World Health Organization

Background documentation for Day 2

This file contains the slides that were shown by the presenters during Day 2 of the meeting as well the background documentation shared with MPAG members ahead of the meeting.

Tuesday, 5 October 2021		
	Session 3	**Open**
12:00 – 12:45	Antimalarial drug resistance in Africa • Presentation by Dr Pascal Ringwald • Background article • Draft meting report	Dr Pascal Ringwald
12:45 – 14:00	Rectal artesunate and quality of care • Presentation	Ms Silvia Schwarte Dr Justin Cohen, CHAI Dr Manuel Hetzel, Swiss TPH Dr Pascal Ringwald
14:00 – 14:30	The relationship between chemoprevention and drug resistance • Presentation by Dr Chris Plowe • Background report	Dr Chris Plowe
	Session 4	**Open**
14:45 – 15:45	Update on the WHO Guidelines for malaria (Vector control, Chemoprevention, Elimination, Treatment) • Presentation	Dr Pedro Alonso Dr Jenny Stevenson Dr David Schellenberg Dr Kim Lindblade Dr Peter Olumese

WHO/UCN/GMP/MPAG/2021.07

Antimalarial drug efficacy and resistance in Africa

C. Rasmussen, GMP/DMR

P. Ringwald, GMP/MDO

Global **Malaria** Programme

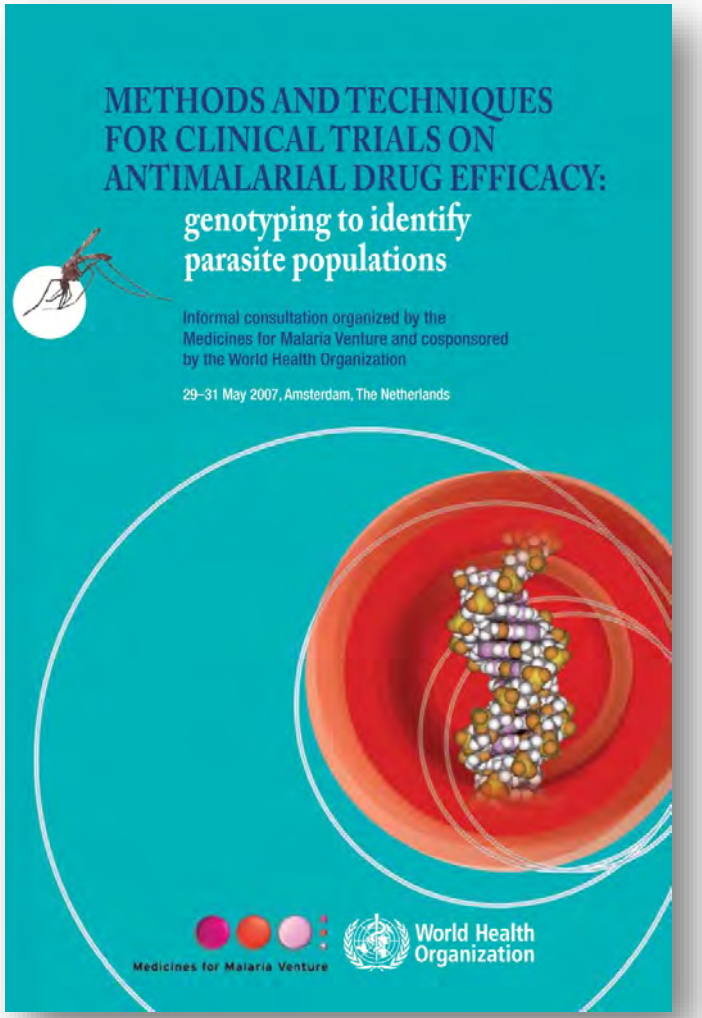
World Health Organization

Outline

- Monitoring antimalarial drug efficacy in Africa: update on PCR correction
- Situation of antimalarial drug efficacy and resistance
  - Artemisinin resistance
  - Partner medicine
  - Prevention and response activities
  - Chemoprevention protocol

Past WHO guidance and discussions

- WHO published guidance in 2008 on genotyping to identify parasite populations for clinical trials using *msp1, msp2* and *glurp.*
- The guidance was reviewed in a meeting of the Technical Expert Group on Drug Efficacy and Response (TEG DER) (2017).
- US CDC Malaria Laboratory has for more than a decade used neutral microsatellites genotyping: (*Pfpk2, Poly-α, TA1, TA109, 313, 383, TA2490*) and probabilistic approach of a Bayesian algorithm quantifying the probability that a sample is either a recrudescence or a new infection.
- Two African countries are using a combination of *msp1* and *msp2* and microsatellites:
  - Mali: *CA1, TA87, TA99*;
  - Uganda: *TA40, TA60, TA81, Pfpk2*.



Updating methodologies to distinguish reinfection from recrudescence in high malaria transmission areas

A virtual consultation was held in 17-18 May 2021 to review methodologies to distinguish reinfection from recrudescence.

The objectives of the consultation were to:

- Review available data and assess the advantages and disadvantages of:
  - changing the markers used to differentiate recrudescence from reinfection;
  - changing the algorithms used to classify recrudescence and reinfection.
- Assess transmission settings where a change in the current methodology could improve the precision of classifying recurrent *P. falciparum* as recrudescence or reinfection.
- Discuss alternative tools for future use and suggest research needed to validate these tools.

Recommendations

Recommendation 1:

- As an interim solution, *msp1* and *msp2* should continue to be used, but *glurp* should be replaced with a panel of two to three carefully chosen microsatellites (such as *Poly-α, Pfpk2* and *TA1*), customized to provide sufficient local diversity at least at the country level or regionally if possible.
- For simplicity and reasons of practical implementation, match-counting should be maintained as the primary analysis methodology for reporting.
- These methods should be applied in both low to moderate and high transmission settings in Africa.
- Bayesian algorithms may be applied for evaluation and comparison, but not for primary reporting.
- Outside Africa, the current method (*msp1/msp2/glurp*) should still be applied.

Recommendations cont'd

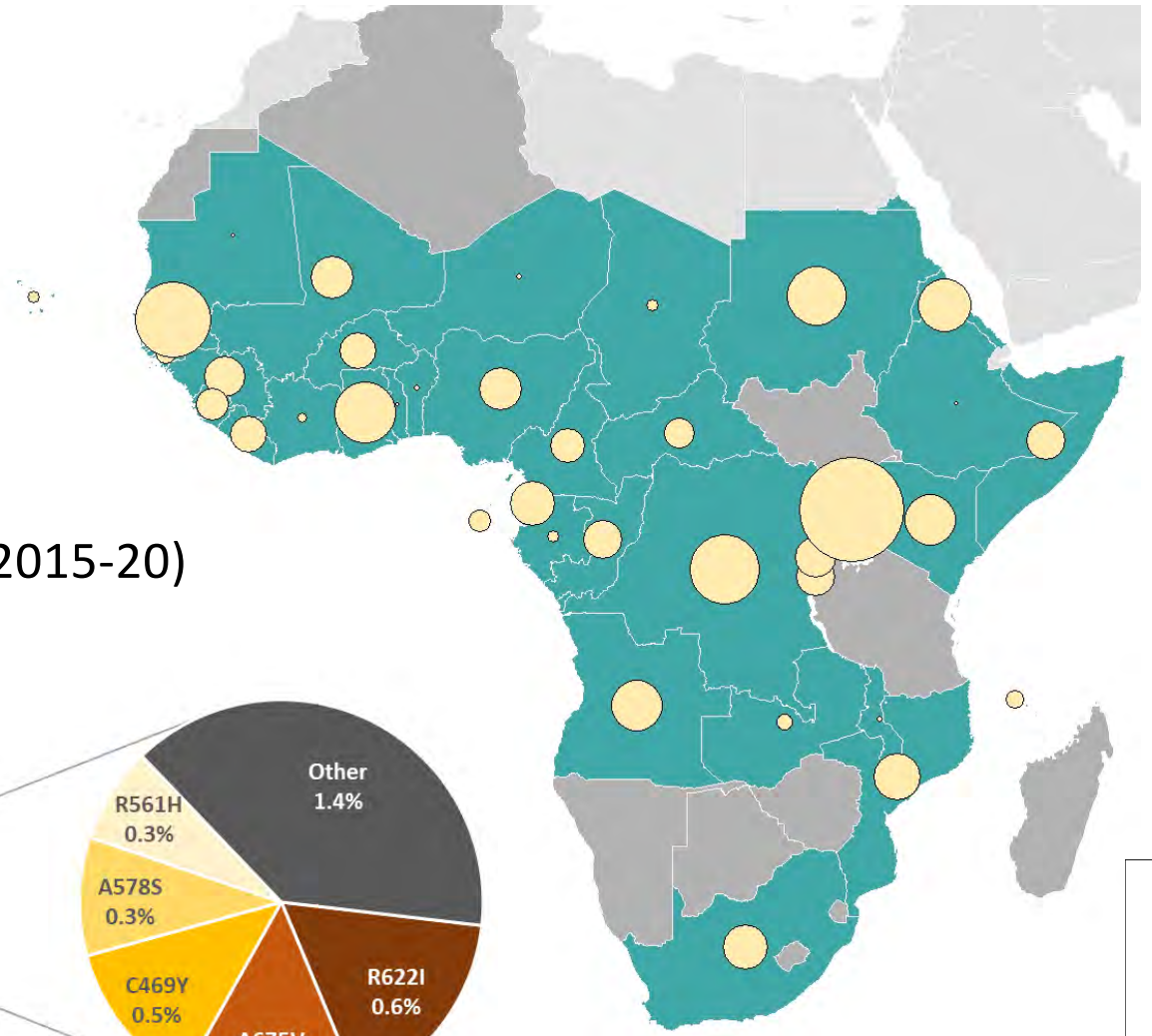
Recommendation 2:

- For a transition period, data should be analysed and reported using both the current (*msp1/msp2/glurp*) and new (*msp1*/*msp2*/microsatellites) methods to enable historical comparison and to understand the implications of the new methods in terms of thresholds for treatment policy change and introduction of new antimalarial drugs.

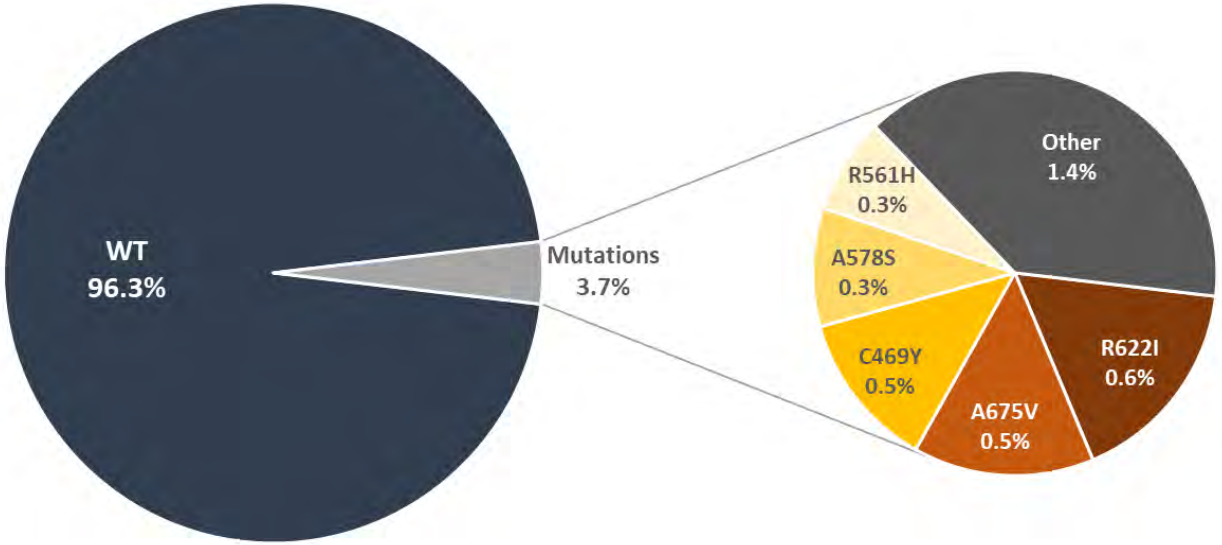
Recommendation 3:

- As a medium-term (five-year) target, AmpSeq should be evaluated in parallel across Africa and outside Africa to compare it with current methods at sites and to validate whether this genotyping methodology should be adopted as the standard.
- A simple match-counting algorithm complemented by a Bayesian algorithm could be paired with this approach, but more comparative data are needed to inform the recommendation on the algorithm for analysing AmpSeq results.

K13 genotypes in African countries (2015-2020)



K13 genotypes in Africa (2015-20)
(n = 18 327)

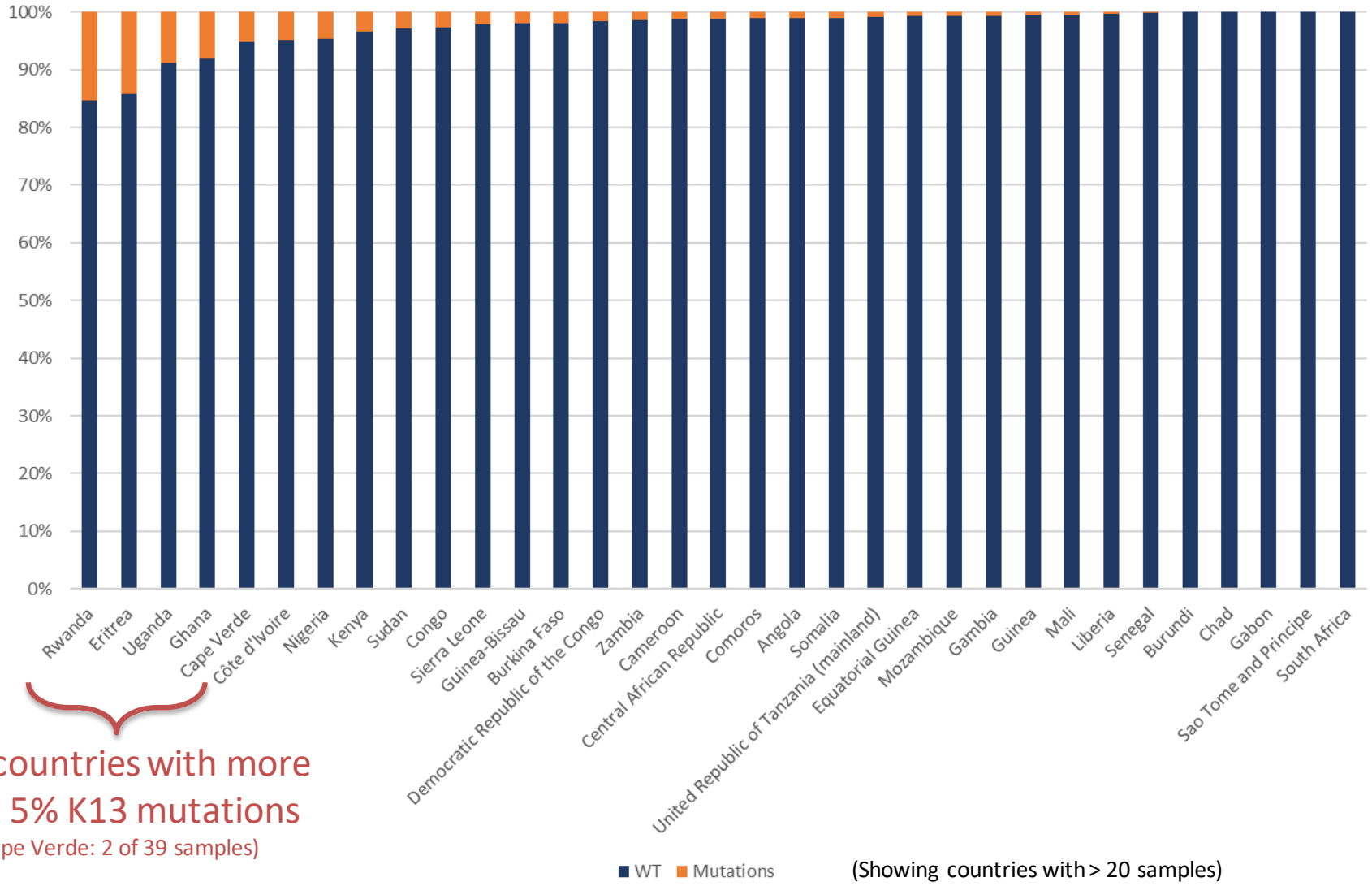


Global **Malaria** Programme

Source: WHO database

World Health Organization

K13 genotypes in African countries (2015-2020)



Five countries with more than 5% K13 mutations

(Cape Verde: 2 of 39 samples)

(Showing countries with > 20 samples)

Global **Malaria** Programme

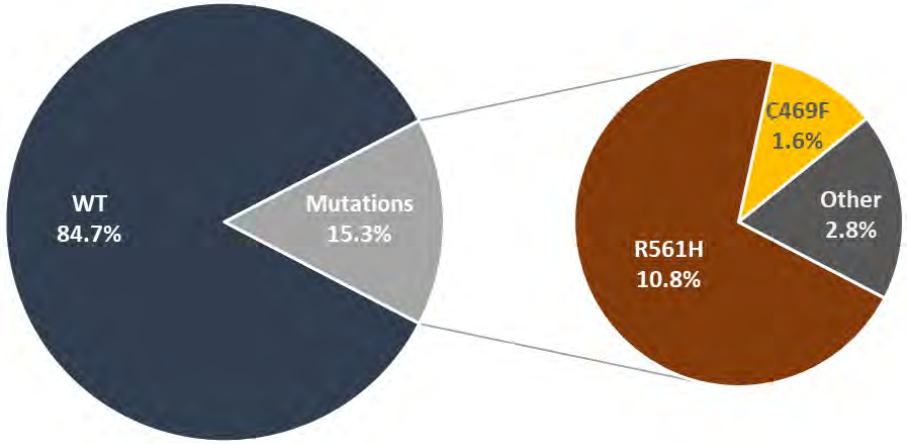
Source: WHO database

World Health Organization

K13 genotypes in 4 African countries (2015-2020)

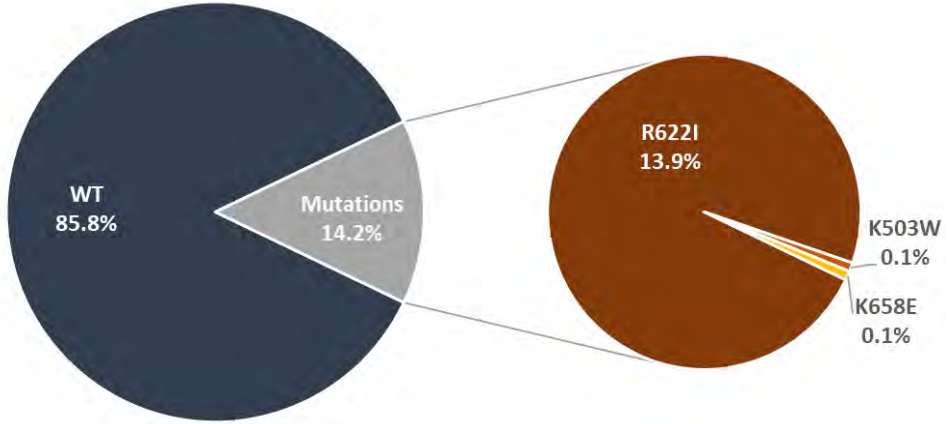
Rwanda (n=425)*

12 different K13 mutations detected



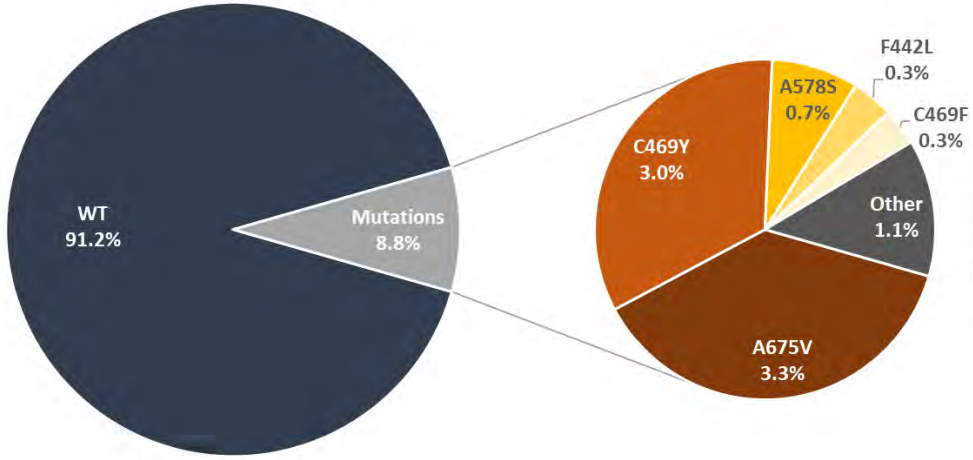
Eritrea (n=769)*

3 different K13 mutations detected



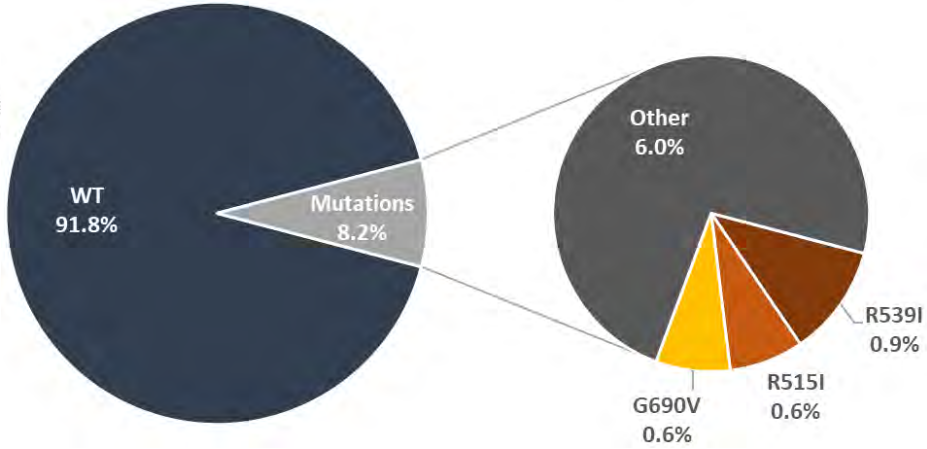
Uganda (n=2872)*

21 different K13 mutations detected



Ghana (n=968)

36 different K13 mutations detected



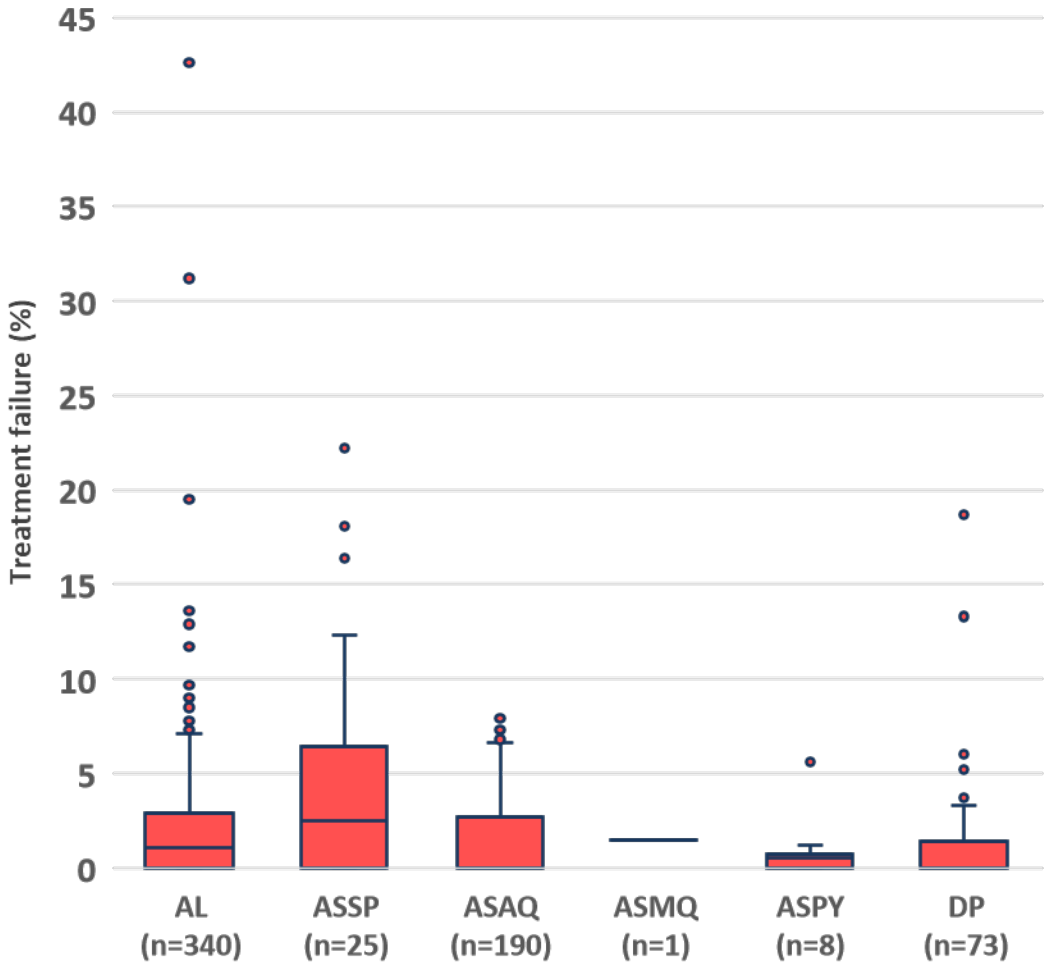
Global **Malaria** Programme

Source: WHO database

World Health Organization

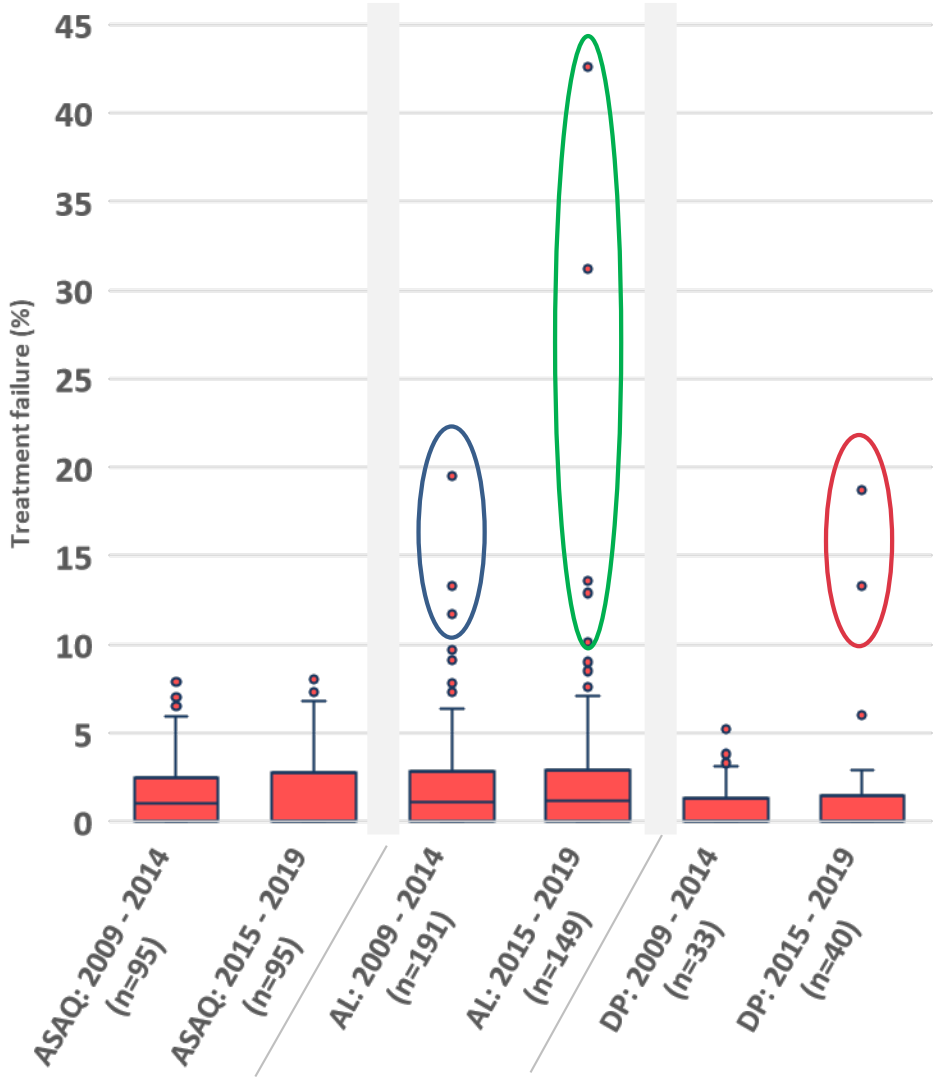
Therapeutic Efficacy Studies in Africa 2009-2019

TES sites in Africa

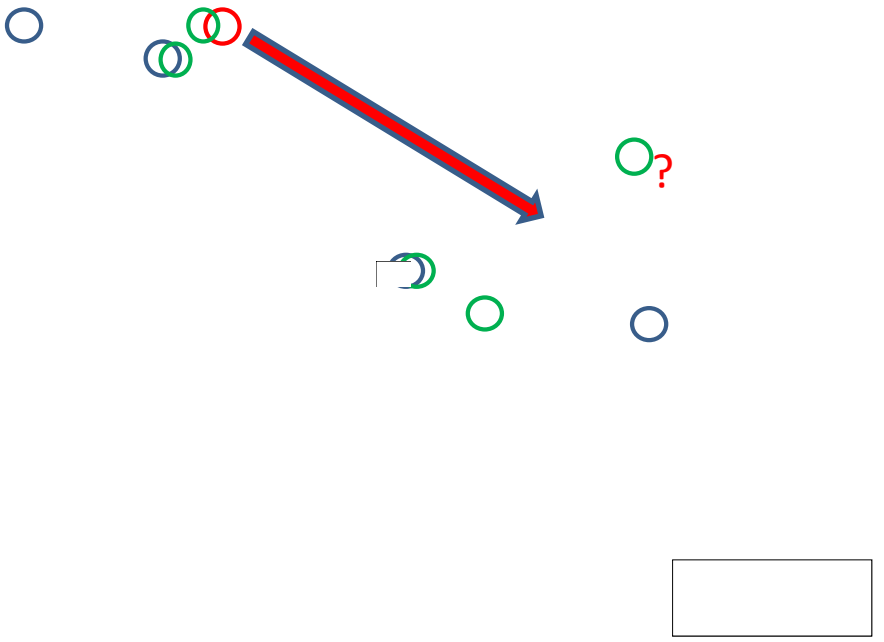


Failure rates for selected ACTs for 2009-14 vs 2015-19

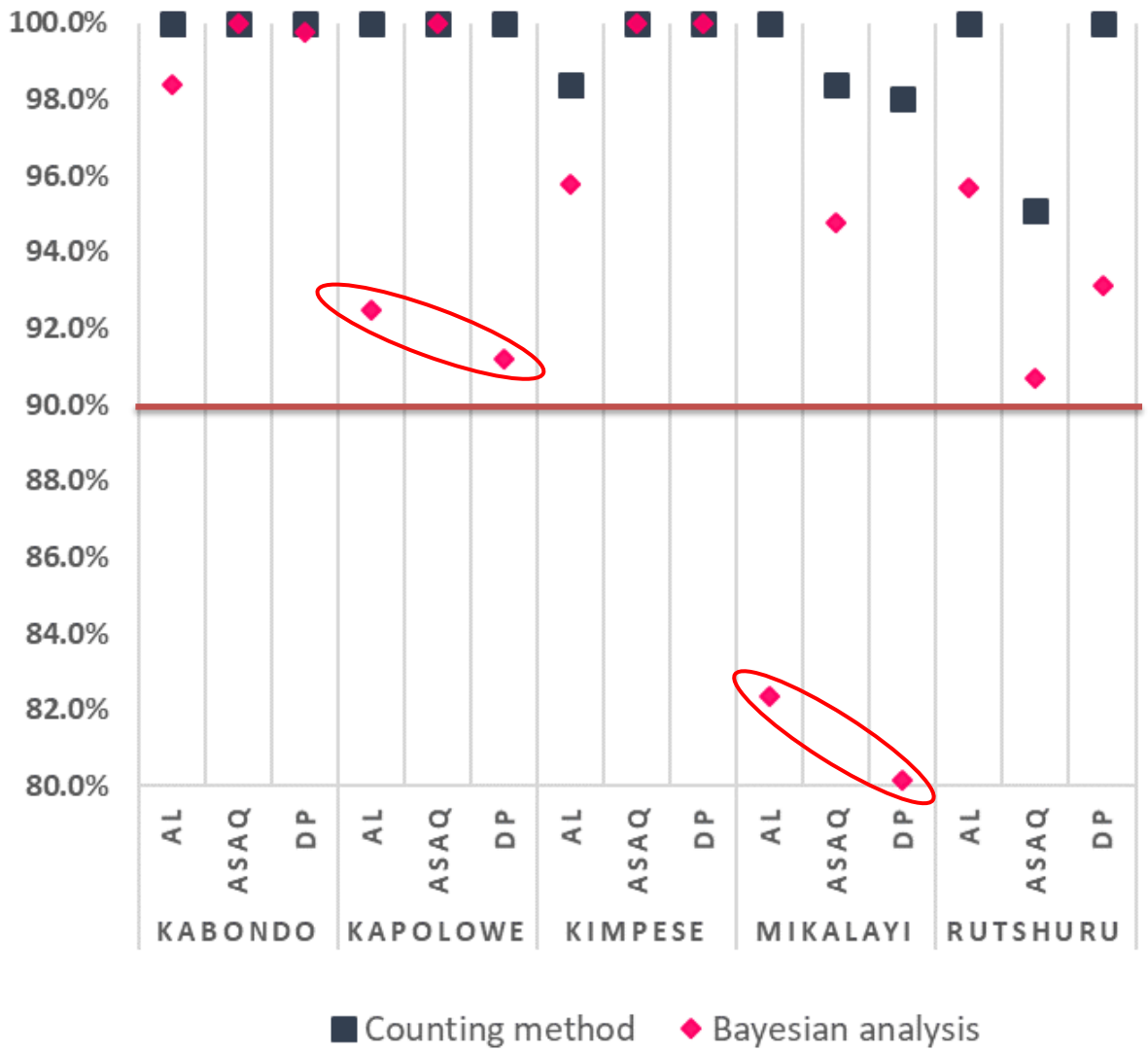
Treatment failure rates in studies in Africa
ASAQ, AL and DP for 2009 - 2014 and 2015 - 2019

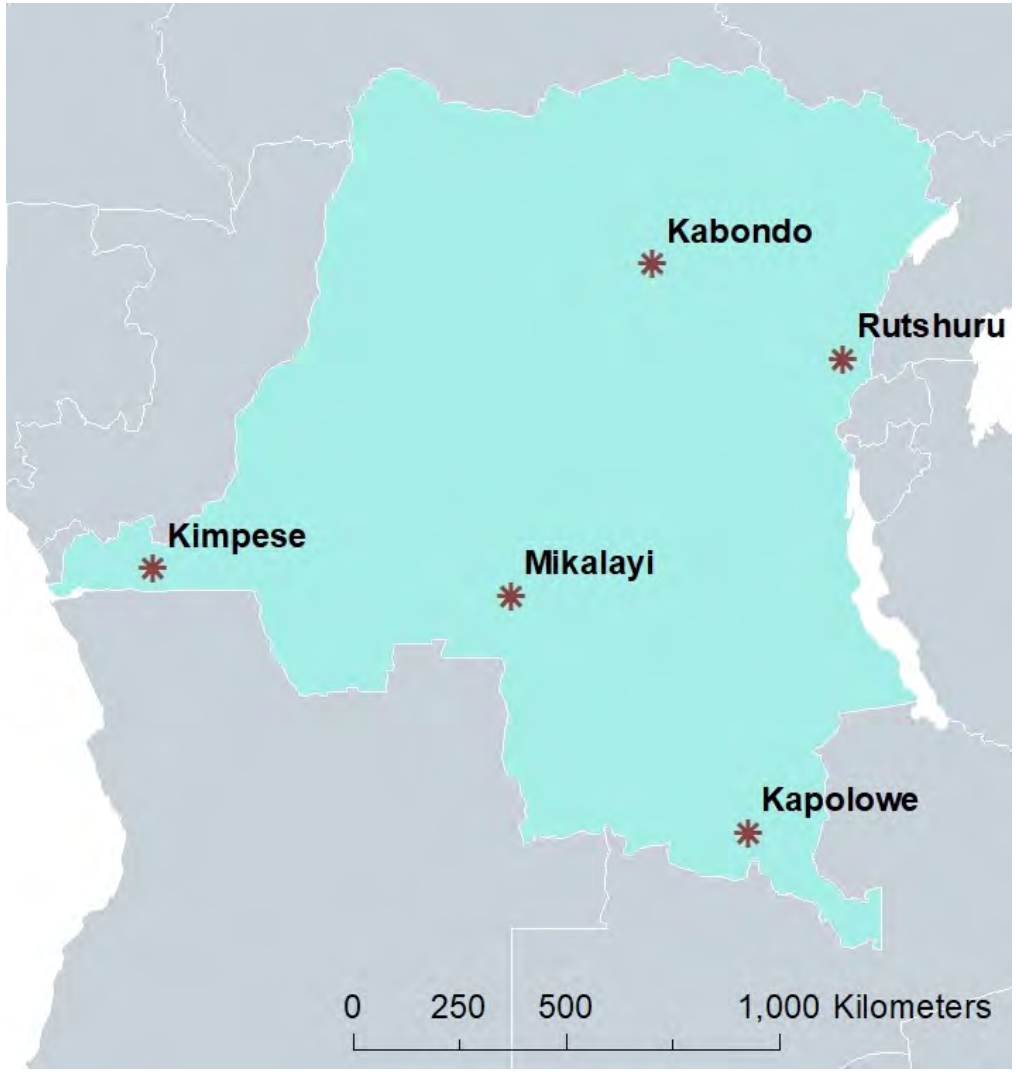


Sites where failure rates of >10% has been detected



TES in DRC: counting vs Bayesian





Moriarty et al., AMJTH, Sep 2021

Is there lumefantrine resistance in Africa?

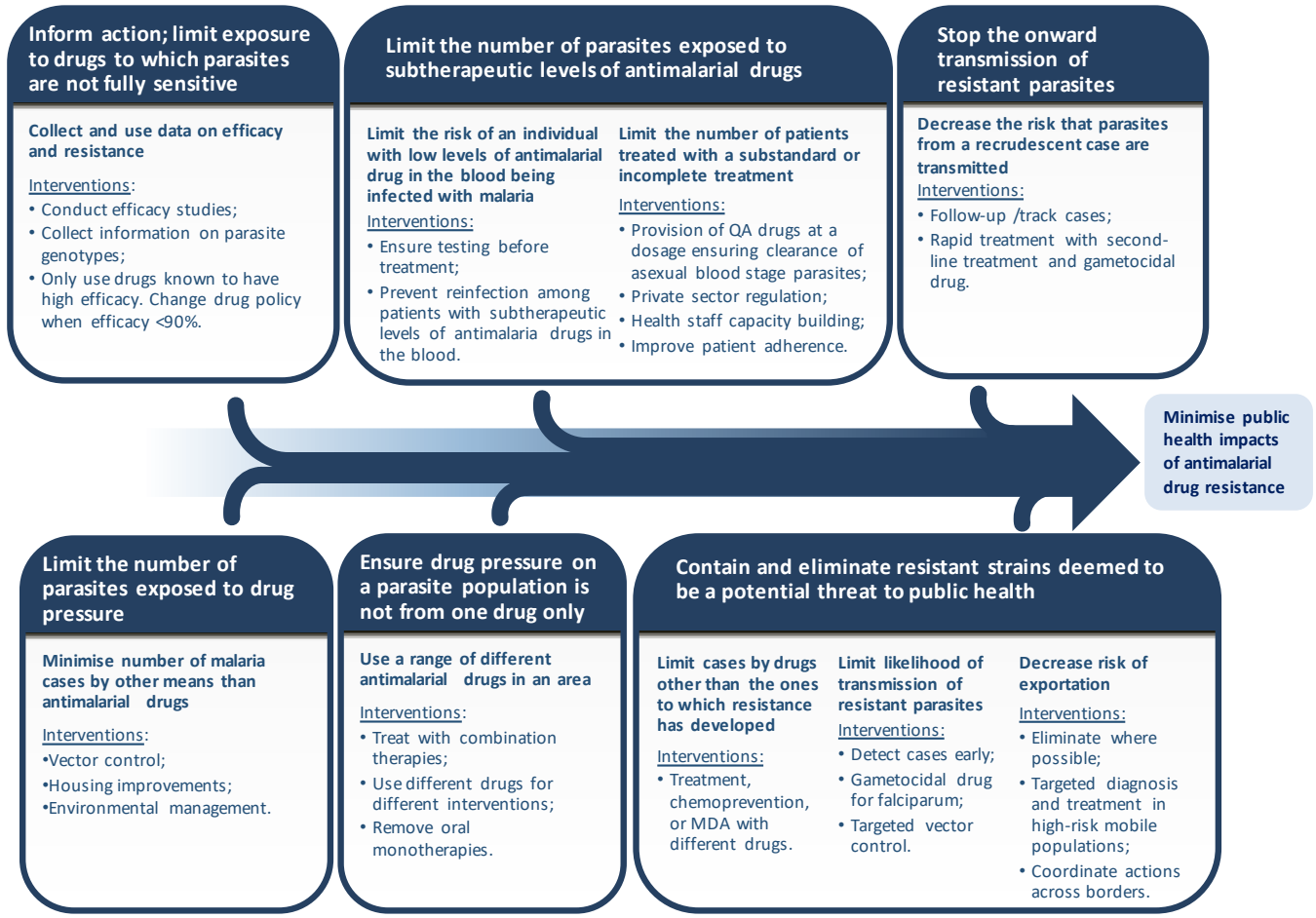
Pros

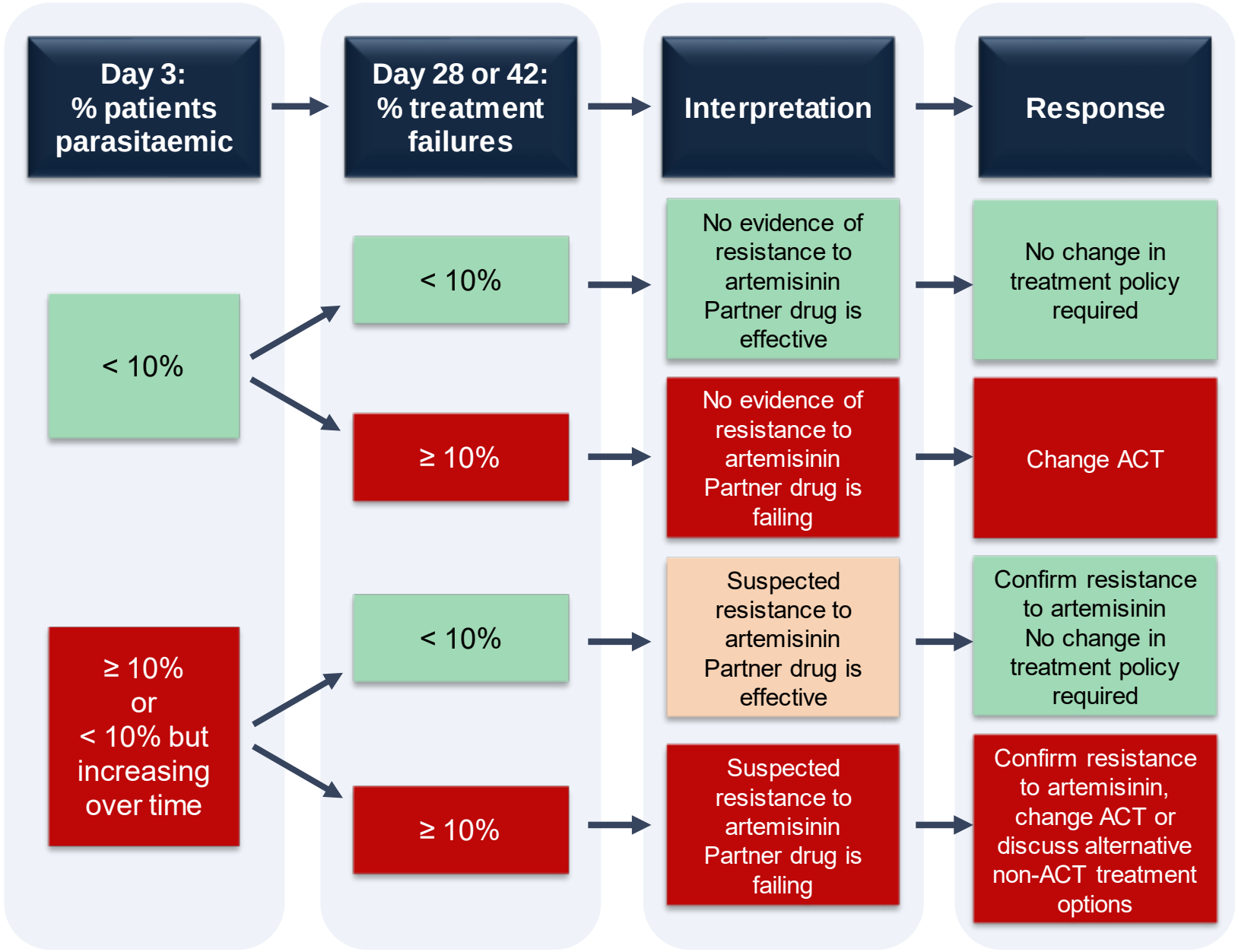
- Reports of treatment failures in travelers coming back to UK, Sweden and Portugal but blood level at day 7 often missing;
- Recurrent signal during TES but deviations from WHO protocol: PCR correction and Bayesian approach;
- Few reports on lumefantrine IC_{50} increase but trends difficult to analyze and no lumefantrine resistance threshold defined.

Cons

- Many confounders possible: poor absorption, 2nd dose per day often unsupervised, short half-life potentially leading to high reinfection rate;
- No high treatment failure rates in Lao PDR and Myanmar despite high prevalence of artemisinin resistance;
- Too much emphasis on N86 *Pfmdr1 (mdr1* CNV);
- AL treatment failures in travelers successfully cured with AL (Turkey and Sri Lanka);
- No clear explanation why AL high failure rate is associated with DP high failure rate in the same sites (Burkina Faso and DRC but not in Angola); cross-resistance?

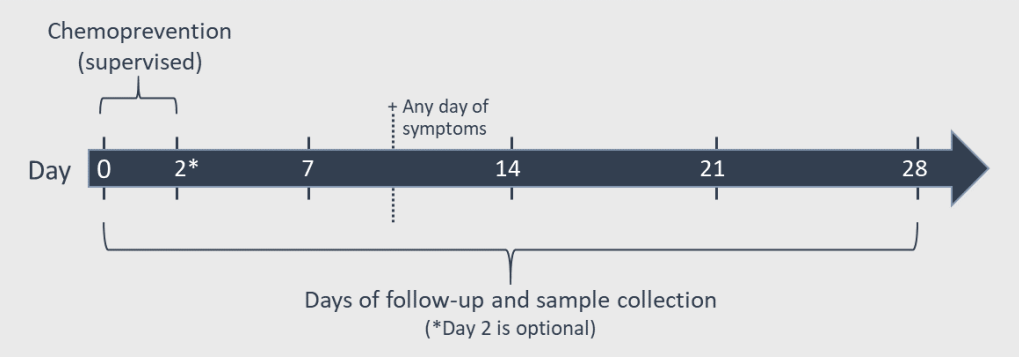
Interventions to prevent and respond to resistance




Day 3: % patients parasitaemic
Day 28 or 42: % treatment failures
Interpretation
Response
< 10%
< 10%
No evidence of resistance to artemisinin Partner drug is effective
No change in treatment policy required
≥ 10%
No evidence of resistance to artemisinin Partner drug is failing
Change ACT
≥ 10% or < 10% but increasing over time
< 10%
Suspected resistance to artemisinin Partner drug is effective
Confirm resistance to artemisinin No change in treatment policy required
≥ 10%
Suspected resistance to artemisinin Partner drug is failing
Confirm resistance to artemisinin, change ACT or discuss alternative non-ACT treatment options

Protocol for surveillance of chemoprevention efficacy (CPES)

- Chemoprevention strategies currently recommended by WHO are IPTp, IPTi and SMC;
- Contrarily to TES, no standardized approaches for monitoring and evaluating the efficacy of malaria chemoprevention strategies;
- Molecular markers have been found to be associated with treatment outcomes, but:
  - Predictive value for the efficacy and usefulness of chemoprevention is still unclear;
  - Not reliable at present to rely only on surveillance of MM to make decisions on chemoprevention efficacy (see C. Plowe's presentation);
- CPES are single arm studies that aim to evaluate the ability of one round of chemoprevention to prevent parasitaemia in a predefined period of follow-up.



Thank you for your attention

Malaria Policy Advisory Committee Meeting
4—7 October 2021, Geneva, Switzerland
Background document for Session 3

Informal consultation on methodology to distinguish reinfection from recrudescence in high malaria transmission areas

17–18 May 2021, Geneva, Switzerland, virtual meeting

Advance copy prepared for the 20th meeting of the Malaria Policy Advisory Group – the final report will be published in the coming weeks.

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Abbreviations

Africa CDC	Africa Centres for Disease Control and Prevention
AL	artemether-lumefantrine
AmpSeq	amplicon sequencing
AS	artesunate
ASAQ	artesunate-amodiaquine
ASSP	artesunate+sulfadoxine-pyrimethamine
bp	base pair
CE	capillary electrophoresis
COI	complexity of infection
DRC	Democratic Republic of the Congo
DP	dihydroartemisinin-piperaquine
glurp	gene of glutamate-rich protein
GMP	Global Malaria Programme
HeOME	heterozygome
IBC	image barcode
SNP	single nucleotide polymorphism
TEG DER	Technical Expert Group on Drug Efficacy and Response
TES	therapeutic efficacy study
MIP	molecular inversion probe
MMV	Medicines for Malaria Venture
MOI	multiplicity of infection
msp1	gene of merozoite surface protein 1
msp2	gene of merozoite surface protein 2
PCR	polymerase chain reaction
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
US-CDC	United States Centers for Disease Control and Prevention
WGS	whole genome sequencing
WHO	World Health Organization

Executive summary

This data-driven meeting assessed the advantages and disadvantages of changing the way in which recurrences are differentiated as reinfection or recrudescence following the treatment of uncomplicated *Plasmodium falciparum* malaria. This has implications for the evaluation of antimalarial efficacy in therapeutic efficacy studies (TESs), as well as in regulatory trials for the development of new antimalarial drugs.

Guidance for discriminating *P. falciparum* recrudescence from reinfection was published by the World Health Organization (WHO) in 2008 *(1)*. Blood samples collected pre-treatment (day 0) and on the day of treatment failure (day X) are compared using three markers: genes for merozoite surface protein 1 (*msp1*), merozoite surface protein 2 (*msp2*) and glutamate-rich protein (*glurp*). A standard genotyping methodology is recommended, including the use of capillary electrophoresis (CE). The decision algorithm (referred to as the 'WHO/MMV' algorithm) requires the three markers to be genotyped and analysed in sequence, starting with *msp1*, followed by *msp2* and then *glurp*, and stopping once a marker has classified the paired samples as a reinfection. In this case, if any marker indicates reinfection, the recurrence is deemed a reinfection. An alternative approach has been suggested (termed the '2/3' algorithm) whereby *msp1/msp2* are evaluated, and only in cases where these two markers are discordant, *glurp* is used as the deciding factor. In this case, even if *msp1* indicates a reinfection, if *msp2* and *glurp* indicate a recrudescence, the recurrence is deemed a recrudescence.

The consultation examined evidence around changes in the genetic markers used to determine the relatedness of initial and recurrent parasites, as well as the algorithms used to analyse these markers to classify recurrences as either a recrudescence or reinfection. In particular, the panel examined the applicability of recent advances in genotyping and analysis. The meeting focused on areas of high transmission in Africa because the high multiplicity of infection (MOI; i.e., the number of concurrent clones in an infection) and high reinfection rates in such areas complicate the discrimination of recrudescence from reinfection.

Summary conclusions

The different methodologies for genotyping and analysis used to differentiate recrudescence from reinfection all have advantages and limitations, and clearly give different results. This has important consequences because, in some cases, the difference in the number of recurrences classified as recrudescence drives the efficacy rate below 90%, which is the currently recommended threshold requiring a change in treatment policy. In addition, this may affect decisions during drug development and adoption of new treatments, where a 95% efficacy threshold is recommended.

The panel considered which methods are most likely to be closest to the 'true' values for reinfection and recrudescence. The most robust and reliable genotyping method is amplicon sequencing (AmpSeq). However, the capacity to apply this technology in Africa needs to be strengthened before this method can be adopted as a standard. This could be achieved by capacity building in African countries and/or offering deep sequencing at core facilities(s) in Africa or elsewhere, which could process samples and return data to countries for analysis – the dual aim being to ensure that drug efficacy is accurately measured and that countries retain ownership of their data. The examined data clearly indicate that *glurp* is not an ideal marker. Therefore, until AmpSeq implementation is feasible, as an interim solution, *glurp* should be replaced with alternative markers. Microsatellites with a diversity relevant to the study site location appear to be the most feasible and reliable option. For the transition period, data from the new methods and the current *msp1/msp2/glurp* markers should be reported to enable historical comparison.

In terms of analysis, the '2/3' approach is comparable to the 'WHO/MMV' algorithm in low to moderate transmission settings, but may overestimate recrudescence rates for artemether-lumefantrine (AL) in high transmission settings. The '2/3' method ignores data from the 'third' discordant marker, which is a reasonable strategy when *msp1* and *msp2* agree; however, ignoring the other markers is not necessary if microsatellites are used in place of *glurp.* Match-counting is simple to use and does not disregard information from any markers; however, this method may underestimate recrudescence. The panel also considered Bayesian analysis, which has been applied to TESs conducted by the United States Centers for Disease Control and Prevention (US-CDC). The main advantage of this approach is that it provides a measure of uncertainty around the results. However, validation of the model used for Bayesian analysis is needed, including when AmpSeq data are used to distinguish between reinfection and recrudescence. Furthermore, the feasibility of using Bayesian analysis at the country level needs to be carefully assessed.

It is unclear what impact a change in methodology would have on the drug efficacy thresholds that are used to establish antimalarial treatment policy in countries and to support new drug approvals. Therefore, it is anticipated that a transition period will be required to generate comparative data for the different uses of this information. This should be considered in the context of expanding expertise and capacity in Africa for next generation sequencing. Furthermore, the broader trend towards genetic analysis of infectious diseases suggests that such approaches are likely to become more widely accepted and better understood over the next few years.

Recommendations

1) As an interim solution, *msp1* and *msp2* should continue to be used, but *glurp* should be replaced with a panel of two to three carefully chosen microsatellites (such as *Poly-α, Pfpk2* and *TA1*), customized to provide sufficient local diversity at least at the country level or regionally if possible. For simplicity and reasons of practical implementation, match-counting should be maintained as the primary analysis methodology for reporting. These methods should be applied in both low to moderate and high transmission settings in Africa. Bayesian algorithms may be applied for evaluation and comparison, but not for primary reporting. Outside Africa, the current method (*msp1/msp2/glurp*) should still be applied.

2) For a transition period, data should be analysed and reported using both the current (*msp1/msp2/glurp*) and new (*msp1/msp2/*microsatellites) methods to enable historical comparison and to understand the implications of the new methods in terms of thresholds for treatment policy change and introduction of new antimalarial drugs. Data transparency will be critical for comparative analysis and to provide a database for analytical methodology development.

3) As a medium-term (five-year) target, AmpSeq should be evaluated in parallel across Africa and outside Africa to compare it with current methods at sites and to validate whether this genotyping methodology should be adopted as the standard. A simple match-counting algorithm complemented by a Bayesian algorithm could be paired with this approach, but more comparative data are needed to inform the recommendation on the algorithm for analysing AmpSeq results.

Research needs

- Identify the most polymorphic microsatellite markers per country or region.
- Develop automated pipelines to enable TES design and interpretation in order to increase the feasibility of new methods.
- Explore alternative methods of model validation and different modelling approaches.
- Define a systematic process for validating genotyping methods and data analysis.
- Compare different genotyping and analysis methodologies using the same datasets.

- Evaluate the impact of Bayesian analysis on recrudescence rates in areas of high transmission, given the trend in increased recrudescence rates using this method, to determine whether this is an artefact of the method, whether there is some reason for higher failure rates in these areas (e.g., a high MOI may be more challenging for antimalarial drugs to clear), or whether there is emergence of true antimalarial resistance that needs rigorous confirmation.
- Evaluate the interaction between increases in the sensitivity of methods and the detection of gametocytaemia (rather than recrudescence).

1 Rationale

The WHO Global Malaria Programme (GMP) organized an informal consultation bringing together researchers and partners to review WHO guidance on the methodologies used to differentiate reinfection from recrudescence in *P. falciparum* antimalarial efficacy trials in sub-Saharan Africa. The methodologies used can affect the estimated drug failure rates in TESs, and thus has implications for recommendations around drugs for the treatment of *P. falciparum* malaria.

2 Background

Evaluating antimalarial drug efficacy in uncomplicated *P. falciparum* malaria is problematic because of the occurrence of new infections, particularly in areas of high transmission. This can lead to inaccurate estimates of therapeutic efficacy rates. Methods that accurately discriminate between recurrences caused by therapeutic failure (recrudescence) and reinfection are therefore needed. However, there is no gold standard for evaluating the ability of the different methodologies to correctly classify reinfection and recrudescence.

The challenges of classifying recrudescence versus reinfection are different in low to moderate and high transmission settings. In low to moderate transmission settings, parasite diversity is often minimal. Therefore, reinfections may be misclassified as recrudescence. High transmission settings present different challenges, given the relatively higher levels of both polygenomic infections in the population and the higher risk of reinfection during the study period. High infection rates increase the possibility that a reinfection will share alleles with the initial infection, causing the reinfection to be misclassified as a recrudescence. Conversely, a recrudescence might be misclassified as a reinfection because, with an MOI >1, low-frequency alleles may not be observed for a particular locus or may be erroneously classified as a different allele due to polymerase chain reaction (PCR) errors *(2,3)*. Consequently, there are biological limitations to the accuracy of drug efficacy estimates due to adjustments for reinfection. The sensitivity of the genotyping methods, the diversity of the markers used, and the classification algorithms applied all affect the degree of uncertainty around these estimates.

There are several different genetic markers and analysis techniques that can be used to differentiate individual parasite infections. Depending on the research question, however, each has both advantages and limitations. Specific laboratory techniques may be more appropriate for given settings and transmission levels. The cost and complexity of each technique and whether it can be performed in most countries or only in specialized research laboratories are key considerations. Recommended techniques and decision-making strategies should be feasible and simple, and provide operationally relevant information for malaria control programmes.

Current guidance

The current guidance for discriminating *P. falciparum* recrudescence from reinfection was published by WHO in 2008 *(1)*. Blood samples collected pre-treatment (day 0) and on the day of treatment failure

(day X) are compared using three genetic markers: *msp1, msp2* and *glurp*. A standard PCR genotyping methodology is recommended, including the use of CE. The decision algorithm (referred to hereafter as the 'WHO/MMV' method) requires the three markers to be genotyped and analysed in sequence, starting with *msp1*, followed by *msp2* and then *glurp*, and stopping once a marker has classified the paired samples as a reinfection. With this method, as soon as a new infection is identified with one marker, the overall outcome is a new infection. WHO has abandoned the sequential analysis and recommended genotyping the three markers systematically, enabling additional and comparative analyses.

Meeting of the Technical Expert Group on Drug Efficacy and Response, June 2017

This 2008 guidance was reviewed in 2017 at a meeting of the Technical Expert Group on Drug Efficacy and Response (TEG DER). The TEG DER also explored the challenges related to the use of *msp1, msp2* and *glurp*. Of the three markers, *glurp* was thought to be the least useful, as it may increase the proportion of true recrudescent infections classified as reinfections during clinical trials in high transmission settings. However, *glurp* could still be a valuable marker in regions of low malaria endemicity. Despite the limitations of *msp1* and *msp2*, continued use of these markers should provide the minimum essential information for correcting the data to reflect an accurate estimate of drug efficacy. While microsatellites may have their own limitations, these genotyping data were considered to provide similar results as those found using *msp1, msp2* and *glurp (4)*.

The TEG DER proposed a revised algorithm to distinguish between *P. falciparum* recrudescence and reinfection, termed the '2/3' algorithm. In this case, both *msp1* and *msp2* are genotyped for all samples. If *msp1* and *msp2* yield congruent results, the result is reported as the overall result of the genotyping (reinfection or recrudescence). However, if there is discordance between the *msp1* and *msp2* markers, a third marker should be genotyped. This marker could be *glurp* or another validated highly diverse gene. If this third marker supports one of the two previous results, the majority result (2/3) is reported as the overall result *(5)*. However, it was recognized that different groups and organizations use different markers, and that even when the same markers are used, genotyping and analysis methods may be inconsistent.

Informal consultation

The aim of this consultation was to collate comparative data, to give experts the opportunity to provide advice on changes needed to currently recommended methodologies, and to provide direction for the development of tools and methods for future use. Through this meeting, WHO sought to address research results generated by different methodologies for distinguishing between recrudescence and reinfection. Some of these research methodologies need further validation before they can be recommended for use in routine TESs. While waiting for the validation of new tools, the key expected outcome was an agreement on an interim common position on the genetic markers and analysis algorithm to be used by both countries and researchers to differentiate recrudescence from reinfection.

3 Introduction and Declarations of Interest

The list of participants is provided in Annex 1. All invitees attended the meeting, except for Daouda Ndiaye who sent apologies. Organizations invited as observers were The Global Fund to Fight AIDS, Tuberculosis and Malaria, Bill & Melinda Gates Foundation, Medicines for Malaria Venture (MMV), and the United States Agency for International Development (USAID). The meeting agenda is provided in Annex 2.

Only members of the expert panel and GMP Secretariat attended and took part in the final discussion and formulation of key recommendations. All 11 members of the expert panel submitted Declarations of Interest, which were assessed by the GMP Secretariat at WHO. Of the 11 experts on the panel, six declared no interest and five reported interests that were summarized in a report, which is available upon request. Based on this assessment, all the experts were able to fully participate in the discussion and deliberation of the consultation. Discussions were conducted under a confidentiality agreement signed by all participants.

4 Objectives

The primary objective was to discuss the most appropriate genotyping and analysis methods for reporting recrudescence and reinfection rates following antimalarial treatment of *P. falciparum* malaria in sub-Saharan Africa, with special focus on high transmission regions.

Specific objectives

- Review available data and assess the advantages and disadvantages of:
  - changing the markers used to differentiate recrudescence from reinfection;
  - changing the algorithms used to classify recrudescence and reinfection.
- Assess transmission settings where a change in the current methodology could improve the precision of classifying recurrent *P. falciparum* as recrudescence or reinfection.
- Discuss alternative tools for future use and suggest research needed to validate these tools.

5 Process and presentation

GMP convened this consultation to review the evidence and advise WHO on the most appropriate methods to differentiate recrudescence from reinfection in sub-Saharan Africa, with special focus on high transmission regions.

Background documents

In preparation for the meeting, WHO collected relevant publications on the topic, and manuscripts were shared by presenters (see Annex 3).

Presentations

Presentations, followed by a brief discussion, were made by Ingrid Felger and Christian Nsanzabana (Swiss Tropical and Public Health Institute, Basel, Switzerland); Eric Halsey, Mateusz Plucinski, and Venkatachalam Udhayakumar (US-CDC and President's Malaria Initiative, Atlanta, United States of America); Ian Hastings (Liverpool School of Tropical Medicine, Liverpool, United Kingdom of Great Britain and Northern Ireland); Jonathan Juliano (University of North Carolina, Chapel Hill, United States of America); Didier Ménard (Institut Pasteur, Paris, France); Daniel Neafsey (Broad Institute of MIT and Harvard, Cambridge, United States of America); and Pascal Ringwald (WHO, Geneva, Switzerland). Summaries of these presentations are included in Annex 4.

6 Evidence available and reviewed

Markers and genotyping

- In the 2008 document, WHO acknowledged the potential use of other markers as an alternative to *msp1*, *msp2* and *glurp*, but did not specifically recommend microsatellites *(1)*.
- For more than a decade, the US-CDC Malaria Laboratory has used neutral microsatellite genotyping, arguing that this approach can include more markers, has higher discriminatory power, and avoids confounding owing to selection pressure on the markers *(6)*.

- Two African countries (Mali and Uganda) use a combination of markers: *msp1*, *msp2*, and between one and four microsatellites including *CA1*, *TA87* and *TA99* (Mali), and *TA40*, *TA60, TA81* and *PfPK2* (Uganda).
-

Analysis algorithms and modelling

- Several molecular correction algorithms were assessed using *msp1, msp2* and *glurp* data provided by WHO from a large trial carried out in Rwanda and Cambodia. The model developed by the Liverpool School of Tropical Medicine indicated that the '2/3' algorithm was the most appropriate. Depending on the parameters and assumptions, a two-fold increase in the recrudescence rate was reported compared to using the 'WHO/MMV' algorithm. There was no significant difference in the recrudescence rate using data from Cambodia, a low transmission setting *(5)*.
- Using the same model developed by the Liverpool School of Tropical Medicine, simulation data were used to compare the two main approaches in order to analyse paired microsatellite data *(7)*:
    - match-counting – all detectable alleles in all tested loci are compared between day 0 and day of recurrent infection;
    - Bayesian algorithm – a probabilistic approach quantifies the probability that a sample is either a recrudescence or a new infection.
- WHO/GMP re-analysed all PCR corrections for which *msp1*, *msp2* and *glurp* data were collected to compare the 'WHO/MMV' and the '2/3' algorithms in different transmission settings.
- In collaboration with the Institut Pasteur Paris, WHO/GMP is evaluating the replacement of the *glurp* marker with one or more microsatellite markers or AmpSeq, as previously suggested *(7,8)*.

7 Conclusions and recommendations

The expert panel addressed the following key questions, with reference to the situation in sub-Saharan Africa, and put forward the following conclusions and recommendations for consideration:

1) What are the advantages and disadvantages of each of the following possible changes to the current methodology (considering simplicity and applicability at the country level)?

Changing markers

- Replacing one or more of the currently recommended markers (in particular *glurp*) with alternatives?
- Basing the reinfection/recrudescence classification on *msp1*, *msp2* and microsatellite data?
- Basing the reinfection/recrudescence classification on *msp1* and *msp2* data and AmpSeq?
- Basing the classification on microsatellite data only?
- Basing the classification on AmpSeq only?

Changing the analysis algorithm

- Changing to a 2/3 algorithm?
- Changing a simple match-counting algorithm to analyse microsatellite data using a Bayesian algorithm?
- Using another algorithm?

Conclusions

Each methodology for genotyping and analysis used to differentiate recrudescence from reinfection has advantages and limitations, and clearly give different results. This has important consequences because, in some cases, the difference in the number of recurrences identified as recrudescence drives the efficacy rate below 90%, which requires a change in treatment policy. In considering changing the current methodology, it is important to consider the following:

- A change in methodology could result in consistently low efficacy estimates for drugs in use or development. This could require revision of the threshold level for drug efficacy that prompts antimalarial treatment policy change in the country.
- It is important to balance the need for a methodology that can be carried out broadly across malaria-endemic countries and that will provide the minimum amount of information needed to inform decisions. Highly specialized laboratories can serve as reference centres to provide additional or more detailed information.
- AmpSeq is considered the method likely to be used for genotyping once this technology is more widely available. The transition to AmpSeq is anticipated once the method is validated and capacity is widely available.

The data examined clearly indicate that *glurp* is not an ideal marker and more discriminatory markers are needed. Microsatellite markers can provide an interim solution and a validated set of microsatellite markers should be evaluated for each country/region.

TESs are expensive and have important implications for drug treatment policy and drug development. It is critical to use methods that are reliable and reproducible across a range of transmission intensities. While underestimating recrudescence could lead to a delayed recognition of spreading drug resistance, it is important to recognize that overestimating recrudescence can also have negative consequences for patients and malaria control efforts. Prematurely and mistakenly changing a drug could mean less access to treatment and increased cost to malaria programmes.

Recommendations

The genetic comparison of initial and recurrent parasites to estimate recrudescence and reinfection rates remains a valuable component of TESs. However, the panel suggested some changes to the current approach.

- *msp1* and *msp2* should continue to be used, but the *glurp* marker should be replaced with a panel of two to three carefully chosen microsatellites (such as *Poly-α, Pfpk2* and *TA1*) with sufficient local diversity at the country level or ideally regional level.
    - The genetic diversity of the microsatellite markers requires verification/validation for each country/region, with a panel of validated microsatellites available from which countries can choose the most appropriate based on marker diversity in that area.
    - This method is currently being used for the analysis of *msp1/msp2/*microsatellites in Mali and Uganda. This approach would ideally use CE, but could also be done with agarose gel electrophoresis with validation.
- For simplicity and reasons of practical implementation, the match-counting analysis algorithm should be maintained as the primary analysis method. The Bayesian analysis approach should be additionally applied, whenever possible, for validation and for more thoroughly evaluating the advantages and limitations of this method compared to simpler approaches.
- For a transition period, data should be analysed and reported using both the current (*msp1/msp2/glurp*) and new (*msp1/msp2/*microsatellites and AmpSeq) methods to enable historical comparison and to understand the implications of the new methods, for example, in terms of thresholds for treatment policy change.
    - This transition period would permit cross-validation of detection methodologies.

    - This transition period would support the move from length polymorphism to microhaplotype methods, and provide evidence to evaluate simple counting analysis versus more complex probabilistic analysis.
- There is a need for transparency on the method used, and methods should be clearly described when reporting the data.
- All genotyping data should be reported in full to enable comparative and exploratory analyses.

2) In which transmission settings would a change in methodology improve the classification of recurrent *P. falciparum* infection as recrudescence or reinfection?

Conclusions

The same general methodology should be applied to all transmission settings within sub-Saharan Africa. However, there needs to be careful investigation of how the different methods perform at different transmission levels. Outside Africa, the methods could remain unchanged as per the current guidance, and countries could transition directly to AmpSeq (see below).

Recommendations

For sub-Saharan Africa, all studies should use *msp1* and *msp2* with a panel of two to three informative microsatellite markers (such as *Poly-α, Pfpk2* and *TA1*). Analysis should use the match-counting method. The details and rationale for this is described above.

3) Are there other tools in development that could be used for this purpose in the future?

- If so, what are the advantages and disadvantages compared to the recommended methodology?
- What research is needed to validate new tools?

Conclusions

AmpSeq appears to be the most robust and reliable genotyping method that could become an affordable and field-deployable technology in the medium term (within five years). However, current capacity in Africa is insufficient to recommend this technology as a standard. This could be addressed by capacity building in African countries or, because AmpSeq is most cost-effective with high throughput, by sending samples to African regional facilities or to central laboratories outside of Africa. However, care must be taken to ensure continued ownership for implementers. This could be achieved by processing samples in regional African centres or at central facilities, but siting the analysis and interpretation of data in the country of origin. Whole genome sequencing (WGS) and molecular inversion probes (MIPs) are perhaps further in the future in terms of deployment in Africa, but could be used in research settings.

From a scientific perspective, the analysis method provides a degree of uncertainty that is associated with the results. Methods that include uncertainty, such as Bayesian analysis, need further validation. There are additional concerns around how these methods can be practically implemented in countries. The communication of uncertainty in the Bayesian analysis may be challenging in a public health context. More comparative data with simpler analysis methods would enable an evaluation of the relative strengths and limitations of this approach.

The trend is moving towards genetic analysis of infectious diseases, and these approaches are likely to become more widely accepted and better understood over the next few years.

Recommendations for the future

- AmpSeq should be targeted as the next method for evaluating recrudescence and reinfection rates in TESs.
- These data could be analysed using simple match-counting at first, although the level of uncertainty for this method is not defined.

- The addition of a Bayesian algorithm provides a measure of uncertainty around the results and is tolerant to errors.
    - The validity and evaluation of the advantages of a Bayesian method need to be demonstrated at different transmission levels.
    - Automation could potentially compensate for the increased complexity of this approach.
- Drug development studies may offer an opportunity for initial application of these new methods in parallel with the standard methodology. Such studies would also generate comparative data for validation and provide evidence to regulatory authorities to increase acceptance of this approach.

Research recommendations

- Identify the most polymorphic microsatellite markers per country or region.
- Develop automated pipelines to enable TES design and interpretation in order to increase the feasibility of new approaches.
- Explore alternative methods of model validation and different modelling approaches.
- Define a systematic process for validating genotyping methods and data analysis.
- Compare genotyping and analysis methodologies using the same datasets.
    - Evaluate the performance of the different approaches in different transmission settings.
    - Use longitudinal data to confirm signals that suggest the emergence and spread of drug resistance.
    - Use fully validated drug resistance markers to improve the confidence in methodology for detecting recrudescence caused by drug resistance (e.g., compare data from day 0 and day of recurrent infection samples).
    - Use drug plasma level or ex vivo data to confirm if 'failing' drugs have reduced efficacy because of poor adherence or absorption, not because of resistance.
- Evaluate Bayesian analysis to more fully investigate initial findings of a trend of increased recrudescence rates in areas of high transmission in order to determine whether these findings represent a methodological artefact, whether there is some reason for higher failure rates in these areas (e.g., a high MOI may be more challenging for antimalarial drugs to clear) or whether there is emergence of true antimalarial resistance that needs rigorous confirmation.
    - Looking at the MOI versus recrudescence rate for the different markers/analysis methods could be informative, as well as for drug types and transmission levels.
- The interaction between increased sensitivity of methods and the detection of gametocytaemia (rather than recrudescence) requires evaluation.

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Annex 1. Participants

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Annex 2. Agenda

Monday 17 May 2021		
14.00–14.10 (CET)	Welcome 10 minutes	P. Alonso, Director GMP D. Wirth, Chair
14.10–14.15	Declarations of Interest	P. Ringwald
14.15–14.40	Objectives of the meeting Presentation of the WHO recommendations and conclusions of the TEG meeting 2017	P. Ringwald
14.40–15.30	New evidence on *msp1, msp2* and *glurp* as markers for reinfection and recrudescence	I. Felger, C. Nsanzabana
15.30–16.20	Microsatellites and Bayesian algorithm in Africa: added value of microsatellites, validation of Bayesian algorithm, comparison with counting methodology and impact on study results on TES in Africa	E. Halsey, M. Plucinski, V. Udhayakumar
16.30–17.20	Reinfection/recrudescence: pros and cons of available and potential new methods and feasibility within national programmes	D. Neafsey
17.20–18.10	Role of modelling to validate algorithms to distinguish reinfection from recrudescence	I. Hasting
Tuesday 18 May 2021		
14.00–14.50 (CET)	Experience of using *msp1, msp2* and *glurp* in different transmissions settings: comparison between sequential and 2/3 algorithm and impact on TES results	P. Ringwald
14.50–15.40	Comparison of *msp1*, *msp2* and *glurp* vs *msp1*, *msp2* and microsatellites vs *msp1*, *msp2* and amplicon sequencing in African samples collected in TES	D. Ménard
15.40–16.30	Comparison of whole sequencing vs *msp1*, *msp2* and *glurp* vs microsatellites using African samples	J. Juliano
16.45–17.45	Overall discussion	Expert panel, Secretariat, and Rapporteur
17.45–18.45	Formulation of recommendations	Expert panel, Secretariat, and Rapporteur
18.45	Closing remarks	P. Alonso, D. Wirth

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Annex 4. Presentations

Day 1

New evidence on *msp1*, *msp2* and *glurp* as markers for reinfection and recrudescence (I. Felger, C. Nsanzabana)

Limitations of current markers and genotyping methods

The currently recommended genotyping protocol is for multiplex nested PCR for *msp1, msp2* and *glurp* with CE high-resolution fragment sizing in an automated sequencer. However, some issues have arisen.

- Confusion around CE: In some cases, this has been interpreted as fragment sizing by Bioanalyzer, which is not sufficiently precise *(1)*. Fragment sizing should be performed in an automated sequencer.
- Definition of genotype bins: CE by automated sequencer discriminates a 1–2 base pair (bp) difference. Genotype bins of 3 bp are adequate for protein coding markers, and their boundaries are carefully selected based on discrete size steps when plotting a large number of fragments by size.
- Competition between allelic families: To avoid amplification bias, accuracy of genotyping outcomes should be improved through separate amplification reactions per allelic family *(2,3)*.
- Within-family competition also exists: PCR is biased towards short fragments. (Such bias in PCR was also reported for microsatellites during the meeting) *(2)*.
- Limitations of *glurp* as a marker: Long allele sizes increase competition; within one allelic family there is direct competition between all alleles; it is prone to stutter peaks, requiring increased cut-off limits and increasing the risk of excluding clones *(2)*.
- Markers *msp1* and *msp2* should be maintained, as both are highly diverse and amplification bias can be reduced (but not fully avoided) by using allelic-family-specific, single-tube PCR.

Adherence to the agreed WHO definitions of new infection and recrudescence is still insufficient, as evidenced by published reports of PCR correction. Automated sequencing should be used for maximum resolution of fragments. All PCRs should be performed separately to reduce template competition. Reporting of findings should include the cut-off for peak height, the strategy used for cut-off validation and the minority clone detection limit. In the wider context, external quality control activities should be encouraged for all laboratories and communication among genotyping laboratories should be enhanced as a platform for improvement.

Other markers and genotyping methods

The development and evaluation of five AmpSeq markers in samples from clinical trials was presented. The three best-performing markers were *cpmp*, *cpp* and *ama1-D3 (4,5)*. Despite high reproducibility in triplicates and robust detection of minority clones as low as 1%, this technique is operationally challenging (laborious pipetting, contamination prevention) and requires substantial technical expertise, particularly in bioinformatics.

The analytical sensitivity of five different genotyping techniques for minority clone detection was compared under standardized conditions at the Swiss Tropical and Public Health Institute using well-characterized laboratory strains (3D7, K1, HB3, FCB1):

- *msp1/2/glurp* by CE *(2)*;
- *msp1/2/glurp* by Bioanalyzer *(6)*;
- *TA60, TA40, TA81, PfPK2* by CE *(7)*;

- *ama-1-D3*, *csp*, *cpmp*, *cpp*, *msp7* by AmpSeq *(4,5)*;
- *msp1*/*msp2* by high-resolution melt analysis *(8).*

For each technique, minority clone detectability, robustness, time and cost were evaluated. CE and AmpSeq showed the highest sensitivity for detecting minority clones and are considered robust techniques. Microsatellites improved sensitivity in minority strain detection versus *glurp*. Note that these results may not be wholly representative of biological samples, and with all techniques the biological constraints remain.

Microsatellites and Bayesian algorithm in Africa: added value of microsatellites, validation of Bayesian algorithm, comparison with counting methodology and impact on study results on TES in Africa (E. Halsey, M. Plucinski, V. Udhayakumar)

Microsatellites

The US-CDC uses seven neutral microsatellites (*TA1*, *Poly-α*, *Pfpk2*, *TA109*, *TA2490*, *313_C2*, and *383_C3*) *(9–11)*. Ideally, reinfections would be identified by finding all different markers between day 0 and day X, and recrudescences by finding identical markers; however, there is usually a mixture. In fact, repeat genotyping of the exact same sample shows that variations can sometimes occur in microsatellite markers, even when none would be expected. These differences could be the result of measurement error, unobserved minority strains (often due to limited DNA concentration), and varying allele frequencies in a population.

Bayesian analysis

A good analytic approach should account for the variability outlined above, be reproducible, enable classifications to be generated based on published genotype data, and provide precision, with a measure of uncertainty around the classifications. To address these needs, the US-CDC has developed a Bayesian statistical approach that outputs a posterior probability of recrudescence based on an estimate of the degree to which the observed evidence supports reinfection versus recrudescence from microsatellite genotyping data. This approach incorporates parameters such as population frequencies, the probability of allelic suppression, and the error rate of fragment length measurement *(12)*.

The algorithm is implemented using a Monte Carlo Markov chain method, an open-source online tool investigators can use to calculate posterior probabilities without the use of additional statistical software. The algorithm has been used to analyse microsatellite data genotyping for US-CDC/PMI studies since 2015. Across 26 sites in nine African countries (Guinea, Benin, Democratic Republic of the Congo [DRC], Angola, Ethiopia, Uganda, Rwanda, Madagascar, Mozambique), the most recent PCR-corrected efficacy rates from each country were all over 90% except for:

- Busia, Uganda (AL 87%);
- Mikalayi, DRC (AL 86%, DP 84%);
- Lunda Sul, Angola (AL 88%).

Conclusions

The advantage of using a Bayesian approach is the ability to jointly estimate the posterior distribution of the probability of recrudescence, as well as the unknown functions and parameters and hidden (unobserved) alleles. Therefore, it is possible to report the uncertainty around antimalarial drug efficacy estimates.

Consistency in approach and transparency in reporting methods are necessary, with the reporting of full genotype data, in order to enable alternative analysis. New genotyping methods require new statistical approaches and algorithms. Because of the differences in methods over time, trends in efficacy should be interpreted with caution.

Reinfection/recrudescence: pros and cons of available and potential new methods and feasibility within national programmes (D. Neafsey)

Comparison of genotyping methods

There are four approaches available: *msp1/msp2/glurp* genotyping, microsatellites, AmpSeq and WGS. The advantages and disadvantages of each were compared based on the following criteria and are summarized below.

Sensitivity

- Absolute sensitivity: Will the assay detect ANY parasites in the sample?
- Minor strain sensitivity: Will the assay detect ALL parasites in the sample? *(8).*

Reproducibility *(7,8,13)*:

- Is the assay reliable?
- Is there opportunity for subjective interpretation?
- How comparable are results between different studies?

Information content:

- Are the assay targets sufficiently diverse in the local population? Marker number and heterozygosity reduce uncertainty, particularly in polyclonal infections *(14)*. AmpSeq targets can be highly diverse *(4,15)*.
- Marker number and heterozygosity are fungible, but higher marker number decreases feasibility.

Feasibility:

- Equipment, expertise, cost and analysis requirements in the setting in which it is to be applied need to be considered.
- Targeted next generation sequencing is uncommon in Africa, but centres of excellence are already being established to provide high-quality sequencing services for several laboratories and research groups *(16)*. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has also highlighted the need for greater capacity for genomic analysis.

Summary:

Method	Sensitivity (absolute)	Sensitivity (minor strains)	Reproducibility	Information content	Feasibility
msp1/msp2/glurp	High	5:1	Poor (gels) Medium (CE)	High	Very high
Microsatellites	High	5:1	Poor (gels) Medium (CE)	High	Very high
AmpSeq	High	100:1	High	High	High
WGS	Medium	~20:1	High	Very high	Medium

Analysis considerations

- Probabilistic infection classifications can capture uncertainty with any method, enabling comparison across studies/methods if genotyping error profiles are understood.
- All probabilistic infection classification approaches require knowledge of the diversity of the markers in the local parasite population. With AmpSeq, this can be done from locally generated data, and whole genome data are available for parasites in many sites.
- Error profiles are differentially quantifiable by genotyping method: AmpSeq error profiles are more measurable and so more easily incorporated into the analysis.
- Marker number and marker heterozygosity reduces uncertainty, particularly in polyclonal infections.
- Cloud-based common analysis pipelines can assist in transparency and portability.

Conclusions

Overall, AmpSeq is the best genotyping method, although issues around affordability and skills acquisition need to be addressed for its use in Africa. However, the advantages of this method should incentivize the community to address the feasibility challenges.

Role of modelling to validate algorithms to distinguish reinfection from recrudescence (I. Hasting)

Mechanistic pharmacokinetic/pharmacodynamic modelling

A model was developed to estimate the antimalarial cure rate on a per-infection (clone) basis *(17)*. This is particularly relevant in high transmission areas where the MOI is >1 and individuals may experience multiple reinfections that may or may not be cleared *(18)*. Genotypes were added to each clone randomly and 5000 patient simulations were conducted. Note that within simulations the 'true' failure rate is known so the performance of the different genotyping methods can be evaluated.

msp1*, *msp2* and *glurp

Four analysis methods using *msp-1*, *msp-2* and *glurp* were evaluated *(17)*:

- WHO/MMV: Initial and recurrent samples must share alleles at all three markers for recrudescence.
- No *glurp*: Same as for WHO/MMV but based on *msp1* and *msp2* only.
- 2/3 markers: Initial and recurrent samples must share alleles for at least 2/3 markers for recrudescence.
- Allelic family switch: Identical alleles in *msp1* and *msp2* indicate recrudescence, and absence of shared alleles in both markers indicate reinfection. If markers are discordant, a complete allelic family shift in the no-sharing marker is required to classify reinfection.

The WHO/MMV method tended to underestimate the true failure rate by a factor of two. The 2/3 method most closely matched the true failure rate because the misclassification of recrudescence as reinfection and vice versa was more closely balanced. None of the algorithms correctly classified all recurrent infections.

Microsatellites

Seven microsatellite markers and their allele distributions from three sentinel sites in Angola were examined and classified using a simple match-counting algorithm or Bayesian analysis *(19)*.

- The match-counting method was unstable and unreliable.

- The Bayesian algorithm was unable to accurately identify low-density recrudescence, but this did not appear to compromise its utility as a highly effective molecular correction method for analysing microsatellite genotypes.

The model findings were largely consistent with field data *(12)*. The US-CDC Bayesian algorithm does not model for the possibility of spurious allele readings in the data (false positive); this effect would increase with the MOI and force Bayesian statistics to infer more 'missing' alleles. Therefore, the distribution of the posterior probabilities should be checked.

Deep sequenced amplicons

Five markers were evaluated (*cpmp*, *ama1-D3*, *cpp*, *csp*, *msp-7*), with allelic diversity obtained from published sources *(20)*. Recrudescence was determined by counting the number of markers with a shared allele between the initial and recurrent samples.

- By using *cpmp*, *ama1-D3* and *cpp* and a matching threshold of ≥2 or =3 to classify recrudescence, the method returned highly robust estimates under a range of MOI distributions and force of infection values.
- This method has not been compared to field data.

Conclusions

- The advantage of using AmpSeq over other markers is the improved ability to detect genetic signals from low-density clones.
- A simple matching algorithm for three amplicons provided robust estimates of recrudescence and reinfection across a range of simulated transmission settings.
- False positives have not been addressed in the Bayesian algorithm.
- Bayesian analysis has not been tested in this model for AmpSeq.

Day 1: Discussion

Sequential WHO/MMV versus 2/3 decision algorithm

The WHO/MMV sequential analysis method allows early stopping. The 2/3 method might have cost implications in some settings. However, with this algorithm, *msp1* and *msp2* can be done together and then *glurp* is only done if the findings from *msp1* and *msp2* are discordant. The primary limitation of *glurp* is its size, although it does have extensive genetic diversity. Therefore, it has some discriminatory value, particularly in low transmission zones where there is no MOI. The advantage of 2/3 is that if the initial and recurrent *msp1* and *msp2* share the same genotype, then the possibility of it being a recrudescence is high and *glurp* can be discounted.

According to modelling, the 2/3 method superficially appears to provide better classification than the WHO/MMV method. This is only because the misclassification errors are more balanced between reinfection and recrudescence, whereas MMV/WHO is more likely to classify recrudescence as reinfection. The major limitation of the 2/3 algorithm is that even if one of the markers clearly indicates a reinfection, it is ignored. This method also appears to overestimate recrudescence for AL in high transmission areas, so would not be appropriate for use across all transmission settings and for all antimalarial drugs. When applied to *msp1/msp2/glurp*, the 2/3 algorithm gives equal weight to *glurp* when there is discordance between *msp1/msp2*, even though *glurp* is a suboptimal marker.

AmpSeq and the detection of minority clones

Although the different genotyping techniques use different methods to identify recrudescent parasites versus reinfection, they can be compared on their ability to detect minority clones.

The ability to more effectively detect minority clones might affect the threshold for determining whether a drug is failing. In addition, low-density recrudescences that would previously have been missed might be identified, although these may not be clinically relevant. AmpSeq is not significantly more sensitive than CE; if only samples from patients with microscopically determined infection are analysed, then these represent clinically significant parasitaemia levels. However, there may be some very low-density minority infections identified that may change the outcome of the genotyping.

The parasite most likely detected on day 0 is the dominant clone responsible for the clinical symptoms of malaria; although minority clones may be present, these parasites were not the cause of the clinical disease. So, if the aim is to determine if the drug cleared the parasite responsible for clinical disease, then only the majority clone at day 0 will be relevant. However, the detection of minority clones is important because a minority clone at day 0 could be the dominant clone at day 28. Additionally, any clone that survives to cause a recurrence is the one most likely to be drug-resistant. Therefore, identifying and clearing the minor clones is important for preventing the spread of resistance within the population.

With more markers, there is the opportunity to resolve parasites that are highly similar to each other but not identical. However, this might be an issue in lower transmission settings where parasite diversity is likely to be more limited. In sub-Saharan Africa, in many settings with moderate to high levels of transmission, most parasites are sufficiently dissimilar so that a modest panel of markers could suffice. Ongoing comparisons with WGS data could help to establish the optimal number of markers.

Implications of sensitivity

The sensitivity required depends on the question being asked, and sensitivity might be limited by the facilities available in a country versus in a central laboratory. However, if a method is too sensitive, there is a possibility that gametocytes might be detected. This would undermine the reliable estimation of drug efficacy, as most antimalarial treatments do not clear gametocytes.

Microsatellite genotyping analysed using a Bayesian algorithm

The variation in microsatellites across the population requires consideration. The Bayesian algorithm is tuned to population-specific allele frequencies of the microsatellite markers, and a match with two frequent alleles is not weighted the same as a match with two rare alleles. The algorithm can also incorporate unpaired day 0 samples to enrich the information on allelic frequencies.

The microsatellite markers used by the US-CDC were chosen after pilot testing of various markers. The combination of the seven markers has a high discriminatory power to identify different clones. The US-CDC has collected a large body of data on the diversity of the seven microsatellites that they use and would be happy to share those data with the group.

The US-CDC switched from *msp1/msp2* to microsatellites because, in highly endemic settings, although they were getting fragment lengths that were identical, when they sequenced the samples, they found that they were different parasites. With seven markers, if there is a high MOI, it may be

difficult to obtain a complete set of information for all clones. However, seven markers were chosen to provide the diversity needed to discriminate parasite types across all study sites, so that one set of markers could be used for all study sites.

Characteristics of the Bayesian algorithm

An advantage of the Bayesian approach is that informative priors can be assigned. The current algorithm is run with uninformed priors, so every treatment failure is treated as equally likely to be a recrudescence or reinfection. However, it is possible to modify the model for the likely outcomes for a particular site. All of the data are run on a site-specific basis at present because of the variability in allele frequencies between sites. The Bayesian approach has been applied to 30 studies across 11 sites, peer-reviewed, made open-source and investigated in the pharmacokinetic/pharmacodynamic model.

The finding that the same samples give different genotyping results indicates either that things are being missed or that these are false positives. The influence of false positives on the Bayesian approach was raised. There are two things that increase the probability of a false positive or a chance match: 1) when there are very frequent alleles; and 2) high MOI because of the high numbers of comparisons. The latter in particular is a characteristic of high transmission settings.

The possibility of automation was discussed. Georgia Tech has already coded the algorithm in Python and it is publicly available and open-source on a web interface. The aim going forward is to integrate this into an analytic pipeline where the data are generated, automatically analysed and outputted in a standardized and understandable format. Explaining the rationale and methods for genotyping to malaria programmes is complex. However, the US-CDC has provided seminars for countries on microsatellite genotyping and Bayesian analysis where further information has been requested.

Pharmacokinetic/pharmacodynamic modelling

The Bayesian algorithm has not been applied to *msp1, msp2* and *glurp* within this model.

The model assumed failure rates of 10%. At this level, the misidentified recrudescences and misidentified reinfections were balanced with the 2/3 method. At higher failure rates, although this balance might be disturbed, the drug would still be identified as a failing drug.

The model assumes that everyone attends their assessments on time. This is because the timing of the assessment influences the number of clones detected. If there is a long gap between follow-up visits, it provides time for the minority clones to emerge and replicate, so the MOI will increase.

In low transmission areas, reinfection by related parasites is more likely, so three amplicons might not be enough to discriminate recrudescence from reinfection. The model is focused on moderate to high transmission, so this aspect has not been considered. However, co-transmission has been observed in high transmission areas, so it might be useful to examine how the degree of relatedness might affect the model outcomes.

Both *msp1* and *msp2* are subject to immune pressure, whereas microsatellites are neutral markers. The effect of immunity on the model has not been investigated relative to the decision algorithm. However, US-CDC has done an in-house adaptation of the Bayesian algorithm for *msp1/msp2/glurp* and the Bayesian estimates very closely match the 2/3 approach.

Immunity is not built into the model because if patients have clinical malaria, it is assumed that they are immunologically susceptible to that parasite. However, this does not consider the possibility that other parasites are present but are immunologically suppressed.

In the model, the Bayesian algorithm can produce profiles with mostly clear recrudescences or reinfections, which is generally consistent with field data. However, some field data show a significant number of intermediate values for the posterior probabilities. At present, the reasons for this pattern have not been explored in the model. However, intermediate results from the Bayesian algorithm are a flag that the genotyping data are not fully discriminatory. This is useful information and can enable identification of samples for further downstream analysis, for example, *msp1/msp2* for samples with intermediate microsatellite results. An advantage of the probabilistic approach is that it incorporates uncertainty into the final efficacy calculations; for example, in the efficacy calculations, a late treatment failure with a posterior probability of recrudescence of 0.35 would count as 35% of a recrudescence.

The key factor determining the outcomes of the model is the sensitivity of the different methods to detect minority clones.

Communicating the outputs of different analysis methods

Regarding interpretation, there was a concern that the Bayesian approach to analysing microsatellites generally reports decreased efficacy for antimalarial drugs compared to the *msp1/msp2/glurp* WHO/MMV approach. Should this decrease be regarded as a concerning sudden drop in drug efficacy, or should it prompt a change in the threshold for drug efficacy (currently 90%)? Although the Bayesian approach does not always decrease efficacy, this issue highlights that any change in methods that gives rise to a change in efficacy presents a communication challenge. The methods and discussion need to make clear that the methods and analysis are not comparable with drug efficacy data obtained in previous studies. However, communicating these issues in a reasonable and credible manner is something that requires careful thought.

Operational considerations

Although AmpSeq appears to be the best method for genotyping, there were questions around whether it would be feasible to deploy AmpSeq to study sites, or whether it would be best at a limited number of central laboratories. There are several examples of Illumina sequencing being performed at central sites in Africa.

Most public health groups would be comfortable sending samples to a central centre for analysis. However, some countries may not want samples sent outside the country for processing, but may not have the capacity for AmpSeq.

There are also supply chain issues (e.g., cold-chain provision of sequencing reagents) that might limit the development of these capabilities in every country with a TES. One issue is that AmpSeq is not cost-effective for small numbers of samples, for example, for repeating an assay; batches of several hundred samples are required. Therefore, for these reasons, a 'centres of excellence' approach or regional hubs for AmpSeq is a more viable strategy in Africa.

A further advantage of AmpSeq is that the same platform can be used to detect mutations associated with drug resistance. This offers the potential to integrate drug efficacy and drug resistance data. SARS-CoV-2 has highlighted the need for capacity in genome sequencing, which can be leveraged to establish AmpSeq as the preferred approach to evaluating drug efficacy.

The Bill & Melinda Gates Foundation has been supporting the Africa CDC to look at the role it could play as a continent-wide organizer for pathogen genomics. Before the SARS-CoV-2 pandemic, they were planning on setting up a series of six laboratory sites across Africa. These are not public health laboratories, but are more closely identified as research institutes. The laboratories are within institutions where genomics research has been previously conducted. In phase I, the plan was for these laboratories to work on a range of pathogens, but their efforts are currently directed mainly towards SARS-CoV-2 genomics. However, the pandemic has accelerated capacity in these laboratories, with tens of thousands of SARS-CoV-2 genomes generated largely on the Illumina platform. It has also driven the establishment of shared procurement networks. Africa CDC is looking to return to conducting routine surveillance for a range of pathogens, including malaria. In particular, histidine rich protein 2 gene deletion and drug resistance surveillance are priorities. It will likely be a least a year before the laboratories can transition from their focus on SARS-CoV-2. However, these laboratories represent a potentially impactful network of sites. There are advantages to being under the umbrella of Africa CDC, such as the authority of the organization, which promotes Member States' willingness to share data and cooperate, and shared procurement mechanisms; procurement is a major challenge for the establishment of any laboratory facility in sub-Saharan Africa. This network of laboratories supported by Africa CDC is a potentially valuable asset for deploying AmpSeq for antimalarial drug efficacy studies across Africa.

Day 2

Experience of using *msp1, msp2* and *glurp* in different transmissions settings: comparison between sequential and 2/3 algorithm and impact on TES results (P. Ringwald)

Methods

For 145 TESs conducted between 2010 and 2019, the results of PCR correction (*msp1, msp2* and *glurp*) using the WHO/MMV algorithm were compared to the 2/3 algorithm for 1086 recurrences of *P. falciparum* malaria (2332 samples). Overall PCR success for each marker was high (*msp1* 96.0%, *msp2* 94.0%, g*lurp* 93.6%).

Low to moderate transmission areas outside Africa (Greater Mekong, Middle East and South America)

A total of 60 TESs were conducted from 2011 to 2019 in Cambodia, Lao People's Democratic Republic (PDR), Myanmar, Viet Nam, Pakistan, Yemen and Guyana monitoring artesunate-amodiaquine (ASAQ), artesunate-mefloquine, artesunate-pyronaridine, artesunate+sulfadoxine-pyrimethamine (ASSP), artemether-lumefantrine (AL), dihydroartemisinin-piperaquine (DP), and artesunate monotherapy (297 patients, 587 samples). Failures classified as recrudescences were 86.5% (244/282) with WHO/MMV versus 94.3% (266/282) with 2/3 (p=0.002); the discrepancy was caused by *glurp* in 72.7% of cases.

- Kaplan–Meier efficacy estimates were ≤2% lower for 2/3 than for WHO/MMV, except:
    - DP in Dak Lak (Viet Nam) in 2019: 35.9% with MMV/WHO versus 28.4% with 2/3.

For 93 samples from Cambodia (2014–2016), *P. falciparum* 10-single nucleotide polymorphism (SNP) barcodes were performed. For two discordant samples (MMV/WHO reinfection; 2/3 recrudescence), one was a reinfection, and one was a recrudescence.

Low to moderate areas in the Horn of Africa

A total of 33 TESs were conducted from 2010 to 2019 in Eritrea, Somalia and Sudan monitoring ASAQ, ASSP, AL and DP (175 patients, 350 samples). Failures classified as recrudescences were 54.4% (92/169) with WHO/MMV versus 71.6% (121/169) with 2/3 (p<0.001); the discrepancy was caused by *glurp* in 58.6% of cases.

- Kaplan–Meier efficacy estimates were ≤3.4% lower for 2/3 than for WHO/MMV, except:
  - ASSP in Sinnar (Sudan) in 2011: 98.9% with MMV/WHO versus 94.4% with 2/3.

Low to moderate transmission areas in West Africa

A total of 10 TESs were conducted from 2010 to 2018 in Gambia and Mauritania monitoring ASAQ, AL and DP (27 patients, 53 samples). Failures classified as recrudescences were 57.7% (15/26) with WHO/MMV versus 69.2% (18/26) with 2/3 (p=NS); the discrepancy was caused by *glurp* in 100% of cases.

- Kaplan–Meier efficacy estimates were ≤3.0% lower for 2/3 than for WHO/MMV.

Moderate to high transmission in West Africa

A total of 14 TESs were conducted from 2011 to 2018 in Liberia, Sierra Leone and Togo monitoring ASAQ, AL and DP (91 patients, 174 samples). Failures classified as recrudescences were 16.7% (14/84) with WHO/MMV versus 50.0% (42/84) with 2/3 (p<0.001); the discrepancy was caused by *glurp* in 89.3% of cases.

- Kaplan–Meier efficacy estimates were ≤2.4% lower for 2/3 than for WHO/MMV, except:
  - ASAQ in Montserrado (Liberia): 93.7% with MMV/WHO versus 84.9% with 2/3;
  - AL in Bo (Sierra Leone): 100% with MMV/WHO versus 94.4% with 2/3;
  - AL in Eastern (Sierra Leone): 100% with MMV/WHO versus 94.6% with 2/3;
  - AL in Kara (Togo): 97.3% with MMV/WHO versus 93.2% with 2/3.

Moderate to high transmission in Central Africa

A total of 13 TESs were conducted from 2010 to 2018 in Congo, DRC and Equatorial Guinea monitoring ASAQ and AL (84 patients, 168 samples). Failures classified as recrudescences were 29.8% (25/84) with WHO/MMV versus 53.6% (45/84) with 2/3 (p<0.001); the discrepancy was caused by *glurp* in 75.0% of cases.

- Kaplan–Meier efficacy estimates were ≤2.3% lower for 2/3 than for WHO/MMV, except:
  - AL in Haut Katanga (DRC): 97.6% with WHO/MMV versus 92.2% with 2/3;
  - AL in Ebibeyin (Equatorial Guinea): 93.7% with WHO/MMV versus 84.5% with 2/3;
  - ASAQ in Ebibeyin (Equatorial Guinea): 98.7% with WHO/MMV versus 93.6% with 2/3.

Low to moderate South-eastern Africa

A total of nine TESs were conducted from 2012 to 2015 in Burundi and Rwanda monitoring ASAQ, AL and DP (218 patients, 420 samples). Failures classified as recrudescences were 14.4% (27/187) with WHO/MMV versus 28.3% (53/187) with 2/3 (p<0.001); the discrepancy was caused by *glurp* in 53.8% of cases.

- Kaplan–Meier efficacy estimates were ≤2.7% lower for 2/3 than for WHO/MMV, except:
  - AL in Eastern 2012 (Rwanda): 97.1% with WHO/MMV versus 92.2% with 2/3;

- AL in Kigali (Rwanda): 95.7% with WHO/MMV versus 90.9% with 2/3;
- AL in Eastern 2013 (Rwanda): 99.3% with WHO/MMV versus 95.4% with 2/3;

Summary

Overall, the proportion of recurrent parasitaemia classified as recrudescence was higher with the 2/3 algorithm (68.8%) than with the WHO/MMV method (46.0%) ($p<0.001$). However, this did not always translate into a significant difference in Kaplan–Meier estimates of treatment outcome (i.e., >4% difference, 12/145 studies).

- Differences in the Kaplan–Meier estimates of treatment outcome were more evident in areas of moderate to high transmission than in areas of low to moderate transmission.
- AL was the most affected drug (66.7%) > ASAQ (16.7%) > ASSP and DP (8.3%) based on a failure rate
  >4%.
- The main marker leading to discordance between the two analyses was *glurp* (68.2%) > *msp2* (16.2%) and *msp1* (15.6%).

Conclusions

- As a marker to discriminate recrudescence from reinfection, *glurp* is inadequate and has a disproportionate influence on the outcome of the analysis algorithms. These data support abandoning *glurp* as a marker.
- The disproportionate effect on AL efficacy from using the 2/3 method versus the WHO/MMV method is concerning, given that lumefantrine has the shortest half-life of any partner drug. In the absence of a 'gold standard', this analysis does not provide evidence that 2/3 is an appropriate method for evaluating antimalarial treatment outcomes in high transmission regions. The other possible interpretation is that lumefantrine resistance has emerged, but it seems odd for it to emerge only in high transmission areas.

Comparison of *msp1*, *msp2* and *glurp* vs *msp1*, *msp2* and microsatellites vs *msp1*, *msp2* and amplicon sequencing in African samples collected in TES (D. Ménard)

Methods

Markers were evaluated from paired samples obtained from TESs conducted between 2016 and 2019 in countries with low and high endemicity.

- *msp1*, *msp2* and *glurp* with bands detected using gel electrophoresis (60%) or CE (40%): For gel electrophoresis, bands were considered different between initial and recrudescent samples if the size of the bands differed >20 bp for *msp1/msp2* and >50 bp for *glurp*, and for CE, if the size of the bands differed >10 bp for msp1/msp2 and > 20 bp for glurp.
- Microsatellites (*Poly*-α, *Pfpk2*, *TA1*) with bands detected using CE *(21)*: Bands were considered different between initial and recrudescent samples if the size of the bands differed >5 bp.
- *ama1*, *cpmp*, *cpp* polymorphic genes detected by AmpSeq *(5)*.

Different combinations of markers were analysed using either the WHO/MMV or the 2/3 algorithm both overall and for low transmission areas (Eritrea [n=23] and Gambia [n=2]) and high transmission areas (Burundi [n=30], Congo [n=3], Equatorial Guinea [n=14], Liberia [n=20]).

Markers

- The degree of concordance within markers was 38% for *msp1/msp2/glurp*, 58% for microsatellites and 63% for AmpSeq.

- The degree of concordance between markers was highest with AmpSeq, with 29% of results classified as 'intermediate' compared to 42% for microsatellites and 62% for *msp1/msp2/glurp*.

Algorithms

There was considerable variation in the percentage recrudescence rates determined using the different combinations of markers and algorithms (MMV/WHO or 2/3):

- 26% *msp1/msp2*/microsatellites (MMV/WHO); 29% *msp1/msp2/glurp* (MMV/WHO); 33% *msp1/msp2*/AmpSeq (MMV/WHO); 37% microsatellites (MMV/WHO); 50% *msp1/msp2* (MMV/WHO); 65% AmpSeq (MMV/WHO); 70% *msp1/msp2/glurp* (2/3).
- In low transmission areas, there was no significant difference in recrudescence rates between the different marker/algorithm combinations, with outcomes ranging from 52% for *msp1/msp2*/microsatellites (MMV/WHO) and microsatellites (MMV/WHO) to 80% for *msp1/msp2/glurp* (2/3) and *msp1/msp2* (MMV/WHO).
- By contrast, in high transmission areas, the range of outcomes was broader, ranging from 13% for *msp1/msp2/glurp* (MMV/WHO) to 66% for *msp1/msp2/glurp* (2/3).

Conclusions

- AmpSeq was the most robust technique with less discordance than with microsatellites or *msp1/msp2/glurp*.
- Adding *msp1/msp2* to microsatellites or AmpSeq increased the percentage of new infections in moderate to high transmission areas.
- AmpSeq is simple, rapid and accurate, but the equipment can be costly and it is more expensive if only a few samples are tested.
- The algorithm applied to determine recrudescence rates had no significant effect in areas of low endemicity, but in areas of high transmission with the same markers (*msp1/msp2/glurp*), the recrudescence rate was 66% with the 2/3 algorithm versus 13% using WHO/MMV.
- Although AmpSeq (WHO/MMV) and *msp1/msp2/glurp* (2/3) gave similar recrudescence rates, the results for the individual samples were not concordant between the two methods (concordance = 61%, 56/92 paired samples).
- In high transmission settings, ideally AmpSeq would be used, but microsatellites could be applied if AmpSeq is not operationally feasible.

Note: This analysis was performed on a random sample and the impact on therapeutic efficacy rates cannot be estimated. However, expanding the study to include all the TES data could provide these data and enable assessment of the effect of the different genotyping and analysis methods on drug efficacy rates.

Comparison of whole sequencing vs *msp1*, *msp2* and *glurp* vs microsatellites using African samples (J. Juliano)

Methods

A TES was conducted at six sites in DRC in 2017/18 evaluating AL, ASAQ and DP in children with uncomplicated *P. falciparum* malaria. PCR correction employed seven neutral microsatellites analysed using two methods:

- Counting-based method: recrudescence defined as a pre-identified number of identical alleles between day 0 and day of failure sampled across the seven loci (7 of 7 match);
- Bayesian statistical method: a statistical algorithm assigns a probability of recrudescence to each late treatment failure given the observed data.

Findings and further investigation

The counting method resulted in higher PCR-corrected efficacy rates than the Bayesian approach, and, in Mikalayi, the Bayesian algorithm returned therapeutic efficacies less than 90% for AL and DP. These findings were investigated further using the following approaches to estimate antimalarial efficacy for AL (n=34), ASAQ (n=20) and DP (n=39) and the complexity of infection (COI) in Mikalayi:

- *msp1/msp2/glurp* using either a strict bandwidth (*msp1* and *msp2* binned at 3±1.5 bp size and *glurp* at 20±10 bp) or a wide bandwidth (*msp1* and *msp2* binned at 3±5 bp size and *glurp* at 20±50 bp) and analysed using the WHO/MMV or the 2/3 algorithm;
- microsatellites (*TA1*, *Poly-α*, P*fpk2*, *TA109*, *TA249*0, *C2M34* and *C3M6*9) analysed using a Bayesian algorithm;
- MIPs *(22)*: A genome-wide SNP image barcode (IBC) was used to examine *P. falciparum* parasite relatedness and COI *(23,24)*, and microhaplotypes from the *P. falciparum* heterozygome (HeOME) were used to examine *P. falciparum* parasite relatedness and COI *(25)*.

Interpretation of COI was defined as:

- WHO: the number of alleles detected at the most diverse of the three genotyped loci;
- microsatellites: the number of alleles detected at the most diverse of the seven genotyped loci;
- HeOME/IBC: calculated using a Markov Chain Monte Carlo method (THE REAL McCOIL) *(26)*.

Summary

- The IBC and HeOME data are incomplete, but at present show similar efficacy rates to the standard *msp1/msp2/glurp* (WHO/MMV) method, and higher efficacy than with microsatellites (Bayesian) or *msp1/msp2/glurp* (2/3) for all three drugs. However, further work is needed to refine the analysis for IBC and HeOME.
- The WHO/MMV method had the highest mean COI (3.4 clones), followed by HeOME (2.8 clones), IBC (2.2 clones), and microsatellites (2.1 clones). The higher value for the WHO/MMV method was driven predominantly by *msp1*.

Conclusions

- In high transmission settings, efficacy estimates are impacted by the genotyping method and the interpretation method, and the 'true' value cannot be determined. Consequently, there is a need to account for uncertainty, and this may become more important in next generation sequencing.
- Probabilistic infection classification will be needed to account for the underlying population distribution of SNP/haplotypes; missing genotype loci or SNP haplotypes in the data; depth of analysis at each loci; and parasitaemia of the sample (risk of false positives).
- Higher multiplex and WGS methods may be more appropriate in low to moderate transmission settings where within-host complexity decreases but parasites within the

population are more highly related. A smaller number of highly diverse targets is likely superior in high transmission settings.

Day 2: Discussion

Microsatellites

Analysis of microsatellites using the Bayesian algorithm for the Mikalayi (DRC) data resulted in a decrease in the adjusted efficacy rate compared to the counting method; this was particularly evident for AL and DP, although the difference for ASAQ was much smaller.

The choice of microsatellites is important, as some are not diverse enough to be useful for this analysis. The distribution of the microsatellite sizes for Mikalayi was around 20–35 bins for most of the microsatellites, following a generally normal distribution; however, one microsatellite was less diverse with seven bin sizes (*TA2490*). Even in high transmission settings, there is not an insignificant amount of sharing of alleles, so the lower efficacies may be partially driven by that effect. For example, in the SNP data, only around 100–200 out of the 500 SNPs analysed show differences; because there are so many that are heterozygous at those locations, there is a lot of sharing, i.e., the high COI causes both alleles to be present in all the samples.

The choice of microsatellites would need to be justified by the data showing the diversity at the sites where the samples were collected. There may be scope for determining a particular combination of microsatellites or choosing which microsatellites to use for a particular area.

The bins used for microsatellite data were 5 bp or 7 bp, while some strains differed for microsatellites with only 3 bp. Therefore, wider bins tend to overestimate recrudescence by including artificially similar strains that are actually different in the same bin. Using a Bioanalyzer, sensitivity is lower, so that a wider range of bins is needed for the different haplotypes. Where the strict and wide binning approaches were applied (J. Juliano presentation), it was clear that the matches differed between the two methods with a wider bin width increasing the number of recrudescences.

Instead of using a wide bin range, the percentage of the highest peak can be used to remove most of the stutter peaks. This strategy was used for *msp1* and *msp2* to try to remove most of the stutter peaks, even though this also removed some of the repeats, with thresholds of 10% for *msp1/msp2* and 20% for *glurp*. Therefore, although this strategy decreases the possibility of false positives, it also decreases the sensitivity, meaning that some minority strains will be missed. It is not clear where the optimal balance lies, but it may depend on the transmission characteristics of the study site.

Use of *glurp*

Although *glurp* has limitations as a marker, when using the 2/3 method, *glurp* impacts the outcome only when *msp1* and *msp2* are discordant. This is in contrast to the WHO/MMV method in which *glurp* can indicate a reinfection even if *msp1* and *msp2* both indicate recrudescence. Therefore, it might be a good compromise to retain *glurp*, but to limit its impact on the overall outcome.

However, the data indicate that *glurp* is not an optimal marker for discriminating reinfection from recrudescence. The main driver of the discordance between the 2/3 and WHO/MMV methods was

glurp, which suggests that there is a problem with this marker. One option is that *glurp* could be abandoned and replaced with, for example, microsatellites as an interim solution. Although there is some template competition for some microsatellites, it is not to the same extent as for *glurp*. The use of *msp1/msp2* plus microsatellites would not cost significantly more than the current genotyping methods. The strategy would then move from length polymorphism to microhaplotype comparisons in the future.

AmpSeq

This appears to be the best method overall for genotyping. It has good resolution when the MOI is >2, so this is particularly relevant to areas of high transmission. Although the AmpSeq method (match-counting) gave similar recrudescence rates to *msp1/msp2/glurp* analysed using the 2/3 algorithm, the results for the individual samples were discordant. This is because the errors in the 2/3 method balance each other out *(17)*, whereas AmpSeq is more accurate.

The cost may be a barrier to implementation at present. In Africa, there are some places where AmpSeq is already being applied. This enables field testing of the methodology during a transition period. The cost of doing large runs is no more than for *msp1/msp2/glurp*. However, the re-sequencing of samples on a small scale is expensive. In addition, there are implementation questions regarding access to reagents in Africa.

The evaluation of new genotyping approaches should be evaluated in tandem with new analysis approaches because they inform each other. Therefore, if AmpSeq is being considered, the analysis method it is paired with should be able to indicate the depth of coverage, how many replicates are required, and therefore what the final cost might be and how sustainable it could be.

When it is done well, CE has similar results to AmpSeq. Therefore, the issue is more which algorithm should be used to interpret the genotyping data (match-counting, 2/3, Bayesian, or another). However, an additional advantage of AmpSeq is that drug resistance markers can be examined on the same platform. It is also possible to quantify whether some molecular markers have been selected following antimalarial treatment. This might help to verify if there is really a recrudescent infection.

Whole genome sequencing

At present it is unknown how minor allele frequency of SNPs in the population impacts the distribution. This will be looked at by J. Juliano when re-sequencing. It is expected that most SNPs will have a very low minor allele frequency, and if those with higher values are selected, it might improve their ability to resolve infections. Furthermore, co-transmission may impact MOI/COI estimations and may need to be considered when interpreting the data in high transmission settings. There will likely be variation by site.

Implications of sensitivity

Increasing sensitivity may reach the point where gametocytes that may survive drug treatment are detected. For example, if there is a higher level of gametocyte carriage with a particular drug, it might lead to a greater likelihood of gametocytes being identified as recrudescence if more sensitive genotyping methods are used. Therefore, it is important to set the sensitivity to a level that best reports drug efficacy against asexual parasites. There is also a danger of increasing sensitivity in the

sample selection. If light microscopy is not used but a more sensitive method, then parasites that are not clinically relevant may be detected.

Conversely, high sensitivity may be needed to detect resistance. One issue with the TES is that it is not sensitive enough to detect resistance; in Africa, there is no correlation between the prevalence of the molecular marker and the result of the TES. So, to detect resistant parasites early, the genotyping methods need to be very sensitive.

Triangulation with other data

Drug resistance markers: The addition of four microsatellite markers to *msp1* and *msp2* genotyping resulted in a reclassification of outcomes that strengthened the association between *dhfr59R*, an anti-folate resistance mutation, and recrudescence *(7)*. This association could be looked at in order to compare the different methods.

High versus low transmission: Parasitaemia levels, MOI, parasite relatedness, immunity, etc. are all factors that can vary by transmission setting. For both field data and modelling, looking at the markers/analysis methods with respect to these factors could indicate where there might be biases.

Managing uncertainty

The Bayesian method has uncertainty already incorporated. However, measures of uncertainty could be generated for other methods based on the degree of discordance in the classification of reinfection or recrudescence, particularly in the case of common alleles that are more likely to re-occur for whatever marker is being used. No data type is perfect, and this is why quantifying uncertainty is important. Probabilistic classification models can incorporate error models that tolerate mistakes, e.g., THE REAL McCOIL method to estimate COI, which has a website with a graphical user interface to encourage wide uptake.

Field data versus modelling

- The data presented by P. Ringwald indicate that the 2/3 algorithm increased recrudescence rates and that this effect was magnified in areas of high transmission. This is consistent with the modelling predictions, with the same magnitude – about a two-fold increase versus the WHO/MMV algorithm.
- The AmpSeq and 2/3 algorithm presented by D. Ménard are aligned with the simulations that show higher recrudescence than with the WHO/MMV method.
- The Bayesian analysis of microsatellite data presented by J. Juliano also show good agreement with the model predictions and again return higher recrudescence rates than with the WHO/MMV method.

All the methods differ, and it is not possible to determine which is the most accurate. As there is no gold standard, the 'true' underlying failure rate cannot be determined. Accordingly, it is a question of what evidence is needed to drive policy.

Note that the model applied a sensitivity analysis using a drug with a low failure rate (1%). The results indicated that none of the methods assessed would incorrectly identify an effective drug as a failing drug. In the model, there was little difference between the 2/3 and the WHO/MMV algorithms in low transmission settings. However, the 2/3 algorithm was closer to the model 'true' rate in high

transmission areas, except in the case of AL (see below) for which the WHO/MMV method was more appropriate, as the 2/3 method overestimated recrudescence. This is in agreement with the TES data presented by P. Ringwald.

Treatment efficacy with artemether-lumefantrine

The 2/3 method and the Bayesian approach both increased recrudescence rates relative to the WHO/MMV method, particularly for AL, but mainly in high transmission settings. Applying the current treatment efficacy thresholds of 90% efficacy could result in AL being removed from common use in some countries. However, there is not enough evidence to support any policy change for AL, which is a very popular and safe drug. Any recommendation to switch from AL cannot be taken lightly. In particular, there are cost and access issues to consider for the management of malaria in the field.

There are several alternative explanations for the findings with AL:

- They could be an artefact of the decision algorithms leading to an overestimation of the recrudescence rate for drugs with a short half-life in areas with high reinfection rates.
    - Lumefantrine has the shortest half-life of all artemisinin-based combination therapies (ACTs) and the effect was only seen in areas of high transmission, i.e., where reinfection rates are higher.
    - It may be possible to investigate this by examining treatment efficacy as a function of the MOI. However, as the MOI increases, failure rates may also increase as more clones have to be cleared *(18)*.
    - The modelling studies predict the higher failure rate of AL using the 2/3 method. When there are more than 10 infections per year, with AL, the WHO/MMV method appears to be more accurate, while the 2/3 method overestimates recrudescence by ~3–4% for that setting/drug (Jones, et al. AAC, 2019: Supplementary file 2, Figure S15) *(17)*.
    - This is consistent with the TES data that indicate apparent reductions in AL efficacy in areas of high transmission when the 2/3 analysis is used. By contrast, the WHO/MMV method indicates higher rates of drug efficacy at these sites.
- AL could be losing efficacy in African high transmission settings because of emergence of resistance to lumefantrine.
    - The available data in Africa do not indicate a change over time in AL efficacy. Regular TESs in the same sites are from Angola collected by the US-CDC: AL efficacy has hovered around the 90% mark since 2013 with no clear trend. However, these TESs may not be large enough to detect small changes in drug efficacy around 90%.
    - Ex vivo data do not suggest AL resistance in Africa.
    - AL efficacy over time could be examined in the WHO database to see whether there is any trend towards reduced efficacy in Africa, which was not really observed in the Greater Mekong Subregion.
    - In high transmission areas, higher numbers of patients need to be enrolled so that by study end there is a sufficient number of patients (at least 100) available for a valid analysis of the primary efficacy endpoint.
    - The possibility of resistance could be further investigated by triangulation with ex vivo data and lumefantrine blood levels.

- Adherence to drug therapy might be lower with AL given the twice-daily dosing regimen compared to once-daily ACTs, causing a higher failure rate. AL also has high inter-patient variation in pharmacokinetics and must be taken with a fatty meal to improve absorption; this could impact effectiveness.
- There is the possibility that gametocytaemia could be greater with AL and this might influence the recrudescence rate.

Thresholds for efficacy

It is clear that there is an interaction between the genotyping/analysis method and the estimated efficacy. Accordingly, efficacy may be below the 90% threshold for some methods and above the threshold for others. Different markers and more complex methods for classifying efficacy rates may require a more nuanced approach to setting the threshold below which a drug is considered to be failing.

More sensitive techniques for detecting minority strains can change the landscape of the investigation beyond drug efficacy, as asymptomatic carriage might also be detected. Consequently, a new approach to considering what is meant by drug efficacy may be required.

If the same markers are used (*msp1/msp2/glurp*) but a different analysis method is applied (2/3 rather than WHO/MMV), perhaps the same threshold should be used. However, the application of thresholds has major implications for drugs currently in use and ones in development. It is not just the percentage value that needs to be considered, but also the context.

Note that WHO studies are now being published using both the 2/3 method and the WHO/MMV method of analysis.

Implications of methods and thresholds for drug development versus TES

Although the differences in treatment efficacy between the different methods are generally <4% and not statistically significant, for drug development, the difference between 96% and 92% efficacy determines whether a drug progresses through the pipeline. Therefore, the interaction between the genotyping methods/analysis and thresholds at which decisions are made regarding a drug's efficacy needs to be examined. Furthermore, in drug development, the intention-to-treat population must be considered, not just the per-protocol population.

Getting towards the 'truth' in terms of showing complete clearance of parasites is important in drug development. The questions around AL are already affecting drug development in terms of how these data should be interpreted considering the uncertainty around the values. In clinical trials for drug development, different standards are required compared to what is acceptable for TESs.

A key consideration is how data that are generated using new methods can be interpreted against historical data. This may require the data to be analysed in two ways – the old way and the new way – while the implications of any changes are being reviewed. In particular, for TESs, there needs to be a mechanism to evaluate if there has been a change in efficacy that indicates resistance emergence.

Regulatory drug trials are comparative, so the methods used within a trial to evaluate reinfection versus recrudescence enables new drugs to be evaluated against existing treatments. However, in countries, the data on new drugs will inevitably be compared with historical data on established therapies. It may, therefore, be difficult to persuade countries that new treatments are effective if the efficacy rates appear to be lower. Even just changing *glurp* to microsatellites changes the efficacy rates, as does using the 2/3 method instead of the WHO/MMV method of analysis. Consequently, genotyping data may need to be generated and analysed in both the 'old' and 'new' ways, at least for a transition period.

Malaria drug treatment can be a challenge for regulators because of the complexities of the disease. At present, the United States Food and Drug Administration (FDA) does not recognize PCR-adjusted efficacy because it has not been validated. There is a risk that the European Medicines Agency (EMA) will also decide that this method is not acceptable. AmpSeq may be the way forward, but it might be too expensive for countries; it can be used in regulatory trials, however. The regulatory authorities may be reassured if it can be shown that the methods being used are state of the art. However, there is a need to compare regulatory studies conducted in different settings, and this may be difficult if different methods have been used. There cannot be one standard for drug registration and another standard for TESs or post-registration studies. However, it may be possible to agree with regulators on a transition method to enable comparisons while AmpSeq capacity is expanded in Africa. In the meantime, AmpSeq and the selected interim TES methodologies could both be reported in the initial post-licensure studies to benchmark the field efficacy of the new drug.

Issues around implementation

The Bayesian approach is complex and may be difficult to implement at the surveillance level in countries within malaria case management groups. Analytical methods need to be as simple as possible for implementation as tools in national malaria control programmes. Not everyone has access to an external research group and they must be able to do the work themselves. However, although uncertainty may take more time to explain, it may help decision-makers to understand the degree of uncertainty around findings, thereby supporting more informed policy decisions.

From an operational perspective, there needs to be one method that applies to both low and high transmission areas. The interim situation may require the best of the available methods to be used, but with a longer term aim to research and validate new technologies.

Improving quality control for laboratory methods in both field and reference laboratories is critical, and control panel parasites are lacking for most in the field. Quality control should include both microscopy and PCR. Moving forward, setting up regional reference laboratories for molecular methods in Africa and other endemic regions is also important.

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Current and emerging strategies to combat antimalarial resistance

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REVIEW

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Current and emerging strategies to combat antimalarial resistance

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ABSTRACT

Introduction: Since the spread of chloroquine resistance in *Plasmodium falciparum* in the 1960s, recommendations have been made on how to respond to antimalarial resistance. Only with the advent of artemisinin partial resistance were large-scale efforts made in the Greater Mekong Subregion to carry out recommendations in a coordinated and well-funded manner. Independent emergence of parasites partially resistant to artemisinins has now been reported in Rwanda.

Areas covered: We reviewed past recommendations and activities to respond to resistance as well as ongoing research into new ways to stop or delay the spread of resistant parasites.

Expert opinion: Inadequate information limits the options and support for a strong, coordinated response to artemisinin partial resistance in Africa, making better phenotypic and genotypic surveillance a priority. A response to resistance needs to address factors that may have hastened the emergence and could speed the spread, including overuse of drugs and lack of access to quality treatment. New ways to use the existing treatments in response to resistance, such as multiple first-lines, are currently impeded by the limited number of drugs available.

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1. Introduction

Malaria remains a major public health problem. In 2019, there were an estimated 229 million malaria cases and 409,000 malaria deaths worldwide. The World Health Organization (WHO) African Region, accounted for about 94% of cases. Two malaria species are the most prevalent: *Plasmodium falciparum* and *Plasmodium vivax*. *P. falciparum* causes most cases and deaths; the estimated proportion of cases due to *P. vivax* in 2019 was 3% [1]. Antimalarial compounds are used to treat malaria and protect at-risk populations; having safe and effective treatments prevents malaria patients from developing severe disease and death. Resistance has repeatedly caused the loss of key drugs resulting in increased morbidity and mortality, and the need to continue the search for new drugs.

Drug resistance in *P. falciparum* has been and remains one of the greatest threat to malaria control and elimination [2]. Since the spread of chloroquine resistance in *P. falciparum* in the 1960s, recommendations have been made regarding the best ways to respond to antimalarial resistance with the aim to save the treatments used and prevent or delay drug resistance. However, only with the advent of artemisinin partial resistance were large-scale efforts made in the Greater Mekong Subregion (GMS)[1] to carry out recommendations in a coordinated and well-funded manner. Recently, a change in *P. falciparum* response to artemisinin was detected outside the GMS in Rwanda [3]. Furthermore, the development of resistance to the partner drugs used in the artemisinin-based combination therapies (ACTs) continues to pose a challenge in the treatment of malaria [4]. This article focuses on resistance in *P. falciparum* and reviews the efforts done since the 1960s to respond to antimalarial resistance and outlines the available tools to respond to the challenges currently faced.

2. Resistance in the era of the Global Malaria Eradication Programme

In 1955, the Global Malaria Eradication Programme (GMEP) was approved by the 8th World Health Assembly. The central and often sole intervention planned in GMEP was insecticide residual spraying (IRS) using dichloro-diphenyl-trichloroethane (DDT). Initially, the use of antimalarial was thought only to have an important role in the later stages of the eradication efforts when few cases remained [5].

Concerns over the development of insecticide resistance and lack of progress in some countries led to explorations of ways to use chemotherapy not only in the last stages of eradication but also as an auxiliary to IRS to more rapidly achieve elimination [6,7]. One method tested in several countries was the introduction of cooking salt medicated with an antimalarial drug. Cooking salt with pyrimethamine or chloroquine was tested in several countries including Brazil, Cambodia, Ghana, Guyana, and Uganda. In Brazil, a large-scale trial was done in 1959 covering the entire Amazon region and a population of 2.5 million, supplying cooking salt containing 0.25% chloroquine [6,8–10].

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Article highlights

- Antimalarial resistance continues to be a threat to public health, compromising the ability to prevent and treat malaria, and necessitating a constant search for new drugs.
- In the past, the antimalarial resistance response has been based on a strategy seeking to isolate areas where resistance has been identified.
- However, the ability to effectively respond to resistance has been impeded by lack of information on the spread of resistance.
- The emergence of artemisinin partial resistance in the countries of the Greater Mekong Subregion prompted a coordinated, well-funded response to resistance which has helped significantly lower the malaria burden making malaria elimination a feasible target.
- Artemisinin partial resistance causing delayed clearance after treatment with an artemisinin has been identified in studies in Rwanda and is probably present in other parts of eastern Africa.
- The recommended first-line artemisinin-based combination therapies (ACTs) are still reported to be efficacious. However, if parasites carrying resistance to both artemisinin and ACT partner drugs spread like it happened in the Greater Mekong Subregion, the consequences would be disastrous.
- The emergence of artemisinin partial resistance in Africa requires a response that includes improved phenotypic and genotypic surveillance and addresses factors that may have hastened the emergence and could speed the spread.
- Investments made due to COVID-19 in laboratory infrastructure and training of staff could potentially be leveraged to improve surveillance of antimalarial resistance and efficacy.

Countries in the GMS employed antimalarial drugs as a central part of their eradication program already from early in the eradication efforts. In Myanmar and Thailand, single doses of chloroquine and pyrimethamine were administered to suspected cases encountered during house visits. In Cambodia, routine administration of chloroquine and pyrimethamine at a fixed interval was used as a supplementary measure to IRS in areas with population movement [11]. Pailin province in eastern Cambodia had attracted gem-miners since the late 1940s coming to mine sapphires and rubies. There was a steady influx of nonimmune young adults living often in only rudimentary shelters and in the presence of *Anopheles dirus*, an exophilic vector difficult to control with residual spraying. Mass drug administration (MDA) with pyrimethamine and chloroquine was carried out in Pailin, twice a year from 1955 to 1957, weekly from 1958 to 1959, and indirectly through medicated salt from 1960 to 1962 [12].

2.1. Emergence of resistance

Cases of *P. falciparum* resistance to proguanil and pyrimethamine had been reported in 1948–50. However, prior to the initiation of the GMEP in 1955, significant drug resistance to chloroquine or amodiaquine had not been reported [13]. Nevertheless, already 2 years after the launch of GMEP, in 1957, resistance to chloroquine was first suspected in eastern Thailand due to delays in the clinical response of *P. falciparum* to chloroquine [14]. Further studies in eastern Thailand found falciparum parasites resistant to all widely used drugs except quinine [15]. Chloroquine resistance and pyrimethamine resistance were identified in Pailin in 1962 [12]. In South America, chloroquine resistance was first observed in Columbia in 1959 [16,17]. The widespread use of chloroquine helped make progress in reducing malaria mortality and morbidity. However, the success of chloroquine also helped facilitate the spread of resistance. By 1964, chloroquine resistance had been reported from Brazil, Cambodia, Columbia, Guyana, Malaysia, Thailand, and Vietnam [16,17].

2.2. Early responses to resistance

The prevalent belief that IRS would pave the way for eradication meant that the appearance of reports of resistance to chloroquine, only a few years after the launch of GMEP, was not given the attention warranted. Global technical recommendations in 1960 in response to the potential emergence of chloroquine resistance stressed that efforts to discover resistance early should be made, and when present, a rapid change to a drug of a different chemical class should occur. It was concluded that it seemed 'that combined use of drugs with different types of actions and at adequate dosage will prevent the development of resistance' [8].

Another WHO meeting was held in 1964 to review resistance of malaria parasites to drugs. While there was an understanding of the urgency in improving the knowledge of the distribution of resistance and the need to establish standard criteria of resistance to chloroquine, it was thought that with intelligent use of drugs, selection of parasites less sensitive to chloroquine could be avoided. The meeting formulated the first proposals for countermeasures to be taken when drug resistance had been confirmed. The priority was given to eliminate the resistant parasites so that foci where resistance had emerged did not remain a threat to the fight against malaria, and to plan future case management in areas of resistance in such a way that effective treatments would be available for acute infections. To eliminate the resistant parasites, it was recommended that every foci of resistance should be treated as a separate problem. Effective treatment of confirmed cases should prevent onward transmission. Presumptive treatment done without microscopic confirmation of malaria should only be done with treatment able to cure the patient and render them noninfectious to mosquitoes. Where possible, MDA schemes using a drug to which resistance had developed should switch to another drug. Other measures proposed included extended use of vector control not only in the areas with resistance but also in the adjacent areas to try and provide a barrier that would contain the spread of resistant parasites. Population movement across this barrier should, to the extent possible, be treated as population movement into areas from which malaria had been eliminated [16]. These proposed countermeasures depended on both up-to-date information on the emergence and spread of resistance and the availability of sufficient resources to react as needed. However, the lack of information on the

developing patterns of resistance and limited alternative drug choices impeded effective responses in the field.

There are records of local responses to resistance. In Guyana, chloroquinized salt was used from 1961 to 1965 in areas with sparse population and considerable population movement. This resulted in a reduction in cases, but chloroquine-resistant strains were imported into areas bordering Brazil in 1962. The use of DDT in house spraying in the border areas complemented by similar operations across the border stopped this spread of the resistant strains [9,18,19].

2.3. Aftermath of the eradication programme

The lack of progress and resurgence of malaria in some areas led to the recognition that there were areas where malaria elimination was not feasible in the short term. Consequently, in 1969, the time-limited goal of eradication was effectively abandoned. Resources and support for malaria activities had decreased and diminished further in the following decades. Due to lack of resources, insecticide resistance and some public concerns over the widespread use of DDT, there was a drastic reduction in the use of residual insecticides and chloroquine-resistant strains of *P. falciparum* spread to many areas, including areas where the malaria prevalence had been greatly reduced during the GMEP. Where there were no longer the resources to continue the IRS campaigns or insecticide resistance hampered the usefulness of these, chemotherapeutic measures became the main tool available [5,20]. This was even more so in remote, forested areas. Economic crisis and conflicts led to increased number of people living in such areas, often working in agriculture or mining. Here, the lack of other malaria control measures and the influx of nonimmune people often led to an extensive and unregulated private sector market trade of antimalarial drugs [5,21].

By 1980, chloroquine resistance had spread through much of South America, Southeast Asia, and North-East India [22,23]. Measures to help contain the spread of resistance were included in some of the national malaria plans developed after the end of the GMEP. In India, the National Malaria Eradication Program had seen early success, bringing the number of cases reported in 1961 to below 50,000. After 1965, malaria resurged and by 1975, 6.5 million cases were reported. In response, the Modified Plan of Operation was launched in 1977 with a *P. falciparum* Containment Programme. This program included the establishment of teams in strategic areas for monitoring of two-way movement from areas known to have foci of drug-resistant strains in North-Eastern regions, and to facilitate actions necessary to prevent spread of the resistant strains [24]. However, by 1977 resistance had likely already spread outside the north-western part of India.

The difficulties in making a sustained impact in the intensive malaria transmission in Africa through large-scale DDT-spraying programs meant that chloroquine was distributed in huge quantities during the GMEP. Reports of nonimmune visitors to Kenya and Tanzania getting malaria and not responding to a standard dose of chloroquine appeared in 1978 [25,26]. Through the 1980s and 1990s, chloroquine resistance spread through Africa from east to west. In the 1970s, the death rate from malaria among children in Africa was almost half the level of the pre-chloroquine years, but hospitalizations and deaths rose again with the spread of chloroquine-resistant parasites [27,28].

In 1981, WHO reviewed the proposed activities to be taken toward prevention and containment of *P. falciparum* resistance. These activities were recommended to be targeted in areas defined by the presence and risk of resistance. In the areas with widespread resistance, the emphasis was on mapping resistance, vector control and providing effective treatment for *P. falciparum* based on parasitological diagnosis, establishment of check posts on known migration routes from chloroquine resistance areas, and establishment of systems with the ability to detect and investigate treatment failures [23].

Pyrimethamine monotherapy had been widely used in the 1950s and 1960s as treatment and for mass prophylaxis. Later, pyrimethamine was reformulated and used in combination with sulphadoxine as sulphadoxine-pyrimethamine (SP). SP became the recommended first-line treatment in many countries, where resistance rendered chloroquine unusable [29,30]. Low-level resistance to pyrimethamine emerges easily; genetic profiling of resistant strains showed that higher-level resistance emerged first in Southeast Asia on the Cambodia–Thailand border areas and then spread to Africa [31]. Pyrimethamine resistance was initially masked by sulphadoxine but resistance to sulphadoxine emerged in both Africa and Asia [29,30].

SP became the first-line treatment for falciparum malaria in Thailand in 1973 and SP was widely available in local pharmacies, used for prophylaxis and treatment of fevers not confirmed to be caused by malaria. In the early 1980s, SP efficacy was so low that Thailand changed initially to a 7-day treatment with quinine-tetracycline. In 1985, mefloquine was introduced, initially in combination with sulfadoxine and pyrimethamine, and from 1991 as monotherapy. Mefloquine was restricted for use by the malaria control program and government hospitals only for the treatment of microscopically confirmed falciparum malaria. However, even before the introduction of mefloquine as a first-line treatment, mefloquine resistance was detected on the Cambodia-Thailand border [21]. Mefloquine was widely available in countries neighboring Thailand, where conflicts and high number of migrants often meant that access to organized malaria control measures was limited. Resistance to mefloquine spread, leading Thailand in 1995 to become the first country to introduce a combination treatment with mefloquine and artesunate (an artemisinin derivative, described in the next section). It was first used only in selected areas deemed to be high-level multidrug resistance zones on the border with Myanmar and Cambodia. Initially, it was given as a two-day treatment and from 2007 as a three-day treatment [21,32,33]. In 2000, Cambodia also introduced the combination treatment with mefloquine and artesunate [34]. In 2000 and 2001, WHO first discussed the use of combination therapies and WHO recommended that countries experiencing resistance to monotherapies should adopt an ACT as first-line treatment for

uncomplicated *P. falciparum*. In 2006, this recommendation was expanded to all countries [35–37].

3. Era of artemisinin

Fueled by the spread of resistance to the most widely used antimalarial drugs, Chinese researchers discovered the antimalarial activity of artemisinin in 1972, based on research on the antimalarial properties of medical plants described in ancient texts [38]. The advantages and disadvantages of artemisinin and artemisinin derivatives (such as artesunate) were clear from the beginning. The drugs were well tolerated and fast-acting, and quickly reduced the number of parasites in the blood. However, effective drug concentration levels were only maintained in the plasma for a relatively brief period after drug administration, and short oral treatment courses resulted in high rates of recrudescence. To prevent recurrent parasitemia, 7 days of treatment was needed when using artemisinin or an artemisinin derivative as a monotherapy [39,40]. Combining an artemisinin derivative with a partner drug with a longer half-life into an ACT takes advantage of the rapid action of the artemisinin derivatives, while the partner drug helps prevent recrudescence, even after a short three-day treatment [41].

3.1. Artemisinin resistance containment

At the time ACTs were introduced, there had been no documented resistance to artemisinin and its derivatives, and it was believed that the rapid elimination of artemisinins from the body would help delay if not prevent the development of resistance. However, from 2003, data began to emerge showing prolonged clearance times after treatment with either artesunate plus mefloquine for 3 days or artesunate monotherapy for 7 days in the areas around the Cambodia–Thailand border [33,42]. In efficacy studies, this was typically seen as a higher than expected proportion of patients with parasites in the blood on day 3 after the start of treatment [2]. Initially, the priority was to confirm if these observations could reflect the emergence of genuine resistance. The Bill & Melinda Gates Foundation funded the Artemisinin Resistance Confirmation, Characterization and Containment (ARC3) project coordinated by WHO that supported treatment efficacy trials. Data collected in 2007 and 2008 found that *P. falciparum* had reduced in vivo susceptibility to artesunate in western Cambodia as compared with north-western Thailand. There were discussions as to what term to use to describe the delayed parasite response. Initially, the term 'artemisinin tolerance' rather than resistance was used. More recently, 'partial resistance' has been used to describe parasites that may not clear as fast as fully sensitive parasites but will still be cleared when treated with a 7-day treatment of artesunate. While Pailin was seen as the epicenter, the actual geographical extent of the problem was still not very clear. However, researchers conducting the studies called for urgent containment measures due to fear of resistance spreading to other countries and continents, with potentially catastrophic global consequences [2,33,42–44].

The ARC3 project also sought to identify strategies to contain the spread of artemisinin-resistant malaria within Southeast Asia. Part of this work was to better understand the factors contributing to the development of drug resistance along the Cambodia–Thailand border. One of the factors identified was the large number of migrants and mobile populations in the areas, many of whom had no immunity and often only had access to expensive and poor quality treatments. In Cambodia, the majority of people with fever sought treatment from the unregulated private sector, where artemisinin monotherapies were widely available [33]. Counterfeit artesunate was widespread [45]; these products have obvious potentially dangerous consequences for patients, and their use can contribute to development of drug resistance. Higher prices of artemisinin and the quick resolution of clinical symptoms also lead to people prematurely stopping treatment, contributing to selection pressure and continued infectiousness. The widespread mefloquine resistance in Cambodia and Thailand when artesunate+mefloquine became the recommended treatment could also have played a role in the decline in parasites' response to artemisinin [32,46]. To reduce the selection pressure globally, WHO Member States had adopted the World Health Assembly resolution WHA60.18 in 2007 calling for a progressive removal of oral artemisinin-based monotherapies from markets [47].

In 2008, the artemisinin resistance containment and elimination (ARCE) project was started. This project was like ARC3 funded by the Bill & Melinda Gates Foundation and coordinated by WHO. The goal of the project was to contain artemisinin-tolerant *P. falciparum* parasites by removing selection pressure and reducing and ultimately eliminating falciparum malaria. Implementation of field activities began in May 2009 and the project ran until November 2011. The activities were coordinated through an international task force, as well as Thai and Cambodian National Task Forces [48,49].

Many of the strategies used were those proposed previously in the 1960s, but these had never been implemented to the same degree as was now envisioned in what was defined as containment zones. Zone 1 was defined as the areas around the Cambodia–Thailand border where there was evidence of artemisinin resistant *P. falciparum*. Zone 2 covered areas where there was no evidence of resistance, but the risk was considered high due to the proximity to Zone 1. Approximately 400,000 people were targeted in areas labeled as zone 1 and 4.86 million people were targeted in areas labeled as Zone 2 [49].

Removing artemisinin selection pressure was not possible as safe, efficacious, and affordable alternatives to ACTs were not widely available. However, mathematical modeling indicated that the most effective intervention to eliminate artemisinin-resistant malaria was to ensure a switch of treatment from artemisinin monotherapy to ACTs. As ACTs were more effective against artemisinin-sensitive parasites, the remaining last parasites were likely the most resistant. Thus, any strategy employing artemisinin needed to be sustained until elimination is achieved [44,50]. During the ARCE project, patients detected in zone 1 in Thailand were treated with atovaquone-proguanil as a directly observed treatment. Artesunate-mefloquine (AS-MQ) remained the first-line treatment in Cambodia until 2009, when co-formulated dihydroartemisinin and piperaquine (DHA-PPQ) became the first-line

BOX 1. Specific objectives of the Artemisinin resistance containment and elimination project (ARCE)

1. To eliminate artemisinin-tolerant parasites by detecting all malaria cases in target areas and ensuring effective treatment and gametocyte clearance.
2. To decrease drug pressure for selection of artemisinin-tolerant malaria parasites.
3. To prevent transmission of artemisinin-tolerant malaria parasites by mosquito control and personal protection.
4. To limit the spread of artemisinin tolerant malaria parasites by mobile/migrant populations.
5. To support containment/elimination of artemisinin-tolerant parasites through comprehensive behaviour change communication (BCC), community mobilization and advocacy.
6. To undertake basic and operational research to fill knowledge gaps and ensure that strategies applied are evidence-based.
7. To provide effective management, surveillance and coordination to enable rapid and high-quality implementation of the strategy.

BOX 2. Main elements of the *Global plan for artemisinin resistance containment* (GPARC)

The GPARC sets out a high-level plan of attack to protect ACTs as an effective treatment for *P. falciparum* malaria. The GPARC has two goals:

- contain or eliminate artemisinin resistance where it already exists;
- prevent artemisinin resistance where it has not yet appeared.

The plan makes five recommendations:

- stop the spread of resistant parasites;
- increase monitoring and surveillance to evaluate the threat of artemisinin resistance;
- improve access to diagnostics and rational treatment with ACTs;
- invest in artemisinin resistance-related research;
- motivate action and mobilize resources.

treatment for *P. falciparum* malaria in zone 1. However, the efficacy of DHA-PPQ declined fast, and in 2012, atovaquone-proguanil also became the first-line treatment in Pailin, Cambodia [49].

The ARCE project employed a variety of new tools and technologies to meet the objectives (BOX 1), including ensuring good access to testing and treatment. More than 3000 village volunteer workers were provided with rapid diagnostic tests (RDTs), thereby extending access to testing and treatment in villages. In addition, 128 migrant workers were trained to provide testing and treatment to migrant populations. Mobile phones and online reporting were tested for use for more responsive surveillance [51]. In Cambodia, the market authorization for all oral artemisinin monotherapies was withdrawn in March 2009, and the ban was enforced by regular inspections by 200 law enforcement officers known as the 'Justice Police' [51]. This did affect the availability of artemisinin monotherapy: of private sector outlets stocking antimalarials, the proportion stocking oral artemisinin monotherapy fell from 20.3% in 2009 to 4.2% in 2011 [52].

Originally, a mass screening and treatment was envisioned, but early pilot schemes could not be scaled up due to lack of human resources [53]. Instead, a focal screening and treatment was carried out in Pailin, Cambodia. A total of 6931 individuals were screened using PCR; the prevalence of *P. falciparum* was found to be less than 1%, 96% of the patients were asymptomatic. There was 1.57% prevalence in villages deemed to be high risk based on surveillance data vs. 0.24% for low-risk villages. One village accounted for 50% of all *P. falciparum* cases detected. The findings and the resources required to do the screening and treatment led to researchers making the case in favor of MDA with treatment given to an entire population without prior screening [49,54].

Long-lasting insecticide-treated nets (LLINs) were used for vector control, and 1 net per person was distributed. Distribution of LLINs was only partially successful; the large distribution campaigns resulted in high levels of coverage, but the coverage and usage quickly dropped off due to the highly mobile population. Hammock nets were distributed to mobile populations on both sides of the Cambodia–Thailand border, but coverage among those groups was difficult to measure and maintain. One innovative approach was lending of nets through employers to workers who often stayed at worksites for short periods. Work was also done to have foremen at farms and plantations send sick workers to treatment posts. Furthermore, almost 800 visits were made to do mass screenings of migrant workers or screening of new incomers in collaboration with employers [48,49].

These efforts did have an impressive impact in terms of the overall burden; from 2008 to 2011 reductions in *P. falciparum* cases of 44% to 57% were seen in zones 1 and 2. In the containment zones in Thailand, *P. falciparum* reduced by around 60% over the 2-year period 2009–2011. This reduction was much greater than what has been observed in the country as a whole. However, in Thailand, surveillance showed that reduction in cases was greater in the population of Thai nationality than in the non-Thai population. In the provinces corresponding to zones 1 and 2 in Thailand, the proportion of cases detected among non-Thais were 14.2% in 2009 and 47.4% in 2011 [48,49].

While progress was made toward elimination in the containment zones, as predicted, a higher proportion of the remaining cases showed delayed clearance after treatment with an artemisinin. Based on data on day 3 positivity rates in Thailand, the Thai zone 1 was expanded to cover two full provinces, Trat and Chantaburi [49]. After the end of the ARCE project, containment activities were continued in Thailand and Cambodia funded by the Global Fund to Fight AIDS, TB, and Malaria. In Thailand, the activities were further expanded and

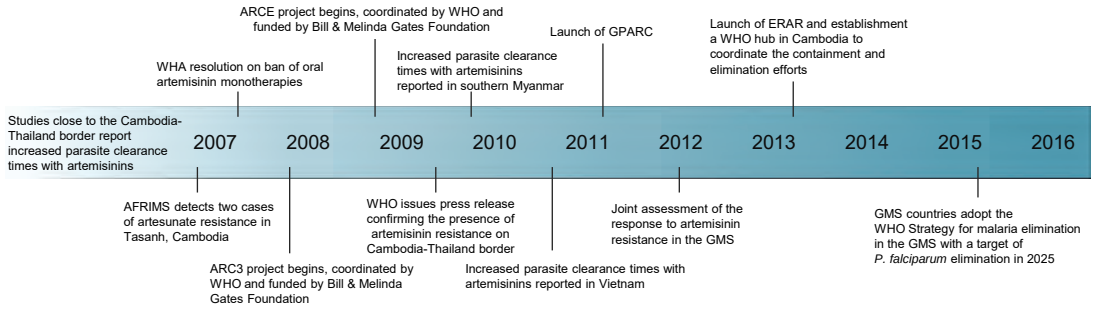

Figure 1. Timeline of events in the emergence and response to artemisinin resistance in the Greater Mekong Subregion.
(AFRIMS: Armed Forces Research Institute of Medical Sciences; ARC3: Artemisinin Resistance Confirmation, Characterization and Containment project; ARCE: Artemisinin Resistance Containment and Elimination project; GPARC: Global Plan for Artemisinin Containment; ERAR: Emergency response to artemisinin resistance in the GMS)

included efforts such as observed treatment for patients and follow-up on day 28 to ensure complete cure.

Learning from the experiences of the containment project, the *Global Plan for Artemisinin Containment* (GPARC) was released in January 2011, having been developed by the WHO Global Malaria Programme through consultation with over 100 malaria experts. The GPARC set out to both contain or eliminate artemisinin resistance where it already existed, and to prevent artemisinin resistance where it had not yet appeared. Five recommendations were formulated to achieve this (BOX 2)

In view of regional differences and varying levels of artemisinin resistance, the GPARC recommendations were to be applied according to an evaluation of level of risk in countries and areas. Different levels of risks were defined as tiers. Areas for which there was credible evidence of artemisinin resistance were defined as 'tier I.' In tier I, an immediate, multifaceted response was recommended to contain or eliminate resistant parasites as quickly as possible. Tier II areas were those with significant inflows of mobile and migrant populations from tier I areas or shared borders with tier I areas. The recommendations for tier II countries were intensified malaria control to reduce transmission and limit the risk of emergence or spread of resistant parasites. In tier III areas, defined as *P. falciparum* endemic areas, which have no evidence of artemisinin resistance and limited contact with tier I areas, prevention and preparedness was focused on scaling up control measures [55].

While containment efforts were ongoing on the Cambodia–Thailand border, in other parts of the GMS, studies were done to collect efficacy data. Evidence started emerging that artemisinin resistance was not only present in the areas defined as zone 1 but also in areas of southern Myanmar bordering Thailand, in Binh Phuoc province in Vietnam bordering Cambodia, and in Yunnan province in China [56]. The areas where resistance was suspected had commonalities: they were typically close to international borders and had high numbers of migrant and mobile populations working in forested areas engaging in, for instance, mining and logging. In Myanmar and Vietnam, plans were developed based on the activities proposed in GPARC and containment activities started in areas where artemisinin resistance was suspected. In Yunnan, malaria elimination was pursued (for timeline, see Figure 1).

In 2011 to 2012, an assessment of the response to artemisinin resistance in the GMS was carried out with the collaboration of WHO, DFID, and USAID/PMI and sponsored by AusAID and the Bill & Melinda Gates Foundation. The joint assessment concluded that a good, if delayed, start had been made to address artemisinin resistance in the GMS. It was found that the approach outlined in GPARC and the associated national level strategies and plans were appropriate but needed modification as new evidence on the nature of artemisinin resistance was produced. Implementation of such a strategy along the Cambodia–Thailand border had significantly reduced the incidence of malaria, especially that caused by *P. falciparum*, and the number of malaria deaths. The joint assessment concluded, however, that 'not enough is yet being done, with enough intensity, coverage and quality, to respond to a problem that could not only slow future progress but also undo the gains already made in malaria control worldwide' [57].

Based on the recommendations from the assessment, the *Emergency response to artemisinin resistance (ERAR) in the Greater Mekong subregion, Regional Framework for Action 2013–2015* was released in April 2013. The framework highlighted key action areas in which progress was urgently needed to contain resistance and move toward elimination of malaria in the GMS. The framework recalled the overarching goal of GPARC in protecting ACTs as an effective treatment for *P. falciparum* malaria. The framework sought to do this by rallying stakeholders to urgently scale-up and increase the effectiveness of interventions to address artemisinin resistance in the GMS [58].

Resistance to partner drugs, as well as to artemisinin, posed a challenge to progress in the GMS. Thailand changed the treatment for *P. falciparum* from AS-MQ to DHA-PPQ in 2015 [59]. However, in Pailin in 2010, just 1 year after DHA-PPQ was introduced, a study recorded a treatment failure rate of 27% (n = 29) [60]. Piperaquine resistance later spread throughout Cambodia, to Lao PDR, Thailand, and Vietnam [4].

Despite some setbacks, the resources invested, and the progress made meant that malaria elimination became within

reach. WHO recommended complete elimination of *P. falciparum* in the GMS based on this progress, along with the reports of resistance to ACT partner drugs and genomic epidemiology studies showing that artemisinin resistance was not only spreading transnationally but also emerging locally at multiple sites [61]. The regional elimination strategy was launched in May 2015 following extensive consultations with countries and partners in the GMS. The ultimate goal of this strategy is to eliminate malaria by 2030 in all GMS countries and, considering the urgent action required against multidrug resistance in the GMS, to eliminate *P. falciparum* by 2025. This goal is within reach; in 2020, 33,781 *P. falciparum* cases were reported from the GMS (see Figure 2).

4. Drug resistance development

Planning measures to prevent and respond to resistance requires an understanding of the causes behind the emergence and spread of resistance and likely scenarios for resistance development. Drug-resistant parasites emerge in two distinct stages: the initial de novo genetic event and a subsequent spread. In vitro studies conducted in the 1990s suggested that *P. falciparum* parasites in Southeast Asia were more readily mutating and developing resistance to structurally and mechanistically unrelated compounds [62]. However, a more recent study of mutation rate variations in Southeast Asian and West African parasites did not find evidence of such hypermutator *P. falciparum* lineages in Southeast Asia. Therefore, other factors that may affect the selection and spread of these mutations must explain the recurring emergences of new drug resistance mutations in Southeast Asia [63].

Drug pressure drives the selection and spread of de novo genetic changes that make a parasite less sensitive to a drug. The selection happens in a 'window of selection' when a drug is present in the blood at levels that inhibit the growth of other sensitive parasites while allowing the nascent resistant parasite to multiply and be transmitted. The window of selection is a function of the half-life of the drug. This half-life is very short for the rapidly eliminated artemisinins but long for the ACT partner drugs [64].

Drug pressure depends not only on the proportion of malaria infections that is treated but also on the overall rate at which people consume antimalarials [65]. In malaria endemic areas, people often have residual drug from previous chemoprophylaxis or treatments. The drug pressure from residual drug probably does not play an important role in the initial de novo selection. The reason is partly numerical; the number of parasites emerging from the liver potentially being exposed to residual drug in a newly infected patient is very low compared with the number of parasites in a hyperparasitemic patient where more than 4% of the red blood cells are infected by malaria parasites [66]. Consequently, treatment failure in a hyperparasitemic patient is the most probable source for the de novo selection [67]. However, residual antimalarial drug is thought to be an important selective force in the spread of resistance when the concentration in the blood prevents new drug-sensitive infections but allows resistant infections to be maintained and transmitted [68].

The risk of resistance spreading and being established in a population is affected by the immunity profile of the community and the local epidemiology [69]. Host immunity kills parasites regardless of drug resistance, so resistance will more easily emerge and spread in a nonimmune population. In low transmission areas with populations with lower acquired immunity, infections are more likely to evolve into clinical diseases requiring treatment, meaning that it is more likely that a resistant parasite will encounter drugs. Furthermore, in high transmission areas, the resistant parasite is more likely to have to compete with a large number of sensitive parasites [67,70].

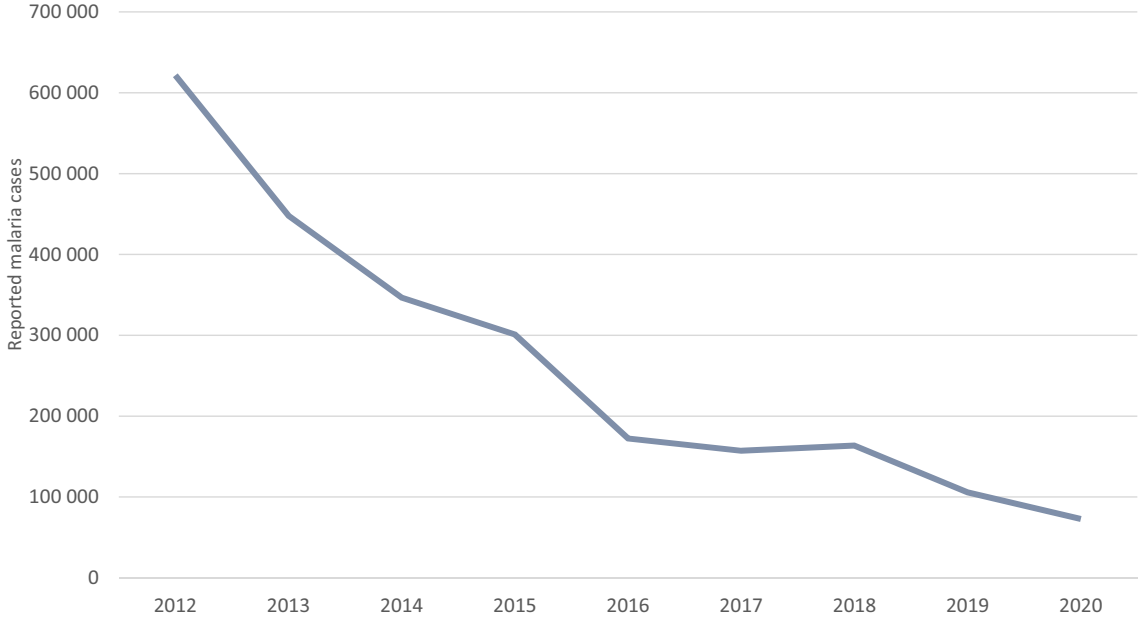

Figure 2. Malaria cases in the six GMS countries[1] (2012–2020) [1].

The survival of resistant parasites is affected by the degree of resistance provided by a given genetic change and the fitness cost associated with this genetic change. Drug resistance mutations often come with a fitness cost giving a disadvantage to the parasite carrying the mutation in the absence of drug pressure, especially in high transmission settings with more frequent intra-host competitions between strains [71,72]. However, continued drug pressure may offer parasites the opportunity to acquire additional mutations that can help compensate for the initial fitness cost. *Plasmodium* reproduces not only asexually but also sexually and the different mutations are less likely to be separated during meiosis and recombination in areas with low transmission, which means that resistance is even more likely to be disseminated in these settings [73–75].

The risk of treatment failure is affected by factors other than parasite resistance and fitness cost, including patient adherence, drug quality, dosing, drug malabsorption, and comorbidities. In controlled trials such as therapeutic efficacy studies, the recommended first-line ACTs are generally found to have high efficacy. Nevertheless, modeling estimated the effectiveness of ACTs in 2016–2019 to be only 71.8% (IQR: 46.9–76.4) largely due to the adjustments applied for drug quality and patient adherence [76]. The consequence is unfortunately that treatment failure is a common occurrence. Just as human behavior affects the likelihood of emergence and selection, it also affects the likelihood of spread between areas through factors, such as migration rates.

Overall, the areas where resistance first emerged have all the hallmarks of resistance breeding grounds – they have been low endemicity areas with an influx of populations with limited immunity, lacking access to quality diagnosis and treatment and with high, unregulated usage of antimalarials.

4.1. Spread or independent emergence

Containment efforts are based on the notion of the development of resistance as rare occurrences where a strain of resistant parasites spread from one area to become prevalent in other areas and threaten the efficacy of first-line treatments. Most of the resistance mutations that in the past have challenged the ability to effectively treat patients have only a few independent origins. Consequently, spread, rather than de novo mutations, appears to be the most important way in which resistant strains are introduced into a parasite population [77].

Chloroquine and pyrimethamine resistance have been used as typical examples of hard selective sweeps where resistant haplotypes spread from a few independent emergences. As noted previously, chloroquine resistance associated with mutations in *Pfcrt* emerged in South America and Asia and spread successfully at least 6 times with resistant strains spreading from Asia to Africa [78,79]. Low-level pyrimethamine resistance associated with the single and double mutations of *Pfdhfr* alleles probably emerged multiple times within Africa in the 1950s and 1960s, but high-level pyrimethamine resistance associated with a triple mutant *Pfdhfr* likely spread from Asia to Africa at the same time as chloroquine resistance in the late 1970s and 1980s [29,80]. Similarly, single mutations in *Pfdhps* conferring low-level resistance to sulfadoxine have been found in many different genetic backgrounds, while parasites with triple mutations appear only to have a few origins [77,81]. Also, mefloquine resistance was first detected in Southeast Asia; in Thailand mefloquine resistance emerged quickly after mefloquine was first introduced in 1985 [21]. However, mefloquine resistance does not appear to have spread west to Myanmar, India, and Africa, probably due to the relatively low mefloquine drug pressure in these areas.

Point mutations in *PfKelch13* have been found to be associated with the delayed parasite clearance after treatment with artemisinins [82]. Analyses have shown that *PfKelch13* mutations were spreading not only in the areas close to the Cambodia–Thailand border but also in western Thailand prior to the start of the artemisinin resistance containment project [83]. At present, at least 135 different *PfKelch13* mutations have been identified in the GMS. Of these, 21 have been shown to be associated with delayed clearance in clinical trials or in vitro [2]. These single point mutations alone have been shown to be enough to affect the clearance rate. However, there are differences in the impact on the clearance rate between the *PfKelch13* mutations, possibly linked to different fitness costs [84].

Pfkelch13 mutations associated with delayed clearance have both spread transnationally and emerged independently within different GMS countries, casting doubt on the potential for successful containment [61]. In parts of the GMS, soft sweeps of different *Pfkelch13* mutations appear to have been replaced with a single hard sweep of a specific *Pfkelch13* mutation, the C580Y [85]. This is partly explained by the rapid spread of a linage carrying both C580Y and genetic changes associated with resistance to piperaquine [86]. *Pfkelch13* mutations have been shown to increase the risk of failure in parasites carrying resistance to piperaquine, as well as in parasites carrying resistance to mefloquine [87,88].

Outside the GMS, *PfKelch13* mutations are frequently identified, but it is rare that these mutations are detected at a prevalence of more than 1% [89]. However, mutations confirmed to be associated with artemisinin partial resistance have been found at higher prevalence in a few locations. In Guyana, *Pfkelch13* mutants were identified in samples collected between 2016 and 2017 among highly mobile patients, most with a recent history of travel in remote gold-mining areas. The mutations were found to have emerged independently rather than having spread from Asia [90]. The mutations have not spread in Guyana at the rates seen in Cambodia, possibly because the mutation was found not only to confer partial resistance to artemisinin but also carry a high fitness cost [91]. Genetic analysis of samples collected in Rwanda between 2013 and 2015 revealed expansion of an indigenous linage carrying a *PfKelch13* mutations, R561H, confirmed to be associated with artemisinin resistance in the GMS [3].

That artemisinin resistance only appears to be spreading in Africa now can have multiple explanations including lower artemisinin drug pressure over the past decades when compared with Southeast Asia, higher immunity,

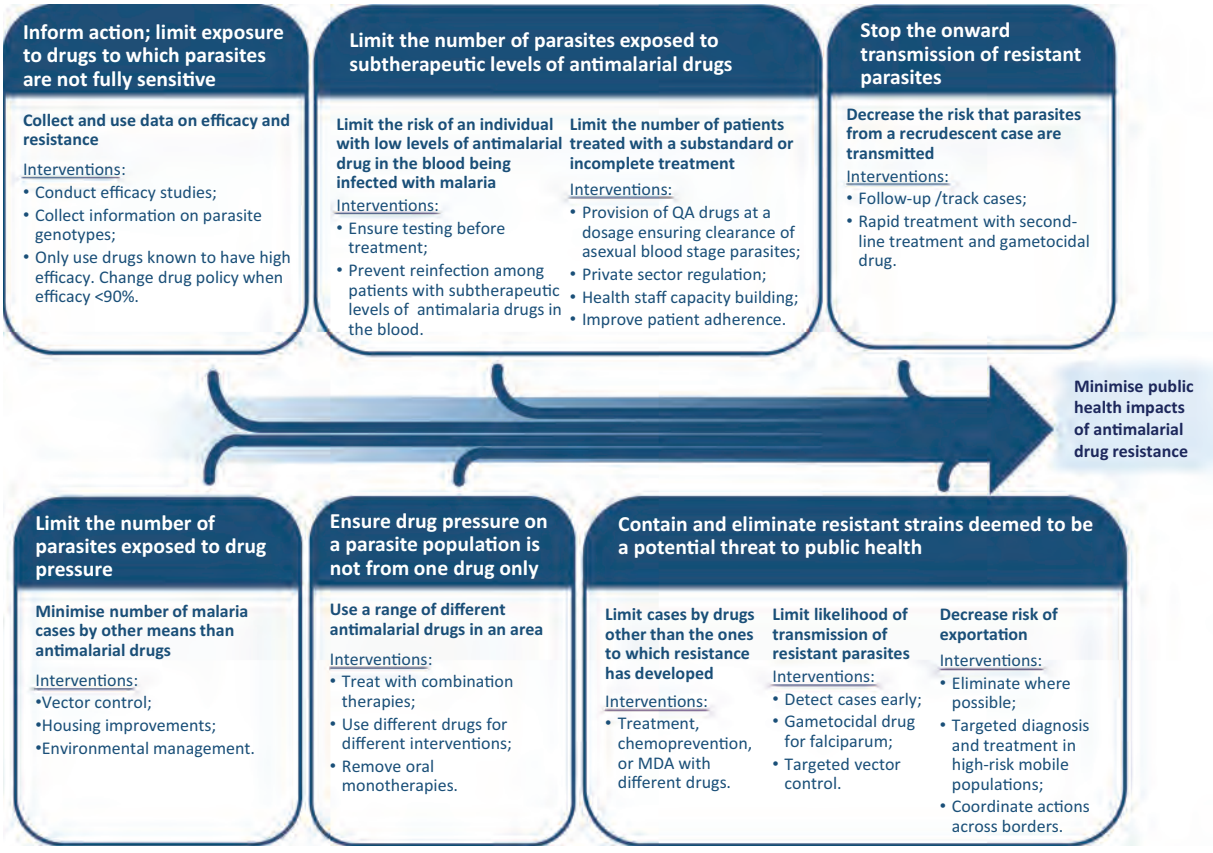

Figure 3. Interventions to prevent and respond to resistance.

and higher transmission level increasing both the competition between parasites and the risk of recombination causing loss of supplementary mutations that could for instance help offset fitness cost. *P. falciparum* strains can vary significantly in their infectivity to *Anopheles* species in vitro. However, available evidence indicates that artemisinin-resistant *P. falciparum* clinical isolates from Cambodia can be transmitted by diverse mosquito vectors of Southeast Asia and Africa [92]. It should be noted that despite the apparent overall differences between the malaria epidemiology in Africa and Southeast Asia, there are lower endemic areas in Africa with an epidemiology more similar to Southeast Asia than to the very high transmission settings in Africa.

Recent developments indicate that the parasite strains that end up posing the biggest risk to the currently used treatments in Africa may emerge there rather than being imported from elsewhere. Nevertheless, it is also possible that what will happen now is multiple soft sweeps of different *Pfkelch13* mutations that are later replaced by a few hard, selective sweeps of haplotypes emerging in low transmission areas. The response to resistance needs to consider these different possible scenarios, seeking both to minimize the impact of the emergence of artemisinin resistance in Africa and the risk of importation of resistant strains from other areas.

Currently, there are no alternatives to ACTs, though the antimalarial drug pipeline is promising [93,94]. The main aim must therefore be to prolong the therapeutic lifespan of the current treatments. The efficacy of ACTs remains high in the areas where delayed parasite clearance after treatment with artemisinins has been detected. The artemisinin component is responsible for most of the short-term clearance of parasites, while the partner drugs are efficacious in the absence of artemisinins and responsible for the overall therapeutic outcome [3]. Consequently, at present, the emergence of artemisinin partial resistance has limited consequences for the patients being treated with ACT as long as the partner drug remains efficacious. The worst scenario would be seeing a pattern similar to what has been seen in the GMS: the emergence of a strain carrying both resistance to artemisinin and to partner drugs.

5. Responding to resistance

Early guidance on the response to resistance recommended treating each focus of resistance as separate problems [16]. The epidemiology of each area differs, as do the main factors driving resistance and the possible responses. In the GMS, the epidemiology, decline in cases, and financial and political support have meant that elimination of *P. falciparum* has been and is a feasible target. However, elimination may not be a feasible target in the short term when resistance emerges in or spreads to higher burden areas. The aim must be to minimize any public health impact where resistance has emerged and limit the risk of spread by activities inside and outside the areas with resistance.

Interventions that decrease the selective advantage of parasites with reduced sensitivity to a drug will slow the

Table 1. Molecular markers of antimalarial drug resistance [2,169] (table adapted based on [2]).

Drug	Molecular markers	
	Gene	Mutation
4-aminoquinolines		
Chloroquine	*Pfcrt*	*K76T + different sets of mutations at other codons (including C72S, M74I, N75E, A220S, Q271E, N326S, I356T, R371I)*
	Pfmdr1 (in combination with *Pfcrt* mutations only)	*N86Y, Y184F, S1034C, N1042D, D1246Y*
Amodiaquine	Yet to be validated	Studies show that amodiaquine selects for *Pfmdr1* mutations
Piperaquine	*Pfpm2-3*	*Pfpm2-3 increased copy number*
	Pfcrt	*T93S, H97Y, F145I, I218F C350R*
Antifolates		
Pyrimethamine	*Pfdhfr*	*N51I, C59R, S108N, I164L*
Sulfadoxine	*Pfdhps*	*S436A/F, A437G, K540E, A581G, A613T/S*
Proguanil	*Pfdhfr*	*A16V, N51I, C59R, S108N, I164L*
Amino-alcohols		
Lumefantrine	Yet to be validated	Studies show that lumefantrine selects for *Pfmdr1* mutations (N86).
Mefloquine	*Pfmdr1*	*Pfmdr1 increased copy number*
Quinine	Yet to be validated	
Mannich base		
Pyronaridine	Yet to be validated	
Naphthoquinone		
Atovaquone	*Pfcytb*	*Y268N/S/C*
Sesquiterpene lactones		
Artemisinin and its derivatives	*PfK13*	List of candidate and validated markers developed

spread of resistance. Consequently, interventions needed include those that decrease drug pressure, ensure that drug pressure on a parasite population is not from one drug only, and that minimizes the risk that parasites from recrudescent cases are transmitted (see Figure 3).

A challenge in every response to resistance has been that the information available has not been sufficiently granular to clearly delineate the spread of resistance. This has hampered the ability to plan and target the response. Lessons learned from the artemisinin resistance containment efforts in the GMS as well as technological advances could help better guide future responses to resistance.

5.1. Surveillance of drug efficacy and resistance

Information on parasite resistance and treatment efficacy has been collected through three main methods: therapeutic efficacy studies (TES), in vitro/ex vivo assays, and molecular markers of drug resistance. WHO recommends that all national malaria programs establish sentinel sites and conduct TES in these sites every 2 years. TES are the gold standard for

informing antimalarial drug policy as outcomes have direct clinical relevance [2]. TES have been a crucial source of information in detecting changes in parasites' response to treatment. Nevertheless, TES are time-consuming and resource heavy. Additionally, the clinical response to treatment depends on factors not related to resistance including immunity. Consequently, detection of changes in parasite genotypes in TES studies may be delayed in highly endemic areas compared to low endemic areas. In vitro and ex vivo assessments of parasites' sensitivity to drugs have the advantage that they are not confounded by host immunity [95]. However, conducting these assessments requires substantial laboratory infrastructure and skilled human resources. Thus, to be able to adequately map the spread of resistance and hopefully detect it before it spreads, phenotypic surveillance needs to be supplemented by genotypic surveillance.

When the response to artemisinin resistance was initiated, there were no known markers of the delayed response to artemisinin. Since then, *PfKelch13* mutations have been identified as molecular markers, and there have been significant advances in sequencing technology. Therefore, mapping the prevalence of *PfKelch13* mutation is feasible and could help national programs to obtain useful information about the extent of resistance and inform appropriate actions. In the past, molecular markers of drug resistance have played a limited role in informing treatment policy. This is partly due to the low predictive value of molecular markers on clinical outcome [96–98]. Nevertheless, molecular surveillance can play an important role in providing early warning of changes happening and, at a minimum, help inform where further phenotypic studies are needed.

Validated molecular markers are not available for key drugs including the ACT partner drugs pyronaridine, lumefantrine, and amodiaquine (Table 1). Advances in sequencing technologies, such as next-generation sequencing (NGS) platforms, allow for targeted deep and whole-genome sequencing and can detect changes in patterns of malaria parasite diversity, potentially providing critical information. Although the costs of NGS technologies have decreased, they still require adequate laboratory infrastructure, expertise in data analysis, and high computing power, not always available in malaria endemic countries. Establishing centers of excellence or regional reference laboratories could help support the overall work. To ensure the accuracy and the comparability of the results from different laboratories, a good external quality assurance system will need to be implemented [99,100]. In some countries, it is possible that investments made due to COVID-19 both in laboratory infrastructure and in training of staff can be leveraged to also strengthen the work for malaria.

Large-scale routine sampling of malaria parasites can be done via existing surveys such as the malaria indicator surveys. Alternatively, dried blood spots or RDTs can be collected from a selection of health centers [101]. One of the challenges in the use of molecular marker data is the time lag to publication, meaning that data is often only publicly available years after the samples have been collected [96,97]. To be of full use for public health, resistance data need to be more readily available and considered part of routine surveillance.

5.2. Reduction of selection pressure

Limited number of drugs means that even when resistance has been detected, halting the use of that drug may not be the best option or even possible. Early guidance recognized that if part of the response requires increased use of the drug to which resistance is developing, then a probable outcome is that the proportion of cases that are resistant will increase. Consequently, seeking to lower transmission and stop the resistance spread with interventions other than drugs must be a basic part of any response to resistance. In the GMS, the usefulness of vector control in some areas has been challenged by the presence of exophagic, early biting mosquitos and transmission taking place in the forest [102,103]. Therefore, efforts have gone into finding new ways of providing protections such as giving forest workers hammock nets, although with mixed success [104–106]. Responses elsewhere will need to be built on an understanding of entomological and human factors contributing to malaria transmission to optimize the use of available tools.

Ensuring that those being treated for malaria are adequately protected by vector control measures could also help lessen the residual drug pressure by limiting the risk that those with low levels of antimalarial drugs in the blood are reinfected with malaria. Previous treatments with antimalarial have been found to be associated with malaria infection [107]; individuals working or living in conditions that once resulted in a malaria infection can be at higher risk of being reinfected.

Other strategies used to seek to reduce the selective drug pressure have been limiting the use of antimalarial drugs to only those who have been confirmed to have malaria by microscopy or RDT. Analyses of data from household surveys in sub-Saharan Africa in 2015–2019 estimates that only 37.7% of children under 5 years with fever who sought care received a finger or heel prick (a surrogate indicator for having been assessed for malaria through RDT or microscopy), while 80.5% received treatment with ACTs [1]. The consequence of an overuse of drugs is increased levels of residual drug pressure. A study in Tanzania in 2015 found that 12.4% of those participating in a cross-sectional survey had lumefantrine or desbutyl-lumefantrine in the blood [108]. The wider availability of RDTs has played a large role in the GMS in making progress toward ensuring that only those with confirmed infection receive antimalarial treatment. The provision of community-based diagnosis and treatment by village malaria workers and community health workers has played a big role in improving access to diagnosis and treatment [109–111] and has been an important component in the response to drug resistance. Combining increased access to quality diagnosis and treatment with training of health staff and information to patients can

help improve treatment adherence and thereby play a role in increasing effectiveness [112,113].

Eliminating substandard medicines, including monotherapies and increasing access to and effectiveness of quality treatment, will minimize the exposure of parasites to subtherapeutic levels of antimalarial drugs. Restricting the availability of substandard drugs and monotherapies requires strengthening the regulatory capacity and quality control laboratories at the national and regional levels [114,115]. A strong regulatory framework needs to be combined with increased capacity to enforce bans. The private sector remains the main source of treatment seeking for febrile illness in many countries. The Affordable Medicine Facility-malaria (AMFm) and Global Fund's Private Sector Co-Payment Mechanism have in some countries improved the quality of treatment provided to patients in the private sector by providing private sector subsidies for quality-assured ACTs, resulting in lower ACT prices and increased availability [116].

5.3. Use combinations of drugs

Ensuring that the drug pressure on a parasite population is not from one drug only can be done both by treating malaria with a combination of different drugs and by using different drugs for different interventions. Interventions currently recommended that do not only use ACTs include chemoprevention strategies such as seasonal malaria chemotherapy (SMC), intermittent preventive treatment in infants (IPTi) and intermittent preventive treatment for pregnant women (IPTp).

5.3.1. Multiple first-line treatments

Currently, countries typically recommend a specific single first-line ACT for the treatment of uncomplicated *P. falciparum*. If efficacy falls below 90%, WHO recommends changing the first-line policy to another ACT with an efficacy above 95% [66]. Having multiple first-line treatments (MFT) has been proposed as a way of delaying the development and spread of resistance and prolonging the useful therapeutic life of the ACTs [117–121]. Most countries have different ACTs registered and permit their use in the private sector. Therefore, where the private sector plays a large role in treatment seeking, different ACTs are commonly used. However, this has not occurred in the context of a planned strategy to prevent resistance by actively promoting heterogeneity of antimalarial drug treatment. The argument in favor of MFL is simple: using different partner drugs will minimize selective drug pressure for a specific drug. However, in 2013, a WHO Technical Expert Group on Drug Resistance and Containment considered the divergent results from mathematical models [117,118] on the benefit of MFT in certain settings, and subsequently refrained from endorsing a general recommendation on the implementation of MFT [122]. MFT strategies were found to delay but not stop the emergence of resistant strains. However, one model found that in areas of high drug usage, MFT performed slightly worse in prolonging the therapeutic life of the used ACTs than did sequential use. Furthermore, inadequate dosage was found to be a much more potent driver of drug resistance than the decision as to whether to deploy drugs as MFL or sequentially [117,118].

Weighing against promoting MFL are both the cost and logistical challenges, as well as the fear that MFL would favor the selection of genotypes resistant to multiple ACT partner drugs earlier than other strategies [118]. In Cambodia, ACTs have been deployed sequentially with DHA-PPQ replacing AS-MQ only to again be replaced by AS-MQ in 2016. This was possible because following the shift to DHA-PPQ, parasites regained susceptibility to mefloquine [123]. In Cambodia, the potential use of MFL is made unfeasible due to the lack of available treatments, and the fear of not having a second-line treatment available to treat failures. At present, the options are limited by the number of ACTs available and by the fact that all the recommended treatments for uncomplicated *P. falciparum* considered for inclusion in an MFL policy contain an artemisinin derivative. There are several new antimalarial compounds in the drug pipeline. When these are ready to be introduced, this must be done in a way to both ensure optimal patient treatment and to maximize the useful therapeutic life, potentially through an MFL policy [121].

5.3.2. Triple combination therapies

The short half-life of artemisinins as one of the two components of the ACTs means that parasites can be exposed to the slowly eliminating partner drug as a monotherapy. Recently, the potential use of triple artemisinin-containing combination antimalarial treatments (TACTs) has been debated [124–132]. TACTs would combine an artemisinin component with two of the currently used ACT partner drugs. The partner drugs most frequently proposed to be combined in TACTs are piperaquine + mefloquine and lumefantrine + amodiaquine due to observations of these drugs having antagonistic resistance mechanisms. Other triple combination therapies that are being tested are an ACT in combination with atovaquone-proguanil [133,134].

A large-scale trial recently evaluated DHA-PPQ, AS-MQ, DHA-PPQ plus mefloquine, artemether–lumefantrine (AL) and AL plus amodiaquine. In countries with piperaquine resistance (Cambodia, Thailand, and Vietnam), the 42-day PCR-corrected efficacy was 48% for DHA-PPQ and 98% for DHA-PPQ plus mefloquine. The trial only has data for AS-MQ in Cambodia, where it was 95%. In Myanmar, piperaquine resistance has not been identified, and the 42-day PCR-corrected efficacy was 100% for DHA-PPQ and 91% for DHA-PPQ plus mefloquine (n = 46). The 42-day PCR-corrected efficacy of AL plus amodiaquine was 98% and similar to AL: 97%; these drugs were tested in Bangladesh, the Democratic Republic of the Congo, Lao PDR, India, and Myanmar [125].

In the GMS, it is not surprising that the addition of mefloquine to DHA-PPQ in areas where parasites are resistant to piperaquine but sensitive to mefloquine results in high efficacy [129]. The main purpose in deploying DHA-PPQ plus mefloquine at present would not be to provide patients with an efficacious treatment, as both AS-MQ and artesunate-pyronaridine remain highly efficacious [2]. Rather, the purpose would be to seek to delay the reemergence of mefloquine resistance and, when it happens, to provide efficacious treatment. However, piperaquine resistance is widespread and parasites carrying resistance to both mefloquine and piperaquine have been identified, meaning that the spread of these

parasites is a risk [135]. Shifting to a triple combination therapies when significant levels of resistance have already been developed to component drugs will mean that the potential benefits of the combination in terms of resistance prevention could be limited [68,130]. Furthermore, while the study reported the TACTs to be safe and well tolerated, adding another drug to established regimens would require further studies on tolerability, toxicity, and drug interactions [131]. The GMS countries aim to eliminate *P. falciparum* by the end of 2023, thus hopefully leaving no need for TACTs or any other new combinations in this region.

A question remaining is the potential usefulness of TACTs outside the GMS. The most likely TACT to be deployed is AL plus amodiaquine as tested in the trial [125]. AL has been observed to select for *Pfmdr1* N86, 184F, and D1246 (the NFD haplotype), while artesunate-amodiaquine (AS-AQ) appears to select for *Pfmdr1* 86Y, Y184, and 1246Y (the YYY haplotype) [136]. The YYY haplotype has been found to be associated with amodiaquine treatment failures in Africa, while the NFD haplotype was associated with AL failures [137]. However, the YYY haplotype is generally not observed in Asia [136]. A study of the efficacy of AS-AQ in Cambodia in 2016 found a high failure rate (19.0%). The parasites were NFD haplotype and amodiaquine resistance was not found to be associated with any of the previously identified molecular markers [138]. The *Pfmdr1* N86Y change has been associated with resistance to both chloroquine and amodiaquine. Countries deploying AL after the withdrawal of chloroquine have seen a return of the wild-type *Pfmdr1* N86 [139]. The same reversal may not happen in countries where AS-AQ is widely used due to the cross-resistance between chloroquine and amodiaquine. It is possible that the opposing selection pressure observed is linked with chloroquine resistance patterns, and the search for good molecular markers for amodiaquine and lumefantrine needs to continue.

Other proposed changes in how the currently available treatments are used include extending ACT regimens from the standard 3 days to 5 or 6 days or using two different ACTs sequentially. An extended 5-day AL regimen was tested in Myanmar [140], and a 6-day AL regime was tested in Tanzania [141] compared with the standard 3-day regime. Both studies found the extended treatment to be safe and well tolerated but not superior to the standard 3-day treatment.

AL and AS-AQ are efficacious in Africa so the purpose of combining the drugs in a TACT or extending the treatment would not be to provide the patients currently being treated with a better treatment but to prevent the emergence and spread of resistance to lumefantrine and amodiaquine. AL and AS-AQ are the most widely recommended first-line treatments in Africa. Losing these drugs before new treatments become available would be devastating. The potential delay in resistance benefiting future patients would have to be balanced with any potentially increased risk for current patients caused by providing an additional drug and drug–drug interaction, and with the resources needed to get co-formulated TACTs with dosing for all age groups or alternative 5 or 6-day regimens ready for and introduced into policy, and the need to allocate more funding to treatment with these drugs versus for instance spending this funding on vector control [126]. Providing combination treatments co-blistered rather than co-formulated could result in more frequent use of one of the drugs alone. Focusing on improving how the currently available treatments and tools are used and developing new classes and combinations of antimalarials rather than spending resources on a temporary solution are more likely to provide the greatest benefit to current and future populations at risk.

5.4. Stopping transmission of resistant parasites

Transmission of malaria from a human is dependent on the presence of the nonpathogenic sexual-stage parasites gametocytes as only gametocytes are infectious to mosquitos. Gametocytes are formed when asexual parasites differentiate into male and female gametocytes. When these are ingested during a blood feeding by a female *Anopheles* mosquito, they are activated in the mosquito midgut into male and female gametes and can fertilize and produce a zygote. This zygote is subsequently transformed into an ookinete, an oocyst, and sporozoites that can be transmitted to humans once again when the mosquito bites and injects saliva [142,143].

Artemisinins are well known for their ability to rapidly kill the asexual parasite in the red blood cells [143]. However, artemisinins have also been shown to be able to block the activation of male gametes, thereby hindering transmission. Some strains carrying *PfKelch13* mutations have been shown to have an increased capability to activate gametes and infect mosquitoes under artemisinin treatment compared with sensitive controls [143]. This could significantly hasten the spread of artemisinin resistance.

Primaquine has been shown to block transmission through its effect on gametocyte persistence and infectivity. Therefore, WHO recommends adding a single low-dose (0.25 mg/kg) of primaquine in combination with ACTs in areas of low transmission or artemisinin-resistant *P. falciparum*. Particularly, areas threatened by artemisinin resistance and areas with elimination programs were expected to benefit from this recommendation [144,145]. Currently, there are no available alternatives to ACTs and the potential role of ACT coverage in driving the spread of *PfKelch13* mutations makes the addition of primaquine in areas of artemisinin resistance a priority.

Treatment failures drive the emergence of resistance by facilitating onward transmission of parasites that have been exposed to drugs [67]. Having systems that follow-up, catch and treat all failures is difficult even in high resource setting. Greater emphasis on identifying treatment failures in the training of staff and in the development of surveillance systems could increase the number of such failures who then receive curative re-treatment before onward transmission. Other options to explore include planning future treatments, so they are not only efficacious in trial setting where adherence is assured; this could be done by reducing the duration of the regimens, ideally to a single dose [146].

5.5. Containing and eliminating resistant strains

Additional interventions proposed in areas where resistance has developed include MDA, which seeks to reduce the number of malaria cases using drugs other than the ones to which resistance has developed. In 2010, an expert meeting was convened to evaluate the appropriateness of including MDA in the strategy to contain artemisinin-resistant parasites in the GMS [147]. The meeting recommended piloting of MDA; this was thought likely to bring about significant reductions in parasite biomass that would diminish the probability that resistant parasites would spread, but MDA was thought unlikely to permanently interrupt *P. falciparum* transmission. The recommended drug of choice was atovaquone-proguanil [147]. Where drugs other than the drug to which resistance has developed are available for the MDA, the rationale is clear: MDA could potentially both lower the malaria burden and the proportion of resistant parasites. The expert group did not endorse the use of ACTs for MDA in the GMS, since the treatment would likely be more effective at targeting artemisinin-sensitive parasites, leading to an increase in the proportion of resistant infections; failure to remove all resistant parasites would eventually allow them to repopulate. No pilot using atovaquone-proguanil was undertaken due to the emergence of a single point mutation in the *cytochrome b* gene conferring high-level atovaquone resistance, which occurred after brief use of this drug as a first-line treatment in parts of Cambodia [49]. Some modeling and reviews argued for the use of MDA with ACTs [148,149], based on the premise that MDA would clear infections among individuals with low-density, blood-stage parasitemia often not detected by RDTs or microscopy. These individuals would not receive treatment when presenting at a health facility with symptoms, would not be detected in any focal or mass screenings, and thus could potentially serve as an infectious reservoir [150–152].

DHA-PPQ was the ACT proposed for MDA due to the long half-life of piperaquine allowing the drug to act as chemoprophylaxis for longer than other partner drugs. It was reasoned that provided full treatment were given, three rounds of MDA would be unlikely to contribute to artemisinin resistance because most individuals would not be hyperparasitemic but rather have low density asymptomatic infections, suggesting that the likelihood of MDA causing de novo emergence would be very low. Furthermore, the short half-life of dihydroartemisinin would provide a too short window of selection for the MDA with DHA-PPQ to drive the spread of artemisinin resistance [148,149].

In a 2015 WHO recommendation on MDA, it was stated that 'Given the threat of multidrug resistance and the WHO call for malaria elimination in the GMS, MDA may be considered as a component of accelerated malaria elimination efforts in areas of the GMS with good access to treatment, vector control and surveillance.' [153]. The recommendation stated that MDA should only be started if there is a good chance that elimination is feasible in the area where it is being administered [153]. Studies were undertaken to assess the effectiveness of MDA with DHA-PPQ in reducing *P. falciparum* incidence and prevalence [53,154–158]. A cluster randomized trial in Myanmar, Vietnam, Cambodia, and the Lao PDR established vector control and community-based case management and provided three monthly rounds of DHA-PPQ MDA in eight villages, while another eight villages served as controls for 12 months. A low dose of primaquine was given on day 1 in all countries except Cambodia. Of the villagers, 87% completed at least 1 round and 57% participated in all 3 rounds. The clearance rate was 87% in Vietnam, 88% in Cambodia, and 100% in Lao PDR and Myanmar.

While the intervention had a substantial impact on the prevalence of *P. falciparum* infections at 3 months, after 12 months, *P. falciparum* infections had returned due to the spread of the remaining infections as well as reintroduction from surrounding areas. The prevalence at 12 months was below baseline levels, and the researchers concluded that MDA might be a useful tool to accelerate falciparum malaria elimination in low endemicity settings. MDA was found to be less effective for *P. vivax* [158].

Modeling was done to compare findings from four established models on the effectiveness of MDA in different settings. It was concluded that while MDA could reduce transmission for a limited time, it has to be repeated regularly for sustained effect [159]. Effective vector control, early diagnosis and treatment, and good surveillance and response systems are prerequisites to achieve and sustain elimination, as well as being a requirement to maintain any reductions gained from an MDA [160]. The potential gain from an MDA needs to be weighed against the considerable resources needed and the potential impact on drug resistance [161]. In large-scale MDA, identifying individuals in whom the treatment fails to clear parasites will be difficult; if the failure to clear parasites is due to partner drug resistance, a large proportion of the remaining parasites will carry this resistance. If the treatment blocks the activation of male gametes only for parasites not carrying mutations conferring artemisinin resistance, successful transmissions are more likely caused by artemisinin-resistant parasites; therefore, primaquine needs to be included [143,145]. Studies are underway on the impact of adding an endectocide treatment, such as ivermectin, to an MDA with an ACT. Endectocides reduce the longevity of *Anopheles* mosquitoes that feed on treated hosts, potentially decreasing transmission and further increasing the impact of the MDA and decreasing the risk of selection of resistant parasites [162,163]. However, with the currently available drugs there are relatively few situations where MDA may play a role in the response to resistance; these will primarily be in isolated, low-transmission settings. Targeted MDA in forest work camps, and other drug-based interventions targeting high-risk groups such as intermittent preventive treatment can play a role in helping to achieve elimination in the GMS as it has proven difficult to reach these population groups [164,165].

In the past, resistance has developed in border areas. Additionally, cross-border migrants often work in areas that put them at high risk of malaria. International coordination and efforts to minimize the barriers to malaria

services faced by migrants are therefore often needed. While artemisinin partial resistance has emerged in multiple locations, the risk of spreading highly resistant strains across continents should not be ignored. In the GMS, actions taken to minimize the risk that resistant strain spreads include attempts to screen migrants and other travelers for malaria and provide them with treatment. A key challenge is that borders are often porous and many of those at highest risk of malaria cross via unofficial routes into neighboring countries [166]. Nevertheless, reducing the risk of spread must be a priority where resistance emerges that is deemed to be a potential threat to public health and a significant number of possible carriers of resistant parasites can be reached. In Cambodia, protocols were developed to screen UN peacekeepers being sent from Cambodia to highly endemic areas in Africa to ensure that resistant parasites were not spread this way.

6. Expert opinion

There is an urgent need to improve the surveillance of resistance. Responses to resistance have built on the idea of treating each focus of resistance as a separate problem; the aim has been to eliminate malaria, where resistance is detected, and limit the number of parasites crossing from the resistant areas to other endemic areas. However, the information available has not been sufficiently granular, and resistance has become discernible too late for containment to be possible.

Data from Rwanda on the emergence of indigenous *PfKelch13* mutations have recently been published from studies starting in 2013. There are indications that *PfKelch13* mutations are spreading elsewhere in Africa. Inadequate information on how widespread these mutations are limits the options and support for a strong, coordinated response. Thus, better phenotypic and genotypic surveillance is a priority. Advances in sequencing technologies and investments made due to COVID-19 both in laboratory infrastructure and in training of staff should be leveraged to also strengthen the work for malaria.

Lack of data should not be used as an excuse for inaction. In the GMS, rapid spread of strains carrying resistance to both piperaquine and artemisinin resulted in DHA-PPQ efficacy falling quickly. The same must not be allowed to happen in Africa; if declining efficacy is observed for an ACT, the ACT needs to be changed rapidly. Where data have confirmed the high prevalence of *PfKelch13* mutations, priority actions include adding a single, low-dose primaquine to ACT treatments. Other activities must be planned based on analyses of key factors that can help explain why resistance emerged where it did and what may cause it to worsen and spread. These activities are likely to include addressing gaps in vector control coverage, and stopping the continued availability of monotherapies and substandard drugs, while at the same time increasing access to early diagnosis and treatment with quality-assured ACTs.

The utility of triple combination treatments based on currently available drugs is affected by both resistance limiting the drug choices and the need for further studies on tolerability, toxicity, and drug interactions before any co-formulated triple combination therapies can become available. Currently, all the possible first-line treatments contain an artemisinin and almost all countries in Africa recommend one of two different ACTs as first-line treatments: AL or AS-AQ. The result is a high degree of vulnerability to resistance to amodiaquine and lumefantrine. Experiences from ACTs in the GMS and TACTs should be used to better combine and make use of current and new compounds to lessen this vulnerability.

Drugs for which resistance has developed have only rarely been used as part of an aggressive strategy to lower transmission. The fear is that doing so would result in a loss of efficacy of the drugs needed to treat patients and that, unless elimination is achieved, there would be an eventual increase in cases and spread caused by parasites with a high level of resistance. At present, MDA can only play a minor role in the response to resistance; this role is limited to isolated low-endemic areas or possibly as a targeted intervention for high-risk groups.

The biggest risk to the currently used treatments in Africa may emerge there rather than being imported from elsewhere. However, the possibility of history repeating itself and parasites with a high level of resistance being imported from elsewhere cannot be ignored. The current efforts to eliminate malaria in the GMS need to continue.

7. Five-year view

Over the last decades, activities and research have focused on how best to respond to resistance in the Southeast Asian context. The progress made toward malaria elimination in the GMS and the emergence of artemisinin partial resistance in Africa means that the focus and funding need to shift. At present, the recommended ACTs are still efficacious in Africa, but resistance to key ACT partner drugs is likely to emerge, and it is possible that artemisinin partial resistance will help fuel the spread of partner drug resistance.

The response to resistance in Africa will to a large extent have to focus on surveillance and getting the basics right: providing access to vector control, diagnosis, and the recommended combination treatments, and eliminating oral artemisinin monotherapies and substandard treatments. If sufficient investments are made, technological advances could mean that the information available in 5 years will be able to better guide the response and improve the use of the tools currently available as well as new tools, such as the vaccine being piloted in young African children. New ways to use the available treatments will continue to be debated, but any benefits of new combination treatments and strategies like multiple first-lines are likely only to be fully realized once new drug compounds are available [167,168].

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1. Covers Cambodia, China (Yunnan province), Lao PDR, Myanmar, Thailand, and Vietnam.

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MPAG Session 3
Rectal artesunate and quality of care

Malaria Policy Advisory Group (MPAG)

Virtual meeting – 5 October 2021

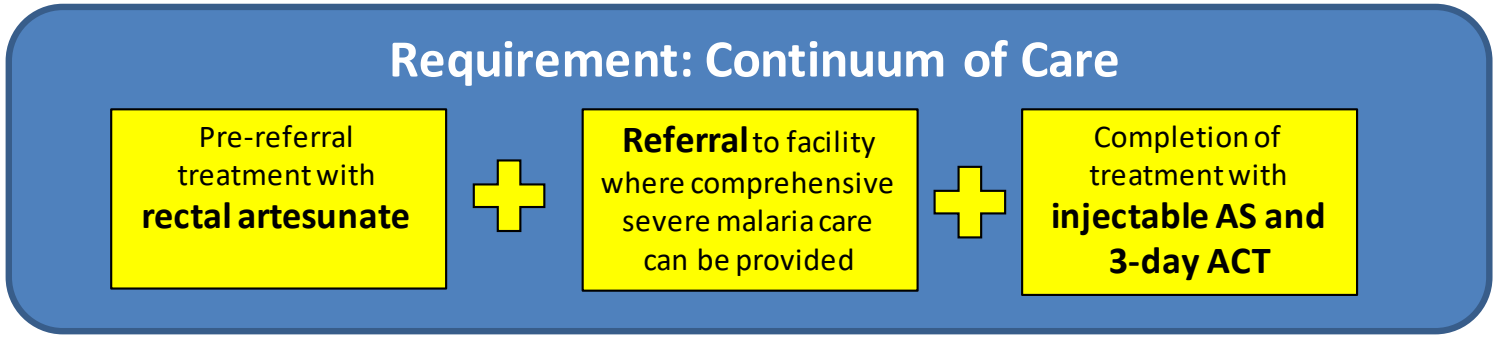
Silvia Schwarte, WHO/GMP (e-mail: schwartes@who.int)

Global **Malaria** Programme

World Health Organization

Background (I)

- Since 2005, WHO has recommended[1] rectal artesunate (RAS) as an effective pre-referral treatment for severe malaria in children less than 6 years of age.



- As of 2017, only 16 countries in Africa had included the use of pre-referral treatment with RAS in their national treatment guidelines, some of them were not fully aligned with WHO guidance.
- RAS was expected to become available at a **quality-assured standard**, acceptable for procurement on a large-scale with international funds, and this was a **critical time to demonstrate how this life-saving medicine can be correctly used across diverse, 'real-life' settings.**

[1] WHO Guidelines for Malaria (https://app.magicapp.org/#/guideline/4870)

Background (II)

Community access to rectal artesunate for malaria (**CARAMAL)** funded by UNITAID in April 2017

CARAMAL consortium:
CHAI (project lead), **UNICEF** (implementer) and **Swiss TPH** (operational research)
The Consortium was supported through the Unitaid-funded MMV Supply Grant and WHO Enabler Grant

Project countries: **DRC, Nigeria, Uganda**

Project goal: Contribute to reducing malaria mortality in children globally with 4 project outputs:

- Quality Assured rectal artesunate available in malaria endemic countries
- Rectal artesunate introduced as pre-referral treatment into strengthened severe malaria management systems in implementation areas
- Evidence generated and shared on effects and rational use of rectal artesunate
- Transition to evidence-based and step-wise scale-up of rectal artesunate in target countries

Malaria cases: 229 million (2019)

- Twenty-nine countries accounted for 95% of global malaria cases; Nigeria (27%), the Democratic Republic of the Congo (12%), Uganda (5%), Mozambique (4%) and Niger (3%) accounted for ~ 51% of global cases
- WHO African Region accounted for about 94% of cases with an estimated 215 million cases in 2019.

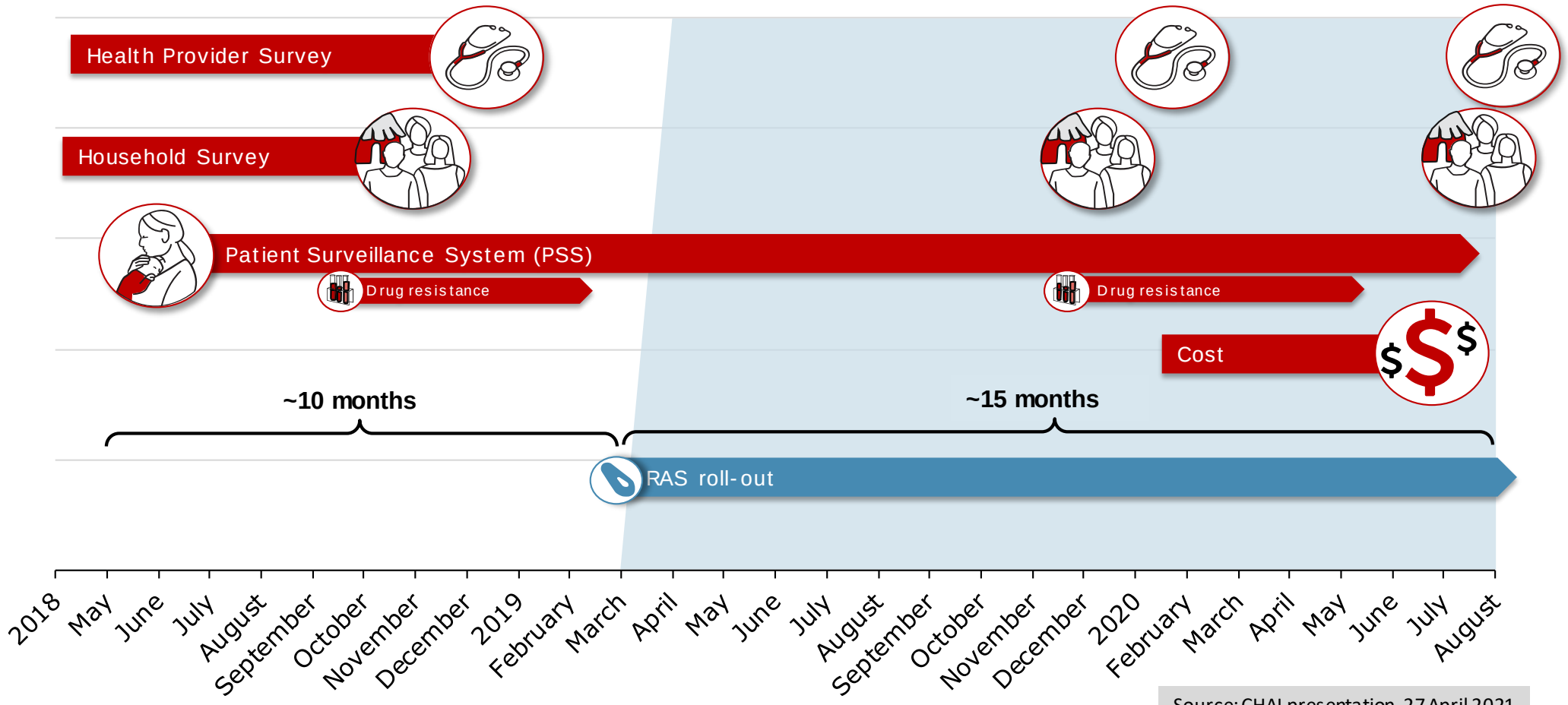
Malaria deaths: 409 000 – 67% in children < 5 years (2019)

- About 95% of malaria deaths were in 31 countries; Nigeria (23%), the Democratic Republic of the Congo (11%), the United Republic of Tanzania (5%), Mozambique (4%), Niger (4%) and Burkina Faso (4%) accounted for about 51% of all malaria deaths in 2019.

Source: WMR 2020

Study design

- **Observational study** alongside the rollout of RAS by UNICEF, government agencies & partners
- **Before-and-after** plausibility design measuring **case fatality** and **predictors along the continuum of care**



Source: CHAI presentation, 27 April 2021

CARAMAL findings

CARAMAL RESEARCH QUESTIONS

1. What are the **minimal requirements** of a community case management system **to ensure that RAS is an effective part of the continuum of care**
2. What are the **unintended consequences** of scaled implementation, such as adverse drug reactions, unforeseen costs, or unforeseen issues in treatment of malaria at all levels of care, and how can they be addressed?
3. Is there any **use of RAS beyond the recommended guidelines**, including full treatment of severe cases with RAS at community level, and the treatment of uncomplicated malaria with RAS? What interventions are necessary to avoid this inappropriate use?

CARAMAL RESEARCH FINDINGS

1. The CARAMAL study helped to highlight **major deficiencies in the quality of the cascade of care** in all three countries. A number of potential corrective measures as well as a set of minimal requirements of a community case management system were identified.
2. **Unintended consequences** of scaled implementation were identified, including
   - a large proportion of children received **RAS pre-referral treatment without meeting criteria of severe** malaria
   - a significant proportion of those that received RAS dose was not in line with their age, resulting in **underdosing**
   - among those that received RAS, there was a **decrease in referral** (as required by WHO recommendations)
   - Many children did not receive the recommended full treatment for severe malaria and were left with monotherapy (RAS and / or injectable artesunate) resulting in an **increase in the use of artemisinin monotherapy**
3. There were **no significant data generated that would allow the expansion of the current WHO** policy recommendation

CARAMAL findings

CARAMAL RESEARCH QUESTIONS

4. Can the introduction of pre-referral QA RAS **reduce severe malaria case fatality ratio** over time under real-world operational circumstances in three distinct settings?
5. What are the **costs and cost-effectiveness** of community and peripheral health facility-based RAS?

CARAMAL RESEARCH FINDINGS

4. The introduction and scale up of RAS was **not associated with a decrease in CFR**. In fact, in two of the countries, the introduction and scaled up use of RAS was followed with an increase in CFR (large and statistically significant in Nigeria). In Uganda, there was no change in CFR before and after the introduction. However, the very low CFR in Uganda is not compatible with previously reported CFR of severe malaria even when treated at excellent referral facilities.
5. The use of monotherapy may **contribute to accelerate the selection of artemisinin partial resistant strains**. The resistance monitoring baseline survey showed that several haplotypes of K13 circulated in all the three sites mainly at low rate without any sign for emergence. The endline data did not show any mutant selection in DRC and Nigeria, however, in **Uganda selection** of K13 mutant with the haplotype C469Y was reported.

CARAMAL Project: Evidence assessment

WHO Technical Consultation, 27-29 April 2021

- Review, discuss and assess the evidence generated from the CARAMAL project: DRC, Nigeria and Uganda
- Review, discuss and assess the evidence obtained from additional countries where RAS is implemented and scaled up: Angola, Malawi, Senegal, Sierra Leone, Zambia
- Discuss findings on artemisinin resistance and impact generated by the CARAMAL project

Ecole de Santé Publique de Kinshasa
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UNIVERSITE DE KINSHASA

akena associates

School of Public Health
College of Health Sciences,
Makerere University

CLINTON HEALTH ACCESS INITIATIVE

unicef

Unitaid
Innovation in Global Health

Swiss TPH

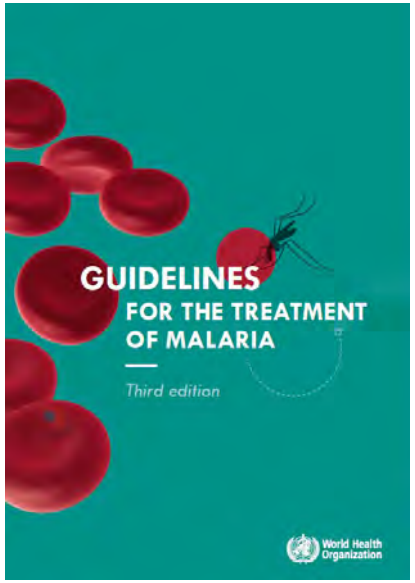
Community Access to Rectal Artesunate for Malaria (CARAMAL)

Evidence from implementing pre-referral rectal artesunate in DR Congo, Nigeria and Uganda

Manuel Hetzel, on behalf of CARAMAL
Swiss Tropical and Public Health Institute

WHO MPAG Meeting, 5 October 2021

Pre-referral rectal artesunate: current recommendations



Where intramuscular injections of artesunate are not available, treat children < 6 years with a single rectal dose (10 mg/kg bw) of artesunate, and refer immediately to an appropriate facility for further care. Do not use rectal artesunate in older children and adults.

- Two WHO pre-qualified rectal artesunate (RAS) products available only since 2018

- Full treatment includes
  1. parenteral artesunate for at least 24 hours, then
  2. full course of artemisinin-based combination therapy (ACT)

Pre-referral rectal artesunate: prior evidence

- In a randomised controlled trial, pre-referral RAS reduced case fatality rate in children <6 years with signs of severe malaria by 26% (Gomes 2009, Okebe 2014)

  Context: CFR (Africa) 4%, >90% referral completion, no data on post-referral treatment, inconsistent follow-up time, larger effect in post hoc sub-group analysis

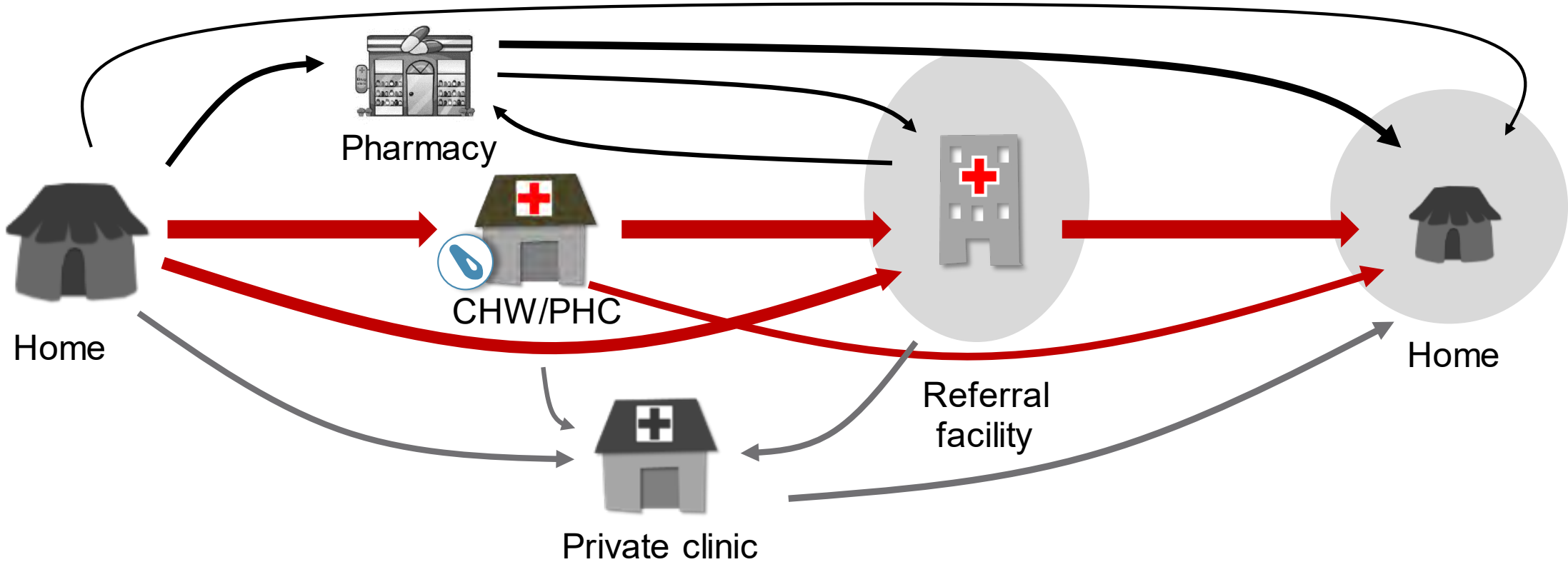
- Pre-referral RAS *in combination with* strengthened referral and post-referral treatment reduced severe malaria CFR in health facilities in Malawi (Green 2019)

  Context: emergency transport system, no drug stock-outs, health care worker training, increase in % malaria cases categorised as 'severe', no user vs. non-user analysis

- **What is the optimal way of rolling out RAS on a large scale?**
- **What is the effect of RAS implemented without major supportive interventions?**

Complexity of treatment pathways

Initial treatment seeking	RAS administration & referral	Referral completion	Post-referral treatment: inj. AS & ACT	Health outcome



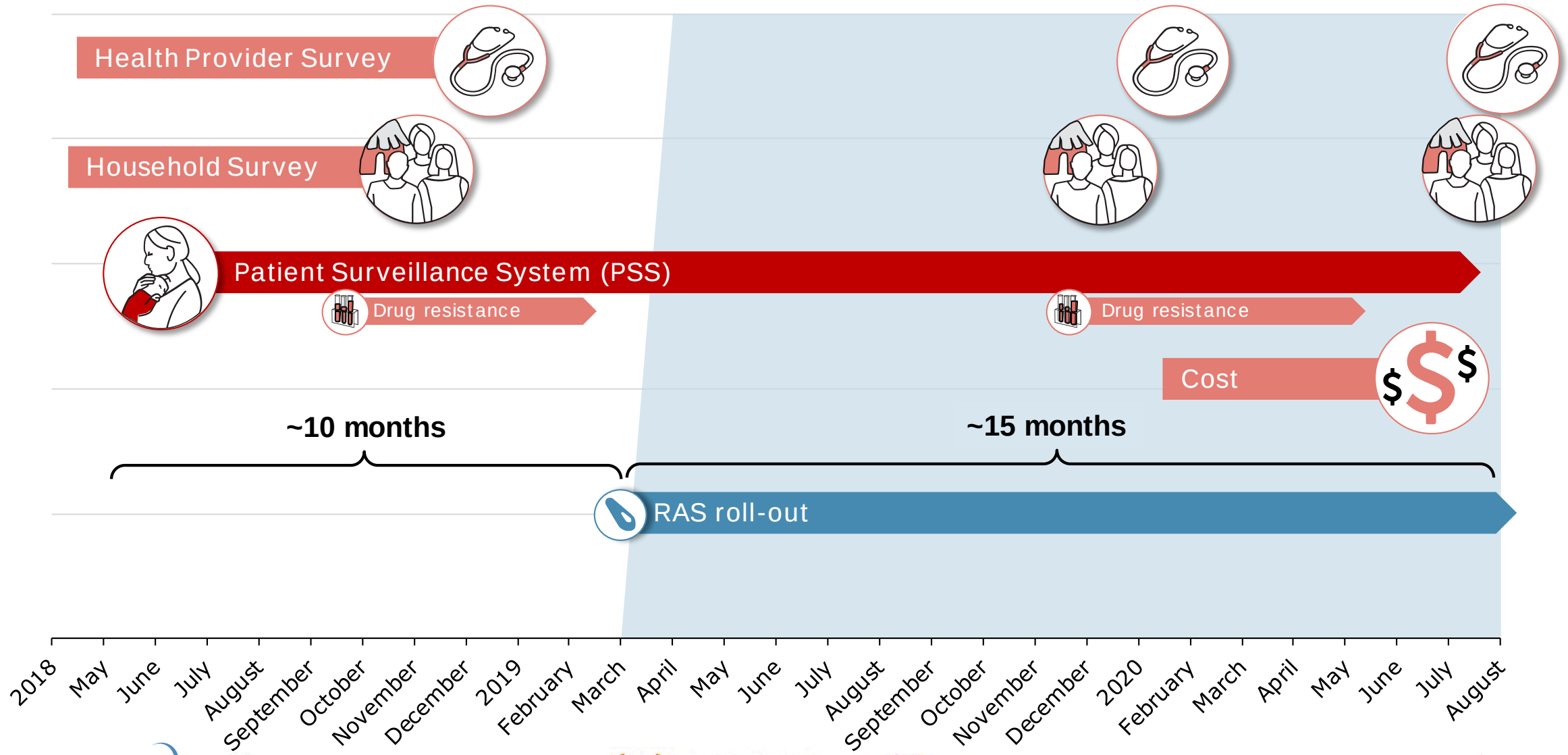
CARAMAL (Community Access to Rectal Artesunate for Malaria)

- Observational study accompanying the large-scale roll-out of pre-referral RAS through community health workers (iCCM) and primary health centres
- Goal: **Contribute to developing operational guidance for the scale-up of pre-referral RAS**

- **Implementation** of pre-referral RAS (children <5) by UNICEF & local health authorities
- **Research** by Swiss TPH, Kinshasa School of Public Health (DRC), Akena Associates (Nigeria), Makerere University School of Public Health (Uganda)

3 health zones/LGAs/districts per country

CARAMAL research activities: children <5 years



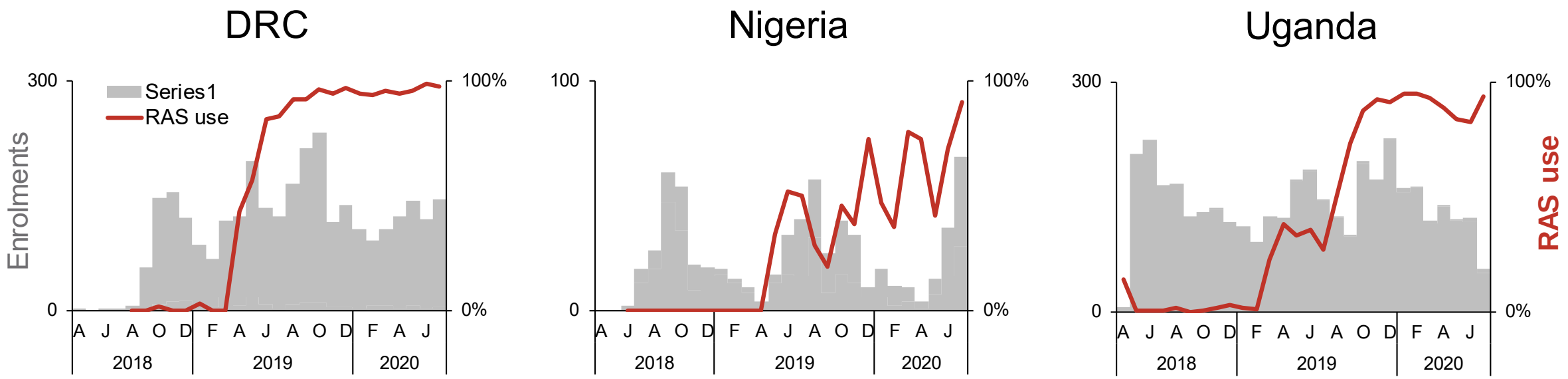
CARAMAL PSS study population

- Children <5 years with fever, danger signs (and positive malaria test)
- Monitored during admission (if applicable); day 28 home visit

Enrolment location	DR Congo		Nigeria		Uganda		Overall
	Pre-RAS	Post-RAS	Pre-RAS	Post-RAS	Pre-RAS	Post-RAS	
Community health workers (CHW)	60	104	178	180	1608	2285	**4415**
Primary health centres (PHC)	701	2177	67	233	1	33	**3212**
Referral health facilities	973	1525	351	496	620	2166	**6131**
Total	**1734**	**3806**	**596**	**909**	**2229**	**4484**	**13758**

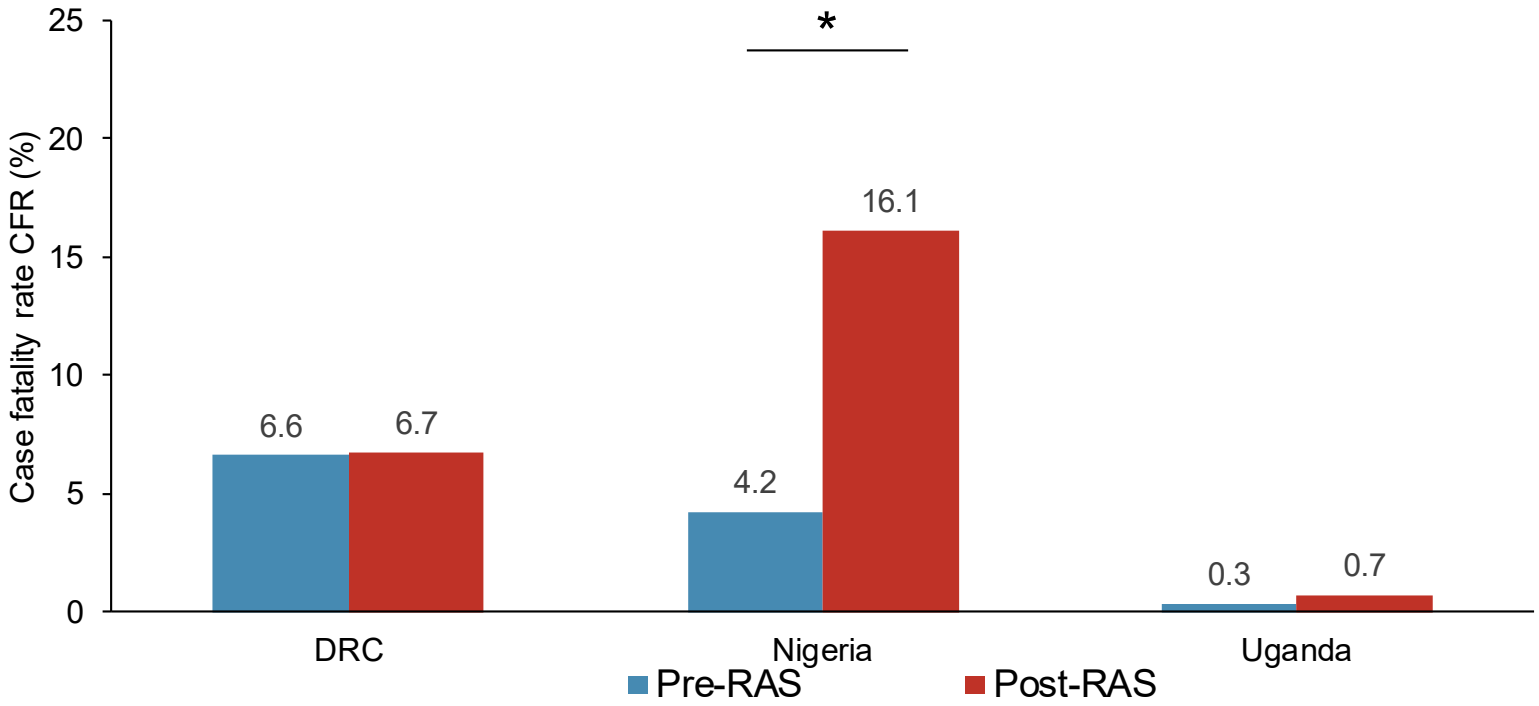
Overall: Pre-RAS 1/3 Post-RAS 2/3

RAS uptake



Lengeler, Burri et al. manuscript in preparation

Day 28 case fatality rate and impact of RAS

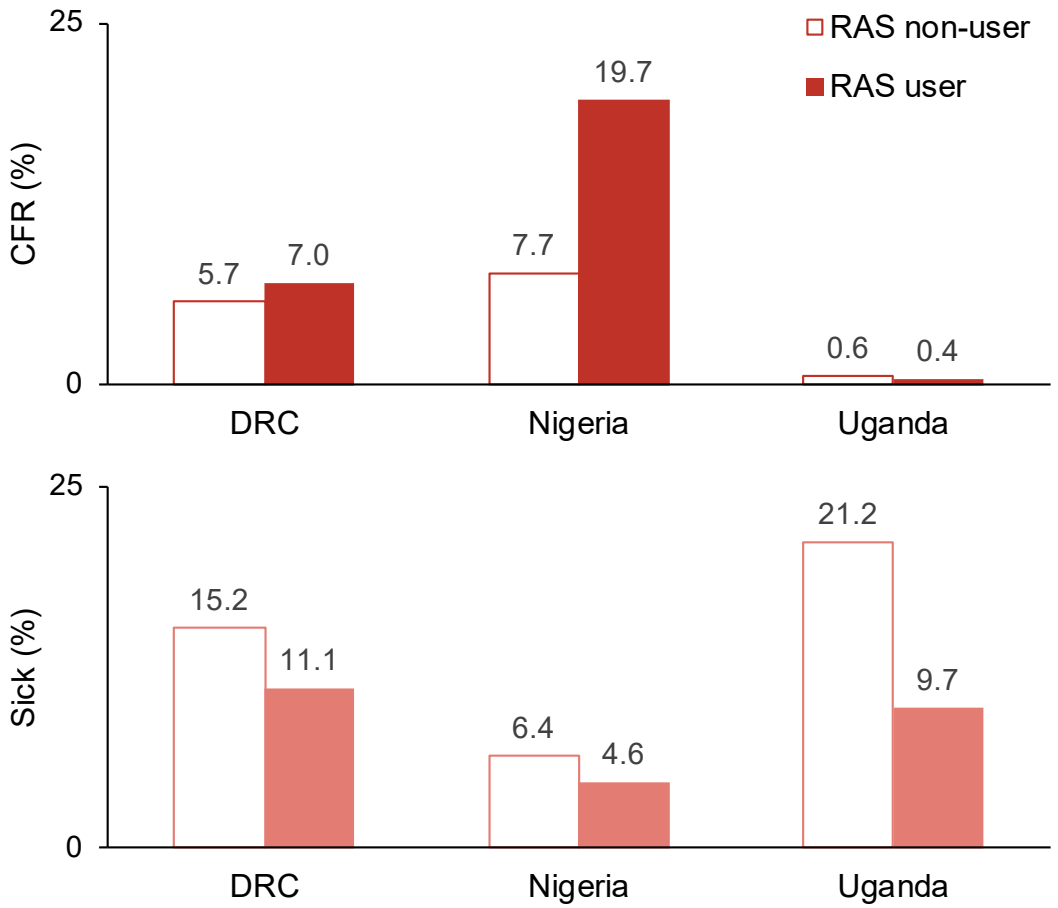


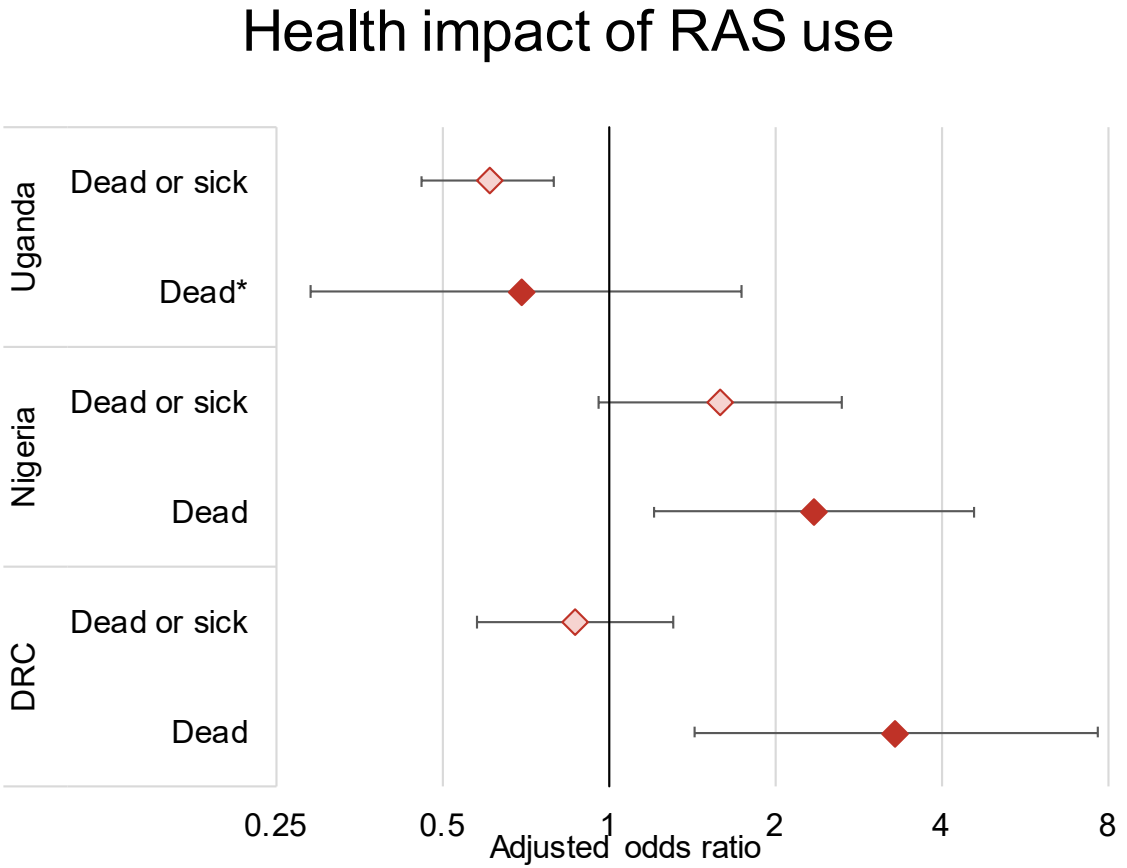
* p < 0.001

Hetzel et al. *medRxiv* 2021 https://doi.org/10.1101/2021.09.24.21263966

Day 28 case fatality rate and impact of RAS

Entire study period (pre and post RAS)

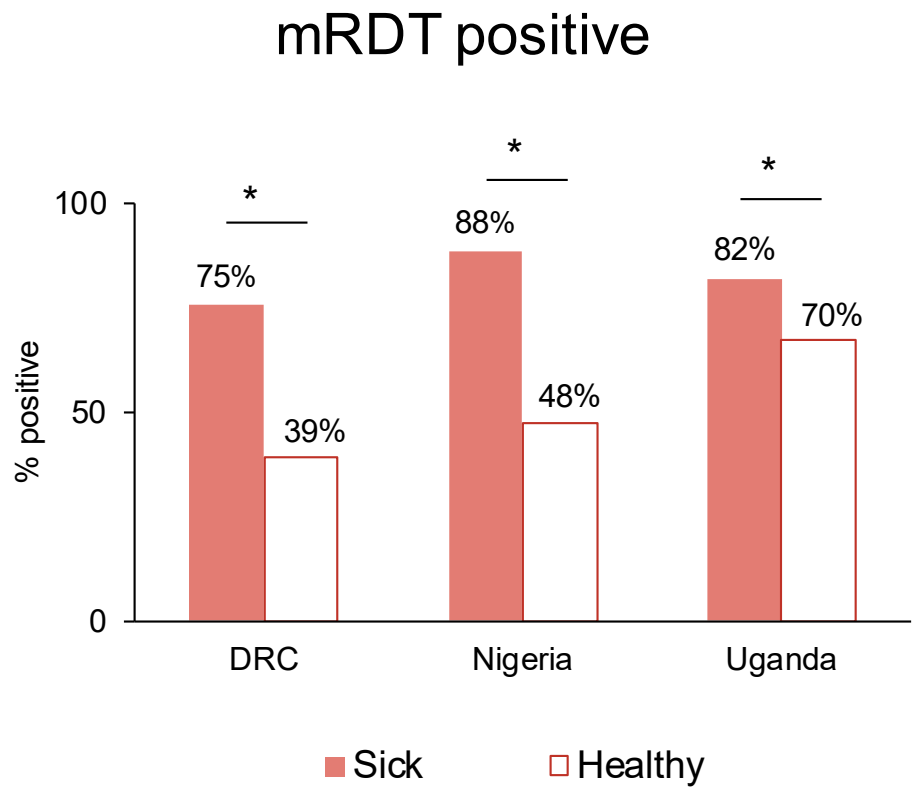


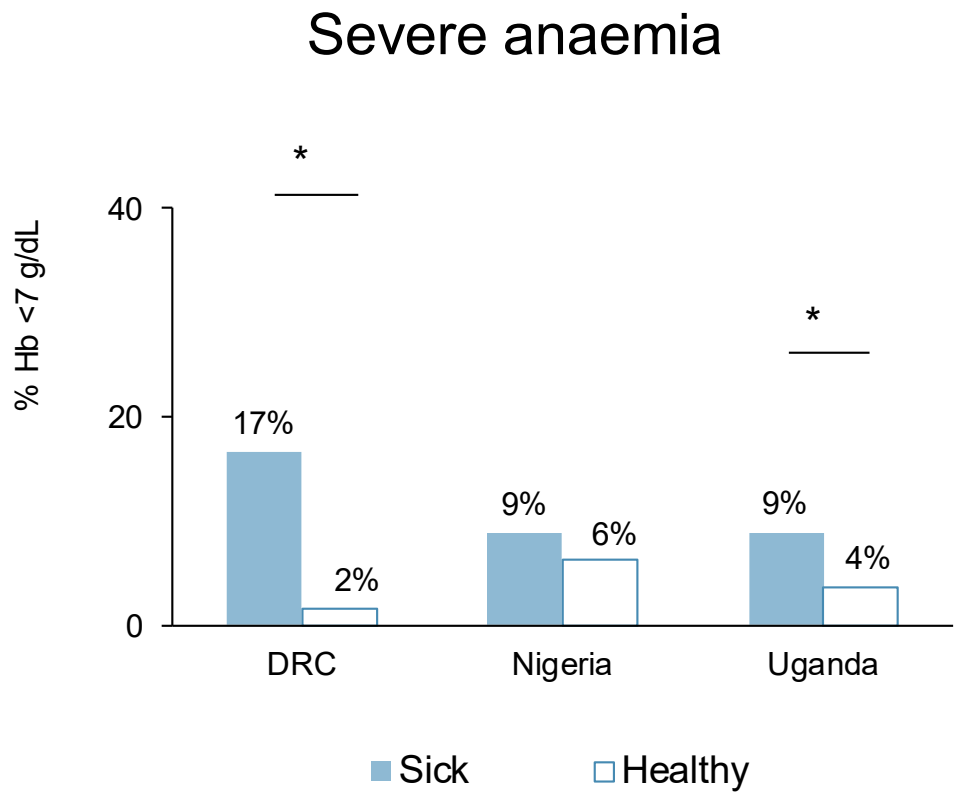


* unadjusted

Hetzel et al. *medRxiv* 2021 https://doi.org/10.1101/2021.09.24.21263966

Day 28 health outcomes





* p < 0.001

Hetzel et al. *medRxiv* 2021 https://doi.org/10.1101/2021.09.24.21263966

Factors associated with day 28 health outcome

DRC (death)

↑ RAS use

↓ Not completing referral

↓ Treatment with inj. antimalarial + ACT

Nigeria (death)

↑ RAS use

↓ Treatment with inj. antimalarial

Uganda (death)

No effect

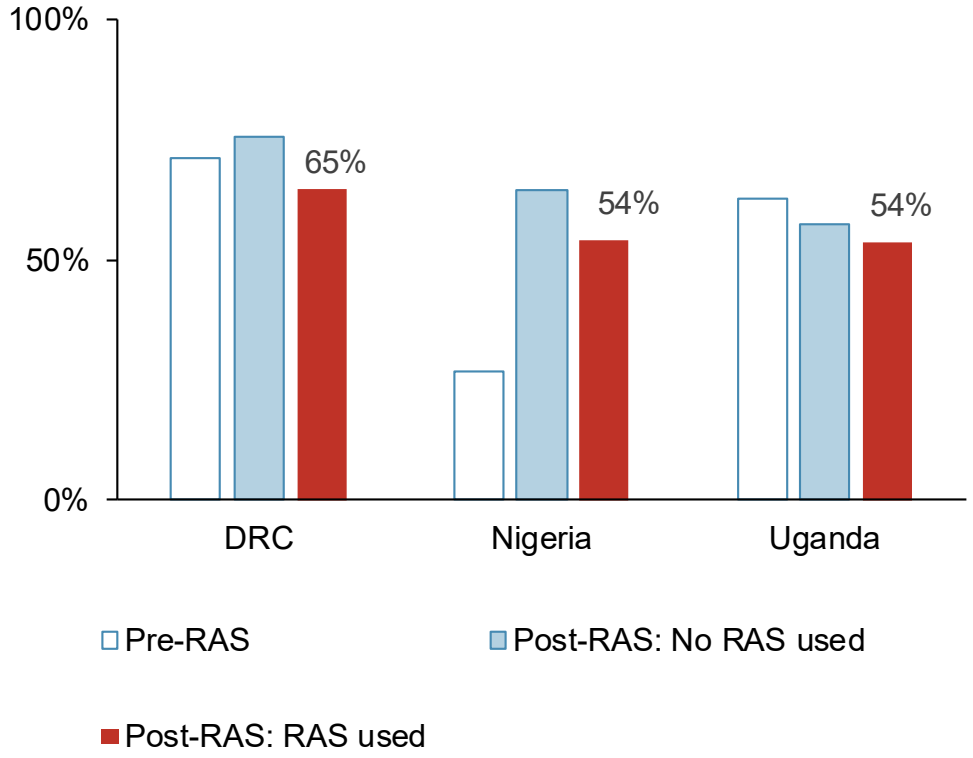
Uganda (death or illness)

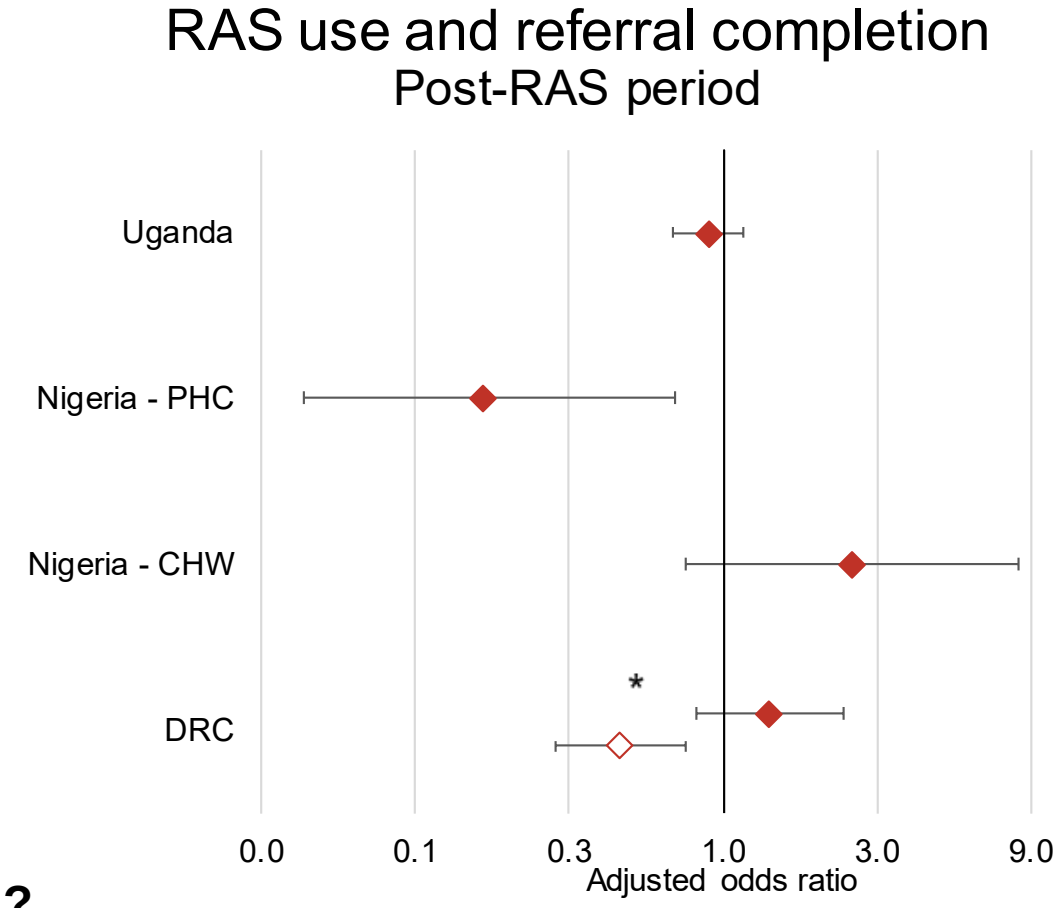
↓ RAS use

↓ Not completing referral

↓ Treatment with inj. antimalarial

Referral completion



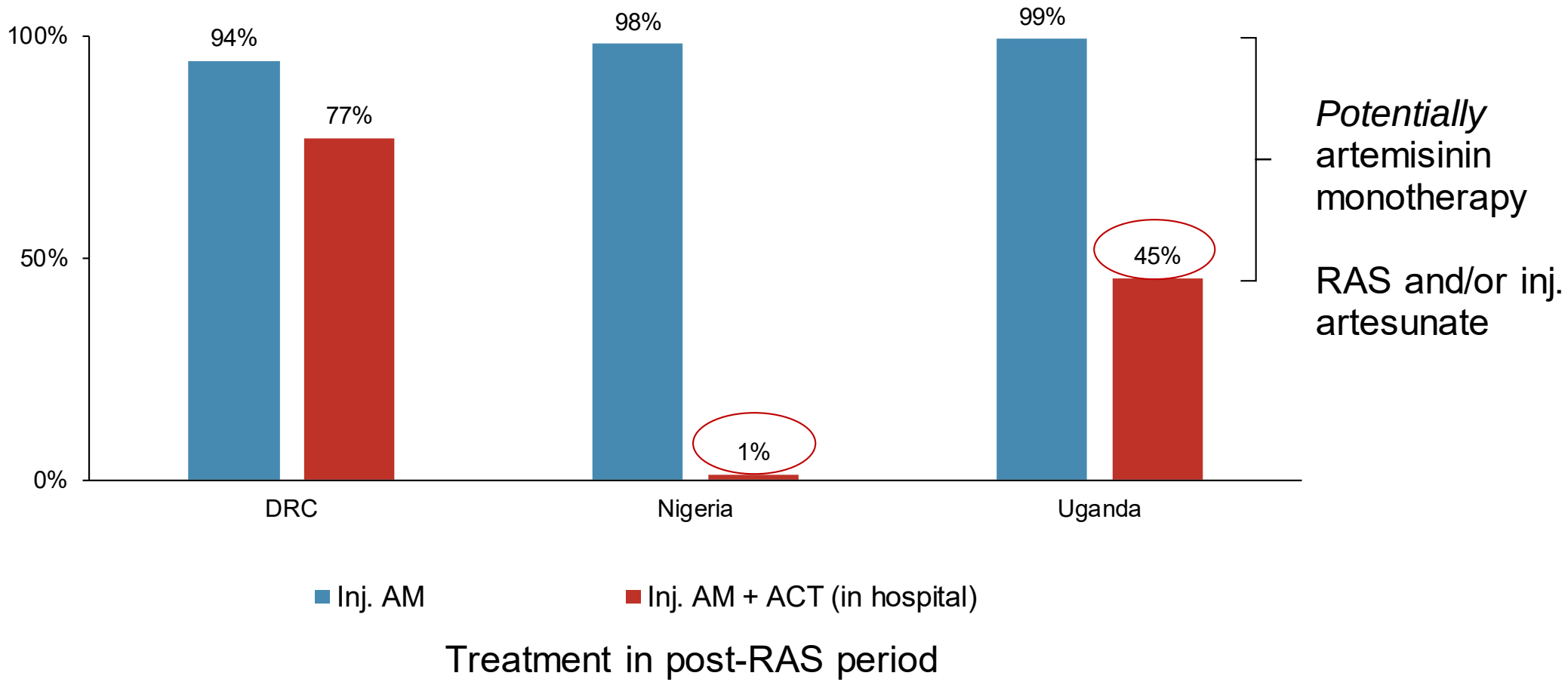


Treatment of children who don't complete referral?

Brunner et al. *medRxiv* 2021 https://doi.org/10.1101/2021.09.27.21264073

* pre-RAS vs post-RAS

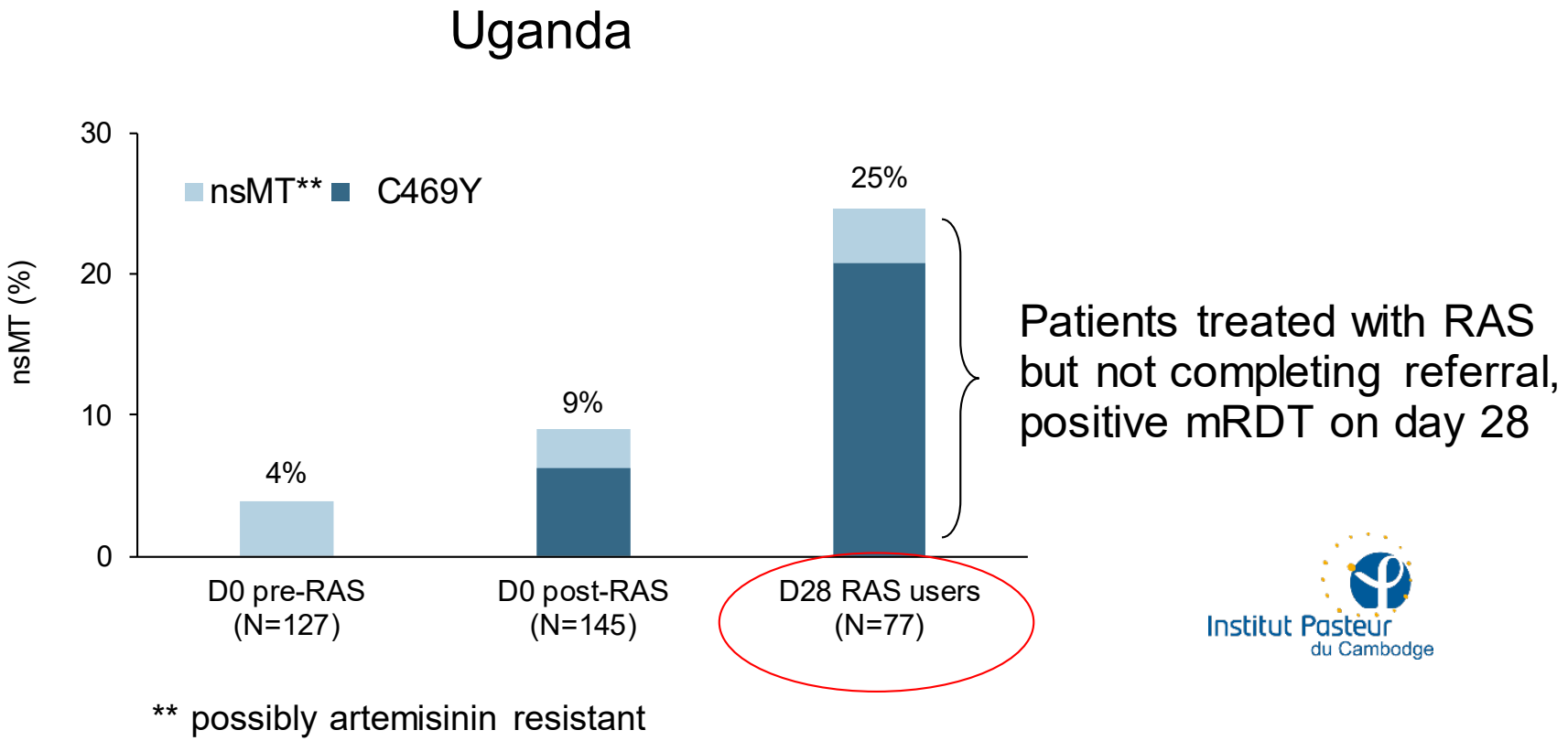
Severe malaria treatment at referral facilities



Signorell et al. manuscript in preparation

Selection of artemisinin resistant parasites

➔ Presentation Pascal Ringwald



Awor, Khim et al. manuscript in preparation

Summary and conclusions: strengthen the system!

- Previously, RAS was shown to reduce deaths in controlled or strongly supported settings which is unlikely to reflect most “real world” implementation scenarios
- CARAMAL found no positive effect of pre-referral RAS on case fatality in three distinct highly malaria endemic settings
- RAS was associated with reduced referral completion
- Not completing referral may contribute to incomplete treatment and missing of other infections and comorbidities
- Post-referral treatment was often incomplete; in particular, ACT was not consistently administered (→ artemisinin monotherapy)
- Higher CFR in children using RAS may be due to secular or seasonal trends, (diagnosis & treatment of) comorbidities and other unaccounted factors
- Incomplete treatment (and introduction of RAS) may exacerbate the selection and spread of artemisinin-resistant parasites

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With funding from Unitaid
Innovation in Global Health

Justin Cohen

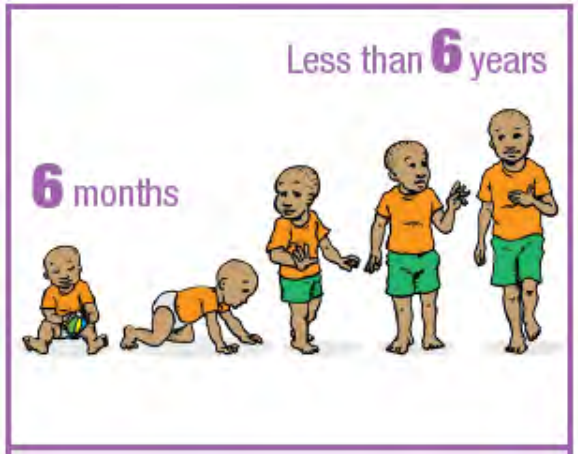
Clinton Health Access Initiative

[In Gomes et al. 2008], "dispensing of artesunate suppositories and referral of patients was done under clinical trial conditions…. How effective will the strategy be in real-life settings?"

-von Seidlein & Deen 2008

Rectal artesunate for pre-referral treatment

MMV job aid (www.severemalaria.org)



ASSESS AGE AND WEIGHT

Between 6 months to less than 6 years.

RECOGNIZE THE DANGER SIGNS

A febrile child or a child with recent history of fever with one or many danger signs:

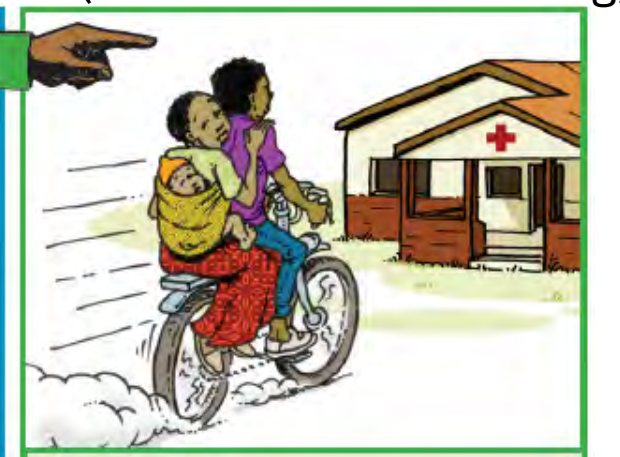
and/or → Unconscious or Lethargic

and/or → Not able to drink or eat

and/or → Vomits everything

ADMINISTER RECTAL ARTESUNATE

The community health worker prepares the child and administers **rectal artesunate**.



TRANSFER URGENTLY

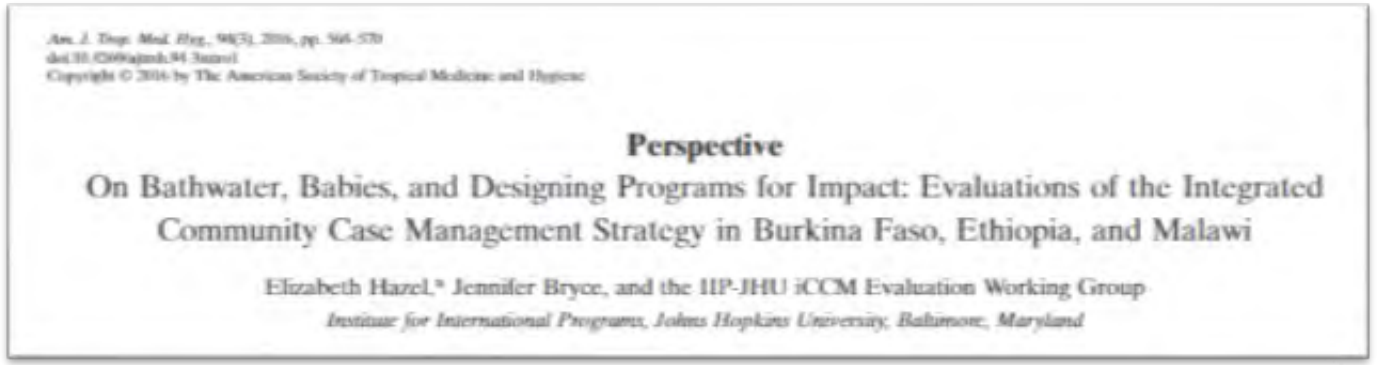
The child must be referred **immediately** to the nearest hospital or health care facility for a full course of antimalarial medicine by IV or IM.

CARAMAL was purposefully designed to introduce rectal artesunate into established community platforms with minimal supportive interventions

Challenges with community health worker networks

% of febrile children reportedly taken for care at a CHW within the study areas of:

- Nigeria: 0% (DHS 2018)
- DRC: 5% (MICS 2017-18)
- Uganda: 24% (DHS 2018-19)



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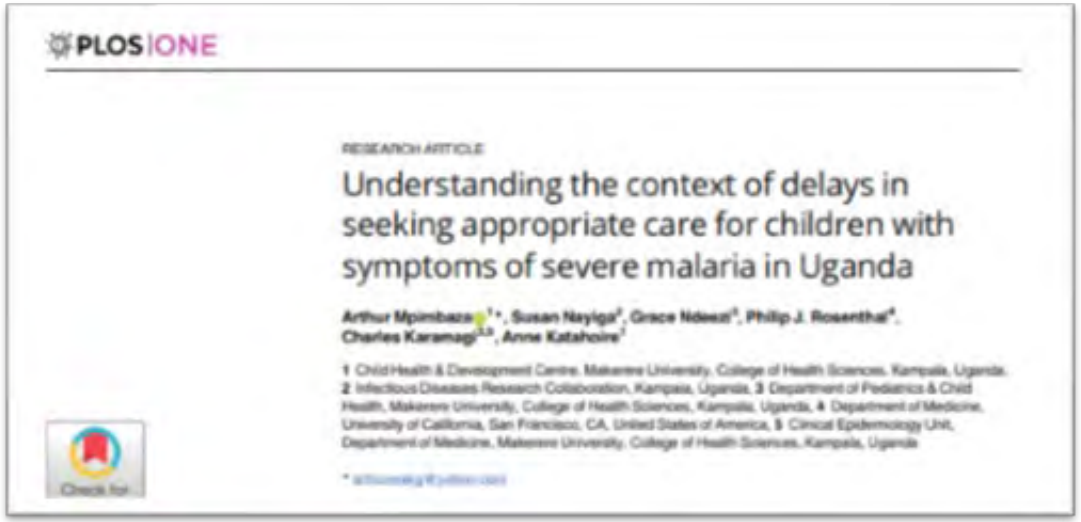
Perspective

On Bathwater, Babies, and Designing Programs for Impact: Evaluations of the Integrated Community Case Management Strategy in Burkina Faso, Ethiopia, and Malawi

Elizabeth Hazel,* Jennifer Bryce, and the IIP-JHU iCCM Evaluation Working Group
Institute for International Programs, Johns Hopkins University, Baltimore, Maryland

Challenges with care at referral facilities



PLOS ONE

RESEARCH ARTICLE

Understanding the context of delays in seeking appropriate care for children with symptoms of severe malaria in Uganda

Arthur Mpimbaza[1]*, Susan Nayiga[2], Grace Ndeezi[3], Philip J. Rosenthal[4], Charles Karamagi[2,3], Anne Katahoire[2]

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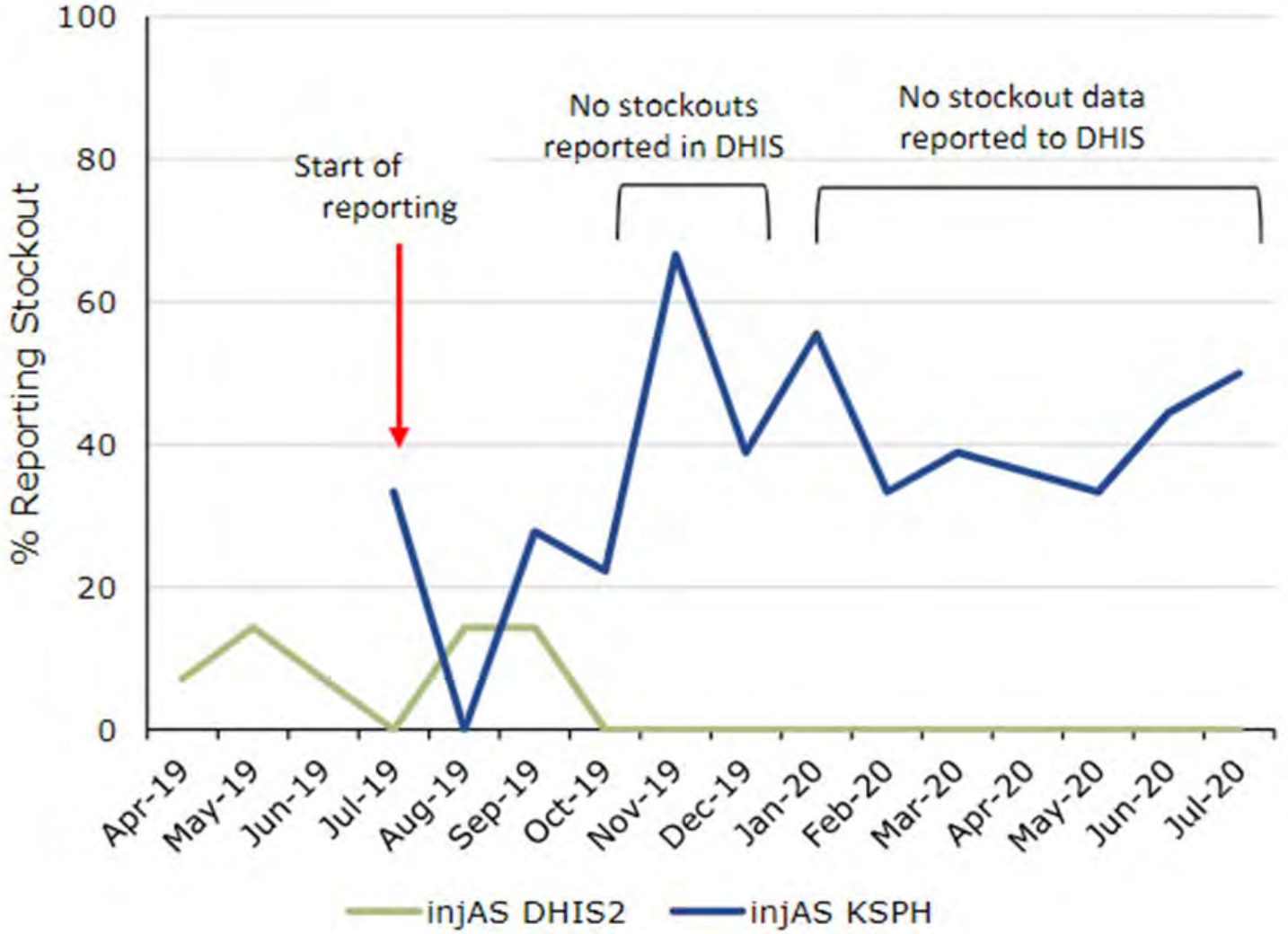
*

"They never have medicine. When you get there they tell you that they do not have drugs. You may get there and there are no health workers."

"Yes, you need transport of 1500 plus it is painful… You find that there are no drugs, even Panadol… that is why we don't go."

Challenges with routine information systems

Example comparison of routine and study reporting systems



[In Gomes et al. 2008], "dispensing of artesunate suppositories and referral of patients was done under clinical trial conditions…. How effective will the strategy be in real-life settings?"

-von Seidlein & Deen 2008

Emergence of artemisinin resistance in Uganda and CARAMAL project

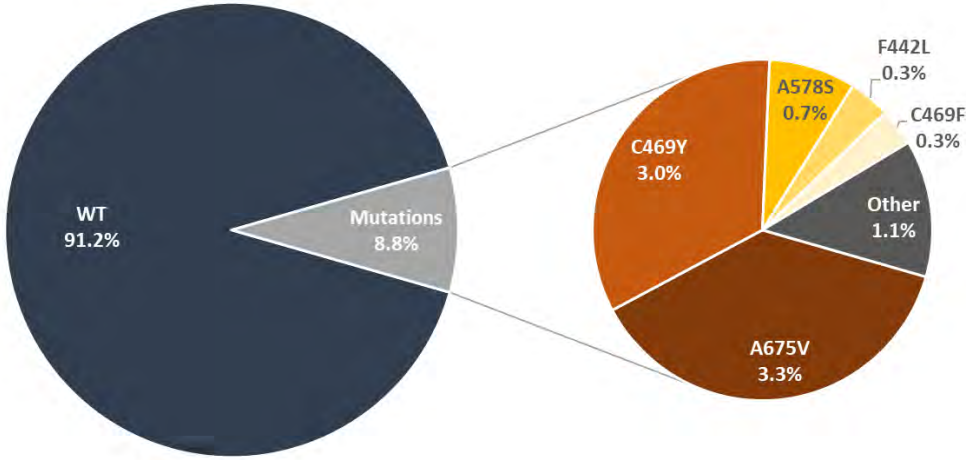
P. Ringwald

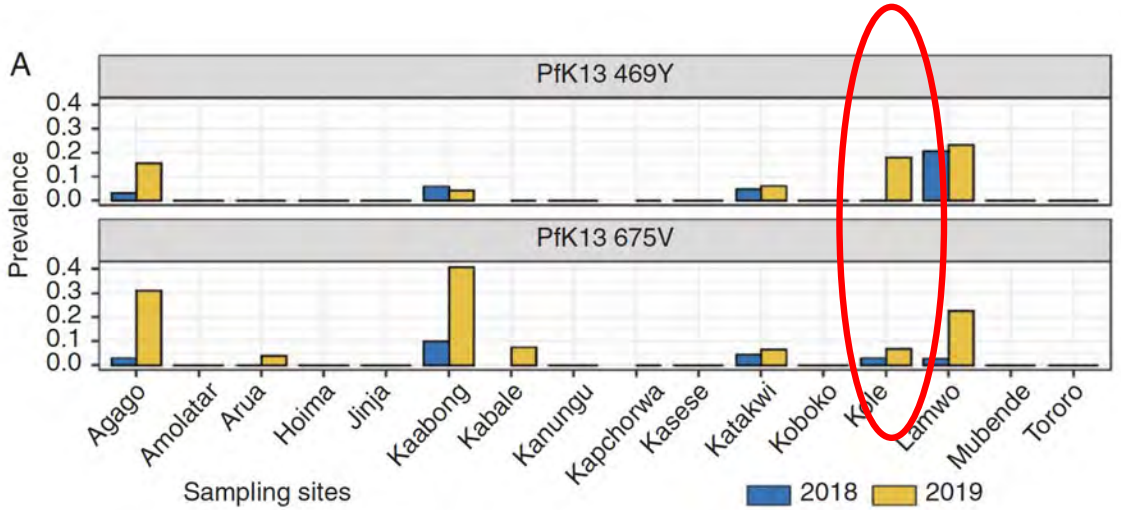
Global **Malaria** Programme

World Health Organization

Study sites of CARAMAL project in Uganda

K13 genotypes in Uganda 2015-2020 (n = 2872)





Prevalence of *Plasmodium falciparum* kelch protein (PfK13) 469Y and 675V mutations

Source: WHO database from studies and surveys
Asua V et al. *J Infect Dis.* 2020

Description of the population

Pre-RAS roll-out (2018-2019)

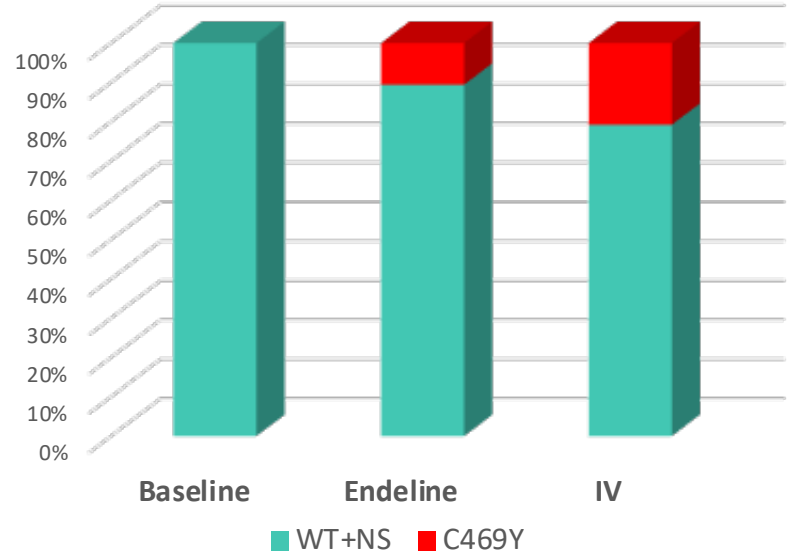
- I. Children presenting directly to a referral health facility without prior administration of RAS (pre-RAS): provides a baseline assessment of artemisinin resistance marker prevalence before the introduction of RAS.

Post-RAS roll-out (2020)

- II. Children presenting directly to a referral health facility without prior administration of RAS (post-RAS): group not receiving pre-referral RAS and hence having baseline pressure for K13 resistance markers.
- III. Children receiving pre-referral RAS from community-based provider and successfully referred to a referral health facility: group receiving pre-referral RAS (monotherapy).
- IV. Children receiving pre-referral RAS from community-based provider but not completing referral to a referral health facility, followed-up at their home on day 28: children malaria-positive on Day 28 may have an increased chance of harboring a resistant infection.

Specific analysis of C469Y mutant

Group		WT	nsMT	C469Y	Total
I	n	139	8	0	147
	%	94.6	5.4	0.0	100
II	n	135	4	9	148
	%	91.2	2.7	6.1	100
III	n	28	0	2	30
	%	93.3	0.0	6.7	100
IV	n	57	4	16	77
	%	74.0	5.2	20.8	100



- C469Y was not detected during baseline investigation;
- Significant statistical differences
  - I vs II/III, p=0.0033
  - I vs IV, p < 0.0001
  - II/III vs IV, p = 0.0003

Thank you

Backup slides

WHO recommendation (I)

https://app.magicapp.org/#/guideline/4870

5.5.3 Pre-referral treatment options 1

Treating cases of suspected severe malaria pending transfer to a higher-level facility (pre-referral treatment) (2015)

Where complete treatment of severe malaria is not possible but injections are available, give **adults and children a single intramuscular dose of artesunate**, **and refer to an appropriate facility for further care**. Where intramuscular artesunate is not available use intramuscular artemether or, if that is not available, use intramuscular quinine.

Where intramuscular injection of artesunate is not available, treat **children < 6 years** with **a single rectal dose** (10mg/kg bw) of artesunate, **and refer immediately to an appropriate facility for further care**. Do not use rectal artesunate in older children and adults.

WHO recommendations (II)

https://app.magicapp.org/#/guideline/4870

5.5 Treating severe malaria

5.5.1 Artesunate 2

Treating severe malaria (2015)

Treat adults and children with severe malaria (including infants, pregnant women in all trimesters and lactating women) with **intravenous or intramuscular artesunate for at least 24 h and until they can tolerate oral medication**. Once a patient has received at least 24 h of parenteral therapy and can tolerate oral therapy, **complete treatment with 3 days of ACT**.

Revised dose recommendation for parenteral artesunate in young children (2015)

Children weighing < 20 kg should receive a higher dose of artesunate (3 mg/kg bw per dose) than larger children and adults (2.4 mg/kg bw per dose) to ensure equivalent exposure to the drug.

5.5.2 Parenteral alternatives when artesunate is not available 1

Parenteral alternatives where artesunate is not available (2015)

If artesunate is not available, use artemether in preference to quinine for treating children and adults with severe malaria.

Agenda – Day 1

Tuesday, 27 April 2021		
Chair: Elizabeth Chizema		
14:00 – 14:10	Welcome and short introduction of participating organisations (10 min)	Pedro Alonso, Director GMP
14:10 – 14:20	Purpose of the meeting and expected outcomes (10 min)	Silvia Schwarte, WHO/GMP
14:20 – 14:30	Brief CARAMAL project overview and data sources (10 min)	Theodoor Visser, CHAI
14:30 – 15:30	**Session 1: Key CARAMAL indicators and cascade of care analysis results,** (90 min: 20 min presentation and 10 min discussion per country) ▪ **DRC** ▪ **Nigeria**	▪ Antoinette Tshefu, Kinshasa School of Public Health, DRC ▪ Elizabeth Omoluabi, Akena Association, Nigeria
15:30 – 15:45	Coffee break	
15:45 – 16:15	Session 1 (continuation) ▪ **Uganda**	▪ Phyllis Awor, Makarere University, Uganda
16:15 – 17:20	**Session 2: CARAMAL implementation experience and supportive interventions,** (45 min presentation, 20 min discussion) ▪ **DRC** ▪ **Nigeria** ▪ **Uganda**	UNICEF ▪ DRC: Lydia Mulongo Kabamba / Francine Kimanuka ▪ Nigeria: Emmanuel Emedo ▪ Uganda: Fred Kagwire / Flavia Mpanga
17:20 – 17:30	Wrap up / Summary of key elements Day 1 Closure of the day	Rapporteur WHO/GMP

Agenda – Day 2

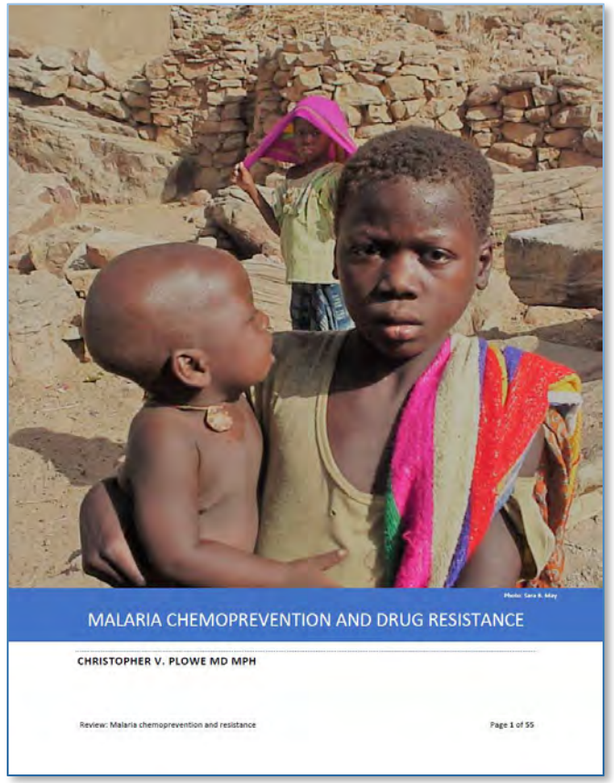
Wednesday, 28 April 2021		
Chair: Dorothy Achu		
14:00 – 14:10	Recap of Day 1 (10 min)	Rapporteur
14:10 – 15:00	**Session 3: Experiences from non-CARAMAL countries** (50 min moderated panel discussion based on country-specific pre-reads. During sessions countries briefly highlight special observations, learnings, challenges, etc for discussion)	Harriet Napier, CHAI Hans Rietveld, MMV Representatives from ▪ Angola: Jose Martins ▪ Malawi: Michael Kayange ▪ Senegal: Doudou Sene ▪ Sierra Leone: Anitta Kamara ▪ Zambia: Mutinta Mudenda
15:00 – 15:30	**Session 4: Cost** of RAS implementation in the CARAMAL project (30 min: 15 min presentation, 15 min discussion)	Mark Lambiris, Swiss TPH
15:30 – 15:45	Coffee break	
15:45 – 16:15	**Session 5: RAS commodity features** including stability, transport and storage, etc (30 min: 15 min presentation, 15 min discussion)	▪ Hans Rietveld, MMV ▪ Andrew Slade, MMV
16:15 – 17:00	**Session 6: Resistance monitoring** (45 min: two 15 min presentations, 15 min discussion) ▪ CARAMAL resistance monitoring ▪ Evidence from non-CARAMAL countries Discussion: Impact for RAS utilization	▪ Benoit Witkowski, IPC ▪ Pascal Ringwald, WHO/GMP
17:00 – 17:15	Wrap up / Summary of key elements Day 2 Closure of the day	Rapporteur WHO/GMP

Agenda – Day 3

Thursday, 29 April 2021		
Chair: Steven Oguche		
14:00 – 14:15	Recap of key points from Days 1 and 2 as basis for Day 3 discussion and conclusions (15 min)	Rapporteur
14:15 – 15:00	**Session 7: Unintended consequences, ADRs, and use of RAS beyond the recommended guidelines** (45 min: 20 min presentation, 25 min discussion)	▪ Aita Signorell, Swiss TPH ▪ UNICEF Country Offices
15:00 – 15:45	**Session 8: Minimal health system requirements for implementation** (45 min: 20 min presentation, 25 min discussion)	▪ Manuel Hetzel, Swiss TPH ▪ Valentina Buj, UNICEF
15:45 – 16:00	Coffee break	
16:00 – 17:00	**Session 9: Discussion of conclusions for development of operational manual** (60 min)	Chair
17:00 – 17:15	Next steps and timelines Wrap up and closure of meeting	Silvia Schwarte, WHO/GMP Pedro Alonso, Director GMP

Agenda – Closed Session on 30 April

Friday, 30 April 2021: CLOSED SESSION	
Chair: Elizabeth Chizema	
10:00 – 12:00	Next steps ▪ Presentation: Proposed structure of the document ▪ Discussion: Proposed structure, content and conclusions, responsibilities, timelines, etc for writing of operational manual / field guide <u>Invited participants:</u> ▪ Technical Consultation Expert Members ▪ WHO Secretariat (HQ, RO, CO) ▪ Rapporteur



The relationship between chemoprevention and drug resistance

Christopher V. Plowe MD MPH

WHO Malaria Policy Advisory Group Meeting

4 October 2021

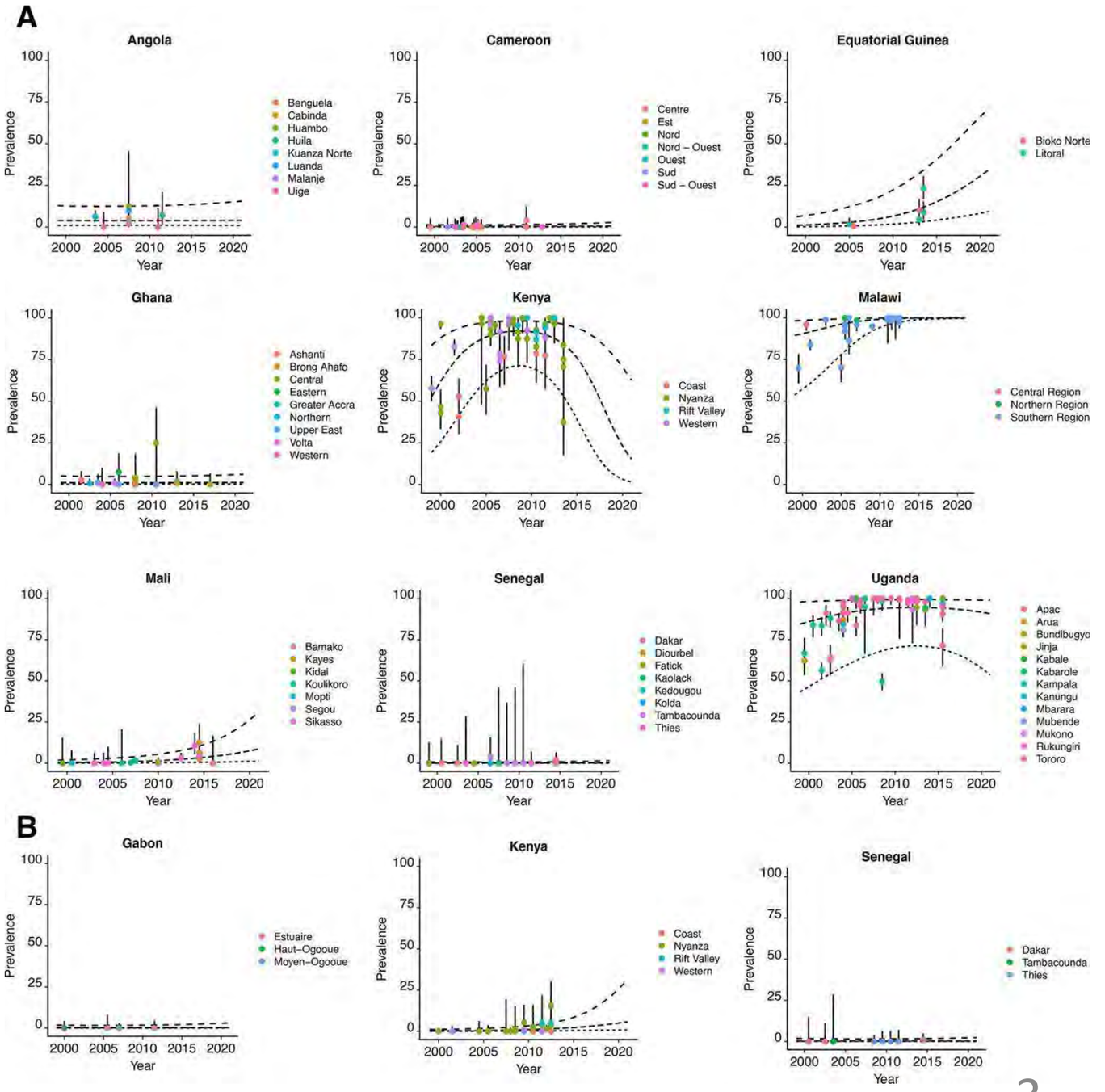
Questions:

1. How do **malaria chemoprevention strategies*** affect, and how are they affected by, **drug resistant malaria**?
2. How is drug resistance best measured and monitored in each chemoprevention scenario?
3. What approaches could mitigate the impact of resistance on chemoprevention efficacy?

*IPTp, IPTi, SMC, MDA

National scale temporal trends and projections of resistance to SP

- "Resistance" defined here as prevalence of dhps A581G
- Prevalence generally higher in East Africa
  - Highly heterogeneous, including within countries
  - Some upward trends in West Africa
  - Some downward trends in East Africa



BMJ Global Health

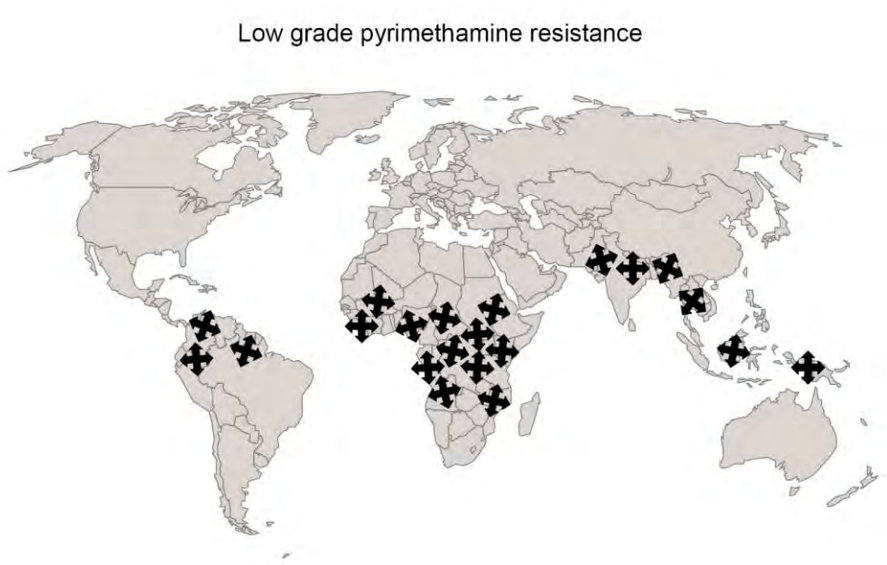
Floriano Amimo et al. BMJ Glob Health 2020;5:e003217

Key findings: Measuring and monitoring resistance

- Drug resistance is but one of many factors that determine the efficacy of IPTp, IPTi, SMC and MDA
- Clinical trials that measure health outcomes are the gold standard for measuring chemoprevention efficacy
- Drug treatment efficacy is not a reliable surrogate for chemoprevention efficacy
- Molecular markers accurately indicate the presence of drug resistant parasites, but are less accurate at predicting chemoprevention efficacy
- Specific resistance markers must be validated independently as predictors of efficacy for each different chemoprevention regimen

Impact of IPTp on resistance

- IPTp-SP appears to select for antifolate resistance mutations associated with low to moderate increases in drug resistance, but there is no convincing evidence of selection favouring the key mutations—like *dhps* A581G—associated with higher level antifolate resistance and loss of ITPp-SP efficacy



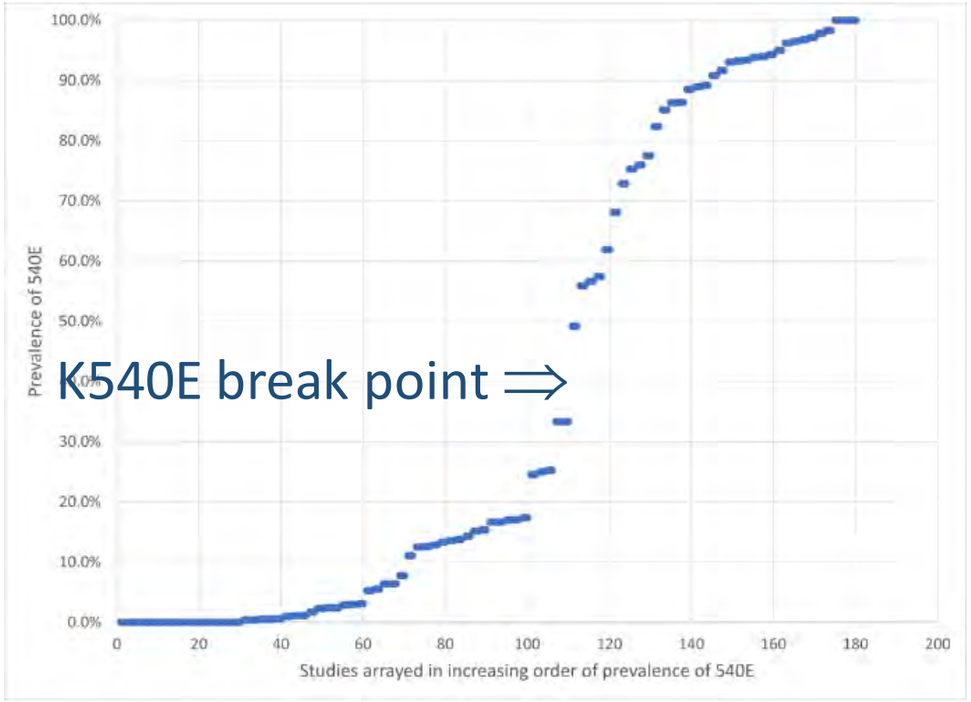
Impact of resistance on IPTp

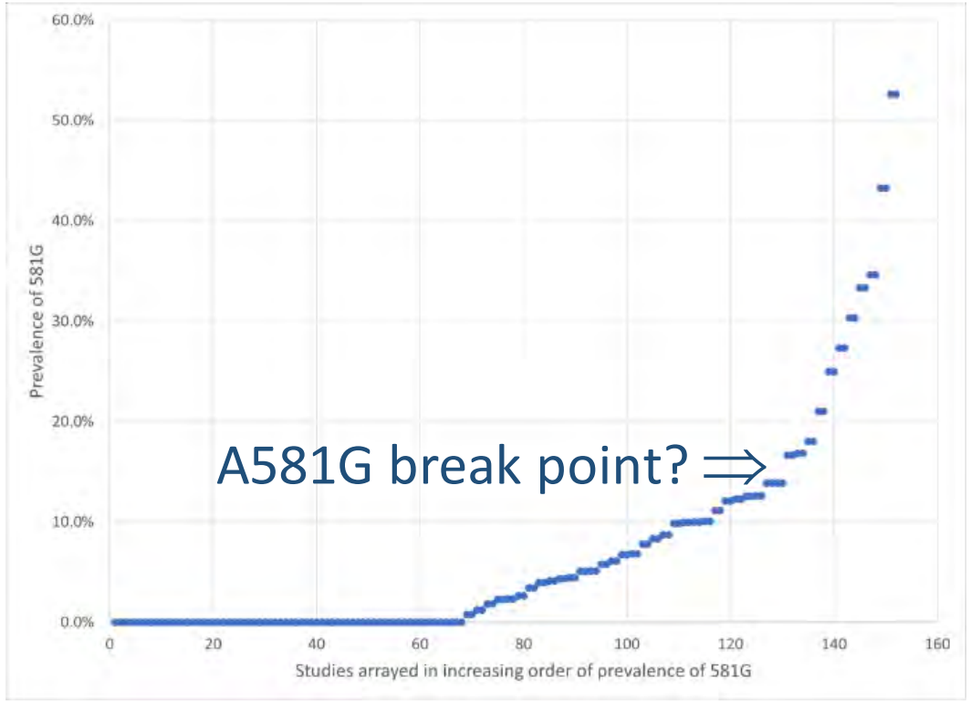
- Despite some evidence that high level antifolate resistance at least partially compromises IPTp-SP efficacy, a worst-case scenario of harmful effects in the presence of SP resistance was not borne out by subsequent studies
- Sensational claims that ITPp is harmful in presence of dhps A581G not borne out by prospective trials or meta-analyses
- Conclusions from large meta-analyses based on as few as 2 studies
- Evidence supporting a recommendation to withhold ITPp-SP where the prevalence of resistance markers exceeds 10% prevalence of *dhps* A581G is not strong

Impact of IPTi on resistance

- IPTi-SP has been accompanied by overall increases in prevalence of some antifolate resistance markers
- But, neither clinical trials of IPTi nor ecological surveys comparing IPTi implementation zones to control areas over time have shown evidence of significant selection of the dhfr/dhps haplotypes associated with SP efficacy for treatment or chemoprevention
- This conclusion is in agreement with that of an Institute of Medicine expert committee, and 2 independent modeling studies

Potential approach to guide policy?





- Frequency distributions of *dhps* K540E prevalence (L) and A581G (R) in Africa 2015-2021
- K540E prevalence tends to cluster below 20% and above 50%
- A581G prevalence estimates lack an obvious break point.

Impact of resistance on IPTi

- Evidence supporting recommendation that IPTi-SP not be deployed where prevalence of *dhps* K540E exceeds 50% was based essentially on just two trials ten years ago
- Little new evidence available to validate this threshold, or to set new criteria to guide IPTi policy (e.g., a prevalence threshold for dhfr I164L, dhps A581G, and/or dhps A613S/T)
- Marker thresholds for implementing IPTi based on natural clustering of prevalence data in recent studies not validated
- In the meantime prevention efficacy studies remain the gold standard to guide policy

Impact of SMC on resistance

- While some studies have reported that SMC is followed by increased prevalence of resistance markers, other studies found no such evidence of selection
- There is no evidence that SMC results in increased prevalence of the higher-level resistance mutations that most severely impair SP efficacy, nor does SMC appear to select for parasites carrying mutations associated with amodiaquine resistance

Impact of resistance on SMC

- Unless and until high-level resistance mutations become more prevalent in areas where SMC is used, it will not be possible to draw conclusions about the impact of resistance on SMC efficacy

What about MDA?

Aspects of Production of Chloroquinated Salt, Amazon Valley, 1953. Courtesy of the Casa de Oswaldo Cruz-Oswaldo Cruz Foundation, Rostan Soares papers.

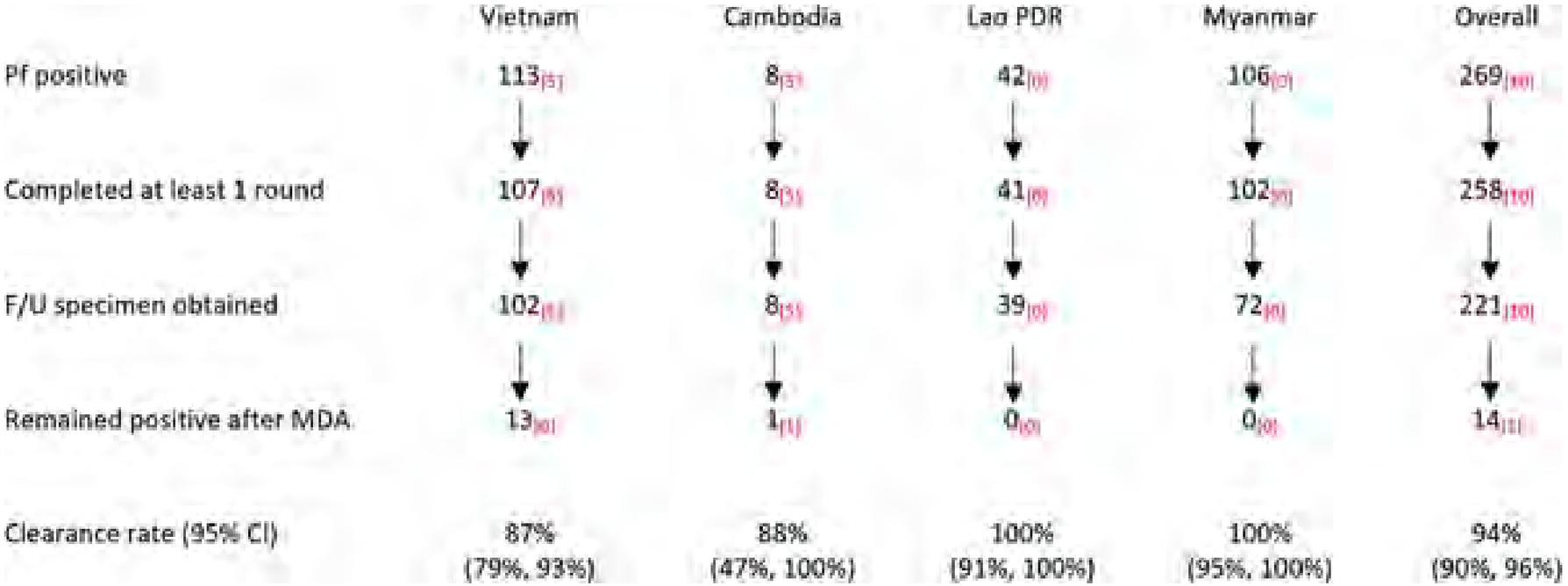
From Autonomy to Partial Alignment: National Malaria Programs in the Time of Global Eradication, Brazil, 1941-1961

GILBERTO HOCHMAN

CBMH/BCHM / Volume 25:1 2008 / p. 161-192

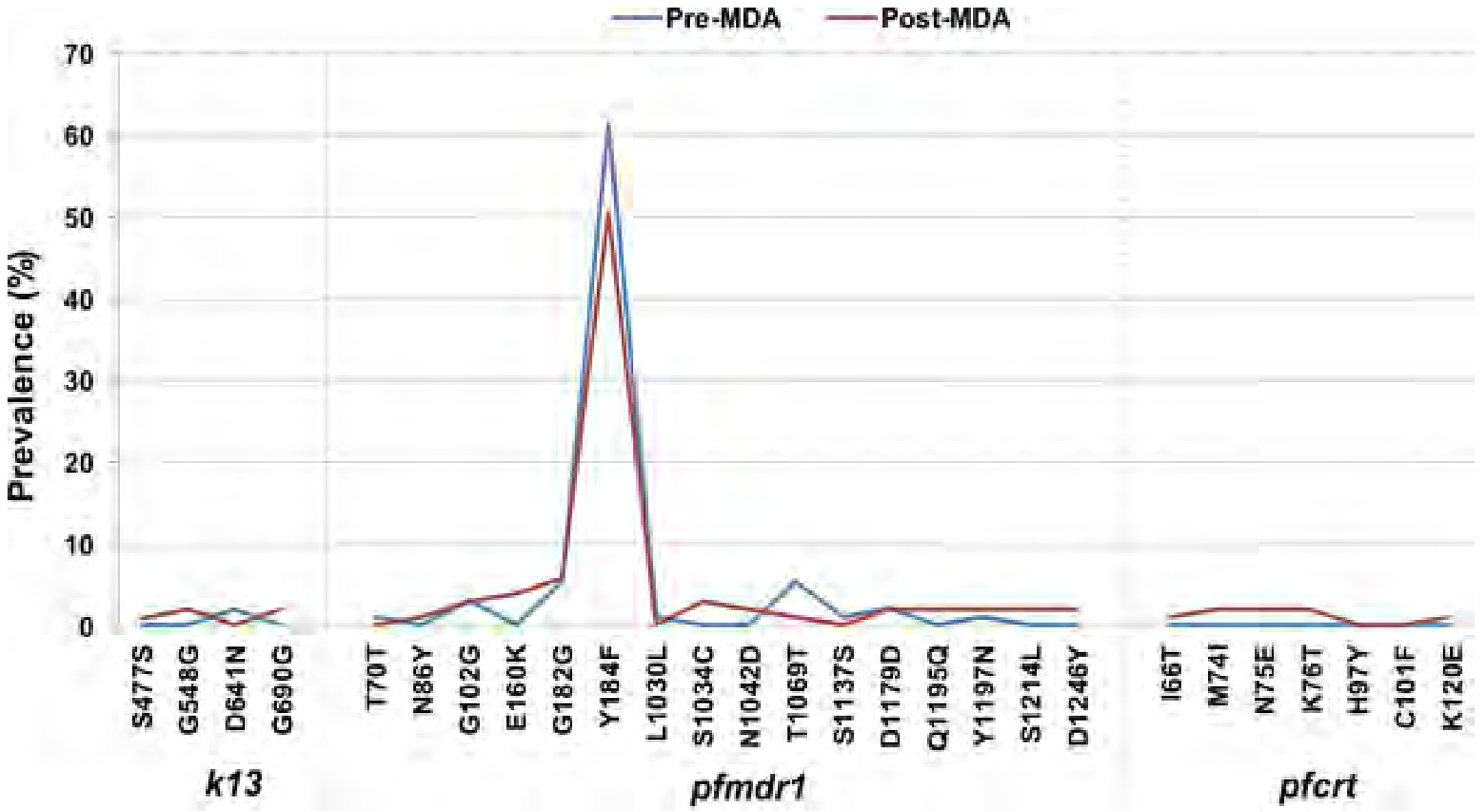
Chloroquinated Salt Distribution, Capim River, Amazon Valley, c. 1955. Courtesy of the Casa de Oswaldo Cruz-Oswaldo Cruz, Aristides Lima Verde Papers.

Fig 5. P. falciparum clearance after MDA: Dihydroartemisinin-piperaquine efficacy against asymptomatic infections estimated from individual-participant-level data from villages randomised to both early and deferred MDA in Myanmar and Vietnam, and from early MDA villages only in Cambodia and Lao PDR. Subscripts in red indicate the number of participants with the P. falciparum PfPailin genotype [8]—a long haplotype containing PfKelch13 C580Y, conferring artemisinin resistance, and multiple copies of the Pfplasmepsin2/3 genotype conferring piperaquine resistance.



von Seidlein L, Peto TJ, Landier J, Nguyen TN, Tripura R, et al. (2019) The impact of targeted malaria elimination with mass drug administrations on falciparum malaria in Southeast Asia: A cluster randomised trial. PLOS Medicine 16(2): e1002745. https://doi.org/10.1371/journal.pmed.1002745
https://journals.plos.org/plosmedicine/article?id=10.1371/journal.pmed.1002745

Does MDA with DHA-piperaquine select for resistance markers?
Distribution of Plasmodium falciparum k13, pfmdr1 and pfcrt polymorphism frequencies among pre- and post-MDA isolates.



Gupta H, Galatas B, Chidimatembue A, Huijben S, Cisteró P, et al. (2020) Effect of mass dihydroartemisinin–piperaquine administration in southern Mozambique on the carriage of molecular markers of antimalarial resistance. PLOS ONE 15(10): e0240174. https://doi.org/10.1371/journal.pone.0240174
https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0240174

Impact of MDA on resistance

- In the GMS, a cluster-randomized trial of MDA with DHA-piperaquine in sites with varying levels of resistance found very few post-MDA infections, and no evidence of selection for resistance markers
- In Mozambique, prevalence of resistance markers was compared before and after MDA with dihydroartemisinin-piperaquine—no evidence of selection for resistance markers
- **There is no evidence that MDA in the modern era using highly effective ACTs results in increased drug resistance**

Impact of resistance on MDA

- In the past, drug resistance has diminished the efficacy of MDA when drugs have been used in sub-curative formulations and dosing regimens
- However, in the 21st century, MDA with highly effective combination drugs has proven efficacious even in the face of high levels of resistance

Managing and mitigating resistance

- Standardized protocols for measuring and monitoring chemoprevention efficacy are needed
- With imperfect evidence, practical considerations can help guide recommendations on when and where to deploy chemoprevention strategies
- Using different drugs for chemoprevention and treatment and combining drugs with countervailing resistance mechanisms may help to preserve efficacy
- The best approach for mitigating and managing drug resistance to protect the efficacy of chemoprevention strategies is to ensure a pipeline of safe and effective new malaria drugs with diverse mechanisms of action and resistance

Questions/Discussion

Merci/Gracias/Zikomo/I Ni Ce/Kyay Zuu/Thank you

Malaria Policy Advisory Group Meeting

4—7 October 2021, Geneva, Switzerland
Background document for Session 3

World Health Organization

Malaria chemoprevention and drug resistance

Dr Christopher V. Plowe, Adjunct Professor of Medicine,
University of Maryland School of Medicine, Baltimore, USA

Photo: Sara B. May

MALARIA CHEMOPREVENTION AND DRUG RESISTANCE

CHRISTOPHER V. PLOWE MD MPH

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ABBREVIATIONS

ACT	artemisinin-based combination therapy
ANC	antenatal clinic
AQ	amodiaquine
DHA	dihydroartemisinin
DHFR	*Plasmodium falciparum* dihydrofolate reductase enzyme
dhfr	*P. falciparum* gene that encodes DHFR
DHPS	*P. falciparum* dihydropteroate synthase enzyme
dhps	*P. falciparum* gene that encodes DHPS
EIR	Entomological inoculation rate
GMP	Global Malaria Programme
IC50	50% inhibitory concentration (measure of in vitro drug resistance)
IPTi	intermittent preventive treatment in infants
IPTp	intermittent preventive treatment in pregnancy
ITN	insecticide-treated net
k13	*P. falciparum kelch 13* gene
MDA	mass drug administration
pfcrt	*P. falciparum* chloroquine resistance transporter gene
pfmdr1	*P. falciparum* multi-drug resistance gene
pfpm2/3	*P. falciparum* plasmepsin 2/3 gene(s)
RDT	rapid diagnostic test
RRR	relative risk reduction
SMC	seasonal malaria chemoprevention
SNP	single nucleotide polymorphism (also known as point mutation)
SP	sulphadoxine-pyrimethamine
WHO	World Health Organization

KEY FINDINGS	
Measuring and monitoring resistance	• Drug resistance is but one of many factors that determine the efficacy of IPTp, IPTi, SMC and MDA • Clinical trials that measure health outcomes are the gold standard for measuring chemoprevention efficacy • Drug treatment efficacy is not a reliable surrogate for chemoprevention efficacy • Molecular markers accurately indicate the presence of drug resistant parasites, and can serve as useful but imperfect means of predicting chemoprevention efficacy • Specific resistance markers must be validated independently as predictors of efficacy for each different chemoprevention regimen
Impact of IPTp on resistance	• IPTp-SP appears to select for antifolate resistance mutations associated with low to moderate increases in drug resistance, but there is no convincing evidence of selection favouring the key mutations associated with higher level antifolate resistance and loss of ITPp-SP efficacy
Impact of resistance on IPTp	• Despite some evidence that high level antifolate resistance at least partially compromises IPTp-SP efficacy, a worst-case scenario of harmful effects in the presence of SP resistance was not borne out by subsequent studies • The evidence supporting a recommendation to withhold ITPp-SP where the prevalence of *dhps* A581G exceeds a threshold of 10% is not strong
Impact of IPTi on resistance	• While IPTi-SP has been accompanied by overall increases in the prevalence of some antifolate resistance markers, there is little evidence of significant selection of the forms of resistance known to compromise SP efficacy for treatment or chemoprevention
Impact of resistance on IPTi	• The evidence supporting a recommendation that IPTi-SP should not be deployed where prevalence of *dhps* K540E exceeds 50% remains limited
Impact of SMC on resistance	• While some studies have reported that SMC is followed by increased prevalence of resistance markers, other studies found no such evidence of selection • There is no evidence that SMC results in increased prevalence of the higher-level resistance mutations that most severely impair SP efficacy, nor does SMC appear to select for parasites carrying mutations associated with amodiaquine resistance
Impact of resistance on SMC	• Unless and until high-level resistance mutations become more prevalent in areas where SMC is used, it will not be possible to draw conclusions about the impact of resistance on SMC efficacy
Impact of MDA on resistance	• There is no evidence that MDA in the modern era using highly effective ACTs results in increased drug resistance
Impact of resistance on MDA	• In the past, drug resistance has diminished the efficacy of MDA when drugs have been used in sub-curative formulations and dosing regimens • However, in the 21st century, MDA with highly effective combination drugs has proven efficacious even in the face of high levels of resistance
Other chemoprevention strategies	• Evidence that seasonal malaria chemoprevention in school-age children increases drug resistance does not stand up to careful scrutiny • Selection of clinically relevant forms of resistance by chemoprevention is not inevitable
Managing and mitigating resistance	• Standardized protocols for measuring and monitoring chemoprevention efficacy are needed • With imperfect evidence, practical considerations can help guide recommendations on when and where to deploy chemoprevention strategies • Using different drugs for chemoprevention and treatment and combining drugs with countervailing resistance mechanisms may help to preserve efficacy • The best approach for mitigating and managing drug resistance to protect the efficacy of chemoprevention strategies is to ensure a pipeline of safe and effective new malaria drugs with diverse mechanisms of action and resistance

BACKGROUND

With 229 million cases and 409,000 malaria deaths worldwide in 2019, malaria remains a major global health burden [1]. More than 90% of these deaths occurred in the WHO Africa Region, where children aged less than five years are the most vulnerable, accounting for 67% of malaria deaths. After decades of dramatic reductions in malaria cases and deaths worldwide, progress toward malaria control and elimination has plateaued, and the 2020 Global Technical Strategy targets for morbidity and mortality reductions have not been met. Ample historical examples support predictions that further erosion of the recent gains in malaria control will lead to resurgences, at great cost to the health, lives, and economies of the world's poorest countries [2]. Current tools and available resources for malaria control need to be optimized and the risks of resistance to antimalarial drugs used for prevention and treatment must be mitigated and managed for momentum to be regained and sustained.

Table 1. Definitions of Malaria Chemoprevention Strategies*

intermittent preventive treatment in pregnancy (IPTp)	A full therapeutic course of antimalarial medicine given to pregnant women at routine prenatal visits, regardless of whether the woman is infected with malaria.
Intermittent preventive treatment in infants (IPTi)	A full therapeutic course of sulphadoxine-pyrimethamine delivered to infants in co-administration with DTP2/Penta2, DTP3/Penta3 and measles immunization, regardless of whether the infant is infected with malaria.
Seasonal malaria chemoprevention (SMC)	Intermittent administration of full treatment courses of an antimalarial medicine during the malaria season to prevent malarial illness. The objective is to maintain therapeutic concentrations of an antimalarial drug in the blood throughout the period of greatest risk for malaria.
Note: This intervention is recommended only for areas with highly seasonal malaria, where transmission occurs during a few months of the year.	
Mass drug administration (MDA)	Administration of antimalarial treatment to all age groups of a defined population or every person living in a defined geographical area (except those for whom the medicine is contraindicated) at approximately the same time and often at repeated intervals.

*Definitions from WHO Malaria Terminology, last updated 2019 [3]

The WHO Global Malaria Programme (GMP) convened a technical consultation in 2019 to review existing WHO recommendations on, and experience with, malaria chemoprevention[1] [4]. The chemoprevention strategies reviewed are defined in **Table 1**, and included intermittent preventive treatment in pregnancy (IPTp), intermittent preventive treatment in infants (IPTi), seasonal malaria chemoprevention (SMC), and mass drug administration (MDA) for the reduction of disease burden in emergency situations. The examination of these strategies side-by-side for the first time was intended to be a key step toward a more holistic and consistent approach to the use of antimalarial medicines to prevent malaria among people living in endemic settings.

[1] Chemoprevention is the use of antimalarial medicines for prophylaxis and for preventive treatment. This review focuses on preventive treatment, and not on prophylaxis for visitors to endemic areas.

From the time that these chemoprevention strategies were first conceived, concerns have been raised both about their potential impact on the development and spread of drug resistance that might compromise the treatment efficacy of the drug classes used, and about the impact of drug resistance on the efficacy of different chemoprevention strategies. This review of what is known about resistance in the context of chemoprevention is intended to serve as an aid in evaluating and updating WHO's chemoprevention recommendations.

APPROACHES TO MEASURING AND MONITORING RESISTANCE

Because most of the chemoprevention strategies currently recommended by the WHO rely on the antifolate combination sulphadoxine-pyrimethamine (SP), antifolate resistance is a main focus of this review. Other drugs and resistance mechanisms are discussed as they relate to the chemoprevention strategies under consideration. Similarly, because *Plasmodium falciparum* malaria is the primary target species for these strategies, nearly all of the reviewed papers focus primarily on *P. falciparum*.

CLINICAL TRIALS REMAIN THE GOLD STANDARD FOR MEASURING AND MONITORING EFFICACY

Concerns about drug resistant malaria arise from the potential for resistant parasites to escape the action of antimalarial drugs, resulting in continued infection, transmission potential, and/or clinical illness. These clinical and parasitological outcomes are best assessed in prospective, randomized clinical trials in malaria-endemic settings. Because their primary outcome measures are affected by a number of host and parasite factors in addition to parasite resistance, clinical trials directly do not measure drug resistance *per se*, but these prospective studies remain the gold standard for measuring the efficacy of both antimalarial drug treatment efficacy and chemoprevention strategies.

Antimalarial drug treatment efficacy is monitored worldwide in single-arm clinical trials known as WHO Therapeutic Efficacy Studies (TES), which follow standardized protocols to provide direct evidence of drug efficacy to guide policy decisions [5]. As they have been developed and implemented, chemoprevention strategies have been evaluated in more complex prospective, controlled trials that typically randomly assign either individuals or clusters (e.g., villages or districts) to receive either the drug prevention regimen being tested, or an alternative regimen, or no preventive regimen. Outcomes are then measured to estimate treatment efficacy. Unlike TES for routine monitoring of treatment efficacy, simplified protocols for routine monitoring of chemoprevention efficacy are not yet in use.

Clinical trials have some limitations. As is described below in more detail for each of the chemoprevention strategies, treatment efficacy trials do not reliably predict the efficacy of chemoprevention strategies, even when the same antimalarial drugs are used for treatment

and chemoprevention at the same time and place. Moreover, even streamlined clinical trials such as TES are laborious and expensive, requiring the on-site presence of experienced researchers for periods of months to years. They can only be done where there is sufficient risk of clinical malaria or other outcomes (e.g., low birth weight for IPTp), making it difficult to enrol enough participants to achieve target sample sizes in areas where the malaria burden is low. Finally, once a treatment or intervention is proven efficacious, it is often considered ethically unacceptable to randomize study participants to receive placebos or unproven alternative interventions.

Because antimalarial drug resistance is considered paramount among the many host, parasite, pharmacological and other factors that affect efficacy and effectiveness of drug treatment and chemoprevention, methods for detecting the presence of resistant parasites have long been employed as a surrogate for efficacy trials.

IN VITRO SUSCEPTIBILITY TESTING IS OF LIMITED USE FOR MONITORING EFFICACY

In vitro assays for measuring drug resistance provide a direct measure of parasite response to drugs [6], but have proven to be even more limited in scope and suitability for surveillance than clinical trials. In vitro testing requires that venous blood with a high parasite density be quickly frozen, or transported cold to a well-equipped laboratory for parasite cultivation. The methods are laborious and require technical expertise, and failure to establish primary parasite growth is common. Micro-test ex vivo assays of fresh parasite isolates provide the clearest assessment of drug susceptibility in clinically relevant parasites, but these tests have limitations: they can only be performed once and thus cannot be repeated to confirm results; polyclonal infections, which are especially common in high transmission areas, may contain both sensitive and resistant parasites, make the tests challenging to interpret; they can be confounded by the presence in the blood of other drugs with antimalarial activity (e.g., antibiotics); and ex-vivo tests are subject to variation and artifact, especially when testing for resistance to drugs that have limited solubility. While more-rigorous in vitro tests of culture-adapted isolates are more reproducible, the processes of freezing, thawing and adaptation to culture also introduce the possibility of selecting sub-populations of parasites that survive best under non-natural in vitro conditions. This means that the parasites ultimately assayed may be genetically and phenotypically unrepresentative of the original parasite population.

In vitro testing is particularly unreliable for antifolate drugs, especially the sulphas, because the tests are exquisitely sensitive to host folate blood levels, which are affected by diet and vary widely among different individuals [7]. For all these reasons, in vitro tests have not played a significant role in assessing resistance in relation to the currently recommended chemoprevention strategies. Nevertheless, in vitro methods are indispensable for confirming and characterizing newly emerging forms of resistance and for establishing and confirming the molecular mechanisms of resistance [8, 9].

MOLECULAR RESISTANCE MARKERS

Elucidation of the molecular basis of in vitro *P. falciparum* resistance to the antifolates made it possible to define the determinants of in vivo resistance to these drugs, and to develop simple assays for molecular markers of antifolate resistance that can potentially serve as surrogate indicators of drug efficacy. Pyrimethamine and other antifolates such as proguanil (via its metabolite cycloguanil) and trimethoprim target *P. falciparum* dihydrofolate reductase (DHFR), while sulphadoxine and other sulphas target dihydropteroate synthase (DHPS). Resistance to DHFR inhibitors and sulpha drugs in vitro is conferred by single nucleotide polymorphisms (SNPs) in *P. falciparum* DHFR and DHPS, respectively. Briefly, a SNP in the *dhfr* gene encoding a Ser→Asn change at codon 108 (S108N) causes up to a 300-fold increase in IC50 in cultured parasites subjected to in vitro drug susceptibility testing. The addition of N51I and/or C59R mutations can confer more than 200-fold higher levels of pyrimethamine resistance in vitro, and DHFR I164L, when combined with S108N and N51I and/or C59R, confers up to 20,000-fold higher IC50s compared to wild-type[2] parasites [10-15]. Mutations in *dhps* associated with decreased susceptibility to sulphadoxine in vitro include SNPs encoding the amino acid changes S436A/F, A437G, K540E, A581G, and A613S/T [16-19]. Both sets of mutations tend to occur in a progressive, step-wise fashion, with higher levels of in vitro resistance occurring in the presence of multiple mutations.

Potential molecular markers have been similarly identified for *P. falciparum* resistance to many but not all other antimalarial drugs (reviewed in [20, 21]), including currently used chemoprevention agents such as amodiaquine (SNPs in *pfcrt* and *pfmdr1*), lumefantrine, mefloquine (copy number variation in *pfmdr1*), piperaquine (copy number variation in *plasmepsin2* and SNPs in *pfcrt)*, and the artemisinins (SNPs in *kelch13*). Several of these putative markers are less well validated than those for SP and chloroquine resistance, and in some cases (e.g., lumefantrine), "resistance" markers are associated only with modest differences in susceptibility in vivo but not with clinical resistance or treatment failure. The current status of molecular markers of resistance to drugs used in malaria chemoprevention is summarized in **Table 2**. As noted earlier, this review focuses primarily on antifolate resistance markers given the key role of SP in the chemoprevention strategies discussed here.

The relationships between resistance mutations and clinical and parasitological outcomes of antimalarial drug treatment and chemoprevention are less straightforward, primarily because intrinsic drug resistance is only one of many factors that affect these outcomes, along with drug quality, intake, absorption, metabolism and clearance; nutritional and other health status indicators; and, especially, naturally acquired immunity to malaria, which can aid in clearing parasites, including drug-resistant parasites [22-26]. Because all these factors vary widely across individuals and populations, validating molecular markers as useful tools for measuring and monitoring drug treatment efficacy and chemoprevention outcomes has been challenging, and no marker or set of markers (haplotype) can reliably predict the outcome of a given drug regimen in an individual person. Nevertheless, many clinical trials and epidemiological studies

[2] **Wild-type** is defined here as parasites in which no resistance mutations are present.

Table 2. Molecular markers of resistance to drugs that may be used in chemoprevention strategies

Drugs	Use in chemoprevention	Resistance polymorphisms	Status of resistance and marker(s)
4-aminoquinolines			
Chloroquine	Potential for use in new combinations where chloroquine-susceptibility has returned (not currently recommended by WHO)	*Pfcr*t 76T primary mediator; *pfmdr1* 86Y and 1246Y; other SNPs in these two genes contribute, mainly outside Africa	Resistance, mediated primarily by *pfcrt* 76T, has been widespread, but reversion to wild-type (sensitive) parasites is ongoing in many areas
Amodiaquine	Potential for use in combination with artesunate	Impacted by same mutations as chloroquine, but active against resistant parasites	Cross-resistance with chloroquine, but artesunate-amodiaquine highly efficacious
Bis-quinoline			
Piperaquine	Used in SMC in combination with DHA	Increased plasmepsin-2 copy number; *pfcrt* SNPs	Highly effective in combination with DHA; resistance polymorphisms seen in southeast Asia are uncommon in Africa
Arylamino alcohols			
Mefloquine	Potential for use in combination with artesunate	Increased *pfmdr1* copy number	Highly effective in combination with artesunate
Lumefantrine	Used in combination with artemether	Resistance not documented; decreased sensitivity associated with *pfcrt* and *pfmdr1* polymorphisms	Highly effective in combination with artemether
Artemisinins			
DHA	Used in combination with piperaquine	K13 mutations	Highly effective as ACT component
Artemether	Used in combination with lumefantrine	K13 mutations	Highly effective as ACT component
Artesunate	Used in combination with amodiaquine, mefloquine, or pyronaridine	K13 mutations	Highly effective as ACT component
DHFR inhibitors			
Pyrimethamine	Used in combination with sulfadoxine	Stepwise resistance with acquisition of *dhfr* mutations (108N, 51I, 59R, and 164L)	Widespread resistance
Sulphas			
Sulphadoxine	Used in combination with pyrimethamine	Stepwise resistance with acquisition of *dhps* mutations (primarily 437G, 540E, 581G)	Widespread resistance

Pfcrt, *Plasmodium falciparum* chloroquine resistance transporter gene; pfmdr1, *P. falciparum* multi-drug resistance-1 gene; SMC, seasonal malaria chemoprevention; DHA, dihydroartemisinin; SNP, single nucleotide polymorphism; K13, *P. falciparum* Kelch-13 gene; ACT, artemisinin-based combination therapy; DHFR, *P. falciparum* dihydrofolate reductase enzyme; dhfr, DHFR gene; dhps, *P. falciparum* dihydropteroate synthase gene; N, asparagine; I, isoleucine; R, arginine; L, leucine; G, glycine; E, glutamic acid. Table adapted from Conrad *et al.* [21] with permission.

have demonstrated strong and consistent associations between the presence of specific *dhfr* and *dhps* mutations and outcomes of interest for both treatment and chemoprevention regimens relying on antifolate drugs. A "quintuple mutant[3]" consisting of the *dhfr* S108N/N51I/C59R triple mutant[4] and the *dhps* A437G/K540E double mutant is strongly

[3] **Quintuple mutant** is defined as *dhfr* N51I/C59R/S108N and *dhps* A437G/K540E2 in the same *P. falciparum* infection. Some publications use "quintuple mutant" (or sextuple or septuple mutant) to refer to any *dhfr/dhps* haplotype containing any combination of five (or six or seven) mutations, but most use the definition applied here.
[4] **Triple mutant** is defined as *dhfr* mutations N51I/C59R/S108N in the same infection.

associated with SP treatment failure in African children [27]. In most settings the presence of *dhps* K540E, which is highly prevalent in East African but scarce in West Africa, reliably signals the presence of the other four mutations, making it possible to use this single marker as a surrogate for the quintuple mutant, a strategy that has been recommended by WHO for monitoring the efficacy of IPTi with SP [28].

In settings where antifolates are used, most of the other *dhfr* and *dhps* mutations that have been reported are too rare to permit a clear understanding of their roles in clinical and parasitological outcomes. However, there is growing concern, reviewed below, that a "sextuple mutant[5]" consisting of *dhps* A581G in the presence of the quintuple mutant may be associated with even higher rates of treatment failure and reduced effectiveness of chemoprevention strategies that rely on SP.

Correlating parasite genetic markers with clinical outcomes is potentially even more challenging for chemoprevention strategies than it is for treatment efficacy. This is because the relationships between parasite genotypes and efficacy outcomes are comparatively more straightforward in the case of drug treatment of clinical malaria. Drugs are administered and parasites are either cleared or not over the ensuing days. While factors other than resistance affect outcomes for both drug treatment and chemoprevention strategies, in the latter instance, the outcome is less immediate (e.g., birth outcomes following two or more doses of SP administered weeks apart), and the other factors influencing outcome (e.g., immunity, pharmacological factors, nutrition, other health indicators), are likely to play a more prominent role in chemoprevention outcomes. For example, these factors that complicate the relationship between resistance and health outcomes likely explain the persistent efficacy of ITPp-SP in the face of high failure rates when SP is used to treat clinical malaria in children (as discussed in detail below).

Additional factors that help explain the lack of concordance between treatment and chemoprevention efficacy include the fact that all patients who receive treatment for acute malaria are infected at the time of treatment, while individuals are often uninfected at the time chemoprevention drugs are administered, and may remain uninfected. In the absence of exposure to malaria parasites, chemoprevention efficacy outcomes (e.g., birth outcomes) are necessarily determined by factors unrelated to parasite resistance. Another difference between treatment and chemoprevention arises from the differential effect that some drugs have on different life cycle stages of the parasite. Treatment regimens work primarily by clearing blood-stage parasites, while chemoprevention regimens may exert their effect on blood-stage and/or pre-erythrocytic stage parasites, so a drug may remain effective against liver stage parasites even when blood stage parasites become resistant.

When using molecular markers to monitor resistance in the context of chemoprevention, the population in which resistance is assessed may influence how resistance estimates are interpreted. For example, implementation of chemoprevention strategies in a limited

[5] **Sextuple mutant** is defined here as *dhfr/dhps* quintuple mutant plus dhps A581G in the same infection.

population (infants, young children, pregnant women) sometimes results in increasing incidence of drug-resistant infections in that population, but not in the general population. In this circumstance, even if some of the same drugs are used for both chemoprevention and clinical treatment, there may be less cause for concerns about selection pressure from chemoprevention resulting in increasing resistance that would compromise the efficacy of antimalarial treatment.

In summary, drug resistance is but one of many factors that determine the efficacy of IPTp, IPTi, SMC and MDA. Clinical trials that measure health outcomes are the gold standard for measuring the efficacy of these chemoprevention strategies. Clinical trials of treatment efficacy cannot be used as a surrogate for chemoprevention efficacy. For antifolates and some other drugs, molecular markers accurately indicate the presence of drug resistant parasites, and are a useful but imperfect means of predicting the efficacy of chemoprevention strategies.

REVIEW OBJECTIVES AND APPROACH

This review summarises the current understanding of drug resistance in relation to the different malaria chemoprevention strategies. Separately, formal systematic reviews will be undertaken to summarise what is known about the safety, efficacy, and effectiveness of each of the individual chemoprevention strategies. While these strategy-specific reviews may capture some information on relationships between drug resistance and chemoprevention, the present review is intended to provide an overarching perspective on drug resistance as it relates to all of the chemoprevention strategies, considered both separately and together. This review thus aims to 1) summarize what is known about how malaria chemoprevention strategies affect, and are affected by, drug resistant malaria; 2) examine how drug resistance is best measured and monitored in each of the chemoprevention scenarios; and 3) consider approaches that can be used to mitigate the potential adverse public health impact of resistance, as related to chemoprevention.

This review considers primarily reports of studies published in the scientific literature, including randomized controlled clinical trials, observational studies including cross-sectional, cohort, and case-control studies, effectiveness studies, monitoring of routine implementation, modelling, and other studies. Relevant publications were identified through searching public databases, e.g., PUBMED, using search terms such as:

> *(drug resistance OR drug resistant) AND (malaria OR plasmodium) AND (SMC OR IPTi OR IPTp OR "mass drug administration" OR "seasonal malaria chemoprevention" OR "seasonal malaria chemoprophylaxis" OR "intermittent preventive"))*

These search terms yielded 379 publications from 1972 through 2021. Strictly laboratory-based studies and other publications that do not pertain directly to the chemoprevention strategies of interest were set aside, leaving 211 potentially relevant papers (including both original research reports and some reviews) that were individually reviewed. Among these 211 potentially relevant papers, 94 focused on IPTp, 23 on IPTi, 17 on SMC, and 16 on MDA. Seven papers focused on IPT in children that was not described as SMC (e.g., studies of non-seasonal chemoprevention in children in settings with year-round malaria transmission), and 54 addressed antimalarial drug resistance in the context of chemoprevention either generally or in relation to multiple chemoprevention strategies. Many studies in this latter category were surveys of resistance markers that considered implications for chemoprevention, but that did not directly study specific chemoprevention strategies.

As the review progressed, additional publications were identified in an iterative fashion from references and other sources, including careful review of key studies driving the results and interpretations of meta-analyses and systematic reviews of chemoprevention efficacy and resistance. Informal consultations with other experts identified yet more relevant publications to include in the review.

Research studies and publications were assessed for the quality and appropriateness of study design and statistical methods, and whether results supported the authors' conclusions. For example, randomized trials that prospectively assessed the impact of drug resistance on malaria chemoprevention efficacy, or vice versa, were given more emphasis than surveys or other observational or ecological studies uncoupled from data on chemoprevention efficacy. Many surveys of molecular marker prevalence were reviewed, but are not discussed in detail unless the results could be related to chemoprevention trials or implementation schemes. Greater weight was also given to results of well-designed, larger studies with sound methods that were conducted in settings and populations most relevant to the chemoprevention strategies under consideration. For example, studies of drug resistance in relation to MDA were prioritized based on their relevance to WHO's current recommendations for MDA to reduce the malaria burden in emergency situations, with lower priority assigned to studies of resistance where MDA has been tested as an elimination intervention in very low burden settings.

IMPACT OF CHEMOPREVENTION ON RESISTANCE

CHEMOPREVENTION SELECTS FOR DRUG RESISTANT PARASITES

More than 60 years ago David Clyde showed that the prevalence of antifolate-resistant parasites increased rapidly and dramatically in Tanzanian villages whose residents received weekly pyrimethamine for malaria prophylaxis [29]. Parasitological evidence of resistance was also detected in nearby villages whose residents did not receive chemoprevention, with the highest rates of resistance found in villages closest to those whose residents received pyrimethamine. The molecular basis for this rapid emergence and spread of resistant parasites was demonstrated 45 years later when this field experiment was repeated in a village in Mali,

where resistance-conferring mutations in *P. falciparum dhfr* rapidly and dramatically increased in prevalence[6] in the village within just a few weeks of starting all consenting villagers on weekly pyrimethamine [30].

Many subsequent studies have confirmed that community use of the antifolate combination sulphadoxine-pyrimethamine (SP), whether for treatment or chemoprevention, is often followed by increases in community prevalence of resistance mutations in both *dhfr* and *dhps*. In many of these studies, only very general temporal or ecological trends are reported that are consistent with, but not proof that, various chemoprevention strategies directly select for forms of resistance that affect clinical outcomes. For example, one report described trends of increasing molecular markers for antifolate resistant *P. falciparum* in Kenya over a 20 year period when SP was in use, initially for treatment, and subsequently for IPTp [31]. While one novel *dhps* mutation, S436H, more than doubled in prevalence between 2010 and 2017/2018, most of "the usual suspects" of *dhfr* and *dhps* mutations that have been associated with reduced efficacy of SP treatment and chemoprevention were already at near-fixation[7] in 2000, and their prevalence rose only marginally: *dhfr* N51I was prevalent at 90% in 2005, 99% in 2010, and 100% in 2017/2018, and *dhps* A437G was prevalent at 98% in 2000 and 2010 and 100% in 2017/2018. Even if the use of SP for treatment and IPTp was chiefly responsible for these increases in prevalence, such small changes in prevalence would have minimal impact on SP efficacy. Another molecular survey pooled data from nearly 40,000 samples collected in 38 African countries between 1998 and 2018 [32]. Generally higher prevalences of *dhps* A581G were seen in East compared to West Africa, with extensive heterogeneity including within countries.

While Clyde's Tanzania study and the oft-seen rapid selection of resistance mutations suggest that new forms of resistance can easily emerge locally under drug pressure, genomic epidemiology surveys have found that the most highly resistant forms of resistance to nearly all antimalarial drugs do not tend to arise *de novo* wherever drugs are used; rather, parasites with levels of resistance sufficient to cause treatment failure have arisen just a few times, usually in Asia, before spreading to Africa [33-36] (reviewed in [26]). This means that before highly resistant parasites have arrived in an area, even heavy drug selection pressure may not lead to loss of efficacy, as may be the case for antifolate resistance in much of West Africa. However, once highly resistant parasites are present, even at low prevalence, they can increase in response to drug pressure, as appears to be the case with antifolate resistance in much of East Africa. As more genomic epidemiology studies are undertaken, they are uncovering exceptions to the general rule that clinically relevant forms of resistance tend to emerge in Asia and spread to Africa. It is also possible for new, highly-resistant variants to arise locally on existing genetic backgrounds, as has been reported for *dhps* A581G in East Africa [37].

[6] In this review, "prevalence" of a given mutation or haplotype is defined as the proportion of infected individuals in whom that marker or haplotype is detected, irrespective of whether other alleles or haplotypes (e.g., wild-type) are also present in the infection. This definition is in line with WHO communications, e.g., *WHO Policy Recommendation on Intermittent Preventive Treatment During Infancy with Sulphadoxine-Pyrimethamine (IPTi-SP) for Plasmodium falciparum Malaria Control in Africa*.

[7] "Fixation" of a given mutation means that it is prevalent at nearly 100%.

One important consideration in evaluating the impact of chemoprevention strategies on resistance is that, if the strategy is effective at reducing malaria infections, the number of resistant infections in a population or setting may decrease even while the proportion of infections that are resistant increases as a result of selection pressure exerted by the chemoprevention drugs. When a chemoprevention strategy is highly effective, such as MDA using an ACT, selection favouring resistant parasites may have minimal public health impact if there are so few post-MDA infections that the resistant parasites in a given individual are rarely if ever transmitted. This is why MDA has tended to work best when it is implemented in parallel with rigorous vector control strategies [38]. Recent experiences with MDA in Southeast Asia (discussed below) are consistent with this scenario. In contrast, chemoprevention strategies that are less effective at reducing infections, such as IPTp-SP, may be more likely to result in increasing not only the proportion but the number of resistant infections, since they exert their effect less by preventing infections than by reducing parasite densities. Thus, the impact of chemoprevention on resistance depends both on the probability that resistant parasites emerge in an individual infection, and the probability that such resistant infections occur and are successfully transmitted. Mathematical models that incorporate these factors can be helpful in assessing the impact of specific chemoprevention strategies on drug resistance and efficacy [39].

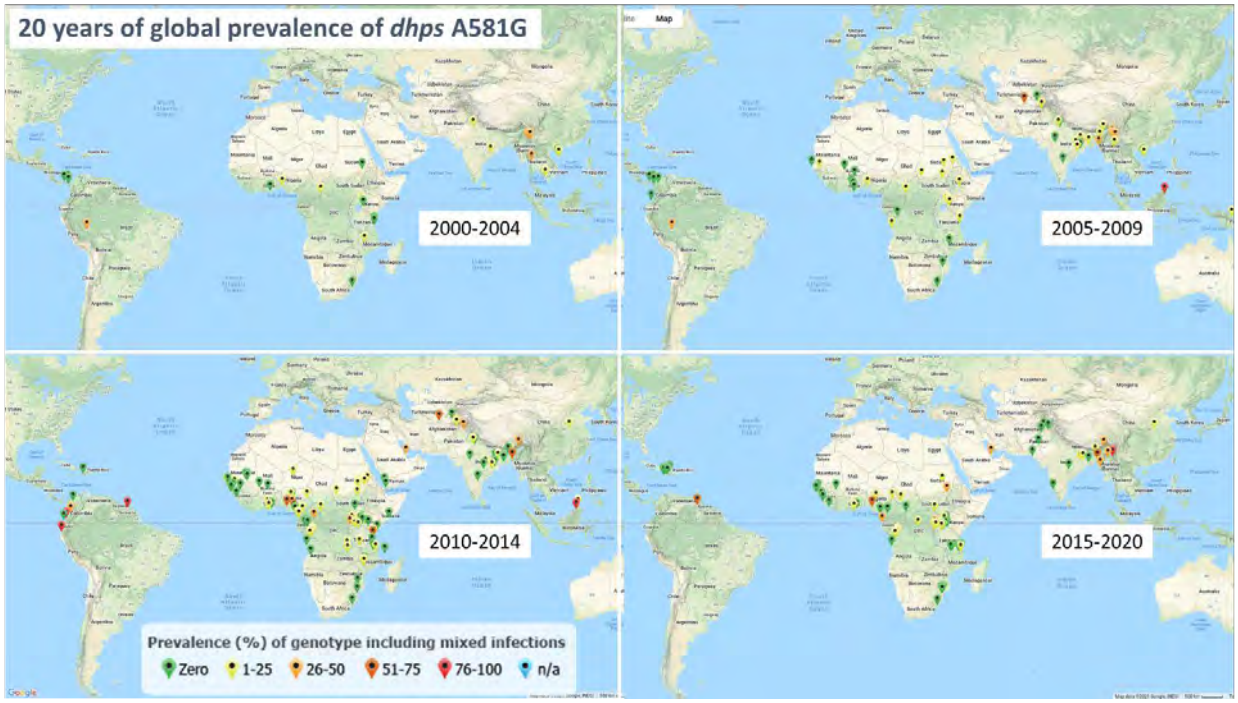

Figure 1. Global map of the prevalence of sulfadoxine-pyrimethamine resistance marker *dihydrofolate reductase* A581G. Data are from published sources and available at http://www.wwarn.org/dhfr-dhps-surveyor/#0 (accessed 12 April 2021).

Published data on the prevalence of resistance markers including A581G have been compiled and made available online. For example, **Figure 1** shows global prevalence data for *dhps* A581G between 2000 and 2020. The geotemporal trends for this mutation are consistent with the generally observed pattern of clinically significant resistance mutations being found earlier and at higher prevalence in East as compared to West Africa [26]. The reasons for this pattern are unclear, but plausible potential explanations include: 1) earlier introduction of resistance as a result of more-frequent human migration between Asia, the most common site of origin of highly resistant parasites, and East Africa; 2) more-rapid spread of mutations as a result of higher and more perennial malaria transmission in East Africa; and/or 3) earlier introduction of next-line antimalarial drugs (first SP, then ACTs) in East Africa owing to the earlier emergence of chloroquine resistance there has resulted in earlier and more intense selection pressure favouring parasites resistant to the new drugs in East Africa before these drugs were widely introduced in West Africa.

These global and regional patterns of emergence and spread of drug resistance illustrate a key point about the impact of chemoprevention on resistance: while there are ample examples of malaria chemoprevention strategies being followed by increased drug resistance, it is clear that not every chemoprevention scheme in every setting and population leads to measurable increases in resistance that lead to meaningful loss of drug efficacy in that setting and population. Moreover, it can be difficult to assess the impact of drug use on resistance, and vice-versa, in the many studies that report only prevalences of individual mutations, which by themselves are less reliable predictors of efficacy than full haplotypes. Other issues that commonly cloud interpretation of chemoprevention's impact on resistance include neglecting to genotype mutations previously believed to be absent from an area, and failure to account for differences in exposure risk among comparator groups in non-randomized observational studies.

IMPACT OF RESISTANCE ON CHEMOPREVENTION EFFICACY

Antifolate-resistant *P. falciparum* was already well established in Africa by the time IPTp, IPTi, and SMC were implemented there. Based on declining SP treatment efficacy in countries that were early adopters of SP following the rise of chloroquine resistance [27, 40], it was reasonable to expect SP-based chemoprevention strategies to follow a similar pattern. However, IPTp performed well even in settings where antifolate resistance led to SP treatment failure rates of 25% or higher in children [41] (discussed in more detail below).

About eight years after IPTp-SP was recommended by WHO in 1998, reports of increasing SP resistance led to renewed concern that, as one publication asserted, "In northern Tanzania, SP is a failed drug for treatment *and its utility for prophylaxis is doubtful*" (italics added) [42]. This assertion was based on the results of an open label single arm trial of SP efficacy for treating *P. falciparum* in symptomatic children and asymptomatic infants in Korogwe District, about 30 km north of Muheza, Tanzania. The trial had been stopped early owing to an early treatment

failure rate of 39% and day 28 failure rate of 82% in the symptomatic children. The authors implicated *dhps* A581G as the culprit in these alarming failure rates, despite multivariate analyses showing that factors associated with treatment failure included young age, high parasite density, and presence of three *dhfr* mutations, but not the presence of *dhps* A581G, which was prevalent at 55%. Notably, the *dhfr* triple mutant had a prevalence of 96% in this study. The findings that this *dhfr* haplotype was at near-fixation in this setting and was nevertheless significantly associated with treatment failure, while *dhps* A581G was not, despite being present in roughly half of the infections, suggests that the lack of association of A581G with treatment failure was real, and not a result of low prevalence or insufficient study power.

This and other studies of SP efficacy for treating clinical malaria in Africa thus raised alarms about the potential impact of antifolate resistance on IPTp and other chemoprevention strategies, but their inconclusive results called for directly examining this question in chemoprevention efficacy trials.

RELATIONSHIPS BETWEEN RESISTANCE AND SPECIFIC CHEMOPREVENTION STRATEGIES

INTERMITTENT PREVENTIVE TREATMENT DURING PREGNANCY AND RESISTANCE

Many studies have demonstrated selection of resistance markers in the context of IPTp-SP, although the specific mutations and haplotypes under apparent selection vary widely across sites and studies. Less clear is whether and under what conditions antifolate resistance affects IPTp efficacy. It may seem unremarkable, even obvious, that ITPp-SP would exert selection pressure on *P. falciparum* parasites resulting in rising prevalence of resistant mutants, which in turn must compromise IPTp efficacy. However, the sheer volume of studies and diversity of both study designs and results make it hard to ascertain clear and reproducible patterns in the relationships between IPTp and resistance. Studies reviewed here include cross-sectional studies in pregnant women, some with a single time point and some with two time points at first antenatal clinic (ANC) visit and at delivery; pregnancy cohort studies; birth cohort studies; retrospective case-control studies nested within these cohort studies; and clinical trials ranging from small open-label single-arm uncontrolled trials to large multi-country prospective randomized clinical trials conducted under rigorous regulatory oversight; to, finally, effectiveness studies.

A multitude of genotyping platforms has been used on a variety of sample types including both peripheral and placental blood obtained through both active surveillance in scheduled study visits and passive surveillance when people seek treatment for malaria illness. These differences in study design, methods, study sites, and populations, can affect study results, hampering cross-study comparisons and generalizability. Several meta-analyses, as well as analytical tools offered by the WorldWide Antimalarial Resistance Network [43], have attempted to mitigate these limitations, with mixed success.

IMPACT OF IPTp ON RESISTANCE

As IPTp-SP was being evaluated and implemented in the early 2000s, studies began to examine selection of resistant parasites by ITPp-SP. When *dhfr* and *dhps* mutations were compared in Malawian women from 2003-2006 before they started SP-IPTp and after delivery, the prevalence of the *dhfr/dhps* quintuple mutation increased significantly, from 81% before the intervention to 100% after delivery [44]. Around the same time, studies in other African countries compared marker prevalences in women receiving SP-IPTp compared with those not receiving treatment. The prevalence of *dhfr* mutations was compared in pregnant Ghanaian women at early gestation who had not received IPTp, and in women at delivery, nearly all of whom had received at least one dose of ITPp-SP [45]. Prevalence of the *dhfr* triple mutant was similar between the two groups and did not increase with an increasing number of IPTp-SP doses. Thus, even though the overall prevalence of *dhfr* mutations in the study population doubled between 1998 and 2006 in parallel with the implementation of SP-IPTp, the authors suggested that SP-IPTp might not be responsible for this increase. Similarly, in a study of peripheral and placental samples obtained from pregnant women over a 13-year period in western Kenya, the prevalence of the *dhfr/dhps* quintuple mutant rose contemporaneously with the implementation of IPTp-SP [46]. However, presence of the quintuple mutant was not associated with IPTp-SP use in multivariate analyses, suggesting that other factors were chiefly responsible for its rising prevalence.

In Mozambique, the prevalence of the quintuple mutant was higher in placentas of women receiving IPTp-SP than those receiving a placebo [47]. This association was only significant in women who had received a dose of SP within the 2.5 months before delivery, reflecting the "selection window" [48, 49] during which blood concentrations of sulphadoxine and pyrimethamine remain sufficient to select resistant parasites. In an ITPp-SP study done in Burkina Faso in 2014-2015, *dhfr* and *dhps* triple mutants were more common at delivery than at first ANC visit, but the same mutations were even more common in the general population than in pregnant women at either encounter, and recent use of ITPp-SP was not associated with increased prevalence of mutations [50]. In this study, *dhps* K540E was very rare, and *dhfr* I164 and *dhps* A581G and A613S/T were not assessed. Another study in Burkina Faso reported a similar increase in lower-level *dhfr* mutations, but no increase in *dhps* mutations [51].

As *dhps* A581G began to rise in prevalence in Africa, more studies focused on this mutation, which typically occurred together with the other *dhfr* and *dhps* mutations comprising the quintuple mutant to form the so-called sextuple mutant. In a Tanzanian study discussed at length in the next section, *dhps* A581G prevalence was significantly higher in IPTp-SP recipients compared to pregnant women who had not received IPTp [52]. Surprisingly, a survey done ten years later found that A581G was rare or absent in all but one of seven sites in Tanzania [53].

The prevalence of resistance markers before and during IPTp with SP or dihydroartemisinin-piperaquine was compared in clinical efficacy trials conducted in two Ugandan districts, Tororo in 2014-2015, and Busia in 2016-2017 [54]. The *dhfr/dhps* quintuple mutant was already near fixation at both sites, while *dhps* A581G was absent in Tororo and prevalent at only 3% in Busia.

Mutations associated with 4-aminoquinoline resistance, *pfmdr* N86Y and Y184F and *pfcrt* K76T, all appeared to be selected in the dihydroartemisinin-piperaquine arms of both trials. The *dhfr/dhps* quintuple mutations were all already prevalent at >90% and did not increase significantly in the SP arms at either site. The prevalence of *dhfr* I164L remained less than 2% both before and during IPTp-SP in Tororo, but I164L rose in prevalence from 4% to 13.7% in Busia. This is consistent with selection by IPTp-SP at this site, but prevalence of this mutation also rose to 9% in women in the dihydroartemisinin-piperaquine arm who were unexposed to SP, so it is not possible to distinguish between selection by ITPp and community-wide trends in prevalence in Busia over the course of the study. The *dhps* A581G mutation remained absent in Tororo and did not increase in prevalence in either arm in Busia, decreasing from 3% at baseline to 0% in the dihydroartemisinin-piperaquine arm and to 1.9% in the SP arm. The authors speculated that the apparent lack of selection of A581G in Busia was due to its low baseline prevalence. This explanation is unconvincing, in that sharp increases in the prevalence of resistance, and resistance markers, is commonly seen under antifolate drug pressure for other antifolate mutations found at low baseline prevalence [29, 30]. Another study reported apparent selection favouring A581G in Uganda after a single dose of IPTp-SP, but this conclusion was based on very small numbers: A581G was found in two of 52 infected women at the first antenatal visit, compared with two of 12 at the second visit [55].

The *dhps* A581G and A613S/T mutations were reported to be selected by IPTp-SP in another study of antifolate resistance marker prevalence conducted in Ghana in 2015-2017. This cross-sectional study compared marker prevalence in pregnant women at their first antenatal visit and at delivery [56]. At delivery more than 70% of women had received at least two doses of IPTp-SP, so parasites were presumed to have been under selection pressure from SP. Unlike in the contemporaneous Ugandan study that found no increase in prevalence in *dhps* A581G, this West African study saw A581G increase from 9% to 16%. Statistical analyses were not presented, but this increase is not statistically significant (uncorrected X^2=2.95, P=.09). While A613S/T had similar prevalence before and after delivery (15.2% to 17.5%, uncorrected X^2= 0.82, P=.37), the authors reported in the abstract that a septuple mutant with both A581G and A613S/T increased significantly from 6.1% at enrolment to 18.2 % at delivery (P=.03). These results are difficult to compare with those of studies in East Africa, most of which have found that A581G is usually accompanied by K540E. In contrast, in this study A581G was always accompanied by the *dhps* triple mutant and *dhps* S436G, A437G and A613S/T, but never by K540E. Another recent West African survey, of asymptomatically infected pregnant women in Nigeria, similarly found that K540E was absent despite high prevalences of A581G (71%), S436A (55%) and A613S/T (36%) [57], and a survey in Ghana reported similar results [58]. This tendency for K540E to be uncoupled from A581G at some West African sites likely reflects the global patterns of spread of *dhps* haplotypes, with more highly resistant forms commonly found in East Africa having Asian origins, while less resistant homegrown *dhps* haplotypes predominate in West Africa [36].

The single study that provides the most convincing evidence that ITPp-SP does not strongly select *dhps* A581G comes from a well-designed randomized clinical trial done in Malawi in 2011-2014 [59]. Pregnant women were randomized to one of two intervention arms: standard

IPTp-SP, or intermittent screening by rapid diagnostic test (RDT), and treatment of RDT-positive infections with dihydroartemisinin-piperaquine. No differences were found in the prevalence of *dhps* A581G in either the peripheral or placental blood among women in the IPTp group who had been exposed to SP, compared to women randomized to the screen-and-treat group who were not exposed to SP.

In summary, IPTp-SP appears to select for antifolate resistance mutations associated with low to moderate increases in drug resistance, but there is no convincing evidence of selection favouring the key mutations—especially *dhps* A581G—associated with higher level antifolate resistance and loss of ITPp-SP efficacy.

IMPACT OF RESISTANCE ON IPTp EFFICACY

The most recent WHO Guidelines for Malaria, published in February 2021, continue to recommend IPTp-SP for women living in areas of moderate-to-high transmission in Africa, including in areas with >90% prevalence of the *dhfr/dhps* quintuple mutant [60]. The guidelines note that where infections with the quintuple mutant plus either *dhfr* I164L or *dhps* A581G are prevalent, "…the efficacy of IPTp-SP may be compromised. It is unclear by how much." The following discussion considers whether currently available evidence can add clarity on this topic.

EARLY STUDIES YIELDED CONFLICTING RESULTS

Many studies of widely varying quality have assessed the impact of SP resistance on IPTp efficacy. An influential systematic review and meta-analysis published in 2007 pooled data from seven clinical trials of IPTp-SP in relation to SP efficacy for treating symptomatic malaria in young children at or near the same times and locations of the IPTp trials [41]. The authors concluded that even in areas where SP had lost treatment efficacy in children (day 14 treatment failure rates of 19-26%), IPTp-SP continued to provide important health benefits to HIV-negative semi-immune pregnant women and their infants. Moreover, they found no evidence of a substantial loss of IPTp efficacy as SP treatment failure rose from 3% to 39% across sites. In women living with HIV, a group in which IPTp benefit is reduced, IPTp efficacy did decline with rising treatment failure.

The discordance between IPTp-SP benefit in HIV-uninfected pregnant women and SP treatment efficacy in children was attributed mainly to greater levels of acquired immunity in pregnant women. This systematic review did not directly address drug resistance as distinct from treatment failure, nor did it examine relationships between *dhfr* and *dhps* mutations and IPTp outcomes. Nevertheless, policymakers were reassured by the persistent benefit of IPTp-SP in the face of high rates of SP treatment failure in children, and WHO recommended adopting IPTp-SP in Africa even where the prevalence of parasitological failure at Day 14 after SP

treatment among children was as high as 50%, or even higher in areas where IPTp was already implemented [61].

DOES *dhps* A581G RENDER IPTp-SP NOT ONLY INEFFECTIVE BUT HARMFUL?

A study done in the same region of Tanzania where earlier studies had found that rising prevalence of *dhps* A581G curtailed the efficacy of both SP treatment of children and IPTp-SP, appeared to support the concern that this mutation boded ill for ITPp-SP [52]. This study, which assessed clinical, parasitological, and histopathological outcomes of IPTp, was even more alarming than the report of very high SP treatment failure rates in Tanzanian children [42]. Based on a study in mice showing that resistant parasites grew to unexpectedly high densities when drug treatment eliminated sensitive parasites [62], the authors hypothesized that, with its compromised efficacy, SP might "select resistant parasites and exacerbate infections in the placenta". SP resistance mutations, placental parasite densities, and placental inflammation were assessed in women enrolled at delivery between 2002 and 2005 who reported having received, or not having received, SP-IPTp. Those who reported receiving IPTp were classified as "recent IPTp" if they had measurable sulpha levels in their blood, and "early IPTp" if sulpha levels were undetectable.

The authors reported that IPTp was associated not only with higher prevalence of *dhps* A581G but with dramatically higher placental parasite density, and, most concerning, with increased placental inflammation. The authors reasoned that inflammation indicates chronic placental malaria infection; that inflammation should thus be absent in acute placental malaria; and that placental parasite density normally decreases as placental inflammation increases. Based on these expectations and the observation that inflammation was more common in women who received IPTp, they deduced that the high parasite densities could not be attributed to new acute infections, and therefore must have resulted from the greater presence of resistant parasites carrying *dhps* A581G in the women who received IPTp. In a subsequent publication of data from the same observational study, the authors reported that IPTp did not reduce the odds of placental malaria, increase mean maternal haemoglobin, or increase birthweight, and IPTp was associated with lower cord haemoglobin and increased risk of foetal anaemia [63]. The implications for ITPp-SP seemed dire.

This study had significant flaws, including the inference that differences in outcomes were the result of IPTp and not other confounding factors in what was essentially a retrospective case-control study nested within a birth cohort. While most baseline characteristics showed no significant differences between women who did or did not report receiving IPTp, there was one important, highly significant difference: only 29% of women who received no IPTp lived in rural areas, while 68% of women who received IPTp lived in rural villages. The authors elided any discussion of differences in malaria epidemiology or risk between the rural and urban sites. An earlier paper describing the parent birth cohort study [64] cited an annual entomological inoculation rate (EIR) of around 400 for the study area of Muheza District, but that paper in turn

cited another paper from a decade earlier that reported heterogeneous transmission in Muheza, with EIRs ranging from 34 to 405 infected bites/person/year [65].

With the only available data on transmission intensity in the study area being a ten-year-old study that reported a more than 10-fold range of EIRs, it was not reasonable to dismiss the baseline observation that significantly more rural women had received IPTp. A plausible alternative explanation for the higher parasite densities and placental inflammation in women who received IPTp would be that rural women may have been exposed to up to 10-fold higher malaria transmission intensity than their peri-urban counterparts, increasing their risk of acute-on-chronic placental infections. Bed net use was also different at baseline: women who reported no IPTp also reported marginally and insignificantly higher use of bed nets (76.5% vs. 64.4%). However, while none of the (more urban) non-IPTp women reported using insecticide-treated nets (ITN), 16% of (more rural) women who had received IPTp reported using ITNs. The complete absence of ITN use among the non-IPTp women is consistent with alternative explanations, including the possibility that the parasitological and histopathological findings attributed to IPTp selection of A581G-carrying resistant parasites were actually a result of baseline differences in malaria risk between women who received IPTp and those who did not.

Subsequent studies failed to replicate the disquieting finding that IPTp-SP led to increased parasite growth in a setting with prevalent *dhfr/dhps* sextuple mutants. In another cohort of pregnant women in Korogwe District, less than 30 km from Muheza, *dhfr* and *dhps* were genotyped in samples from women who had *P. falciparum*-positive RDTs, and pregnancy outcomes were assessed [66]. During the study period of 2008-2010, the prevalence of the sextuple mutant with *dhps* A581G in Korogwe was 44%, slightly lower than in nearby Muheza several years earlier. The presence of the sextuple mutant was associated with substantially lower birthweights. However, in contrast to the Muheza cohort, the presence of the sextuple mutant was not associated with whether or not women had received IPTp-SP or with how many doses they received; peripheral parasite density tended to be lower, not higher, in women with the sextuple mutant; and there was no relationship between early or recent IPTp and the effect of *dhfr/dhps* haplotypes on birth weight. Studies in Mozambique [47] and Malawi [67] similarly failed to support the notion that IPTp was harmful in settings with high levels of antifolate resistance, although *dhps* A581G was rare (but present) in both studies.

Another systematic review was published in 2013 by the same group that conducted the 2007 review of IPTp efficacy. A meta-analysis of data from seven trials, one each in Kenya, Tanzania, Zambia, Burkina Faso, and Mali, and two in Malawi, found higher average birthweights and lower risk of low birthweight in women who had received three or more doses of IPTp-SP, compared with those who received only two doses [68]. This association was consistent across sites where the prevalence of the *dhfr/dhps* quintuple mutant—as indicated by the presence of *dhps* K540E—ranged from 0-96%. The *dhps* A581G mutant was not prevalent at any of the study sites. Based on these relatively encouraging findings, WHO recommended at least three doses of IPTp-SP irrespective of the presence of *dhfr/dhps* quintuple mutants [69].

To recap, as of 2013, two high quality systematic reviews [41, 68] and more recent clinical trials [47] and surveys [67] supported the WHO position that IPTp-SP was beneficial and should be used across a wide range of antifolate resistance and SP treatment efficacy. On the other hand, an open label SP efficacy study in children [42] and two observational studies in pregnant women [52, 63, 66] suggested that the sextuple mutant represented a dangerous threat to IPTp-SP efficacy, and might be causing IPTp to be not only ineffective but harmful in pregnancy. Each of these three studies portending bad news for IPTp had significant limitations and flaws in design and interpretation, and all three were conducted in two adjacent districts in Tanzania, limiting their generalizability to other sites in Africa, where the sextuple mutant remained mostly rare or absent.

WELL-DESIGNED STUDIES CHALLENGE THE INDICTMENT OF A581G

Aiming to resolve these discrepant results, an observational study followed by a clinical trial in Malawi and a multi-country efficacy trial of IPTp-SP efficacy directly examined the relationship between SP resistance and IPTp outcomes. The effectiveness of IPTp-SP was assessed in 2009-2011 in Malawi, where the prevalence of the sextuple mutant was 8.4% [70]. The presence of A581G was associated with an approximately 3-fold increase in the occurrence of “patent” infections (both PCR and microscopy positive) in both peripheral and placental blood, and with higher parasite densities. However, A581G was not associated with any of the following: 1) histological evidence of active placental infection; 2) mean haemoglobin; 3) anaemia; 4) severe anaemia; 5) pre-term delivery; or 6) infants born small for gestational age. Furthermore, women infected with parasites carrying *dhps* A581G gave birth to infants with slightly higher birthweights and had a nearly 2-fold lower incidence of low birthweight, although these trends did not achieve statistical significance. And, the finding of higher parasite densities in A581G-carrying infections disappeared when the analysis was limited to women with “patent” infections, i.e., when infections that were PCR-positive but microscopy-negative were excluded from the analysis.

Some methodological issues cloud the interpretation of these results. For example, even though more than 90% of both patent (PCR and microscopy-positive) and “subpatent” (PCR-positive, microscopy-negative) infections were successfully genotyped, the prevalence of A581G was reported to be 10-fold lower in subpatent infections, a surprising finding that is not explained by the authors. Further muddying the picture, most of the data are presented as pooled results from two study sites, one rural and one urban, even though the prevalence of A581G was more than twice as high at the rural site. Different microscopy staining and reading protocols were used at the two sites, with more rigorous standards at the urban site in Blantyre, and no quality control procedures were described, raising the possibility that rural-urban differences in both malaria epidemiology and the quality of microscopic diagnosis could account for some of the study findings. As with the flawed Tanzanian study described above [52], it is possible that higher parasite densities attributed to resistant parasites were actually a reflection of higher transmission intensity or other epidemiological differences at the rural site where more A581G-carrying infections were found.

A subsequent trial, also done in Malawi by the same group, randomized pregnant women at three sites in 2011-2014 either to receive standard IPTp-SP or intermittent screening with RDTs and treatment of RDT-positive infections with dihydroartemisinin-piperaquine [59]. By the time of this study, the *dhfr/dhps* quintuple mutant was at near-fixation, and the *dhfr* I164L mutation was absent, while *dhps* A581G had a prevalence of 4% in infections found at enrolment in the IPTp-SP group, and 6% in placental infections at delivery. The presence of A581G in placental infections was associated with a significant decrease in gestational age and lower birthweights, but not with parasite placental density, placental inflammation, maternal haemoglobin level, or weight-for-age Z score. Overall, the timing of SP exposure had no impact on birth outcomes, but in the small group of women who had placental infections with A581G, more recent SP exposure was associated with significantly longer pregnancies and higher birthweights. However, a sensitivity analysis showed that this result was driven by a single premature birth of a very small infant to a woman who had received only a single dose of SP; when this outlier was accounted for, the association between recent SP and birth outcomes among women with A581G was not significant.

Taken together, the results of these two studies in Malawi confirmed a partial diminution of IPTp efficacy against A581G-containing placental malaria, but they did not support findings of the studies that had raised the alarm about ITPp-SP causing harm where A581G is prevalent.

PROSPECTIVE, MULTI-COUNTRY TRIAL OF IMPACT OF RESISTANCE ON IPTp-SP BIRTH OUTCOMES

None of the studies described so far that promoted the notion that antifolate resistance in the form of *dhps* A581G spelled doom for IPTp-SP in Africa were prospective, controlled trials designed specifically to address this question. In contrast, the relationship between antifolate resistance mutations and efficacy of IPTp-SP was prospectively assessed in a multi-country trial among asymptomatic, microscopy-confirmed *P. falciparum*-infected, HIV-uninfected, pregnant women. Prospective efficacy studies were undertaken between 2009 and 2013 at eight sites in six African countries spanning the continent and a range of prevalences of mutations in *dhfr* and *dhps* [71]. With weekly follow-up, treatment failure was defined as smear-positive *P. falciparum* on or after day 4, with both uncorrected and PCR-corrected efficacy estimates. Resistance genotyping was done using pooled sequencing, so any novel mutations should have been detected.

Study sites were characterized as having low, moderate, or high SP resistance, based on prevalence of *dhps* K540E of <10%, 10-90% or >90%, respectively. Defining SP resistance on the basis of this one mutation was reasonable, in that K540E serves as a good surrogate for the *dhfr/dhps* quintuple mutant in settings where *dhps* A581G is absent or rare, as it was in the study sites. A581G, the surrogate for the *dhfr/dhps* sextuple mutant, was absent in the moderate (Zambia) and low (Burkina Faso and two sites in Mali) resistance sites. A581G was found in each of the high resistance sites, with prevalence of less than 0.25% in Uganda, less than 2% in both Malawian sites, and just over 5% in Kenya. At these low levels, it was not

possible to assess the relationship between the sextuple mutant and IPTp-SP outcomes in this study.

While SP resistance mutations in this multicentre study did appear to compromise parasite clearance and result in more reinfections as well as a shorter time to reinfection, resistance did not appear to affect birth outcomes. As the authors note, prevalence of resistance mutations was not the only difference across the sites. Although transmission intensity was not measured in this study, malaria transmission has historically been reported to be lower and more sharply seasonal in the West African low-resistance sites, and higher and more year-round in the moderate- and high-transmission sites in East and Southern Africa. This pattern makes it difficult to attribute outcomes solely to resistance. Moreover, lower resistance and transmission might lead us to expect better IPTp outcomes in West Africa, but this same lower transmission may also mean lower natural immunity contributing to poorer outcomes, making it challenging to sort out the relative contributions of these countervailing effects of resistance, exposure risk, and immune protection on IPTp birth outcomes. Finally, women who had been enrolled in the in vivo SP efficacy study were excluded from the birth outcome study. If women who had patent infections early in pregnancy were at higher risk of malaria, their exclusion from the birth outcome study might have reduced the study power to detect SP's antimalarial efficacy, lending more weight to non-malaria factors in determining outcomes. Nevertheless, this well-designed study further added to the growing body of evidence that the benefits of IPTp persist in the face of high rates of SP resistance as conferred by *dhfr/dhps* quintuple mutants. The impact of the sextuple mutant (the addition of *dhps* A581G) on IPTp outcomes remained unaddressed by this study, which finished data collection in 2013.

One potential factor contributing to the apparent lack of impact of antifolate resistance mutations on IPTp-SP efficacy is suggested by studies showing that IPTp with either dihydroartemisinin-piperaquine or SP results in comparable pregnancy outcomes despite dihydroartemisinin-piperaquine's superior efficacy at clearing and preventing malaria infection [72-74]. Studies are underway to assess whether some of SP's impact on pregnancy outcomes is mediated by non-malaria benefits, e.g., antibacterial activity.

RECENT SYSTEMATIC REVIEWS AND META-ANALYSES

Two additional systematic reviews have attempted to identify a threshold prevalence of *dhps* A581G above which IPTp-SP efficacy is lost. One pooled data from nine IPTp studies (five clinical trials and four observational studies completed at a total of 12 sites) to assess the impact of malaria transmission intensity on IPTp outcomes, as modulated by A581G prevalence [75]. Transmission intensity did not appear to influence the efficacy of IPTp on low birth weight. Data on A581G prevalence collected within two years and 250 miles of the IPTp studies were pooled. Two sites had >50% prevalence of A581G, and the others had 10% prevalence or less, with four sites having no A581G. Among women who had received two or more doses, IPTp-SP efficacy was preserved at sites with 10% or lower prevalence of A581G. At the two sites with >50% prevalence, efficacy was diminished but still significant among primigravid and secundigravid

women, and absent in multigravid women. The authors concluded that the A581G prevalence threshold above which IPTp-SP should not be used was somewhere between 10-52%.

Another, larger, meta-analysis and systematic review was recently published by the same group that performed the two earlier meta-analyses discussed above, each of which pooled data from just seven studies [41, 68]. This meta-analysis pooled data from 57 studies done in 17 African countries between 1994 and 2014, and confirmed the relationship between prevalence of the quintuple mutant (signalled by *dhps* K540E) and diminished IPTp-SP efficacy [76]. The *dhps* A581G mutation, used as a surrogate for the *dhfr/dhps* sextuple mutant, was present in 16 of the studies, at prevalences ranging from 2.5% to 47%. The pooled analysis thus overcame the sample size limitations of the individual studies that had made it difficult to draw definitive conclusions about the impact of the sextuple mutant in IPTp-SP outcomes. However, the authors' conclusion that IPTp-SP effectiveness is lost where *dhps* A531G prevalence exceeds 10% is based on just five of the 57 studies included in the meta-analysis.

Three of these five studies had small sample sizes in the reference group and a pooled prevalence of A581G of 21%, and together yielded a relative risk reduction (RRR) of 35%. This means that IPTp-SP retained good effectiveness comparable to that seen in low resistance sites, despite A581G prevalence greater than 20%. Of these three studies, two were from Tanzania and had limitations and discordant results that are discussed at length above [52, 63, 66]. Interpreting results from the third study, from Uganda, is confounded by the unusual finding that both *dhps* A581G and *dhfr* I164L had 36% prevalence, occurring together almost as often as not [77]. The *dhfr* I164L mutation, which has been largely absent or unreported in other studies of IPTp and resistance, is found in parasites with the highest measured pyrimethamine IC50s, approximately 10-fold higher than IC50s of parasites carrying the *dhfr* triple mutant. The frequent occurrence of parasites carrying both of these mutations prevents clear attribution of IPTp outcomes in this study to A581G.

Of the five studies that provided the basis for concluding that IPTp-SP efficacy is lost above a 10% prevalence threshold for A581G, the remaining two were from the Democratic Republic of Congo [78] and Uganda [79]. Both of these studies had larger sample sizes and were conducted in areas with a pooled prevalence of A581G of 46%, much higher than that seen in the three smaller studies. Together, these two studies had an RRR of -2% for low birthweight (compared with 35% for the other three studies), signifying a complete loss of IPTp-SP efficacy. This means that among the 57 studies include in the meta-analysis, just two reported that IPTp-SP efficacy was lost where A581G prevalence exceeds 10%. Both studies were also done in areas where the prevalence of K540E was above 90%, making it difficult to attribute the loss of IPTp efficacy to the presence of A581G. Neither of these studies included molecular analyses of *dhfr* or *dhps* mutations—the meta-analysis relied on molecular data collected as close as possible in space and time to the field studies [80-82]. While some of these molecular studies did report prevalence of *dhfr* I164L, the meta-analysis only used data on *dhps* mutations.

In summary, despite some convincing evidence that the presence of *dhps* A581G at least partially compromises the efficacy of IPTp-SP, the worst-case scenario [52] was not borne out

by subsequent trials [59, 70, 71]. The evidence supporting a recommendation to withhold ITPp-SP where the prevalence of *dhps* A581G exceeds a threshold of 10% is not strong.

INTERMITTENT PREVENTIVE TREATMENT DURING INFANCY AND RESISTANCE

As with IPTp, resistance has been of concern since IPTi was first conceived and evaluated. Most early studies incorporated molecular surveillance to assess the relationships between resistance markers and IPTi-SP efficacy, and to measure the impact of IPTi on the prevalence of resistance markers.

IMPACT OF IPTi ON RESISTANCE

In a 2003-2005 trial of IPTi-SP in aparasitaemic Ghanaian infants, the incidence of *dhfr/dhps* quintuple mutants during two months after the third dose of IPTi was twice as high in the treatment group compared a placebo group [83]. In contrast, the prevalence of *dhfr* triple and *dhfr/dhps* quadruple[8] mutants remained stable over a one-year period as IPTi-SP was implemented in Mali in 2006-2007, with no differences in marker prevalences between 11 IPTi implementation zones and 11 control zones [84].

Ecological surveys accompanied IPTi-SP evaluations in Tanzania (2004-2007) [85] and Senegal (2006-2008) [86]. Two years after implementation of IPTi-SP, prevalence of the *dhfr* triple mutant in both countries was significantly higher in areas subjected to IPTi compared to nearby control areas. Prevalence of *dhps* A437G was also significantly higher in IPTi areas in Senegal, where *dhps* K540E was absent. While the prevalence of the *dhps* A437G/K540E double mutant was also higher in IPTi areas than in control areas in Tanzania, this difference was not significant.

Follow-up surveys in Senegal in 2009-2010 confirmed that *dhps* K540E remained absent both in zones where IPTi-SP (or IPTc-SP, as seasonal malaria chemoprevention was then called) had been implemented, as well as in control zones. The *dhps* A437G mutation decreased in prevalence in both IPT and control zones between 2009-2010. In the IPT zone both A581G and A613S declined from 3% to 0% and from 5% to 0%, respectively, while in the control zone A581G and A613S both rose from 0% to 3% in prevalence during the same two-year period.

In summary, while IPTi-SP has been accompanied by overall increases in the prevalence of some antifolate resistance markers, neither clinical trials of IPTi nor ecological surveys comparing IPTi implementation zones to control areas over time have shown evidence of significant selection of the *dhfr/dhps* haplotypes associated with reduced SP efficacy for treatment or chemoprevention. This conclusion is in agreement with that of an Institute of

[8] **Quadruple mutant** is defined here as *dhfr* triple mutant plus *dhps* A437G in the same infection.

Medicine expert committee discussed in the next section [87], and with results of two independent modelling studies [88, 89].

IMPACT OF RESISTANCE ON IPTI EFFICACY

In 2008, the Institute of Medicine (now the National Academy of Medicine) in the United States convened an expert committee to evaluate the evidence concerning IPTi-SP efficacy, including an assessment of the impact of antifolate resistance [87]. The committee undertook a detailed review of published and unpublished data, including new meta-analyses of pooled data from pilot studies of IPTi-SP done at six sites in Tanzania, Ghana, Mozambique, and Gabon. They concluded that: 1) SP treatment efficacy for clinical malaria is not a reliable indicator of IPTi effectiveness; and 2) IPTi-SP retained 20-30% efficacy in the face of 40-80% prevalence of the *dhfr* triple mutant. Among the six sites where IPTi showed measurable efficacy were Ashanti, Ghana, where more than 60% of baseline infections had four or more *dhfr*/*dhps* mutations [90]; Tamale, Ghana, where the *dhfr* triple mutant plus *dhps* A437G (i.e., the quadruple mutant) was found in 44% of infected children aged less than five years [91]; and Manhica, Mozambique, where prevalence of the *dhfr/dhps* quintuple mutant in the placebo arm was 44% [92]. The committee was unable to evaluate the impact of *dhfr* I164L because it was absent or rare at African sites where studies had looked for this mutation. The committee did not consider the role of *dhps* mutations in IPTi efficacy in more detail, owing to the paucity of available data at a time when few studies had examined the role of *dhps* K540E in IPTi efficacy, and the A581G and A613S/T mutations were still rare in Africa.

Subsequent studies found that IPTi-SP efficacy diminished in parallel with rising prevalence of the *dhfr/dhps* quintuple mutant. While the sample sizes were small, all nine genotyped baseline infections had the quintuple mutant in an IPTi trial Korogwe, Tanzania, where four infections (44%) also carried the *dhps* A581G mutation [42]. Although marker prevalence was not reported for infants participating in a trial in Same, Tanzania, a 2001 survey of two sites nearby had found a 60-75% prevalence of the *dhfr* triple mutant and a 43-55% prevalence of the *dhps* double mutant [93]. Neither of these two trials demonstrated significant protective efficacy of IPTi-SP [94].

A pooled analysis of results from seven IPTi trials conducted between 1999 and 2008 found that the duration of protective efficacy was shortened by 50% in the presence of quintuple mutant parasites, from 42 days in Navrongo, Ghana (no *dhfr/dhps* quintuple mutant), to 21 days in Korogwe, Tanzania (89% prevalence of the quintuple mutant) [95]. This meta-analysis also found that protective efficacy in the 35-day period after the 9-month dose of IPTi-SP decreased with an increasing number of resistance markers, although there were not enough data points to determine the effects of specific markers. These data are consistent with a meta-analysis that found that the duration of post-treatment chemoprophylaxis for different ACTs was shorter when the prevalence of markers of resistance to the ACT partner drug was higher [96].

Based on the data available at the time, a 2009 WHO technical consultation recommended that IPTi-SP be implemented only "when parasite resistance to SP in the area is not high", adding that "Precise cut-offs cannot be defined on the basis of available data." [28] Just a few months later another technical consultation that included expertise in drug resistance reviewed essentially the same body of evidence and recommended that IPTi-SP not be implemented where prevalence of *dhfr* K540E exceeded 50% [28]. This recommendation was based on just two IPTi-SP trials, one showing 21% protective efficacy in Mozambique where baseline prevalence of *dhfr* K540E was 55%, and one in Tanzania showing no significant efficacy where K540E prevalence was 94%.

A subsequent analysis of molecular marker data collected across Africa from 2005-2011 found that, based on the 50% threshold for K540E prevalence, eight East African countries were classified as unsuitable for SP-IPTi; 14 Central and West African countries were classified as suitable; and seven countries could not be classified owning to a lack of available contemporary data [97]. A cost-effectiveness analysis concluded that IPTi-SP remained cost-effective across a range of SP resistance levels, but the analysis did not consider the high-level resistance conferred by *dhps* K540E, A581G and A613S/T, limiting relevance of the study for areas where these mutations are prevalent [98].

In the last decade, few new studies that inform the impact of resistance on IPTi efficacy have been published. When a cluster randomized trial of IPTi-SP in Tanzania found no survival benefit, the authors speculated that drug resistance was one of many possible factors that could account for this finding, along with operational deficits, decreasing malaria transmission, improving vector control, and better case management [99]. A recent Cochrane review of IPTi noted overall trends of declining IPTi efficacy in parallel with increasing antifolate resistance in Africa, but no new data on SP resistance markers underly this observation, so this meta-analysis does not help in more precisely defining a resistance threshold to guide IPTi implementation decisions [100].

As countries consider implementing IPTi or introducing new drug combinations where IPTi-SP has lost efficacy in the face of resistance, studies directly assessing not only efficacy but duration of protection against both asymptomatic infection and clinical malaria episodes in relation to the prevalence of resistance markers would be of value. As was shown for different ACT treatment regimens [96], the benefits of different chemoprevention regimens may be different in areas with different resistance patterns.

Given the continued paucity of data on the relationships between SP resistance markers and IPTi efficacy to justify a threshold of resistance above which IPTi should not be implemented or continued, more creative approaches may be needed. For example, **Figure 2** shows the frequency distribution of prevalence measures of *dhps* K540E and A581G for studies completed in sub-Saharan African countries between 2015 and 2021, arranged in increasing order of prevalence. The prevalence of the K540E mutation ranged from 0-100%, with a clear "break point" (sharp change in slope) at around 40% prevalence, providing a natural point for grouping sites with prevalences above and below that point. In contrast, the prevalence of A581G ranged

from 0-53%, with a less obvious break point around 15%. When there is a wide range between prevalence levels at which IPTi efficacy persists or is lost, it might be reasonable to choose thresholds based on these break points in the data, to reflect naturally occurring clustering of prevalence levels. This approach might help policy makers avoid difficult decisions when measured prevalences lie very close to the thresholds.

In the case of IPTi efficacy and *dhps* K540E prevalence, based on the observation that IPTi retained 21% efficacy where K540E was prevalent at 55% but not where it was 94%, any threshold between 55-94% could have been selected to segregate sites where IPTi-SP might be expected to retain and lose efficacy. The WHO technical consultation recommended a 50% threshold based on the assumption that where prevalence was less than 50%, efficacy should be at least 21%. Based on this new analysis of more recent marker prevalence data as shown in **Figure 2**, a case could be made for implementing IPTi where K540E has a prevalence of 40% or lower.

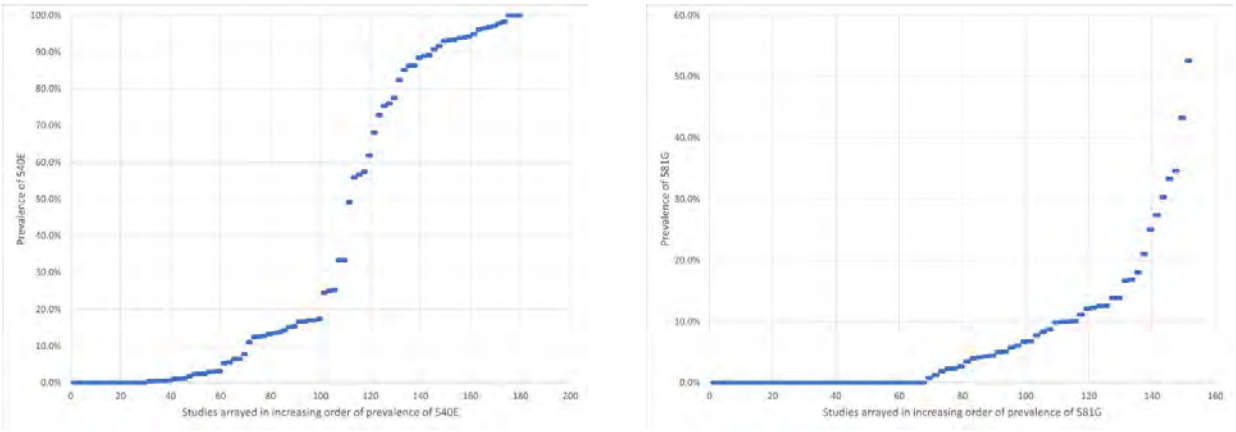

Figure 2. Frequency distributions of prevalence estimates of *dhps* K540E (L) and A581G (R) mutations measured in studies completed in sub-Saharan Africa from 2015-2021. Data were downloaded from http://www.wwarn.org/dhfr-dhps-surveyor and studies completed before 2015 and outside of Africa were excluded. Recent measures of K540E prevalence tend to cluster below 20% and above 50%, while A581G prevalence estimates lack an obvious break point.

The prevalence of A581G is generally lower, with many studies having 0% prevalence and only two of 152 studies having more than 50% prevalence. Choosing a 10% threshold of A581G for implementing IPTi-SP would be problematic in that eight studies had measured prevalences between 9.9% and 10.1%. Choosing a prevalence threshold of 15% would make it easier for policy makers to segregate sites where IPTi should or should not be used, based on available recent data.

With additional analysis it might be possible to select thresholds based not only on clustering of prevalence estimates, but also geographical clustering. The intent would be to avoid having geographically adjacent areas with prevalence estimates just above and just below a given threshold. Having different IPTi policies in areas that are both geographically close and with

similar malaria epidemiology could be confusing to policy makers. Selecting thresholds that would group countries or regions in a logical, understandable fashion could make recommendations easier to understand and follow. For example, choosing a threshold that results in IPTi being recommended in most of francophone West Africa but not in anglophone East Africa would be more palatable than one that results in different policies being recommended in coastal and western Kenya, or in northern and southern Tanzania.

These potential new approaches to setting guidelines for chemoprevention when data on resistance and efficacy are limited could be assessed in both field and modelling studies to gauge their utility and feasibility.

In summary, the evidence supporting a recommendation that IPTi-SP not be deployed where prevalence of *dhps* K540E exceeds 50% was thin when an expert group identified this threshold based essentially on just two trials ten years ago, and little new evidence is available to validate this threshold, or to set new criteria to guide IPTi policy (e.g., a prevalence threshold for *dhfr* I164L, *dhps* A581G, and/or *dhps* A613S/T). Efficacy studies of potential new IPTi drug regimens should include assessments of efficacy and duration of protection in relation to resistance markers. Until more evidence is available on the relationship between SP resistance and IPTi-SP efficacy, an alternative approach would be to select thresholds for implementing IPTi based in part on natural clustering of prevalence data in recent studies.

SEASONAL MALARIA CHEMOPREVENTION AND RESISTANCE

In 2012 WHO recommended another chemoprevention strategy, seasonal malaria chemoprevention (SMC, formerly called Intermittent Preventive Treatment in Children or IPTc). SMC with SP and amodiaquine (SP-AQ) is recommended for children aged less than five years in regions of the West African Sahel with intense seasonal malaria transmission. As recommended by WHO, SMC consists of a complete treatment course of SP-AQ administered to children aged 3-59 months at monthly intervals, beginning at the start of the transmission season, up to a maximum of four doses during the malaria transmission season. The relatively lower levels of antifolate resistance in West Africa, and the addition of amodiaquine to the regimen, gave rise to optimism that SMC might be less threatened by resistance than IPTp was in East Africa.

IMPACT OF SMC ON RESISTANCE

In a 2008 trial in Burkina Faso, after three monthly rounds of SMC with SP-AQ the prevalence of infections with *dhfr*/*dhps* quadruple mutants (triple *dhfr* and *dhps* A437G mutants) was comparable in the treatment and placebo arms, with an overall increase over baseline prevalence in both groups [101]. In contrast, a contemporaneous trial in Mali appeared to show SMC selection of low- and mid-level antifolate resistance markers. While the *dhfr/dhps*

quintuple mutant (quadruple plus *dhps* K540E) was absent, the prevalence of quadruple mutants was significantly higher in the SP-AQ group than in the placebo group, and prevalence increased from baseline in the SMC group but not in the placebo group [102]. In a trial of SMC with SP plus artesunate in Senegal, the post-intervention prevalence of quadruple mutants was also significantly higher in the intervention arm than the placebo arm, again with an increase in both groups from baseline [103]. Prevalence of resistance markers continued to rise in both groups, and no difference between the intervention and placebo arms was detected after the second year of follow up, possibly as a result of increased SP use in the general population following a change in national first-line treatment policy to SP-AQ [103]. A subsequent comparison of SMC with SP-AQ and dihydroartemisinin-piperaquine in Burkina Faso similarly found evidence of modest selection *dhfr* S108N and C59R and *pfcrt* K76T in the SP-AQ arm of the trial [104].

As noted above, the impact of SMC on resistance is related not only to the proportion of infections that carry resistant parasites, but on the proportion of people who become infected. Modelling studies may be useful in assessing whether SMC's efficacy at reducing the prevalence of infection mitigates the risks posed by its effect of increasing the prevalence of resistance (defined here as the proportion of infections carrying resistant parasites).

Based on these early studies, it appeared that at least short-term selection of resistance markers may follow SMC implementation. Surveys of health districts that had or had not implemented SMC or IPTi in Senegal found significant selection of the *dhfr* triple mutant, but not for *dhps* mutations [105]. An ecological survey in Ghana that included areas where SMC had and had not been implemented reported similar increases in *dhfr/dhps* quintuple mutants, but this study did not test for the higher-level resistance mutations *dhfr* I164L and *dhps* A581G and A613S/T [106]. Another prospective SMC trial done in Mali in 2014 found that prevalence of the quintuple mutant remained similar and below 5% before and after IPTp-SP was implemented in two districts (this trial also did not assess higher-level SP resistance mutations) [107]. The Mali trial also reported no increases in the prevalence of *pfcrt* or *pfmdr1* polymorphisms associated with diminished AQ susceptibility.

A large observational study of the scale-up of SMC with SP-AQ in seven Central and West African countries measured the prevalence of resistance markers in 2016 and 2018 among 10-30 year-olds to assess the overall trends in resistance markers in communities where under-fives were given SMC [108]. The *dhfr* triple mutant was already prevalent at more than 90% across the sites, and increased yet more; and *dhps* mutations were initially lower and increased proportionally more, with up to 4-fold increases in prevalence over time. However, AQ resistance markers in *pfmdr1* and *pfcrt* decreased modestly during the scale-up period. These results are consistent with SMC with SP-AQ selecting for antifolate resistance but not 4-aminoquinoline resistance. However, other plausible reasons for these changes in marker prevalence include reduced CQ use in the region resulting in reduced selection pressure for resistance to 4-aminoquinolines, and other sources of selection pressure favouring antifolate resistance by the use of SP or other antifolates such as trimethoprim-sulfamethoxazole (co-trimoxazole) for antibacterial treatment or chemoprevention. Notably, the fold-increases in the

prevalence of *dhps* markers as well as various *dhfr-dhps* haplotypes associated with intermediate to high antifolate resistance were all lower (in many cases, 2-3-fold lower) in the under-fives than in 10-30 year-olds, despite the younger group being subjected to direct selection for antifolates under SMC. This marked age difference further clouds the interpretation that SMC was solely responsible for the rise in antifolate markers over the study period.

In summary, while some prospective trials and ecological studies of SMC with SP-AQ in West Africa have reported increased prevalence of the *dhfr/dhps* quadruple and quintuple mutants, other studies found no such evidence of selection. No evidence has been reported of SMC being followed by increased prevalence of the higher-level resistance mutations that most severely impair SP efficacy, nor does SMC appear to select for parasites carrying mutations associated with diminished AQ susceptibility.

IMPACT OF RESISTANCE ON SMC EFFICACY

While the *dhfr/dhps* quadruple mutant was already prevalent in West Africa as SMC was being tested and implemented, *dhps* K540E was still rare in the region [109]. SMC efficacy using SP combined with either amodiaquine or artesunate ranged from 70-87% at sites in Senegal, Mali, and Burkina Faso with baseline prevalences of 32-58% of the *dhfr* triple mutant and 22-29% for *dhps* A437G [101-103], suggesting that SMC benefit persists in the face of moderate levels of the quadruple mutant. A meta-analysis of SMC trials was conducted [110], but because baseline prevalence of resistance markers prior to implementation was generally not reported, marker prevalence could not be associated with efficacy, nor could selection be measured. Putative molecular markers for amodiaquine resistance, including mutations in *pfcrt* and *pfmdr1*, have generally not proven reliable predictors of SMC efficacy. For example, a clinical trial of SP, AQ and SP-AQ for treatment of clinical malaria in Cameroon found that prevalence of *pfcrt* and *pfmdr1* mutations thought to be associated with reduced susceptibility to AQ was higher at sites where AQ and SP-AQ treatment failures were lower [111].

In summary, unless and until high-level resistance mutations become more prevalent in areas where SMC is used, it will not be possible to draw conclusions about the impact of resistance on SMC efficacy.

MASS DRUG ADMINISTRATION AND RESISTANCE

Mass drug administration (MDA) refers to mass drug treatment of an entire population, irrespective of the presence of symptoms and without individual testing for malaria [38, 112]. During the last century MDA schemes often led to declines in malaria rates, but gains were usually temporary [113]. Exceptions to this pattern include instances of MDA being deployed in combination with aggressive vector control and rigorous surveillance in low-transmission areas,

and in geographically conscribed areas such as islands [38, 114]. MDA was blamed for driving drug resistance, most notably after introduction of antimalarial drugs in table salt in the 1950s [115], and WHO stopped recommending it. However, in response to the renewed call for malaria eradication and the emergence of artemisinin resistance, MDA has been reexamined [112, 114, 116]. Trials and implementation projects have been undertaken both in low burden settings slated for elimination such as the Greater Mekong Subregion [117] as well as in Africa [118]. These more recent experiences with MDA have provided the opportunity to gain a better understanding of the impact of MDA on the emergence and spread of resistance, and the impact of drug resistance on MDA efficacy.

IMPACT OF MDA ON RESISTANCE

In MDA, every consenting member of a malaria-exposed population is administered curative doses of antimalarial drugs, irrespective of infection status. This is often repeated at intervals, e.g., two monthly cycles repeated annually for two years. It would seem obvious that such a massive drug exposure would exert powerful selection pressure favouring resistant parasites—and, indeed, MDA has been indicted for hastening resistance throughout the history of malaria control. Malaria icon Walther Werndsorfer (who literally wrote the book on malaria) asserted 40 years ago that "Mass drug administration in its various forms, and insufficient treatment are obviously the most important motors of selection." [119] While this is an oft-repeated sentiment, the evidence is less clear cut.

Theoretical arguments have been made that MDA prevents rather than fosters resistance, based on calculating probabilities of emergence and spread of resistance in relation to parasite density [120]. This prediction appears to be supported by recent well-executed MDA schemes in low-transmission elimination zones with highly efficacious drugs that found no evidence of selection for drug resistant parasites. For example, mutations in *P. falciparum kelch13* associated with artemisinin resistance were already prevalent when MDA with dihydroartemisinin-piperaquine and low-dose primaquine was evaluated in eastern Myanmar, where a piperaquine resistance marker (multiple copies of the *P. falciparum* genes *plasmepsin2/3*, or *pfpm2/3*) was absent at baseline. There was no evidence of selection of resistance by MDA: after MDA, the piperaquine resistance marker was still absent, and *kelch13* mutations had decreased in prevalence from 86% to 57% [121].

A cluster-randomized trial of MDA with dihydroartemisinin-piperaquine included Southeast Asian sites with varying levels of resistance. MDA was randomly either initiated or delayed in 16 villages with about 500 residents each [122]. A highly resistant parasite lineage with both the *kelch13* artemisinin-resistance mutation C580Y and the piperaquine resistance marker, multiple copies of *pfpm2/3*, was absent at baseline in Myanmar and Lao PDR, but present in Vietnam and Cambodia at prevalences of 4% and 63% of genotyped infections, respectively. Only 14 of the 258 individuals who were infected with *P. falciparum* at baseline and completed three rounds of MDA were persistently infected a month later, 13 in Vietnam and one in Cambodia. Only the single persistent infection in Cambodia carried the highly resistant haplotype.

In Mozambique, where malaria transmission and parasite densities are much higher than in Southeast Asia, the prevalence of resistance markers was compared before and after two annual cycles of two monthly rounds of MDA with dihydroartemisinin-piperaquine [123]. No evidence of selection was found for markers of resistance to artemisinins (*k13*) or piperaquine (*pfpm2* and *pfcrt*).

Modelling studies have both supported and undermined the notion that MDA is a potent force driving resistance. One study concluded that the "windows of selection" for drugs used in chemoprevention were longer than estimated based on clinical data, leading the authors to assert that MDA and other chemoprevention strategies using full treatment regimens "will be far more potent drivers of resistance than previously thought" [124]. However, another modelling study that also incorporated pharmacodynamic properties as well as resistance mechanisms of MDA drugs came to different conclusions. This study found that while MDA using drugs to which parasites can become highly resistant with a single mutation, such as atovaquone, would result in high levels of resistance even after a single round, MDA with artemisinin-based combination therapies (ACTs) would retain efficacy because of the lower grade of resistance generated by more complex and therefore less frequently occurring genetic mechanisms [125]. The latter model appears to align better with the results of recent MDA experiences with ACTs in both low and high malaria transmission settings.

In summary, there is no evidence that MDA in the modern era using highly effective ACTs results in increased drug resistance, although studies addressing this topic are limited.

IMPACT OF RESISTANCE ON MDA EFFICACY

In early experiences with MDA using sub-curative drug regimens, MDA quickly selected for resistance, which in turn compromised efficacy [29, 38]. However, in more recent MDA schemes in Southeast Asia, the high efficacy of ACTs has been preserved, even in areas with more than 60% prevalence of artemisinin resistance, and efficacy has been stable across sites with low and high rates of resistance to both artemisinins and ACT partner drugs [117, 122]. MDA with ACTs has been less efficacious in Africa, not because of drug resistance but because of epidemiological and parasitological factors that differ from low-transmission areas slated for elimination. For example, MDA has either failed or been followed by rebounding malaria incidence when it has been attempted in limited areas adjacent to non-MDA areas that serve as a source for rapid re-introduction of malaria to the populations subjected to MDA [38, 114]. Even in lower transmission settings, MDA's effects are short-lived if it is applied with less-than-ideal rigor in the absence of effective vector control methods [126]. The near-complete absence of clinically relevant levels of resistance to ACT drugs in Africa precludes any assessment of the impact of resistance on MDA efficacy there.

In summary, in the past drug resistance diminished the efficacy of MDA when drugs were used in sub-curative formulations and dosing regimens (e.g., single drugs used at doses that fail to clear infection). However, in the 21st century, MDA with highly effective combination drugs has proven efficacious even in the face of high levels of resistance. Nevertheless, policy makers continue to express worries about MDA promoting resistance [127].

OTHER POTENTIAL USES OF CHEMOPREVENTION AND RESISTANCE

While other potential uses of chemoprevention for malaria control and elimination are not presently recommended by WHO, evidence from evaluations of new chemoprevention strategies can shed light on the relationships between drug resistance and the WHO-recommended strategies reviewed here. For example, several studies have explored the benefits of preventive drug treatment for malaria among school-age children in East and Southern Africa, where malaria transmission tends to be more perennial than in the West African countries where SMC has been tested and implemented.

A recent systematic review and meta-analysis of preventive treatment among school-age children in Africa that pooled data from 13 studies [128] noted that "…the only study to measure directly the effect of school-based treatment on drug resistance showed that recent treatment with dihydroartemisinin–piperaquine was associated with higher prevalence of molecular markers of drug resistance." The study in question, from Uganda [129], measured the proportion of *P. falciparum* infections carrying only the "pure mutant" forms of known resistance markers in relation to the time of the most recent dose of dihydroartemisinin–piperaquine given as monthly chemoprevention to school-age children. This analysis thus combined mixed infections (containing both resistant mutant parasites and sensitive wild-type parasites) with pure wild-type infections in the reference (ostensibly non-resistant) group. This analytical approach limited the "resistant" outcome to those infections in which only "pure mutant" forms were detected. For the purpose of assessing selection of resistance and risk of treatment failure, arguably the more appropriate analysis would have been to compare the proportion of infections containing any resistant parasites, whether or not wild-type parasites were also present in the infection. This is because it is the presence of resistant parasites (irrespective of the presence or absence of sensitive parasites) that signals the risk of treatment failure—the additional presence of wild-type sensitive parasites should have no effect on whether or not the resistant parasites are cleared by drug treatment.

As shown in **Figure 3**, when the data in the Uganda paper [129] are re-analysed using this approach of assessing the presence or absence of resistant parasite genotypes (irrespective of presence of wild-type genotypes), there is no suggestion of increased prevalence of any resistance markers in infections occurring further in time from dihydroartemisinin-piperaquine administration. In fact, one of the resistance markers, *pfmdr1* N86Y, appears to be significantly less prevalent in infections that occurred sooner after drug treatment, consistent with selection favouring wild-type parasites. The other marker that had appeared in the original analysis to be

selected by chemoprevention in this setting, *pfcrt* K76T, was prevalent at near-fixation levels in all infections, irrespective of temporal proximity to drug treatment, as shown in **Figure 3**.

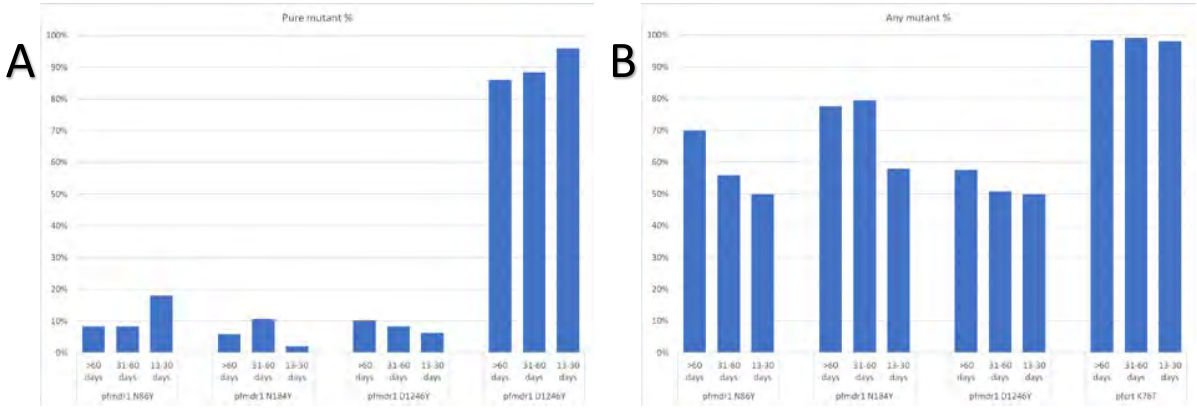

Figure 3. Re-analysis of data purportedly showing selection of resistance markers by monthly seasonal malaria chemoprevention in school-age Ugandan children. For each resistance marker, the three bars represent proportion of infections containing mutant genotypes at increasingly distant times from last drug treatment with Dihydroartemisinin-piperaquine. **Panel A** shows the original analysis, depicted here in graph form, and showing apparent selection of "pure mutant" genotypes of *pfmdr1* N86Y and *pfcrt* K76T based on their increasing in prevalence after drug treatment. **Panel B** depicts a re-analysis of the same data showing no evidence of positive selection for mutant genotypes when all infections containing the mutation in question are considered to have resistant parasites. Data from Nankabirwa *et al.* Antimicrob Agents Chemother 2016, 60:5649-54.

In summary, the evidence that malaria chemoprevention in school-age children increases drug resistance does not stand up to careful scrutiny. This example illustrates the importance of rigorous study design and analysis in assessing the relationships between drug resistance and malaria chemoprevention strategies and lends further support to the notion that selection of clinically relevant forms of resistance by chemoprevention is not inevitable.

POTENTIAL APPROACHES TO MANAGE AND MITIGATE THE RISK OF RESISTANCE

The history of antimicrobial use is rife with examples of drugs being used in inappropriate ways that hasten the emergence and spread of resistance, such as overprescribing antibacterial drugs for viral illnesses, or adding antibiotics to livestock feed to enhance animal growth. In the case of malaria, it is hard to dispute the inadvisability of practices like adding antimalarial drugs to table salt [115] and the unfettered sale and use of drugs of questionable quality in the private sector [130]. Concerns about resistance can trigger policymakers to resist new or expanded uses of valuable drugs. While this protective urge is understandable, and can lead to useful initiatives such as expanding diagnostic capacity to reduce empiric malaria treatment for all fever cases, it comes with a risk of restricting access to beneficial drugs that could be

deployed in ways that do not appreciably shorten their useful lifespans. Understanding of resistance mechanisms may offer potential approaches for finding the optimal balance between treating and preventing malaria and preserving drug efficacy.

CAN COUNTERVAILING RESISTANCE MECHANISMS BE EXPLOITED TO PRESERVE EFFICACY?

WHO and others have recommended that the risk of chemoprevention hastening the demise of treatment drugs should be mitigated by using different drugs for chemoprevention and first-line treatment. IPTp, IPTi and SMC programmes generally follow this recommendation, as SP and SP-AQ are not recommended first-line treatments in countries where these strategies are deployed. Recent MDA programs have been less compliant with this advice, in that MDA with ACTs has been used in areas where ACTs are also the first-line malaria treatment. This means that the same class of drug—the artemisinins—are subjected to potential selection pressure for resistance in both treatment and chemoprevention regimens, in the same areas if not in the same populations. ACTs are likely to remain the first choice for MDA until other equally highly efficacious and well-tolerated regimens are available.

In the meantime, one approach for reducing the potential for MDA to select forms of resistance that impair ACT efficacy is to use different regimens for MDA and first-line treatment, with ACT partner drugs that have antagonistic resistance mechanisms. For example, resistance to mefloquine has been associated with increased copy number of the *pfmdr1* gene [131-133] and piperaquine resistance is associated with increased copy number of the *pfpm2* and *pfpm3* genes [134, 135]. Parasites with increased copy numbers of *pfpm2/3* signalling piperaquine resistance usually occur together with the wild-type single-copy *pfmdr1* associated with mefloquine sensitivity. These antagonistic resistance mechanisms could potentially be exploited to preserve efficacy by deploying ACTs with countervailing resistance selection pressure, e.g., using dihydroartemisinin-piperaquine for MDA and artesunate-mefloquine or artemether-lumefantrine for treatment. A recent trial of ITPp with mefloquine reported apparent selection against the *pfmdr1* N86Y mutation that is associated with chloroquine resistance, raising the possibility that IPTp-mefloquine could drive selection of mefloquine-resistant but chloroquine-sensitive parasites [136].

The two antimalarial drugs for which counter-resistance is best documented, chloroquine and mefloquine, have recovered efficacy after being withdrawn in some areas and are being evaluated for reintroduction into use. When chloroquine was withdrawn and replaced with SP as the first-line drug in Malawi, chloroquine resistance disappeared over a period of about eight years [137]. Chloroquine was shown to be highly efficacious once again for malaria treatment [138], and weekly and intermittent chloroquine chemoprevention had similar efficacy to IPTp-SP in pregnant women [139]. Chloroquine resistance also declined dramatically after chloroquine was no longer recommended in Tanzania [140] and Zambia [141]. Similarly, after six years as first-line treatment in Thailand, mefloquine efficacy declined from 98% to up to 50% [142]. When dihydroartemisinin-piperaquine was used in the region, mefloquine efficacy recovered, and it is now being studied in the region as a component of a triple ACT [143].

Whether recovery of efficacy results from counter-resistance favouring drugs lost to resistance, or simply resurgence of sensitive parasites in the absence of drug pressure [144], rotating or alternating antimalarial drugs could be a useful approach for managing resistance. Alternatively, drugs with countervailing resistance profiles could be deployed in parallel: A strategy of "multiple first line therapies" has been proposed to preserve efficacy of treatment drugs [145], and the rationale for "triple therapy" ACTs includes the possibility of using drugs with antagonistic resistance profiles [143, 146].

Triple therapy in the form of dihydroartemisinin combined with piperaquine and mefloquine has been proposed as a way to protect ACT partner drug efficacy [147] and is being evaluated for malaria treatment in western Cambodia [143]. Where ACT efficacy is severely compromised triple drug therapy offers a valuable option for malaria treatment, but the added expense and safety considerations make triple therapy less viable for chemoprevention strategies. A recent systematic review of mefloquine for preventing malaria in pregnancy found that while it had superior efficacy to ITPp-SP, high rates of mefloquine-related adverse events limit its potential effectiveness [148]. Other proposed approaches for mitigating or overcoming the impact of resistance on chemoprevention include using antibacterial drugs that have modest antimalarial efficacy and are thought to be refractory to resistance, such as azithromycin [149, 150], doxycycline [151], or trimethoprim-sulfamethoxazole [152]; increasing the dosage or changing the dosing interval to protect against resistant parasites [153]; and adopting screen-and-treat instead of intermittent treatment [59, 72, 154-156]. None of these approaches has gained acceptance as a viable alternative to IPTp-SP.

Another potential approach for deterring resistance is matching pharmacokinetic properties of drugs used in combination, so that longer-acting partner drugs are not left "unprotected" by persisting at levels that select for resistance after the shorter-acting partner drug has been eliminated [157-159]. Matching half-lives and elimination curves is an attractive approach that should ideally be incorporated into the design of future antimalarial drug combinations. In the meantime, with the limited number of effective drugs currently available, most drug combinations in use now, and all ACTs, include partner drugs with grossly mis-matched pharmacokinetic profiles. Compared to ACTs, which all pair longer-acting partners with extremely rapidly cleared artemisinins, SP and SP-AQ are reasonably well-matched combinations.

Each of these approaches to mitigating and deterring resistance comes with significant challenges. In discussions about multiple first line therapies[9], National Malaria Control Program managers explained to researchers and modellers that implementing changes in first-line malaria treatment drugs is not simply a matter of issuing recommendations—doing so effectively requires major investment of resources, time, and effort in training health providers, educating the public, and establishing new procurement and distribution systems. With

[9] Conference, "Anti-malarial Drug Strategies: Getting the Most from Anti-malarial Drugs," Prestana Kruger Lodge, Kruger National Park, South Africa, March 30-April 3, 2008

mathematical models yielding divergent predictions about the benefits of multiple first-line therapies [160], policy makers remained understandably sceptical about this approach.

Proposed chemoprevention strategies that rely on drugs with adverse effects that are tolerable when treating ill patients (e.g., doxycycline, mefloquine) may not be acceptable to the healthy people who are the target population for chemoprevention strategies. Increasing drug dosages to overcome resistance likewise increases safety concerns, especially for use in infants, children, and pregnant women. Screen-and-treat strategies are appealingly efficient, in that they avoid treating uninfected individuals, but they also miss the large reservoir of sub-patent infections and miss out on the post-treatment prophylaxis benefit for people infected shortly after treatment that accounts for much of the benefit of IPT and SMC.

In summary, standardized protocols for measuring and monitoring chemoprevention efficacy are needed. With imperfect evidence, practical considerations such as known prevalence patterns can help guide recommendations on when and where to deploy chemoprevention strategies. Using different drugs for chemoprevention and treatment and combining drugs with countervailing resistance mechanisms may help to preserve efficacy. The best approach for mitigating and managing drug resistance to protect the efficacy of chemoprevention strategies is to ensure that there is a pipeline of safe and effective new malaria drugs, ideally with diverse mechanisms of action and resistance, to replace those lost to resistance.

FINAL PERSPECTIVES

The evidence reviewed here about the relationships between drug resistance and malaria chemoprevention strategies comes from a patchwork of studies of diverse designs and varying quality that sometimes yield conflicting results. Studying the relationships between resistance and efficacy is only possible where there is both a high enough prevalence of resistance and high enough level of efficacy to measure associations with adequate statistical power. The heterogeneous settings, populations, and malaria epidemiologies where chemoprevention strategies are tested and used limit the generalizability of individual studies.

Meta-analyses of pooled data have been helpful in guiding policy recommendations, but even large meta-analyses are limited by the small numbers of well-designed studies in which data on resistance were directly collected. This can mean that seemingly robust analyses that draw conclusions based on pooled data from dozens of studies may in fact base those conclusions on just a handful of studies. Most of the meta-analyses reviewed here also pooled molecular marker data from separate surveys that were done as close as possible in time and space to chemoprevention trials. Conclusions thus rely on the suspect assumption that the prevalence of molecular markers is stable across time, space, and populations.

These limitations in the quality and comparability of the available data mean that it is much easier to draw conclusions about what is not known than to develop clear evidence-based

guidance based on what we do know. Ultimately, health policy makers must make decisions in the face of substantial uncertainty. For example, WHO has recommended specific molecular marker prevalence thresholds above which certain chemoprevention strategies should not be implemented. The available evidence may support only wide ranges—if the data tell us that a given strategy is likely to retain efficacy if the prevalence of a given marker is somewhere between 10-50%, do we recommend a threshold of 10%, or 50%, or something in between? Recommendations may need to be tempered to offer broader guidelines than precise prevalence thresholds for resistance markers. For example, guidelines may include statements along the lines of: "IPTx has been shown to be efficacious in settings with a prevalence of [resistance marker] up to XX% but not in a setting with a [resistance marker] of YY%. The relationship between efficacy and mutation frequencies between XX% and YY% remains unknown. Resistance may not have been the only factor influencing efficacy in these settings."

When selecting thresholds for recommending where and when chemoprevention strategies should be used, practical factors unrelated to evidence about resistance and efficacy should also be considered, such as whether or not current or future treatment drugs share resistance mechanisms with chemoprevention drugs, or whether a given threshold might result in confusing situations such as different recommendations in adjacent areas with similar malaria epidemiologies that happen to have resistance prevalences just above and below the threshold.

These limitations can be mitigated to some extent by standardizing study designs and coordinating multi-centre trials and pooled analyses, as has been done by consortia that have formed to test and implement some chemoprevention strategies. WHO recommendations on research priorities can also guide researchers to conduct studies that will yield data useful to policy makers, to the extent that researchers are made aware of and follow such recommendations. For example, a standardized protocol for "Preventive Efficacy Studies (PES)" akin to TES studies is currently being developed by GMP.

It is somewhat encouraging that malaria chemoprevention does not inevitably lead to meaningful increases in resistance, and even high rates of resistance do not necessarily impair chemoprevention efficacy. At the same time, it can reasonably be anticipated that, over time, as drugs are widely used, resistance will generally increase, and sooner or later efficacy will be lost. Decisions about whether, where and when chemoprevention strategies should be deployed will continue to need to be made on the basis of imperfect evidence. It is hoped that this assessment of what is known about the relationships between resistance and chemoprevention will be useful as WHO evaluates and updates its chemoprevention recommendations.

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WHO Guidelines for malaria

Malaria Policy Advisory Group meeting

5 October 2021

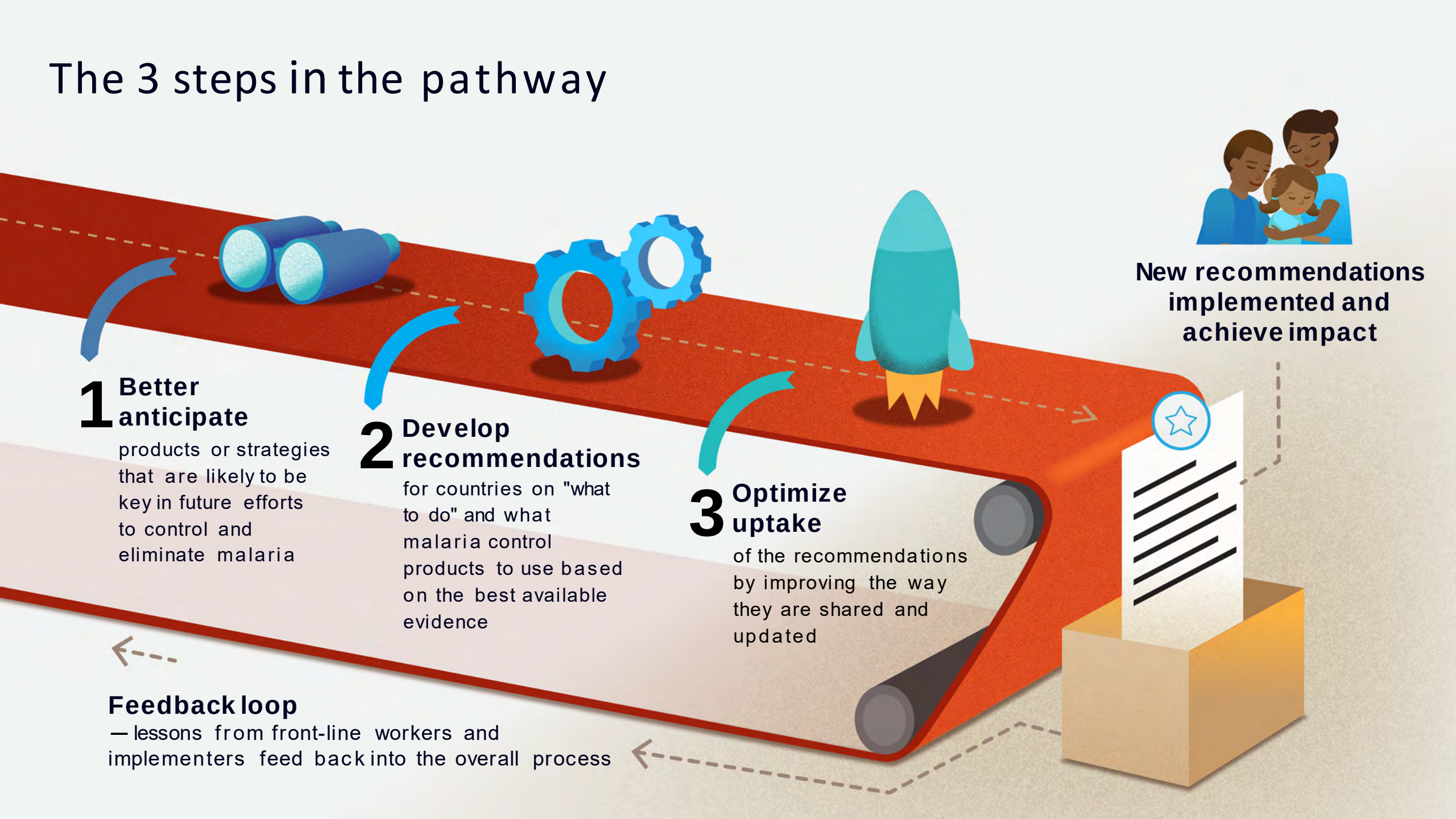
Dr Pedro Alonso, Dr Jenny Stevenson, Dr Kim Lindblade,

Dr David Schellenberg, Dr Peter Olumese, and Dr Jane Cunningham

Global **Malaria** Programme

World Health Organization

The 3 steps in the pathway



1 Better anticipate

products or strategies that are likely to be key in future efforts to control and eliminate malaria

2 Develop recommendations

for countries on "what to do" and what malaria control products to use based on the best available evidence

3 Optimize uptake

of the recommendations by improving the way they are shared and updated

New recommendations implemented and achieve impact

Feedback loop

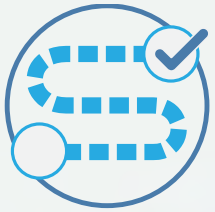
— lessons from front-line workers and implementers feed back into the overall process

2 Develop recommendations

WHO's evidence-informed recommendations on malaria guide national ministries of health as they develop polices and strategic plans to combat the disease; they support decisions around "what to do".

WHO also develops implementation guidance - such as operational and field manuals - to advise countries on "how to" deliver the recommended tools and strategies.

Step 2 in the pathway involves:

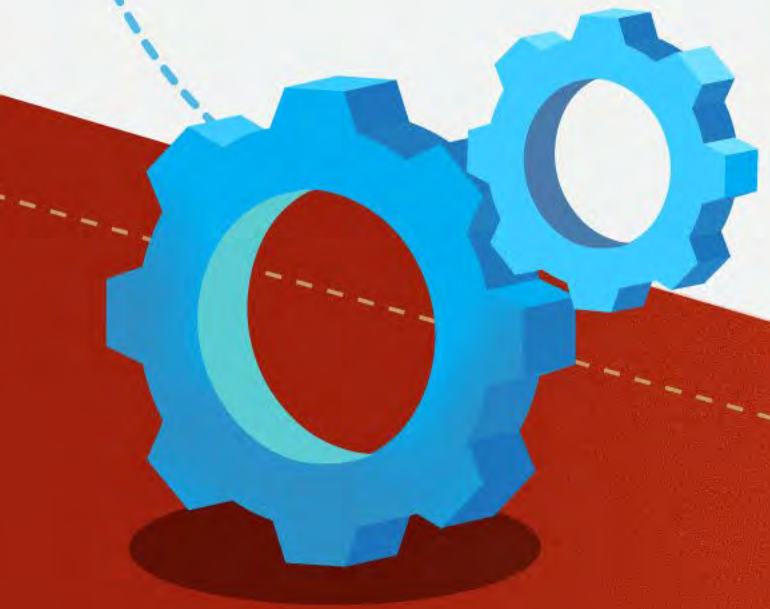


Developing recommendations for new tools and strategies through WHO's transparent, predictable and rigorous guideline development process

Ensuring that any recommendation around the use of a specific product is developed in parallel with its prequalification assessment

The WHO prequalification process ensures that diagnostics, medicines and other disease control products meet global standards of quality, safety and efficacy.

Issuing WHO recommendations and their related prequalification listings at the same time



Develop recommendations

- WHO Guidelines for Malaria
  - 4 Guidelines Development Groups established – Vector control, Elimination, Chemoprevention & Treatment
  - 1 Joint Guideline Development process - Vaccine
  - 1 Planning proposal in development – Diagnosis
- Published in February 2021; first update July 2021
- French version published; Spanish and Arabic in development
- Mobile app available for download (WMR, Threats Map and Guidelines)

Continued innovations

- Living Guidelines – a combination of continuous literature surveillance, rapid updating of prioritized systematic reviews and virtual consultations with GDGs
- Dissemination Strategy – to improve the way that WHO's recommendations on malaria are packaged and shared with end users to optimize uptake by national programmes
- Sub-national tailoring of malaria interventions: an operational manual

Vector Control

Key Questions for Vector Control Guidelines (approved 2020)

- Background information on ‘how nets work’: Personal protection and **community effects of ITNs**
- **Housing modifications**
- **Larval habitat manipulation and modification**
- Vector control interventions in **humanitarian emergencies**
- **Pyrethroid-PBO nets**
- **Co-deploying IRS and ITNs**
- **Topical repellents**
- **Residual treatments (including indoor, and outdoor)**
- **Cost and cost-effectiveness** of vector control and **resource considerations**

Updating the recommendations for vector control

1st set: GDG meeting Nov 2020, updates published July 2021

- Background information on 'how nets work': Personal protection and community effects of ITNs
- Edited section on insecticide resistance monitoring and interpretation
- **Housing modifications -new**

House screening (2021)

WHO conditionally recommends the use of untreated screening of residential houses for the prevention and control of malaria in children and adults living in areas with ongoing malaria transmission.

Conditional recommendation, low to moderate-certainty evidence

https://www.cochranelibrary.com/cdsr/doi/10.1002/14651858.CD013398.pub3/full

- **Larval habitat manipulation and modification (LHMM) – insufficient evidence**

Larval habitat modification and/or larval habitat manipulation (2021)

No recommendation can be made because the evidence on the effectiveness of a specific larval habitat modification and/or larval habitat manipulation intervention for the prevention and control of malaria was deemed to be insufficient.

No recommendation, very low-certainty evidence

- **Resources listed**

Updating the recommendations for vector control

2nd set: GDG meeting June 2021, planned for publication Q1 2022

- Pyrethroid-PBO nets - update
  - Two trials now complete, systematic review updated and published
  - Studies conducted in 'high' resistance areas
  - Considerations of cost
- Co-deploying IRS and ITNs -update
  - Systematic review updated with inclusion of other insecticides, other nets
  - Unclear if addition of IRS is filling a coverage gap
  - Considerations of cost
- Vector control interventions in complex emergencies – new
  - New systematic review to include ITNs, IRS, ITC, repellents, ITPs, treated cattle
  - Limited evidence available except for ITNs and IRS
  - Considerations of logistical issues
- Cost and cost-effectiveness

Use of Pyrethroid-PBO nets published published May 2021
https://www.cochranelibrary.com/cdsr/doi/10.1002/14651858.CD012776.pub3/full

Cost effectiveness of malaria control interventions published May 2021
https://www.sciencedirect.com/science/article/pii/S1098301521001479

Updating the recommendations for vector control

3rd set: Systematic reviews ongoing or to start, PICOs to be developed. GDG meeting Q1 & Q3 2022

PICOs developed:

- Topical repellents – update to include studies that may estimate personal protection effects
- Residual treatments (including full indoor, selective indoor, and outdoor treatments) – update and new. To include new insecticides, new application methods

Potential new PICO

- New nets (2 trials completed Q2/3 2022)
  - Interceptor® G2 : alphacypermethrin & chlorfenapyr (BASF)
  - Royal Guard ®: alphacypermethrin & pyriproxyfen (DCT)

Timeline : 2nd set

2 – 4 Nov '20

GDG meeting: PICOs finalized with systematic review teams

Nov '20

VC humanitarian emergencies protocol review: methodologist and SR team

May '21

VC humanitarian emergencies review of results: methodologist and SR team

May – June '21

Systematic reviews concluded set 2

June '21

GDG meeting: PICOs for set 3 finalized , GRADE tables, formulation of recommendations for set 2

July - Sep'21

Full narrative drafting with GDG, approval by GDG

Oct – Nov '21

External review, feedback incorporated, guidelines edited and finalized

Dec '21- Jan '22

GRC internal approval

Feb '22

Publication of revised guidelines in MAGICapp

Systematic review commissioned March 2020: 14 months

Systematic reviews on **pyrethroid-PBO nets, combining IRS and ITNs, vector control in complex emergencies, cost-effectiveness**

Elimination

Key Questions for Malaria Elimination Guidelines

ACCELERATOR STRATEGIES

- Mass drug administration (MDA)
- Mass (screen) test and treat (MTaT)
- Mass relapse prevention

TARGETED STRATEGIES

- Targeted drug administration
- Targeted test and treat
- Targeted test and treat at points of entry (border screening)

REACTIVE STRATEGIES

- Reactive drug administration
- Reactive test and treat (reactive case detection)
- Reactive indoor residual spraying

Developing recommendations for elimination

1st set: GDG meeting 23-27 August 2021, publication Q1 2022

- Mass drug administration divided into 3 questions
  - MDA for Pf in very low to low transmission settings
  - MDA for Pf in moderate to high transmission settings
  - MDA for Pv
- Mass relapse prevention
- Background information on clustering of cases at household or neighbourhood level
- Reactive case detection, reactive drug administration and reactive IRS

2nd set: GDG meeting planned for Q4 2021, publication Q1 2022

- Mass test and treat
- Targeted drug administration
- Targeted test and treat
- Targeted test and treat at points of entry (border screening)

Elimination Timeline

Oct 20

GDG finalized PICOs

Nov 20 –Feb 21

Protocols for systematic reviews

Jan & Mar 21

Touchpoints

Inclusion/exclusion criteria for sets 1 and 2, and included/excluded studies for set 1

Jul 21

Systematic reviews concluded set 1

Set 1: 9 months

Aug 21

Review of GRADE tables and formulation of recommendations for set 1

Oct 21

Touchpoints

Included/excluded studies for set 2

Nov-Dec 21

Review of GRADE tables and formulation of recommendations for set 2

Set 2: 12 months

Q1 22

Publication of elimination recommendations

Chemoprevention

Abbreviated PICO questions for Chemoprevention GDG

1. Should children living in settings with **perennial malaria transmission** be given anti-malarial medicines as chemoprevention?

2. Should children living in settings with **seasonal malaria transmission** be given anti-malarial medicines as chemoprevention?

4. Is **mass drug administration** (MDA) a safe and effective approach to reduce the burden of malaria in moderate and high transmission settings?
   - During emergencies or periods of health service disruption, should people living in malaria-endemic settings be given anti-malarial medicines for chemoprevention?

5. Should women be given anti-malarial medicines as chemoprevention during **pregnancy**?
6. Should **school-age children** living in settings with malaria transmission be given anti-malarial medicines as chemoprevention to reduce disease burden?
7. Should children hospitalized with severe anaemia in malaria-endemic settings be given anti-malarial medicines as **chemoprevention post-discharge**?
8. In areas of moderate to high malaria transmission, should residents known to be at increased risk of clinical malaria, severe malaria, death, or other adverse effects of *P falciparum* infection, be given anti-malarial medicines as chemoprevention?

Chemoprevention GDG meetings

- November 2020 - PICO questions developed
- July 2021
  - Training: interpreting evidence profiles, the evidence to decision framework
  - Background papers: drug resistance & chemoprevention; malaria risk groups; ethical considerations
  - Chemoprevention in young children in perennial transmission settings, based on updated Cochrane review of IPTi
  - Considerations for generic chemoprevention recommendation and development of PICO for post-discharge chemoprevention
    - Prioritisation of outcomes & potential effect modifiers
- October 2021
  - Assess evidence for MDA for burden reduction & chemoprevention for children in seasonal transmission settings

Chemoprevention guideline timeline

GDG Meetings for **evidence review** and **formulation of recommendations**

Perennial use

Seasonal use & MDA for burden reduction

July 2021

Oct 2021

Systematic review on **IPTi**

Systematic reviews on **SMC & MDA** for burden reduction

Oct – Nov 2021

Full narrative drafting with GDG, approval by GDG

External review feedback incorporated, guidelines edited and finalized

Nov - Dec 2021

Dec – Jan 2021

GRC internal approval

Publication of revised guidelines as pdf and in MAGICapp

Q1 2022

Systematic reviews → GDG evidence review →

IPTp, school children +/- post discharge chemoprevention

Intermittent preventive treatment for malaria in infants (v 17 July 2021)

https://doi.org/10.1002/14651858.CD011525.pub3

Treatment

Treatment process updates and timeline

- Planning proposal approved by GRC *(December 2020)*

- 1st GDG Meeting: Finalization of PICO Questions *(4-5 May 2021)*
  - For uncomplicated Pf malaria, is AS-Pyr an effective and safe option for treatment?
  - For uncomplicated malaria during the first trimester of pregnancy, is any artemisinin -based combination therapy (ACT) as safe and efficacious as quinine-based therapies?
  - For radical cure of Pv/o malaria, can the currently recommended total dose be given safely and effectively over a shorter period than 14 days?

- Systematic reviews based on the PICO questions *(June 2021)*

Treatment process updates and timeline

- 2nd GDG meeting - formulation of recommendations *(11-12 Nov 2021)*
  - For uncomplicated Pf malaria, is AS-Pyr an effective and safe option for treatment?
  - For radical cure of Pv/o malaria, can the currently recommended total dose be given safely and effectively over a shorter period than 14 days?

- 3rd GDG meeting - formulation of recommendation *(Feb 2022)*
  - For uncomplicated malaria during the first trimester of pregnancy, is any artemisinin -based combination therapy (ACT) as safe and efficacious as quinine-based therapies?

- External Review of draft recommendations; finalization of recommendations and clearance through GRC *(December 2021- May 2022)*

Diagnosis

Diagnosis updates and timeline

- Planning proposal drafted for submission in October 2021
  - Scope limited to recommendations concerning use of near patient G6PD tests
- Cochrane systematic review of diagnostic test accuracy of near-patient G6PD tests in people undergoing treatment or prophylaxis with primaquine or tafenoquine or in people susceptible to malaria
  - Protocol published January 2021
  - Analysis October 2021
- 1st GDG Meeting: Finalization of PICO Questions – December 2021
- Reviews of contextual factors and exploring linked evidence approach (LEA) – Q1 2022

Cross-cutting Challenges

Challenges

- Certainty of evidence and when to use the ‘No recommendation’ option
- Lack of data
- Multiple interventions applied at the same time
- Shaping research space when more evidence is needed so that the recommendation is not a disincentive
- Consistency in wording
- Balance between being prescriptive and flexibility
- Learning curve