



Technical Expert Group on Drug Efficacy and Response

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Abbreviations

ACT	artemisinin-based combination therapy
AL	artemether-lumefantrine
AP	atovaquone-proguanil
AS-AQ	artesunate-amodiaquine
AS-MQ	artesunate-mefloquine
AS-PY	artesunate-pyronaridine
CYP2D6	cytochrome 2D6 activity
CYP2C19	cytochrome 2C19 activity
DER	Drug Efficacy and Response
DHA-PIP	dihydroartemisinin-piperaquine
GMP	Global Malaria Programme
GPARC	Global Plan for Artemisinin Resistance Containment
GTS	Global Technical Strategy for Malaria 2016–2030
G6PD	glucose-6-phosphate dehydrogenase
GMS	Greater Mekong Subregion
IC ₅₀ or 90	inhibitory concentration 50% or 90%
PCR	polymerase chain reaction
<i>Pfcr</i>	<i>P. falciparum</i> chloroquine resistance transporter
<i>Pfcy</i>	<i>P. falciparum</i> cytochrome b
<i>Pfdhfr</i>	<i>P. falciparum</i> dihydrofolate reductase
<i>Pfdhps</i>	<i>P. falciparum</i> dihydropteroate synthase
<i>Pfkelch 13</i>	<i>P. falciparum</i> kelch propeller domain on chromosome 13
<i>Pfmdr1</i>	<i>P. falciparum</i> multidrug resistance protein 1
PSA	piperaquine survival assay
RSA	ring-stage survival assay
SMC	seasonal malaria chemoprevention
SNP	single nucleotide polymorphism
TEG	Technical Expert Group
TES	therapeutic efficacy study

Summary and recommendations

Piperaquine resistance

There is sufficient evidence to confirm *Pfplasmepsin 2-3* increased copy number as a marker of piperaquine resistance in Asia. *Pfplasmepsin 2-3* increased copy number should be incorporated into surveillance and monitoring activities globally where piperaquine is being used or considered for use. Although other mutations/amplifications may be involved in piperaquine resistance, including novel *Pfcr* mutations, these require further research and validation before recommendations can be made.

Based on the proportion of clinical treatment failures determined in a therapeutic efficacy study (TES), no threshold for the prevalence of *Pfplasmepsin 2-3* increased copy number was recommended for treatment policy change. Nonetheless, the predictive value of *Pfplasmepsin 2-3* increased copy number prevalence with respect to clinical failure could be useful in informing the threshold at which a TES should be conducted. *Pfkelch 13* prevalence and a growing prevalence of *Pfplasmepsin 2-3* increased copy number should be considered in situations where a TES might not be feasible.

Piperaquine survival assay should be the standard in vitro assessment for piperaquine phenotype. However, IC₉₀ obtained from conventional in vitro drug sensitivity assays also represents a valid method.

Markers of reinfection and recrudescence for *P. falciparum*

With regard to the current guidance on *P. falciparum* genotyping in clinical trials:

- The use of capillary electrophoresis for *msp1*, *msp2*, and *glurp* assessment should be promoted.
- Both molecular markers *msp1* and *msp2* should be genotyped for all samples. If *msp1* and *msp2* yield congruent results, this result should be reported as the overall result of the genotyping. In the few cases where there is a discrepancy between the outcomes of markers *msp1* and *msp2*, a third marker should be genotyped. This marker could be *glurp* or another validated highly diverse gene. This marker will then automatically support one of the two previous results; the majority result will then be reported as the overall result for PCR correction.
- PCR of different allelic families of *msp1* and *msp2* should be performed in different tubes in order to avoid template competition.

In terms of assessing new techniques for distinguishing recrudescence from reinfection, it was agreed that data from barcoding and amplicon sequencing could be incorporated into the planned modelling studies, along with the current length polymorphism approach. This would allow for further evaluation of the relative merits of each laboratory technique. WHO will provide data from clinical studies. New algorithms for interpreting data will be compared for their best fit to simulated data. The Technical Expert Group (TEG) recommends that once the new analysis has been completed, the guidance on *P. falciparum* genotyping should be reviewed and revised if necessary.

***P. vivax* molecular markers**

There are no markers that can be used to differentiate between recrudescence, relapse, and reinfection, which makes it difficult to interpret primaquine efficacy and blood stage resistance studies.

There are no molecular markers of *P. vivax* resistance to chloroquine, mefloquine, or primaquine. Only markers of pyrimethamine and sulfadoxine resistance have been validated, although that treatment is not recommended for acute vivax malaria under almost all circumstances. Clinical trials of therapies for acute vivax malaria with robust therapeutic response phenotyping protocols are needed in order to inform the search for much-needed validated molecular markers of resistance.

Low/intermediate CYP2D6 activity has predictive value for recurrent *P. vivax* infections treated with effective blood schizontocides and primaquine. CYP2D6 genotyping should be included in primaquine clinical trials. The prevalence of impaired CYP2D6 varies widely among ethnic groups and may be quite common, e.g., in Southeast Asian populations the impaired *10 allele may occur in as much as 40–60% of the population. Surveys of the frequencies of impaired CYP2D6 alleles in populations exposed to risk of vivax malaria are needed.

Monitoring the efficacy of seasonal malaria chemoprevention (SMC)

An update on the TEG's previous recommendations regarding SMC is presented below.

Recommendation	Status
The ratio of malaria cases in children under 5 years versus children over 10 years	Yes
The occurrence of clinical malaria relative to the time of the previous SMC dose	Yes
The incidence of severe malaria at sentinel sites	Yes
Case–control sampling before each dose for microscopy	Case–control studies recruit clinical cases as they report to health facilities
Gametocytes	Possible, but not done
PCR relative to the time of previous SMC dose	Planned
Molecular markers of sulfadoxine-pyrimethamine and amodiaquine resistance pre-SMC	Yes, except for Senegal
Molecular markers of sulfadoxine-pyrimethamine and amodiaquine resistance post-SMC	Planned
Local capacity-building for the monitoring of molecular markers	Analyses are done in London using high throughput facility. Researchers from each country have been trained in the laboratory methods, and the project is building capacity for the design and monitoring of programmes, analysis, and interpretation
Standard membrane feeding assay	Yes

Complexity of infection from studies of parasite genetics	Possible, but not done
Changes in parasite diversity	Possible, but not done
Drug policy effects: the impact of SMC on first-line ACT diversity	AL is the first-line in areas where SMC is implemented

Data on the effect of SMC on molecular markers of resistance are not yet available, although baseline data indicate that parasites resistant to either sulfadoxine-pyrimethamine or amodiaquine exist at low frequencies in asymptomatic individuals. Data from Mali suggest that the risk of developing drug resistance is higher with sulfadoxine-pyrimethamine than with amodiaquine.

Strategy for antimalarial drug resistance management

The TEG agreed that it would be valuable to have a new strategy for antimalarial drug resistance management, and this should be developed and made available as soon as possible. The scope and components of the strategy presented were considered appropriate and should include the following:

- Scenario-planning, for instance in case of outbreaks of falciparum malaria in areas with multidrug resistance;
- Guidance on *P. vivax* resistance;
- New information and approaches since the GPARC;
- Distinct scenario-planning for different resistance situations;
- Consideration of all interventions using antimalarial drugs, their potential impact on resistance development, and actions that might mitigate this risk;
- Measures for containment across borders;
- Guidance on the management of suspected and confirmed treatment failures, including diagnostics and alternative treatments that can be used in remote or resource-poor areas.

An ideal format would include a generic section building on what is in the GPARC and what has been learned more recently, plus scenarios that can change over time as new evidence and tools become available.

Update on antimalarial drug efficacy and drug resistance

The TEG recommends that all putative *Pfkelch13* mutants conferring artemisinin resistance be independently verified as being associated with resistance both in genetic studies and in the RSA, ideally before publication claiming such association.

Planned activities (TES and survey) to investigate *Pfkelch13* C580Y in South America are sufficient. However, whole genome sequencing may be useful to examine backbone mutations. Ideally, resistant parasites should be collected for culture adaption.

The presence of multicopy *Pfplasmepsin 2-3* in Africa is a potential concern in terms of the use of dihydroartemisinin-piperaquine (DHA-PIP). However, additional information is required regarding the in vivo and ex vivo piperaquine-resistant phenotype in African parasites. Additional African data

are needed to assess the relationship between DHA-PIP treatment failures and molecular markers (*Pfkelch13*, *Pfplasmepsin 2-3*, and *Pfcrt*).

Triple therapies

Although TRAC 2 data are preliminary, the data support the testing of triple therapies as a potential strategy against multidrug-resistant *P. falciparum*. In areas where DHA-PIP is failing, the effectiveness of DHA-PIP+mefloquine treatment would rely to a large extent on the mefloquine component; as such, artemether-lumefantrine+amodiaquine may be the preferred triple therapy and should be tested in Cambodia and Viet Nam. The initial rationale of combining DHA-PIP with mefloquine assumed that the drugs have competing resistance mechanisms need to be reassessed in light of the data presented on parasites from Cambodia with both multicopy *Pfmdr1* and multicopy *Pfplasmepsin 2-3*.

Given the concern over QTc interval prolongation interval and the issues regarding the measurement of changes in QTc as malaria symptoms resolve, further analysis of QTc using alternative methods was requested.

An alternative treatment option for multidrug-resistant *P. falciparum* is to use two sequential artemisinin-based combination therapies. This approach should be tested in clinical trials.

Atovaquone-proguanil

In the GMS, there may be a role for atovaquone-proguanil (AP) in combination with an ACT. artesunate-mefloquine+AP and artesunate-pyronaridine+AP are two options for testing.

Further studies are required to validate mutations as a clinically relevant molecular marker of atovaquone resistance. There may be other mutations contributing to resistance besides the *Pfcytb* mutation at position 268.

Until there is stronger evidence that a *P. falciparum* *Pfcytb* Y268C/N/S mutant is not transmissible, it cannot be concluded that atovaquone resistance is not transmissible.

1 Welcome and introduction of guest speakers

The list of participants is provided in Annex 1. All Technical Expert Group (TEG) members attended the meeting. Welcome to new members David Fidock, Daouda Ndiaye, and Neena Valecha, and to the invited speakers Ingrid Felger, Paul Milligan, and Mariusz Wajnarski. The Bill & Melinda Gates Foundation, Department for International Development (DFID), and Medicines for Malaria Venture were invited as observers, and representatives attended the meeting. The Global Fund to Fight AIDS, Tuberculosis and Malaria and the United States Agency for International Development were invited as observers, but were unable to attend. The meeting agenda is provided in Annex 2.

Thanks were expressed on behalf of WHO to all TEG members past and present and to the sponsors, DFID.

The role of this TEG is to advise the Drug Efficacy and Response (DER) Unit at the Global Malaria Programme (GMP) on policy and recommendations regarding drug efficacy and response. Questions directed at the TEG from the DER Unit are listed in Annex 3.

2 Declarations of interest

TEG members participating in the meeting submitted declarations of interest, which were assessed by the DER Unit at GMP.

3 Minutes and action points of TEG 2015

The minutes of the 2015 TEG were accepted and can be found at:

<http://www.who.int/malaria/mpac/mpac-mar2016-teg-der-report-session3.pdf?ua=1>

4 Session 1. Molecular markers: genotyping and monitoring drug resistance

4.1 Molecular markers of piperaquine resistance

Presentations

Pfplasmepsin 2-3 copy number

In 2010, dihydroartemisinin-piperaquine (DHA-PIP) was adopted as the national first-line antimalarial therapy in Cambodia. However, *P. falciparum* piperaquine resistance spread rapidly and parasites resistant to both artemisinin and piperaquine began circulating. This resulted in high DHA-PIP treatment failure rates, leading to a shift in treatment policy in these areas to artesunate-mefloquine (AS-MQ) from 2014. Although mutations in the *Pfkelch 13* propeller region have been validated as molecular markers of artemisinin resistance, there is still no molecular marker of piperaquine resistance.

The piperaquine survival assay (PSA) was developed to address the limitations of the classical isotopic in vitro assay in characterizing piperaquine-resistant isolates. This assay is able to define a reliable in vitro phenotype and has been validated retrospectively and prospectively. In studies, all isolates collected from patients presenting a recrudescence within 42 days of follow-up had PSA survival rates $\geq 10\%$.

Based on findings from the PSA, next-generation sequencing was performed on eight piperazine-sensitive and 24 piperazine-resistant strains, all with the same *Pfkelch 13* C580Y allele. Strong signals of gene amplification were detected in two genes encoding hemoglobin-digesting proteases: *Pfplasmepsin 2* and *Pfplasmepsin 3*

Note that the *Pfmdr1* gene was amplified in 5/8 piperazine-sensitive lines, but in 0/23 piperazine-resistant lines. Recent epidemiological studies in Cambodia have shown that most piperazine-resistant isolates characterized by *Pfplasmepsin 2-3* gene amplification have a single *Pfmdr1* copy, while a small proportion (1%) have both *Pfplasmepsin 2-3* and *Pfmdr1* multiple copies.

Subsequent experiments demonstrated that the amplification of these genes leads to *Pfplasmepsin 2-3* mRNA overexpression, with at least 2-fold more protein in piperazine-resistant parasites than in piperazine-sensitive ones. In Cambodian isolates, a strong correlation was observed between *Pfplasmepsin 2-3* gene amplification (by qPCR) and PSA, with an increased *Pfplasmepsin 2-3* copy number predicting PSA survival rates $\geq 10\%$ with a sensitivity of 97% and specificity of 98%. Clinical data from 725 patients showed that the cumulative incidence of DHA-PIP treatment failure increased with the *Pfplasmepsin 2-3* copy number, with a hazard ratio for failure of 32.2 for single versus two copies, 49.0 for single versus $\geq$ three copies, and 1.5 for two versus $\geq$ three copies. Multivariate analysis indicated that *Pfplasmepsin 2-3* copy number (single versus multiple) was the most important determinant of DHA-PIP treatment failure (adjusted hazard ratio 20.4).

A review of 405 samples collected in Pailin and 324 in Rattanakiri between 2002 and 2015 showed an increase in *Pfplasmepsin 2-3* copy number over time, mirroring the increase in treatment failures observed over this period. Notably, in Pailin, about 2% of parasites exhibited increased *Pfplasmepsin 2-3* copy number in 2002, suggesting either that this increase may be a natural polymorphism or that piperazine-resistant parasites emerged after the massive use of DHA-PIP in the area between 2001 and 2003. Overall, data from areas of Cambodia where the *Pfkelch 13* mutation has a prevalence of $> 50\%$ indicate that the clinical efficacy of DHA-PIP at day 42 fell below 90% when the proportion of multicopy *Pfplasmepsin 2-3* parasites rose to 22% ($p < 0.0001$). Notably, multicopy *Pfplasmepsin 2-3* has no longer been detected in *Pfkelch 13* wild type parasites, contrary to data reported 5-10 years ago.

In conclusion, these findings indicate a strong association between multicopy *Pfplasmepsin 2-3* with in vitro resistance and DHA-PIP clinical failure rates. These findings have been confirmed in another set of studies conducted by a different group using another dataset and alternative approaches. In addition, multicopy *Pfplasmepsin 2-3* parasites have been reported in Viet Nam and Thailand, clustering in areas where both artemisinin resistance and piperazine resistance are common. There is also a biological rationale for *Pfplasmepsin 2-3* to be associated with resistance, as piperazine affects hemoglobin degradation and heme detoxification processes.

To fully validate *Pfplasmepsin 2-3* copy number as a molecular marker of piperazine resistance, genome edited *P. falciparum* with single/multicopy *Pfplasmepsin 2-3* would be an invaluable tool, even though editing *Pfplasmepsin 2-3* copy number is technically challenging. However, progress has been made in developing stable strains with knock down of *Pfplasmepsin 2-3* from a multicopy parasite.

Pfcr

Four mutations in *Pfcr* have been identified in piperazine-resistant parasites. All of these, namely H97Y, F145I, M343L, and G353V, are exclusively on the Dd2 background and are only found in Cambodia. They have all been detected at low abundance. Genome-wide association studies with *Pfcr* F145I found an association between these mutations and DHA-PIP treatment failure, after adjusting for the presence of amplified *Pfplasmepsin 2-3*. Introduction of the F145I mutation into Dd2 parasites also conferred piperazine resistance in vitro and impacted the potency of multiple other antimalarial medicines. Attempts to introduce F145I into the Cambodian artemisinin-resistant background Cam3.II (harbouring the *Pfkelch 13* C580Y mutation associated with artemisinin resistance) have been unsuccessful, perhaps because of the reduced transfection efficiency observed with this strain whose cell cycle is longer than the conventional 48 hours.

Gene editing experiments on the Dd2 background showed that an experimental *Pfcr* C101F mutation conferred piperazine resistance and sensitized *P. falciparum* to chloroquine, quinine, and amodiaquine but not to mefloquine or lumefantrine.

The introduction of M343L and G353V into Dd2 parasites produced a piperazine-resistant phenotype, as measured using the PSA. Dd2 parasites expressing the *Pfcr* G353V variant had an unusually large and translucent digestive vacuole. The removal of the *Pfcr* M343L or H97Y mutations from Cambodian piperazine-resistant isolates restored sensitivity to piperazine, as measured using the PSA. These mutations were also found to affect sensitivity to chloroquine, quinine, and amodiaquine, depending on the variant.

Discussion

PSA should be used if possible for in vitro phenotyping. It is difficult to get the kill curve to 0 in the isotopic assay, even when varying piperazine doses and durations, and parasitaemia. However, the area under the inhibitory concentration curve correlates well with PSA and can provide a more dynamic range.

There are two formats for the PSA: in vitro for laboratory samples (generally done after culture adaption and synchronization) and ex vivo for fresh samples. It is not known whether these two formats provide different results. With the ring-stage survival assay (RSA) used for assessing artemisinin resistance, for example, survival is higher in the ex vivo format.

The data supporting *Pfplasmepsin 2-3* copy number, as a molecular marker of piperazine resistance in the Greater Mekong Subregion (GMS) appear to be very strong. However, these data have been found almost exclusively in association with *Pfkelch 13* mutations, and there is no evidence that *Pfplasmepsin 2-3* copy number is a molecular marker of piperazine resistance outside the GMS. Although treatment policy decisions should be based on clinical data from therapeutic efficacy studies (TESs), it may be possible to define a threshold for the prevalence of *Pfplasmepsin 2-3* multicopy number at which a TES should be triggered.

Although rare, the existence of parasites with multiple copies of both *Pfplasmepsin 2-3* and *Pfmdr1* is worrying. In many areas of Cambodia, the prevalence of multicopy *Pfplasmepsin 2-3* parasites remains high. DHA-PIP has been removed only recently, so it may be too early to observe a decrease in the prevalence of multicopy *Pfplasmepsin 2-3* parasites in areas where DHA-PIP is no longer used. The propensity to lose multiple copy numbers in the absence of selective pressure might depend on

the parasite's genetic background. The dynamics of *Pfplasmepsin 2-3* de-amplification in the absence of drug pressure has not been established; for other *P. falciparum* genes, the de-amplification or silencing of copies tends to be quite a rapid process, as is the opposite process of re-expressing or re-amplifying once selective pressure is restored.

As with mefloquine in Cambodia and Thailand, in China, where piperaquine was used as a monotherapy, piperaquine resistance emerged in the absence of artemisinin resistance. It is not yet known whether piperaquine resistance can emerge in the absence of artemisinin resistance where DHA-PIP is used, for example in Africa. Since areas of low transmission and high DHA-PIP use are most vulnerable to the development of piperaquine resistance, monitoring in these areas of Africa would be valuable.

In Cambodia, patterns of *Pfcr* mutation may be very regional. It is possible that the *Pfcr* mutations listed above have been missed because there has been no analysis of the individual variants of Dd2 and examination of how they map individually to the PSA. The genome-wide association studies should be re-analysed and compared, taking into account the Dd2 variants.

Evidence suggests that parasites with *Pfcr* mutation are often less fit (potentially due to high levels of accumulated globin-derived peptides). Fitness might be restored in such parasites harbouring multiple copies of *Pfplasmepsin 2-3* (where hemoglobin processing might be accelerated). It is possible that these additional mutations drive high-level piperaquine resistance.

The *Pfcr* C350R mutation, identified by the Institut Pasteur de Cayenne in South America, has been shown to reverse chloroquine resistance, but in vitro has induced a significant decrease in piperaquine susceptibility. There is no evidence that this mutation is present in Asian parasite strains.

4.2 New evidence on *msp1*, *msp2*, and *glurp* as markers of reinfection and recrudescence

Presentation

In 2007, WHO published guidance on genotyping to identify parasite populations for clinical trials on antimalarial efficacy (Methods and techniques for clinical trials on antimalarial drug efficacy: genotyping to identify parasite populations. Geneva: World Health Organization, 2008). Recommendations were to compare *P. falciparum* parasite genotypes sequentially in pre- (day 0) and post-treatment samples (day X of treatment failure) using *msp1*, *msp2*, and *glurp* as markers of new infection vs. recrudescence. Given the experience gained by different groups and advances in laboratory techniques over the last 10 years, a reappraisal of the 2007 guidance may be required.

Issues that have been identified include poor quality of PCR execution and analysis (especially with respect to reading the agarose gels), PCR bias towards short fragments, template competition, and limitations in the use of the sequential decision algorithm for deciding on recrudescence or reinfection, particularly in high transmission areas where multiplicity of infections is high and many coinfection clones compete with each other during PCR amplification.

A plan for examining the validity of revised algorithms that differentiate between *P. falciparum* recrudescence and reinfection was presented, along with a comparison of different methods for

generating and interpreting results, and modelling studies and statistical methods to support a genotyping strategy that best fits the simulated data.

4.3 Reinfection/recrudescence: pros and cons of other methods (microsatellites, barcoding, and amplicon sequencing)

Presentation

There are a number of different markers and techniques that can be used to differentiate individual parasite infections. Depending on the research question, however, all have their advantages and limitations.

Single nucleotide polymorphism (SNP)-based genotyping leverages independent, neutral alleles with a high frequency of variation within or between populations. This approach may be useful in low transmission settings with evidence of single-clone infections; it may be more difficult to use in areas of higher transmission dominated by polygenomic infections, or in areas of very low transmission with highly inbred parasite populations in which gene variation is greatly reduced.

Microsatellites have the same limitations as *msp1*, *msp2*, and *glurp*. In addition, microsatellites suffer from stutter peaks (meaning loss of many minority clones) requiring a high cut off setting.

Amplicon sequencing requires that a reinfection exhibit a distinct amplicon haplotype that is not represented in the initially treated infection. The technique has greater discriminatory power if two amplicons are used rather than one. There is the possibility to multiplex PCRs of many samples and several molecular markers in order to investigate recrudescence versus reinfection and drug-resistance markers simultaneously. Since the PCR products are of a similar size, there is also less size-length polymorphism bias than with *msp1*, *msp2*, and *glurp* genotyping. Although this approach is likely to be highly cost-efficient, it is currently less deployable in most field settings, since the approach relies on deep sequencing strategies.

4.4 Barcoding to genotype *Plasmodium* in TES: experience in South-East Asia

Presentation

A 10-SNP barcode has been validated using DNA from reference strains. The barcode was applied to clinical data from Cambodian clinical trials, using *msp1*, *msp2*, and *glurp* genotyping in parallel, with good concordance between the two techniques. The results from the barcoding were easier to interpret in the low transmission area characterized by limited parasite diversity; this would not be the case, however, with polygenomic infections. Furthermore, in very low transmission settings with limited parasite types, it may be challenging to distinguish reinfection by the same parasite type. Compared to PCR gels, the 10-SNP barcode requires more sophisticated equipment and is more expensive.

Global discussion of the three presentations

Many laboratories are able to obtain consistent PCR results. However, high transmission settings present greater challenges, given the relatively higher levels of polygenomic infections. There should be continued efforts to build capacity and improve training and quality control for PCR in local laboratories. Blood samples should be sent to a centralized laboratory for quality control in order to improve quality.

Capillary electrophoresis was recommended in the 2007 WHO document. In many cases, this procedure can be outsourced to a site that has such facilities available. For example, in surveillance studies, PCR can be performed locally, with subsequent capillary electrophoresis conducted at a separate site. Local investigators can download the electronic capillary electrophoresis results as one file per sample for analysis, which also serves as a quality control method for the PCR.

Re-examination of the genotyping guidelines is warranted, and the plan to validate new approaches seems reasonable. Data from various sources will be used, comparing results obtained through the current length polymorphism approach (using *msp1*, *msp2*, and *glurp*) and, if possible, amplicon sequencing and barcoding. A pharmacokinetic/pharmacodynamic model will be used to simulate recrudescence and reinfection following drug treatment. The performance of the different methodologies and algorithms will be assessed in terms of their ability to distinguish between new infection and recrudescence in settings of different transmission intensity. Note that the model can incorporate the genetic structure of the local population, so background allele frequency estimates will be needed. Different laboratory techniques may be more appropriate given the setting and transmission level. The following points should be considered when developing new guidelines:

- In very low transmission settings, where only a few *P. falciparum* strains are circulating, it may be difficult to use molecular markers to distinguish recrudescence from reinfection, since reinfection can be with the same parasite strain or a closely related one. In these settings, methods with a higher resolution, such as genetic barcoding, are likely to perform better than conventional *msp1*, *msp2*, and *glurp* genotyping; however, these methods will also fail to distinguish recrudescence from reinfection when the reinfecting genotype is the same.
- The cost and complexity of each technique and whether it can be performed locally or centrally should also be considered. Quality control should always be centralized and should start from blood samples, not from extracted DNA.
- For any new laboratory procedures, there should be guidance on the workflow of samples and if/when it might be appropriate to centralize them. Local ownership of data and analysis should be ensured, even if the laboratory procedures are carried out elsewhere.
- The interpretation of molecular markers may be aided by understanding the background diversity of these markers at the study site. For routine surveillance in very low transmission settings, it might be appropriate to assume that a recurrent infection is a recrudescence without having to genotype the infecting strains; for research and TES studies that have potential impact on drug policies, however, the best available genotyping method should be used. These methods require proper validation and should be reproducible by other laboratories.
- Techniques and decision-making should be simple and clear-cut.

4.5 Update on *P. vivax* molecular markers

Presentation

The hypnozoite dictates *P. vivax* malaria epidemiology, diagnosis, treatment, prevention, control, and elimination. However, it is not yet possible to detect hypnozoites, and there is little

understanding of the potential size of the hypnozoite reservoir. Available studies suggest that over 80% of acute attacks derive from relapses (Thai and Papua New Guinea cohort studies). Relapse latency varies between 17 days and 5 years, and the risk, timing, and number of relapses varies both geographically and temporally. Relapse latency is a key consideration when assessing the effectiveness of anti-hypnozoite drugs, as a long latency period and short follow-up will fail to detect relapses. Studies indicate that treatment failures occur mostly within 6 months and more rarely within 8 months with rapid-relapsing tropical Asian strains. Temperate strains almost certainly require at least 8 months follow-up and ideally 12 months.

The radical cure of *P. vivax* requires blood schizontocidal therapy plus primaquine. *P. vivax* resistance to chloroquine has been confirmed in many sites throughout the world, although data are relatively sparse. A recurrence before day 17 is likely to be recrudescence; between days 17 and 28, it could be recrudescence, relapse, or reinfection. Chloroquine resistance is defined as any recurrence with chloroquine+desethylchloroquine whole blood concentrations > 100 ng/ml at the time of recurrence. Primaquine treatment before day 28 creates a bias in the in vivo test, as primaquine also has blood schizonticidal activity. There is no validated genetic marker of chloroquine resistance in *P. vivax*.

There is no information on parasite resistance to primaquine, as recrudescence, relapse, and reinfection cannot be differentiated genetically. Determining whether the recurrence is homologous or heterologous is irrelevant for assessing primaquine anti-hypnozoite efficacy. Consequently, there is no primaquine-resistant phenotype for *P. vivax*, either clinically or in animal models, and molecular markers of *P. vivax* primaquine resistance cannot currently be validated.

There is some evidence that there are two distinct populations of sporozoites, destined to either progress through the life cycle (tachysporozoites) or enter dormancy as hypnozoites (bradysporozoites). The ratio of these populations appears to be strain-specific. It is not yet known whether the fate of each population is a genetic or epigenetic trait; if genetic, there might be the possibility of using a genetic marker to distinguish relapse from reinfection or recrudescence.

In order to assess primaquine efficacy, high-dose primaquine must be used (30 mg x 14 days), with supervised adherence and no exposure to reinfection. Follow-up must be at least 4 months, preferably 12 months. Ideally, a rapidly eliminated schizonticide medicine should be used (quinine or artesunate). Moreover, failure to relapse may be caused by a lack of hypnozoites. Although such studies are challenging to conduct, available data from infections acquired in Indonesian Papua indicate that primaquine anti-hypnozoite efficacy is about 85% after 1 year of follow-up.

However, primaquine resistance may not be the cause of the 15% treatment failure rate. Importantly, primaquine is a prodrug, and its efficacy apparently depends on cytochrome 2D6 activity (CYP2D6) for conversion to the active metabolites. Poor/intermediate CYP2D6 metabolizers are at significantly greater risk of relapse compared to normal metabolizers. Thus, there appears to be a population of patients who will be naturally less responsive to primaquine. As such, primaquine resistance can only be concluded if therapeutic levels of the active metabolites are achieved. As currently those metabolites cannot be readily measured, CYP2D6 genotyping provides important clues in the form of predicted metabolic activity levels using the AS-Model scoring system.

Discussion

Trials of anti-hypnozoite therapy must include a relapse control arm with only effective blood schizontocidal activity in order to identify the natural relapse behaviour of local strains. In areas of chloroquine resistance, chloroquine alone should not be used, as chloroquine-resistant *P. vivax* could confound the results.

Chloroquine+primaquine will have greater blood schizontocidal efficacy than chloroquine alone. Therefore, assessing chloroquine efficacy without primaquine therapy is essential for identifying chloroquine resistance as a clinical and public health problem. Although chloroquine+primaquine is almost universally recommended, in practice, the inclusion of primaquine carries the risk of intravascular hemolysis due to glucose-6-phosphate dehydrogenase (G6PD) deficiency; consequently, primaquine is often not applied.

It may be important to understand that patients in endemic areas may experience relapse infections due to hypnozoites from one or more prior infections. In other words, it is possible to have relapses at any time from hypnozoites, so it cannot be assumed that failure before day 17 is a recrudescence and chloroquine failure. Nonetheless, any persistent or rapidly reappearing parasitaemia (< 17 days) that is overcoming relatively high levels of chloroquine in the blood is certainly resistant to the drug, regardless of the parasite source (i.e., from hypnozoites or recrudescence infections).

The evidence for tachysporozoite and bradysporozoite phenotypes comes from human infection studies conducted in the 1940s/50s. In the studies, a North Korean late relapse strain with no primary infection was thought to have a dominant population of bradysporozoites, unlike the rapidly relapsing tropical Chesson strain that had a dominance of tachysporozoites. In addition, Thai strains distinguished by circumsporozoite markers consistently produced fixed proportions of hypnozoites versus tissue schizonts. There are various explanations for these findings, including potential interactions with the hepatocyte and genetic/epigenetic differences. It is not clear whether the predisposition exists at the sporozoite stage (genetically programmed) or after entering the hepatocyte (epigenetic commitment phenomena). Gametocyte production is epigenetically regulated and commits a consistent proportion of sporozoites to sexual differentiation; this may also be the case for hypnozoites.

At present, strains with the potential for primaquine resistance are not under significant selection pressure, as primaquine is likely not being applied at sufficient levels to sustain the selection pressure to drive resistance. Chloroquine resistance has been identified at many sites, including the Indonesian archipelago and malarious Western Pacific, where high rates of resistance have necessitated the adoption of an artemisinin-based combination therapy (ACT) as first-line *P. vivax* therapy. Elsewhere, however, chloroquine remains an affordable and convenient treatment option and should not be abandoned without firm evidence that treatment failure is occurring at rates exceeding 10% of acute attacks. However, in the future, a single treatment protocol for *P. falciparum* and *P. vivax* may be appropriate, including a presumptive radical cure with primaquine where these two species are sympatric (i.e., almost everywhere). Such a practice would require great improvements in the safety of primaquine hypnozoitocidal therapy, e.g., with robust G6PD deficiency screening or close clinical monitoring. Primaquine is too dangerous a drug to apply broadly without the necessary measures in place to prevent serious harm, including possible loss of life.

4.6 Recommendations: Session 1

Piperaquine resistance

There is sufficient evidence to confirm *Pfplasmepsin 2-3* increased copy number as a marker of piperaquine resistance in Asia. *Pfplasmepsin 2-3* increased copy number should be incorporated into surveillance and monitoring activities globally where piperaquine is being used or considered for use. Although other mutations/amplifications may be involved in piperaquine resistance, including novel *Pfcr* mutations, these require further research and validation before recommendations can be made.

Based on the proportion of clinical treatment failures determined in a TES, no threshold for the prevalence of *Pfplasmepsin 2-3* increased copy number was recommended for treatment policy change. Nonetheless, the predictive value of *Pfplasmepsin 2-3* increased copy number prevalence with respect to clinical failure could be useful in informing the threshold at which a TES should be conducted. *Pfkelch 13* prevalence and a growing prevalence of *Pfplasmepsin 2-3* increased copy number should be considered in situations where a TES might not be feasible.

PSA should be the standard in vitro assessment for piperaquine phenotype. However, IC₉₀ obtained from conventional in vitro drug sensitivity assays also represents a valid method.

Markers of reinfection and recrudescence for *P. falciparum*

With regard to the current guidance on *P. falciparum* genotyping in clinical trials:

- The use of capillary electrophoresis for *msp1*, *msp2*, and *glurp* assessment should be promoted.
- Both molecular markers *msp1* and *msp2* should be genotyped for all samples. If *msp1* and *msp2* yield congruent results, this result should be reported as the overall result of the genotyping. In the few cases where there is a discrepancy between the outcomes of markers *msp1* and *msp2*, a third marker should be genotyped. This marker could be *glurp* or another validated highly diverse gene. This marker will then automatically support one of the two previous results; the majority result will then be reported as the overall result for PCR correction.
- PCR of different allelic families of *msp1* and *msp2* should be performed in different tubes in order to avoid template competition.

In terms of assessing new techniques for distinguishing recrudescence from reinfection, it was agreed that data from barcoding and amplicon sequencing could be incorporated into the planned modelling studies, along with the current length polymorphism approach. This would allow for further evaluation of the relative merits of each laboratory technique. WHO will provide data from clinical studies. New algorithms for interpreting data will be compared for their best fit to simulated data. The TEG recommends that once the new analysis has been completed, the guidance on *P. falciparum* genotyping should be reviewed and revised if necessary.

***P. vivax* molecular markers**

There are no markers that can be used to differentiate between recrudescence, relapse, and reinfection, which makes it difficult to interpret primaquine efficacy and blood stage resistance studies.

There are no molecular markers of *P. vivax* resistance to chloroquine, mefloquine, or primaquine. Only markers of pyrimethamine and sulfadoxine resistance have been validated, although that treatment is not recommended for acute vivax malaria under almost all circumstances. Clinical trials of therapies for acute vivax malaria with robust therapeutic response phenotyping protocols are needed in order to inform the search for much-needed validated molecular markers of resistance.

Low/intermediate CYP2D6 activity has predictive value for recurrent *P. vivax* infections treated with effective blood schizontocides and primaquine. CYP2D6 genotyping should be included in primaquine clinical trials. The prevalence of impaired CYP2D6 varies widely among ethnic groups and may be quite common, e.g., in Southeast Asian populations the impaired *10 allele may occur in as much as 40–60% of the population. Surveys of the frequencies of impaired CYP2D6 alleles in populations exposed to risk of vivax malaria are needed.

5 Session 2. Monitoring the prophylactic effect of preventive treatment

5.1 Monitoring efficacy of seasonal malaria chemoprevention in the ACCESS-SMC project

Presentation

Seasonal malaria chemoprevention (SMC) was scaled up in 2015 and 2016, having been recommended by WHO in 2012. A total of about 26 million monthly treatments with sulfadoxine-pyrimethamine plus amodiaquine were administered to about 6.7 million children in 2015, and about 60 million treatments (15 million children) were administered in 2016. In 2016, 12 countries (Burkina Faso, Cameroon, Chad, Gambia, Ghana, Guinea, Guinea-Bissau, Mali, Niger, Nigeria, Senegal, and Togo) had SMC programmes. Funding has been secured to maintain similar coverage in 2017. In 2015 and 2016, about half of the treatments were delivered through the UNITAID-funded ACCESS-SMC project (3.8 million children in 2015 with 14.5 million treatments) and 8 million in 2016 with 30 million treatments) in seven countries (Burkina Faso, Chad, Gambia, Guinea, Mali, Niger, and Nigeria). Children < 5 years have been included, except in Senegal and parts of Mali where treatment is for children under 10. In parts of northern Mali, SMC is provided for all ages.

Nearly 100 million SMC treatments have been delivered since 2012. SMC has been deployed most extensively in Mali (33 million treatments), Burkina Faso (15 million), Niger (12.5 million), and Nigeria (12.2 million). In most countries, in ACCESS-SMC implementation areas between 2015 and 2016, more than 80% of children have received SMC. Overall coverage with at least three monthly treatment cycles in ACCESS-SMC areas has reached 73%.

The London School of Tropical Medicine and Hygiene is working with research groups in Burkina Faso, Chad, Gambia, Guinea, Mali, Niger, Nigeria, and Senegal to measure SMC coverage and quality of delivery, assess the impact of SMC on malaria, support pharmacovigilance, monitor the efficacy of

SMC drugs, and evaluate the prevalence of markers of sulphadoxine-pyrimethamine and amodiaquine resistance. The baseline data on molecular markers of resistance in Burkina Faso, Chad, Gambia, Guinea, Mali, Niger, and Nigeria were presented.

The impact of SMC can be seen in comparison to control districts with no SMC, for example a 45% reduction in cases in children under 5 in Burkina Faso. The impact can also be seen with a reduction in malaria cases in children under 5 versus children over 10. Malaria mortality in children under 5 has also been reduced relative to pre-SMC data and relative to children over 10. In Senegal, a reduction in the number of malaria deaths in hospital in children under 10 was observed following introduction of SMC for that age group in the southern regions of the country.

In 2015, baseline community surveys to monitor drug-resistance markers were conducted in areas that were yet to start SMC (with the exception of Gambia, which started SMC in 2014) in children under 5 and those aged 10–30 years. A total of 2000 samples were collected in each group in each area, with a total target sample size of 28 000. Markers were *Pfcr*t (CVMNK, CVIET, and SVMNT), *Pfmdr*1 (86, 184, and 1246), *Pfdhfr* (51, 59, and 108), and *Pfdhps* (431, 436, 437, 540, 581, and 613). Of the 21 024 samples tested, 3448 (16.4%) were *P. falciparum* positive and 2324 have been genotyped so far.

The prevalence of *Pfcr*t and *Pfmdr*1 markers reflects the drug combinations most used for first-line malaria treatment in recent years. *Pfcr*t CVIET was most prevalent in Chad (54%) and Gambia (53%), and at very low prevalence in Nigeria (5%).

Amodiaquine resistance was defined as *Pfcr*t CVIET plus *Pfmdr*1 86Y+184Y. *Pfmdr*1 YY haplotypes were uncommon, seen in only five samples (one in Burkina Faso, four in Niger). One of the samples from Niger had *Pfcr*t CVIET and *Pfmdr*1 YY.

Sulfadoxine-pyrimethamine resistance was defined as *Pfdhfr* 51+59+108 plus *Pfdhps* 437+540. Eight samples (0.33%) – seven from Guinea and one from Niger – carried *Pfdhfr* triple and *Pfdhps* double mutations. None of these samples carried *Pfmdr*1 YY. Molecular marker data from Senegal were not presented.

In conclusion, mutations associated with sulfadoxine-pyrimethamine- and amodiaquine-resistant phenotypes were at low prevalence in the study areas before the roll-out of the SMC intervention. Further analyses will examine samples from malaria cases in relation to the period of time since SMC. Surveys will be repeated after the 2017 transmission season with complete results in 2018. There is a need for a longer term plan to monitor resistance.

Discussion

The data represent an excellent baseline set, and the TEG would be grateful to see post-SMC data when they become available. The only trends observed so far have been from Mali, where molecular markers of sulfadoxine-pyrimethamine resistance increased after SMC. For example, the *Pfdhfr*-*Pfdhps* quintuple mutant genotype increased from 1.6% to 7.1% ($p = 0.02$). However, the prevalence of *Pfmdr*1 86Y decreased from 26.7% to 15.3% ($p = 0.04$), with no change for *Pfcr*t K76T.

The presence of *Pfdhfr*-*Pfdhps* quintuple mutants, even at low prevalence, is a concern, as it might reflect other selection pressures on sulfadoxine-pyrimethamine, for example from intermittent preventive treatment during pregnancy; in this case, looking for markers in pregnant women would be valuable. Note that artemether-lumefantrine (AL) is being used as first-line malaria treatment in

the SMC countries and thus may be deterring the development of amodiaquine resistance. Given that artesunate-amodiaquine (AS-AQ) is difficult to buy in the SMC areas, there is probably little use of amodiaquine other than for SMC. However, there is nothing to deter the development of resistance to sulfadoxine-pyrimethamine.

Increased prevalence of resistance markers alone will not trigger policy change; this will depend on SMC efficacy determined through case–control studies.

It is not clear whether the prevalence of resistance markers will be the same in asymptomatic cases as in malaria patients. However, there are some surveys being conducted in patients presenting to health centres, and rapid diagnostic test cassettes have been retained from health facilities near survey sites. Resistance markers from these sources should be compared with those from asymptomatic infections. Although drugs for SMC are being used for their preventive effect, they must also have efficacy to clear parasites from asymptomatic individuals.

Membrane-feeding studies to assess impact on transmission are being performed in Burkina Faso, but results are not yet available. With only children under 5 included in SMC, there will be no effect on transmission. However, where children up to 10 are included, transmission may be reduced. In many areas with SMC programmes, there is a substantial burden of severe disease and malaria mortality in older children. Accordingly, there is a rationale for extending SMC to older children in these areas. At present, however, the goal of SMC is to reduce clinical cases and infant mortality.

5.2 Recommendations: Session 2

Monitoring the efficacy of SMC

An update on the TEG's previous recommendations regarding SMC is presented below.

Recommendation	Status
The ratio of malaria cases in children under 5 years versus children over 10 years	Yes
The occurrence of clinical malaria relative to the time of the previous SMC dose	Yes
The incidence of severe malaria at sentinel sites	Yes
Case–control sampling before each dose for microscopy	Case–control studies recruit clinical cases as they report to health facilities
Gametocytes	Possible, but not done
PCR relative to the time of previous SMC dose	Planned
Molecular markers of sulfadoxine-pyrimethamine and amodiaquine resistance pre-SMC	Yes, except for Senegal
Molecular markers of sulfadoxine-pyrimethamine and amodiaquine resistance post-SMC	Planned

Local capacity-building for the monitoring of molecular markers	Analyses are done in London using high throughput facility. Researchers from each country have been trained in the laboratory methods, and the project is building capacity for the design and monitoring of programmes, analysis, and interpretation
Standard membrane feeding assay	Yes
Complexity of infection from studies of parasite genetics	Possible, but not done
Changes in parasite diversity	Possible, but not done
Drug policy effects: the impact of SMC on first-line ACT diversity	AL is the first-line in areas where SMC is implemented

Data on the effect of SMC on molecular markers of resistance are not yet available, although baseline data indicate that parasites resistant to either sulfadoxine-pyrimethamine or amodiaquine exist at low frequencies in asymptomatic individuals. Data from Mali suggest that the risk of developing drug resistance is higher with sulfadoxine-pyrimethamine than with amodiaquine.

6 Session 3. Prevention and treatment of multidrug-resistant malaria

6.1 Outline of a strategy for antimalarial drug resistance management

Presentation

The purpose of the presentation was to discuss the potential development of a new strategy for antimalarial drug resistance management.

Recent WHO guidance on how to manage and respond to antimalarial drug resistance has mainly focused on the threat of artemisinin resistance. This includes guidance given in the *Global plan for artemisinin resistance containment* (GPARC), released in 2011, and the *Emergency response to artemisinin resistance in the Greater Mekong Subregion, regional framework for action 2013–2015*, released in 2013.

The *Global technical strategy for Malaria 2016–2030* (GTS), adopted at the World Health Assembly in 2015, highlights the potential of antimalarial drug resistance to seriously weaken the effectiveness of malaria responses and to erode the gains achieved. Therefore, the GTS recommends the monitoring and management of antimalarial drug resistance.

Since the development of the GPARC, the understanding of artemisinin resistance has improved, and the growing impact of resistance to ACT partner drugs is being recognized. Furthermore, while the GPARC focused on the risk of spread of resistance, resistance to antimalarial medicines can emerge independently in different locations. In December 2016, a WHO Expert Review Group concluded that there is "a significant risk of artemisinin and partner drug resistance outside the GMS – either via spontaneous emergence or importation, and spread". In part because of these developments, countries have been requesting concrete guidance on the management of antimalarial drug resistance.

The proposed new strategy for antimalarial resistance management aims to protect the efficacy of all malaria treatments. The key target audience is Ministries of Health and partners mainly working at the country level. The presentation outlined the suggested components of the strategy and placed added emphasis on the planning and implementation of activities at the country level.

GMP will develop the strategy with potential support from the TEG. Feedback from countries will be sought at regional and subregional meetings, with an online consultation for additional feedback from a range of stakeholders.

Discussion

The TEG agreed that a new strategy for antimalarial drug resistance management would be valuable. Scenario-planning for different eventualities was thought to be particularly useful, potentially with some regional adaptations. While there is already a strategy for malaria elimination for the GMS, additional technical recommendations focusing on problems related to antimalarial drug resistance are needed, for instance recommendations on how to detect and manage treatment failures. Planned components laid out in the new strategy should be detailed enough for programmes to verify what additional activities might be needed in order to avert or manage resistance. It would be useful if the scenario-planning component could be in a format that is easy to update.

The TEG thought the following issues should be included in the strategy:

- Scenario-planning, for instance in case of outbreaks of *P. falciparum* malaria in areas with multidrug resistance;
- *P. vivax* resistance to antimalarial drugs;
- The factors contributing to the emergence and spread of resistance;
- New information and approaches since the GPARC and the rationale for changes since the GPARC was published;
- The potential impact on the risk of resistance following changes in transmission and immunity.

In addition, while resistance may emerge independently, the spread of resistance remains a major threat. In light of this, population movements and border areas require special attention, and regional (rather than country-level) strategies may be required. The strategy should also outline the current landscape of use of antimalarial medicines, not just for treatment. All interventions using antimalarial drugs should be considered, as should the potential impact on resistance development and actions that might mitigate this risk. The risk–benefit of interventions using antimalarial drugs should be taken into account, since any use of drugs can carry a risk of resistance. However, this risk does not mean that the intervention should be discouraged. Ensuring effective quality control of drugs is a key factor in resistance management, and the strategy should include practical steps to monitor and improve quality control. Finally, guidance on the management of suspected and confirmed treatment failures should be provided, including diagnostics and alternative treatments that can be used in remote or resource-poor areas.

6.2 Update on antimalarial drug efficacy and drug resistance

Presentation

All ACTs are vulnerable to artemisinin resistance, though even in areas with a high prevalence of *Pfkelch13* mutations, ACTs retain treatment efficacy in the absence of resistance to the partner drug. In the event of partner drug resistance, treatment failure rates increase and, unless containment measures are in place, resistance will spread. However, containment can work. For example, malaria elimination efforts at the Thai–Myanmar border have resulted in a large decrease in malaria cases, reducing the potential for spread to other regions.

In Cambodia, DHA-PIP failure rates at day 42 are < 5% for parasites that have wild type for piperazine resistance markers with regardless presence of *Pfkelch13* wild type or *Pfkelch13* mutant (mainly C580Y). However, failure rates increase to around 20% for parasites with *Pfkelch13* wild type or multicopy *Pfplasmepsin 2-3*, and to 45% for those harbouring both markers.

In 2015–2016, a TES was conducted at five sites in Cambodia. At three of the sites, parasites were found that had both multicopy *Pfmdr1* and multicopy *Pfplasmepsin 2-3* at frequencies of 5.8% (Siem Reap), 4.2% (Pursat), and 2.0% (Kampong Speu). These frequencies are worrying as *Pfmdr1* increased copy number had virtually disappeared in Cambodia. Therefore, the appearance of the double mutant with markers of both piperazine and mefloquine resistance is concerning. It was hypothesized earlier that the two drugs might have competing resistance mechanisms that allowed DHA-PIP to drive multicopy *Pfmdr1* out of the population and restore sensitivity to mefloquine in the region after it had been failing.

Four ACTs have already failed in Cambodia, and if AS-MQ efficacy is lost, there are limited alternative options available. Studies are underway with artesunate-pyronaridine (AS-PY) in Eastern Cambodia, but previous data showed high failure rates in Western Cambodia with subsequent failure on DHA-PIP retreatment. These failures remain unexplained, as there is no cross-resistance between piperazine and mefloquine with pyronaridine.

In Africa, *Pfkelch13* mutations are rare and have not been associated with artemisinin resistance. In 2013, *Pfkelch13* M579I was isolated from a Chinese worker who had malaria 8 weeks after returning from Equatorial Guinea; however, the circumstances of the case and the parasite origin are not well documented.

Partner drug resistance is an emerging issue in Africa, with treatment failure rates increasing for AL. Treatment failures have also been observed in travellers. Cases are not well documented for resistance, and in some instances antimalarial drug levels may have been sub-therapeutic. However, treatment failure in travellers should be considered a red flag for reduced sensitivity. As in the GMS, AL treatment failures in Africa are not associated with *Pfmdr1* increased copy number.

Multicopy *Pfplasmepsin 2-3* was detected at a rate of 8.7% in the Comoros in 2013 and 5.3% in Mozambique in 2016.

In South America, *Pfkelch13* C580Y mutants were detected in Guyana in 2010. These mutants had a distinct microsatellite profile, indicating an origin independent of those observed in South-East Asia. While a TES in 2014 found no *Pfkelch13* mutants, a 2016 survey (n = 691) confirmed the presence of *Pfkelch13* C580Y in two neighbouring regions in the north-west of the country at a prevalence of 9.4% (9/96) in region 1 Barima-Waini, 0.6% (3/477) in region 7 Cuyuni-Mazaruni, and 1.9% (13/691)

overall. The prevalence of *Pfkelch13* mutants appears to be seasonal, as they were detected between April and September, with a peak in June. TES is planned in region 1 and/or 7 and in the capital city, Georgetown. The survey will continue in 2017.

In conclusion, the data reaffirm the need for an urgent and continued intensive regional malaria elimination campaign in the GMS both to prevent a resurgence of malaria caused by multidrug-resistant parasites and to contain these parasites, thereby preventing their spread to other areas. Surveillance for artemisinin and partner drug resistance needs to be continued and strengthened in the GMS. Outside the GMS, surveillance is needed to detect potential de novo resistance or introduction of resistant parasites. Where surveillance signals a potential threat to leading ACTs, effective alternative ACTs should be identified and implemented before resistance reaches critical levels.

Discussion

The report of the *Pfkelch13* M579I parasite isolated from a Chinese worker who had returned from Equatorial Guinea provides insufficient information to conclude that this parasite was acquired in Africa or was of African origin, or that this mutation confers artemisinin resistance, as the RSA value is marginal. The TEG recommends that all putative *Pfkelch13* artemisinin-resistant mutants be independently verified both for genetics and in the RSA, ideally before publication. It is reassuring that, despite two surveys, no further *Pfkelch13* mutants have been detected in Equatorial Guinea. Thus, there is no evidence that an artemisinin-resistant *Pfkelch13* mutant parasite population has become established or spread in the country.

The relevance of multicopy *Pfplasmepsin 2-3* to piperazine resistance in African strains that lack mutant *Pfkelch13* is unknown. Therefore, it should not be concluded that DHA-PIP would fail in areas where these parasites have been detected. If multicopy *Pfplasmepsin 2-3* is a natural polymorphism, as suspected, then there will be a small background prevalence of these parasites.

The presence of multicopy *Pfplasmepsin 2-3* in Africa is a potential concern in terms of the use of DHA-PIP. Drug pressure from DHA-PIP (through its use in mass drug administration and via the private sector) could further select multicopy *Pfplasmepsin 2-3*. However, additional information is required as to the in vivo and ex vivo piperazine-resistant phenotype in African parasites. Data are needed on the relationship between DHA-PIP treatment failures and molecular markers (*Pfkelch13*, *Pfplasmepsin 2-3*, and *Pfcrt*). Such data may have already been collected in various clinical trials of DHA-PIP in Africa, but will need to be examined.

Policy change should be based on therapeutic efficacy. However, a high prevalence of multicopy *Pfplasmepsin 2-3* or a trend of increasing prevalence should trigger further investigation.

Given the diversity of *Pfkelch13* mutations in Cambodia conferring artemisinin resistance, it is interesting that *Pfkelch13* C580Y is the only *Pfkelch13* mutation that has been detected in South America. In Cambodian parasites, *Pfkelch13* C580Y is the most fit *Pfkelch13* mutation. It is possible that this is the only mutation with great enough fitness to survive in the South American setting. The findings could also be the result of a homogeneous population structure with few different circulating parasites. However, the situation in Cambodia today may not be so relevant to the South American setting, as *Pfkelch13* mutations have been evolving in South-East Asia for 15 years.

The malaria situation in Venezuela has gained recent attention, as cases have soared following the breakdown of malaria control and health care provision. The regions of Guyana in which *Pfkelch13* C580Y has been detected border Venezuela. Although microsatellites have been examined, whole genome sequencing may be useful to examine backbone mutations. Ideally, resistant parasites should be captured for culture adaption, but this presents logistical challenges. There are also plans to obtain *P. falciparum* and *P. vivax* samples from a regional hospital in a mining area of Venezuela.

6.3 Update on TRAC 2: preliminary results of triple therapies

Presentation

The TRAC 2 study is evaluating triple therapies for uncomplicated *P. falciparum* malaria. The study aims to enroll 1800 subjects at 15 sites in eight countries, mostly in South-East Asia and India, but with one site in the Democratic Republic of Congo. Treatment comparisons are DHA-PIP versus DHA-PIP+mefloquine, AL versus AL+amodiaquine, and AS-MQ versus DHA-PIP+mefloquine, depending on the malaria treatment guidelines in the country. So far, 294 patients have been enrolled. The study will be completed in mid-2018.

Preliminary data indicate high failure rates for DHA-PIP in Cambodia, Viet Nam, and Thailand, but 100% efficacy with DHA-PIP+mefloquine. In Cambodia, the control arm was switched to AS-MQ after the start of the study, in line with the malaria treatment policy, with an efficacy rate of 100%. In Myanmar, efficacy was found to be 100% with both DHA-PIP and DHA-PIP+mefloquine. Data with AL have shown a few recurrent infections, but so far there have been no recurrent infections in the AL+amodiaquine arm in South-East Asia or India. In Democratic Republic of Congo, recurrent infections with both AL and AL+amodiaquine appear to be high, but these are likely reinfections; PCR correction has not yet been done.

Although data remain limited, all triple therapies have generally been well tolerated with no concerning laboratory parameters. No further prolongation of QTc interval has been observed with DHA-PIP+mefloquine compared to DHA-PIP without mefloquine. A slight QTc prolongation has been found with amodiaquine added to AL, although this is attributable to amodiaquine rather than to an interaction between the drugs.

Discussion

In areas where DHA-PIP is failing, the effectiveness of DHA-PIP+mefloquine treatment would rely to a large extent on the mefloquine component; as such, AL+amodiaquine may be the preferred triple therapy. Also, the initial rationale of combining DHA-PIP with mefloquine assumed that the drugs have competing resistance mechanisms. In light of the data presented at the TEG on parasites from Cambodia with both multicopy *Pfmdr1* and multicopy *Pfplasmepsin 2-3*, this rationale might need to be reassessed.

AS-AQ have limited efficacy in Cambodia. However, lumefantrine and amodiaquine have opposing resistance mechanisms, and this combination should now be tested in Cambodia and Viet Nam – countries with high DHA-PPQ failure rates.

Mefloquine has been found to increase the risk of QTc interval prolongation when associated with halofantrine and quinine. However, this does not seem to be the case for mefloquine and

piperaquine. QTc prolongation with DHA-PIP+mefloquine has been evaluated in healthy volunteers and is now being assessed in patients, with reassuring preliminary results.

Multidrug-resistant *P. falciparum* threatens to undermine all current treatment options in Cambodia. If triple therapies were to be introduced into treatment protocols, the components would need to be co-packaged. For the potential introduction of triple therapies, not only must the necessary data be obtained, but also appropriate product presentation must be developed and manufacturing and distribution logistics put in place; all aspects need to be worked on simultaneously.

Ideally, alternative approaches using currently approved drugs should be tested along with triple therapies. One option for multidrug-resistant *P. falciparum* is to use two sequential ACTs. As there are issues with neutropenia at cumulative doses of artemisinin over 24 mg/kg, further safety data are needed. Adherence may be an issue, although co-packaging will help. If there are no alternatives, however, adherence problems will have to be overcome.

6.4 Use of atovaquone-proguanil in the context of a containment project in Cambodia

Presentation

In 2011, atovaquone-proguanil (AP) became the first-line treatment in Pailin because of high treatment failure rates with DHA-PIP. Although AP's efficacy was initially 100% in 2008–2009 and 2010, a study conducted in 2012–2013 reported high AP treatment failure rates. Whereas 4/24 patients had wild type parasites at day 0 and Y268N (n = 3) or F263T (n = 1) mutants at day 28, 1/24 treatment failures had the *Pf*cytb Y268C mutation present at day 0 and day 28. The remaining 19 patients had wild type parasites at codon 268 at day 0 and day 28. A survey found *Pf*cytb mutant parasites in 13/295 samples, but it is unknown whether these were 'day 0' or recrudescence samples. A temporal relationship was found between the use of AP in Pailin and the increase in the frequency of AP treatment failures or prevalence of *Pf*cytb mutant parasites.

In Africa, it was reported that atovaquone-resistant mutations can occur spontaneously and that another mutation can be involved (I258M) in treatment failure. In addition, there is some evidence that atovaquone-resistant *P. berghei* parasites are not transmissible to mosquitoes.

6.5 Role of atovaquone-proguanil and artesunate+atovaquone-proguanil for the treatment of multidrug-resistant malaria in Cambodia

Presentation

A TES was conducted in Cambodia comparing AP and artesunate+AP in 205 volunteers with *P. falciparum* or mixed *P. falciparum*/*P. vivax* infection. Primaquine 15 mg was co-administered with the first dose of study medicine to all patients by direct-observed treatment. The study was conducted at two sites: Anlong Veng in the north and Kratie in the east. Treatment efficacy at day 42 was just above 90% with no difference between arms. Efficacy at Kratie was higher than at Anlong Veng. Artesunate+AP appeared to have some beneficial effects on gametocyte carriage relative to AP alone, but the effect on transmission has not been evaluated.

The ex vivo susceptibility of parasites did not differ between patients treated with AP and those treated with artesunate+AP. IC₅₀ values for AP were low, suggesting that treatment failures could be caused by inadequate atovaquone exposure; however, pharmacokinetic data are pending and IC₅₀s could not be established for all recrudescence samples. Cytochrome 2C19 (CYP2C19) metabolizes proguanil into the active metabolite cycloguanil, which has limited antimalarial activity against parasites carrying *Pf dhfr* mutations. However, there is an unknown mechanism by which proguanil potentiates the antimalarial activity of atovaquone. Thus, theoretically, slow CYP2C19 metabolizers should have higher proguanil levels and consequently higher atovaquone antimalarial activity. Conversely, rapid metabolizers should have lower proguanil levels and therefore lower atovaquone potentiation. No data were available on the CYP2C19 status of patients in this study.

Nearly all isolates at both sites carried *Pf kelch13* C580Y. *Pf cytb* Y268C was detected in only 1/14 treatment failures. Based on the amplicon deep sequencing of the blood sample with confirmed *Pf cytb* mutation at recrudescence, it appears that resistance developed during AP therapy. There may be other atovaquone-resistant *Pf cytb* mutations not detected through Sanger sequencing.

Combinations of AP and other antimalarial drugs could be investigated as a stop-gap measure against multidrug-resistant *P. falciparum*.

In conclusion, both regimens were well tolerated, but there was no clinical benefit to artesunate+AP over AP alone, probably because of extensive artemisinin resistance in the region. However, the addition of artesunate may reduce gametocyte carriage, and the potential impact on transmission requires evaluation. The probability of selecting for atovaquone-resistant mutants has been estimated as 1 in 500 treatments in other studies. The emergence of AP resistance would have important implications for chemoprophylaxis in South-East Asia.

Discussion

In published case reports, the presence of the *Pf cytb* Y268C mutation in the samples from patients with treatment failure was associated with a significant rise of atovaquone IC₅₀s (at least 1000-fold). However, this marker has not been validated and it is not known whether *Pf cytb* mutations affect resistance at the population level.

Most treatment failures with AP appear to be occurring without detectable *Pf cytb* mutations. Therefore, treatment failures may also be related to the pharmacokinetics of AP, but drug levels in the blood are needed to further investigate these failures. Even in the absence of detectable *Pf cytb* mutations, failure rates appear to be around 10%, which may potentially be related to CYP2C19.

Until there is additional evidence that *P. falciparum Pf cytb* Y268C/N/S mutants are not transmissible, it cannot be concluded that atovaquone resistance is not transmissible. However, membrane feeding assays or direct feeding studies would be needed to assess the true risk of infectivity of field parasites with *Pf cytb* mutations.

Where artemisinin is failing, combining AS with AP, which has an intrinsic failure rate of 10% (even in the absence of detectable *Pf cytb* mutation), will not work. If AP were to be redeployed, it would need to be in combination with an ACT (not doxycycline), preferably one with a partner drug with a long half-life, e.g., either AS-MQ or AS-PY. Drug–drug interactions may limit the potential combinations available. The safety of multiple combination drugs is always a concern.

Currently, the efficacy of AS-MQ is 100% in Cambodia. Although adding AP to this would not improve efficacy, it might protect the combination from developing resistance and provide safety information on the triple combination.

The efficacy of AS-PY in Western Cambodia is not as high as it is elsewhere, although the reasons for this are unclear. Ideally, AP should be partnered with a drug with high efficacy so as to minimize the risk of resistance development. However, if it becomes the case that there are no other options in Cambodia, it might be useful to know now whether an AS-PY+AP combination has higher efficacy than AS-PY alone and to obtain additional safety data.

6.6 Recommendations: Session 3

Strategy for antimalarial drug resistance management

The TEG agreed that it would be valuable to have a new strategy for antimalarial drug resistance management, and this should be developed and made available as soon as possible. The scope and components of the strategy presented were considered appropriate and should include the following:

- Scenario-planning, for instance in case of outbreaks of falciparum malaria in areas with multidrug resistance;
- Guidance on *P. vivax* resistance;
- New information and approaches since the GPARC;
- Distinct scenario-planning for different resistance situations;
- Consideration of all interventions using antimalarial drugs, their potential impact on resistance development, and actions that might mitigate this risk;
- Measures for containment across borders;
- Guidance on the management of suspected and confirmed treatment failures, including diagnostics and alternative treatments that can be used in remote or resource-poor areas.

An ideal format would include a generic section building on what is in the GPARC and what has been learned more recently, plus scenarios that can change over time as new evidence and tools become available.

Update on antimalarial drug efficacy and drug resistance

The TEG recommends that all putative *Pfkelch13* mutants conferring artemisinin resistance be independently verified as being associated with resistance both in genetic studies and in the RSA, ideally before publication claiming such association.

Planned activities (TES and survey) to investigate *Pfkelch13* C580Y in South America are sufficient. However, whole genome sequencing may be useful to examine backbone mutations. Ideally, resistant parasites should be collected for culture adaption.

The presence of multicopy *Pfplasmepsin 2-3* in Africa is a potential concern in terms of the use of DHA-PIP. However, additional information is required regarding the in vivo and ex vivo piperaquine-resistant phenotype in African parasites. Additional African data are needed to assess the

relationship between DHA-PIP treatment failures and molecular markers (*Pfkelch13*, *Pfplasmepsin 2-3*, and *Pfcrt*).

Triple therapies

Although TRAC 2 data are preliminary, the data support the testing of triple therapies as a potential strategy against multidrug-resistant *P. falciparum*. In areas where DHA-PIP is failing, the effectiveness of DHA-PIP+mefloquine treatment would rely to a large extent on the mefloquine component; as such, AL+amodiaquine may be the preferred triple therapy and should be tested in Cambodia and Viet Nam. The initial rationale of combining DHA-PIP with mefloquine assumed that the drugs have competing resistance mechanisms need to be reassessed in light of the data presented on parasites from Cambodia with both multicopy *Pfmdr1* and multicopy *Pfplasmepsin 2-3*.

Given the concern over QTc interval prolongation interval and the issues regarding the measurement of changes in QTc as malaria symptoms resolve, further analysis of QTc using alternative methods was requested.

An alternative treatment option for multidrug-resistant *P. falciparum* is to use two sequential ACTs. This approach should be tested in clinical trials.

Atovaquone-proguanil

In the GMS, there may be a role for AP in combination with an ACT. AS-MQ+AP and AS-PY+AP are two options for testing.

Further studies are required to validate mutations as a clinically relevant molecular marker of atovaquone resistance. There may be other mutations contributing to resistance besides the *Pfcytb* mutation at position 268.

Until there is stronger evidence that a *P. falciparum* *Pfcytb* Y268C/N/S mutant is not transmissible, it cannot be concluded that atovaquone resistance is not transmissible.

Annex 1: List of participants

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Annex 2: Meeting agenda

Thursday 1 June 2017		
09:00–09:20	Welcome P. Alonso – Director GMP A. Dondorp – Chair TEG DER	
09:20–09:30	Declaration of interest P. Ringwald	
09:30–09:50	Minutes and action points of the last TEG meeting A. Dondorp	→ For information
Session 1: Molecular markers: genotyping and monitoring drug resistance		Purpose of session and expected outcomes
09:50–11:00	i) Molecular markers of piperaquine resistance D. Ménard 20' D. Fidock 5' C. Plowe 5' Discussion 40'	→ For information and decision
11:00–11:30	Coffee/tea break	
11:30–12:40	ii) New evidence on <i>mps1</i> , <i>msp2</i> , and <i>glurp</i> as markers of reinfection and recrudescence I. Felger 30' iii) Reinfection/recrudescence: pros and cons of other methods (microsatellites, barcoding, and amplicon sequencing) S. Volkman 30'	→ For information and decision

	iv) Barcoding to genotype <i>Plasmodium</i> in TES: experience in South-East Asia D. Ménard 10'	
12:40–14:00	Lunch	
14:00–15:00	Discussion 60'	
15:00–16:00	v) Update on <i>P. vivax</i> molecular markers: - genotyping to differentiate homologous and heterologous infections; - molecular markers of drug resistance. K. Baird 30' + 30'	→ For information and decision
16:00–16:30	Coffee/tea break	
Session 2: Monitoring the prophylactic effect of preventive treatment		Purpose of session and expected outcomes
16.30–17:20	Monitoring efficacy of seasonal malaria chemoprevention in the ACCESS-SMC project P. Milligan 25' + 25'	→ For information
Session 3: Prevention and treatment of multidrug resistant malaria		Purpose of session and expected outcomes
17:20–18:00	Outline of a strategy for antimalarial drug resistance management C. Rasmussen 20' + 20'	→ For information and comments
Friday 2 June 2017		
Session 3: Prevention and treatment of multidrug-resistant malaria		Purpose of session and expected outcomes
8:30–9:00	Update on antimalarial drug efficacy and drug resistance P. Ringwald 30'	→ For information
9:00–09:30	Update on TRAC 2: preliminary results of triple therapies A. Dondorp 30'	→ For information

9:30–10:30	Discussion 60'	
10:30–11:00	Coffee/tea break	
11:00–11:15	Use of atovaquone-proguanil in the context of a containment project in Cambodia P. Ringwald 15'	→ For information and comments
11:15–11:45	Role of atovaquone-proguanil and artesunate+atovaquone-proguanil for the treatment of multidrug-resistant malaria in Cambodia M. Wojnarski 30'	→ For information and decision
11:45–12:30	Discussion 45'	
12:30–14:00	Lunch	
14:00–17:00	Formulation of TEG recommendations A. Dondorp	Closed session
17:00	Closing remarks A. Dondorp/P. Alonso	Closed session

Annex 3: List of questions

Session 1

Piperaquine resistance

- Is there sufficient evidence to confirm that *Pfplasmepsin 2-3* increased copy number is a marker of piperaquine resistance?
- Could other mutation(s), including in *Pfcrtr*, be involved in piperaquine resistance?

Markers of reinfection and recrudescence for *P. falciparum*

- Can the WHO recommendation on distinguishing *P. falciparum* recrudescence from reinfection using *msp1*, *msp2*, and *glurp* be improved? Are any changes to the recommendations required?
- Are there other molecular markers/tools that could be used for this purpose? If so, what are the advantages and disadvantages compared to existing WHO recommendations?

P. vivax molecular markers

- Are there reliable molecular markers that can be used to distinguish between reinfection, recrudescence, and relapse during *P. vivax* clinical trials?
- Are there validated molecular markers of *P. vivax* resistance to chloroquine, mefloquine, pyrimethamine, sulfadoxine, or other antimalarial medicines?

Session 2

Monitoring the efficacy of seasonal malaria chemotherapy (SMC)

- Were the recommendations of the last Technical Expert Group (TEG) used in the efficacy monitoring of SMC?
- Is there evidence showing that SMC deployment is causing resistance to emerge or increasing pre-existing resistance to one or both drugs used for SMC?
- If yes, has resistance affected the effectiveness of SMC interventions?

Session 3

Strategy for antimalarial drug resistance management

- Is there a need for a strategy on antimalarial drug resistance management to guide activities at the country level?
- Are the scope and components of the strategy, as presented to the TEG, appropriate?

Update on antimalarial drug efficacy and drug resistance

- What actions need to be taken by WHO and by the NMCPs in response to the *Pfkelch13* C580Y mutants reported outside the Greater Mekong Subregion (GMS)?

Triple therapies

- Is there sufficient evidence for the combination to be recommended/used in further trials?
- Are there additional investigations that need to be conducted on cardiotoxicity, in particular based on the latest ERG conclusions on the cardiotoxicity of antimalarial medicines?

Atovaquone-proguanil

- What could be the role of atovaquone-proguanil in the GMS?
- Are there any other combinations treatments that would be worth testing in the GMS?