Understanding artemisinin-resistant malaria: what a difference a year makes

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Purpose of review
The emergence of artemisinin resistance in Southeast Asia (SEA), where artemisinin combination therapies (ACTs) are beginning to fail, threatens global endeavors to control and eliminate *Plasmodium falciparum* malaria. Future efforts to prevent the spread of this calamity to Africa will benefit from last year's tremendous progress in understanding artemisinin resistance.

Recent findings
Multiple international collaborations have established that artemisinin resistance is associated with slow parasite clearance in patients, increased survival of early-ring-stage parasites *in vitro*, single-nucleotide polymorphisms (SNPs) in the parasite's kelch protein gene (*K13*), parasite 'founder' populations sharing a genetic background of four additional SNPs, parasite transcriptional profiles reflecting an 'unfolded protein response' and decelerated parasite development, and elevated parasite phosphatidylinositol-3-kinase activity. In Western Cambodia, where the K13 C580Y mutation is approaching fixation, the frontline ACT is failing to cure nearly half of patients, likely due to partner drug resistance. In Africa, where dozens of *K13* mutations have been detected at low frequency, there is no evidence yet of artemisinin resistance.

Summary
In SEA, clinical and epidemiological investigations are urgently needed to stop the further spread of artemisinin resistance, monitor the efficacy of ACTs where *K13* mutations are prevalent, identify currently-available drug regimens that cure ACT failures, and rapidly advance new antimalarial compounds through preclinical studies and clinical trials.

Keywords
artemisinin, parasite kelch protein, malaria, *Plasmodium falciparum*, resistance

INTRODUCTION

For decades, Southeast Asia (SEA) has been ground zero for the evolution of drug-resistant *Plasmodium falciparum* malaria. After spawning generations of parasites resistant to chloroquine, sulfadoxine-pyrimethamine, quinine, and mefloquine, this region has now given rise to parasites resistant to artemisinins – the world's frontline antimalarial drugs. These include artemisinin and its derivatives artesunate and artemether, all of which are metabolized to the active compound dihydroartemisinin (DHA) *in vivo*. Parenteral artesunate has been highly efficacious in reducing malaria morbidity and mortality in SEA [1] and Africa [2]. Artemisinin combination therapies (ACTs) – oral coformulations of a potent, short-acting artemisinin and a less-potent, long-acting partner drug – have effectively reduced the world's malaria burden, but now face the clear and present danger of artemisinin resistance [3]. This is because higher numbers of parasites that survive exposure to the artemisinin component are now exposed to the partner drug alone. This larger parasite biomass is, thus, more likely to develop partner-drug resistance, which is readily defined by a triad of findings following directly-observed treatment with a high-quality ACT: recrudescent parasitemia within 28–42 days (depending on the ACT), identified by expert microscopic examination of weekly blood smears; adequate partner drug exposure, confirmed by measurement of drug plasma concentrations on day 7; and decreased in-vitro susceptibility of recrudescent parasites to the

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KEY POINTS

- Artemisinin resistance is defined as a parasite clearance half-life at least 5 h in patients, and a parasite survival rate at least 1% in the ring-stage survival assay *in vitro*.
- Parasite kelch protein (K13) mutations that confer artemisinin resistance are widespread in Southeast Asia; others are present at low frequency in Africa but are not yet associated with resistance.
- Artemisinin resistance in Southeast Asia is associated with parasite 'founder' populations that share a genetic background of K13 polymorphism and four other single-nucleotide polymorphisms.
- Artemisinin resistance is associated with an 'unfolded protein response' and decelerated parasite development, as well as elevated phosphatidylinositol-3-kinase activity and phosphatidylinositol-3-phosphate levels.
- In areas of Cambodia where artemisinin resistance is entrenched, the frontline artemisinin combination therapy dihydroartemisinin-piperaquine is failing fast, likely due to emerging piperaquine resistance.

partner drug, detected by an increase in its median inhibitory concentration (IC_{50}).

In contrast, artemisinin resistance has been more challenging to define, mostly because artemisinins act potently and rapidly to clear parasites from the bloodstream by a unique mechanism involving the spleen [4,5]. Artemisinins are considered prodrugs that are activated by heme iron-mediated cleavage of their endoperoxide moiety within the parasite, a process that forms reactive oxygen intermediates that target nucleophilic groups in parasite proteins and lipids. In patients with *P. falciparum* infection, this killing process can only be observed in the blood wherein intraerythrocytic ring-stage parasites develop and circulate for 24 h before they sequester in microvessels. When these ring stages are exposed to artemisinins, they consolidate into pyknotic forms resembling Howell–Jolly body inclusions that are efficiently cleared from the bloodstream by 'pitting,' a process whereby parasites are squeezed out of their host red blood cells (RBCs) as they pass through tight endothelial slits in the spleen, and the resultant 'once-infected' RBCs return intact to the peripheral blood. In patients treated with artemisinins, artemisinin-sensitive parasites rapidly undergo pyknosis and pitting, and, thus, show fast parasite clearance rates. These rates are measured by making frequent parasite counts until parasites are undetectable, log-transforming these counts and plotting them against time, identifying the linear portion of the resultant parasite clearance curve [6] using a 'Parasite Clearance Estimator' tool (http://www.wwarn.org/tools-resources/toolkit/analyse/parasite-clearance-estimator-pce) [7], and then calculating the parasite clearance half-life (in hours) from the slope of this line.

Artemisinin resistance was first reported from Pailin Province, Western Cambodia, as a slow parasite clearance rate in 2009 [8]. Since then, this clinical phenotype has been documented elsewhere in Cambodia [9,10▪▪], Vietnam [10▪▪,11,12], Thailand [10▪▪,13], Myanmar [10▪▪,14], and China [15▪]. Here, I review a recent series of clinical, epidemiological, genomics, and in-vitro studies that has rapidly transformed our understanding of artemisinin resistance in the human and parasite populations of these Southeast Asian countries.

WHAT IS THE DEFINITION OF ARTEMISININ RESISTANCE?

In Southeast Asian patients with uncomplicated *P. falciparum* malaria and a starting parasite count at least 10 000 parasites per μl of blood, artemisinin resistance is defined as a parasite clearance half-life at least 5 h following treatment with artesunate monotherapy or an ACT (http://www.who.int/malaria/publications/atoz/status_rep_artemisinin_resistance_sep2014.pdf?ua=1). This 5-h cutoff value reflects the upper limit of parasite clearance half-lives in areas without artemisinin-resistant parasites [10▪▪]. Importantly, parasite clearance half-lives in SEA are not significantly modified by age [13]; hemoglobin E, a polymorphism carried by 50% of Cambodians [9]; starting parasite density [9,13]; or relatively lower drug exposures, that is, parasite clearance half-lives were similar in patients randomized to receive 2 or 4 mg/kg artesunate [10▪▪]. Although immunity likely plays a role in parasite clearance in SEA, this has not yet been adequately studied, largely because age is a poor surrogate of adaptive immunity and no in-vitro correlate of parasite-clearing immunity has been established in this region.

To investigate whether adaptive immunity accelerates parasite clearance, two recent studies were conducted in a Malian village where artemisinin resistance is absent and age-dependent reductions in both malaria incidence and parasite density were clearly demonstrated [16]. In the first study [17], parasite clearance half-lives decreased significantly as age increased, suggesting that age-dependent immunity is involved in clearing ring-infected RBCs within hours of artesunate exposure. In the second study [18▪], younger children cleared their parasites mostly by pitting, suggesting they lacked immune responses that rapidly clear ring-infected RBCs, whereas older children cleared their parasites mostly

by a nonpitting, artemisinin-independent mechanism, suggesting they possessed such immune responses. As parasite clearance half-lives are likely influenced by immunity in many areas of Africa, site-specific, age-stratified data are needed to define baseline cutoff values for suspected artemisinin resistance in the future. In areas of Africa where malaria is being eliminated, the progressive loss of immunity may cause a lengthening of parasite clearance half-lives over time, which would not necessarily herald emerging artemisinin resistance.

Artemisinin resistance in *P. falciparum* has also been defined as a parasite survival rate at least 1% in the ring-stage survival assay ($RSA^{0-3\,h}$) *in vitro* [19]. In this assay, clinical parasite isolates are adapted to culture, synchronized at the early-ring stage (0–3 h postinvasion of RBCs), exposed to a pharmacologically-relevant dose of DHA for 6 h, and then cultured for an additional 66 h. The percentage of parasites surviving DHA exposure is then calculated as the ratio of parasites surviving exposure to DHA versus those surviving exposure to dimethyl sulfoxide (the DHA solvent). This assay discriminates two groups of parasites, one with less than 1% survival and another with at least 1% survival, which are generally defined as artemisinin sensitive and artemisinin resistant, respectively [19,20▪▪,21,22▪▪]. Importantly, this assay is unable to discriminate these two groups of parasites at the mid-ring and late-ring stages [19], suggesting that artemisinin resistance is an early-ring-stage phenotype. This finding may account for some discrepancies between parasite clearance half-lives and parasite survival rates in the $RSA^{0-3\,h}$ [19]. For example, parasite isolates that are artemisinin resistant in the $RSA^{0-3\,h}$ may clear rapidly in patients if they are circulating as mid-to-late ring stages during the time that parasite clearance is measured.

WHAT ARE THE GENETIC DETERMINANTS OF ARTEMISININ RESISTANCE?

Initial genome-wide association studies of parasite clearance half-life implicated two regions of parasite chromosome 13 in artemisinin resistance [23,24], but the specific genetic determinant(s) remained elusive. In a parallel investigation, Ariey *et al.* [20▪▪] successfully induced artemisinin resistance in a Tanzanian parasite line by exposing it to increasing doses of artemisinin *in vitro*. By comparing the whole-genome sequences of drug-selected and unselected parasite lines, they identified a single-nucleotide polymorphism (SNP) in the *PF3D7_1343700* gene on chromosome 13, which encodes a M476I substitution in the propeller domain of a kelch protein. When compared with a known mammalian ortholog Keap1, the parasite kelch protein ('K13') consists of *Plasmodium*-specific sequences, a bric-à-brac, tramtrack, broad-complex/poxvirus zinc fingers (BTB–POZ) domain, and a six-blade propeller domain (Fig. 1). Validation of K13-propeller polymorphism as a molecular marker of artemisinin resistance in Cambodia was achieved by showing that 17 different K13 mutations were present in parasites from this country (with each parasite clone carrying only one mutation); that the predominant C580Y mutation had rapidly increased in prevalence in areas of Western Cambodia where artemisinin resistance had become common; and that the C580Y, Y493H, and R539T mutations were associated with long parasite clearance half-lives and elevated $RSA^{0-3\,h}$ survival rates.

Multiple groups have since made rapid progress in demonstrating K13-propeller polymorphism as a marker of artemisinin resistance elsewhere in SEA, including Vietnam, Thailand, Myanmar, and China by associating the same and additional K13-propeller mutations with slow parasite clearance [10▪▪,15▪,25,26▪]. Molecular surveillance studies have greatly expanded the map of K13-propeller polymorphism to include additional areas of Cambodia [27], Thailand [28], Myanmar [29▪,30], China [30,31], and Bangladesh [32]; some of these mutations have been previously associated with slow parasite clearance at other sites, but most have not and require validation. Currently, C580Y predominates in Cambodia [20▪▪,26▪,27,33,34▪] and along the Thailand–Myanmar border [25,26▪,29▪], whereas F446I predominates along the Myanmar–China border [15▪,29▪, 31]. At present, it is unclear how C580Y is approaching fixation in Western Cambodia given that this mutation does not seem to confer higher $RSA^{0-3\,h}$ survival rates than other prevalent mutations (e.g., R539T and R543T) [20▪▪, 21,22▪▪]. Multiple studies in Africa have detected dozens of K13-propeller mutations – many of which have not yet been observed in SEA – at very low frequency in 17 countries [35▪,36–40] (http://biorxiv.org/content/early/2015/05/22/019737). Whether these K13-propeller mutations cause artemisinin resistance in patients and *in vitro* also awaits further investigation. Table 1 [10▪▪,15▪,20▪▪,21,22▪▪,25,26▪,27,28,29▪,30–32, 34▪,35▪,36–40] (http://biorxiv.org/content/early/2015/05/22/019737) lists all K13-propeller mutations discovered to date, according to their geographic location and association with artemisinin resistance.

In population genetics studies of artemisinin resistance, several surprising and unprecedented findings were made [41,42▪▪]. First, multiple parasite 'founder' populations were identified in Cambodia and Vietnam. These groups of highly-differentiated, clonal subpopulations are as different from each other as each of them is to African parasites, suggesting they have undergone extreme recent bottlenecking and subsequent expansion. Second, seven of the 11

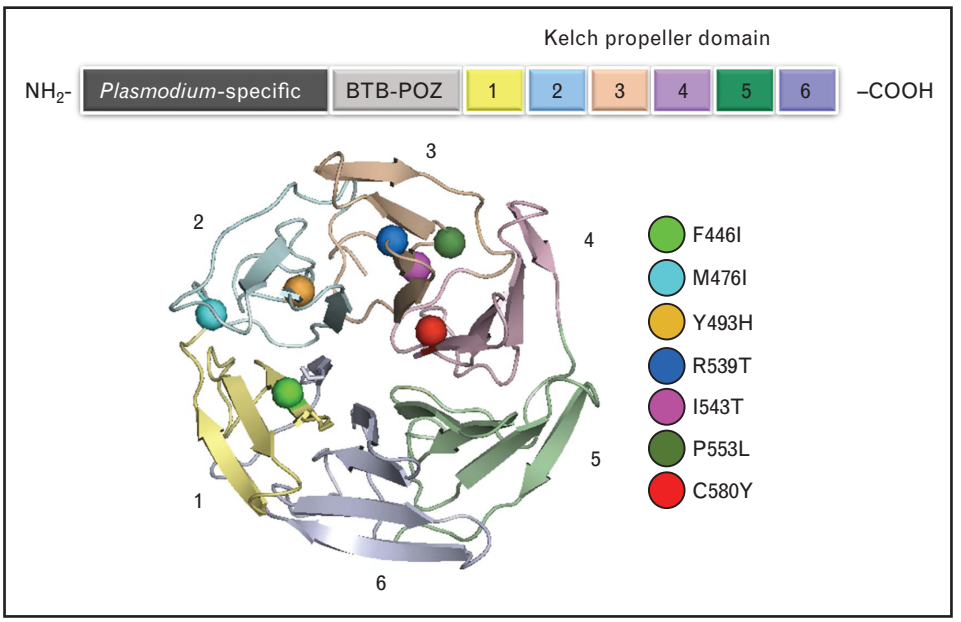

FIGURE 1. *Plasmodium falciparum* kelch13 protein (K13). K13 consists of *Plasmodium*-specific sequences, a bric-à-brac, tramtrack, broad-complex/poxvirus zinc fingers (BTB-POZ) domain, and six kelch domains that are predicted to form a six-blade propeller. In the structural model, the original M476I mutation discovered by Ariey *et al.* [20▪▪] and six other mutations associated with artemisinin resistance in Southeast Asia are shown. Adapted from [20▪▪]. Molecular structure courtesy of Dr Odile Puijalon, Institut Pasteur, Paris, France.

founders were found to be artemisinin resistant in patients [41,42▪▪], and three of them were additionally confirmed to be artemisinin resistant in the $RSA^{0-3\,h}$ [21], suggesting that most of them were naturally selected by artemisinins. Third, each founder was tagged by a single K13-propeller mutation, with the C580Y mutation independently emerging on three different founders in Cambodia. Fourth, all seven artemisinin-resistant founders share a common genetic background comprised of four SNPs in genes encoding apicoplast ribosomal protein s10 (*arps10* V127 M), ferredoxin (*fd* D193Y), multidrug resistance 2 transporter (*mdr2* T484I), and chloroquine resistance transporter (*crt* N326S) [42▪▪]. The roles of these mutations in the natural selection of these founders are unknown, but are likely to include increases in fitness. Some possibilities are that they compensate for putative deleterious effects of K13-propeller mutations; potentiate resistance to artemisinins; mediate resistance to previously used drugs (i.e., chloroquine, sulfadoxine-pyrimethamine, quinine, and doxycycline) or currently used ACT partner drugs (i.e., mefloquine, piperaquine, and lumefantrine); or increase parasite transmission to *Anopheles* mosquito vectors.

WHAT IS THE MOLECULAR MECHANISM OF ARTEMISININ RESISTANCE?

As mammalian kelch proteins can detect oxidants and other stressors, K13-propeller mutations were reasonably implicated in mediating resistance to artemisinin [20▪▪], a prooxidant drug. In one hypothetical model of artemisinin sensitivity and resistance (Fig. 2a), wildtype K13 binds a putative transcription factor and delivers it to ubiquitin ligase, which targets it for proteosomal degradation. When wildtype K13 senses oxidants like artemisinins, it undergoes a conformational change to liberate the transcription factor, which then upregulates the expression of genes involved in counteracting oxidative damage. In this model, the response of wildtype parasites is believed to be too little too late, such that the action of artemisinins is too potent and too rapid for parasites to successfully overcome. In artemisinin-resistant parasites, on the contrary, K13-propeller mutations destabilize the K13-transcription factor interaction, leading to constitutive activation of transcriptional changes that 'prime' the parasite to withstand oxidative damage caused by artemisinins.

Given the logical assumption that K13-propeller mutations mediate artemisinin resistance, Straimer *et al.* [22▪▪] tested this hypothesis directly by using zing-finger nuclease technology to edit the *K13* locus in contemporary Cambodian parasite isolates. When three different K13-propeller mutations (C580Y, R539T, and I543T) were edited to the wildtype sequence, the artemisinin-resistance phenotype – as measured in the $RSA^{0-3\,h}$ – was completely lost. They also showed that the introduction of five

Table 1. K13-propeller mutations, according to propeller blade number, geographic location, and artemisinin-resistance association

1	**P441L**	F442	P443S	L444	V445	**F446I**	C447	I448	**G449A**	G449S
	G449D	G450	F451	D452E	G453	V454I	E455	Y456	L457	**N458Y**
	N458I	S459L	M460	E461	L462	L463S	D464	I465T	S466	Q467
	Q468	**C469Y**	C469F	W470	R471	M472	C473	T474I		
2	P475	*M476I*	S477	T478P	K479I	K480	**A481V**	Y482	483S	G484
	S485N	A486	V487I	L488	N489	N490T	F491	L492S	***Y493H***	V494I
	F495L	G496F	G497	N498	N499D	Y500	D501G	Y502	K503	A504
	L505	F506	E507	T508N	E509	V510	Y511M	D512	R513	L514
	R515T	D516Y	V517	W518	Y519	V520I	V520A	S521	**S522C**	N523
	L524	N525D	I526							
3	P527H	R528T	R529	N530	N531	C532S	G533A	G533S	V534L	V534I
	T535	S536	N537I	**G538V**	***R539T***	I540	Y541	C542Y	***I543T***	G544R
	G545E	Y546	D547	G548S	S549	S550	I551	I552	**P553L**	N554D
	V555	E556D	A557S	Y558H	D559	H560	**R561H**	R561C	M562	K563
	A564	W565	V566I	E567	**V568G**	A569T	P570	L571	N572	T573S
4	**P574L**	R575K	R575G	S576L	S577	A578S	M579	***C580Y***	V581F	A582
	F583	**D584V**	D584N	N585	K586	I587	Y588	V589I	I590	G591
	G592	T593S	N594	G595S	E596	R597	L598	N599	S600	I601
	E602	V603	Y604	E605	E606	K607	M608L	N609	K610	W611
	E612D	Q613E	Q613L							
5	F614L	P615	Y616	A617T	A617V	L618	L619S	E620	A621F	R622
	S623C	S624	G625	A626P	A626T	A627	F628	N629Y	Y630F	L631
	N632	Q633	I634	Y635	V636	V637I	V637A	V637D	G638R	G639V
	I640	D641	N642	E643	H644	N645	I646	L647	D648	S649
	V650	E651	Q652	Y653	Q654	P655	F656	N657	K658	R659
	W660	Q661	F662	L663	N664	G665				
6	V666A	P667A	P667L	E668	K669	K670	M671	N672	**F673I**	G674
	A675V	A676S	A676D	T677	L678	S679	D680N	S681	Y682	I683
	I684	T685	G686	G687	E688	N689	G690	E691	V692	L693
	N694	S695	C696	H697	F698	F699	S700	P701	D702	T703
	N704	E705	W706	Q707	L708	G709	P710	S711	L712	L713
	V714	P715	R716	F717	G718	**H719N**	S720	V721	L722	I723
	A724	N725	I726							

Plasmodium falciparum kelch13 protein (K13)-propeller amino acids are stratified by propeller blade numbers 1 through 6. All mutations are compared with the *P. falciparum* 3D7 reference sequence, version 3. Colors indicate mutations observed only in Southeast Asia (blue), only in Africa (yellow), and in both Southeast Asia and Africa (green). Bold type indicates mutations associated with parasite clearance half-life at least 5 h in at least one Southeast Asian patient with uncomplicated malaria [10▪▪,15▪,20▪▪,25,26▪,27,28,29▪,30–32,34▪,35▪,36–38,40] (http://biorxiv.org/content/early/2015/05/22/019737). The A578S mutation has been associated with parasite clearance half-life at least 5 h in three Ugandan children with severe malaria [39]. Italic type indicates mutations associated with more than 1% survival in the ring-stage survival assay $RSA^{0-3\,h}$ [20▪▪,21,22▪▪].

different K13-propeller mutations confer increasing levels of resistance to the Indochinese Dd2 parasite line (Y493H<C580Y<M476I<R539T<I543T), and that introduction of the C580Y mutation confers higher levels of resistance to contemporary parasite isolates from Cambodia than to older parasite lines from Indochina. These data provide compelling evidence that different K13-propeller mutations mediate different levels of artemisinin resistance, and that the level of resistance can be influenced by parasite genetic background. Evidence that C580Y confers artemisinin resistance to the African NF54 parasite line has also been reported [43].

Although these studies established that K13 mutations confer artemisinin resistance to a variety of parasite clinical isolates and laboratory lines, additional studies were needed to further define the molecular mechanism. In a large population

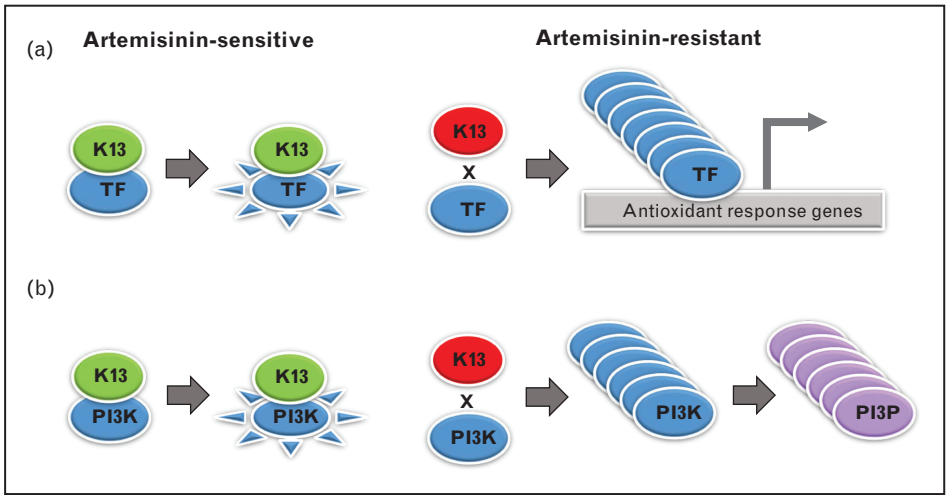

FIGURE 2. Two proposed mechanisms of artemisinin sensitivity and resistance in *Plasmodium falciparum*. (a) In artemisinin-sensitive parasites, wildtype *Plasmodium falciparum* kelch13 protein (K13) (green) binds a putative transcription factor and targets it for degradation. In artemisinin-resistant parasites, on the contrary, mutant K13 (red) fails to bind this putative transcription factor, which is then free to upregulate genes involved in the antioxidant response. In this 'protected' state, parasites are better prepared to handle the oxidative stress imposed by activated artemisinins, for example, by refolding oxidatively-damaged proteins. (b) In artemisinin-sensitive parasites, wildtype K13 binds phosphatidylinositol-3-kinase (PI3K) and targets it for degradation. In artemisinin-resistant parasites, on the contrary, mutant K13 fails to bind PI3K, leading to increased PI3K activity and phosphatidylinositol-3-phosphate (PI3P) levels. In this 'protected' state, high PI3P levels are presumably able to promote the survival of parasites exposed to artemisinins, for example, my mediating membrane fusion events involved in parasite growth. Adapted from [47▪▪].

transcriptomics study of *P. falciparum* isolates obtained directly from Southeast Asian patients with malaria [44▪▪], Mok *et al.* first identified a subset of parasites that was collected at the early-ring stage of development, that is, when the artemisinin-resistance phenotype is expressed. In analyzing the transcriptional profiles of these isolates against a wide range of corresponding half-lives, these investigators found that artemisinin resistance is associated with increased expression of an 'unfolded protein response' pathway involving two major chaperone complexes: *Plasmodium* reactive oxidative stress complex (PROSC) and TCP-1 ring complex (TRiC). Artemisinin-resistant clinical isolates also showed transcriptional evidence of delayed progression through the intraerythrocytic lifecycle upon cultivation *ex vivo*. These two transcriptional phenotypes are closely linked to K13-propeller mutations, and may enable parasites to survive artemisinin by first repairing their oxidatively-damaged proteins before progressing through their cell cycle. Future work is needed to integrate these findings with those of two more-recent studies that describe an enhanced cell-stress response [45] and altered patterns of development [46] in artemisinin-resistant parasites.

Further progress in exploring mechanisms of artemisinin sensitivity and resistance was recently provided by Mbengue *et al.* [47▪▪], who report evidence that artemisinins target the sole *P. falciparum* phosphatidylinositol-3-kinase (PI3K), and that PI3K is the putative binding partner of K13. In their model of artemisinin sensitivity (Fig. 2b), wildtype K13 binds PI3K and delivers it to ubiquitin ligase, which polyubiquitinates K13 and marks it for proteosomal degradation. As these parasites have low basal levels of phosphatidylinositol-3-phosphate (PI3P), the product of PI3K activity, they are highly sensitive to artemisinins, which inhibit PI3K and, thus, prevent the increase in PI3P levels that is presumably needed for parasite growth (PI3P levels normally increase as parasites develop from rings to schizonts). In their corresponding model of artemisinin resistance, mutant K13 fails to bind PI3K. PI3K, thus, avoids being degraded, resulting in high basal levels of PI3K and its product PI3P. As resistant parasites already have high basal levels of PI3P when exposed to artemisinin, they can better withstand the PI3K-inhibiting effects of this drug and, thus, continue their PI3P-dependent growth. How elevated levels of PI3P might mediate artemisinin resistance is not known, but one possibility is that PI3P is involved in membrane biogenesis and fusion events required for parasite growth. Future work is needed to integrate these findings with those of the aforementioned population transcriptomics study [44▪▪], which found no association between PI3K transcript levels and either parasite

clearance half-lives or K13-propeller mutations, and to reconcile two very disparate artemisinin modes of action: namely, nonspecific oxidation of multiple parasite proteins versus specific inhibition of PI3K.

WHAT IS THE CLINICAL IMPACT OF ARTEMISININ RESISTANCE?

It is important to emphasize that ACTs still cure patients with slow parasite clearance, provided that the partner drug remains effective. However, slow parasite clearance in ACT-treated patients causes more parasites to be exposed to partner drugs alone, increasing their chance of developing resistance to these drugs and causing ACT failures. As predicted, DHA-piperaquine is now failing to cure malaria in Western Cambodia, where artemisinin resistance is most entrenched. Compared with earlier studies that documented 98% DHA-piperaquine efficacy, three recent studies have reported reduced efficacy in this region. In the first study [48], efficacy in Pailin and Pursat Provinces was 75 and 89% in 2008–2010. In the second study [33], in which data were pooled by region, efficacy was 85% in four Western Cambodian provinces (where artemisinin resistance is common) compared with 98% in four eastern Cambodian provinces (where artemisinin resistance is uncommon) in 2011–2013. In this study, the most significant risk factor for treatment failure was the presence of a resistance-associated K13-propeller mutation. In the third study [34▪], efficacy in Oddar Meancheay Province was 46% in 2012–2014. In this study, a significant risk factor for treatment failure was the presence of the K13 C580Y mutation and two other SNPs on chromosomes 10 and 13 that were previously associated with slow parasite clearance [23]. Surprisingly, all three studies were unable to associate treatment failures with elevated piperaquine IC_{50} values *in vitro*. As high ACT failure rates in SEA have only been observed in areas where resistance to the partner drug exists, it is likely that piperaquine resistance has indeed emerged. Although this possibility is further suggested by increasing piperaquine IC_{50} values within multiple study sites over time (unpublished data; [49]), more robust evidence of piperaquine resistance is needed to identify its genetic markers, elucidate its molecular mechanism, and discover new drugs that circumvent it. Meanwhile, artesunate-mefloquine may be an effective treatment for DHA-piperaquine failures, as suggested by a contemporaneous reduction in mefloquine IC_{50} values and disappearance of the multicopy *pfmdr1* genotype – a molecular marker of mefloquine resistance [33,49,50].

CONCLUSION

The aggressive global use of ACTs was expected to weaken malaria's stranglehold on the health and economies of the world's most impoverished communities. Unfortunately, the eventual spread of artemisinin resistance from SEA, where ACTs have begun to fail, to Africa, where the world's greatest malaria transmission, morbidity, and mortality occur, seems likely. Multiple international collaborations have defined in-vivo and in-vitro correlates of artemisinin resistance, identified its causal genetic determinant, begun to elucidate its molecular mechanism, and assessed its clinical impact. These collaborative efforts should now be extended to monitor ACT efficacy in areas where K13-propeller mutations are prevalent, test whether currently available drugs cure ACT failures, and advance newly-developed antimalarial compounds into clinical trials.

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Conflicts of interest

There are no conflicts of interest.

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Papers of particular interest, published within the annual period of review, have been highlighted as:

- ▪ of special interest
- ▪▪ of outstanding interest

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This study mapped artemisinin resistance (parasite clearance half-life $\geq$5 h) in multiple sites across SEA, showing that K13-propeller polymorphism is also a molecular marker of this phenotype outside Cambodia. It greatly increased the number of K13-propeller mutations associated with long parasite clearance half-life, and associated K13-mutant, slow-clearing parasites with increased pretreatment and posttreatment gametocytemia, suggesting that artemisinin-resistant parasites have a potential transmission advantage. This study, thus, argues strongly for the use of single low-dose primaquine to kill mature gametocytes and stop the spread of artemisinin-resistant parasites.

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This study associated the K13 F446I mutation with artemisinin resistance in Southern China, along its border with Myanmar. The prevalence of F446I is very high (36.5%) in this region, placing it at risk of ACT failures in the near future.

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18. ■ Ndour PA, Lopera-Mesa TM, Diakite SA, *et al.* Plasmodium falciparum clearance is rapid and pitting independent in immune Malian children treated with artesunate for malaria. J Infect Dis 2015; 211:290–297.

This study showed that in Mali, where *P. falciparum* is artemisinin sensitive, older children clear their parasites following artesunate treatment faster than younger children. This clearance is due to a nonpitting, artemisinin-independent mechanism (likely age-dependent adaptive immunity), which removes ring-infected RBCs from circulation and, thus, hastens parasite clearance. Such immunity may forestall the development of ACT resistance in Africa by helping to decrease the parasite biomass exposed to artemisinins and ACT partner drugs.

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20. ■■ Ariey F, Witkowski B, Amaratunga C, *et al.* A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. Nature 2014; 505:50–55.

This study described K13-propeller polymorphism as a molecular marker of artemisinin resistance *in vivo* and *in vitro* by associating several mutations with long parasite clearance half-lives and elevated $RSA^{0\text{-}3\,h}$ survival rates in Cambodia. Although the C580Y mutation predominated in Western Cambodia, 16 additional mutations were identified in the country, thus indicating that molecular surveillance for artemisinin resistance will require sequencing the entire K13-propeller region. These discoveries enabled the Tracking Resistance to Artemisinin Collaboration and other research groups to rapidly map the contemporary prevalence of this marker throughout Southeast Asia and Africa.

21. Amaratunga C, Witkowski B, Dek D, *et al.* Plasmodium falciparum founder populations in western Cambodia have reduced artemisinin sensitivity in vitro. Antimicrob Agents Chemother 2014; 58:4935–4937.
22. ■■ Straimer J, Gnadig NF, Witkowski B, *et al.* Drug resistance. K13-propeller mutations confer artemisinin resistance in Plasmodium falciparum clinical isolates. Science 2015; 347:428–431.

This study showed that K13-propeller polymorphism is not only a marker of artemisinin resistance in SEA, but also causes this phenotype. Using zinc-finger nucleases to edit K13-propeller mutations to the wildtype sequence in parasite clinical isolates from Cambodia, this study established that K13-propeller polymorphism confers artemisinin resistance (increased $RSA^{0\text{-}3\,h}$ survival rate). The study also showed that different K13-propeller mutations confer different levels of artemisinin resistance to the Indochinese Dd2 parasite line, and that the predominant C580Y mutation confers greater levels of resistance to contemporary clinical isolates than to older laboratory-adapted lines from Indochina. Collectively, these data suggest that the emergence of artemisinin resistance is driven not only by K13-propeller mutations, but also parasite genetic background.

23. Takala-Harrison S, Clark TG, Jacob CG, *et al.* Genetic loci associated with delayed clearance of Plasmodium falciparum following artemisinin treatment in Southeast Asia. Proc Natl Acad Sci U S A 2013; 110:240–245.
24. Cheeseman IH, Miller BA, Nair S, *et al.* A major genome region underlying artemisinin resistance in malaria. Science 2012; 336:79–82.
25. Nyunt MH, Hlaing T, Oo HW, *et al.* Molecular assessment of artemisinin resistance markers, polymorphisms in the k13 propeller, and a multidrug-resistance gene in the eastern and western border areas of Myanmar. Clin Infect Dis 2015; 60:1208–1215.
26. ■ Takala-Harrison S, Jacob CG, Arze C, *et al.* Independent emergence of artemisinin resistance mutations among Plasmodium falciparum in Southeast Asia. J Infect Dis 2015; 211:670–679.

This study showed that some K13-propeller mutations (e.g., C580Y) have independently emerged in different parasite populations in different regions of SEA, and that parasites do not seem to be spreading from Cambodia to Myanmar. These findings have important implications for the containment of artemisinin resistance in SEA, in that they increase the focus on preventing the local emergence of resistant parasites.

27. Bosman P, Stassijns J, Nackers F, *et al.* Plasmodium prevalence and artemisinin-resistant falciparum malaria in Preah Vihear Province, Cambodia: a cross-sectional population-based study. Malar J 2014; 13:394–403.
28. Talundzic E, Okoth SA, Congpuong K, *et al.* Selection and spread of artemisinin-resistant alleles in Thailand prior to the global artemisinin resistance containment campaign. PLoS Pathog 2015; 11:e1004789–e1004803.
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This study showed that K13-propeller mutations are highly prevalent in Myanmar, being found in 39% of samples from 55 sites in 10 administrative districts in 2013–2014. This prevalence is even higher (47%) in a region only 25 km from the Myanmar–India border, suggesting the very real possibility that artemisinin-resistant parasites have already spread locally into India.

30. Feng J, Zhou D, Lin Y, *et al.* Amplification of pfmdr1, pfcrt, pvmdr1, and K13 propeller polymorphisms associated with Plasmodium falciparum and Plasmodium vivax isolates from the China-Myanmar border. Antimicrob Agents Chemother 2015; 59:2554–2559.
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This study showed that DHA-piperaquine treatment failures are occuring in 54% of patients in Oddar Meanchey Province in 2013–2014, thus confirming ACT resistance in a fifth Western Cambodian province. The presence of the K13-propeller C580Y mutation and two additional SNPs was associated with a 5.4-fold greater risk of treatment failure and higher piperaquine IC_{50} *in vitro*, linking artemisinin resistance to the evolution of piperaquine resistance.

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This study showed that multiple K13-propeller mutations are present at low frequency throughout sub-Saharan Africa, and that none of them have been associated with artemisinin resistance in SEA. Given that many of these mutations have not been observed in SEA (where other mutant alleles have been naturally selected to high frequency) and there is limited allele sharing between parasite populations, these data suggest a large reservoir of naturally-occurring K13 variation in Africa from which resistance alleles may be selected in the future.

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38. Ouattara A, Kone A, Adams M, *et al.* Polymorphisms in the K13-propeller gene in artemisinin-susceptible Plasmodium falciparum parasites from Bougoula-Hameau and Bandiagara, Mali. Am J Trop Med Hyg 2015; 92:1202–1206.
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This study associates artemisinin-resistant founder populations with the presence of K13-propeller polymorphism and a particular genetic background comprised of four SNPs in genes encoding apicoplast ribosomal protein s10 (*arps10* V127M), ferredoxin (*fd* D193Y), multidrug resistance 2 transporter (*mdr2* T484I), and chloroquine resistance transporter (*crt* N326S). This finding, which was observed both in the Greater Mekong Subregion and along the Thailand–Myanmar border, strongly suggests that K13-propeller mutations by themselves are not naturally selected in the human and mosquito populations of SEA. Instead, it seems that additional SNPs are needed to increase fitness in these geographical settings. The finding that this genetic background is not seen in Africa suggests that the naturally-occurring K13-propeller mutations observed on this continent cannot yet give rise to artemisinin-resistant subpopulations. The genetic background of four SNPs may, therefore, be considered a molecular marker for identifying areas most at risk of artemisinin resistance in the future.

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44. Mok S, Ashley EA, Ferreira PE, *et al.* Drug resistance. Population transcrip-
■■ tomics of human malaria parasites reveals the mechanism of artemisinin resistance. Science 2015; 347:431–435.
This study reports the largest ex-vivo transcriptomics study of any infectious disease. By analyzing the transcriptional profiles of more than 1000 *P. falciparum* clinical isolates, this study identified parasites at the early-ring stage of parasite development that expressed a transcriptional profile associated with long parasite clearance half-lives and K13-propeller mutations. This profile indicates that parasites, before they have encountered artemisinin in the patient, have already decelerated their growth and upregulated their 'unfolded protein response' pathway. Together, these findings suggest that parasites have evolved to anticipate artemisinin exposure, and are, thus, better able to first repair oxidative protein damage before progressing through their lifecycle. The identification of two upregulated chaperone complexes (PROSC and TRiC), with multiple molecular components, has opened up myriad new avenues for future investigation.

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■■ artemisinin resistance in Plasmodium falciparum malaria. Nature 2015; 520:683–687.
This study reports a novel candidate mechanism of artemisinin action and resistance. Artemisinins are proposed to target *P. falciparum* PI3K, leading to decreased PI3K activity, decreased PI3P levels, and inhibition of parasite growth. To overcome this effect, artemisinin-resistant parasites have evolved mutant K13-propeller proteins that fail to bind PI3K and to mark it for degradation. The resultant increases in PI3K activity and PI3P levels enable artemisinin-exposed parasites to continue their intraerythrocytic development until drug levels decrease and PI3K activity is restored. Future studies are needed to reconcile these findings with those of a recent population transcriptomics study showing that the PI3K transcript levels of artemisinin-sensitive and artemisinin-resistant parasites are not significantly different, and the widely-held belief that artemisinins act through an oxidation mechanism.

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