Annals of Parasitology 2022, 68(2), 215–225
doi: 10.17420/ap6802.427

Review article

Antimalarial drugs resistance genes of *Plasmodium falciparum*: a review

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ABSTRACT. Malaria remains the most common parasitic disease on the planet, with 229 million cases and 409,000 deaths worldwide in 2019, including 274,030 children under the age of 5. It is one of the most important infectious diseases in the world and its control is compromised by the spread of the parasite's resistance to antimalarial drugs. This study aims to review the literature of resistant *Plasmodium falciparum* genes over the past twenty years. One hundred and five (105) articles were collected and read while the resistance of *P. falciparum* was being studied. Several *P. falciparum* gene resistances antimalarial drugs were discovered over the past twenty years. The most recent one is the *Kelch13* gene of *P. falciparum* (*Pfkelch13*) which has showed resistance to artemisinin in Asia. In Africa, this gene represents a potential candidate for resistance to artemisinin, although no resistance was reported.

Keywords: gene, *Plasmodium falciparum*, resistance, antimalarial, drug

Introduction

Malaria is a disease caused by a parasite of the genus *Plasmodium* transmitted to humans by the bite of a female mosquito of the genus *Anopheles*. Malaria remains the most common parasitic disease on the planet, with 229 million cases and 409,000 deaths worldwide in 2019, including 274,030 children under the age of 5. The majority of malarial cases were recorded on the African continent [1]. The management of these cases remains based on chemotherapy and the hope of control based on vaccine candidates still at the trial phase. The spread of the parasite's resistance to antimalarial drugs is a phenomenon, which seriously compromises the control of the fight against malaria [2]. Several studies conducted to understand this phenomenon showed that *Plasmodium falciparum* has a genetic polymorphism resulting in a variety of strains, some of which are involved in the process of chemoresistance [3]. The increase in the *P. falciparum* resistance to the usual antimalarial drugs (chloroquine, sulfadoxine-pyrimethamine) had led the World Health Organization (WHO) in 2001 to recommend a rapid substitution of monotherapy for artemisinin-based combination therapy (ACT) in the treatment of uncomplicated malaria. In this dynamic in 2005, the National Malaria Control Program of Burkina Faso revised its strategy by introducing dual therapy with ACTs in the treatment of uncomplicated

malaria [4]. The emergence of an artemisinin-resistant phenotype on the Thai-Cambodian border is raising new concerns for the fight against malaria today, threatening all efforts to control and eliminate malaria [5]. Mutations in several candidate genes were associated with *in vitro* resistance to artemisinin. Recently, Ariey et al. [6] identified a marker for resistance to artemisinin and showed a strong association between the existence of mutations in the „K13-propeller" gene, the *in vitro* survival of the parasites and the parasite clearance *in vivo*. In Africa, Miotto [7] showed an independent acquisition of the mutation of the K13 gene at a low level recognized as the cause of resistance to artemisinin in Southeast Asia. This study aims to make a review of *P. falciparum* gene-resistance to antimalarial drugs over the past twenty years.

Data collecting

The study took place in Burkina Faso in West Africa where malaria is endemic. This was a retrospective study based on articles on the *P. falciparum* gene-resistance to antimalarial drugs and published between the years 2000 and 2020. The documentary research was done with research engines such as: Google Scholar and PubMed. The keywords used were „Gene, *Plasmodium falciparum*, resistance, antimalarial drug".

In total, 105 articles were collected. For each article selected, parameters such as the name of the main author, date of publication, *P. falciparum* resistance gene and antimalarial drugs used were taken into account.

Resistance genes according to antimalarial drugs

In the last twenty years, many antimalarial drug resistance genes have been observed in *Plasmodium falciparum* (Tab. 1).

P. falciparum chloroquine resistance transporter gene (*Pfcrt*)

An intensive use of chloroquine (CQ) resulted in the emergence of resistant *P. falciparum* strains. Studies of genetic crosses between sensitive strain HB3 (Honduras) and a resistant strain Dd2 (Indochina) allowed to highlight a region of chromosome 7 responsible for the parasite resistance to CQ with an involvement of the gene *Pfcrt* which is highly polymorphic and coding for a transmembrane carrier of the digestive vacuole [21].

Some nucleotide substitutions showed a close link with the resistance phenotype of most strains of *P. falciparum* in the laboratory and in the field. However, only the *Pfcrt* K76T mutation identified in 2000, has a key role in the emergence of the CQ resistance phenotype [21]. The presence of mutations would prevent the CQ from accumulating in the vacuole due to *Pfcrt* ability to export the CQ outwardly [22]. Three models were proposed to explain the resistance mechanism of *Pfcrt* :

1. the partitioning model: the faster efflux of CQ outside the vacuole was initially attributed to a change in the pH of the vacuole in resistant CQ strains [22]. Other teams later demonstrated that resistant strains had the same pH, but must have a mechanism for efflux of CQ in the vacuole membrane that increased the permeability of CQ in a particular form. Although it may be conceivable that pH may play a role in some resistant strains, it is generally accepted that resistance to CQ is caused by an increased efflux capacity of CQ outside the vacuole;

2. the channel model: in this model, mutated *Pfcrt* would act as a channel for passive efflux of CQ outside the vacuole;

3. the carrier model: this model presents mutated *Pfcrt* as a carrier facilitating the active or passive diffusion of the molecule through the vacuole. The *Pfcrt* K76T mutation is observed alone or in combination with other mutations. More than 30 *Pfcrt* mutation points have been described in the literature (Fig. 1) [23]. Still requiring the presence of *Pfcrt* K76T to induce the resistance phenotype, these mutations have geographical specificities and could allow for the maintenance of protein function in the presence of the *Pfcrt* K76T mutation [24].

Pfcrt haplotypes have been implicated in reducing the sensitivity of certain antimalarial drugs. The *Pfcrt* K76T mutant haplotype is thought to induce cross-resistance between amodiaquine (AQ), quinine (QN), and CQ. High levels of AQ resistance have been specifically associated with the SVMNT haplotype on *Pfcrt* 72-76. This haplotype was first described in Tanzania in 2004 and then in Angola in 2007 [26]. It is mainly observed in Asia and South America and was selected by the AQ early use as first-line treatment.

P. falciparum multi-drug resistance 1 gene (*Pfmdr1*)

The *Pfmdr1* gene, originally called the CQ

Table 1. Molecular resistance markers of *P. falciparum* depending on antimalarial drugs

Antimalarial	Resistance marker		Validation level	References
	Gene	Allele associated with resistance or decrease sensitivity		
Chloroquine (CQ)	***Pfcrt***	Lys76Thr	*in vivo* association [8,9]	
	Pfmdr1	His191Tyr and Ser437Ala	*in vitro* association	
Amodiaquine (AQ)	***Pfmdr1***	Asn86Tyr	*in vitro* association	[10]
	Pfcrt	His191Tyr and Ser437Ala	*in vitro* association	
Mefloquin (MQ)	***Pfmdr1***	Number of copy > 1	*in vivo* association	[11]
Sulfadoxine-pyrimethamine (SP)	***Pfdhfr***	Ser108Asn	*in vivo* association	[12,13]
	Pfdhfr	Triple mutation : Ser108Asn + Asn51Ile + Cys59Arg	*in vivo* association	
	Pfdhps	Ala437Gly	*in vivo* association	
	Pfdhps	Double mutation : Ala437Gly + Lys540Glu	*in vivo* association	
	Pfdhfr* + *Pfdhps	Quintuple mutation : Ser108Asn (*dhfr*) + Asn51Ile (*dhfr*) + Cys59Arg (*dhfr*) + Ala437Gly (*dhps*) + Lys540Glu (*dhps*)	*in vivo* association	
	Pfmrp	Lys1466Arg	*in vivo* association	
Proguanil (cycloguanil)	***Pfdhfr***	Double/triple mutation : Ser108Asn + Asn51Ile and/or + Cys59Arg	*in vivo* association	[12]
Atovaquone (ATQ)	***Pfcytb***	Tyr268Asn or Tyr268Ser	*in vivo* association	[14]
Luméfantrine (LUM)	***Pfmdr1***	Asn86 and number of copy > 1	*in vivo* association	[15,16]
Quinine (QN)	***Pfnhe-1* (ms4760)**	Number of motifs : DNNND > 2 ; NHNDNHNNDDD < 3	*in vitro* association	[17,18]
	Pfmdr1	His191Tyr and Ser437Ala	*in vitro* association	
Doxycycline (DOX)	***PftetQ***	Number of motif : KYNNNN < 3	*in vitro* association	[19]
	PftetQ	Number of copy > 1	*in vitro* association	
	Pfmdt	Number of copy > 1	*in vitro* association	
Artemisinin (ART)	***K13 propeller***	M476I, C580Y, R539T, Y493H	*in vitro* association	[6]
Dihydoartemisinin-piperaquine (DHA-PPQ)	***Pfcrt***	K76 76T Mixed (K76T)	*in vitro* and *in vivo* association	[16]
	Pfmdr1	N86 86Y Mixed (N86Y)		[20]
	Pfpm2	(r = 0.7, p = 0.0008, N= 20)		

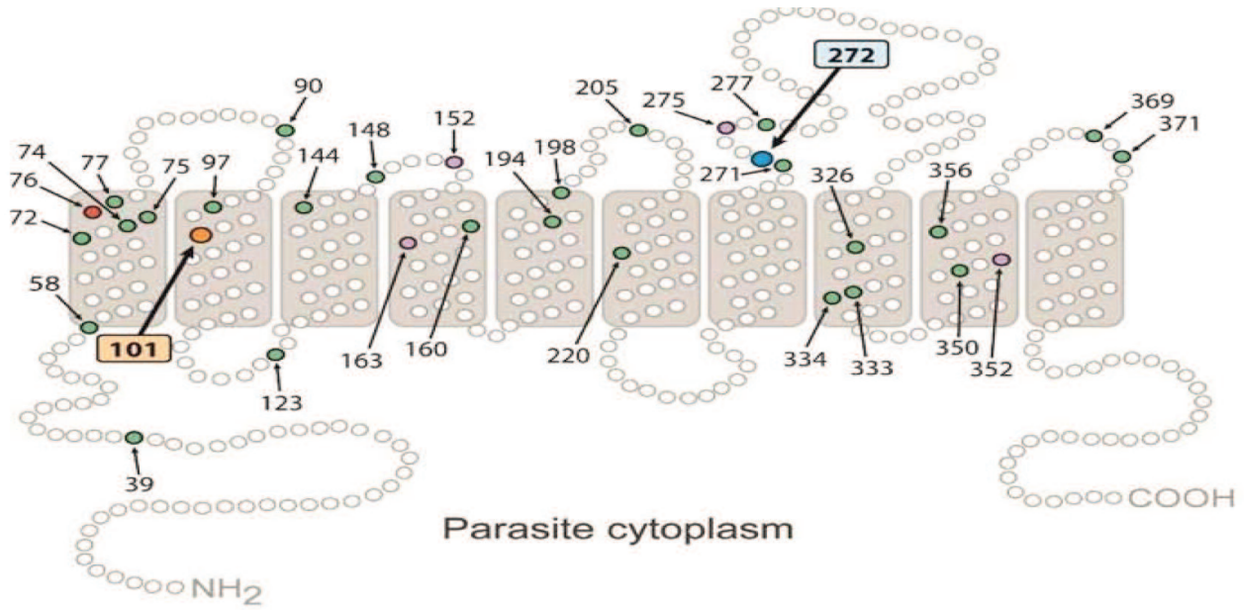

Figure 1. Schematic representation of *Pfcrt* and positions of identified mutations [25]

resistance candidate gene, was identified by looking for homologs in the mdr (multi-drug resistance) transporter family involved in the resistance of mammalian cancer cells. This *Pfmdr1* gene, located on chromosome 5, codes for a second carrier, the *P. falciparum P-glycoprotein* (Pgh1) counterpart 1 located in the digestive vacuole membrane with its ATP binding domain directed towards the cytoplasm [27]. In *Plasmodium*, this support would induce an auxiliary mechanism allowing molecules to enter the digestive vacuole. Sanchez et al. showed that Pgh1 was capable of carrying CQ and that the polymorphism of the *Pfmdr1* gene affected the specificity of the substrate [28]. Five punctual mutations, N86Y, Y184F, S1034C, N1042D and D1246Y, were detected and would impair the physicochemical properties of *Pgh1* due to substitutions for more polar amino acids. Thus, these modifications would affect the binding capacity, the molecule transfer, the influence sensitivity to LUM, ART, QN, MQ, halofantrine, and CQ [28,29]. Polymorphisms on the genes *Pfmdr1* and *Pfcrt* as well would also affect sensitivity to artemisinin-based combination therapy (ACT)), including the combination *Pfcrt* 76T and the haplotype *Pfmdr1 86*Y-*164*Y-*1246*Y which would be associated with recrudescence and reinfection after artesunate-amodiaquine (AS-AQ) treatment. Similarly, the wild alleles *Pfcrt k76*, *Pfmdr1* N86 and D1246 associated with the mutation *Pfmdr1* 184F would be selected after a treatment on artemether-lumefantrine. However, the joint role of these mutations remains to be substantiated and the number of associated molecules to treat patients complicates their study. Indeed, the mutations *Pfmdr1* N86Y and D1246Y would modulate the level of resistance to CQ of parasites already mutation carrying on the gene *Pfcrt* [30].

Plasmodium falciparum Na+/H+ exchanger-1 (*Pfnhe*-1)

The polymorphism of the *Pfnhe-1* gene appears to be associated with resistance to QN. Tandem repeats of *Pfnhe-1* peptide patterns (coded by the intra-gene microsatellite region ms4760) were found associated with decreases *in vitro* sensitivity of *P. falciparum* isolates or clones suitable for cultivation. This gene encodes Na+/H+ exchanger located in the plasma membrane of the parasite (unlike other carriers involved in resistance to quinoleins that are localized in the vacuolar membrane) that could regulate the cytoplasmic pH or digestive vacuole of the parasite. Disturbances of this pH related to this carrier could alter the activity of the QN [18]. If these observations are confirmed *in vivo*, repeat profiles could become molecular markers of decreased sensitivity or resistance to QN.

Plasmodium falciparum multidrug resistance protein-1 gene (*Pfmrp1*)

P. falciparum multidrug resistance protein-1 is a protein of the super family ABC carriers [31]. Two

common mono-nucleotide polymorphisms (SNP) frequent in Africa, I876V and K1466R, were selected by artemether/lumefantrine treatments and SP, but were never associated with altered sensitivity of African isolates [32]. Different *Pfmrp1* mutations were observed in isolates in northeastern Myanmar and were associated with reduced sensitivities to CQ, MQ, and lumefantrine (LUM) [33]. Impaired parasitic growth and an increase in sensitivity to CQ observed in *Pfmrp1*-deficient parasites, suggest that the protein is involved in parasitic fitness and antipaludic efflux [34].

P. falciparum dihydrofolate reductase gene (*Pfdhfr*) *and P. falciparum dihydropteroate synthase gene* (*Pfdhps*)

The *P. falciparum in vitro* resistance to antifolates (pyrimethamine, proguanil) and sulfonamides (sulfadoxine) is due to a sequential acquisition of specific point mutations on the genes *Pfdhfr* and *Pfdhps*, respectively [35]. The N51I, C59R, and S108N substitutions of the *Pfdhfr* gene confer resistance to pyrimethamine (PYR) while A16V and S108T confer resistance to cycloguanil [36]. The A437G, K540E and A581G substitutions of the *Pfdhps* gene are mostly involved in sulfadoxine resistance. Mutations on these target enzymes would alter the conformation of the active site where the inhibitors get attached, thereby decreasing their binding affinities, while retaining the main function of these enzymes [37].

The mutation *Pfdhfr S108N* is, in general, the first to appear when parasites are exposed to SP. This mutation results in a moderate level of resistance that increases when the parasite accumulates new mutations: A16V, N51I, C59R and I164L [36]. Prolonged exposure results in the accumulation of mutations on the 2 enzymes *DHFR* or *DHPS*. The haplotype A437G/K540E is the most common in Africa. The combination of this haplotype with the three mutations *Pfdhfr N51I*, *C59R,* and *S108N* forms a quintuple mutant highly resistant to SP, which now widely spread in Africa. Each mutation gives a reduction in sensitivity. Recently, a notion of „super-resistant" genotypes was introduced when the quintuple genotype is supplemented by the mutations *Pfdhps* A581G or A613S/T*,* or the mutation *Pfdhfr* I164L combines with the allele *Pfdhfr* N51I, C59R*,* S108N. The variation in the number of copies of the *GTP Cyclohydrolase* 1 (*gch1*) encoding the enzyme that catalyzes the first stage of the folate biosynthesis pathway, could compensate for the effect of mutations in the genes *Pfdhfr* and *Pfdhps*. Indeed, the increase in the number of copies of *gch1* would facilitate the development of highly resistant parasites by promoting the folate synthesis pathway through a greater supply of substrates. Contrary to what is observed for CQ-resistant parasites, the persistence of mutated strains on the genes *Pfdhfr* and *Pfdhps* and overexpressing *gch1*, in the absence of antifolate pressure, indicates a beneficial effect of *gch1* on the fitness of resistant parasites [37].

Plasmodium falciparum cytochrome *b* gene (*Pfcyt b*)

The cytochrome *bc1* complex (or complex III) is a key mitochondrial enzyme that catalyzes electron transfer from ubiquinol to cytochrome c, thereby maintaining mitochondrial membrane potential [38]. Atovaquone or hydroxy-1, 4-naphthoquinone is the only clinically used molecule that targets this enzyme. This molecule is a structural analogue of the parasitic ubiquinone. Ubiquinone accepts electrons from dehydrogenase enzymes and transfers them to cytochromes. Atovaquone (ATQ) is therefore a potent inhibitor that blocks this electron transfer chain and causes parasitic death [39].

Numerous SNPs in the Q0 domain cytochrome b gene (*cytb*) of *Plasmodium* spp. are involved in resistance to ATQ. Whereas M133I and L271V mutations are involved in mouse models of malaria and L144S, K272R, and V284F were observed in cultures exposed to high concentrations of ATQ [14,40]. SNPs at position 268 (Y268C, Y268S, Y268N) were associated with treatment failures with the ATQ-proguanil combination. These *cytb* mutations emerge and are rapidly selected in patients treated with ATQ due to a large number of *cytb* copies, the ease with which this gene mutates, and the low levels of electron transport required during the erythrocyte phase [41].

P. falciparum tetracycline resistance TetQ gene (*PftetQ*) *and P. falciparum multidrug transporter gene* (*Pfmdt*)

P. falciparum has the *PftetQ* gene of the *GTPase* family and the *Pfmdt* gene of multi-drug resistance. These genes both have strong sequence homologies with bacterial genes encoding, respectively, cytoplasmic proteins involved in the protection of ribosomes against the action of tetracycline and efflux pumps [42]. An increase in the number of

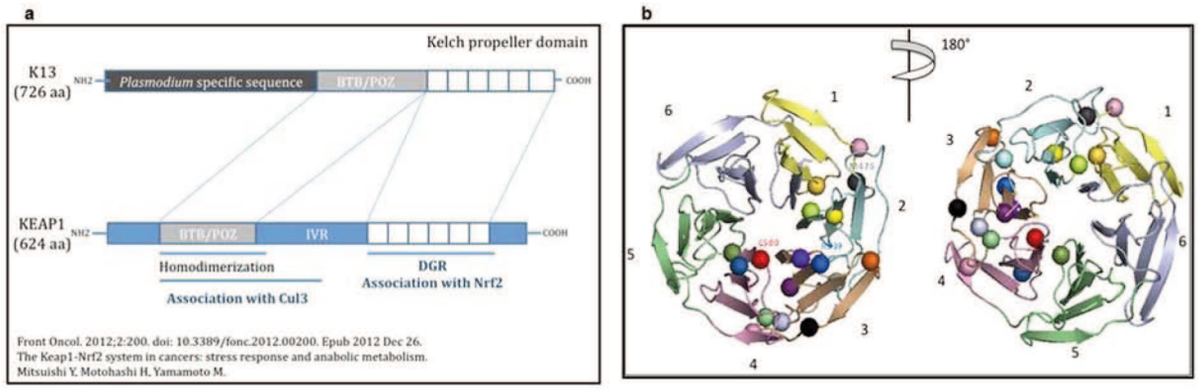

Figure 2. Schematic representation of K13 and its human orthologist KEAP1 [6]
(a) 3D modeling of the structure of K13, (b) Circles represent the different mutations

copies of the *PftetQ* and *Pfmdt* genes and a sequence polymorphism of the *PftetQ* gene were also shown to be associated with a decrease in *vitro Plasmodium* sensitivity to doxycycline (DOX) [24].

Recently, a case of prophylactic failure to DOX in an observing patient has been/ *were* postponed. This patient's isolate had 2 copies of the *PftetQ* and *Pfmdt* genes as well as 2 repeated KYNNNN patterns on the *PftetQ* gene confirming the previous results. However, in other studies published in the same year, no association was found between these markers and resistance observed in Thai isolates [43].

Plasmodium falciparum kelch13 gene (*Pfk13*)

An approach combining *in vitro* resistance selection and genomic analysis revealed a polymorphism on the gene *PF3D7_1343700* of chromosome 13, *Pfkelch13*, which correlated with a decrease in parasitic clearances in Southeast Asia (Fig. 2) [6].

K13 mutant strain generation studies revealed different resistance levels depending on the mutation introduced: the R539T and I543T mutations confer a higher resistance level than the M476I, C580Y, and Y493H mutations. In addition, the resistance levels induced by a mutation are influenced by the genetic background of the strains in which they are present [44]. In Africa, *Pfk13* mutations remain rare, varied; different from those observed in Asia and have never been associated with increased parasitic half-lives or RSA0-3h survival rates (synchronized ring-stage survival test between 0 and 3 hours), or with clinical failures [45].

Pfk13 is described as the orthologist of the *Keap 1* gene in humans, which codes for a constitutively sequestering protein, the Nrf2 transcription factor

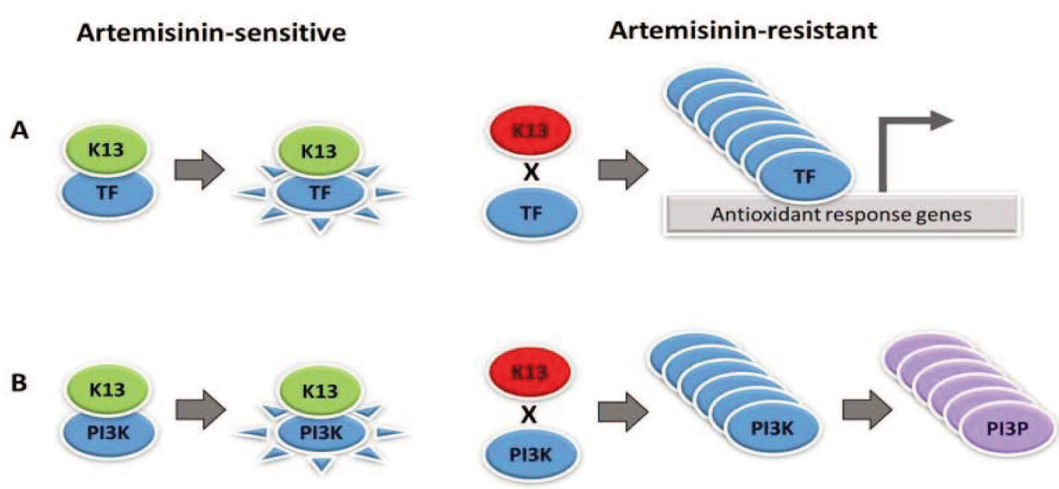

Figure 3. Assumed mechanisms of susceptibility and resistance of *P. falciparum* [46]
TF: Transcription Factor

Figure 4. Prevalence of Kelch 13 mutations involved in artemisinin derivative resistance between 2000 and 2017 worldwide [48]

on the cell membrane. Under oxidative stress, the *Nrf2/KEAP1* complex is cleaved and Nrf2 translocates to the nucleus where it induces transcription of genes involved in cellular protection from stress. It is assumed that the protein encoded by the *K13* gene could have functions similar to *KEAP1* in *P. falciparum* by delivering a potential transcription factor to an ubiquitin ligase so that it becomes the target of degradation. During oxidative stress, *K13* would release the transcription factor which, thus, would no longer be degraded by the proteasome but would accumulate in the nucleus to positively regulate about 200 genes involved in oxidative stress responses, such as that induced by artemisinin treatment [10]. In artemisinin-resistant parasites, mutations on the *K13* gene would constitutively prevent the interaction of *K13* with its partner and cause deregulation of the antioxidant response (Fig. 3).

Mok et al. [46] were able to associate artemisinin resistance with overexpression of the unfolded protein response (UPR) pathway and delayed progression of the intra-erythrocyte cycle, allowing parasites to repair damaged proteins before progressing in the cell cycle. Mbengue et al. [47] recently proposed another artemisinin mode of action model that would target a *K13* partner, *P. falciparum phosphatidylinositl-3-kinase* (*PI3K*). In artemisinin-sensitive parasites, *Pfk13* would be fixed to *PI3K* and become the target of proteasome degradation, limiting *PI3-phosphate* (*PI3P*) reserves. Thus, parasites subjected to *PI3K* inhibition related to the presence of artemisinin can no longer generate sufficient *PI3P* levels necessary for their growth. The study showed that a mutation of *Pfk13* by preventing ubiquitinylation of *PfPI3K*, caused its accumulation as well as that of *PI3P*, thus conferring the parasites, the resistance to ART. It should be noted that an increase in the product PI3P alone is sufficient to confer the resistance to ART of parasites carrying a wild *Pfk13* gene**.** [48].

Plasmepsin 2 (*Pfpm2*)

High frequency of isolates with multiple copies of the *Pfpm2* has already been reported in recent studies conducted in Cambodia. The emergence of piperaquine resistance among *P. falciparum* in Cambodia raises concerns the usefulness of drugs such as DHA-PPQ in the treatment of malaria in Southeast Asia [49]. Unexpectedly, amplification of *Pfpm2* gene in African isolates was shown to occur at a much higher frequency (27% on average across clinical sites in Africa, reaching 30.5% in Burkina Faso and 33.9% in Uganda) than was recently described (from 11.1 to 13.8% in Uganda, 10% in Mali and 1.1% in Mozambique). Considering the geographical extent and the diversity of the clinical sites in Africa, the high frequency reported at sites

distant to each other suggests that amplification of *Pfpm2* gene occurred independently in each site. More importantly, since in Southeast Asia most parasites with multiple copies of *Pfpm2* also display *kelch13* resistance mutations, which is not the case in African samples, it is likely that *Pfpm2* amplification originated in Africa, independently of Southeast Asia [50].

As to the mechanism of PPQ resistance involving plasmepsin II–III, PPQ is thought, like CQ, to accumulate in the food vacuole (FV) and inhibit conversion of toxic heme moieties to non-toxic hemozoin crystals during hemoglobin digestion. We have shown that PPQ is acting on all stages of the parasite life cycle but is least effective at the latest stages. High-resolution live-cell imaging has shown that hemoglobin degradation is initiated early after invasion of the RBC by the parasite [20].

In conclusion, the antimalarial resistance genes of *P. falciparum* observed over the past twenty years are a real challenge in the global antimalarial fight. In Africa, resistance to artemisinin has yet to be demonstrated. However, candidate genes for artemisinin resistance were demonstrated in several studies in Africa. Recent research into the identification and development of new molecules should allow for a greater antipaludic choice. There is a need to effectively monitor the emergence and spread of resistance to antimalarial drugs in order to control or eliminate malaria, which kills thousands of people every year, especially children. The implementation of RTS, S vaccine against malaria in children in 2021 could contribute to a gradual decrease in infant mortality.

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Received 31 October 2021
Accepted 15 February 2022