

Antimalarial Drug Resistance: A Threat to Malaria Elimination

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Increasing antimalarial drug resistance once again threatens effective antimalarial drug treatment, malaria control, and elimination. Artemisinin combination therapies (ACTs) are first-line treatment for uncomplicated falciparum malaria in all endemic countries, yet partial resistance to artemisinins has emerged in the Greater Mekong Subregion. Concomitant emergence of partner drug resistance is now causing high ACT treatment failure rates in several areas. Genetic markers for artemisinin resistance and several of the partner drugs have been established, greatly facilitating surveillance. Single point mutations in the gene coding for the Kelch propeller domain of the K13 protein strongly correlate with artemisinin resistance. Novel regimens and strategies using existing antimalarial drugs will be needed until novel compounds can be deployed. Elimination of artemisinin resistance will imply elimination of all falciparum malaria from the same areas. In vivax malaria, chloroquine resistance is an increasing problem.

The two main pillars for malaria control and beyond remain targeting the anopheline mosquito vector and effective case management, which is crucially dependent on the efficacy of the deployed antimalarial drugs (Bhatt et al. 2015). Antimalarial drug resistance in *Plasmodium falciparum* tends to emerge in low-transmission settings, in particular in Southeast Asia or South America, before expanding to high-transmission settings in sub-Saharan Africa (White 2004). Resistance to chloroquine and later to sulfadoxine–pyrimethamine have followed this route and have contributed to millions of excess malaria attributable mortality in African children (Trape et al. 1998; Trape 2001;

Korenromp et al. 2003). At the end of the last century, introduction of the artemisinin combination therapies (ACTs) provided a much needed, highly efficacious antimalarial treatment, which became the first-line treatment for uncomplicated falciparum malaria in all endemic countries (WHO 2001, 2015a). Parenteral artesunate became the first-line treatment for severe malaria. However, partial artemisinin resistance characterized by much slower clearance of parasitemia in the first 3 days of treatment following artemisinin monotherapy or ACT was identified in western Cambodia in 2008–2009 (Noedl et al. 2008; Dondorp et al. 2009), and subsequently in all countries of the Greater

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D. Menard and A. Dondorp



Mekong Subregion (Amaratunga et al. 2012; Hien et al. 2012; Phyo et al. 2012; Ashley et al. 2014; Huang et al. 2015). Artemisinin resistance has selected for concomitant resistance to ACT partner drugs, resulting in high late treatment failure rates with dihydroartemisinin–piperaquine in Cambodia (Leang et al. 2013, 2015; Lon et al. 2014; Saunders et al. 2014; Duru et al. 2015; Spring et al. 2015; Amaratunga et al. 2016) and with artesunate–mefloquine on the Thai–Myanmar border (Carrara et al. 2013). Close surveillance of the emergence and the distribution of artemisinin and partner drugs resistance are important to guide public health measures. This will require drug efficacy studies in sentinel sites, but can be greatly facilitated by the increasing availability of genetic markers for antimalarial drug resistance. New antimalarial treatments are urgently needed. It is expected that new compounds will not be ready for deployment before 2020 (Wells et al. 2015). Until then, novel strategies and regimes using existing antimalarial drugs will have to be implemented to ensure effective treatment. Elimination of artemisinin resistance will imply elimination of all falciparum malaria from the same areas before falciparum malaria becomes untreatable (Maude et al. 2009). This paradigm has contributed to the adoption of a malaria elimination agenda for the Greater Mekong Subregion, which also includes vivax malaria (WHO 2015b). In this respect, increasing resistance to *Plasmodium vivax* to chloroquine in Indonesia and beyond is an important notice.

This article discusses driving forces of antimalarial drug resistance, the global antimalarial drug resistance situation for *P. falciparum* and *P. vivax*, current insights in the molecular markers and mechanisms of antimalarial drug resistance with a focus on the artemisinins, and possible strategies for the treatment of artemisinin and multiple drug-resistant malaria in the context of malaria elimination.

ORIGINS OF ANTIMALARIAL DRUG RESISTANCE

De novo emergence of antimalarial drug resistance requires the spontaneous arising of mu-

tations or gene duplications conferring reduced drug susceptibility, which is then selected in the individual by the presence of antimalarial drug concentrations sufficient to kill or inhibit the growth of sensitive parasites, but allowing expansion of the resistant clone. For the resistant parasite to be successful, the gene alterations conferring resistance should not affect parasite fitness to a large extent (White 2004; Barnes and White 2005). Drug-resistant mutations can arise in the sexual parasite stages in the mosquito (where diploidy and meiosis occur), in the preerythrocytic liver stages or in the asexual erythrocytic parasite stages, and there has been much debate on the most likely source (Pongtavornpinyo et al. 2009). It seems that resistant parasites are most likely to emerge during high levels of asexual-stage parasitemia in patients with subtherapeutic drug levels and, less likely, in the liver stages (Pongtavornpinyo et al. 2009; White et al. 2009). Antimalarial drugs will be more prone to resistance when requiring a limited number of genetic events conferring a considerable level of resistance (such as atovaquone or mefloquine), and when its pharmacokinetic properties include a long terminal half-life translating into a long period of subtherapeutic drug levels (such as piperaquine). Once resistance starts emerging, its transmission and, thus, spread are facilitated by the increased production of gametocytes in partial resistant strains, as shown, for instance, for sulfadoxine–pyrimethamine (Barnes et al. 2008).

Although the total number of circulating *P. falciparum* parasites and, thus, the number of spontaneous genetic events is much higher in high transmission settings in sub-Saharan Africa, history shows that antimalarial drug resistance is much more likely to emerge successfully in low transmission settings. In particular, Southeast Asia has in the last decades been the cradle for the emergence of *P. falciparum* resistance to chloroquine (Eyles et al. 1963; Young et al. 1963), sulfadoxine–pyrimethamine (Hofler 1980; Hurwitz et al. 1981), mefloquine (Boudreau et al. 1982; Smithuis et al. 1993), and more recently to artemisinins (Noedl et al. 2008; Dondorp et al. 2009) and piperaquine (Leang et al. 2013; Saunders et al.

2014). An important reason for this apparent brake on resistance emergence in regions with high stable transmission is host immunity, which can contribute substantially to parasite clearance of partial resistant parasite and also makes that older children and adults can carry substantial numbers of parasites without causing illness (Sarda et al. 2009; Lopera-Mesa et al. 2013). Because these individuals will not seek treatment, the associated large asymptomatic reservoir dilutes the selective pressure provided by antimalarial drugs at the population level (White 2004). In addition, in high transmission areas, patients have multiple strain infections transmitted to the mosquito vector. Crossing over of genes during meiosis in the mosquito can then break up resistance and compensatory mutations, and this greater opportunity for recombination will result in increased parasite diversity and direct competition between different parasite strains, with less opportunity for resistant alleles to become fixed (Jiang et al. 2011; Takala-Harrison and Laufer 2015). This is not the case in low transmission areas where multiple infections are much less common, infected individuals are less preimmune, usually more prone to be symptomatic, and, as a consequence, to be treated with possible poor-quality antimalarial drugs, incomplete treatment courses, or (artemisinin) monotherapies. *P. falciparum* parasite populations in Southeast Asia are highly structured with high rates of parasite inbreeding; particular genetic background alleles seem to predispose to the development of resistance-causing mutations through multi-stage processes in natural parasite populations (Miotto et al. 2013). Moreover, hemoglobinopathies (mainly HbAE or HbEE) and glucose-6-phosphate dehydrogenase deficiency, which are highly prevalent in Southeast Asian human populations, may have selected parasites less susceptible to oxidative stress while most antimalarial drugs currently in clinical use exert their activities, at least in part, by increasing oxidative stress in the parasitized erythrocyte (Becker et al. 2004).

Poor drug stewardship has been an important driver of antimalarial drug resistance, and in particular the emergence of artemisinin re-

sistance in Southeast Asia. In the early 1960s, pyrimethamine and later chloroquine were added to salt for consumption as a measure of population malaria prophylaxis in Cambodia (Verdrager 1986). Although the artemisinins have been deployed as combination therapies in ACTs, unregulated artemisinin or artesunate monotherapy has been available since the mid-1970s in the region. In most countries, including Cambodia where artemisinin resistance was first recognized, the majority of patients obtain their antimalarial treatment through the private sector, which consisted until recent years mainly of artesunate monotherapy (Yeung et al. 2008). A ban on artemisinin monotherapies and deployment of fixed dose combinations for the majority of ACTs have been an important step forward. Counterfeited or substandard drugs that contain less active ingredients than stated are additional sources of subtherapeutic dosing of artemisinins, which may also have contributed to the selection of resistant parasite strains (Newton et al. 2003). Moreover, it is possible that the different pharmacokinetic properties of artemisinins in subgroups of the population, such as pregnant women and children, have resulted in underdosing (Kloprogge et al. 2015). It is thought that an important driver of the rapid spread of resistance to sulfadoxine-pyrimethamine in Africa has been underdosing of the drug in children with falciparum malaria (Barnes et al. 2006).

CURRENT MAP OF ANTIMALARIAL DRUG RESISTANCE IN *P. falciparum* AND *P. vivax*

The emergence and spread of antimalarial drug resistance is a dynamic process that can change by year. Figure 1 provides an overview of the current situation of falciparum artemisinin resistance (Fig. 1A) and vivax chloroquine resistance (Fig. 1B). For updated information, there are several sources intending to provide information in real time on the global antimalarial drug-resistance situation. The World Health Organization (WHO) maintains a network of sentinel sites in malaria-endemic countries performing therapeutic efficacy studies of first- and second-line antimalarial drugs using a

D. Menard and A. Dondorp

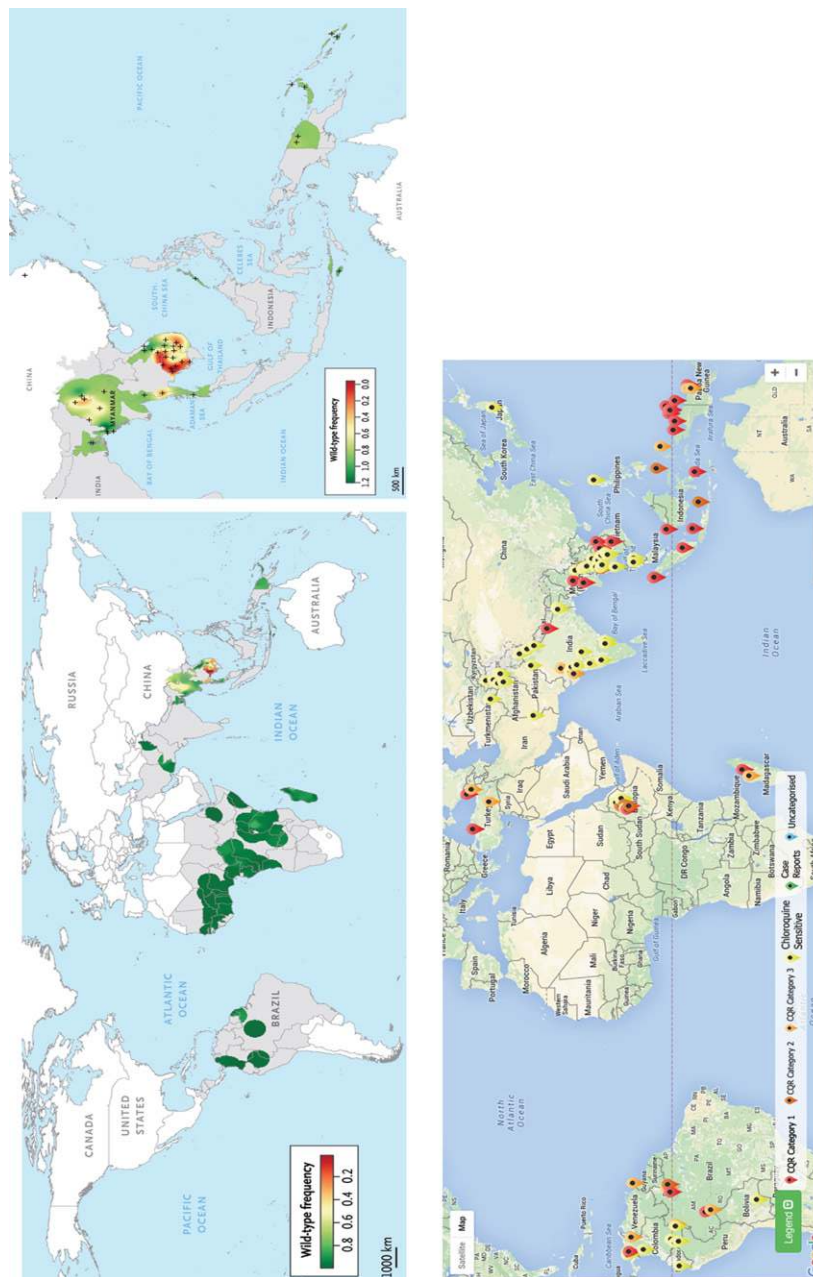


Figure 1. Overview of the current situation of falciparum artemisinin resistance (A), and vivax chloroquine resistance (B). (A) Frequency distribution of the wild-type K13 allele worldwide (left panel) and in Asia (right panel). Malaria-endemic areas are considered malaria free. The mean frequency of the wild-type allele is shown using the color code shown in the inset. Data were interpolated using two different approaches and the map censored for regions with very low to nil reported malaria prevalence. To generate the world map (left panel), we used an inverse distance-weighted interpolation method with the gstat package, where the inverse distance weighting power was arbitrarily set to 5. (Legend continues on following page.)



standard protocol. Combined with information from national malaria control programs, the findings are regularly updated on the WHO website (www.who.int/topics/malaria/en). The WorldWide Antimalarial Resistance Network (WWARN) provides updated maps on antimalarial drug resistance from clinical and laboratory studies, including molecular markers, with a focus on academic and other research groups (www.wwarn.org). The KARMA international consortium (K13 Artemisinin Resistance Multicenter Assessment) launched in 2014 and led by the Institut Pasteur and the WHO provides a worldwide map of the polymorphism in the propeller domain of the *P. falciparum* K13 gene (see below) (Menard et al. 2016). The International Centers of Excellence for Malaria Research also established a network for monitoring of antimalarial drug efficacy (Cui et al. 2015). A number of research groups monitor drug efficacy through clinical and laboratory studies, which are published in peer-reviewed journals. Close cooperation between academic and research groups, and national malaria control programs is important for quick incorporation of results into drug policy.

ASSESSING ANTIMALARIAL DRUG EFFICACY

Sources of information on antimalarial drug efficacy include clinical drug efficacy studies, ex vivo and in vitro assessments of drug sensi-

tivity, and molecular markers. Regarding clinical studies, it is important that follow-up of patients is sufficiently long to assess appropriate parasitological and clinical cure, in particular when trialing antimalarial drugs with long terminal half-lives, such as mefloquine or piperaquine. Short follow-up will miss up to 90% (at 14 d) or 50% (at 28 d) of late recrudescence infections, which can occur up to 63 d after the start of therapy (Stepniewska and White 2006). In trials on drug efficacy in falciparum malaria, it is important to distinguish between reinfection and recrudescence as the source of recurrent infection, using genotyping methods of parasite strains (WHO 2007). This is a major issue in vivax malaria, as genotyping cannot reliably classify recurrent infection into a relapsing infection (parasites released from liver hypnozoites) or recrudescence (resistant parasites), because parasites from relapse infection can be issued from a similar or a different hypnozoite clone than the initial clone (Chen et al. 2007; Imwong et al. 2007).

In vitro assays assessing the sensitivity of *P. falciparum* malaria parasites to antimalarial drugs is a research tool, which is frequently used to complement data from clinical studies and for providing data on the epidemiology of drug-resistant malaria. In vitro sensitivity testing can contribute to the early detection of emergence of drug resistance, changing trends in parasite drug susceptibility over time and space, or changes in the in vitro responses of indiv-

Figure 1. (Continued) A 100-km radius surrounding the coordinate sampling site(s) was used. To generate the Asia map (right panel), we used the well-established spatial statistical interpolation of ordinary kriging using a 50-km radius for the area surrounding the coordinate sampling site(s). The individual sites of sample collection are indicated with a cross (reproduced, with permission). (B) Chloroquine resistance in *Plasmodium vivax* infections. The map summarizes evidence from published and unpublished data from 1980 to 2015 compiled by the WWARN, available at www.wwarn.org. Category 1: >10% recurrent infections by day 28 (with a lower 95% CI of >5%), irrespective of confirmation of adequacy of blood chloroquine concentrations at the moment of recurrence. The presence of >10% recurrent infections is highly suggestive of chloroquine resistance. Category 2: parasitological confirmed recurrent infection within 28 d, in the presence of adequate whole-blood chloroquine concentrations (>100 nM) at the moment of recurrence. This confirms chloroquine resistance. Category 3: >5% recurrent infections by day 28 (lower 95%, CI of <5%), irrespective of confirmation of adequacy of blood chloroquine concentrations at the moment of recurrence. In this category, the contribution of other factors than drug resistance, such as poor drug absorption or drug quality, cannot be ruled out. Category 4: chloroquine-sensitive *P. vivax* infections, concluded from studies reporting on ≥ 10 symptomatic patients with symptomatic malaria, treated with chloroquine monotherapy (without early primaquine treatment), showing fewer than 5% recurrent infections within 28 d. (Reproduced, with permission, from WWARN.)

D. Menard and A. Dondorp

idual drugs currently deployed in combination therapies) (Guiguemde et al. 1994; Philipps et al. 1998; Ringwald et al. 2000; Menard et al. 2013). In vitro assessments are also useful for drug development (drug screening, isobologram studies for drug combinations, cross-resistance studies, in vitro phenotype comparisons of pre- and posttreatment isolates, baseline parasite susceptibility to a new drug before country implementation) and for validating candidate molecular markers associated with drug resistance. However, a limited number of laboratories in malaria-endemic countries have the capacity to perform in vitro assays, which requires sophisticated equipment, extensive resources, training, and expertise. There is no universally accepted, standardized protocol for in vitro drug sensitivity assays available, yet, but most protocols are based on the assessment of the 48-h *P. falciparum* in vitro development of isolates freshly collected in the field (ex vivo) or of short-/long-term culture-adapted parasite strains (in vitro), in the presence of increasing concentrations of antimalarial drugs (Basco 2007). The procedures for the parasite culture are those defined by Trager and Jensen (1976). The readout of the ex vivo or in vitro tests is parasite growth (at 48 h or later), evaluated by various methods including microscopy (Rieckmann et al. 1978), radioisotopic activity (isotopic test) (Desjardins et al. 1979), colorimetry (ELISA based on HRP2 and pLDH detection) (Makler and Hinrichs 1993; Brasseur et al. 2001; Noedl et al. 2002), fluorescence (Pico Green or Sybr Green dyes) (Smilkstein et al. 2004; Bacon et al. 2007; Rason et al. 2008), or flow cytometry (Pattanapanyasat et al. 1997; Contreras et al. 2004). In vitro susceptibility parameters of *P. falciparum* isolates are expressed as the 50% inhibitory concentration (IC₅₀) or the 90% inhibitory concentration (IC₉₀) defined as the minimal concentration of antimalarial drug that inhibits parasite growth by 50% or 90% compared with the development in drug-free control wells. IC₅₀ and IC₉₀ estimations can be calculated by a variety of means, including algorithms within software packages or freely available tools based on log-probit (Grab and Wernsdorfer 1983), polynomial (Noedl et al. 2002),

and sigmoid inhibition (Le Nagard et al. 2011) models. Easy-to-use online tools, such as ICE-stimulator 1.2 (www.antimalarial-icestimator.net) or IVART (www.wwarn.org/tools-resources/toolkit/analyse/ivart), are available for free.

The main advantage of in vitro susceptibility testing is that inhibitory constants calculated from the parasite growth are an inherent trait of the parasite and are not affected by host factors, such as acquired immunity, bioavailability, or pharmacokinetics of antimalarial drugs (e.g., low drug absorption or metabolic alterations) (Woodrow et al. 2013). However, for some drugs, classical in vitro assays have limited sensitivity for detecting resistant parasites. This is, in particular, the case for artemisinin derivatives. Most of the initial studies investigating in vitro susceptibility to artemisinin showed that the delayed parasite clearance phenotype does not correspond to increased artemisinin 50% inhibitory concentration (IC₅₀) values. Slightly increased IC₅₀ values for dihydroartemisinin (the active metabolite of all artemisinins) were reported for slow-clearing parasites (Noedl et al. 2008), but they substantially overlap with those for fast-clearing parasites (Dondorp et al. 2009; Amaratunga et al. 2012). Studies with culture-adapted resistant parasite lines showed that artemisinin resistance was associated with decreased susceptibility of ring stages (Witkowski et al. 2010, 2013a,b; Cui et al. 2012; Klonis et al. 2013) and in some lines to mature stages (Cui et al. 2012; Teuscher et al. 2012). A novel in vitro assay (ring-stage survival assay [RSA]) that measures susceptibility of 0–3 h postinvasion *P. falciparum* ring stages to a pharmacologically relevant, short exposure (700 nM for 6 h) to dihydroartemisinin developed by Witkowski and colleagues demonstrated significant higher survival rates of culture-adapted parasites (in vitro RSA^{0–3 h}) or fresh isolates (ex vivo RSA) in slow-clearing *P. falciparum* infections (threshold >1%) compared with isolates collected from fast-clearing infections (Witkowski et al. 2013a). In contrast, late rings and trophozoites from slow- and fast-clearing infections showed no difference in their susceptibility to dihydroartemisinin. Parasite survival rates in the RSA also significantly corre-

lated with parasite clearance half-life, including in areas where artemisinin-resistant parasites had not yet been described, designating this assay as the current reference platform to detect in vitro artemisinin resistance.

Molecular markers associated with antimalarial drug resistance, when they are validated, are highly relevant to detect and monitor in real time the geospatial distribution of resistant parasites. To date, known molecular signatures are mutations in genes or changes in the copy number of genes encoding to the drug's parasite target or to transport proteins involved in intraparasitic influx/efflux of the drugs. Markers represent useful surveillance tools as their prevalence in a parasite population is often a good indicator of the level of clinical resistance. Different methods, including classical polymerase chain reaction (PCR), followed by direct sequencing, allele-specific PCR, PCR-RFLP, PCR-SSOP, FRET-MCA, PCR with molecular beacons, SNPE, PCR-LDR-FMA, and real-time PCR can be used depending on resources (Wilson et al. 2005; Barnadas et al. 2011). Their main advantage is that they allow us to test thousands of small volumes of blood samples (capillary blood collected by finger prick, spotted into filter paper, and stored at ambient temperature) at a wide scale by high-throughput automated approaches (Menard and Ariey 2015). Validated or candidate genetic markers are available for a limited number of antimalarial drugs, as described in Tables 1 and 2. Current molecular markers associated with antimalarial drug resistance are summarized in Table 1. These include markers for resistance to chloroquine (*Pfcr*, *Pfmdr-1*), sulfonamides, and sulfones, including sulfadoxine (*Pfdhps*), pyrimethamine, cycloguanil and chlorocycloguanil (*Pfdhfr*), atovaquone (*Pfycytb*), mefloquine and halofantrine (*Pfmdr-1* amplification), amodiaquine (*Pfcr*, *Pfmdr-1*), quinine (*Pfcr*, *Pfmdr-1*, *Pfnhe-1*), and, most recently, artemisinins (*PfK13*).

Located on chromosome 13 (*PfK13* gene, PF3D7_1343700, previous ID: PF13_0238), *PfK13* is a single exon gene, which encodes a 726 amino acid protein that constitutes three domains, including a *Plasmodium*-specific/

Apicomplexa-specific domain, a BTB/POZ domain, and a six-blade β -propeller Kelch domain. Seminal studies performed by Ariey et al. (2014) demonstrated that a single mutation in the β -propeller domain of the K13 gene was a major determinant of resistance to artemisinin derivatives (see paragraph below). The identification of K13 mutant-allele parasites in patients with a slow parasite clearance rate and site-specific genome-editing experiments using zinc-finger nucleases (Straimer et al. 2015) or the CRISPR-Cas9 system (Ghorbal et al. 2014) provided final evidence that this molecular marker was a major determinant of resistance to artemisinin derivatives. However, because this is a laborious process, to date only four mutant alleles have been validated by genome editing (580C \rightarrow Y, 539R \rightarrow T, 543I \rightarrow T, and 493Y \rightarrow H) among the 173 nonsynonymous mutations described to date (Straimer et al. 2015; Menard et al. 2016). The discovery of K13 polymorphism as the major determinant of *P. falciparum* artemisinin resistance opened unprecedented opportunities for resistance monitoring and soon after this discovery several molecular epidemiology studies were conducted to map the extended artemisinin resistance (Ashley et al. 2014; Takala-Harrison et al. 2015; Tun et al. 2015). The KARMA project is the largest study, yet, and provides critical information for drug policymakers in the following years, by clarifying the roadmap for future surveillance activities involving samples collected across 59 malaria-endemic countries (Menard et al. 2016).

CURRENT INSIGHTS IN ARTEMISININ RESISTANCE

The clinical phenotype of artemisinin resistance is characterized by delayed parasite clearance after treatment with artemisinin monotherapy or an ACT. Delayed clearance can be assessed as an increased parasite half-life assessed from the log-linear part of the peripheral blood parasite clearance curve or as persistence of parasitemia at 72 h after the start of treatment (Flegg et al. 2011; White et al. 2015). In addition to resistance of the parasite to the artemisinins, para-

D. Menard and A. Dondorp

Table 1. Catalogue of the current molecular markers associated with antimalarial drug resistance***Plasmodium falciparum* chloroquine resistance transporter (PfCRT)**

Located on chromosome 7 (*Pfcr* gene, PF3D7_0709000, previous ID: MAL7P1.27), *Pfcr* encodes a food vacuole membrane transporter protein, member of the drug/metabolite transporter superfamily (Tran and Saier 2004). The mutation at codon 76 (K → T) always associated with other nonsynonymous mutations (at codons 72, 74, or 75) (Warhurst 2001; Sidhu et al. 2002) is the primary mediator of chloroquine resistance, by increasing the export of chloroquine from the food vacuole, away from its target (Sanchez et al. 2007). Laboratory experiments have shown that *Pfcr* is also involved in decreasing parasites' susceptibility to monodesethylamodiaquine (SVMNT, 7G8 allele) and quinine (Cooper et al. 2007; Tinto et al. 2008) and mediates increased susceptibility to mefloquine and artemisinins. In areas where *Pfcr* mutant-type alleles are not fixed, like Africa, an increase in the frequency of the wild-type allele has been observed after the discontinuation of chloroquine (Laufer et al. 2006; Noranate et al. 2007). In South America, where SVMNT alleles are almost fixed, emergence of a mutation at codon 350 (C → R) mediates both increase susceptibility to chloroquine and resistance to piperazine (Pelleau et al. 2015).

***P. falciparum* multidrug resistance protein 1 (PfMDR1)**

Located on chromosome 5 (*Pfmdr-1* gene, PF3D7_0523000, previous ID: MAL5P1.230, PFE1150w), *Pfmdr-1* encodes an ABC transporter (ATP-binding cassette, P-glycoprotein homolog). MDR1, located in the membrane of the food vacuole, is involved in the modulation of the susceptibility to several antimalarial drugs and, more particularly, in the hydrophobic antimalarial efflux (Duraisingh and Cowman 2005). Resistance mechanisms are associated to (1) increased copy number of *Pfmdr-1* leading to an increase in the expression of the protein (Nishiyama et al. 2004) and resistance to mefloquine, lumefantrine, quinine, and artemisinins (Cowman et al. 1994; Pickard et al. 2003; Price et al. 2004; Sidhu et al. 2006), and (2) mutations at codons 86N → Y and 1246D → Y (found in Africa) mediating decreased susceptibility to chloroquine and amodiaquine, but increased sensitivity to lumefantrine, mefloquine, and artemisinins (Duraisingh et al. 2000; Reed et al. 2000; Mwai et al. 2009) or at codons 1034C → S and 1042N → D (observed outside Africa), which have been associated with altered sensitivity to lumefantrine, mefloquine, and artemisinins (Reed et al. 2000; Pickard et al. 2003; Sidhu et al. 2005, 2006). Opposite effects on different drugs have been reported between chloroquine and mefloquine: the 86N → Y mutation decreases the parasite susceptibility to chloroquine, but increases mefloquine sensibility (Duraisingh and Cowman 2005). Similarly, increased copy number of *Pfmdr-1* increases resistance to mefloquine, but conversely increases the sensitivity to chloroquine and to piperazine (Leang et al. 2013, 2015; Duru et al. 2015; Lim et al. 2015; Amaratunga et al. 2016)

***P. falciparum* bifunctional dihydrofolate reductase-thymidylate synthase (PfDHFR-TS)**

Located on chromosome 4 (*Pfdhfr-ts* gene, PF3D7_0417200, previous ID: MAL4P1.161, PFD0830w), *Pfdhfr-ts* encodes an enzyme involved in the pathway of the folate synthesis (Foote and Cowman 1994; Gregson and Plowe 2005). DHFR-TS is the target of the antifolate drugs such as pyrimethamine and proguanil (metabolized in vivo to the active form cycloguanil). Antifolate drugs act by inhibiting DHFR-TS activity, blocking the pyrimidine synthesis, and the replication of the parasite DNA (Hankins et al. 2001; Sibley et al. 2001). The accumulation of several specific nonsynonymous mutations in *Pfdhfr-ts* mediates high clinical treatment failure rates and increase in vitro susceptibility to pyrimethamine (codons 51N → I, 59C → R, 108 S → N, and 164I → L) and to cycloguanil (codons 16A → V, 108S → T).

***P. falciparum* hydroxymethyl–dihydropterin pyrophosphokinase–dihydropteroate synthase (PfPPPK-DHPS)**

Located on chromosome 8 (*Pfhppk-dhps* gene, PF3D7_0810800, previous ID: PF08_0095), *Pfhppk-dhps*, this gene encodes another parasite-specific enzyme involved in the de novo synthesis of essential folate coenzymes. Resistance to sulfa drugs (sulfonamide, sulfadoxine, sulfone, and dapsone), most commonly involves the changes at codons 436S → A, 437K → G, 540K → E, 581A → G, and 613A → S/T (Hyde 2002; Gregson and Plowe 2005). Accumulation of mutations in *Pfdhfr-ts* and *Pfhppk-dhps* genes is strongly associated to clinical failure rates in patients treated with sulfadoxine–pyrimethamine combination, widely used in Africa in pregnant women or in children in the intermittent preventive treatment strategy (Kublin et al. 2002). The most frequent resistant combination in HPPK-DHPS and DHFR-TS (quintuple mutant for which frequencies of 70% or higher in some areas of East Africa is currently

Continued

Table 1. *Continued*

observed) is the double-mutant form 437A → G, 540K → E with the triple-mutant form of DHFR (51N → I, 59C → R, 108 S → N) (Pearce et al. 2009; Naidoo and Roper 2010).

***P. falciparum* multidrug resistance-associated protein 1 (PfMRP1)**

Located on chromosome 1 (*Pfmrp1* gene, PF3D7_0112200, previous ID: MAL1P3.03, PFA0590w), PfMRP1 is an ABC transporter (Koenderink et al. 2010). The *Pfmrp1* gene knockout in culture-adapted parasite lines causes a reduction in parasite growth and increased susceptibility to chloroquine, suggesting that MRP1 is involved in the efflux of antimalarial drugs from the parasite and is important for parasite fitness (Raj et al. 2009). Polymorphisms in *Pfmrp1* have been associated to decreased sensitivity to chloroquine and quinine (Mu et al. 2003) in in vitro susceptibility assays of culture-adapted cloned isolates and mefloquine, pyronaridine, and lumefantrine from Southeast Asian field isolates (Gupta et al. 2014).

***P. falciparum* cytochrome *b* (PfCYTB)**

Located on the mitochondrial genome (*Pfcytb* gene, mal_mito_3), the *Pfcytb* gene encodes the mitochondrial cytochrome *bc1* complex involved in the electron transport and ATP synthesis, and is the target of atovaquone (Fry and Pudney 1992; Birth et al. 2014; Siregar et al. 2015). A single mutation at codon 268 (Y → N/S/C) highly decreases sensitivity to atovaquone (Korsinczky et al. 2000; Fivelman 2002; Farnert 2003), in combination with proguanil (Malarone) currently widely used for malaria chemoprophylaxis in travelers (Kain et al. 2001). Mutations at codon 268 are rarely detected in field isolates, and are mostly intrahost selected following atovaquone–proguanil treatment in patients experiencing clinical failure (Musset et al. 2007; Nuralitha et al. 2015).

***P. falciparum* sodium–hydrogen exchanger gene Na⁺, H⁺ antiporter (PfNHE)**

Located on chromosome 13 (*Pfnhe-1* gene, PF3D7_1303500, previous ID: PF13_0019), *Pfnhe-1* encodes a putative sodium–hydrogen exchanger protein involved in parasite homeostasis by increasing the cytosolic pH (pH_{cyt}) and compensating acidosis caused by anaerobic glycolysis (Bosia et al. 1993; Nkrumah et al. 2009). Using quantitative trait loci analysis of the genetic cross of the HB3 and Dd2 clones, it has been demonstrated that three genes including *Pfnhe-1* were associated with quinine-reduced susceptibility (Ferdig et al. 2004). Sequencing analysis of *P. falciparum* culture-adapted isolates and reference lines from Southeast Asia, Africa, and South America revealed significant associations between variations in ms4760 intragenic microsatellite (alleles with >2 DNNND repeat motifs in block II, such as ms4760–1), and in vitro quinine response. However, the reliability of polymorphisms in the *Pfnhe-1* gene as molecular markers of quinine resistance appeared restricted to endemic areas from Southeast Asia or possibly east African countries and needs to be confirmed (Menard et al. 2013a).

***P. falciparum* non-SERCA-type Ca²⁺-transporting P-ATPase (PfATP4)**

Located on chromosome 12 (*Pfatp4*, PF3D7_1211900, previous ID: 2277.t00119, MAL12P1.118, PFL0590c), *Pfatp4* encodes a plasma membrane protein involved in the sodium efflux (Spillman et al. 2013). Recent laboratory investigations demonstrated that nonsynonymous mutations in this gene were associated to the resistance of new antimalarial compounds, including the spiroindolones, the pyrazoles, and the dihydroisoquinolones (Rottmann et al. 2010; Jimenez-Diaz et al. 2014; Vaidya et al. 2014; Spillman and Kirk 2015).

site clearance dynamics are also, to some extent, affected by the differences in host immunity (causing a variance of 0.5–1 h in parasite half-life), partner drug efficacy, splenic function, and other factors (Dondorp et al. 2010). Persistence of parasitemia at 72 h as a measure of artemisinin resistance is much dependent on the initial parasitemia and on the sensitivity of the method assessing parasitemia at 72 h (White et al.

2015). Because *P. falciparum* parasites in the second half of their asexual-stage development sequester in the microcirculation, delayed clearance suggests that ring-stage sensitivity is affected by artemisinin resistance. Artemisinins are the only class of antimalarial drugs with potent and rapid parasitocidal action against ring-stage parasites translating to a 10,000-fold decrease in parasitemia 48 h after the start of treatment

D. Menard and A. Dondorp

Table 2. Recommended antimalarial drugs: epidemiological, biological, and molecular characteristics

Artemisinin derivatives (artesunate, artemether, dihydroartemisinin)	
Chemical structure	Sesquiterpene lactone endoperoxide
Introduced in	1980s (monotherapy), 2000s (combined with a partner drug in ACT)
First report of resistance in	2008 (partial resistance)
Half-life	0.5–2.0 h (artesunate, dihydroartemisinin) 5–7 h (artemether)
Mode of action	Not fully understood. Active against blood-stage parasites, from the ring stages to early schizonts as well as young gametocytes, involving cation-mediated generation of reactive intermediates and reduction of the peroxide bridge.
Molecular signatures of resistance	
Validated	<i>PfK13</i> gene at codons 580 (C → Y), 539 (R → T), 543 (I → T), 493 (Y → H), 561 (R → H)
Associated	<i>PfK13</i> gene at codons 441 (P → L), F446 → I, G449 → A, N458 → Y, M476 → I, N537 → D, P553 → L, V568 → G, P574 → L, M579 → I, D584 → V, A675 → V, H719 → N
In vitro susceptibility threshold value for resistance	Survival rate > 1% in the RSA ^{0–3 h}
In vitro cross-resistance (IC ₅₀ correlation)	–
Spatial distribution of confirmed resistance	Southeast Asia
Quinine	
Chemical structure	Aryl-amino alcohol
Introduced in	1632 (cinchona), 1820 (quinine)
First report of resistance in	1908
Half-life	3–36 h
Mode of action	Active against large rings and trophozoites by inhibiting intraparasitic haem detoxification in the parasite's digestive vacuole. Active against gametocytes (except for <i>Plasmodium falciparum</i>).
Molecular signatures of resistance	
Validated	None
Associated	<i>PfCRT</i> at codon 76 (K → T) Pfmdr-1 at codons 1042 (N → D), 1034 (S → C) or 1246 (D → Y) ms4760 variation in Pfhel1 gene (increase in DNNND repeats in block II) (in Asian parasite populations)
In vitro susceptibility threshold value for resistance	IC ₅₀ > 500–800 nM
In vitro cross-resistance (IC ₅₀ correlation)	Positively correlated with chloroquine, lumefantrine, mefloquine, halofantrine

Continued

Table 2. *Continued*

Spatial distribution of confirmed resistance	Sporadic worldwide cases (but mainly in Southeast Asia)
Chloroquine	
Chemical structure	4-Aminoquinoline
Introduced in	1945
First reports of resistance in	1957
Half-life	5–12 d (chloroquine and monodesethylchloroquine)
Mode of action	Active by inhibiting intraparasitic haem detoxification in the parasite's digestive vacuole. Chloroquine may also act on the biosynthesis of nucleic acids.
Molecular signatures of resistance	
Validated	<i>Pfprt</i> at codon 76 (K → T) with other mutations: Dd2 Southeast Asian allele (74 M → I, 75 N → E) or 7G8 South American allele (72 C → S)
Associated	<i>Pfmdr-1</i> a codons 86 (N → Y), 1034 (S → C), 1042 (N → D), and 1246 (D → Y)
In vitro susceptibility threshold value for resistance	IC ₅₀ > 100 nM
In vitro cross-resistance (IC ₅₀ correlation)	Positively correlated with quinine, monodesethylamodiaquine, piperazine. Negatively correlated with mefloquine.
Spatial distribution of confirmed resistance	Worldwide
Amodiaquine	
Chemical structure	4-Aminoquinoline
Introduced in	1945
First reports of resistance in	1990s
Half-life	3–12 h (amodiaquine) 4–10 d (monodesethylamodiaquine)
Mode of action	Active by accumulation in the parasite's digestive vacuole and inhibition of the haem detoxification
Molecular signatures of resistance	
Validated	<i>Pfprt</i> at codons 72 (C → S) and 76 (K → T) (7G8 South American allele SVMNT)
Associated	<i>Pfmdr-1</i> at codons 86 (N → Y) and 1246 (D → Y) (on the <i>Pfprt</i> CVIET allele genetic background)
In vitro susceptibility threshold value for resistance	Amodiaquine > 80 nM monodesethylamodiaquine IC ₅₀ > 60 nM
In vitro cross-resistance (IC ₅₀ correlation)	Positively correlated with chloroquine and quinine
Spatial distribution of confirmed resistance	South America, Asia, and East Africa

Continued

D. Menard and A. Dondorp

Table 2. Continued

Mefloquine	4-methanolquinoline
Chemical structure	1984
Introduced in	1991
First reports of resistance in	8–15 d
Half-life	Active by inhibiting intraparasitic haem detoxification in the parasite's digestive vacuole and endocytosis of the cytosol by the parasite
Mode of action	Increase expression of the amplified (≥ 2 wild-type <i>Pfmdr-1</i> gene copy) <i>Pfmdr-1</i> gene
Molecular signatures of resistance	–
Validated	IC ₅₀ > 30 nM
Associated	Positively correlated with halofantrine and lumefantrine and negatively correlated with chloroquine and piperazine
In vitro susceptibility threshold value for resistance	–
In vitro cross-resistance (IC ₅₀ correlation)	–
Spatial distribution of confirmed resistance	Positively correlated with halofantrine and lumefantrine and negatively correlated with chloroquine and piperazine
Lumefantrine	Southeast Asia, and sporadically in South America, India, Africa
Chemical structure	Aryl-amino alcohol
Introduced in	2000s (combined with artemether)
First reports of resistance in	–
Half-life	2–11 d
Mode of action	Active by inhibiting intraparasitic haem detoxification in the parasite's digestive vacuole and endocytosis of the cytosol by the parasite
Molecular signatures of resistance	–
Validated	<i>Pfmdr-1</i> at codons 184 (Y → F), 1034 (S → C) and 1042 (N → D) (on the <i>Pfcr1</i> CVIET allele genetic background)
Associated	–
In vitro susceptibility threshold value for resistance	–
In vitro cross-resistance (IC ₅₀ correlation)	Positively correlated with mefloquine and halofantrine and negatively correlated with chloroquine and piperazine
Spatial distribution of confirmed resistance	–
Piperaquine	Bis-4-aminoquinolin
Chemical structure	1960s (monotherapy), 2008 (combined with dihydroartemisinin)
Introduced in	1970s
First reports of resistance in	–

Continued

Table 2. *Continued*

Half-life	13–28 d
Mode of action	Not fully understood. Active by inhibiting intraparasitic haem detoxification in the parasite's digestive vacuole. Chloroquine may also act on the biosynthesis of nucleic acids.
Molecular signatures of resistance	–
Validated	–
Associated	<i>Pfcr</i> at codon 350 (C → R). Deamplification of an 82-kb region of chromosome 5 (including <i>Pfmdr-1</i> gene) and amplification of an adjacent 63-kb region of chromosome 5.
In vitro susceptibility threshold value for resistance	Survival rate > 10% in the PSA (piperazine survival assay)
In vitro cross-resistance (IC ₅₀ correlation)	Positively correlated with chloroquine and negatively correlated with mefloquine
Spatial distribution of confirmed resistance	Southeast Asia, China
Primaquine	
Chemical structure	8-Aminoquinoline
Introduced in	1950
First reports of resistance in	–
Half-life	4–9 h
Mode of action	Active by disrupting the metabolic processes of <i>Plasmodium</i> mitochondria and by interfering with the function of ubiquinone as an electron carrier in the respiratory chain and by producing highly reactive metabolites generating toxic intracellular oxidative potentials
Molecular signatures of resistance	–
Validated	–
Associated	–
In vitro susceptibility threshold value for resistance	–
In vitro cross-resistance (IC ₅₀ correlation)	–
Spatial distribution of confirmed resistance	–
Sulfadoxine	
Chemical structure	Sulfonamide
Introduced in	1937
First reports of resistance in	1970s (in association with pyrimethamine)
Half-life	4–11 d

Continued

D. Menard and A. Dondorp

Table 2. *Continued*

Mode of action	Active by inhibiting the enzyme dihydropteroate synthase (DHPS), a component of the folate biosynthetic pathway and the replication of the parasite DNA
Molecular signatures of resistance	<i>Pfdhfrs</i> at codons 436 (S → A), 437 (K → G), 540 (K → E), 581 (A → G), and 613 (A → S/T)
Validated	–
Associated	–
In vitro susceptibility threshold value for resistance	–
In vitro cross-resistance (IC ₅₀ correlation)	–
Spatial distribution of confirmed resistance	Worldwide
Pyrimethamine	
Chemical structure	Diaminopyrimidine derivative
Available since	1940s
First report of resistance in	1952 and 1970s (in association with sulfadoxine)
Half-life	2–19 d
Mode of action	Active by inhibiting the bifunctional dihydrofolate reductase–thymidylate synthase activity, blocking the pyrimidine synthesis and the replication of the parasite DNA
Molecular signatures of resistance	<i>Pfdhfr</i> at codons 51 (N → I), 59 (C → R), 108 (S → N), and 164 (I → L). In South America, mutation at codon 50 C → R instead of 59 (C → R).
Validated	–
Associated	–
In vitro susceptibility threshold value for resistance	IC ₅₀ > 100 nM
In vitro cross-resistance (IC ₅₀ correlation)	–
Spatial distribution of confirmed resistance	Worldwide
Proguanil	
Chemical structure	Biguanide
Available since	1940s
First report of resistance in	1949
Half-life	Proguanil (8–18 h); cycloguanil (16–23 h)
Mode of action	Active through its active triazine metabolite (cycloguanil) by inhibiting the bifunctional dihydrofolate reductase–thymidylate synthase activity, blocking the pyrimidine synthesis and the replication of the parasite DNA

Continued

Table 2. Continued

Molecular signatures of resistance	<i>Pf</i> dhfr at codons 16 (A → V) and 108 (S → T)
Validated	–
Associated	–
In vitro susceptibility threshold value for resistance	IC ₅₀ > 15 nM
In vitro cross-resistance (IC ₅₀ correlation)	Sporadic worldwide cases
Spatial distribution of confirmed resistance	
Atovaquone	
Chemical structure	Hydroxynaphthoquinone
Available since	1996
First report of resistance in	1996
Half-life	1–6 d
Mode of action	Active by inhibiting the transport of several parasite enzymes and by interfering with the cytochrome electron transport system, resulting in the collapse of the mitochondrial membrane potential
Molecular signatures of resistance	<i>Pf</i> cytb at codon 268 (Y → N/S/C)
Validated	–
Associated	–
In vitro susceptibility threshold value for resistance	IC ₅₀ > 10 nM
In vitro cross-resistance (IC ₅₀ correlation)	–
Spatial distribution of confirmed resistance	Sporadic worldwide cases

D. Menard and A. Dondorp

(White 2008). This sensitivity of ring-stage *P. falciparum* parasites seems what is primarily affected in artemisinin resistance as suggested by the clinical observations and later confirmed by ring-stage-specific sensitivity tests described above (Dondorp et al. 2009; Flegg et al. 2011; Saralamba et al. 2011; Ariey et al. 2014). The ring-stage survival assay performed on early ring parasites (0–3 h postinvasion, RSA^{0–3 h}) showed a strong correlation between clinical data (parasite clearance half-life) and in vitro parasite survival rates (Witkowski et al. 2013a; Amaratunga et al. 2014). Transcriptomic and cell biological studies suggest that the important contributors to reduced ring-stage sensitivity are a deceleration in ring-stage development (early ring forms are intrinsically less susceptible) and an up-regulation of the “unfolded protein” stress response (UPR) (Dogovski et al. 2015; Mok et al. 2015). This was confirmed by additional population transcriptional studies (Mok et al. 2015; Shaw et al. 2015), which showed an increased expression of the UPR pathways involving the major PROSC and TRiC chaperone complexes to mitigate protein damage caused by artemisinin.

As discussed above, mutations in the K13 gene coding for the propeller region of the *P. falciparum* Kelch protein are a cause of artemisinin resistance. The Kelch protein has a wide range of biological functions, one of which is facilitating polyubiquitination leading to protein degradation in the proteasome (Dogovski et al. 2015; Mbengue et al. 2015). In Kelch-mutated parasites, lower levels of ubiquitinated proteins can be observed, which is in accordance with UPR up-regulation (Dogovski et al. 2015). In addition, it was recently shown that artemisinins target *P. falciparum* phosphatidylinositol-3-kinase (PfPI3K) and Kelch-mutated parasites, through reduced ubiquitination, have increased levels of PfPI3K and its lipid product phosphatidylinositol-3-phosphate (PI3P), conferring reduced artemisinin sensitivity (Mbengue et al. 2015).

Following the discovery of the K13 genetic marker, additional genomewide studies suggested a specific genetic background in Southeast Asia parasite populations associated to ar-

temisinin resistance (Miotto et al. 2015). This genetic background on which *kelch13* mutations are particularly likely to arise includes several nonsynonymous mutations: 193D → Y in PF3D7_1318100 (ferredoxin putative gene), 127V → M in PF3D7_1460900 (apicoplast ribosomal protein S10 precursor gene), 484T → I in PF3D7_1447900 (multidrug resistance protein 2 gene), and 356I → T in PF3D7_0709000 (chloroquine resistance transporter gene). Further research on defining this genetic backbone is ongoing.

Genetic studies also showed that two different foci of resistance originated in Asia, with virtually no overlap between the sets of mutations and haplotypes in Thailand–Myanmar–China and Cambodia–Vietnam–Lao PDR, confirming recent observations (Takala-Harrison et al. 2015; Menard et al. 2016). In Cambodia where artemisinin-resistant mutants in western provinces are almost fixed, haplotyping of K13 neighboring loci revealed multiple independent origins of common mutations alongside numerous sporadic localized mutational events, creating a large repertoire of mutants (Menard et al. 2016). The independent emergence of various K13 mutations will have to be reconciled with the observation that the area in Southeast Asia harboring the artemisinin-resistant phenotype is expanding over time.

South America, Oceania, Philippines, and Central/South Asia are currently areas free of K13 mutant parasites. In Africa, highly diverse and low-frequent K13 mutant alleles have been observed, with no evidence of selection, and none of these were associated with clinical artemisinin resistance assessed by the presence of parasites on day 3 following artesunate monotherapy or a 3-d ACT course. It is thought that artemisinin resistance has not been established in Africa, supported by the additional absence of evidence of invasion by Asian K13 alleles validated as molecular marker of artemisinin resistance (C580Y, R539T, I543T, Y493H), confirming previous smaller-sized studies (Conrad et al. 2014; Torrentino-Madamet et al. 2014; Cooper et al. 2015; Escobar et al. 2015; Hawkes et al. 2015; Isozumi et al. 2015; Kamau et al. 2015; Taylor et al. 2015). Haplotyping studies on the

most common African mutant 578A → S does not show evidence of selection of the mutation in the African parasite population. In addition, the 578A → S mutation seems phenotypically neutral, because genome editing of the Dd2 line indicated that this mutation did not affect artemisinin susceptibility in *in vitro* sensitivity testing with the RSA^{0–3 h} assay, whereas this was clearly the case for other Kelch mutations (Straimer et al. 2015; Menard et al. 2016). Figure 2 summarizes current insights in artemisinin resistance from the molecular to the public health level.

***P. vivax* ANTIMALARIAL DRUG RESISTANCE**

Vivax malaria is treated with antimalarial drugs highly effective against blood-stage parasites. For radical cure, which includes sterilization of liver hypnozoites, primaquine has to be added to the drug regimen. To date, in *vivax* malaria resistance has only emerged against chloroquine, a drug used worldwide for decades. Chloroquine resistance was first recognized in the late 1980s in New Guinea, 30 years after the emergence of *P. falciparum* chloroquine resistance (Rieckmann et al. 1989) and later in Eastern Indonesia, and nowadays in many countries in which *vivax* malaria is endemic (Price et al. 2014). Until now, the detection of chloroquine resistance is challenging, as recurrence after treatment may be a recrudescence (true resistance), a relapse or a reinfection (false resistance). As no reliable genotyping method is available, caution is required to conclude arrival of resistance. A consensus definition of resistance is the capability of a parasite strain to grow in the presence of an adequate drug blood concentration (100 ng/mL in whole blood). Unfortunately, this information is often missing in clinical studies, because of technical constraints. *In vitro* drug sensitivity testing is an alternative option to assess drug resistance for *P. vivax*, but only “one-shot” *ex vivo* drug sensitivity assays can be performed yet, because continuous culturing is not possible for *P. vivax* (Russell et al. 2012). Such assays are difficult to implement (the assay needs to be conducted within few hours of blood collection) and to

interpret, because isolates from patients generally contain a mixture of parasite stages ranging from early ring stages to mature trophozoites and sensitivity of *P. vivax* to chloroquine depends on its parasite stage: ring forms are highly sensitive, whereas trophozoites are more resistant (Kerlin et al. 2012). To date, no validated molecular marker associated with chloroquine resistance in *vivax* malaria has been identified, and the mechanisms of parasite resistance to this drug remain unknown. However, current ACTs remain fully effective to kill blood-stage *P. vivax* parasites and through their posttreatment prophylactic effect protect against relapses for weeks after treatment (Gogtay et al. 2013). Thus, in chloroquine-resistance areas, ACTs provide an effective alternative treatment, decreasing the risk of chloroquine resistance spreading.

FUTURE PERSPECTIVES: TOWARD THE ELIMINATION OF ARTEMISININ AND MULTIPLE DRUG-RESISTANT FALCIPARUM MALARIA IN SOUTHEAST ASIA

Because the artemisinins have much shorter plasma half-lives (~1 h) compared with the ACT partner drugs (days to weeks), the reduction in artemisinin sensitivity has left partner drugs exposed to a larger biomass of parasites after the usual 3-d ACT course. For this reason, artemisinin resistance contributes to the selection for partner drug resistance (Dondorp et al. 2010). Indeed, an increase in concomitant partner drug resistance has been observed in recent years and, as a consequence, treatment failures after ACTs are becoming more widespread in Southeast Asia. Late failure rates (within 21–28 d after the initial treatment) of >30% for dihydroartemisinin–piperaquine and mefloquine–artesunate have been documented in western Cambodia (Leang et al. 2013, 2015; Lon et al. 2014; Saunders et al. 2014; Duru et al. 2015; Spring et al. 2015; Amaratunga et al. 2016) and the western Thailand border areas (Carrara et al. 2013), respectively. Failure of first-line ACTs will damage current control and elimination efforts and accelerate the emergence and spread of resistance. Although

D. Menard and A. Dondorp

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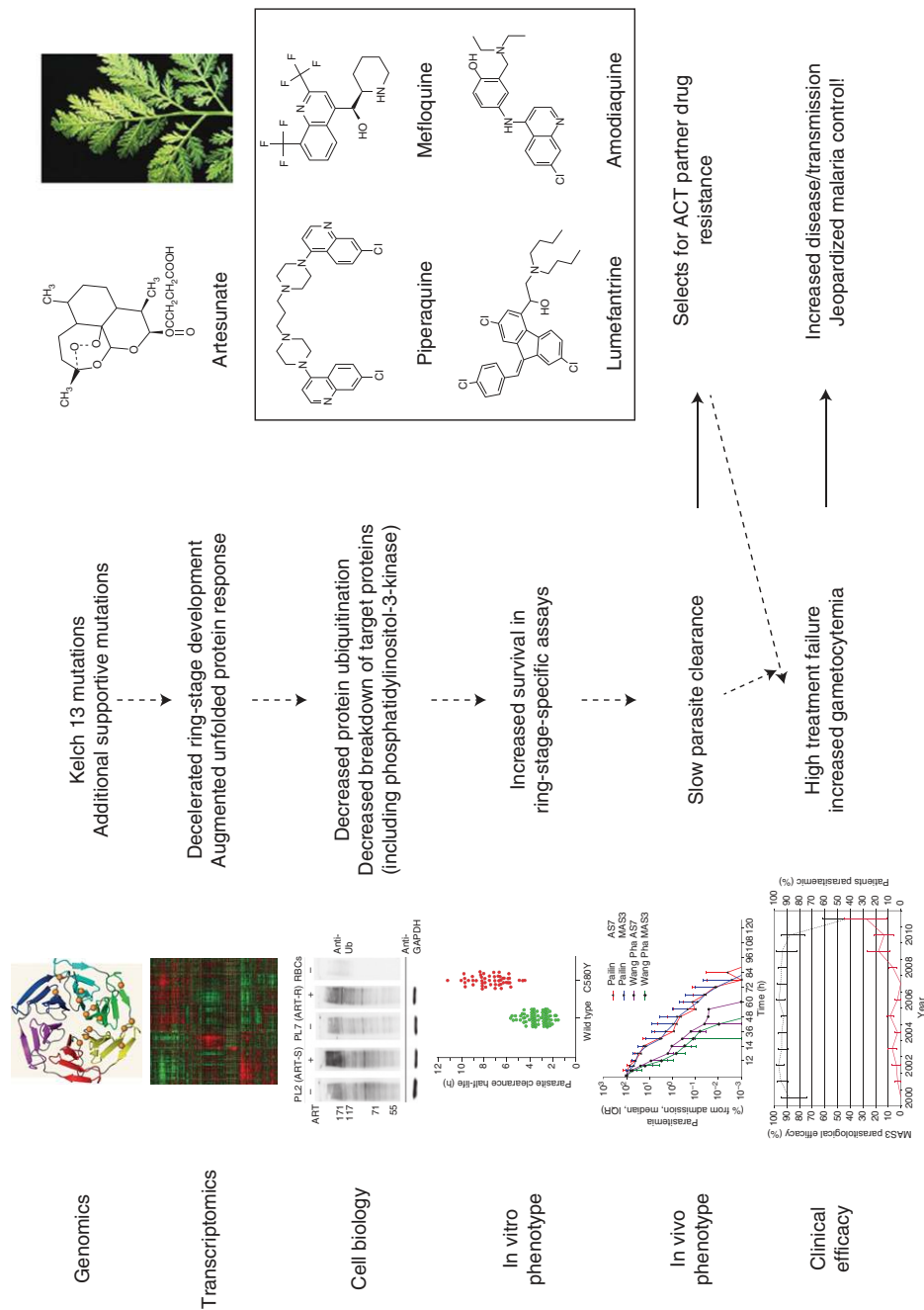


Figure 2. Current insights in artemisinin resistance from the molecular to the public health level.

promising new compounds are currently in phase II and phase III trials, their deployment is not expected before 2020. Promising new drug classes include ozonides, spiroindolones, and imidazole piperazines (Wells et al. 2015). There is an urgent need to evaluate alternative treatments where standard courses of ACTs are failing, and to develop combinations of existing drugs that will not fall rapidly to resistance and can be deployed immediately. Possible strategies include drug rotation between different ACTs, in particular DHA–piperaquine and artesunate–mefloquine. It has been shown that withdrawal of mefloquine as antimalarial treatment is followed by the recovery of mefloquine sensitivity in *P. falciparum*, resulting from the quick loss of *mdr-1* gene amplification, which exerts an important fitness cost in the absence of drug pressure (Preechapornkul et al. 2009; Leang et al. 2013, 2015; Duru et al. 2015; Lim et al. 2015; Amaratunga et al. 2016). This strategy is currently implemented in large parts of Cambodia suffering from high failure rates with DHA–piperaquine. Another possibility is extension of the usual 3-d ACT course to 5 or 7 d, for instance, using artemether–lumefantrine. A 5-d course of the latter drug combination is currently (2015) being trialed. A novel ACT, artesunate–pyronaridine, was recently trialed in western Cambodia, but showed sub-optimal efficacy in an area of artemisinin and piperaquine resistance (Leang et al. 2016). A synthetic endoperoxide trioxane, arterolane, which is marketed in India in combination with piperaquine, might be efficacious in areas with high ACT failure, but cross-resistance with the artemisinins cannot be excluded. Sequential deployment of two alternative full ACT courses could likely restore cure rates. Adherence to the longer treatment course might hinder adherence, and interaction of the long half-life partner drugs will need to be assessed. It should also be noted that a total cumulative dose >20 mg/kg of artesunate has been associated with bone marrow toxicity (Bethell et al. 2010; Das et al. 2013). Finally, a promising approach is the combination of artemisinin derivatives with two slowly eliminated partner drugs in a 3-d triple ACT. The principle of combining three anti-

microbial drugs is a standard approach for the treatment of HIV and tuberculosis. Several groups have advocated for the same approach as the new paradigm for the treatment of falciparum malaria (Shanks et al. 2015). There is a fortuitous inverse correlation between susceptibility to amodiaquine and lumefantrine and between piperaquine and mefloquine, which in addition have reasonably well-matching pharmacokinetic profiles. The combinations artemether–lumefantrine–amodiaquine and DHA–piperaquine–mefloquine are currently studied for their efficacy and safety in the treatment of uncomplicated falciparum malaria in areas of artemisinin and partner drug resistance.

CONCLUDING REMARKS

Artemisinin and partner drug resistance in *P. falciparum* are an increasing problem in Southeast Asia, causing high failure rates with ACTs in several countries of the Greater Mekong subregion. This jeopardizes the malaria elimination agenda of the region. Arrival in sub-Saharan Africa of these very difficult-to-treat parasite strains can have a huge impact on malaria morbidity and mortality, and intense surveillance is indicated. Monitoring of the genetic marker for artemisinin resistance, K13, has greatly facilitated surveillance, supplementing the more labor-intensive clinical studies identifying the slow clearance phenotype. The ring-stage-specific assay, RSA^{0–3 h}, has become the reference in vitro sensitivity test, which has helped to uncover the important aspects of the underlying biological mechanisms conferring artemisinin resistance. Until new antimalarials become available, creative deployment of existing drugs will be essential, which could include triple combination therapies. Accelerated elimination of all falciparum malaria in the Greater Mekong subregion will be needed to counter the threat of artemisinin and partner drug resistance. In vivax malaria, increasing chloroquine resistance is an increasing problem. Its surveillance is hampered by the absence of validated molecular markers or easy deployable in vitro sensitivity assays. ACTs are an effective alternative treat-

D. Menard and A. Dondorp

ment for *P. vivax*, with the addition of primaquine for radical cure of the infection.

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D. Menard and A. Dondorp



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D. Menard and A. Dondorp

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