



# Minutes of the Evidence Review Group meeting on the emergence and spread of multidrug- resistant *Plasmodium falciparum* lineages in the Greater Mekong subregion

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## Abbreviations

ACT	artemisinin-based combination therapy
ERG	evidence review group
GMS	Greater Mekong subregion
MDA	mass drug administration
MPAC	Malaria Policy Advisory Committee
<i>Pfcr</i>	<i>P. falciparum chloroquine resistance transporter</i>
<i>Pfdhfr</i>	<i>P. falciparum dihydrofolate reductase</i>
<i>Pfdhps</i>	<i>P. falciparum dihydropteroate synthase</i>
<i>Pfmdr1</i>	<i>P. falciparum multidrug resistance protein 1</i>
<i>PfKelch13</i>	<i>P. falciparum Kelch propeller domain on chromosome 13</i>
RSA	ring stage survival assay
SNP	single nucleotide polymorphism

## Executive summary

At the request of the Malaria Policy Advisory Committee, an Evidence Review Group conducted a review of the evidence on the spread of multidrug-resistant *P. falciparum* in the Greater Mekong subregion. The regional and global risks this development poses to the efficacy of artemisinin-based combination therapies were assessed in the context of contemporary and historical patterns of emergence and spread of drug-resistant malaria.

### Summary conclusions

- Multiple instances of independent emergence and transnational spread of different lineages of artemisinin-resistant parasites have occurred throughout the Greater Mekong subregion (GMS).
- One specific artemisinin-resistant lineage that is dominant at sites in western Cambodia, north-eastern Thailand and southern Lao PDR is also resistant to piperazine in western Cambodia and north-eastern Thailand.
- These multidrug-resistant parasites have been responsible for increasing dihydroartemisinin-piperazine failure rates across Cambodia over the last 5 years, rendering this important artemisinin-based combination therapy ineffective in affected areas.
- Artesunate-mefloquine is currently efficacious in Cambodia, with cure rates >95%, and is being used as first-line treatment in Cambodia as an intermediate solution.
- The risk of a highly fit multidrug-resistant lineage spreading widely in higher transmission settings cannot be discounted; however, in the case of artemisinin and piperazine multidrug resistance, this risk is mitigated by the likelihood that resistance and/or fitness mutations residing on different chromosomes would be rapidly broken up by recombination in multiclonal infections.
- Changing transmission dynamics in Africa have resulted in larger regions of lower malaria transmission intensity similar to the situation in South-East Asia.
- The shift in Africa towards higher drug pressure and less outcrossing increases the potential for the selection and spread of locally generated resistant strains.
- There is also a possibility for parasites from the GMS to potentially become established and spread following importation. Nevertheless, issues of fitness, genetic complexity of the multi-resistant parasites and reduced prevalence of malaria in the GMS mitigate this risk.

### Recommendations

- The new data reaffirm the need for an urgent and continued intensive regional malaria elimination campaign in the GMS.
- Surveillance for artemisinin and partner drug resistance needs to be continued and strengthened in the GMS.
- There is a critical need for surveillance outside the GMS to detect potential de novo resistance or the potential introduction of resistant parasites.
- Where surveillance signals a potential threat to leading ACTs, efficacious alternative ACTs should be identified and implemented before resistance reaches critical levels.

## 1. Rationale

At the Malaria Policy Advisory Committee (MPAC) meeting, 14–16 September 2016, Professor N. White (Mahidol Oxford Tropical Medicine Research Unit) cited new evidence of a multidrug-resistant *P. falciparum* parasite lineage that has developed resistance to both artemisinin and piperaquine in the Greater Mekong subregion (GMS). This lineage has been observed spreading geographically and replacing other *P. falciparum* parasites in the process. WHO called for the new evidence to be submitted for review. MPAC requested that an Evidence Review Group (ERG) assess the relevance of the information and report the potential implications to MPAC at the next meeting in March 2017.

## 2. Background

Artemisinin resistance in *P. falciparum* has arisen and evolved in the GMS over the past decade. Artemisinin resistance is strongly associated with point mutations in the propeller region of the *PfKelch13* gene. Other genetic changes may also be associated with artemisinin resistance, contributing to resistance and/or compensating for any fitness disadvantage. Initially, many independently arising mutations in *PfKelch13* were observed in the GMS. However, investigation of materials collected over more than a decade shows that the relative frequency of certain mutations has progressively increased to become the dominant mutation in some locations.

Resistance to artemisinin-based combination therapy (ACT) partner drugs, including piperaquine and mefloquine, has emerged in the GMS (1, 2), resulting in the declining efficacy of some of the recommended ACTs. In Cambodia, high treatment failure rates have been observed with dihydroartemisinin-piperaquine, while artesunate-mefloquine currently is highly efficacious. In Viet Nam, dihydroartemisinin-piperaquine has started to show increasing rates of treatment failure. In Thailand, high treatment failure rates have been observed following treatment with artesunate-mefloquine. In areas of southern Lao PDR, the therapeutic efficacy of artemether-lumefantrine has declined.

In response to the drug resistance situation and the declining number of malaria cases, in May 2015, GMS Ministers of Health adopted the *Strategy for malaria elimination in the Greater Mekong subregion 2015–2030* (3). The strategy aims to eliminate *P. falciparum* malaria from the GMS by 2025 and all species of human malaria by 2030. The six GMS countries reduced their malaria case incidence by 54% between 2012 and 2015. Reported malaria death fell by 84% over the same period (4). In 2015, a total of 305 027 malaria cases were reported from health facilities and at the community level in the five GMS countries Cambodia, Lao PDR, Myanmar, Thailand and Viet Nam. Of these, approximately 59.7% were *P. falciparum* (~ 182 069 cases).

## 3. Introduction and declarations of interest

An ERG met 20–21 December 2016 to review new evidence on the emergence and spread of multidrug-resistant *P. falciparum* lineages in the GMS and to advise WHO on the risks posed by artemisinin- and piperaquine-resistant *P. falciparum* parasites.

A list of participants is provided in Annex 1. All ERG members attended the meeting, with the exception of C. Chitnis, K. Marsh and S. Tishkoff. A. Clark participated in the meeting through telephone conference, as he was unable to travel due to bad weather. Organizations invited as observers were the Medicines for Malaria Venture and the Wellcome Trust Centre for Human Genetics, Oxford. The meeting agenda is provided in Annex 2.

All ERG members participating in the meeting submitted a declaration of interest that was assessed by the Drug Efficacy and Response Unit, Global Malaria Programme at WHO. None of the members of the ERG were deemed to have conflicts of interest related to the topics for discussion during the ERG meeting. Unpublished data were presented at the meeting, and discussions were conducted under a confidentiality agreement signed by all participants.

## 4. Objectives

The primary objective of the meeting was to discuss the emergence and spread of multidrug-resistant *P. falciparum* lineages in the GMS.

### Specific objectives

- To review new evidence on the emergence and spread of multidrug-resistant *P. falciparum* lineages with the *PfKelch13* C580Y mutation and the *Pfplasmepsin 2-3* gene amplification in the GMS;
- To assess the risk posed by these parasites in terms of malaria control and elimination in the GMS and in other parts of the world;
- To identify evidence gaps and provide recommendations for further research.

### Key definitions

- Artemisinin resistance: partial/relative resistance, described phenotypically as a delay in parasite clearance (in vivo and in vitro).
- ACT resistance: partial resistance to artemisinin plus resistance to a partner drug.
- ACT failure: treatment failure following ACT therapy, regardless of the presence of drug resistance.

## 5. Process and presentation

The Global Malaria Programme (GMP) convened this ERG meeting to review the evidence and to advise WHO on the risks posed by *P. falciparum* parasites resistant to artemisinin and piperaquine.

### Background documents

In preparation for the meeting, WHO collected relevant publications on the topic, and manuscripts were shared by the relevant research groups (Mahidol Oxford Tropical Medicine Research Unit, Bangkok, Thailand, and Southwest Foundation for Biomedical Research, Texas, USA). Annex 3 presents a list of all documents shared by the presenters and provided by WHO.

### Presentations

Presentations, followed by a brief discussion, were made by N. White (Mahidol Oxford Tropical Medicine Research Unit, Bangkok, Thailand); T. Anderson (Texas Biomedical Research Institute, San Antonio, USA); A. Dondorp (Mahidol Oxford Tropical Medicine Research Unit, Bangkok, Thailand); and P. Ringwald (World Health Organization, Geneva, Switzerland). Additional information and data were provided by T. Wells (Medicines for Malaria Venture, Geneva, Switzerland); D. Kwiatkowski (Wellcome Trust Centre for Human Genetics, Oxford, UK); and C. Plowe (University of Maryland, Baltimore, USA). Summaries of these presentations are compiled in Annex 4.

## 6. Evidence reviewed

### Situation of drug resistance in the GMS

Multiple instances of independent emergence and transnational spread of different lineages of artemisinin-resistant parasites have occurred throughout the GMS. There are at least three haplotypes of *PfKelch13* C580Y in South-East Asia, although it is not known whether these haplotypes are functionally equivalent. The prevalence of one specific *PfKelch13* C580Y haplotype is increasing, replacing other haplotypes in an area that includes sites in western Cambodia, north-eastern Thailand and southern Lao PDR. This indicates a selective sweep in this part of the GMS.

The fact that different *PfKelch13* C580Y haplotypes are spreading at different rates suggests that additional mutations in these haplotypes confer differences in other fitness attributes. In vitro competition experiments with genetically edited parasites from recently acquired and culture-adapted Cambodian isolates provide evidence that there is no in vitro fitness cost with *PfKelch13* C580Y, unlike *PfKelch13* R539T, which does appear to have a fitness cost (D. Fidock, personal communication). Compared to *PfKelch13* C580Y, the *PfKelch13* R539T mutation mediates a higher level of in vitro resistance, as defined using ring-stage assays (RSAs) with parasites starting at 0–3 hours post-invasion.

Piperaquine resistance is associated with *Pfplasmepsin 2-3* gene amplification. The proportion of parasites resistant to both artemisinin and piperaquine is increasing in Cambodia and areas of Thailand bordering Cambodia. In these areas, it has been found that coincident *PfKelch13* C580Y and increased *Pfplasmepsin 2-3* copy number is associated with dihydroartemisinin-piperaquine treatment failure rates often reaching >25%. In addition to increased *Pfplasmepsin 2-3* copy number, *Pfcr1* could contribute to piperaquine resistance. There may be direct, mechanistic cross-resistance between chloroquine and piperaquine, or compensatory mutations associated with *Pfcr1* that also contribute to the success of piperaquine-resistant strains.

The rapid increase in dihydroartemisinin-piperaquine failure rates across Cambodia over the last 5 years is serious from an operational standpoint. Artesunate-mefloquine currently is efficacious in Cambodia, with cure rates >95%. As an intermediate solution, artesunate-mefloquine is being used as first-line treatment in Cambodia, particularly in areas with high levels of piperaquine resistance.

The use of mass drug administration (MDA) campaigns with dihydroartemisinin-piperaquine in the region could hasten the spread of resistant parasites. In addition, the presence of parasites resistant to dihydroartemisinin-piperaquine limits the drugs available for MDA. Consequently, the use of dihydroartemisinin-piperaquine for MDA should be avoided in situations where dihydroartemisinin-piperaquine is the only available treatment or where high levels of dihydroartemisinin-piperaquine resistance have been reported.

Although malaria is decreasing overall in the GMS, there have been some outbreaks; it is not known whether these outbreaks were in any way caused by resistance. There is a need to continue to strengthen surveillance in the region in order to ensure the quick detection of outbreaks.

### **Situation of drug resistance outside the GMS**

Research has shown that *PfKelch13* mutations can be selected in vitro in an African parasite (5). Accordingly, South-East Asian parasites do not necessarily have a unique feature associated with the emergence of artemisinin resistance. Polymorphisms in *PfKelch13* have been detected at low frequencies globally. There is no evidence of expansion of these parasite lineages, with the possible exception of the independent emergence of *PfKelch13* C580Y mutants in Guyana.

There is evidence of parasites with increased *Pfplasmepsin 2-3* copy number outside the GMS in areas where piperaquine has been used. However, no functional analysis has been performed on these isolates, so there is no proven relationship to piperaquine resistance. So far, there appears to be no relationship between increased *Pfplasmepsin 2-3* copy number and dihydroartemisinin-piperaquine treatment failure outside the GMS.

There are too many unknowns to reliably predict if and when resistance to artemisinin and/or partner drugs (lumefantrine, mefloquine or piperaquine) will become established outside the GMS. However, there is no reason to believe that with sufficient drug pressure, artemisinin and/or partner drug resistance could not become established outside the GMS, either by spontaneous emergence or by importation, and subsequently spread outside the GMS.

### **Resistance emergence and spread**

There are several historical examples of resistance emerging in Asia and spreading to Africa. *Pf dhfr* single and double mutations were able to develop in African parasites. However, the triple mutation never developed spontaneously in Africa, but rather was imported from Asia. Modelling work has shown that

achieving high-level pyrimethamine resistance requires alleles with multiple mutations, the evolutionary trajectories of which are constrained by the need to mediate less drug susceptibility with each new mutation without too great a fitness deficit. Similarly, single mutations in *Pfdhps* evolved and spread in Africa, and just two highly resistant haplotypes containing >2 mutations in *Pfdhps* spread from Asia to Africa (6).

Separate origins of chloroquine resistance in South-East Asia and South America have been established; a single *Pfcr* chloroquine-resistant allele migrated from South-East Asia to East Africa in the 1970s and became established across the continent.

It is difficult to reconstruct from historical data the exact sequence of events that caused Asian antimalarial drug-resistant parasites to spread to Africa. Although the evolution of artemisinin resistance can be observed in real time in South-East Asia, it may not be possible to completely understand the potential for spread outside the GMS before it is too late. Thus, an urgent and continued intensive regional malaria elimination campaign in the GMS is needed.

It should be noted that since the spread of chloroquine resistance and sulfadoxine-pyrimethamine resistance to Africa, many features of the malaria epidemiology and health systems in Africa and Asia have changed.

It may be that conditions in Asia are particularly suited to the development of successful resistant parasites, which are subsequently able to invade Africa. There may be epidemiological barriers to the development of antimalarial resistance in Africa, such as multiple infection and increased rates of outbreeding. Nevertheless, historical examples indicate that if a resistant parasite is imported from elsewhere, it can spread. Therefore, the risk of a highly fit multidrug-resistant lineage spreading widely in higher transmission settings cannot be discounted. However, such a risk is mitigated by the likelihood that multilocus extended haplotypes, especially those involving resistance and/or fitness mutations on different chromosomes, would be rapidly broken up by recombination in multiclonal infections. Thus, the risk of transnational spreading of parasites with both artemisinin resistance (encoded by mutations on chromosome 13) and resistance to piperazine or mefloquine (likely encoded to an important extent by copy number variation on chromosomes 14 and 5, respectively) may be lower in moderate- and high-transmission areas of South-East Asia and sub-Saharan Africa than in the GMS. This is especially the case when the drug pressure in these areas of high transmission is relatively low.

Notably, it took several years from the emergence of *PfKelch13* mutations to the point at which they became clinically relevant and detected at significant prevalence in field surveys. This suggests that there was a latent period during which additional compensatory adaptations were acquired, enabling the parasites to spread; the *PfKelch13* C580Y selective sweep may be the most recent evidence of this process. For *PfKelch13* mutations, fitness costs seem to be greater in older isolates than in more recent isolates, also indicating the accumulation of additional compensatory mutations (D. Fidock, unpublished data). The later introduction of ACTs in Africa relative to Asia may explain the lack of *PfKelch13* mutations expanding in the population.

The successful establishment of a resistant parasite reduces variation around the genetic target. This loss of variation could be a fitness disadvantage if the environment were to change, for example by switching antimalarial therapy or changing transmission dynamics. In the absence of drug pressure, for instance, both increased *Pfmdr1* copy number in Cambodia and chloroquine-resistant *Pfcr* in Africa appear to be lost.

Although the artemisinin resistance phenotype is limited to one single life cycle stage at present (the early ring stage), there is the potential that, in the future, artemisinin resistance in other *P. falciparum* life cycle stages in humans will emerge.

For *P. vivax*, no artemisinin-resistant strains and no *PvKelch13* mutations have been reported. Artemisinins have greater activity against *P. vivax* than against *P. falciparum*, and *P. vivax* has a much lower parasite biomass than *P. falciparum*. Therefore, compared to *P. falciparum*, the capacity for de novo resistance selection in *P. vivax* is relatively low.

## 7. Conclusions and recommendations

The ERG addressed the following key questions and made the following conclusions and recommendations for consideration:

### **Is there new evidence of selection and spread of specific artemisinin-resistant genotype(s) in the GMS?**

There is evidence that selective sweeps at *PfKelch13* have occurred throughout the GMS. Currently, the *PfKelch13* C580Y mutation can be found in several genetic backgrounds (haplotypes) throughout the GMS. The prevalence of one specific *PfKelch13* C580Y haplotype is increasing, replacing other haplotypes in an area that includes sites in western Cambodia, north-eastern Thailand and southern Lao PDR. This indicates a selective sweep in this part of the GMS. However, the frequencies of different *PfKelch13* C580Y haplotypes vary by region, and no single haplotype is dominant throughout the GMS. The emergence and spread of *PfKelch13* C580Y haplotypes is a dynamic process, and continued surveillance is essential in order to detect the emergence of a region-wide sweep of a single haplotype.

Although some *PfKelch13* mutants, in absence of partner drug resistance, have increased gametocytaemia compared to wild-type parasites, it is not clear whether this characteristic actually increases transmission. Membrane feeding studies would be able to determine whether there is a significant transmission advantage with the various *PfKelch13* mutations.

### **If yes, what would be the consequences of the selection and spread of specific artemisinin-resistant genotype(s)?**

The consequences of the selection and spread of specific artemisinin-resistant genotype(s) could include:

- Partial or total loss of efficacy to artemisinin treatments;
- Global spread of a dominant haplotype that would increase levels of resistance (following the history of chloroquine resistance and sulfadoxine-pyrimethamine resistance);
- A common genetic background could accumulate mutations at other (i.e., loosely or unlinked) loci that might encode potential compensatory factors, such as ACT partner drug resistance, fitness or transmissibility;
- Alternatively, the spread of *PfKelch13* C580Y haplotypes could result in a loss of within-population genetic diversity. This loss of variation could become a fitness disadvantage for *P. falciparum* should the environment change, for example, by switching antimalarial therapy or changing transmission dynamics.

### **Is there evidence that artemisinin resistance has facilitated the emergence of partner drug resistance in the GMS? If yes, for which partner drug(s)?**

There is evidence of parasites with resistance to both artemisinin and piperaquine in Cambodia and areas of Thailand bordering Cambodia. Piperaquine resistance has occurred in the past in China and Cambodia independently of artemisinin resistance. Therefore, artemisinin resistance is not a prerequisite for the initial appearance of piperaquine resistance.

In the GMS, the perception is that piperaquine resistance occurred after the initial discovery of artemisinin resistance; however, it is not possible to determine the temporal relationship between the emergence of either resistance (i.e., piperaquine resistance could have already been present at low levels). The molecular and physiological mechanisms of piperaquine and artemisinin resistance appear to be independent. There is no evidence that *PfKelch13* C580Y confers any resistance to piperaquine. Instead, the mechanism of piperaquine resistance appears to involve gene amplification of *Pfplasmepsin 2-3*. This type of genetic change (i.e., copy number variation) is unstable and allows rapid back mutation to single copy, particularly during meiosis. As a result, the temporal tracking of piperaquine resistance is difficult.



**Is there evidence that artemisinin resistance has facilitated the selection and spread of partner drug resistance in the GMS? If yes, for which partner drug(s)?**

Recent results clearly show the spread of dual-resistant phenotypes and genotypes (i.e., resistant to both piperazine and artemisinin) in Cambodia, where dihydroartemisinin-piperazine was the first-line treatment for *P. falciparum* malaria. As the resistance mechanism is not understood, it is difficult to understand the spread of resistance in the different genetic backgrounds. It is possible that there is an epistatic effect<sup>a</sup> that changes the synergisms between the two types of drug resistance in different genetic backgrounds, but there is as yet no evidence of this hypothesis.

Different factors affect the selection and spread of piperazine resistance:

- Artemisinin resistance may increase the exposure of the parasite population to piperazine;
- Since piperazine has a long half-life, after treatment with dihydroartemisinin-piperazine, piperazine is effectively present as a monotherapy for about 1 month. During this time, exposure to piperazine may be low enough to allow the survival of piperazine-resistant merozoites emerging from the liver, further fostering the selection of resistance;

Conversely, it is possible that the reduced efficacy of piperazine has facilitated the selection of artemisinin-resistant mutations.

With regard to the selection and spread of resistance to partner drugs other than piperazine:

- Artesunate-pyronaridine: Not enough data are available to draw any conclusions;
- Artemether-lumefantrine: Efficacy was always suboptimal in Cambodia and in Thailand; consequently, few data are available for these countries. Artemether-lumefantrine is still highly efficacious in Myanmar, and was so in Lao PDR until recently;
- Artesunate-mefloquine: As there was a background of mefloquine resistance in the GMS prior to the introduction of artemisinin drugs, it is not possible to assess the role of artemisinin resistance in the emergence and selection of mefloquine resistance. Recent data demonstrate that the use of dihydroartemisinin-piperazine has coincided with a reversion of mefloquine resistance to sensitivity. It is not known whether this is because mefloquine is no longer used as a treatment, or whether this is a direct consequence of dihydroartemisinin-piperazine treatment. The recent decision to use artesunate-mefloquine as first-line treatment in Cambodia will enable us to determine whether this treatment results in an increase in mefloquine resistance in the parasite population, particularly in populations where mefloquine resistance was previously prevalent. This could provide key information for the utility of alternating drug strategies in combating resistance.

Risk factors for the development of ACT partner drug resistance include use of partner drugs as monotherapy; a long half-life of the partner drug; resistance to artemisinin; and non-adherence or use of substandard drugs, particularly for combination therapy, resulting in inadequate dosage of the partner drug to entirely clear the parasite load. Little analysis of these risk factors has been done.

**Is there evidence of the geographical extent of artemisinin resistance outside the GMS?**

No evidence was presented to indicate artemisinin resistance outside the GMS. There is evidence of multiple *PfKelch13* mutations in many geographic regions. However, none of these are associated with a haplotypic expansion of *PfKelch13*. One exception is the possible independent emergence of *PfKelch13* C580Y in Guyana.

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<sup>a</sup> Epistasis is where the phenotypic effect of one mutation differs depending on the presence of another mutation.

**What is the risk (and risk factors) of the spontaneous emergence, selection and spread of artemisinin resistance and/or resistance to ACT partner drugs outside the GMS?**

Historically, chloroquine, sulfadoxine and pyrimethamine resistance emerged in South-East Asia, and those drug-resistant haplotypes spread to Africa. Thus, there is an historical precedent to support concerns that this could happen for artemisinin resistance and/or for resistance to a partner drug(s).

Nevertheless, mefloquine resistance also emerged in the GMS, but it has not spread to other regions, most likely because mefloquine has not been used extensively outside of the GMS. In addition, low-level resistant haplotypes encoded by single mutations in *Pf dhfr* and *Pf dhps* have had multiple African origins and were not acquired from South-East Asia.

There may be epidemiological barriers to the development of antimalarial resistance in Africa, such as multiple infection and increased rates of outbreeding. Although the risk of a highly fit multidrug-resistant lineage spreading widely in higher transmission settings cannot be discounted, this risk is mitigated in the case of artemisinin and piperaquine multidrug resistance by the likelihood that resistance and/or fitness mutations residing on different chromosomes would be rapidly broken up by recombination in multiclonal infections.

It may be that conditions in Asia are particularly suited to the development of successful resistant parasites, which are subsequently able to invade Africa. However, changing transmission dynamics in Africa have resulted in larger regions of lower malaria transmission intensity similar to the situation in South-East Asia. The shift in Africa towards higher drug pressure and less outcrossing increases the potential for the selection and spread of locally generated resistant strains. There is also a possibility for parasites from GMS to potentially become established and spread following importation. Nevertheless, issues of fitness, genetic complexity of the multi-resistant parasites and reduced prevalence of malaria in the GMS mitigate this risk.

The selection and spread of drug resistance in the GMS is presumably related to the high drug pressure that has been present in the region over an extended period of time (particularly for dihydroartemisinin-piperaquine in Cambodia), and to the historical use of monotherapy in South-East Asia, which selected for specific drug-resistant variants.

*PfKelch13* mutations are present at low frequencies in *P. falciparum* outside the GMS. This includes the *PfKelch13* C580Y mutation that has been found in Africa and elsewhere. However, the fact that the *PfKelch13* C580Y mutation exists outside the GMS but is not spreading suggests that additional mutations may be necessary to modulate the potential fitness costs of the *PfKelch13* C580Y mutation or may reflect the relatively lower exposure of parasite populations to artemisinin derivatives. Under drug pressure, however, the necessary compensatory mutations might be acquired, enabling the spread of artemisinin-resistant parasites of African origin.

Unlike the complex loci (multiple mutations) needed for clinically relevant levels of chloroquine and sulfadoxine-pyrimethamine resistance, data from the GMS indicate that the primary resistance mutations for artemisinin, piperaquine and mefloquine are single nucleotide polymorphisms (SNPs) or copy number variations. Consequently, there is a high probability that copy number variations of *Pfplasmepsin 2-3* and *Pfmdr1* needed for clinically relevant resistance key ACT partner drugs already exist at low frequencies in the parasite population outside the GMS. These parasite populations could remain at low frequencies or disappear in the absence of selecting factors. Nevertheless, there is the potential that these variants may rapidly select and spread under increased drug pressure. The massive and uncontrolled use of dihydroartemisinin-piperaquine in settings outside the GMS may thus lead to resistance and loss of efficacy of this treatment, even if resistant parasites are not imported from the GMS.

Overall, there is a significant risk of artemisinin and partner drug resistance outside the GMS – either via spontaneous emergence or importation, and spread. Therefore, resistance surveillance in regions outside the GMS is critical.

To summarize, risk factors contributing to the potential for resistant strains to emerge locally or for imported resistant parasites to spread are multifactorial and interdependent (Table 1).

**Table 1. Factors affecting the potential for resistant strains to emerge or for imported resistant parasites to spread to Africa**

	Increase the potential	Decrease the potential
<b>Health system</b>	Poor access to diagnostics and quality drugs	Improvement in access to diagnostics and quality drugs
<b>Antimalarial drugs</b>	Widespread use of antimalarial drugs, for example MDA; use of substandard antimalarial drugs, available primarily on the private markets; treatment non-adherence	Limited use of antimalarial drugs in immune adult populations and asymptomatic carriers
<b>Human</b>	Increased population movement between Africa and Asia; increased exposure to mosquitoes; low immunity	Reduction in malaria in the GMS <sup>b</sup> potentially leading to a reduction in migration of infected people; reduced exposure to mosquitoes;
<b>Parasite population structure</b>	Changing (decreasing) malaria prevalence leading to changes in parasite population structure and the consequent reduction of the possibility of multiple infection, limiting the outbreeding between different genotypes	Extensive parasite diversity in African parasite populations and evidence of outcrossing in parasite populations, even in regions of low transmission
<b>Vector</b>	The ability of African vectors to transmit Asian parasites; receptivity in areas where importation happens	Greater vector control measures lowering receptivity
<b>Parasite</b>	High transmission potential of <i>P. falciparum</i> -resistant strains; low fitness costs of imported versus local strains	Low transmission potential of <i>P. falciparum</i> -resistant strains; high fitness costs of imported versus local strains

**Identify research questions that might improve our understanding of artemisinin resistance, the selection of specific artemisinin-resistant genotypes, and the role that artemisinin resistance plays in the emergence and selection of resistance to ACT partner drugs.**

1. Biologically characterize artemisinin resistance mutations and mechanisms of artemisinin resistance – both as they occur and using CRISPR/Cas9 to study mutations in a single genetic background:

- Measure fitness both in vitro and using longitudinal population data in vivo;
- A standing hypothesis is that artemisinin resistance is primarily conferred by *PfKelch13* mutations, and other aspects of fitness are modulated by the genetic background. However, this hypothesis needs to be tested and quantified by performing whole-genome sequencing of defined strains in order to determine relevant mutations for testing: (i) by examining the dose-response curve of

<sup>b</sup> In recent years, the access to malaria treatment and reporting of malaria have improved significantly. In 2015, a total of 305 027 malaria cases were reported from health facilities and at the community level in the five GMS countries Cambodia, Lao PDR, Myanmar, Thailand and Viet Nam. Of these, approximately 59.7% were *P. falciparum* (~ 182 069 cases). In 2015, the estimated total number of cases in the five countries was 515 015 (range: 360 000–764 000) (World Malaria Report 2016). In 2010, the five countries reported 858 713 malaria cases, of which approximately 69.9% were *P. falciparum* (~ 600 458 cases). In 2000, the five countries reported 1 418 098 malaria cases, of which approximately 83.5% were *P. falciparum* (~ 1 184 253 cases). In 1990, the five countries reported 1 532 558 malaria cases, of which approximately 81.1% were *P. falciparum* (~ 1 243 103 cases) (World Malaria Report 2011). Data reporting before 1990 is limited.

each parasite life stage with respect to the drug; (ii) by determining the genetic components of fitness in competitive growth studies;

- Measure the effect on transmissibility of the specific mutation and the genetic background in both Asian and African vectors;
- Explore mechanisms of action for a better understanding of how resistance emerges and spreads. A hallmark of this resistance, in contrast to other known resistant mechanisms, is that multiple mutations (>25 SNPs associated with delayed clearance but not necessarily validated as markers of artemisinin resistance) in the *PfKelch13* propeller domain result in a resistance phenotype;
- Explore whether this is a loss-of-function mechanism and identify the underlying pathways;
- From the whole-genome sequencing analysis, develop a targeted set of genetic markers across the genome: (i) include both fitness and molecular markers; (ii) focus on new, emerging resistance markers;
- Validate a better assay as a surrogate of phenotypic resistance. Both RSA and clearance time estimation are limited to detecting ring stage activity, and neither are useful in large-scale surveillance (i.e., RSAs require specialist laboratories, and clearance times require frequent observations of single patients);
- There is some evidence of delayed clearance that is not obviously *PfKelch13*-mediated. There is a need to understand or find other markers that produce this phenotype.

2. Investigate the impact of parasite population characteristics, such as population genetic structure, on the emergence and spread of drug resistance using modelling approaches and resistance mechanism interactions; the simplicity of the *PfKelch13* mutations that result in resistance raises the question as to why resistance has emerged and spread in the GMS and not elsewhere.

3. Assess the role of human mobility and drug use in the emergence and spread of resistance:

- Explore alternative epidemiological approaches to surveillance, for example, using social scientific approaches to identify the movement of human populations at risk of transporting resistant parasites from Asia to Africa;
- Study special populations with little access to health facilities.

4. Explore alternative drug regimens: As new drugs are unlikely to be available within the next 5 years, existing drugs should be evaluated for use in MDA and as treatment. This could include a re-examination of atovaquone-proguanil; in particular, investigate whether *cytochrome b* atovaquone resistance mutations are transmissible.

5. Determine the contribution of artemisinin resistance to the spread of multidrug-resistant malaria parasites:

- Explore the potential impact of the increased use of ACTs in Africa on the emergence and spread of artemisinin resistance.

6. Investigate the contribution of partner drug resistance to the spread of multidrug-resistant parasites:

- Determine whether the loss of *Pfmdr1* copy number following the replacement of artesunate-mefloquine with dihydroartemisinin-piperaquine in Cambodia has been because of competing drug resistance mechanisms and/or the removal of mefloquine drug pressure;
- Explore the potential impact of the increased use of dihydroartemisinin-piperaquine in Africa, particularly in MDA, on the emergence and spread of artemisinin and piperaquine resistance.

7. Conduct thorough and well-coordinated surveillance to enable early identification of the spread of resistant strains and to enact changes in drug strategies that may delay the spread. Faster identification of resistant mutations, haplotypes or strain(s) allows for an earlier start to characterizing the mechanism of resistance. Priorities for surveillance for genetic evidence of artemisinin resistance inside and outside the GMS include:

- Active engagement of Ministries of Health/national malaria control programmes;
- Active surveillance, especially in areas of decreasing transmission or under MDA;
- Coordinated sampling and sharing of data in real time from research groups and nongovernmental organizations to Ministries of Health/national malaria control programmes and WHO;
- Continued and extended surveillance for the emergence and spread of partner drug (lumefantrine, mefloquine or piperaquine) resistance in Africa; this is particularly important in countries where dihydroartemisinin-piperaquine is used for treatment (in private or public sector) or being used as MDA;
- Identification of potential evidence of Asian parasitic genetic backgrounds in the African setting; the gold standard would be whole genome sequencing of *Plasmodium* from multiple regions.

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## Annex 1: Participants

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## Annex 2: Agenda

Monday 19 October 2016		
09:00–09:15	Welcome	P. Alonso, Director GMP D. Wirth, Chair ERG
09:15–09:20	Objectives of the meeting and declaration of interest	P. Ringwald
09:20–10:00	Intrahost selection and spread of antimalarial drug resistance (basic principles-PK-PD-ACT rationale)	N. White
10:00–10:45	Evolution of antimalarial drug resistance (population genetics principles, selective sweeps, history of chloroquine and SP resistance emergence and spread, longitudinal studies on the Thai–Myanmar border)	T. Anderson
10.45–11.15	<i>Coffee/tea break</i>	
11:15–12:00	Development of artemisinin and partner drug resistant falciparum malaria in the GMS (emergence and spread of artemisinin and partner drug resistance in the GMS, recent transnational spread)	A. Dondorp
12:00–12:45	World-wide situation of drug efficacy and drug resistance outside GMS	P. Ringwald
12:45–14:00	<i>Lunch</i>	
14:00–14:30	Questions and answers from review panel	
14:30–16:00	Discussion	
16:00–16:30	<i>Coffee/tea break</i>	
16:30–18:30	Discussion (continued)	
Tuesday 20 December 2016		
09:00–12:30	Formulation of ERG recommendations (Closed session)	D. Wirth, Chair ERG
12:30	Closing remarks (Closed session)	P. Alonso



## Annex 3: Supporting documents

### Documents shared by presenters

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## Annex 4: Presentations

### Intrahost selection and spread of antimalarial drug resistance (N. White)

Antimalarial drugs reduce parasite multiplication, with activity against specific stages of the malaria parasite life cycle. Antimalarial pharmacokinetics and pharmacodynamics are diverse. Clearance half-lives vary from less than 1 hour (artemisinins) to around 1 month (chloroquine). Different parasite clearance time profiles reflect differing pharmacodynamics; of clinically available agents, artesunate has the most rapid parasite clearance time. Drug resistance causes the dose-response curve to shift to the right and may also change the shape of the curve. As the parasite drug resistance level increases, recrudescence occurs earlier following treatment, and drugs can eventually lose all activity.

Antimalarial drugs differ in their propensity to generate de novo resistance. For example, although highly potent against susceptible parasites, proguanil resistance is rapidly generated and selected, is transmissible, and the consequent malaria infection is refractory to treatment. Similarly, resistance to pyrimethamine, mefloquine or atovaquone is readily generated and selected. However, the barriers to resistance for some antimalarial drugs (quinine, artemisinin) appear to be higher, requiring more extensive and sustained drug pressure.

Most identified mechanisms of antimalarial drug resistance involve genetic mutation. Resistance mechanisms include changes to the target (*Pf dhfr*, *Pf dhps*, *Pf plasmeprin 2-3*, *cytochrome b*, *Pf ATPase4*), mutation or amplification of drug pumps (*Pf crt*, *Pf mdr1*), and unknown mechanisms (*Pf Kelch13*, *Pf carl*).

Transmission of de novo resistance requires recrudescence. Therefore, treatment failures drive the spread of resistance. There is some evidence of increased gametocytaemia following the initial treatment of resistant infections, which may facilitate the spread of resistant strains.

De novo resistance is most likely to arise and subsequently spread from a hyperparasitaemic individual receiving inadequate treatment. Such patients have more parasites and consequently more genetic variation in their parasite population; moreover, they have inadequate host defense against the infection. In addition, treatment failure is more likely in hyperparasitaemic individuals with the potential for transmission of resistant parasites.

Symptomatic malaria is generally caused by between  $10^7$  and  $10^{12}$  parasites. The rationale for ACTs is that the artemisinin reduces parasite numbers quickly to around  $10^4$ , while the partner drug kills the remaining parasites and provides protection over several parasite cycles. For ACTs, combination therapy protects the artemisinin and partially protects the partner drug against de novo selection of resistance owing to the reduction in parasitaemia achieved with the artemisinin. However, once artemisinin resistance develops, the unprotected partner drug is exposed to larger numbers of parasites, accelerating the selection of partner drug resistance. The long terminal elimination phase of partner antimalarial drug provides a selective filter that amplifies the spread of resistance to the partner compound.

### Evolution of antimalarial drug resistance (T. Anderson)

The selection of antimalarial drug resistance can be considered:

- 'Hard', whereby genetic variation around the selected gene is purged, thereby producing a strong signature in the genome that is easy to find by association; or
- 'Soft', whereby multiple genetic backgrounds are associated with the selected gene, leaving a weak signature in the genome that is difficult to find by association.

Hard selection events are more easily detected and tracked than soft events. As a result, the initial emergence of resistance as a soft event may go undetected until more competitive variants begin to replace less competitive ones. A hard event becomes evident as diverse genetic backgrounds are purged. Whether an event appears hard or soft, therefore, may depend on when and where you look.

**Pyrimethamine:** Low-level resistance to pyrimethamine is easily generated. Thus, multiple independent emergences of pyrimethamine resistance would be expected. However, data from South-East Asia show that, for parasites with two or more *Pfdhfr* mutations, variation around *Pfdhfr* is very low, with a single dominant microsatellite haplotype associated with resistant *Pfdhfr* alleles across five South-East Asian countries (7). This indicates a hard selective sweep at this locus and a single origin of resistant alleles. These strains that are highly resistant to pyrimethamine subsequently spread from South-East Asia to Africa (8), mirroring the emergence and spread of chloroquine resistance (9).

It is not clear why high-level resistance has so few origins. In every malaria patient, an estimated 100–1000 parasites contain point mutations at each position in the genome. However, although resistance mutations may be common, successful resistance alleles are rare. Perhaps this is because multiple simultaneous changes are required for resistance to arise or because compensatory mutations are needed elsewhere in the genome.

**Artemisinin:** The emergence and spread of artemisinin resistance in the Thai–Myanmar border area allows for the examination of an ongoing selective event. In 2014, around 90% of parasites had *PfKelch13* mutations. The sequencing of *PfKelch13* ( $n = 1876$ ), genotyping of 75 flanking SNPs and investigation of parasite clearance rates ( $n = 3552$ ) revealed 32 independent coding mutations, including those outside the propeller region, associated with significant reductions in the parasite clearance rate (10). These represent soft selective events.

The first *PfKelch13* mutations along the Thai–Myanmar border were described in 2003. Allele diversity increased until 2012, with the *PfKelch13* E252Q mutation dominating until 2010. However, since the emergence of *PfKelch13* C580Y in 2006, it has progressively replaced other mutations to become the dominant mutation, present in around 70% of parasites in 2014 (10). Thus, the selection signature is becoming harder as *PfKelch13* C580Y apparently outcompetes other variants. Whether this will lead to the spread of the *PfKelch13* C580Y allele outside of the GMS is unknown, but the experience of pyrimethamine and chloroquine resistance suggests that this is a possible scenario.

Many different *PfKelch13* mutations result in phenotypic artemisinin resistance. As other mutations are associated with greater delays in parasite clearance rates, the relative selective advantage of *PfKelch13* C580Y is unclear. Furthermore, around 4% of *PfKelch13* wild-type alleles have extended parasite clearance times, indicating the possibility that other loci or contributing factors may be involved in artemisinin resistance (10).

The large mutational target size for *PfKelch13* (87–163 base pairs) makes multiple origins of resistance likely. In addition, the frequency distribution of artemisinin-resistant alleles leads to an estimated short-term effective parasite population size of 88 000 to 1.2 million. This figure is greater than previously estimated and indicates a higher adaptive capacity in *P. falciparum*. In light of this, to avoid the development of antimalarial drug resistance, combination therapies need to be more complex.

*PfKelch13* mutations are currently being detected in Africa and elsewhere, but have not yet become established or begun to spread. The reasons for this are unclear, but may depend on the fitness costs of resistance mutations, as well as higher transmission, lower drug pressure, differences in the response of the local vectors, the higher proportion of multiple genotype infections leading to stronger intrahost competition, and the predominance of outbreeding and high recombination rates, resulting in the breaking up of multilocus genotypes.

Other loci show a temporal change in frequency similar to that of *PfKelch13* and may also be associated with artemisinin resistance. The strongest signature was an SNP in phosphatidylinositol 4-kinase (PI4K) (11). However, no functional validation of this locus has been performed, for example using CRISPR/Cas9.

## Conclusion

There are clear historical examples of antimalarial drug-resistant parasites arising in Asia and spreading to Africa, in some cases replacing indigenous alleles. As we do not fully understand the process by which this happens, the intercontinental spread of artemisinin-resistant parasites also appears to be a possibility. Therefore, the elimination of South-East Asian parasite populations should be prioritized, as this would remove key sources of artemisinin and piperaquine resistance.

## Discussion

The studies discussed were representative of the population along the Thai–Myanmar border. The relative frequencies of mutations appear to be similar in hyperparasitaemic patients, those with ‘normal’ parasite loads, and asymptomatic individuals identified using quantitative real-time polymerase chain reaction (qPCR). The majority of malaria parasites in the region carry *PfKelch13* mutations. Owing to malaria elimination efforts, there were very few cases of *P. falciparum* malaria in this specific region (Thai–Myanmar border) in 2015–2016, reducing the potential for spread to other regions. However, the potential remains for other resistant haplotypes to develop elsewhere independently.

The spread of certain *PfKelch13* mutants is dependent on whether they affect the recrudescence rate; there is some evidence that gametocytaemia may also be increased with these mutations.

## Development of artemisinin- and partner drug-resistant falciparum malaria in the GMS (A. Dondorp)

When ACTs were introduced as first-line therapy along the Thai–Myanmar border, it was against a background of widespread antimalarial drug resistance to chloroquine, sulfadoxine-pyrimethamine, mefloquine and (to a lesser extent) quinine ± tetracycline. Artesunate-mefloquine was introduced in the early 1990s at a time when mefloquine resistance was at quite a high level, but despite this the combination remained efficacious for >15 years. Drug pressure on the artemisinins and partner drugs in the GMS is in part derived from the poor quality of drugs and the availability of artemisinin monotherapy. Artemisinin resistance, evident phenotypically as a delay in parasite clearance, was described in 2009 in western Cambodia; however, it was retrospectively identified as having already been present at the beginning of the millennium, at a time when higher than expected ACT failure rates were intermittently reported in the same areas. Declining clinical efficacy with artesunate-mefloquine resulted in a change to dihydroartemisinin-piperaquine in western Cambodia in 2008 and elsewhere in 2010, although this transition was not complete until 2012.

**Artesunate-mefloquine:** In Cambodia, infections from parasites with *PfKelch13* C580Y and a single *Pfmdr1* copy number had a 100% cure rate. Along the Thai–Myanmar border, artesunate-mefloquine cure rates were the lowest (~60%) in patients with multiple *Pfmdr1* copies and any *PfKelch13* propeller region SNP. In addition, cure rates declined as the proportion of *PfKelch13* mutations increased (observed in patients with single *Pfmdr1* copy number, although this does not exclude low-level mefloquine resistance caused by other mechanisms). By 2013, around 84% of infections had *PfKelch13* mutations and 65% had multiple *Pfmdr1* copies (12).

**Dihydroartemisinin-piperaquine:** Piperaquine has a long half-life, and because of its bi-phasic pharmacokinetic profile, a small increase in the minimum inhibitory concentration can lead to a large reduction in the time the parasite is exposed to parasitocidal drug concentrations. Resistance to dihydroartemisinin-piperaquine has rapidly emerged in western Cambodia, with cure rates of <70% in Pursat in 2012–2013 (13). Resistance appears to be spreading from western Cambodia to eastern Cambodia, and increases in the proportion of parasites with *PfKelch13* mutations appear to have preceded the emergence of piperaquine resistance (14). However, of note, there was some use of piperaquine in western Cambodia around 2000 to 2003, and piperaquine resistance was likely to have pre-existed at low levels in the region.

Increased *Pfplasmepsin* 2-3 copy number predicts dihydroartemisinin-piperaquine treatment failure and has been closely associated with in vitro piperaquine resistance. In 2014–2015, the proportion of parasites with both *PfKelch13* and *Pfplasmepsin* 2-3 copy number amplification had increased to around 50% in Cambodia. Treatment failure rates with these parasites are around 35% at day 28 and 65% at day 42. The *Pfplasmepsin* 2-3 amplification is almost invariably observed in association with *PfKelch13* mutated parasites.

**ACT efficacy:** Even in the presence of artemisinin resistance, efficacy rates may still be adequate. It is only once resistance to the partner drug emerges that treatment failure rates increase significantly. Artemisinin resistance, as indicated by an increase in day-3 slide positivity rates, is expanding across the GMS (14). Treatment failure rates are >10% to four ACTs in Cambodia; two ACTs in Thailand, Lao PDR and Viet Nam; and one ACT in Myanmar, India and China (Yunnan Province). Increased ACT failure rates are driving the onward transmission of resistant parasites, facilitating the rapid spread of resistance. In addition, gametocytaemia increases with artemisinin resistance, as well as with mefloquine resistance and possibly piperaquine resistance, which potentially increases transmission (15).

There is some suggestion that piperaquine and mefloquine may have opposing resistance mechanisms, with the potential for drug cycling to combat resistance. Furthermore, these opposing effects have been attributed to changes in *Pfmdr1* copy number: Whereas mefloquine resistance seems to be associated with an increased *Pfmdr1* copy number, piperaquine resistance seems to be found when the *Pfmdr1* copy number is 1. However, it cannot be excluded that the disappearance of mefloquine resistance in areas where dihydroartemisinin-piperaquine is used may be caused by the removal of drug pressure, as gene amplifications are easily lost. A trial of triple therapy with dihydroartemisinin-piperaquine-mefloquine is being conducted, which may inform this discussion.

**Artemisinin resistance:** The area of South-East Asia where *PfKelch13* mutations have been detected is expanding (16). There is variation in the dominant phenotype, for example *PfKelch13* C580Y in Cambodia and *PfKelch13* F446I in upper Myanmar, and resistance appears to have emerged in multiple locations (17, 18). In Africa, despite the presence of *PfKelch13* mutations, there is no evidence for selection of these mutations (18, 19). This may be because additional permissive or compensatory mutations might be needed for the spread of *PfKelch13* mutations (18). In Cambodia, the *PfKelch13* C580Y mutation has risen to dominance and spread across the country. A recent observation is that a single lineage of *PfKelch13* C580Y mutant parasites has spread across an area encompassing western Cambodia, north-eastern Thailand and southern Lao PDR. This implies a single origin of these apparently fit parasites (within the context of these GMS countries). Some of these emerging parasites have acquired the *Pfplasmepsin* 2-3 marker for piperaquine resistance. In experimental models of transmission, Cambodian artemisinin-resistant clinical isolates were able to infect diverse mosquito vectors of South-East Asia and Africa, suggesting the potential for expansion of resistant parasites to regions outside the GMS (20). In the GMS, *P. falciparum* resistant to artemisinin and/or partner drugs represents an emergency requiring the high-quality and urgent execution of the GMS malaria elimination agenda.

## Conclusion

The slow parasite clearance phenotype that emerged and was selected following artemisinin treatment resulted in multiple soft sweeps of various *PfKelch13* mutations. These appear to have been overtaken by a hard sweep of the *PfKelch13* C580Y mutation, with a single dominant haplotype spreading across a wide geographic area involving three countries in the central part of the GMS. In the GMS, the presence of artemisinin-resistant *P. falciparum* malaria, which is increasingly compounded by partner drug resistance, is an emergency and should be treated as such. It requires the rapid, high-quality execution of the malaria elimination agenda in the GMS with a genuine sense of urgency.

## Discussion

Although piperazine-resistant strains are generally susceptible to mefloquine, mefloquine can select for resistance very rapidly. In the triple therapy trial (dihydroartemisinin-piperazine-mefloquine), there have so far been no reports of resistance to all three agents. Monitoring studies of artesunate-mefloquine efficacy in Cambodia indicate 100% treatment efficacy, except in Ratanakiri, where efficacy is around 95%. Of concern is that in 250 samples in 2015, around 2.5% (six cases) had increased copy numbers for both *Pfmdr1* and *Pfplasmepsin 2-3*. There is no hard evidence that these parasites cannot achieve resistance to both mefloquine and piperazine (as well as to the artemisinins).

Unlike in Cambodia, in Africa there has been no significant drug pressure from piperazine, which may explain why indigenous resistant haplotypes have not yet spread, or why imported resistant parasites have not become established. Selection under increasing use of dihydroartemisinin-piperazine could lead to the spread of these Asian parasites to Africa or the emergence of locally generated resistant strains.

## Worldwide situation of drug efficacy and drug resistance outside the GMS (P. Ringwald)

More than 200 non-synonymous mutations in the *PfKelch13* propeller region have been reported worldwide. However, only *PfKelch13* N458Y, Y493H, R539T, I543T, R561H and C580Y have been validated through RSA as contributing to artemisinin resistance, while *PfKelch13* A578S and E252Q have been confirmed as not associated in vitro with artemisinin resistance. The impact of other *PfKelch13* propeller mutations on artemisinin resistance is still unknown.

The *PfKelch13* C580Y mutation, which has become dominant in western Cambodia, has also been identified in Viet Nam, Thailand, Lao PDR and Myanmar. Although this mutation has been detected sporadically outside the GMS, including in Africa, there is no evidence of expansion elsewhere. A possible exception is Guyana (see below).

Despite the high proportion of parasites harbouring artemisinin resistance and delaying clearance, the actual number of parasites on day 3 is low. Data from Cambodia show that patients treated with an ACT on day 3 have a parasitaemia representing 1–3% of initial parasitaemia (day 0). There is currently no evidence of the emergence of high-level artemisinin resistance.

**India:** *PfKelch13* mutations are rare and heterogeneous, and do not lead to treatment failure. In northern India, where the risk of importation of artemisinin-resistant haplotypes from the GMS is highest, surveillance has infrequently identified *PfKelch13* mutations; to date, no C580Y has been detected. However, artesunate-sulfadoxine-pyrimethamine treatment failures have occurred in India because of a shift from double and triple *Pf dhfr* and *Pf dhps* mutants to quadruple and quintuple mutants, respectively. These parasites have also been observed in Somalia and Sudan, but are still rare in Afghanistan, the Islamic Republic of Iran, and Pakistan.

**Africa:** Non-synonymous *PfKelch13* mutations are still rare and highly diverse in Africa (16). The most frequent allele observed in Africa is A578S, which is not associated with clinical or in vitro artemisinin resistance. Those mutations that are known to be associated with artemisinin resistance have not expanded in African parasite populations.

Amodiaquine resistance and sulfadoxine-pyrimethamine resistance emerged in Africa before the introduction of ACTs. ACT efficacy across Africa remains generally high. Artemether-lumefantrine efficacy is >90%, although investigation into the possible presence of lumefantrine resistance in Angola continues. Artesunate-amodiaquine is used in areas where amodiaquine remains efficacious. The two remaining countries using artesunate-sulfadoxine-pyrimethamine (Somalia and Sudan) will cease treatment with this combination in the near future due to sulfadoxine-pyrimethamine resistance. For dihydroartemisinin-piperazine, increased *Pfplasmepsin 2-3* copy number has been detected infrequently, although these cases have yet to be confirmed. If confirmed, this indicates the potential for piperazine resistance to emerge and spread in Africa.

**South America:** In Guyana, a retrospective analysis of blood samples collected in 2010 for a *Pfhrp2* surveillance study detected five samples with *PfKelch13* C580Y. All five samples had nearly identical haplotypes, suggesting a common origin, distinct from the South-East Asian *PfKelch13* C580Y haplotype. In 2014, a 7-day trial of artesunate 4 mg/kg/day + primaquine ( $n = 50$ ) resulted in a 2% day-3 positivity rate and 100% efficacy; all samples were *PfKelch13* wild-type. A survey conducted in 2016 identified *PfKelch13* C580Y at a frequency of 3.3%, although this frequency was 11.9% in one sample area. As far as is known, ACT efficacy has not been affected, although there are no clinical data for the parasites identified with *PfKelch13* C580Y. Quality control and flanking microsatellite profiles are ongoing. This case may, therefore, represent an independent emergence and limited spread of a *PfKelch13* C580Y haplotype unrelated to that observed in South-East Asia. The proportion of parasites with increased *Pfplasmepsin 2-3* copy number in South America is currently unknown.

## Conclusion

Outside the GMS, there is little evidence of artemisinin resistance or the proliferation of *PfKelch13* mutant parasites, except perhaps in Guyana.

## Discussion

Although it is clear that the *PfKelch13* C580Y mutation detected in Guyana in 2010 is not an Asian parasite, it is not yet known whether the *PfKelch13* C580Y mutation detected in 2016 represents a single haplotype or whether this mutation arose several times independently on different background haplotypes.

Studies to further characterize the functional impact of *PfKelch13* mutations are ongoing. However, there are many different mutations to be tested. Gene-editing and transfection studies are challenging, although there is a new laboratory parasite that may be more amenable to transfection. Knockout studies may provide enough information to determine whether a mutation has functional relevance to artemisinin resistance.

If artemisinin resistance were to extend from the ring stage to other life cycle stages, it would be evident with increasing  $IC_{50}$  values using traditional in vitro assays. A trend of increasing artemisinin  $IC_{50}$  values has been observed in Cambodia, but with high variation and a large degree of overlap between resistant and sensitive parasites. A 7-day artemisinin trial can be used to investigate whether artemisinin resistance can cause delayed clearance and clinical failure. So far, there have only been four cases where artemisinin resistance has led directly to both.

## Drug development landscape (T. Wells)

In the GMS, it may be necessary to move to triple combination therapy in order to sustain efficacy in some regions. In Africa, the emergence of dihydroartemisinin-piperaquine treatment failure may be related to the poor stability of the drugs, as non GMP-certified agents are available in this region.

For new combination therapies (21).

- OZ439/ferroquine: There is a risk of cross-resistance between ferroquine and piperaquine/chloroquine/amodiaquine, although no strong correlation has been found in vitro. To date, resistance to OZ439 has not been induced in the laboratory. However, parasites harbouring a *PfKelch13* mutation exhibit reduced sensitivity to OZ439, although this is mitigated by a longer half-life than for dihydroartemisinin. This should result in the significantly improved efficacy of OZ439 against *PfKelch13* mutant parasites (22).
- KAF156/lumefantrine: This combination is being developed as a single-exposure cure and prophylaxis agent, and as a 3-day treatment for multidrug-resistant parasites. Phase II will be completed in 2018. This combination is susceptible to pre-existing lumefantrine resistance. KAF156 has a mild resistance risk evaluated in vitro.
- In addition, KAE609 (cipargamin), DSM265, MMV048 and SJ733 are in human studies.



Thus, it is possible to develop combinations with two novel drugs or even triple therapies. In the absence of partner drug resistance, treatment efficacy rates with existing ACTs remain high in most regions. Demonstrating the efficacy of new combinations requires larger studies with non-inferiority endpoints. Following this conventional path, the earliest registration for a new combination therapy is anticipated in 2022–2023. However, should drug-resistant malaria be prevalent enough to conduct clinical trials, conditional approval might be possible based on Phase IIb data, thereby accelerating registration. The current low number of clinical malaria cases with ACT resistance and their occurrence in countries not amenable to clinical research does not facilitate large-scale clinical trials. Therefore, it is important to preserve the efficacy of currently available agents and investigate novel deployment strategies.

### **Situation in Cambodia (D. Kwiatkowski)**

There are multiple origins of artemisinin resistance in Cambodia, but *PfKelch13* C580Y is spreading faster than other *PfKelch13* mutations. Although there are several haplotypes for *PfKelch13* C580Y, one origin accounts for more than half of all *PfKelch13* mutations in Cambodia. This dominant haplotype has spread to multiple locations and has recombined onto other genetic backgrounds. In western Cambodia, C580Y is approaching fixation.

*Pfplasmepsin 2-3* amplification has been observed mainly, but not exclusively, in parasites that have the *PfKelch13* C580Y mutation. *Pfplasmepsin 2-3* amplification is a strong marker for dihydroartemisinin-piperaquine treatment failure. In western Cambodia, the proportion of parasites with multiple *Pfplasmepsin 2-3* copy number increased from 0% in 2007 to around 75% in 2013. *Pfplasmepsin 2-3* copy numbers have also increased from double to triple to quadruple. In the same dataset, the proportion with *PfKelch13* mutations rose from 20% in 2007 to around 90% from 2008 to 2013. By contrast, the proportion of parasites with multiple *Pfmdr1* copy number decreased from around 55% in 2007 to 5% in 2013.

*Pfplasmepsin 2-3* amplifications in western, northern and north-eastern Cambodia have a single main origin, as indicated by the same haplotypes and breakpoints. However, across a wider area, multiple origins are probable and have been detected in other datasets from Cambodia. *Pfplasmepsin 2-3* amplifications are spreading across Cambodia and increasing in copy number over time. In northern Cambodia, parasites without *Pfplasmepsin 2-3* amplification have mainly north-Cambodian ancestry, whereas those with amplifications have mainly west-Cambodian ancestry. This indicates the invasion of resistant parasites that are replacing the indigenous piperaquine-susceptible parasites. Further studies are examining samples from Viet Nam. Although there may be additional resistance mechanisms, coincident *PfKelch13* C580Y and *Pfplasmepsin 2-3* amplification have been found to cause high rates of dihydroartemisinin-piperaquine treatment failure (>25%). This selective sweep is spreading very rapidly across Cambodia, most likely due to extensive drug pressure.

### **Independent emergence and spread of artemisinin resistance (C. Plowe)**

In the GMS, haplotype network analysis has indicated that different *PfKelch13* mutations have arisen independently on many different genetic backgrounds. Some mutations have independently emerged ('popped') and not spread, whereas others have emerged and spread ('jumped') between different sites. The *PfKelch13* C580Y mutation has both emerged independently multiple times and spread transnationally multiple times, including one instance where a lineage spread from the southern tip of the Myanmar peninsula to Cambodia, traversing the non-malarial region of Thailand (23). The *PfKelch13* F446I mutation has similarly spread over a large area along the China–Myanmar border and in northern and north-eastern Myanmar, areas where the *PfKelch13* C580Y mutation remains rare. These examples suggest that there have been multiple instances of different mutations spreading widely on different genetic backgrounds. This does not preclude the possibility that a single highly resistant parasite haplotype could become dominant throughout the GMS and spread outside the GMS, but to date the patterns of emergence and spread are complex and geographically variable.

There is evidence of other loci rising to high frequency in the parasite population, and these may have enabling or compensatory functions with regard to artemisinin resistance. Notably, a minority of parasites have an artemisinin-resistant phenotype (delayed parasite clearance), but no mutations in *PfKelch13*. Conversely, not all *PfKelch13* mutations confer the artemisinin-resistant phenotype, suggesting that additional genetic changes may be necessary to achieve resistance.

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