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Polymorphism of *Pfatpase6* in Côte d'Ivoire: Detection of a four new point mutations

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Over the past decade, the number of malaria cases has dropped by more than half in many malaria-endemic countries. However, recent parasite resistance to artemisinin undermines that progress. Artemisinin-based combination therapy (ACTs) is recommended for the treatment of *Plasmodium falciparum* malaria. Among the potential genes that are associated to resistance of *P. falciparum* to artemisinin include *PfATPase6* gene that encodes the protein SERCA: the specific target of drugs in the parasite. *PfATPase6* was the subject of many studies across the world to highlight its' involvement in the resistance of *P. falciprum* to artemisinin. It was found in this work that this gene has a polymorphism but its' involvement in the resistance of the parasite has not been demonstrated. The objective of this study was to describe the basic polymorphism of clinical isolates of *P. falciparum* in Côte d'Ivoire during the period when the country national anti- malaria program introduced ACTs in the treatment of malaria. Thus, 82 DNA fragments from 41 clinical isolates divided into regions A and B were analyzed using automatic sequencing method. The results show more points mutation of DNA fragments of PfATP6 but the most significant are D734Y (29.2%), Q254H (9.7%), N669Y (14.6%) and S670C (12.2%). Other mutations emerged in marginal proportions. We therefore recommend strict monitoring of gene polymorphism in *PfATPase6* in as much as the effectiveness of artemisinin derivatives is concerned; but the fact remains that their involvement in the resistance of *P falciparum* to artemisinin is still very low.

Key words: Côte d'Ivoire, detection, mutations, *PfATPase6*, polymorphism.

INTRODUCTION

Artemisinin (ART) and its derivatives play an indispensable role in the malaria elimination campaigns currently being unfolded in many regions where malaria is endemic. To reduce the chance of resistance development and prolong the life span of this group of drugs, the World Health Organization (WHO) has endorsed ART-based combination therapies (ACTs) as the first-line treatment for *Plasmodium falciparum* malaria

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(Nosten and White, 2007). Since the adoption of the ACT policy in many regions where *P. falciparum* malaria is endemic (Bosman and Mendis, 2007), a trend of steady reduction in global malaria incidence has been observed (WHO, 2011). However, the recent detection of emerging low-grade resistance to ARTs in Western Cambodia, which manifested as delayed parasite clearance, has raised a major concern (Noedl et al., 2008; Dondorp et al., 2009). As recommended by WHO Global Plan for Artemisinin Resistance Containment (GPARC), research aimed to decipher the underlying mechanisms of ART resistance has become a priority. ARTs contain an endoperoxide bridge that is essential for the parasite-killing activities (White, 2008).

Although the structure of ART was solved over three decades ago, the mode of action of this group of drugs has not been unequivocally determined (Cui and Su, 2009; O'Neill et al., 2010; Ding et al., 2011). The most-studied model suggests that heme-mediated activation of ARTs results in C-centered free radicals that alkylate biomolecules in the parasite, leading to parasite death (Meshnick, 1994; Meshnick, 2002; Krishna et al., 2004). Evidence supporting the involvement of heme in the action of ARTs includes antagonistic actions of iron chelators and the requirement of hemoglobin digestion for the activity of ART (Meshnick et al., 1993; Klonis et al., 2011). This also correlates with the tolerance phenomenon of ring-stage parasites to ARTs, when hemoglobin digestion activity is low. The reduced metabolic activity at the ring stage is reflected further in ART induced temporary arrest of growth (dormancy) at this stage (Chavchich et al., 2010; Witkowski et al., 2010). Whereas, this may partially explain the prolonged parasite clearance observed in clinical studies (Nosten, 2010), the possibility of host factors that may play a crucial role in determining prolonged parasite clearance times observed *in vivo* has not been investigated (Vattanaviboon et al., 1998; Charoenteeraboon et al., 2000). In addition, it has been proposed that ARTs may interfere with the mitochondrial function of the parasite (Li et al., 2005; Wang et al., 2010). Other postulated cellular targets of ARTs include the multidrug resistance 1 (*mdr1*) gene, ABC transporter genes *G7* and *G49* (Anderson et al., 2005), translationally controlled tumor protein (Bhisutthibhan et al., 1998), and the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) ortholog PfATP6 (Eshetu et al., 2010). Moreover, none of these candidate genes appears to be responsible for the observed ART resistance in areas of endemicity (Dondorp et al., 2009; Imwong et al., 2010). The proposal of PfATP6 as the primary target of ARTs in malaria parasites was initially based on the structural resemblance of ARTs to thapsigargin, a specific inhibitor of mammalian SERCAs. Since PfATP6 is the only SERCA-type Ca^{2+}-ATPase in the malaria parasite's genome, it was evaluated as the target of ARTs. When expressed in *Xenopus laevis* oocytes, PfATP6ase can be specifically inhibited by ART as well as thapsigargin (Eckstein-Ludwig et al., 2003). Modeling of PfATP6 and docking simulations suggest that ARTs bind to PfATP6 through hydrophobic interactions (Jung et al., 2005; Naik et al., 2011). Variations at a single residue, 263, located in the predicted ART-binding pocket of PfATP6ase, tremendously affect the sensitivity of the enzyme to ARTs (Uhlemann et al., 2005). When assayed in *X. laevis* oocytes, the introduction of a single substitution, L263A or L263S (residues in *Plasmodium vivax* and *Plasmodium berghei* SERCAs, respectively) resulted in an approximately 3-fold increase or decrease of sensitivity to ARTs, respectively. Furthermore, the L263E replacement led to complete abolishment of inhibition by ART (Uhlemann et al., 2005). However, this observation was not extended to *P. falciparum*, where introduction of the L263E mutation through transgenics resulted in borderline non-significant changes in the 50% inhibitory concentrations (IC_{50}s) for ART and its derivatives (Valderramos et al., 2010).

P. falciparum resistance to drugs remains a major public issue in Côte d'Ivoire. The therapeutic failure index for chloroquine and sulphadoxine-pyrimethamine are respectively 62.2 and 35.4% (Ouattara et al., 2010). Since the year 2005, Côte d'Ivoire switched as its first-line treatment policy for uncomplicated cases of malaria to the use of artemisinin-based combination therapy (ACT). Having started in 2006, the implementation has now reached the whole country. The main goal of the study, performed at a time when the national implementation of the ACT policy had just begun, was to describe the genetic polymorphism of the *PfATPase6* gene. This should provide useful baseline data for the Ivorian Malaria Control Program.

MATERIALS AND METHODS

Blood collection and DNA extraction

This study was carried out in two health centers of Abobo Township, El Rapha and Anokoua Kouté in the Northern of Abidjan district at 15 km from the center of the capital city. The study took place from February to December, 2006. During the study, only infected patients with *P. falciparum* diagnosis was carried out by microscopic examination of Giemsa-stained thick blood films as recommended by the Ivorian Malaria Control Program. Patients were aged between 2 to 45 years. Being given that it is a molecular study, only finger-pricked capillary blood was imbibed onto filter paper (Isocode Stix®, Schleichler and Schuell, Ecquevilly, France) obtained from infected patients with haemoglobin rate > 6 g/dL. However, all patients were treated after blood was taken free of charge with artemether-lumefantrine (AL) recommended by the Ivoirian Malaria Control Program. Imbibed blood filter paper samples were dried, and conserved until molecular analysis at Paris-Sud XI University, UMR 8080, Orsay (France). DNA was extracted from filter papers by the boiling method as follows. After rinsing two times with 500 µL of distilled water, the filter papers were immersed in 75 µL of distilled water in a 0.5 mL micro-tube and incubated at 99°C for 30 min. For each PCR, 12.5 µL of the supernatant was used. Informed consent was obtained from

Table 1. Oligonucleotide primers used for PCR amplification of *PfATPase6* gene.

Primer pair	Sequence 5' ⟶ 3'	Target region (pb)	Size (pb)
Sense PFATP-1 Antisense PFATP-2R	ATPGAAGAAGAGGTTATTAAGAATGCTCATACA ATTCATGGTTCATTTTTATATGGTTGTTTA	1-871 (A)	871
Sense PFATP-4 Antisense PFATP-5R	GATTCTTTAACAGAATACCAACTATGTCAA TGCCATATGGCTGGTATACGTGTATTTATG	1138-2431 (B)	1294
Sense PFATP-3 Antisense PFATP-2R	GGTTTGAATGAATTAGAAGTAGAAAAGAAG ATTCATGGTTCATTTTTATATGGTTGTTTA	121-871 (A)	751
Sense PFATP-6 Antisense PFATP-5R	ACAGAATACCAACTATGTCAAAAAGGGGAT TGCCATATGGCTGGTATACGTGTATTTATG	1147-2431 (B)	2431

Table 2. Single nucleotide polymorphisms (SNPs) and their corresponding amino acid point mutations in *PfATPase6* gene.

Wide/mutant type	SNPs	Frequency (%)
TAT- T**T**T[§]	Y148 F	1 (2.4)
GAT- GA**A**	D153E	1 (2.4)
AGT- A**A**T	S158N	1 (2.4)
GGT- GG**A**	G160G*	1 (2.4)
GGT- GG**A**	G223 G*	1 (2.4)
CAA- CA**C**	**Q254H**	**4 (9.7)**
CAT- C**C**T	H638P	1 (2.4)
TCA- TC**C**	S658S*	1 (2.4)
AAT- **U**AT	N663Y	1 (2.4)
AAT- **T**AT	N668Y	1 (2.4)
AAT- **T**AT	**N669**Y	**6 (14.6)**
AGT- **T**GT	**S670**C	**5 (12.2)**
TGG- **G**GG	W681G	1 (2.4)
GAT-**T**AT	**D734Y**	**12 (29.2)**

***Synonymous polymorphisms do not lead to any amino acid change. [§]Transferred nucleotides are in greasiness. N = 41. **Bold:** Sites where mutation have occurred

patients or guardians accompanying the sick children. The study was approved by the National Ethics Committee of Côte d'Ivoire.

PCRs amplication and sequencing

The following mixture was prepared in 50 µL final volume: genomic DNA extracted as described above, specific primers [SIGMA® Aldrich] (10 pmol), buffer (10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTPs and one unit of Taq DNA polymerase. Given the large size of the gene (3000 bp) and the points mutations described above, *PfATPase6* was divided into two regions A and B. For the primary PCR, the primers pairs used were PFATP-1/PFATP-2R (region A) and PFATP-4/PFATP-5R (region B). The PTC-100 thermal cycler (MJ Research, Watertown, MA) was programmed to carry out 30 cycles of 94°C for 2 min (first cycle) or 1 min (rest of the cycle), 50°C x 1 min, and 72°C x 1 min, followed by 72°C x 12 min at the end of 30 cycles. Due to the small quantity of DNA imbibed onto filter paper, nested PCR was necessary to obtain sufficient amount of amplified products for direct PCR sequencing or PCR-RFLP. The primary amplification was performed as described above, using 12.5 µL of DNA suspension. The nested-PCRs were performed with primers pairs PFATP-3/PFATP-2R for region A (900 bp fragment) and PFATP-6/PFATP-5R for region B (1359 bp fragment). All the primers used are presented in the Table 1. A known DNA of *P. falciparum* was used as positive control during the performed PCRs. Amplification products were sequenced by an automated sequencer (MWG Biotech.) and electropherograms were analyzed by using 4 peaks, DNA Strider and Ape software.

Analysis

Data were included in a data file with EPI-INFO v. 6.0 and bivariate relations were analysed by the kappa test of Cohen. The degree of agreement (k) was scored as follows: 0-0.20, slight agreement; 0.21-0.4, fair agreement; 0.41-0.60, moderate agreement; 0.61-0.80, good agreement; and > 0.81, very good agreement.

RESULTS

Samples characteristics

Samples from 41 patients with acute *P. falciparum* malaria ranging from 2 to 45 years old (48% of which were males) were studied. Four samples (8.8%) of the 45 collected isolates had parasites densities lower than 4,000 ring/µL and had been excluded. Sequencing was successful in the all 82 DNA fragments (41 fragments for each region). The size of region A was 900 bp while the region B was 1359 bp. The mean age was 12 years old and the H/F sex ratio was 1.07. The mean parasitemia was 54,831 ring/µL.

Genetic polymorphism and global prevalence of mutation

Genetic polymorphism was found at 14 point mutations. All the mutations were rare except four codons of the *PfATPase6* gene (Table 2). In our analysis, we have considered only four major mutations as follow: Q254H,

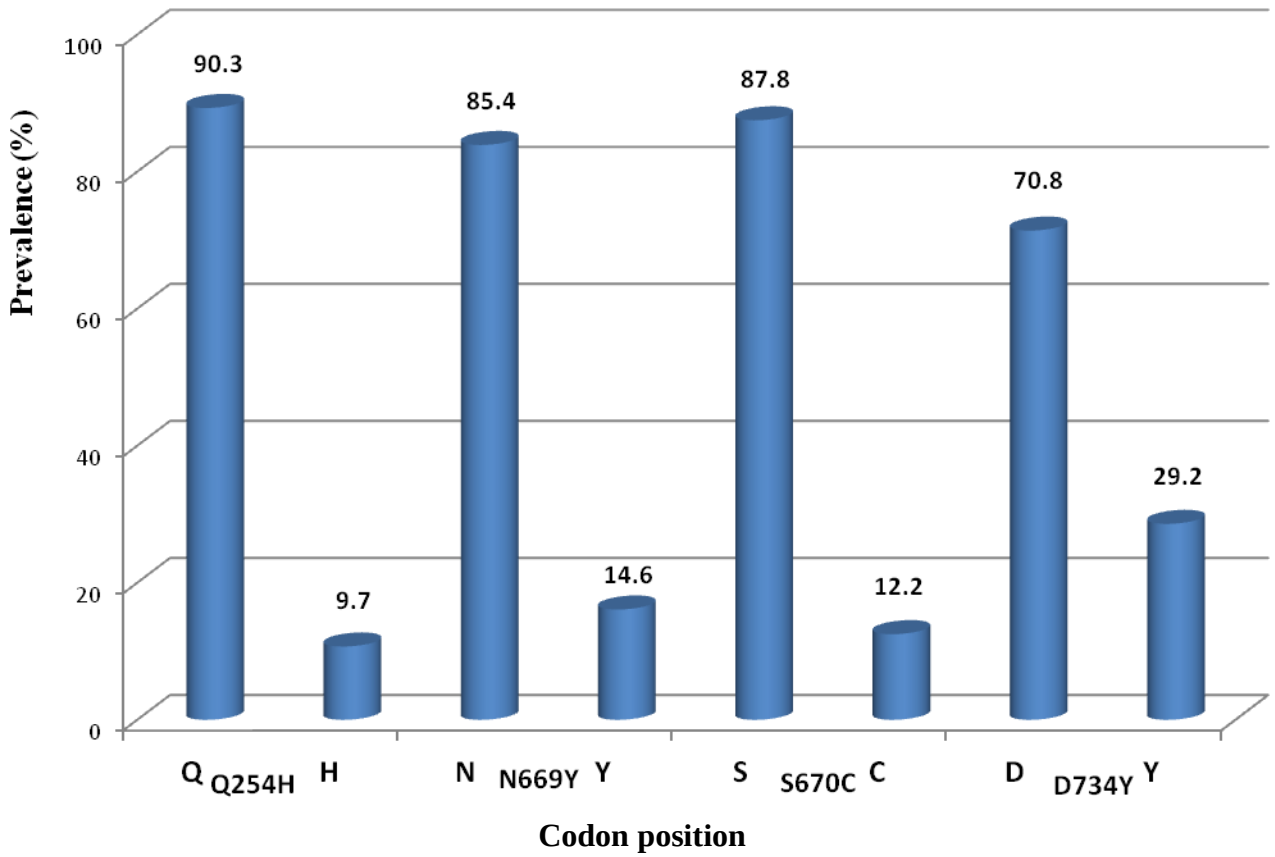

Figure 1. Prevalence of genetic polymorphism in *PfATPase* 6 gene.

N669H, S670C and D734Y. The mutation at codon 254 lead to the amino acid change glutamine in histidine. While the second and third mutations at codons 669 and 670 lead to the amino acid change asparagine in histidine and serine in cysteine, respectively. The fourth mutation at 734 lead to the amino acid change of aspartic acid in tyrosine. The prevalence of each mutation was: 9.7% for Q254H, 14.6% for N669H, 12.2% for S670C and 29.2% for D734Y (Figure 1). The G2306A mutation (encoding S769N) was absent in all the samples.

Bivariate relation of *Pfatpase* mutation according to parasitemia, age and blood collection area

Neither the parasitemia, nor the sex, nor the age, nor the blood collection area was related to the *Pfatpase* mutations (k<0.1).

DISCUSSION

The study on the evaluation of polymorphism in *PfATPase6* gene is the first of its kind to be undertaken in Côte d'Ivoire. This explains why local data is rarely available; but, this gene was tipped to be the genetic support of *P. falciparum* resistance to artemisinin (Imwong et al., 2010). In Côte d'Ivoire, several genetic markers of *P. falciparum* resistance to antimalarial drugs have regularly been the subject of study (Djaman et al., 2010). Those studies have shown, for example, the link between PfcrtK76T mutation and resistance to chloroquine, thus justifying the withdrawal of this molecule as first-line anti-malarial drug. Following example of molecular studies carried out in other countries, we wanted to evaluate the polymorphism in *PfATPase6* gene in Côte d'Ivoire in order to make local data available. This work took place just after the introduction of ACTs as first-line anti-malaria treatment in Côte d'Ivoire in 2005. The previous data indicated that *PfATPase6* gene is polymorphic at codons 89, 243, 263, 401, 431 568, 623, 630, 642, 769 and 898, and that these mutations would be responsible for the decreased in sensitivity of *P. falciparum* to artemisinin derivatives (Ulhemann et al., 2005; Jambou et al., 2005, Mugittu et al., 2006).

Grouping the gene into regions A and B has highlighted these haplotypes described above. Thus, all codons implicated in the resistance to artemisinin were explored in this present study. Apart from these haplotypes, sequencing was used to analyze more than 2400 base pairs (bp). Our results reveal 14 mutations at various

points; meanwhile, four codons had mutated in relatively high proportions. They are 254, 669, 670 and 734. None of the previously described haplotypes has mutated. These point mutations are new compared to the data available on the polymorphism in *PfATPase6* gene. Similar studies were conducted in Sao Tome and Principe and showed a silent mutation of T2694A (Ferreira et al., 2007). Ferreira et al. (2008) also published a similar paper with Brazilian samples (Ferreira et al., 2008). They described the analysis of four SNPs in isolates from Pará in nucleotide positions: 110, 1916, 2306 and 2694. The same previous study was checked for five *PfATPase*6 gene SNPs (538, 574, 623, 683 and 769) by DNA-microarrays (Crameri et al., 2007). By DNA-microarrays, the *PfATPaseA623E* mutation was found in 4.7% of the Niger samples, but sequencing did not confirm this. These abundant and often conflicting data on the polymorphism of *PfATPase6* gene clearly show the non-involvement of this gene in the resistance of *P. falciparum* to artemisinin. Recently, S769N mutation tipped to be the key mutation was found not associated with resistance of *P. falciparum* to artemether (Cui et al., 2012); meanwhile, the efficacy of artemisinin the current first molecule in malaria treatment is in sharp decline (Witkowski et al., 2013). Therefore, we must look for other molecular markers associated with *P. falciparum* resistance to artemisinin to better understand the mechanisms of resistance and thus improve malaria treatment (Ariey et al., 2014; Kamau et al., 2014).

Conclusion

The present work did not show the haplotypes; presently candidates as molecular markers for artemisinin resistance. However, the molecular diversity of *PfATP6* seems more pronounced than previously demonstrated as 14 mutations were detected with four relatively frequent. Whereas these mutations may be spontaneous and as such without any particular role in the parasite response to artemisinin, continual monitoring of *P. falciparum* susceptibility to artemisinin is highly welcome. In addition, molecular epidemiology should be part of routine surveillance to produce complementary information to assess the appropriateness of the current national anti-malarial drug policy.

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REFERENCES

Anderson TJ, Nair S, Qin H, Singlam S, Brockman A, Paiphun L, Nosten F (2005). Are transporter genes other than the chloroquine resistance locus (pfcrt) and multidrug resistance gene (pfmdr) associated with antimalarial drug resistance? Antimicrob. Agents Chemother. 49:2180-2188.

Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, Kim S, Duru V, Bouchier C, Ma L, Lim P, Leang R, Duong S, Sreng S, Suon S, Chuor CM, Bout DM, Sandie Menard S, Rogers WO, Genton B, Fandeur T, Miotto O, Ringwald P, Le Bras J, Berry A, Barale JC, Fairhurst RM, Benoit-Vical F, Mercereau-Puijalon O, Menard D (2014). A molecular marker of artemisininresistant *Plasmodium falciparum* malaria. Nature. 505:50-55.

Bhisutthibhan J, Pan QX, Hossler PA, Walker DJ, Yowell CA, Carlton J, Dame JB, and S. Meshnick R *(*1998). The *Plasmodium falciparum* translationally controlled tumor protein homolog and its reaction with the antimalarial drug artemisinin. J. Biol. Chem. 273:16192-16198.

Bosman A, Mendis KN **(**2007). A major transition in malaria treatment: the adoption and deployment of artemisinin-based combination therapies. Am. J. Trop. Med. Hyg. 77:193-197.

Charoenteeraboon J, Kamchonwongpaisan S, Wilairat P, Vattanaviboon P, Yuthavong Y **(**2000). Inactivation of artemisinin by thalassemic erythrocytes. Biochem. Pharmacol. 59:1337-1344.

Chavchich M, Gerena L, Peters J, Chen N, Cheng Q , Dennis E. Kyle E (2010). Role of pfmdr1 amplification and expression in induction of resistance to artemisinin derivatives in *Plasmodium falciparum*. Antimicrob. Agents Chemother. 54:2455-2464.

Crameri A, Marfurt J, Mugittu K, Maire N, Beck HP (2007). Rapid microarray- based method for monitoring of all currently know single nucleotide polymorphisms associated with parasite resistance to antimalaria drugs. J. Clin. Microbiol. 45:3685-3991.

Cui L, Su XZ **(**2009). Discovery, mechanisms of action and combination therapy of artemisinin. Expert Rev. Anti Infect. Ther. **7**:999-1013.

Cui L, Wang Z, Jiang H, Parker D, Wang H, Su ZX, and Cui L (2012). Lack of Association of the S769N Mutation in *Plasmodium falciparum* SERCA (PfATP6) with Resistance to Artemisinins, Antimicrob. Agents Chemother. 56 (5):2546-2552.

Ding XC, Beck HP, Raso G **(**2011). *Plasmodium* sensitivity to artemisinins: magic bullets hit elusive targets. Trends Parasitol. 27:73-81.

Djaman AJ, Ahibo H, Yapi HF, Bla KB, Ouattara L, Yavo W, Yapo A, Mazabraud A (2010). Molecular monitoring of *falciparaum* malaria isolates in Côte d'Ivoire: genetic markers (*dhfr-ts*, *dhps*, *pfcrt*, *pfmdr-1*) for antimalarial-drugs resistance. Eur. J. Sci. Res. 40(3):461-470.

Dondorp AM, Nosten F, Yi P, Das, D, Phyo A.P, Tarning J, Lwin, KM., Ariey F, Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An SS, Yeung S, Singhasivanon P, Day NP, Lindegardh N, Socheat D, White NJ (2009). Artemisinin resistance in *Plasmodium falciparum* malaria N. Engl. J. Med. 361(5):455-467.

Eckstein-Ludwig U, Webb RJ, Van-Goethem ID, East JM, Lee AG, Kimura M, O'Neill PM, Bray, PG, Ward SA, Krishna S (2003). Artemisinins target the SERCA of *Plasmodium falciparum*. Nature. 424 (6951):957-961.

Eshetu T**,** Berens-Riha N**,** Fekadu S**,** Tadesse Z**,** Gürkov R**,** Hölscher M**,** Löscher T**,** Miranda IB (2010). Different mutation patterns of *Plasmodium falciparum* among patients in Jimma University Hospital, Ethiopia. Malar. J. 9:226.

Ferreira ID, Lopes D, Martinelli A, Ferreira C, Rosario VE, Cravo P (2007). *In vitro* assessment of artesunate, artemether and amodiaquine susceptibility and molecular analysis of putative resistance-associated mutations of *Plasmodium falciparum* from Sao Tomé and Principe. Trop. Med. Int. Health. 12:353-362.

Ferreira ID, Martinelli A, Rodrigues LA, do Carmo EL, Rosario VE, Povoa MM, Cravo P (2008). *Plasmodium falciparum* from Para state (Brazil) shows satisfactory in vitro response to artemisinin derivatives and absence of the S769N mutation in the SERCA-type PfATPase6. Trop. Med. Int. Health. 13:199-207.

Imwong M, Dondorp AM, Nosten F, Yi P, Mungthin M, Hanchana S, Das D, Phyo AP, Lwin KM, Pukrittayakamee S, Lee SJ, Saisung S, Koecharoen K, Nguon C, Day NP, Socheat D, White NJ (2010). Exploring the contribution of candidate genes to artemisinin resistance in *Plasmodium falciparum*. Antimicrob. Agents Chemother. 54:2886-2892.

Jambou R, Legrand E, Niang M, Khim N, Lim P, Volney B, Ekala MT, Bouchier C, Esterre P, Fandeur T, Mercereau-Puijalon O (2005). Resistance of *Plasmodium falciparum* field isolates to *in-vitro* artemether and point mutations of the SERCA-type *PfATPase6*. Lancet. 366 (9501):1960-1963.

Jung M, Kim H, Nam KY, No KT **(**2005). Three-dimensional structure of *Plasmodium falciparum* Ca^{2+}-ATPase (PfATP6) and docking of artemisinin derivatives to PfATP6. Bioorg. Med. Chem. Lett. 15:2994 -2997.

Kamau E, Campino S, Amenga-Etego L, Drury E, Ishengoma D, Johnson, Mumba D, Mihir Kekre2, William Y, Mead D, Bouyou-Akotet M, Apinjoh T, Golassa L, Randrianarivelojosia M, Andagalu B, Maiga-Ascofare O, Amambua-Ngwa A, Tindana P, Ghansah A, MacInnis B, Kwiatkowski D, Djimde A (2014). K13-propeller polymorphisms in *Plasmodium falciparum* parasites from sub-Saharan Africa. J. Infect. Dis. doi: 10.1093/infdis/jiu608.

Klonis N, Crespo-Ortiz MP, Bottova I, Abu-Bakar N, Kenny S, Rosenthal PJ, Tilley L (2011). Artemisinin activity against *Plasmodium falciparum* requires hemoglobin uptake and digestion. Proc. Natl. Acad. Sci. U. S. A. 108:11405-11410.

Krishna S, Uhlemann AC, Haynes RK **(**2004). Artemisinins: mechanisms of action and potential for resistance. Drug Resist. Update. 7:233-244.

Li W, Mo W,Shen D, Sun *L*, Wang J, Lu S, Gitschier JM, Zhou B (2005). Yeast model uncovers dual roles of mitochondria in action of artemisinin. PLoS Genet. 1:e36.

Meshnick SR **(**1994). The mode of action of antimalarial endoperoxides. Trans. R. Soc. Trop. Med. Hyg. 88 (Suppl. 1):S31-S32.

Meshnick SR **(**2002). Artemisinin: mechanisms of action, resistance and toxicity. Int. J. Parasitol. 32:1655-1660.

Meshnick SR, Yang YZ, Lima V, Kuypers F, Kamchonwongpaisan S, Yuthavong Y (1993). Iron-dependent free radical generation from the antimalarial agent artemisinin (qinghaosu). Antimicrob. Agents Chemother. 37:1108 -1114.

Mugittu K, Genton B, Mshinda H, Beck HP (2006). Molecular monitoring of *Plasmodium falciparum* resistance to artemisinin in Tanzania. Malar. J. 5:126.

Naik PK, Srivastava M, Bajaj P, Jain S, Dubey A, Ranjan P, Kumar R, Singh H (2011). The binding modes and binding affinities of artemisinin derivatives with *Plasmodium falciparum* Ca^{2+}-ATPase (PfATP6). J. Mol. Model. 17:333-357.

Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM (2008). Evidence of artemisinin-resistant malaria in western Cambodia. N. Engl. J. Med. 359:2619-2620.

Nosten F **(**2010). Waking the sleeping beauty. J. Infect. Dis. 202:1300-1301.

Nosten F, White NJ **(**2007). Artemisinin-based combination treatment of *falciparum* malaria. Am. J. Trop. Med. Hyg. 77:181-192.

O'Neill PM, Barton VE, Ward SA **(**2010). The molecular mechanism of action of artemisinin: the debate continues. Molecules. 15:1705-1721.

Ouattara L, Bla KB, Assi SB, Yavo W, Djaman AJ (2010). *pfcrt* and *dhfr-ts* sequences for monitoring drug resistance in Adzopé Area of Côte d'Ivoire after the withdrawal of chloroquine and pyrimethamine. Trop. J. Pharm. Res. 9(6):565-572.

Uhlemann, AC, Cameron A, Eckstein-Ludwig U, Fischbarg J, Iserovich P, Zuniga FA, East M, Lee A, Brady L, Haynes RK, Krishna S (2005). A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. Nat. Struct. Mol. Biol. 12(7):628-629.

Valderramos SG, Valderramos JC, Musset L, Purcell LA, Mercereau-Puijalon O, Legrand E, David A. Fidock DA (2010). Identification of a mutant PfCRT-mediated chloroquine tolerance phenotype in *Plasmodium falciparum*. PLoS Pathog. 6:e1000887.

Vattanaviboon P, Wilairat P, Yuthavong Y **(**1998). Binding of dihydroartemisinin to hemoglobin H: role in drug accumulation and hostinduced antimalarial ineffectiveness of alpha-thalassemic erythrocytes. Mol. Pharmacol. 53:492-496.

Wang J, Huang L, Li J, Fan Q, Long Y, Li Y, Zhou B (2010). Artemisinin directly targets malarial mitochondria through its specific mitochondrial activation. PLoS One 5:e9582.

White NJ **(**2008). Qinghaosu (artemisinin): the price of success. Sci. 320:330-334.

WHO **(** 2011). World malaria report 2010. World Health Organization, Geneva, Switzerland. http://www.who.int/malaria/world_malaria_report_2010/en/index.html.

Witkowski B, Lelièvre J, Barragán JM, Victor Laurent V, ZX, Antoine Berry A, Françoise Benoit-Vical F (2010). Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism. Antimicrob. Agents Chemother. 54:1872-1877.

Witkowski B, Khim N, Chim P, Kim S, Ke S, Kloeung N, Chy S, Duong S, Leang R, Ringwald P, Dondorp AM, Tripura R, Benoit-Vical F, Berry A, Gorgette O, Ariey F, Barale JC, Mercereau-Puijalon O, Menard D (2013). Reduced artemisinin susceptibility of *Plasmodium falciparum* ring stages in Western Cambodia, Antimicrob. Agents Chemother. 52 (2):914-923.