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Genetic markers of artemisinin resistance in *Plasmodium* spp. parasites

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The vast majority of malaria patients worldwide are currently treated with combination therapy comprising one of the artemisinin family of drugs, characterised by rapid action and short plasma half-life, co-formulated with a longer-lasting drug from the amino aryl-alcohol or quinoline families. There is now a widely perceived threat to treatment efficacy, as reduced susceptibility to rapid artemisinin clearance *in vivo* has become prevalent among populations of *Plasmodium falciparum* in the Greater Mekong subregion since 2008. *In vitro* and *in vivo* drug selection studies, heterologous cell expression experiments and genetic epidemiology have identified many candidate markers of reduced ring-stage susceptibility to artemisinin. Certain variants of the *P. falciparum pfk13* gene, which encodes a kelch domain protein implicated in the unfolded protein response, are strongly associated with slow parasite clearance by artemisinin in the Mekong subregion. However, anomalies in the epidemiological association of *pfk13* variants with true treatment failure *in vivo* and the curious cell-cycle stage specificity of this phenotype *in vitro* warrant exploration in some depth. Taken together, available data suggest that the emergence of *P. falciparum* expressing K13 variants has not yet precipitated a public health emergency. Alternative candidate markers of artemisinin susceptibility are also described, as K13-independent treatment failure has been observed in African *P. falciparum* and in the rodent malaria parasite *Plasmodium chabaudi*.

A new artemisinin susceptibility phenotype arises in Asia

Noedl et al. [1] first described microscopically detectable *Plasmodium falciparum* parasites persisting in the peripheral blood of artesunate-treated Cambodian malaria patients in 2008. This was soon followed by more extensive observations from the randomised study of Dondorp et al. [2]. Importantly, the latter study demonstrated that slow parasite clearance was observed in malaria patients from western Cambodia irrespective of whether they had been treated with monotherapy, or with artemisinin-based combination therapy (ACT), but this was not observed in patients from the Thailand–Myanmar border to the northwest. Extensive multicentre studies led by the UK-funded TRAC consortium then provided important evidence, just a few years later, that the slow-clearance phenotype was spreading through *P. falciparum* populations in the Greater Mekong subregion (GMS) in association with certain variants of the *pfk13* gene, but that in parallel African studies any treatment failures seen were not associated with this genetic marker [3].

Identification of *pfk13* was the culmination of a search for a marker or markers to better identify *P. falciparum* with reduced susceptibility to artemisinin, a search that had been hampered by the lack of an *in vitro* correlate. Curiously, the *in vivo* slow-clearance phenotype of *P. falciparum*, spreading through the GMS, was not consistently reflected in estimates of the 50% effective concentration (EC_{50}) of dihydroartemisinin (DHA, the dominant parasiticidal artemisinin metabolite *in vivo*) against parasites cultured *in vitro* [2,4]. Only after the Pasteur Institute teams in Phnom Penh and Paris had devised an *in vitro* DHA susceptibility test specific for the earliest blood-stage parasites, the ring-stage survival assay (RSA) [5], Ariey et al. [6] were then able to demonstrate that certain mutations in the

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pfk13 locus correlated with the *ex vivo* parasite phenotype in the RSA. This key locus was identified as a candidate for testing not by genome-wide association studies (GWASs), an approach that promised much [7] but delivered little [8] in terms of identifying specific markers of artemisinin susceptibility, but through 5 years of painstaking drug selection in the laboratory [6]. *Pfk13* was one of a few loci in the genome of the culture-adapted Tanzanian *P. falciparum* isolate F32 that, during this extensive selection process, had accumulated mutations in concert with decreasing DHA susceptibility as measured by the RSA. Alone among these few loci, mutations in *pfk13* were also found to correlate with *ex vivo* DHA susceptibility of Cambodian parasites in the RSA, although the exact mutation identified in mutant F32 parasites has still not been encountered in any wild *P. falciparum* isolates to date [9]. The identification of key variants of *pfk13* that correlate both with slow clearance in patients receiving artesunate monotherapy [3] and with *ex vivo* RSA data [6] has enabled subsequent research on *pfk13*. This includes:

- studies of the distribution of polymorphisms in the field, particularly in Africa [9–13];
- groundbreaking genome-editing studies *in vitro* demonstrating that single-nucleotide changes in *pfk13*, mimicking GMS variants, alter *P. falciparum* DHA susceptibility *in vitro* [14,15];
- GWAS demonstrating the importance of genetic background, including novel alleles at several loci, in the K13-mediated phenotype [16].

The perceived threat to artemisinin-based malaria therapeutics

There is considerable concern that, faced with the spread of *pfk13* variants to the wider GMS, the proportion of slow-clearing phenotypes is increasing, thus posing an immediate threat to ACT efficacy within the region and more globally in the future [17–20]. In fact, some authors have called on the WHO to declare artemisinin 'resistance' to be a "public health emergency of international concern' [18]. However, a careful overview of data from the GMS and beyond provides several arguments against this emotive response. These include the following:

- It is postulated that a single lineage of multidrug-resistant variant K13 parasites, dubbed *PfPailin*, underly this proposed emergency. However, despite the claim that 'transnational spread of this single fit multidrug-resistant malaria parasite lineage is of international concern' [20], these authors' own data show this not to be the case. To quote: 'Parasites with the same C580Y (*pfk13*) haplotype were not clonal since there was significant diversity in microsatellites elsewhere in the genome and in the polymorphic loci in the *msp1*, *msp2*, and *glurp* genes that showed an overall mean He of 0.529 (SE 0.030) for three loci combined.' [19]. This indicates a relatively high level of genetic recombination among a variety of parasite genomes, with the *pfk13* variant locus (and one other gene of interest, *pfplasmepsin2*) showing a selective sweep across these various genomes. Therefore, there is no single multidrug-resistant lineage and no 'super-parasite' emergency.
- The association between *pfk13* carriage and either slow-clearance phenotypes measured 3 days after treatment or parasite recrudescence after 28 or 42 days of active follow-up in drug efficacy studies is elusive. Firstly, parasite persistence and slow clearance in African studies are not associated with the *pfk13* polymorphism [10,21]. Secondly, K13 variants do not determine parasite recurrence after treatment with the ACT DHA–piperaquine (DP), as recently reported for three sites in Vietnam [17]. The Gia Lai site, in which 67% of *P. falciparum* malaria patients carried the C580Y variant of *pfk13*, showed 89% treatment success ('adequate clinical and parasitological response', ACPR), whereas the ACPR among patients enrolled at Ninh Thuan was 78% (C580Y prevalence rate 4%), and among patients enrolled at Binh Phuoc, the ACPR was a very disappointing 56% (C580Y prevalence rate 73%). In fact, the best interpretation of this study is that it is falling partner drug efficacy that has led to the low ACPR in some sites [17]. Furthermore, in Myanmar, despite a prevalence rate of 25–65% of the C580Y variant *pfk13* in 2012 and 2013 in Kawthaung and Myawaddy, 3 days of standard ACT delivered ≥96% ACPR [22].
- *P. falciparum* is rapidly disappearing from the GMS. Despite an increasing proportion of *P. falciparum* isolates in the region carrying multiple determinants of reduced susceptibility to ACT, the overall numbers of falciparum malaria cases have fallen dramatically; as an example, between 2007 and 2015 malaria hospital admissions fell from 25 to 2 per 100 000 (92% fall) and deaths from 0.15 to 0.05 per 100 000 (67% fall) in

Thailand, and only 42% of infections are *P. falciparum* (WHO World Malaria Report 2016; http://www.who.int/malaria/publications/world-malaria-report-2016/en/). Thus, the malaria control effort in this region needs to be applauded and continued. If there is any public health malaria emergency of concern, it is surely in sub-Saharan Africa, where, in Burkina Faso in 2015, ~2000 people were hospitalised for malaria, and 30 malaria deaths were estimated to have occurred, per 100 000 population (ibid.).

- *P. falciparum* harbouring variant K13 alleles, and/or demonstrating slow clearance at day 3, are clearly susceptible to extended-duration artemisinin-based treatment, as an ACPR of more than 90% has been achieved in the GMS with 6 days of artesunate monotherapy [23,24] or 3 days of artesunate monotherapy followed by 3 days of ACT [3]. Thus, regimens such as two sequential ACTs with different partner drugs have been postulated as a rational response to signs of falling 3-day ACT efficacy, and a protocol has been developed to evaluate such regimes before ACT failure becomes a serious problem [25].

In fact, considering all these factors, and the inability of variant *pfk13* parasites to survive 48 h of artemisinin *in vitro* (see below), it seems prudent to cease the practice of calling *pfk13* a marker of artemisinin resistance, but rather see it as an important modulator of susceptibility that represents a parasite adaptation to the short treatment regimens deployed against *P. falciparum* infections *in vivo*. In the next section, arguments based on the parasite cell cycle will be used to support this unorthodox suggestion, and lend further support to the strategy of extended artemisinin regimens to radically cure *P. falciparum* infections [25].

Variants of pfk13 and the parasite cell cycle *in vitro*

There is very good evidence from laboratory studies of *P. falciparum* isolates grown in culture that artemisinin susceptibility varies greatly across the cell cycle [26,27]. The patterns as currently understood for wild-type parasites are represented in cartoon form in Figure 1A. Although all stages are susceptible to artemisinin, a small proportion of the least susceptible stages may survive a single pulse of artemisinin *in vitro*. Figure 1B illustrates some of the findings for Cambodian-origin parasite clones harbouring K13 variants [27]. Most notably, the exquisite susceptibility of wild-type ring-stage trophozoites is ablated by the *pfk13* variant, and this fully resistant stage is extended in duration, thus minimising the proportion of the life cycle spent at the more susceptible stages [mature trophozoite (MT) and early schizont (ES)]. This simplified conceptualisation of the impact of artemisinin on the parasite at different points in the life cycle can be extrapolated, with some caution, to consider the impact of a standard artesunate monotherapy regimen (3 days) on a typical asynchronous natural infection of *P. falciparum*. Presented as a pictorial model in Figure 2, this provides interesting inferences of what may happen in a treated malaria patient with all stages of the parasite life cycle present (although some stages will be sequestered in the tissues, these are drug-exposed).

- Firstly, in wild-type parasites, a small proportion of parasites at the LRT (late ring-stage trophozoite) stage at the time of the first artesunate dose may survive (dark grey shading), particularly considering that the terminal elimination half-life of DHA (administered as DP) is less than 2 h [28]. These will be schizonts by the time of the next administration, but if this is not exactly spaced by 24 h, some of this cohort of parasites may be mature enough to also have a chance of escaping DHA killing. The third dose will find any remnants of this cohort as LRT again, and hence, some may yet survive. Interestingly, at the day 3 follow-up time point, any survivors will be schizonts and fully sequestered, so peripheral blood microscopy will indicate the patient is fully cured. In this way, the drug treatment is seen to effectively synchronise any survivors into a co-ordinated cohort. Is this an argument for a day 4 time point?
- Secondly, in the K13 variant parasites, the benefit of the mutation is entirely due to survival of that proportion of parasites at the most resistant stage — ERT (early ring-stage trophozoite). The pictorial model suggests that, again, day 3 is not the best time point for peripheral microscopy, as survivors will be late trophozoites or schizonts sequestered in the periphery. The second artesunate dose will find relatively vulnerable MT and ES among the resistant survivors, but the third dose again would encounter highly resistant ERT in the resistant cohort (Figure 2, bottom panel, top row). If the perturbations of cell cycle dynamics described by Hott et al. [27] hold *in vivo*, it may be that resistant rings are present for longer periods during the cell cycle, which may increase the overall survival.

The fact that *pfk13*-mediated phenotypes are advantageous solely at the early ring stage of the cell cycle (Figure 2) draws attention to the remarkable spike in susceptibility at this stage in wild-type parasites (Figure 1A). It is widely considered that the endoperoxide bridge in artemisinin is strongly activated by haem

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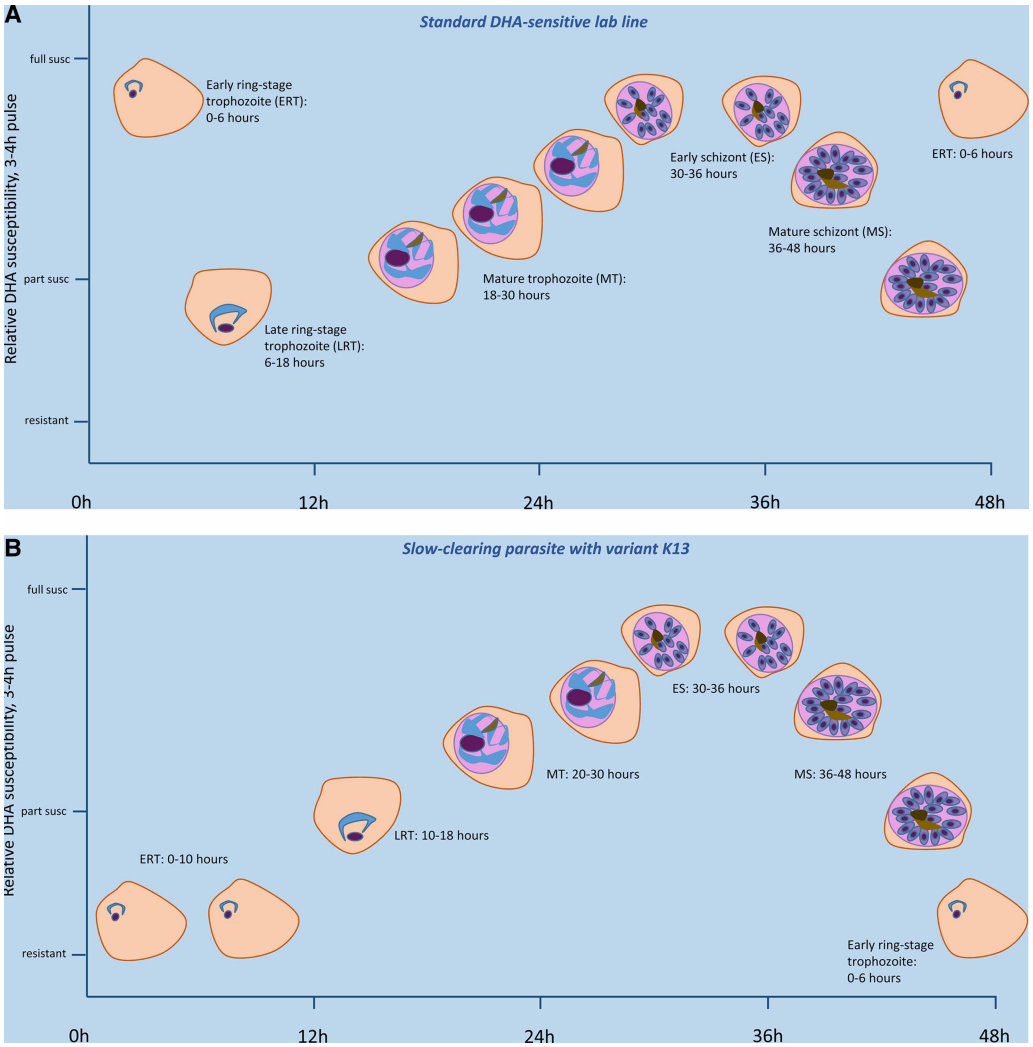

Figure 1. Asexual life-cycle stage specificity of *in vitro* artemisinin susceptibility of *P. falciparum* parasites with or without variant *pfk13* alleles.

Cartoon figures depict in a stylised manner 5 of the morphological stages recognised in fixed thin films of cultured parasites as labelled [ERT, LRT, MT, ES, and mature schizont (MS)], with the number of repeat cartoons approximating the observed duration of that stage during the 48-h life cycle. *Y*-axis reflects estimated stage-specific inhibition/killing of a short (3–4 h) pulse of 700 nM DHA from published sources. All wild-type parasite stages are killed by artemisinin, but the short exposure to drug *in vivo* due to rapid metabolism and extinction of DHA may allow a small proportion of the least susceptible stages to survive. (**A**) Standard DHA-sensitive cultured laboratory parasite line, as shown for 3D7 by Klonis et al. [26]. All stages are susceptible to artemisinin killing; ERT briefly exhibits exquisite susceptibility, while LRT is the least susceptible stage. (**B**) Slow-clearing parasite line of Cambodian origin, exemplified by ARC08-88 (clone 4G) described by Hott et al. [27]. It is notable that other artemisinin-tolerant clones described by these authors displayed a shortened *in vitro* life cycle of under 40 h. Abbreviations: DHA: dihydroartemisinin; h: hours post-invasion in a synchronised ring-stage culture; full susc: development stage is fully susceptible to a 3–4 h pulse of 700 nM DHA, and no further growth is detected after the drug pulse; part susc: partly susceptible to the 700 nM DHA pulse, such that some continued growth occurs after the drug is washed away.

metabolism in the food vacuole, leading to parasite killing [29], a process that barely occurs during the first few hours of the life cycle, so it is unclear how activation of artemisinin, so lethal to parasites in these first few hours, occurs [18,26]. One plausible hypothesis is that a specific parasite cellular process in the first few hours post-invasion provides a finite burst of activated artemisinin; variant K13 renders ERT resistant to artemisinin simply by preventing this specific event. As haemozoin formation in the growing food vacuole proceeds,

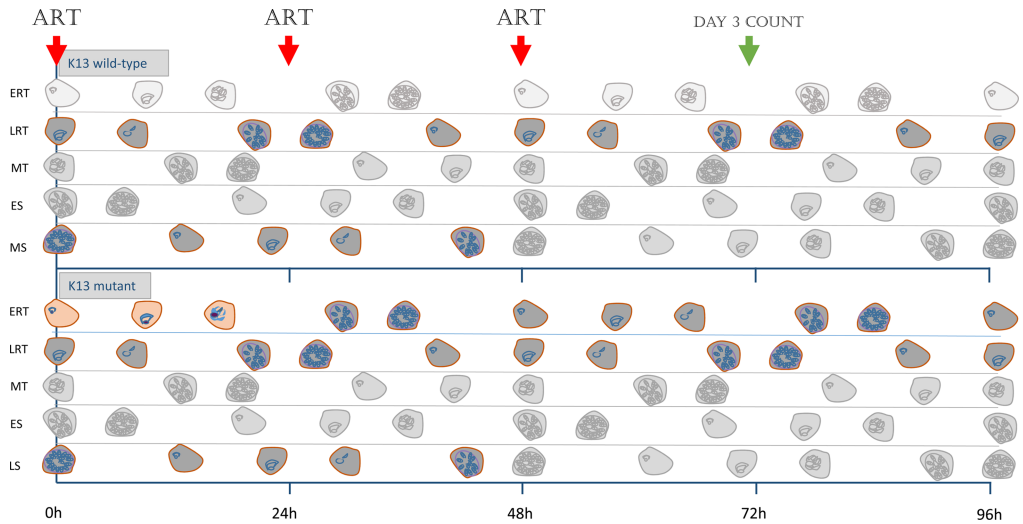

Figure 2. Pictorial model describing the likely impact of non-synchrony in natural infections: three doses of daily artemisinin leave survivors *in vivo*.

Cartoon figures representing ERT, LRT, MT, ES and MS are shown in Figure 1. Two hypothetical malaria patients infected with a single, but asynchronous, clone of *P. falciparum* are depicted under 3 days of artesunate (ART) monotherapy [1,2]. All five developmental periods (represented by the five different cartoon shapes from Figure 1) are assumed to be present at the moment of first treatment. It is not assumed that these are present in equal proportions. Parasites killed or prevented from developing by the current or most recent ART dose are depicted as pale grey ghosts. Viable parasites are presented in full colour. Mid-grey parasites with orange border are drug-affected and significantly reduced in number; these may die or may survive and progress to the next stage. In this figure, altered temporal development as depicted in Figure 1 and seen *in vitro* has not been depicted, as this is not proved *in vivo*. Day 3 count: key follow-up time point to identify slow-clearing parasites for *in vivo* drug trials.

artesunate is activated for the rest of intra-erythrocytic development, and thus, K13 variant parasites are fully susceptible to prolonged exposure to artemisinin, as clearly seen *in vitro* [2,4,5] and *in vivo* [3,22,23].

K13-independent artemisinin susceptibility phenotypes in *Plasmodium* spp

As we have seen, painstaking drug selection in the laboratory was the experimental path that led to identification of the *pfk13* locus as an important modulator of artemisinin susceptibility. The mode of action of artesunate in killing malaria parasites is almost certainly multifactorial, as the activated endoperoxide moiety causes widespread non-specific damage to intracellular proteins [26,29]. Thus, artemisinin selection is expected to identify other mechanisms that reduce susceptibility, and indeed, this has been the case. The best example is the work of Hunt, Cravo and colleagues in the rodent parasite *Plasmodium chabaudi*, in which variants in the C-terminus of *pcubp1*, encoding a ubiquitin carboxyl-terminal hydrolase, were fully validated *in vivo* by whole-genome sequencing and through reverse genetics in a genetic cross, as a true determinant of artemisinin and chloroquine resistance [30,31]. Subsequent *in vitro* and *in vivo* studies have supported, but not yet validated, variants of the orthologous *P. falciparum* locus *pfubp1* as determinants of artemisinin susceptibility [32,33]. An additional locus identified as contributing to high-level artemisinin resistance in the *P. chabaudi* studies, *pcap2mu*, encoding a putative adapter protein subunit involved in clathrin-mediated endocytosis [34], has also been partially validated as a key modulator of ACT treatment outcome in *P. falciparum in vivo* [33] and of DHA susceptibility by generating transgenic parasites *in vitro* [35]. Further studies of these candidate molecular markers for artemisinin susceptibility, including any yet to be identified, are keenly awaited. Certainly, there is no doubt that K13-independent artemisinin susceptibility phenotypes have arisen [10,36], and may continue to arise in the future, under universal selection pressure from ACT deployment worldwide. This threat makes it

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imperative that strategies to preserve the efficacy of our current regimens are vigorously pursued in the short term, as we await the next generation of antimalarial drugs [25].

Summary

- Mutations in pfk13 do not explain all artemisinin treatment failures.
- The mechanism of action of artemisinin is highly stage-specific.
- K13 mutations reduce parasite susceptibility to artemisinin by rendering a single stage, very young trophozoites, resistant.
- Increasing the duration of artemisinin exposure overcomes K13-mediated parasite phenotypes.

Abbreviations

ACPR, adequate clinical and parasitological response; ACT, artemisinin-based combination therapy; DHA, dihydroartemisinin; DP, DHA–piperaquine; ERT, early ring-stage trophozoite; ES, early schizont; GMS, Greater Mekong subregion; GWASs, genome-wide association studies; LRT, late ring-stage trophozoite; MS, mature schizont; MT, mature trophozoite; RSA, ring-stage survival assay.

Competing Interests

The Author declares that there are no competing interests associated with the manuscript.

References

1 Noedl, H., Se, Y., Schaecher, K., Smith, B.L., Socheat, D. and Fukuda, M.M. (2008) Artemisinin Resistance in Cambodia 1 (ARC1) Study Consortium. Evidence of artemisinin-resistant malaria in western Cambodia. *N. Engl. J. Med.* **359**, 2619–2620 https://doi.org/10.1056/NEJMc0805011
2 Dondorp, A.M., Nosten, F., Yi, P., Das, D., Phyo, A.P., Tarning, J. et al. (2009) Artemisinin ressitance in *Plasmodium falciparum* malaria. *N. Engl. J. Med.* **361**, 455–467 https://doi.org/10.1056/NEJMoa0808859
3 Ashley, E.A., Dhorda, M., Fairhurst, R.M., Amaratunga, C., Lim, P., Suon, S. et al. (2014) Spread of artemisinin resistance in *Plasmodium falciparum* Malaria. *N. Engl. J. Med.* **371**, 411–423 https://doi.org/10.1056/NEJMoa1314981
4 Chotivanich, K., Tripura, R., Das, D., Yi, P., Day, N.P.J., Pukrittayakamee, S. et al. (2014) Laboratory detection of artemisinin-resistant *Plasmodium falciparum*. *Antimicrob. Agents Chemother.* **58**, 3157–3161 https://doi.org/10.1128/AAC.01924-13
5 Witkowski, B., Amaratunga, C., Khim, N., Sreng, S., Chim, P., Kim, S. et al. (2013) Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: *in-vitro* and *ex-vivo* drug-response studies. *Lancet Infect. Dis.* **13**, 1043–1049 https://doi.org/10.1016/S1473-3099(13)70252-4
6 Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A.-C., Khim, N. et al. (2014) A molecular marker of artemisinin-resistant *Plasmodium falciparum*. *Nature* **505**, 50–55 https://doi.org/10.1038/nature12876
7 Miotto, O., Almagro-Garcia, J., Manske, M., MacInnis, B., Campino, S., Rockett, K.A. et al. (2013) Multiple populations of artemisinin-resistant *Plasmodium falciparum* in Cambodia. *Nat. Genet.* **45**, 648–655 https://doi.org/10.1038/ng.2624
8 Volkman, S.K., Herman, J., Lukens, A.K. and Hartl, D.L. (2017) Genome-wide association studies of drug-resistance determinants. *Trends Parasitol.* **33**, 214–230 https://doi.org/10.1016/j.pt.2016.10.001
9 Ménard, D., Khim, N., Beghain, J., Adegnika, A.A., Shafiul-Alam, M., Amodu, O. et al. (2016) A worldwide map of *Plasmodium falciparum* K13-propeller polymorphisms. *N. Engl. J. Med.* **374**, 2453–2464 https://doi.org/10.1056/NEJMoa1513137
10 Muwanguzi, J., Henriques, G., Sawa, P., Bousema, T., Sutherland, C.J. and Beshir, K.B. (2016) Lack of K13 mutations in *Plasmodium falciparum* persisting after artemisinin combination therapy treatment of Kenyan children. *Malar. J.* **15**, 36 https://doi.org/10.1186/s12936-016-1095-y
11 Cooper, R.A., Conrad, M.D., Watson, Q.D., Huezo, S.J., Ninsiima, H., Tumwebaze, P. et al. (2015) Lack of artemisinin resistance in *Plasmodium falciparum* in Uganda based on parasitological and molecular assays. *Antimicrob. Agents Chemother.* **59**, 5061–5064 https://doi.org/10.1128/AAC.00921-15
12 Kamau, E., Campino, S., Amenga-Etego, L., Drury, E., Ishengoma, D., Johnson, K. et al. (2015) K13-propeller polymorphisms in *Plasmodium falciparum* parasites from sub-Saharan Africa. *J. Infect. Dis.* **211**, 1352–1355 https://doi.org/10.1093/infdis/jiu608
13 Taylor, S.M., Parobek, C.M., DeConti, D.K., Kayentao, K., Coulibaly, S.O., Greenwood, B.M. et al. (2015) Absence of putative artemisinin resistance mutations among *Plasmodium falciparum* in sub-Saharan Africa: a molecular epidemiologic study. *J. Infect. Dis.* **211**, 680–688 https://doi.org/10.1093/infdis/jiu467
14 Ghorbal, M., Gorman, M., Macpherson, C.R., Martins, R.M., Scherf, A. and Lopez-Rubio, J.-J. (2014) Genome editing in the human malaria parasite *Plasmodium falciparum* using the CRISPR-Cas9 system. *Nat. Biotechnol.* **32**, 819–821 https://doi.org/10.1038/nbt.2925
15 Straimer, J., Gnadig, N.F., Witkowski, B., Amaratunga, C., Duru, V., Ramadani, A.P. et al. (2015) K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates. *Science* **347**, 428–431 https://doi.org/10.1126/science.1260867

16 Miotto, O., Amato, R., Ashley, E.A., MacInnis, B., Almagro-Garcia, J., Amaratunga, C. et al. (2015) Genetic architecture of artemisinin-resistant *Plasmodium falciparum*. *Nat. Genet.* **47**, 226–234 https://doi.org/10.1038/ng.3189
17 Thanh, N.V., Thuy-Nhien, N., Tuyen, N.T.K., Tong, N.T., Nha-Ca, N.T., Dong, L.T. et al. (2017) Rapid decline in the susceptibility of *Plasmodium falciparum* to dihydroartemisinin–piperaquine in the south of Vietnam. *Malar. J.* **16**, 27 https://doi.org/10.1186/s12936-017-1680-8
18 Woodrow, C.J. and White, N.J. (2017) The clinical impact of artemisinin resistance in Southeast Asia and the potential for future spread. *FEMS Microbiol. Rev.* **41**, 34–48 https://doi.org/10.1093/femsre/fuw037
19 Imwong, M., Suwannasin, K., Kunasol, C., Sutawong, K., Mayxay, M., Rekol, H. et al. (2017) The spread of artemisinin-resistant *Plasmodium falciparum* in the Greater Mekong subregion: a molecular epidemiology observational study. *Lancet Infect. Dis.* **17**, 491–497 https://doi.org/10.1016/S1473-3099(17)30048-8
20 Imwong, M., Hien, T.T., Thuy-Nhien, N.T., Dondorp, A.M. and White, N.J. (2017) Spread of a single multidrug resistant malaria parasite lineage (*PfPailin*) to Vietnam. *Lancet Infect. Dis.* **17**, 1022–1023 https://doi.org/10.1016/S1473-3099(17)30524-8
21 Beshir, K.B., Sutherland, C.J., Sawa, P., Drakeley, C.J., Okell, L., Mweresa, C.K. et al. (2013) Residual *Plasmodium falciparum* parasitemia in Kenyan children after artemisinin-combination therapy is associated with increased transmission to mosquitoes and parasite recurrence. *J. Infect. Dis.* **208**, 2017–2024 https://doi.org/10.1093/infdis/jit431
22 Nyunt, M.H., Cueto, C., Smith, J.L., Hwang, J., Gosling, R. and Bennett, A. (2017) Clinical and molecular surveillance of artemisinin resistant falciparum malaria in Myanmar (2009–2013). *Malar. J.* **16**, 33 https://doi.org/10.1186/s12936-017-1983-9
23 Bethell, D., Se, Y., Lon, C., Tyner, S., Saunders, D., Sriwichai, S. et al. (2011) Artesunate dose escalation for the treatment of uncomplicated malaria in a region of reported artemisinin resistance: a randomized clinical trial. *PLoS ONE* **6**, e19283 https://doi.org/10.1371/journal.pone.0019283
24 Kyaw, M.P., Nyunt, M.H., Chit, K., Aye, M.M., Aye, K.H., Aye, M.M. et al. (2013) Reduced susceptibility of *Plasmodium falciparum* to artesunate in southern Myanmar. *PLoS ONE* **8**, e57689 https://doi.org/10.1371/journal.pone.0057689
25 Schallig, H.D., Tinto, H., Sawa, P., Kaur, H., Duparc, S., Ishengoma, D.S. et al. (2017) Randomised controlled trial of two sequential artemisinin-based combination therapy regimens to treat uncomplicated falciparum malaria in African children: a protocol to investigate safety, efficacy and adherence. *BMJ Global Health* **2**, e000371 https://doi.org/10.1136/bmjgh-2017-000371
26 Klonis, N., Xie, S.C., McCaw, J.M., Crespo-Ortiz, M.P., Zaloumis, S.G., Simpson, J.A. et al. (2013) Altered temporal response of malaria parasites determines differential sensitivity to artemisinin. *Proc. Natl Acad. Sci. U.S.A.* **110**, 5157–5162 https://doi.org/10.1073/pnas.1217452110
27 Hott, A., Casandra, D., Sparks, K.N., Morton, L.C., Castanares, G.-G., Rutter, A. et al. (2015) Artemisinin-resistant *Plasmodium falciparum* parasites exhibit altered patterns of development in infected erythrocytes. *Antimicrob. Agents Chemother.* **59**, 3156–3167 https://doi.org/10.1128/AAC.00197-15
28 Birgersson, S., Van Toi, P., Truong, N.T., Dung, N.T., Ashton, M., Hien, T.T. et al. (2016) Population pharmacokinetic properties of artemisinin in healthy male Vietnamese volunteers. *Malar. J.* **15**, 90 https://doi.org/10.1186/s12936-016-1134-8
29 Tilley, L., Straimer, J., Gnädig, N.F., Ralph, S.A. and Fidock, D.A. (2016) Artemisinin action and resistance in *Plasmodium falciparum*. *Trends Parasitol.* **32**, 682–696 https://doi.org/10.1016/j.pt.2016.05.010
30 Hunt, P., Afonso, A., Creasey, A., Culleton, R., Sidhu, A.B.S., Logan, J. et al. (2007) Gene encoding a deubiquitinating enzyme is mutated in artesunateand chloroquine-resistant rodent malaria parasites. *Mol. Microbiol.* **65**, 27–40 https://doi.org/10.1111/j.1365-2958.2007.05753.x
31 Hunt, P., Martinelli, A., Modrzynska, K., Borges, S., Creasey, A., Rodrigues, L. et al. (2010) Experimental evolution, genetic analysis and genome re-sequencing reveal the mutation conferring artemisinin resistance in an isogenic lineage of malaria parasites. *BMC Genomics* **11**, 499 https://doi.org/10.1186/1471-2164-11-499
32 Borrmann, S., Straimer, J., Mwai, L., Abdi, A., Rippert, A., Okombo, J. et al. (2013) Genome-wide screen identifies new candidate genes associated with artemisinin susceptibility in *Plasmodium falciparum* in Kenya. *Sci. Rep.* **3**, 3318 https://doi.org/10.1038/srep03318
33 Henriques, G., Hallett, R.L., Beshir, K.B., Gadalla, N.B., Johnson, R.E., Burrow, R. et al. (2014) Directional selection at the *pfmdr1*, *pfcrt*, *pfubp1*, and *pfap2mu* loci of *Plasmodium falciparum* in Kenyan children treated with ACT. *J. Infect. Dis.* **210**, 2001–2008 https://doi.org/10.1093/infdis/jiu358
34 Henriques, G., Martinelli, A., Rodrigues, L., Modrzynska, K., Fawcett, R., Houston, D.R. et al. (2013) Artemisinin resistance in rodent malaria — mutation in the AP2 adaptor μ-chain suggests involvement of endocytosis and membrane protein trafficking. *Malar. J.* **12**, 118 https://doi.org/10.1186/1475-2875-12-118
35 Henriques, G., van Schalkwyk, D.A., Burrow, R., Warhurst, D.C., Thompson, E., Baker, D.A. et al. (2015) The mu subunit of *Plasmodium falciparum* clathrin-associated adaptor protein 2 modulates *in vitro* parasite response to artemisinin and quinine. *Antimicr. Agents Chemother.* **59**, 2540–2547 https://doi.org/10.1128/AAC.04067-14
36 Sutherland, C.J., Lansdell, P., Sanders, M., Muwanguzi, J., van Schalkwyk, D.A., Kaur, H. et al. (2017) *Pfk13*-independent treatment failure in four imported cases of *Plasmodium falciparum* malaria treated with artemether-lumefantrine in the United Kingdom. *Antimicrob. Agents Chemother.* **61**, e02382-16 https://doi.org/10.1128/AAC.02382-16