Minutes of the Expert Review Committee on K13 molecular marker of artemisinin resistance

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Summary and recommendations

Molecular markers of artemisinin resistance: scientific evidence and public health potential

Molecular markers have been thus far under-utilized to guide policy. It will be important to engage local malaria control program managers to guide the research agenda and include molecular epidemiology into surveillance efforts, with the aim to provide actionable information for artemisinin-based combination therapy (ACT) policies and malaria elimination strategies. Messages on how to use the results of molecular markers surveillance to control programs need to be simple, and need to distinguish between research priorities and public health recommendations. K13 mutations are associated with delayed parasite clearance in genome-wide association studies in South-East Asia, but potential secondary loci have also been implicated. There is evidence for both spread and independent emergence of K13 mutations, supporting the need for elimination in areas of artemisinin resistance as opposed to containment. More research is needed to validate the relationship between African K13 mutations and resistance phenotypes.

Definition of artemisinin resistance

The panel suggested adding to the definition of suspected resistance ≥ 5% prevalence of any one single K13 propeller mutation, or ≥ 10% prevalence of all K13 propeller mutations as emergence of a clonal population could represent emergence of resistance. If suspected resistance is observed through a survey with molecular data only, resistance should be confirmed by obtaining from the same parasite strain information on: the clinical phenotype (clinical study to estimate day 3 parasitemia, parasite clearance half-life), the K13 genotype and if possible validation of the K13 mutant as resistance marker with an in vitro assay (e.g. ring-stage assay (RSA) $_{0\text{-}3h}$ or transfection). If resistance is suspected by the observation of slow clearance in a clinical trial or therapeutic efficacy study, K13 marker analysis should be performed urgently, e.g. from filter paper blood spots. Validating resistance mutations is different than establishing whether there is suspected or confirmed resistance in a given geographic region. The criteria for calling a K13 propeller SNP "candidate" include: 1) a statistically significant association ($p < 0.05$) between a K13 mutation and clearance half-life > 5 hours or day 3 parasitemia via a chi-squared test or appropriate multivariable regression model on a sample of at least 20 clinical cases, or 2) > 1% survival using the $RSA_{0\text{-}3h}$ in at least 5 individual isolates with a given mutation or a statistically significant difference ($p < 0.05$) in the $RSA_{0\text{-}3h}$ assay between culture-adapted recombinant isogenic parasite lines, produced using transfection and gene editing techniques, which express a variant allele of K13 as compared to the wild-type allele. The criteria for calling a K13 propeller SNP "validated" are when bothrequirements 1) and 2)are met.

The artemisinin resistance phenotype

Parasite clearance half-life best defines the in vivo phenotype of artemisinin resistance. A clearance half-life cut-off of 5 hours is currently being used to define resistance in South-East Asia; however, this cut-off depends on the underlying proportions of sensitive and resistant parasite strains and the estimator used, and can thus not be considered as an absolute cut-off. The $RSA_{0\text{-}3h}$ is currently the reference assay to define the in vitro phenotype. Day 3 parasitemia is a useful phenotype for surveillance, whereas, parasite clearance half-life and the ring-stage assay would be more difficult to implement as part of surveillance but are important for confirmation of resistance and research.

The KARMA project

Preliminary results from the K13 Artemisinin Resistance Multicentre Rapid Assessment (KARMA) study were presented. This study aims to provide a worldwide mapping of the distribution of K13 alleles in blood samples collected after 2012 from symptomatic and asymptomatic individuals infected with *P. falciparum* and to build capacity at participating study sites to perform molecular monitoring of K13 using Sanger sequencing. The role of African K13 mutations in artemisinin resistance needs to be determined. K13 mutations have thus far not been detected in South America. In addition to the KARMA project, other groups are providing or will provide data on K13 genotypes throughout endemic countries. It will be important that these data become available in the public domain in a timely fashion, since it has direct consequences for malaria policies.

The genetic architecture of artemisinin resistant *P. falciparum*

The results of a genome-wide association study using samples from the Tracking Resistance to Artemisinins Collaboration (TRAC) were presented. These results suggest that, although K13 mutations are a prerequisite, artemisinin resistance is likely multigenic, and that background loci may serve as useful secondary markers. There is evidence of both spread and independent emergence of K13 mutations. The amount and type of mutations observed in K13 in Africa is what would be expected for a gene with this level of conservation, and therefore the presence of K13 mutant alleles alone is insufficient for suspecting the emergence of artemisinin resistance.

PCR sequencing and genotyping assays

K13 mutations are still being discovered (particularly in Africa) and PCR sequencing (rather than SNP-typing) is the preferred method for genotyping K13. For now, the propeller domain will be the focus for surveillance. Sequencing of day 0 samples is required, but sequencing of parasites from day 3 or day of failure (if present) may show selection for certain subpopulations of parasites. Pooled deep sequencing may be useful for screening large numbers of samples for discovery of new mutations; however, reads would need to be barcoded to link to individual patients in order to determine mutation prevalence. Once a longer list of known mutations is compiled, a SNP chip may be used to genotype known mutations. Whether sequencing is performed locally or outsourced depends on the amount of existing infrastructure. Outsourcing of sequencing may increase efficiency, but there is a need to balance efficiency with capacity building in malaria endemic countries.

Population transcriptomics of artemisinin resistance of *P. falciparum*

Preliminary results of transcriptomics work performed on parasites collected during the TRAC studies were presented. Artemisinin resistant parasites over-express proteins involved in the unfolded protein response and under-express proteins involved in DNA replication, leading to a deceleration of life cycle progression. It is hypothesized that K13 might be a negative regulator of the unfolded protein response with K13 mutations affecting regulatory function leading to over-expression of downstream targets.

Transfection studies and K13 mutations

Additional K13 is sufficient to cause resistance in vitro in transfection studies, as defined using the $RSA_{0\text{-}3h}$ assays. Secondary loci may play a role in augmenting the resistance phenotype, as evidenced by the finding that Cambodian isolates harboring K13 mutations have higher $RSA_{0\text{-}3h}$ survival values as compared to older parasite reference lines. Fitness studies examining the impact of K13 mutations are pending. More research is required to understand the role of secondary loci. Not all K13 mutations have the same impact, in that different mutations confer different levels of resistance in vitro and are predicted to have different evolutionary action. Evolutionary action scores provide one approach to prioritize which mutations to follow up on first in validation studies. With regard to the transfection work, it may be helpful to divide the labor between a few groups that coordinate which strains and

methods to use, if funding sources are willing to support this important line of research. The transfection data clearly document a central role for K13 mutations in mediating ring stage resistance, which has been shown to associate with the clinical resistance phenotype of delayed parasite clearance rates.

Partner drug resistance markers

Potential molecular markers for common partner drugs for artemisinins, including amodiaquine, mefloquine, lumefantrine, and piperaquine, were discussed. There are theoretical advantages to be gained by rotating use of ACTs containing partner drugs with resistance mechanisms that are opposing (e.g. artesunate-amodiaquine and artemether-lumefantrine) or independent (artesunate-mefloquine and dihydroartemisinin-piperaquine). Monitoring and interrogation of possible partner drug compromise should be included where possible.

RSA and K13 mutations

$RSA_{0\text{-}3h}$ can be used for in vitro validation of K13 resistance mutations. In order to validate a mutation, $RSA_{0\text{-}3h}$ values > 1% (or greater than +/- 2 standard deviations of the mean value for K13 wild type parasites from the same area) must be observed in at least five independent isolates. A small number of $RSA_{0\text{-}3h}$ reference laboratories are needed, including some close to field sites, using standard parasite controls for quality control.

Translational aspects

If the K13 criteria for suspected artemisinin resistance criteria are met, phenotypic studies should be initiated (day 3 parasitemia or clearance half-life). $RSA_{0\text{-}3h}$ should be performed if cryopreserved parasite samples are available as well as screening for partner drug resistance markers. If criteria for confirmed resistance are met, it will be necessary to conduct further therapeutic efficacy studies, additional molecular surveys, screening for partner drug resistance markers, and gene flow estimates.

Mapping molecular markers of drug resistance

Gene flow and population structure are useful to plan regional surveillance frameworks and containment efforts; their role in routine surveillance will need to be further defined. Parasite DNA should be collected as part of routine surveillance for K13 mutations and partner drug markers. More research is needed to validate estimates of gene flow/parasite migration as markers of the spread of resistance. Such estimates may help define at-risk populations, along with transmission level and amount of drug pressure.

Reference centre for K13

WHO is interested in the establishment of a reference centre for K13 genotyping and will further discuss with interested partners.. The reference centre will coordinate/standardize methods between laboratories by providing a reference genotyping protocol and by providing guidance regarding the pros and cons of different methodologies. The reference centre will rely on collaborative laboratories, which could serve as advisory centres for national malaria control programmes and WHO to analyze the results of molecular surveillance. A list of laboratories willing to provide quality assured K13 sequencing will be required. The data gathered through the collaborative laboratories and the reference centre should be shared in the public domain through an independent agency. Such data will include the K13 genotyping data, as well as available metadata (e.g. location, year, and whether the infection was symptomatic or asymptomatic).

Acknowledgments

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Introduction of guest speakers, declaration of interest, agenda and minutes of ERG on K13

All invited experts attended the meeting, except D. Kwiatkowski. The full list of participants is provided in Annex 1. All members of ERG participating in the meeting submitted their declaration of interest, which was assessed by the Drug Resistance and Containment Unit at GMP and by Legal at WHO. All the reported relevant interests were read to participants. The agenda is provided in Annex 2.

Molecular markers of artemisinin resistance: scientific evidence and public health potential

Presentation

The important question regarding molecular markers of antimalarial drug resistance is what they tell us about drug efficacy. In the past, mutations within *pfcrt,* particularly 76T, have been associated with chloroquine treatment failure, likewise, triple mutant *dhfr* and double mutant *dhps* are associated with sulfadoxine-pyrimethamine treatment failure in therapeutic efficacy studies. However, to date, existing molecular markers of antimalarial drug resistance have been underutilized in influencing policy, with two notable exceptions in Mali[1] and Tanzania[2].

A genome-wide association study (GWAS) of artemisinin resistance identified a region of *P. falciparum* chromosome 13[3,4]. Subsequently, mutations in a gene encoding a kelch propeller domain (K13) were observed to confer artemisinin resistant[5]. Multiple mutations in K13 were found to characterize the founder populations of artemisinin resistant *P. falciparum* previously identified in Cambodia[6]. A replication GWAS showed that mutations within the K13 gene are associated with delayed parasite clearance in Myanmar and Vietnam, as well as in Cambodia, and identified potential secondary/compensatory loci[7]. A larger GWAS, using 1063 samples from across South-East Asia and Africa, identified K13 mutation C580Y as the most strongly associated mutations to parasite clearance half-life, and revealed multiple mutations, describing a genetic background that accompanies and appear to underpin artemisinin resistant K13 mutations[8]. Similar results were reported by a large multi-centre study of artesunate efficacy, with the distribution of parasite clearance half-lives showing association with K13 propeller mutations in multiple South-East Asian countries[9]. In general, mutations downstream of amino acid position 350 (including BTB/POZ region) were associated with delayed parasite clearance after artesunate treatment, and those upstream of this position were not, and tended to be more prevalent in sites with no resistance (e.g. Africa). In Southern China, the 446I mutation within K13 is most prevalent and is associated with parasitemia on day 3 after treatment. In addition, there is considerable variation in the prevalence of K13 mutations at different study sites and even at the same study site at similar time periods. One recent study showed a low prevalence of

[1] Djimdé AA et al. (2004).J Infect; 190(4):853-5.
[2] Mugittu K et al. (2005). Malaria J; 4:55.
[3] Takala-Harrison S et al. (2013). Proc Natl Acad Sci U S A; 110(1):240-5.
[4] Cheeseman et al. (2012). Science;336(6077):79-82.
[5] Ariey F et al. (2014). Nature;505(7481):50-5.
[6] Miotto O et al. (2013). Nature Genet; 45(6):648-655.
[7] Takala-Harrison S et al. (2014). J Infect Dis; in press.
[8] Miotto O et al. (2014). Nature Genet; in press.
[9] Ashley EA et al. (2014). N Engl J Med;371(5):411-23.

different mutations throughout sub-Saharan Africa[10]. These mutations are often different than those in Asia, but there are many low prevalence K13 propeller mutations in African parasites. A recent study shows similar results, with multiple low prevalence K13 mutations in both contemporary samples as well as samples from a decade ago[11].

What do K13 mutations tell us about drug efficacy? Drug efficacy was high in the papers that first reported artemisinin resistance in western Cambodia[12,13]. Analyses of heritability show that about half of the variation in parasite clearance half-life is due to parasite genetics. Preliminary data show that patients with delayed parasite clearance have less immunity based on a protein array containing diverse variants of malaria surface proteins, suggesting that immunity may be an important confounder in studies of parasite clearance. The association between K13 and drug efficacy is also confounded by the presence of partner drugs.

How do molecular markers assist with elimination efforts? Looking at flanking SNPs in linkage disequilibrium with K13 ("paternity testing" the resistant parasites) can indicate whether K13 mutations are arising locally or spreading from other geographic regions ("popping" versus "jumping"). Two studies show evidence for both independent emergence, as well as spread, of the common resistance-associated K13 mutations[14,15]. There is presently no evidence of spread from western Cambodia to Myanmar. The most common resistance mutation in Cambodia, 580Y, did spread between Cambodia and southern Vietnam, but emerged independently in Myanmar. Preliminary gene flow analyses also show historical evidence of considerable amounts of past parasite migration from western Cambodia to Bangladesh, suggesting a risk of artemisinin resistance spreading along this route. Both understanding parasite migration patterns and origins of K13 mutations could aid containment and elimination efforts.

Discussion

The K13 story is more complex than that for other markers owing to several factors including, 1) the presence of dozens of mutations that are associated with delayed parasite clearance, 2) an unclear relationship between individual K13 mutations and delayed parasite clearance, 3) the role of partner drugs and immunity in the measurement of the in vivo phenotype, 4) the role of secondary and compensatory mutations, 5) both spread and independent emergence of K13 mutations, and 6) the relative contributions of artemisinin resistance and partner drug resistance to artemisinin-based combination therapy (ACT) efficacy. The utility of K13 as a marker for guiding ACT treatment policies and elimination strategies will depend on its ability to predict delayed parasite clearance. K13 mapping may be most important as an early warning of declining ACT efficacy, but we need to know how to interpret the data, particularly the relevance of K13 propeller mutations in Africa. Gene flow and population structure measurements have the potential to provide valuable information but the relevance of genomic information—which reflects an evolutionary time scale—to recent and current emergence and spread of drug resistance, needs to be demonstrated. It is important to engage local malaria control workers in planning, conducting and analyzing molecular surveillance. The messages to control programs need to be simplified and should involve cross border/cross-sectorial cooperation and local leadership.

Conclusions and recommendations

Molecular markers have been thus far under-utilized to guide policy. It will be important to engage local malaria control program managers to guide the research agenda and plan for surveillance,

[10] Taylor SM et al. (2014). J Infect Dis; in press.
[11] Kamau E et al. (2014). J Infect Dis; in press.
[12] Noedl H et al. (2008). N Engl J Med ;359(24):2619-20.
[13] Dondorp A et al. (2009). N Engl J Med; 361(5):455-67.
[14] Miotto O et al. (2014). Nature Gen; in press.
[15] Takala-Harrison S et al. (2014). J Infect Dis; in press.

so that molecular surveillance data will provide actionable information for ACT treatment policies and malaria elimination strategies. Messages to control programs need to be simple, and need to distinguish between research priorities and public health recommendations. K13 is associated with delayed parasite clearance in GWAS, but potential secondary loci have also been implicated. There is evidence for both spread and independent emergence of K13 mutations, supporting the need for elimination as opposed to containment. More research is needed to validate the relationship between African K13 mutations and resistance phenotypes.

Definition of artemisinin resistance

Presentation

Antimalarial drug resistance is the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject[16]. However, not all treatment failures are necessarily due to drug resistance. Therefore, other tools, such as molecular markers, in vitro drug efficacy measurements and measuring levels of antimalarial drug in the blood are also needed to confirm resistance. The current definition of suspected artemisinin resistance is: ≥ 5% of patients carrying K13 resistance-associated mutations, or ≥ 10% of patients with persistent parasitemia by microscopy on day 3 after treatment with an ACT or artesunate monotherapy, or ≥ 10% of patients with a parasite clearance half-life of ≥ 5 hours after treatment with an ACT or artesunate monotherapy. The current definition of confirmed artemisinin resistance is: ≥ 5% of patients carrying K13 resistance-associated mutations, all of whom have been found, after treatment with an ACT or artesunate monotherapy, to have either persistent parasitemia by microscopy on day 3, or a parasite clearance half-life of ≥ 5 hours. Confounding factors in these definitions include the presence of partner drugs, immunity, insufficient levels of drug in the blood, and non-validated K13 mutations.

Artemisinin resistance is defined as delayed parasite clearance, which represents a partial resistance. The majority of patients who have delayed parasite clearance following treatment with an ACT clear their infections (except in Cambodia). Consequences of delayed parasite clearance could include: 1) total artemisinin resistance leading to difficulty in treating severe malaria and/or 2) selection for partner drug resistance. It is not clear if artemisinin has precipitated the emergence of piperaquine resistance or that artemisinin resistance has helped to further select already existing piperaquine resistant parasites, which preceded the implementation of ACTs similarly to mefloquine. Further research is needed to evaluate the role of artemisinin resistance in the development of drug resistance to partner drugs outside Cambodia. WHO messages about spread of resistance to Africa are modest and include: 1) firewall approach is not effective due to independent emergence of K13 mutations in the Greater Mekong subregion (GMS), 2) patients with delayed parasite clearance are still able to clear their infections if the partner drug is efficacious, 3) focus on malaria elimination in the GMS and 4) prevention of partner drug resistance in other parts of the world.

Discussion

Extension of the treatment course with an artemisinin derivative improves drug efficacy, even in the face of delayed clearance, but there is concern that a marker of artemisinin resistance will driven be to fixation. Does artemisinin resistance select for partner drug resistance, or alternatively does failing partner drug use promote artemisinin resistance? Elimination efforts are currently focused on preventing multidrug resistance. Mefloquine was already failing before combination with artemisinins. The 5-10% cut-off for K13 mutations as part of the definition of suspected resistance may need revision as new information comes from ongoing surveillance activities.

[16] WHO (1973).

Conclusion and recommendations

The panel suggested adding to the definition of suspected resistance ≥ 5% prevalence of any one single K13 propeller mutation, or ≥ 10% prevalence of all K13 propeller mutations as emergence of a clonal population could represent emergence of resistance*.

If suspected resistance is observed through a survey with molecular data only, resistance should be confirmed by obtaining from the same parasite strain information on: the clinical phenotype (clinical study to estimate day 3 parasitemia, parasite clearance half-life), the K13 genotype and if possible validation of the K13 mutant as resistance marker with an in vitro assay (e.g. ring-stage assay (RSA) $_{0\text{-}3h}$ or transfection). If resistance is suspected by the observation of slow clearance in a clinical trial or therapeutic efficacy study, K13 marker analysis should be performed urgently, e.g. from filter paper blood spots.

Validating resistance mutations is different than establishing whether there is suspected or confirmed resistance in a given geographic region. The criteria for calling a K13 propeller SNP "candidate" include: 1) a statistically significant association ($p < 0.05$) between a K13 mutation and clearance half-life > 5 hours or day 3 parasitemia via a chi-squared test or appropriate multivariable regression model on a sample of at least 20 clinical cases, or 2) > 1% survival using the $RSA_{0\text{-}3h}$ in at least 5 individual isolates with a given mutation or a statistically significant difference ($p < 0.05$) in the $RSA_{0\text{-}3h}$ assay between culture-adapted recombinant isogenic parasite lines, produced using transfection and gene editing techniques, which express a variant allele of K13 as compared to the wild-type allele. The criteria for calling a K13 propeller SNP "validated" are when bothrequirements 1) and 2)are met. A current list (which will have to be updated regularly) of candidate or validated K13 propeller mutations can be found in Table 1.

Table 1. Associated and validated K13 resistance mutations

K13 Mutation	Reference	Classification
441L	17	Associated
446I	18	Associated
449A	17	Associated
458Y	17	Associated
493H	19, 20	Confirmed
539T	19, 20	Confirmed
543T	19, 20, 21	Confirmed
553L	17	Associated
561H	17	Associated
568G	21	Associated
574L	17	Associated
580Y	19, 20	Confirmed
675V	17	Associated

[17] Ashley EA et al. (2014). N Engl J Med;371(5):411-23.
[18] Huang F et al. In preparation.
[19] Ariey F et al. (2014). Nature;505(7481):50-5.
[20] Straimer et al. (2014). Science; in press.
[21] Takala-Harrison S et al. (2014). J Infect Dis; in press.

* This definition needs most probably to be simplified for national malaria control programmes and will be discussed at the next Technical Expert Group on Drug Resistance and Containment (TEG). With the new tools and information available, the TEG will also have to discuss whether and which containment efforts should be recommended if artemisinin resistance is suspected or confirmed in a certain area.

The artemisinin resistance phenotype

Presentation

The two major ways to measure delayed parasite clearance in vivo include day 3 parasitemia (i.e. persistence of parasitemia 72 hours following treatment with an artemisinin derivative or ACT) and parasite clearance half-life. Day 3 parasitemia is correlated with parasite clearance half-life, but is highly dependent on initial parasitemia. Parasite clearance half-life is the reference in vivo phenotype. A half-life of > 5 hours is used as the standard cut-off for resistance in South-East Asia, but this cut-off is somewhat arbitrary, and depends on the underlying proportions of resistant versus sensitive parasite strains and the estimator used, and can thus not be considered as an absolute cut-off. Conventional inhibitory concentration 50% (IC_{50}) measurements are poorly correlated with delayed clearance. However, $RSA_{0\text{-}3h}$ provide much better sensitivity and correlation with parasite clearance. The $RSA_{0\text{-}3h}$ developed by Pasteur Institute is currently the reference in vitro test.

The Tracking Resistance to Artemisinins Collaboration (TRAC) showed increased gametocyte carriage at day 0 in patients with parasites having K13 propeller mutations. There is also evidence that K13 mutations are both spreading and emerging de novo in different geographic areas[14], which has implications for how to define Tier 1 and Tier 2 regions. The role of African K13 mutations has to be investigated in order to assess their relationship with artemisinin resistance.

Discussion

Which phenotypes are most appropriate to use depends on the questions being asked, as different methods may be more appropriate for surveillance versus research.

Conclusions and recommendations

Parasite clearance half-life best defines the in vivo phenotype of artemisinin resistance. A clearance half-life cut-off of 5 hours is currently being used to define resistance in South-East Asia; however, this cut-off depends on the underlying proportions of sensitive and resistant parasite strains and the estimator used, and can thus not be considered as an absolute cut-off. The $RSA_{0\text{-}3h}$ is currently the reference assay to define the in vitro phenotype. Day 3 parasitemia is a useful phenotype for surveillance. Whereas, parasite clearance half-life and the $RSA_{0\text{-}3h}$ would be more difficult to implement as part of surveillance, they are important tools for confirmation of resistance and research.

KARMA Project

Presentation

The K13 Artemisinin Resistance Multi-Centre Rapid Assessment (KARMA) study aims to: 1) provide a worldwide mapping of the distribution of K13 alleles in blood samples collected after 2012 from symptomatic and asymptomatic individuals infected with *P. falciparum*, and 2) build capacity at participating study sites to perform molecular monitoring of K13 using Sanger sequencing (nested PCR). The study is led by investigators at the Institut Pasteur in collaboration with WHO. Each participating study site is provided with a K13 toolkit, which includes reagents for DNA extraction and PCR, as well as six quality control blood spots. Sequencing can either be done on site or sent out if institutions do not have access to sequencing. Alternatively, sites that have already completed K13 sequencing are invited to share data.

Preliminary data have been obtained from 42 institutions, with 9491 samples tested to date, 92% having been collected after 2012. Final data are expected by the end of October. Of the 9491 samples sequenced and analyzed to date, 8524 have provided interpretable data (90%). Several countries including most in South America, Gabon, Ethiopia, Senegal and Tanzania showed no K13 mutants. One mutant parasite was observed in Brazil, from among 1008 samples. A total of 83 different K13 mutant alleles were found. 55% of mutations observed are new and never described, and 80% of

new alleles are private SNPs found in Africa (68%), in Asia (30%) and in Oceania (2%). Three alleles are shared between Asia and Africa. Only 10 K13 mutants are shared between African countries. Half of the SNPs observed in African samples are silent mutations. The 446I allele was observed in China, as were 11 other new alleles. Most Asian mutations are found in blade 4 of the propeller domain, but African K13 mutations seemed to be distributed more evenly among the different propeller blades.

Discussion

More research is needed to determine whether African K13 mutations are associated with resistance, including in vitro phenotypes ($RSA_{0\text{-}3h}$) and genotyping of loci flanking K13 to determine origins of K13 alleles, especially those mutations also observed in Asia. All therapeutic efficacy studies require K13 genotyping now. To date, there do not appear to be K13 mutants in South America, save for one sample from Brazil. To assess whether a parasite is from Asia or not, flanking loci (e.g. SNPs or microsatellites) in linkage disequilibrium with K13 can be genotyped and compared to Asian haplotypes either through a haplotype network or chromosome painting. Mitochondrial haplotypes may also be useful, as they are non-recombining.

Conclusions and recommendations

The role of novel and emerging African K13 mutations in artemisinin resistance needs to be determined. K13 mutations do not appear to be present in South America.

Genetic architecture of artemisinin resistant *Plasmodium falciparum*

Presentation

A genome-wide association study was conducted using among others samples from the TRAC including 1063 samples from 15 study sites[22]. The analysis used mixed models to estimate the association between approximately 18,000 SNPs (minor allele frequency > 0.01) and parasite clearance half-life. The top "hit" in this GWAS was the K13 580Y mutation. There were at least 7 distinct loci with a p-value < 10^{-7}, including SNPs within *arps10*, *ferredoxin*, *pfmdr2*, and *pfcrt*, which may represent loci with a secondary or compensatory effect on artemisinin resistance. Based on this study, at least 20 K13 mutations within both the propeller and BTB/POZ domains are relevant to artemisinin resistance.

An analysis of common haplotype lengths in regions surrounding K13 indicates a variety of genetic backgrounds on which the same mutation has occurred, confirming the presence of both independent emergence and smaller-scale spread of artemisinin resistance mutations. In an extended analysis of 1612 samples, the Oxford/Sanger research team has identified at least 4 founder populations in Vietnam and 7 in Cambodia. Most founder populations, but not all, are associated with artemisinin resistance. K13 mutations and secondary/background loci (including *arps10*, *ferredoxin*, *pfmdr2*, and *pfcrt*) have very similar geographical distribution. These loci may form part of a genetic background on which K13 mutations emerge or which augment parasite fitness in some way. Even though parasites from Thailand and Myanmar are genetically different from parasites from the rest of the GMS, the background alleles co-occur with K13 mutations across regions.

Analyses of the conservation between *P. falciparum* and *P. chabaudi* indicate high conservation in the BTB/POZ and kelch propeller regions of the K13 protein, where most resistance mutations are located. When examining conservation versus the ratio of synonymous to non-synonymous mutations in African versus Asian parasites, Asian parasites have an excess of non-synonymous mutations within the gene encoding K13, whereas the number of mutations observed in Africa is consistent with the level of conservation. Because of the diversity within the African parasite populations (larger effective population size), it is expected that one would observe a larger number of low frequency alleles, as is the case with K13 in Africa. In addition, radical mutations in hydrophobic regions of the protein circulate

[22] Miotto O et al. (2014). Nature Genet; in press.

in South-East Asia, while in Africa mutations are more conservative and less likely to affect hydrophobicity, which probably indicates they are less likely to modify the binding properties of K13. Most Asian kelch propeller mutations map to a region between *beta* strand 1 and 2 of each blade that exhibits a hydrophobicity peak, suggesting that this region may have an important effect on functional properties of the propeller.

Discussion

The geographical distribution of markers may be driven by drug pressure. Background loci were significantly associated with parasite clearance half-life, even after adjustment for the presence of K13 mutations. Although not all founder populations are necessarily driven by artemisinin resistance, identification of founder populations as they emerge may allow investigation into the drivers of their rapid expansion. It may be interesting to analyze haplotypes flanking secondary loci and contrast with K13 haplotypes to attempt to establish the time of their emergence. There is an ubiquitin-ligase (MAL7P1.19) near *pfcrt* that has been implicated in artemisinin resistance. It will be important to make sure that the GWAS signal is coming from *pfcrt* and not this nearby locus.

Conclusions and recommendations

Artemisinin resistance is likely multigenic, and background loci may serve as useful markers for predicting the risk of emergence of K13 mutations. There is evidence of both spread and independent emergence of K13 mutations. The amount and type of mutations observed in K13 in Africa is what would be expected for a gene with this level of conservation, and therefore the presence of K13 mutant alleles alone is insufficient for suspecting the emergence of artemisinin resistance.

PCR sequencing and genotyping assays

Presentation

The team at Harvard/Broad has culture adapted 66 parasites from the TRAC study. These parasites are from the border between Thailand and Myanmar and western Cambodia (specifically Pailin and Pursat, Cambodia). The parasites are monogenomic and come from patients who demonstrated a range of parasite clearance half-lives, but enriched for samples from the extremes of the distribution. The whole reading frame of the gene encoding K13 (2.2kb) was amplified via PCR and sequenced in three overlapping PCR reactions. The PCR is based on a modified K13 amplification protocol using species-specific primers. In these 66 parasites, no SNPs were detected outside of the propeller region. Three parasites had no K13 mutations but demonstrated delayed clearance in the $RSA_{0\text{-}3h}$. A positive correlation was observed between K13 mutations and in vitro drug sensitivity as measured using the $RSA_{0\text{-}3}$. A novel K13 mutation (D584V) was correlated with $RSA_{0\text{-}3h}$ phenotype from among these parasites. A correlation was also observed between the $RSA_{0\text{-}3}$ phenotype and oxidative stress, suggesting that artemisinin resistant parasites scavenge reactive oxygen species more efficiently than susceptible parasites.

Discussion

A strategy is necessary to characterize which African K13 mutations are relevant to the resistance phenotype. More research is also necessary to understand the mechanism of K13. How will polyclonal infections be dealt with in K13 surveillance? Comparison of day 0 genotypes to day 3 or day of failure genotypes may show whether certain clones are being selected. Which sequencing/genotyping methods are preferred (e.g. capillary sequencing or short read Illumina sequencing)? Different methods might be used for different purposes. Pooled deep sequencing is useful for a broader scan, but it is important to be careful that read depth is not interpreted as prevalence.

Conclusions and recommendations

At this time that K13 mutations are still being discovered (particularly in Africa), PCR sequencing is the preferred method for genotyping K13. For now, the propeller domain will be the focus for surveillance. Sequencing of day 0 samples is required, but sequencing of day 3 or day of failure parasites (if present) may show selection for certain subpopulations of parasites. Pooled deep sequencing may be useful for screening large numbers of samples for discovery of new mutations; however, reads would need to be coded to link findings to individual patients in order to determine prevalence. Once a longer list of known mutations is compiled, a SNP chip may be used to genotype known mutations. Whether sequencing is performed locally or outsourced depends on the amount of existing infrastructure. Outsourcing of sequencing may increase efficiency, but there is a need to balance efficiency with capacity building in malaria endemic countries.

Table 2. Pros and cons of different sequencing/genotyping methodologies

Method	Pros	Cons
Sanger sequencing	Can discover novel K13 mutations; Allows accurate estimates of prevalence in populations; Commercially available.	Relatively high cost per sample; Low cost effectiveness in areas where prevalence of mutants is very low; Quality control can be difficult.
Deep sequencing of individual samples using next generation technologies	Can discover novel K13 mutations; Can accurately assess mixed infections; Genotyping results supported by many sequencing reads; Allows accurate estimates of prevalence in populations Produces surrounding haplotype information, that may inform origin of mutation; Produces genetic information across other loci of interest.	High cost per sample; Low cost effectiveness if one is only interested in K13; Considerable pre-processing and post-processing requires bioinformatics support.
Pooled deep sequencing of multiple samples using next generation technologies	Can discover novel K13 mutations; Genotyping results supported by many sequencing reads; Lower per-sample cost compared to deep sequencing of individual samples.	Cannot reliably estimate allele prevalence; Rare novel alleles may be missed and may be indistinguishable from sequencing errors; Quality control can be difficult.
PCR-based SNP genotyping	Can be very cost-effective and may scale to large-scale surveillance; Can be used to monitor mutations known to be frequent; Allows accurate estimates of prevalence in populations; Straightforward to implement for many molecular labs.	Limited discovery capability of novel K13 mutations; May require design of multiple protocols, which can be customized by geographical region; Cannot ascertain true wild-types.
SNP arrays	Can genotype a large number of mutant alleles; Allows accurate estimates of prevalence in populations; May produce surrounding haplotype information, informative of mutation origin; May produce genetic information across other loci of interest.	Relatively high cost per sample Cannot discover novel K13 mutations; Cannot ascertain true wild-types; Not flexible if redesign is required (e.g. to deploy in a different geographical regions, or if prevalence changes);
Multi-SNP assays (Sequenom)	Can be very cost-effective; Allows accurate estimates of prevalence in populations; Flexible redesign of assay; Extensible: can add more SNP assays, with linear cost scaling.	Cannot discover novel K13 mutations; Not all polymorphisms can be tested (but K13 mutations generally perform well); Cannot ascertain true wild-types.

Population transcriptomics of artemisinin resistance of *P. falciparum*

Presentation

The research team from Nanyang University has generated transcriptomes for 1043 samples from the TRAC study. Analyses focused on a group of parasites, which have just reinvaded the erythrocyte. Overall, increased expression of proteins with endoplasmic reticulum (ER) retention sequences and decreased expression of DNA repair proteins in resistant parasites was observed. Genes that are overexpressed in resistant parasites encode for proteins involved in protein metabolism, pathways related to things going on in the ER, and the unfolded protein response. This function appears to be directed by K13 and not by secondary/background loci identified in recent GWAS. It is hypothesized that K13 may be a negative regulator of the unfolded protein response pathway, and that mutations within the protein affect its regulatory function, leading to over-expression of downstream targets. Genes that are under-expressed in resistant parasites include those encoding proteins related to DNA replication. Resistant parasites appear younger and display a deceleration in progression of the ring stage and recover during the trophozoite and schizont stages. It is possible that triggering of repair mechanisms (e.g. the unfolded protein response) may signal a stall in progression of the ring stage. Clearance half-life is associated with cyclophilin B and other protein folding genes within the reactive oxidative stress complex (PfROSC).

Discussion

PfROSC proteins are reporters of the unfolded protein response. PfROSC is induced by treatment with artemisinins. The relationship between negatively and positively regulated genes could be a marker of resistance (e.g. the ratio of up versus down regulation). K13 itself is not up or down regulated in resistant parasites.

Conclusions and recommendations

Artemisinin resistant parasites over-express proteins involved in the unfolded protein response and under-express proteins involved in DNA replication, leading to a deceleration of life cycle progression. It is hypothesized that K13 might be a negative regulator of the unfolded protein response with K13 mutations affecting regulatory function leading to over-expression of downstream targets.

Transfection studies and K13 mutations

Presentation

The research team at Columbia University has been performing zinc finger nuclease (ZFN)-based editing of K13 in Cambodian parasites and reference laboratory strains[23]. This approach employs ZFNs to introduce mutations into drug sensitive parasites or remove them from resistant isolates. Silent mutations are included to protect the edited locus from re-cleavage. These silent mutations do not significantly alter ring-stage susceptibility to artemisinins based on the RSA_{0-3}. Removal of K13 from clinical isolates ablates the RSA_{0-3} resistance phenotype, with a 70-140-fold decrease in resistance for the 539T mutation and a 5-fold decrease in resistance for 580Y. Introducing individual K13 mutations into Cambodian isolates and laboratory lines leads to an increase of RSA_{0-3} survival rates; however, recently obtained Cambodian isolates and the multidrug resistant V/1S laboratory strain yielded higher RSA_{0-3} values than the reference Dd2 and FCB strains, implying the presence of additional parasites factors or genetic loci that modulate resistance.

In addition, the Columbia team has developed an evolutionary action algorithm to predict the functional impact of mutations. This algorithm combines an estimate of how similar amino acids are that are substituted with an estimate of the evolutionary importance of the site (based on its level of conservation between multiple species) to derive an evolutionary action score. Resistant parasites tend

[23] Mok et al. (2014) Science; in press.

to have high evolutionary action scores, while sensitive parasites have low evolutionary action scores. Evolutionary action score is strongly correlated with parasite clearance half-life (r^2 = 0.84). Using this evolutionary action score, the Columbia team has developed a list of African K13 mutations with high evolutionary action values to follow up on in subsequent transfection experiments.

Discussion

To date there are only a few natural isolates associated with artemisinin resistance that contain more than one mutation in the K13 locus but clonality needs to be assessed[24]. The Columbia team has not yet tried to generate a K13 double mutant. It is hypothesized that double mutants may lead to a markedly unfit parasite. Competition experiments to assess the fitness of different K13 mutations are underway. It is possible that secondary loci (e.g. those identified in recent GWAS) may augment the resistance phenotype, as evidenced by the greater degree of resistance conferred by introducing K13 into contemporaneous Cambodian clinical isolates compared to laboratory strains harvested in the early 1980s, which show increased $RSA_{0\text{-}3}$ values, but to a lesser degree. It is also possible that secondary loci could be involved in protecting the trophozoite stage from either artemisinins or partner drug.

Conclusions and recommendations

K13 is sufficient to cause resistance in transfection studies, but secondary loci may well play a role in augmenting the resistance phenotype. More research is required to understand the role of secondary loci. Not all K13 mutations have the same biological impact, in that different mutations confer different levels of resistance in $RSA_{0\text{-}3h}$ and are predicted to have different evolutionary action. Evolutionary action scores can be used to prioritize which mutations to prioritize for validation studies. With regard to the transfection work, it may be helpful to divide the labor between a few groups that coordinate which strains and methods to use, if funding sources are willing to support this important line of research. The transfection data clearly document a central role for K13 mutations in mediating ring stage resistance, which has been shown to associate with the clinical resistance phenotype of delayed parasite clearance rates.

Partner drug resistance markers

Presentation

The recommended partner drugs for artemisinins are amodiaquine, lumefantrine, mefloquine, piperaquine and sulphadoxine-pyrimethamine. The worldwide extent of chloroquine resistance is considered relevant to understanding resistance to several partner drugs. Chloroquine resistance primarily involves mutant haplotypes in *pfcrt*, as well as secondary mutations in *pfmdr1*. Amodiaquine shows cross-resistance with chloroquine; however, the degree of resistance associated with specific *pfcrt* haplotypes varies significantly between the two drugs. For example, parasites with South American *pfcrt* haplotypes are relatively more resistant to amodiaquine. In addition, there is a greater effect of *pfmdr1* mutations on amodiaquine susceptibility than on chloroquine. Chloroquine resistant parasites are generally hypersensitive to mefloquine and lumefantrine, and use of these drugs first results in selection for wild-type *pfmdr1* and *pfcrt* (if not fixed). Longer-term use leads to amplification of *pfmdr1* copy number and decreased susceptibility. Hence, mefloquine/lumefantrine and amodiaquine may drive selection in different directions. Piperaquine susceptibility could be affected by resistance to chloroquine or mefloquine, as a C101F mutation within *pfcrt* and changes in *pfmdr1* copy number have been implicated in piperaquine resistance selected in vitro. No markers have been identified for the piperaquine resistance phenotype recently detected in Cambodia.

[24] Ashley EA et al. (2014). N Engl J Med;371(5):411-23.

Conclusions and recommendations

There are theoretical advantages to be gained by rotating use of ACTs containing partner drugs with resistance mechanisms that are opposing (e.g. artesunate-amodiaquine and artemether-lumefantrine) or independent (artesunate-mefloquine and dihydroartemisinin-piperaquine).

Table 3. Resistance markers for the main ACT partner drugs

Partner drug	Marker
Amodiaquine	*pfcrt* and *pfmdr1* mutant haplotypes
Mefloquine and lumefantrine	*pfmdr1* amplification
Sulphadoxine-pyrimethamine	*dhps* and *dhfr* mutant haplotypes
Piperaquine	Unknown

Markers are genes encoding the *P. falciparum* chloroquine resistance transporter (*pfcrt*), multidrug resistance 1 (*pfmdr1*), dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*).

RSA and K13 mutations

Presentation

There are two RSA that differ based on the level of synchronization of the rings. The first generation assay contains rings between 0-12 hours, with a 6-hour pulse with 700 nM of dihydroartemisinin[25]. The second generation $RSA_{0\text{-}3h}$ improves synchronization to 0-3 hour rings, with the same 6-hour pulse of 700 nM of dihydroartemisinin[26]. The observed cut-off for resistance for the $RSA_{0\text{-}3h}$ is 1% of parasites surviving (or greater than +/- 2 standard deviations of the mean value for K13 wild type parasites from the same area). All K13 wild type parasites presented a $RSA_{0\text{-}3}$ value below this cut off[27]. There is also an ex vivo $RSA_{0\text{-}3h}$, but ex vivo $RSA_{0\text{-}3h}$ values tend to be usually higher than those for the in vitro $RSA_{0\text{-}3h}$. There are occasionally discordances between $RSA_{0\text{-}3h}$ and clearance half-life. $RSA_{0\text{-}3h}$ values vary for different K13 mutations, but all are > 1%. The 441L mutation seems to be sensitive based on $RSA_{0\text{-}3h}$ of two isolates, while 458I appears to be resistant based on $RSA_{0\text{-}3h}$ on 3 isolates. If cryopreserved parasites are collected, $RSA_{0\text{-}3h}$ can be used to validate African K13 alleles or K13 alleles from other areas.

Discussion

The association between K13 mutations and $RSA_{0\text{-}3h}$ seems to be stronger than the association between K13 mutations and half-life, likely due to host confounding factors or testing conditions. However, it is not practical for $RSA_{0\text{-}3h}$ to be used as part of surveillance. $RSA_{0\text{-}3h}$ is appropriate in a research setting to determine if a given parasite is resistant or not. However, to assess whether resistance is present in a population, then in vivo phenotypes are more appropriate. How many isolates need to be tested in order to validate a resistance mutation via $RSA_{0\text{-}3h}$? With high levels of antimalarial immunity in Africa, would in vitro phenotypes (e.g. $RSA_{0\text{-}3h}$) be preferred over in vivo phenotypes? As with other in vitro drug susceptibility assays, results can vary from laboratory to laboratory, which argues for having a small number of $RSA_{0\text{-}3h}$ reference labs with standardized methods, preferably near field sites.

[25] Witkowski et al. (2013). Antimicrob Agents Chemother;57(2):914-23.
[26] Witkowski et al. (2013). Lancet Infect Dis;13(12):1043-9.
[27] Amaratunga et al. (2014). Antimicrob Agents Chemother;58(8):4935-7.

Conclusions and recommendations

$RSA_{0\text{-}3h}$ can be used for in vitro validation of K13 resistance mutations. In order to validate a mutation, $RSA_{0\text{-}3h}$ values > 1% (or greater than +/- 2 standard deviations of the mean value for K13 wild type parasites from the same area) must be observed in at least five independent isolates. A small number of $RSA_{0\text{-}3h}$ reference laboratories are needed, including some close to field sites, using standard parasite controls for quality control.

Translational aspects

Presentation

If the K13 criteria for suspected artemisinin resistance criteria are met, phenotypic studies should be initiated (day 3 parasitemia or clearance half-life. $RSA_{0\text{-}3h}$ would be performed if cryopreserved parasites are available as well as screening for partner drug resistance markers should be performed. If criteria for confirmed resistance are met, a similar approach would be taken including initiation of more therapeutic efficacy studies, more molecular surveys, screening for partner drug resistance markers. For planning of containment/elimination efforts, genetic epidemiology including on gene flow could be useful.

Discussion

We do not have good markers for all partner drugs.

Conclusions and recommendations

Recommendations are described in the description of the presentations above.

Mapping molecular markers of drug resistance

Presentation

The advantage of mapping molecular markers of drug resistance is that it allows us to understand patterns of dispersal of drug-resistant parasites and to integrate principles into policy. Local and global patterns of pyrimethamine resistance dispersal were reviewed. Resistant parasites can either "jump" (i.e. long range importation, a rare event that's difficult to predict) or "spread" (i.e. local multiplication and spreading to adjacent geographic areas). Operational limitations prevented more widespread use of molecular markers to first-line drugs in the past, and these included: 1) resistance being already widespread by the time markers were identified, 2) data often obsolete by the time of publication, 3) little systematic surveillance coverage, and 4) no formal framework for information flow to national programs. Some of these limitations do not apply in the case of artemisinin resistance markers. For example, artemisinin resistance is not widespread at this time, and advances in sequencing technologies allow for faster turn-around of data and publications. Design of a surveillance framework should be defined based on regional population structure and not national boundaries. There are regional associations between countries sharing *dhfr* resistance alleles. Similarly, lineages of *dhps* alleles have spread regionally and the resistance allele sharing patterns among countries broadly correspond to the regional networks previously established for purposes of drug resistance surveillance. Estimates of gene flow/parasite migration may help guide definition of Tier 1 and Tier 2 regions for elimination. Long range importation is more difficult to predict, but we should try to anticipate it as well as possible.

Discussion

Routine molecular surveillance should include K13 sequencing and typing of partner drug markers. It is important to identify at risk populations (based on malaria transmission levels, drug pressure, and movement of people) and use that to guide surveillance and containment. Outsourcing of sequencing to commercial centres may speed up data generation and publication, but there are cost limitations and there is a need to balance with regional capacity building in malaria endemic countries. If sequencing was outsourced, quality assurance/control (QA/QC) could be done in local laboratories to

promote education and sustainability. Gene flow and population structure are more of a research priority than part of routine surveillance, but depending on the platform used, if DNA is being collected for K13 genotyping as part of routine surveillance, the same DNA may be able to be used to estimate gene flow and population structure. More research is needed to better understand the relationship between gene flow/parasite migration and its relation to the movement of people and the spread of resistance.

Conclusions and recommendations

Parasite DNA should be collected as part of routine surveillance for K13 mutations and partner drug markers. Outsourcing of K13 sequencing from surveillance may help with faster turnaround of results to allow information to be available in near real-time; however, there may be cost limitations, and there is a need to incorporate local capacity building. To allow for education and sustainability, QA/QC could be done in local laboratories, with cooperation between local control programs and local research institutions.

Gene flow and population structure are useful to plan regional surveillance frameworks and containment efforts although informative population genetic data is unlikely to be generated through routine surveillance. More research is needed to validate predictive estimates of gene flow/parasite migration based on historical analyses of previous patterns of spread of resistance. Such estimates may help define at-risk populations, along with transmission level and amount of drug pressure.

K13 reference centre

Presentation

Based on what has been done with the KARMA project, the reference centre should be responsible for the standardization of procedures, coordination of sample shipment and training. The reference centre should rely on collaborative laboratories, be reactive and secure. All K13 reference laboratories partners will agree to respect the confidentiality of the data collected and not use the data collected for publication. For reasons of safety, quality and clarity, each reference laboratory should submit its reports and make recommendations to WHO which will have the authority to redistribute to the appropriate national malaria control programmes.

This network could be organized around two structures: an "operational" structure will consist of contributor laboratories that will collect samples and field information, and collaborating laboratories will be responsible for producing data, training, and QA/QC. Contributors and collaborating laboratories must be able to respond to requests for characterization of the phenotype and genotype. This implies compliance with international quality standards for the receipt of blood samples, delivery of biological results (e.g. traceability, quality improvement, validation and reporting of results, storage) as well as training (e.g. infrastructure, coaching, and biological materials). Each of the collaborating laboratories will be responsible for setting up reference collections (standardized DNA and culture-adapted strains) and securing data (e.g. secure back up computer).

A second "analytical" structure will bring together a group of correspondents who will bring their expertise to the analysis of data produced by the operational structure hosted by the K13 reference centre and/or WHO. The web site could be created and organized around an interactive blog that will provide secure access to the database. A facilitator will be responsible for validation and implementation of the latest data, management discussions; addressing questions from the national malaria control programmes; and, management of interactions with partners.

Conclusions and recommendations

WHO is interested in the establishment of a reference centre for K13 genotyping and will further discuss with interested partners. WHO needs to rely on a reference centre that will coordinate/standardize methods between laboratories by providing a reference genotyping protocol

and by providing guidance regarding the pros and cons of different methodologies. The reference centre, relying on collaborative, collaborator and correspondent laboratories that could analyze the results of molecular surveillance and serve as advisory centres for WHO and national malaria control programmes, would be an asset. A list of laboratories willing to do quality assured K13 sequencing would be required.

EXPERT REVIEW GROUP ON K13

15-16 SEPTEMBER 2014, GENEVA, SWITZERLAND

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EXPERT REVIEW GROUP MEETING ON K13

15-16 September 2014, Geneva, Switzerland

Monday 15 September 2014		
09:00–09:15	Welcome **J. Reeder** **A. Dondorp**	
09:15–09:30	Declaration of interest, agenda **P. Ringwald**	
09:30–10:00	Molecular markers of artemisinin resistance: Scientific evidence and public health potential **C. Plowe**	
10:00–10:45	Definition of artemisinin resistance **P. Ringwald**	
10:45–11:15	**Coffee/tea break**	
11:15–12:00	KARMA project **D. Ménard**	
12:00-12:45	TRAC project **A. Dondorp**	
12:45–14.00	**Lunch**	
14:00–14:45	Genetic architecture of artemisinin resistant *Plasmodium falciparum* **O. Miotto**	
14:45-15:30	PCR sequencing and genotyping assays **S. Volkman**	
15.30-16.00	**Coffee/tea break**	
16:00–16:45	Transcriptional up-regulation and decelerated blood stage progression in artemisinin resistance of Plasmodium falciparum **Z. Bozdech**	

16:45–17:30	Transfection studies and K13 mutations **D. Fidock**	
17:30-18.15	Molecular markers for resistance to other antimalarial drugs **C. Woodrow**	
18:30–20:00	**Reception**	
Tuesday 16 September 2014		
09:00–9:30	RSA and K13 mutations **D. Ménard**	
9:30-10:00	Translational aspects **A. Dondorp**	
10:00-10:45	Mapping of molecular markers for drug resistance **C. Roper**	
10:45–11:15	**Coffee/tea break**	
11:15-12:30	Reference centre **F. Ariey**	
12:30–14:00	**Lunch**	
14:00-15:30	Recommendations including research agenda	
15:30-16:00	**Coffee/tea break**	
16:00-17:00	Recommendations	
17:00-17:30	Closure	