefantrine. Wij vonden geen fenotypische (verlengde 'parasite clearance' halfwaardetijd) of genotypische (*kelch13* mutaties) aanwijzingen voor artemisinine resistentie. Arterolane-piperaquine en de Triple arterolane-piperaquine plus mefloquine bleken veilig en werden goed verdragen. De incidentie van braken was vergelijkbaar na behandeling met arterolane-piperaquine en arterolane-piperaquine plus mefloquine. In beide gevallen was de incidentie van braken hoger in vergelijking met de incidentie na artemether-lumefantrine. Vergelijkbaar met de resultaten van **hoofdstuk 5** leidde het toevoegen van mefloquine aan arterolane-piperaquine niet tot een verdere verlenging van het QTc-interval, hetgeen suggereert dat de combinatie arterolane-piperaquine plus mefloquine veilig is voor jonge kinderen wat betreft cardiotoxiciteit. Wij vonden geen verschil in totale blootstelling aan arterolane tussen patienten behandeld met arterolane-piperaquine of arterolane-piperaquine plus mefloquine.

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PORTFOLIO

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PhD period: 2014-2021
Name PhD supervisor: Arjen M. Dondorp

1. PhD training

General courses	Location	Year	ECTS
Good Clinical Practice Oxford University	Oxford, UK	2015	0.5

Specific courses	Location	Year	ECTS
Epidemiology of infectious diseases	University of Hong Kong	2014	0.5
ASTMH course 'Malaria a moving target'	Philadelphia, USA	2015	0.5
Programming in R, online course	John Hopkins University USA	2016	1
Summer school of computational biology	Hanoi, Vietnam	2016	0.5
Introduction to modeling of infectious diseases	Ho Chi Minh, Vietnam	2016	0.5
Genetic Analysis of Plasmodium falciparum	Bangkok, Thailand	2017	0.5
Genetic epidemiology	Amsterdam, Netherlands	2017	0.5
Bioinformatics Sequence Analysis	Amsterdam, Netherlands	2017	0.5
Observational Epidemiology: Effects/Effectiveness	Amsterdam, Netherlands	2017	0.5
Basic to advanced survival methods	Bangkok, Thailand	2017	0.5
Advanced Biostatistics: Correlated data analysis	Bangkok, Thailand	2018	0.5

Seminars, workshops and master classes	Location	Year	ECTS
Masterclass HIV, Tuberculosis and Hepatitis	Utrecht, Netherlands	2020	1

Presentations	Location	Year	ECTS
Co-chair TRACII investigators meeting	Bangkok, Thailand	2015	0.5
Speaker at symposium ASTMH	Philadelphia, USA	2015	0.5
Presentation JITMM conference	Bangkok, Thailand	2015	0.5
Co-chair of the 2nd TRACII investigators meeting	Bangkok, Thailand	2016	0.5
Symposium Genomic Epidemiology of Malaria	Hinxton, UK	2016	0.5
Speaker at symposium ASTMH	Atlanta, USA	2016	0.5
Presentation at Oxford Tropical Network Meeting	Oxford, UK	2017	0.5
Co-chair of the 3rd TRACII investigators meeting	Bangkok, Thailand	2017	0.5
Presentation at MMV stakeholders meeting	Bali, Indonesia	2017	0.5
Presentation 'Future of Malaria Research'	Baltimore, USA	2017	0.5
Speaker at symposium ASTMH	Baltimore, USA	2017	0.5
Presentation at JITMM conference	Bangkok, Thailand	2017	0.5
Presentation at PACES dissemination meeting	Siem Reap, Cambodia	2018	0.5
Presentation at Oxford Tropical Network Meeting	Ho Chi Minh, Vietnam	2018	0.5
Co-chair of the 4th TRACII investigators meeting	Bangkok, Thailand	2018	0.5

Presentations	Location	Year	ECTS
Presentation at MIM meeting: TRACII project	Dakar, Senegal	2018	0.5
Presentation at MIM meeting: DeTACT project	Dakar, Senegal	2018	0.5
Presentation at Epicentre headquarter (MSF)	Paris, France	2018	0.5
Presentation at annual meeting on drug policy	Phnom Penh, Cambodia	2018	0.5
Presentation at First Malaria World Congress	Melbourne, Australia	2018	0.5
Presentation Nat. Con. Tropical Med and Tox	Chittagong, Bangladesh	2018	0.5
Speaker at symposium ASTMH	New Orleans, USA	2018	0.5
Presentation at TACT-CV investigators meeting	Bangkok, Thailand	2019	0.5
Presentation at DeTACT investigators meeting	Bangkok, Thailand	2019	0.5
Presentation All-Party Parliamentary Group	London, UK	2019	0.5
Presentation at LSHTM	London, UK	2019	0.5
Medical Microbiology Association meeting	Utrecht, Netherlands	2020	0.5

(Inter)national conferences	Location	Year	ECTS
ASTMH conference	Philadelphia, USA	2015	1
JITMM conference	Bangkok, Thailand	2015	0.5
Symposium Genomic Epidemiology of Malaria	Hinxton, UK	2016	0.5
ASTMH conference	Atlanta, USA	2016	1
Oxford Tropical Network Meeting	Oxford, UK	2017	0.5
Future of Malaria Research conference	Baltimore, USA	2017	0.5
ASTMH conference	Baltimore, USA	2017	1
JITMM conference	Bangkok, Thailand	2017	0.5
Oxford Tropical Network Meeting	Ho Chi Minh, Vietnam	2018	0.5
MIM meeting	Dakar, Senegal	2018	0.5
First Malaria World Congress	Melbourne, Australia	2018	0.5
National Conference Tropical Medicine and Toxicology	Chittagong, Bangladesh	2018	0.5
ASTMH conference	New Orleans, USA	2018	1

2. Teaching

Training of study staff in study protocol procedures	19 study sites	2015-2019

3. Parameters of esteem

Grant application	Awarded	Year
TACT grant, Bill and Melinda Gates Foundation	Yes	2016
DeTACT grant, Department for International Development	Yes	2017

LIST OF PUBLICATIONS

Included in this thesis

1. **An open-label randomized clinical trial evaluating the efficacy, safety, tolerability and pharmacokinetic interactions of arterolane-piperaquine, arterolane-piperaquine plus mefloquine and artemether-lumefantrine for uncomplicated Plasmodium falciparum malaria in Kenyan children.**
   Lancet Infectious Diseases 2021. 2021 Jun 7;S1473-3099(20)30929-4
   Mainga Hamaluba, **Rob W. van der Pluijm,** Joseph Weya, Patricia Njuguna, Mwanajuma Ngama, Peter Kalume, Gabriel Mwambingu, Caroline Ngetsa, Juliana Wambua, Mwanamvua Boga, Neema Mturi, Altaf A. Lal, Arshad Khuroo, Walter R.J. Taylor, Sónia Gonçalves, Olivo Miotto, Mehul Dhorda, Brian Mutinda, Mavuto Mukaka, Naomi Waithira, Richard M. Hoglund, Mallika Imwong, Joel Tarning, Nicholas Day, Nicholas J. White, Philip Bejon, Arjen M. Dondorp.

2. **Triple Artemisinin-Based Combination Therapies for Malaria – A new paradigm?**
   Trends in Parasitology 2021 Jan;37(1):15-24
   Rob W van der Pluijm, Chanaki Amaratunga, Mehul Dhorda, Arjen M Dondorp.

3. **Triple Artemisinin-Based Combination Therapies Versus Artemisinin-Based Combination Therapies for Uncomplicated Plasmodium Falciparum Malaria: A Multicentre, Open-Label, Randomised Clinical Trial.**
   Lancet 2020 Apr 25;395(10233):1345-1360
   Rob W van der Pluijm, Rupam Tripura, Richard M Hoglund, Aung Pyae Phyo, Dysoley Lek, Akhter Ul Islam, Anupkumar R Anvikar, Parthasarathi Satpathi, Sanghamitra Satpathi, Prativa Kumari Behera, Amar Tripura, Subrata Baidya, Marie Onyamboko, Nguyen Hoang Chau, Yok Sovann, Seila Suon, Sokunthea Sreng, Sivanna Mao, Savuth Oun, Sovannary Yen, Chanaki Amaratunga, Kitipumi Chutasmit, Chalermpon Saelow, Ratchadaporn Runcharern, Weerayuth Kaewmok, Nhu Thi Hoa, Ngo Viet Thanh, Borimas Hanboonkunupakarn, James J Callery, Akshaya Kumar Mohanty, James Heaton, Myo Thant, Kripasindhu Gantait, Tarapada Ghosh, Roberto Amato, Richard D Pearson, Christopher G Jacob, Sónia Gonçalves, Mavuto Mukaka, Naomi Waithira, Charles J Woodrow, Martin P Grobusch, Michele van Vugt, Rick M Fairhurst, Phaik Yeong Cheah, Thomas J Peto, Lorenz von Seidlein, Mehul Dhorda, Richard J Maude, Markus Winterberg, Nguyen Thanh Thuy-Nhien, Dominic P Kwiatkowski, Mallika Imwong, Podjanee Jittamala, Khin Lin, Tin Maung Hlaing, Kesinee Chotivanich, Rekol Huy, Caterina Fanello, Elizabeth Ashley, Mayfong Mayxay, Paul N Newton, Tran Tinh Hien, Neena Valecha, Frank Smithuis, Sasithon Pukrittayakamee, Abul Faiz, Olivo Miotto, Joel Tarning, Nicholas PJ Day, Nicholas J White, Arjen M Dondorp, Tracking Resistance to Artemisinin Collaboration

4. **Determinants of dihydroartemisinin-piperaquine treatment failure in Plasmodium falciparum malaria in Cambodia, Thailand, and Vietnam: a prospective clinical, pharmacological, and genetic study.**
   Lancet Infectious Diseases 2019 Sep;19(9):952-961
   Rob W van der Pluijm, Mallika Imwong, Nguyen Hoang Chau, Nhu Thi Hoa, Nguyen Thanh Thuy-Nhien, Ngo Viet Thanh, Podjanee Jittamala, Borimas Hanboonkunupakarn, Kitipumi Chutasmit, Chalermpon Saelow, Ratchadaporn Runjarern, Weerayuth Kaewmok, Rupam Tripura, Thomas J Peto, Sovann Yok, Seila Suon, Sokunthea Sreng, Sivanna Mao, Savuth Oun, Sovannary Yen, Chanaki Amaratunga, Dysoley Lek, Rekol Huy, Mehul Dhorda, Kesinee Chotivanich, Elizabeth A Ashley, Mavuto Mukaka, Naomi Waithira, Phaik Yeong Cheah, Richard J Maude, Roberto Amato, Richard D Pearson, Sónia Gonçalves, Christopher G Jacob, William L Hamilton, Rick M Fairhurst, Joel Tarning, Markus Winterberg, Dominic P Kwiatkowski, Sasithon Pukrittayakamee, Tran Tinh Hien, Nicholas PJ Day, Olivo Miotto, Nicholas J White, Arjen M Dondorp

5. **Evolution and expansion of multidrug-resistant malaria in southeast Asia: a genomic epidemiology study.**
   Lancet Infectious Diseases 2019 Sep;19(9):943-951
   William L Hamilton, Roberto Amato, **Rob W van der Pluijm**, Christopher G Jacob, Huynh Hong Quang, Nguyen Thanh Thuy-Nhien, Tran Tinh Hien, Bouasy Hongvanthong, Keobouphaphone Chindavongsa, Mayfong Mayxay, Rekol Huy, Rithea Leang, Cheah Huch, Lek Dysoley, Chanaki Amaratunga, Seila Suon, Rick M Fairhurst, Rupam Tripura, Thomas J Peto, Yok Sovann, Podjanee Jittamala, Borimas Hanboonkunupakarn, Sasithon Pukrittayakamee, Nguyen Hoang Chau, Mallika Imwong, Mehul Dhorda, Ranitha Vongpromek, Xin Hui S Chan, Richard J Maude, Richard D Pearson, T Nguyen, Kirk Rockett, Eleanor Drury, Sónia Gonçalves, Nicholas J White, Nicholas P Day, Dominic P Kwiatkowski, Arjen M Dondorp, Olivo Miotto

6. **Sequential Open-Label Study of the Safety, Tolerability, and Pharmacokinetic Interactions between Dihydroartemisinin-Piperaquine and Mefloquine in Healthy Thai Adults**
   Antimicrobial Agents and Chemotherapy 2019 Jul 25;63(8):e00060-19
   Borimas Hanboonkunupakarn, **Rob W van der Pluijm**, Richard Hoglund, Sasithon Pukrittayakamee, Markus Winterberg, Mavuto Mukaka, Naomi Waithira, Kesinee Chotivanich, Pratap Singhasivanon, Nicholas J. White, Arjen M. Dondorp, Joel Tarning, Podjanee Jittamalaa.

Not included in this thesis

7. **Deploying Triple artemisinin-based combination therapy (TACT) for malaria treatment in Africa: ethical and practical considerations**
   Malaria Journal 2021 Feb 27;20(1):119
   Paulina Tindana, Freek de Haan, Chanaki Amaratunga, Mehul Dhorda, **Rob W van der Pluijm**, Arjen M Dondorp AM, Phaikyeong Cheah

8. **Modulation of Triple Artemisinin-Based Combination Therapy Pharmacodynamics by Plasmodium falciparum Genotype.**
   ACS Pharmacology & Translational Science 2020 Nov 2;3(6):1144-1157
   Megan R. Ansbro, Zina Itkin, Lu Chen, Gergely Zahoranszky-Kohalmi, Chanaki Amaratunga, Olivo Miotto, Tyler Peryea, Charlotte V. Hobbs, Seila Suon, Juliana M. Sá, Arjen M. Dondorp, **Rob W. van der Pluijm**, Thomas E. Wellems, Anton Simeonov, and Richard T. Eastman

9. **Triple artemisinin-based combination therapies for malaria: proceed with caution – Authors' reply**
   Lancet. 2021 Dec 19;396(10267):1976-1977
   Rob W. van der Pluijm, Aung Pyae Phyo, Dysoley Lek, Nicholas J White, Arjen M Dondorp.

10. **A comprehensive RNA handling and transcriptomics guide for high-throughput processing of Plasmodium-stage samples.**
    Malaria Journal 2020 Oct 9;19(1):363
    Michal Kucharski, Jaishree Tripathi, Sourav Nayak, Lei Zhu, Grennady Wirjanata, **Rob W. van der Pluijm**, Mehul Dhorda, Arjen Dondorp, Zbynek Bozdech

11. **Molecular epidemiology of resistance to antimalarial drugs in the Greater Mekong subregion: an observational study**
    Lancet Infectious Diseases 2020 Dec;20(12):1470-1480
    Mallika Imwong, Mehul Dhorda, Kyaw Myo Tun, Aung Myint Thu, Aung Pyae Phyo, Stephane Proux, Kanokon Suwannasin, Chanon Kunasol, Suttipat Srisutham, Jureeporn Duanguppama, Ranitha Vongpromek, Cholrawee Promnarate, Aungkana Saejeng, Nardlada Khantikul, Rungniran Sugaram, Supinya Thanapongpichat, Nongyao Sawangjaroen, Kreepol Sutawong, Kay Thwe Han, Ye Htut, Khin Linn, Aye Aye Win, Tin M Hlaing, **Rob W van der Pluijm,** Mayfong Mayxay, Tiengkham Pongvongsa, Koukeo Phommasone, Rupam Tripura, Thomas J Peto, Lorenz von Seidlein, Chea Nguon, Dysoley Lek, Xin Hui S Chan, Huy Rekol, Rithea Leang, Cheah Huch, Dominic P Kwiatkowski, Olivo Miotto, Elizabeth A Ashley, Myat Phone Kyaw, Sasithon Pukrittayakamee, Nicholas P J Day, Arjen M Dondorp, Frank M Smithuis, Francois H Nosten, Nicholas J White

12. **Safety, pharmacokinetics, and mosquito-lethal effects of ivermectin, dihydroartemisinin-piperaquine and primaquine in healthy adult Thai subjects**
Clinical pharmacology and therapeutics 2020 May;107(5):1221-1230
Kevin Kobylinski, Podjanee Jittamala, Borimas Hanboonkunupakarn, Sasithon Pukrittayakamee, Kanchana Pantuwatana, Siriporn Phasomkusolsil, Silas Davidson, Markus Winterberg, Richard Hoglund, Mavuto Mukaka, **Rob W van der Pluijm**, Arjen M Dondorp, Nicholas PJ Day, Nicholas J White, Joel Tarning

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14. **The origins of malaria artemisinin resistance defined by a genetic and transcriptomic background.**
Nature Communications 2018 Dec 4;9(1)5158
L. Zhu, J. Tripathi, FM Rocamora, O Miotto, **Rob W van der Pluijm**, TS Voss, S Mok, DP Kwiatkowski, F Nosten, NPJ Day, NJ White, AM Dondorp, Z Bozdech; Tracking Resistance to Artemisinin Collaboration I.

15. **Investigating the efficacy of Triple artemisinin-based combination therapies (TACTs) for treating Plasmodium falciparum malaria patients using mathematical modelling**
Antimicrobial Agents and Chemotherapy 2018 Oct 24:62(11)e01068-18.
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16. **The sensitivity and specificity of a urine based Rapid Diagnostic Test for the diagnosis of plasmodium falciparum in a malaria endemic area in Odisha, India.**
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17. **Antimalarial Resistance Unlikely To Explain U.K. Artemether-Lumefantrine Failures.**
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18. **The spread of artemisinin-resistant Plasmodium falciparum in the Greater Mekong Subregion: a molecular epidemiology observational study.**
Lancet Infectious Diseases 2017 May;17(5):491-497
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19. **The effect of iron loading and iron chelation on the innate immune response and subclinical organ injury during human endotoxemia, a randomized trial.**
Haematologica 2014 Mar;99(3):579-87
L.T. van Eijk, S. Heemskerk, **Rob W van der Pluijm**, S.M. van Wijk, J.G. van der Hoeven, M. Cox, D.W. Swinkels, P. Pickkers

20. **Body mass index is not associated with cytokine induction during experimental human endotoxemia.**
Innate immunity 2014 Jan;20(1):61-7
L.T. van Eijk, **Rob W van der Pluijm**, B.P. Ramakers, M.J. Dorresteijn, J.G. van der Hoeven, M. Kox, P. Pickkers

21. **A female with a leiomyosarcoma presenting with acute thoracic pain and dyspnoea.**
Netherlands Journal of Medicine. 2012 Oct;70(8):377-80
Rob W van der Pluijm, M.J. Lamers, M. de Boer, W.T. van der Graaf, H.W. van Laarhoven

22. **The methodological quality of clinical guidelines of the European Society of Human Reproduction and Embryology (ESHRE).**
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W.L.D.M. Nelen, **Rob W van der Pluijm**, R.P.M.G. Hermens, C. Bergh, P. de Sutter, K.G. Nygren, A.M.M. Wetzels, R.P.T.M. Grol, J.A.M. Kremer

ACKNOWLEDGEMENTS

The TRACII project was a collaboration between researchers and research groups all over the world and was coordinated by the Mahidol Oxford Tropical Medicine Research Unit (MORU) unit in Bangkok.

The TRACII project was funded by the **Foreign, Commonwealth & Development Office (FCDO)**. I would like to thank the FCDO for their support throughout the years and for recognizing antimalarial resistance as an important threat to global health.

Before thanking anyone else I want to thank all **study participants**, **their parents** and **other family members** for participation in and contributions to the trials in this PhD. Participation entailed an hospital admission of at least 3 days and a weekly follow up for the next 6 weeks. In a setting where many are dependent on public transportation each visit was challenging. At each visit the participants would get blood drawn and would undergo a full physical examination and answer a long symptom questionnaire. The impact of participation cannot be underestimated. Yet almost all participants completed the study until the end. Clinical trials are crucial for the development of new antimalarials, and I want to thank all of the participants for their contribution to trialling Triple ACTs.

Stepping on the plane to Thailand in September 2014 was exciting. My supervisor **Michèle van Vugt** had predicted that I would come back with a PhD, memories for a lifetime and probably a wife and children… Michèle, you were right! Thank you for your support and supervision throughout the years! Your 'yes' mentality to any project or challenge is extremely contagious and inspiring, and I will never forget our chance encounter in Pyin Oo Lwin, Myanmar!

Michèle inspired me to pursue a PhD abroad and she was also the one to introduce me to **Arjen Dondorp.** Arjen, it has been an absolute honour to work with you over the last 7 years. Your kindness, people skills, work ethos and ethics, patience and knowledge have been an example throughout the years. I would like to thank you for all the ways you have supported me in developing at a professional and personal level. You are a true source of inspiration and I look forward to continuing to work together. The same can be said of **Martin Grobusch**, who also supervised my PhD. Your experience and knowledge in the field of tropical medicine and your scientific achievements are truly inspiring. It was great fun to have our beers together in Dakar, Senegal!

I want to thank **Nick White** for our close collaboration throughout the years. I learned a lot through our discussions, your feedback on manuscripts and your endless ideas for new projects. Thank you, **Nick Day,** for welcoming me in the MORU unit and your support throughout the years, and of course for the cosy Friday whiskey evenings.

A special thank you to **Phung** for tackling all administrative hurdles and thereby making my stay in Thailand and the MORU unit so smooth and enjoyable. **Elizabeth Ashley**, the network you created in the first TRAC project was the basis for the TRACII project. Thank you for your support and advice.

Thank you, **Charlie Woodrow** for our intensive collaboration and discussions at the beginning of the project. Thank you **Phaik Yeong Cheah** and **Zoe Doran** for supporting the project as heads of the MORU Clinical Trial Support Group (CTSG). **Naomi Waithira**, thank you for the support of you and your team in the data management of the trial. I have very good memories of our night in Calcutta, where we danced the night away together with **Mehul Dhorda**. Mehul, thank you for everything you have done as the head of the Asian Regional Centre of WWARN. Because of you and your team (especially **Cholrawee Promnarate**!) the TRACII project has been a success. **Mavuto Mukaka**, you have taught me so much about statistics and clinical trials in general. Thank you for our brainstorm sessions, day-long sessions of analysis and lots of laughter! **Prayoon Yuentrakul**, **Jaruwan Tubprasert** and **Salwaluk Panapipat**, thank you for your incredible guidance and support as CTSG members. **Patrick Hannay**, thank you for your guidance in all the financial aspects of the project. We also found lots of time to talk about politics, history, the natural world, geography. You are one of the most well-read men I have ever met!

I want to thank **Sasithon Pukrittayakamee** and **Pratap Singhasivanon** for the support of the faculty of tropical medicine of the Mahidol University to all the healthy volunteer studies and field studies undertaken for this project. **Borimas Hanboonkunupakarn** and **Podjanee Jittamala**, it has been an absolute pleasure to work together on these healthy volunteer studies on the ward and travelling to field sites in Thailand. Thank you **Kesinee Chotivanich** for the support of you and your team in training study staff and setting up the laboratories in the field and for the hard work of the team in the study sites in Thailand. Thank you **Mallika Imwong**, for all the work you and your team have performed on DNA extraction and genotyping. The speed at which your team works is unimaginable.

I want to thank **Richard Hoglund, Markus Winterberg** and **Joel Tarning** for performing the pharmacological measurements and analysis, and the fruitful collaboration over the last years.

The Sanger institute has been crucial for the genetic analysis in this work. I want to thank **Olivo Miotto** specifically for the pleasant collaboration over the last years and for his patience in explaining to me the ins and outs of malaria genetics. And of course, thank you to **William Hamilton**, **Roberto Amato**, **Sónia Gonçalves,** and **Chris Jacob** for all their work. **Richard Maude**, thank you for your support in creating the maps that are included in this book. And of course, thank you **Zbynek Bozdech** for our ongoing collaboration regarding the transcriptomic analysis of antimalarial resistance. I look forward to our next UK road trip!

And thank you **Brian Angus**, **Ric Price**, and **Christian Holm Hansen** for your guidance in their role as Data and Safety Monitoring Committee of the clinical trials.

I had the pleasure to work with teams of doctors, nurses, laboratory staff and field workers in 18 hospitals in 9 different countries, who worked on the project with great enthusiasm and energy. Working together with such a diverse group of scientists has been an honour and a once in a lifetime experience. I want to thank all team members for their hard work and the warm welcome that the MORU team received during each site visit. In each country I want to thank the following collaborators specifically:

Vietnam: **Nguyen Hoang Chau**, **Tran Tinh Hien**, **Ngo Viet Thanh** and **Nguyen Thanh Thuy-Nhien.**

Cambodia: **Dysoley Lek**, **Cheah Huch, Rekol Huy**, **Rithea Leang**, **Sivanna Mao**, **Sokunthea Sreng**, **Seila Suon**, **Sovannary Yen and Sovann Yok.**

Laos: **Mayfong Mayxay**, and **Paul Newton**.

Myanmar: **Tin Maung Hlaing, Khin Lin, James Heaton**, **Frank Smithuis**, **Khin Lin**, **Myo Thant,** and **Aung Pyae Phyo.**

Bangladesh: **Abul Faiz**, **Akhter Ul Islam**, **Sukanta Das, Abul Khair Mohammad Shamsuzzaman**, **Amir Hossain**, **Ridwanur Rahman**, **Rasheda Samad**, **Aniruddha Ghose**, **Abdus Sattar**, **Mohammad Jahirul Karim**, **Abdullah Abu Sayeed**, **Rafiqul Hasan**, and **Ma-Yin-Nu**.

India: **Anupkumar Anvikar**, **Subrata Baidya**, **Prativa Behera**, **Akshaya Kumar Mohanty**, **Parthasarathi Satpathi**, **Sanghamitra Satpathi**, **Kripasindhu Gantait**, **Tarapada Ghosh**, **Amar Tripura** and **Neena Valecha.**

Kenya: **Mainga Hamaluba**, **Brian Mutinda**, **Patricia Njuguna** and **Philip Bejon**.

Democratic Republic of the Congo: **Caterina Fanello** and **Marie Onyamboko**

During my time in Asia, I was lucky to make some great friends. My first field trip was to Pailin in Cambodia, the cradle of antimalarial resistance. There I met **Tom Peto** and **Rupam Tripura**, who have been a crucial part of the work in this PhD. Tom, thanks for being a great friend ever since. I really miss our night-long banter over some ice-cold Chang beers in Ari. I am sure we will have some soon! Rupam, you have shown me what can be achieved through hard work and persistence, no matter the circumstance. I want to thank **Kevin Kobylinski**, **Frank Kloprogge**, **Els van Veelen**, **Thomas Althaus**, **Stije Leopold**, **Christine Ng**, **Daphne Francois**, **James Watson**, **James Callery**, **Andrea**

Ruecker, Bipin Adhikari and **Chanaki Amaratunga** for their friendship and making my time in Bangkok such a wonderful experience.

Being abroad is not always easy, and I was very happy to stay in touch with my friends in Amsterdam **Gijs**, **Menno**, **Reitse** and **Nick**. It has been a while, but I hope we will find ourselves in the Bastille again very soon!

I feel very lucky to have such a great group of cousins, and our annual 'neven en nichten dag' is one of the highlights of the year. **Michel**, **Dave**, **Boris**, **Martijn**, **Sylvie**, **Koen**, **Rick** and **Erwin**, let's make sure we continue the tradition!

A special thanks to **Gerben Hartman** and **Dirk Ottink** for your friendship throughout the last two decades and for being my paranymphs. It means a lot for me to have you by my side.

I want to thank **Mustafa**, **Jihad**, **Latifa**, **Layan**, **Noura**, **Lawrence**, **Rafeef**, **Andalus** and **Mohammad** for welcoming me in your family. As I have said many times before, Beirut has always felt like a second home to me.

Pim, my brother. Thank you so much for being such a great friend. I could not have had a better brother growing up and I am very proud of you. And now it is fantastic to see your family of **Rachael**, **Hugo** and **William** grow.

Pa and **Ma**, words cannot describe how much you have done for me and Pim. You have given us the best start we could wish for and never stopped supporting us. Pa, we have cycled numerous mountains all over Europe, something a son can only hope to do with his father. Ma, you have been a true source of inspiration, as you have reinvented yourself so many times and never stopped learning.

My beautiful wife **Hala**, thank you for being my everything since the moment we met in Casa Azul. Three years of long distance were followed by the three most intense years of our lives. We made it! I cannot wait to see what adventures will follow. Ad astra! **Kamiel** and **Sabine**, you have no idea how happy and proud you make papa. One day I will try to explain.

ABOUT THE AUTHOR

Rob van der Pluijm was born in Tilburg on the 22nd of July in 1984. He and his younger brother grew up in Moergestel. He attended grammar school at the Odulphus Lyceum in Tilburg and graduated in 2002. During his medical studies at the Radboud University in Nijmegen, he completed internships at the emergency departments of the 'Hospital Humberto Alvarado' in Masaya, Nicaragua and the 'Westmead Hospital' in Sydney, Australia.

After obtaining his medical degree in 2010, he worked at the Intensive Care Unit of the Radboud University Medical Center in Nijmegen. He worked as an internal medicine resident between 2011 and 2014 at the Radboud University Medical Center in Nijmegen and the Amsterdam University Medical Center.

Between 2014 and 2019 he lived in Bangkok, Thailand and worked at the Mahidol Oxford Tropical Medicine Research Unit under supervision of Prof. Dr. Arjen Dondorp. As a PhD student, he coordinated an antimalarial efficacy and safety trial in 17 health clinics and hospitals in 8 countries. During this time, he also co-coordinated a follow-up study in Cambodia and Vietnam and was closely involved in the fundraising for, and design and start of the DeTACT-project, which will further develop and trial Triple Antimalarial Combination Therapies in Asia and Africa. He is currently an Infectious Disease fellow at the Amsterdam University Medical Center. Rob and his wonderfull wife Hala have two children, Kamiel and Sabine.

Cover photo: Dr Alexander Kumar