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Predominance of Wild *K13 Propeller* Haplotypes in three localities of Côte d'Ivoire one decade after the adoption of Therapeutic Combinations based on Artemisinin Derivatives

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A B S T R A C T

The emergence of *P. falciparum* resistance to artemisinin-based combination therapy (ACT) threatens malaria control in Africa. The monitoring of polymorphisms in molecular markers associated with antimalarial drug resistance is essential for malaria control and elimination efforts. The purpose of this study is to analyze the polymorphism of the *pfK13 propeller* gene of *P. falciparum* to artemisinin-based combination therapy (ACT) in three sites in southern Côte d'Ivoire. After obtaining informed consent, blood samples were collected from patients of any sex and aged over 2 years with uncomplicated *P. falciparum* malaria in Anonkoua-Kouté, Port-Bouët and Ayamé. *P. falciparum* genomic DNA was extracted and amplified by Nested-PCR using Pfk13 propeller gene-specific primers. The amplification products were sequenced using the Sanger method at Eurofins Genomics. The key codons (493, 539, 543 and 580), molecular markers of the resistance of *P. falciparum* to ACTs and the additional codons (476 and 561) were analyzed. A total of 186 DNA extracts from blood samples collected from the three study sites were amplified and sequenced. Of the 93 PCR products sequenced, 84.94% (158/186), 91.39% (170/186), 95.69% (178/186), 100% (186/186), 82.79% (154/186) and 94.62% (178/186) corresponding to codons 493, 539, 543, 580, 476, 561, respectively, were successfully analyzed. Thus, the prevalences of wild-type alleles were 92.40% (Tyr493), 94.11% (Arg539), 94.38% (Ile543), 91.39% (Cys580), 89.61% (Met476) and 88.63% (Arg561) against 1.26% (His493), 3.52% (Thr539) and 1.29% (Ile476) of mutant alleles. The mutant alleles Thr543, Tyr580, and His561 were not observed in all three study sites. The analysis indicated a predominance of the YRICMR allelic form representing a susceptible haplotype (78.49%). No significant difference was observed between the prevalences of the wild-type alleles determined in the three study sites ($p>0.05$). More than a decade after the adoption of ACTs for the management of uncomplicated malaria, these combinations are still effective in Anonkoua-Kouté, Port-Bouët and Ayamé in the south of Côte d'Ivoire.

Introduction

Malaria is a serious public health problem in the world. According to the World Health Organization, approximately 247 million cases of malaria were recorded in 2021, of which 619,000 caused deaths, 95% of which occurred in Africa (WHO, 2022). The vast majority of deaths were among children under five years of age, accounting for 80% of all malaria deaths (WHO, 2022).

For nearly 15 years, the treatment of *Plasmodium falciparum* malaria has been based on the use of artemisinin-based combination therapy (ACT). The main objective of treatment is to ensure rapid and complete elimination of malaria parasites to avoid the progression of a simple case to severe or fatal malaria. However, the emergence of *P. falciparum* resistance to ACTs that has been reported since 2008 in Southeast Asia poses a serious threat to malaria control worldwide, particularly in Africa, where malaria transmission is most prominent (WHO, 2018; Noedl *et al.,* 2008; Haldar *et al.,* 2018; Ashley *et al.,* 2014). In Côte d'Ivoire, artesunate-amodiaquine (AS-AQ) and artemether-lumefantrine (AL) combinations have been the first- and second-line treatments for uncomplicated *P. falciparum* malaria since 2004, respectively. Both ACTs have been shown to be highly effective against acute uncomplicated *P. falciparum* malaria (Toure *et al.,* 2014).

Clinical artemisinin resistance is characterized by delayed parasite clearance, and *P. falciparum* isolates from responding patients with a long parasite clearance half-life are characterized by high *in vitro* survival rates using the ring survival assay (RSA_{0-3h}) (Witkowski *et al.,* 2013). Several studies have demonstrated that artemisinin resistance is primarily due to mutations in the *Plasmodium falciparumkelch13 propeller* gene (pfk13) (Ariey *et al.,* 2014; Ashley *et al.,* 2014; MalariaGEN, 2016; Didier Menard *et al.,* 2016; Siddiqui *et al.,* 2020). Recent data suggest that these mutations result in reduced endocytosis of host-derived hemoglobin and, consequently, decreased release of the Fe2+-heme fraction (activating artemisinin), which reduces the efficacy of artemisinin (Yang *et al.,* 2019; Birnbaum *et al.,* 2020).

For example, mutations C580Y, N458Y, M476I, Y493H, R539T, I543T, and R561H) in the *pfk13* gene have been validated as mutations conferring artemisinin resistance (Straimer *et al.,* 2015; Siddiqui *et al.,* 2020; Uwimana *et al.,* 2020; Bergmann *et al.,* 2021). Multiple other mutations have been associated with the delayed clearance phenotype and have been proposed as candidate markers for artemisinin resistance (Group, 2019; WHO, 2019).

Several studies conducted in African countries to establish the presence of *pfk13 propeller* gene mutations led to the discovery of non-synonymous mutations (M472I; Y558C; K563R; P570L; P615S) in Niger (Laminou *et al.,* 2017), a novel mutation (R622I) in Ethiopia (Bayih *et al.,* 2016), 15 nonsynonymous mutations in Senegal (Talundzic *et al.,* 2017), the R561H mutation in Rwanda (Uwimana *et al.,* 2020), the P553L mutation in Mali, Kenya and Malawi (Taylor *et al.,* 2015). However, these mutations have not been linked to delayed parasite clearance except for the one discovered in Rwanda. Thus, there is concern that ACT resistance may spread to other regions such as sub-Saharan Africa as has occurred in the past with chloroquine, amodiaquine, and sulfadoxine-pyrimethamine; hence, the need to strengthen surveillance of artemisinin resistance in Africa. This study, conducted in three locations in southern Côte d'Ivoire, aims to determine the prevalence of selected mutations associated with *Plasmodium falciparum* resistance to ACTs in subjects with uncomplicated malaria.

Materials and Methods

Study site

This was a prospective study that took place from February to August 2015 at the AnonkouaKouté health center and the general hospitals of Port-Bouët

and Ayamé. These sites are located in the southern region of Côte d'Ivoire where the climate is dominated by annual rainfall that exceeds 1700 mm with a temperature that varies between 27 and 33 °C. Malaria is seasonal; predominantly in the rainy season from June to September with peaks in prevalence and incidence in October-November. *Plasmodium falciparum* is the dominant species with over 90% of the parasite formula. The main malaria vectors in this study area (the forested south of Côte d'Ivoire) are members of the *An. gambiaesl* and *An. funestussl* complexes (Adja *et al.,* 2011). The Anonkoua-Kouté health center and the Ayamé general hospital were selected because of their high annual incidences of malaria cases. In addition, these health facilities have been considered for several years as the main sites for multicenter clinical efficacy testing by the Malaria Unit of the Pasteur Institute of Côte d'Ivoire. The Port Bouët General Hospital was selected for this study not only because of its consistently high annual incidence of malaria cases, but also and especially because of its swampy environment used for market gardening.

Study population and sample collection

All patients clinically suspected of having malaria at the Anonkoua-Kouté health center, the general hospitals of Port-Bouët and Ayamé during our study period were eligible. However, after informed consent, blood samples were collected from patients older than 2 years with axillary or rectal temperature higher than 37.5°C and with microscopically confirmed uncomplicated *P. falciparum* malaria.

Blood collection

From each patient with microscopically confirmed malaria, approximately 2 to 5 mL of venous blood was drawn and collected in an EDTA tube. Approximately 50 µL of whole blood was spotted on Whatman 3 MM filter paper using a micropipette with filter cones. The paper containing the blood spots was dried for approximately 60 to 120 minutes at room temperature in a dust-free environment. Blood not used after confettiing and contained in the EDTA tube was stored in cryotubes at -20°C for possible future use.

Amplification of the pfk13 gene

The *pfk13 propeller* gene (pfK13) was amplified by nested PCR using a specific primer pair and a commercial DNA polymerase kit named 5X FIREPol® Blend Master Mix with mM MgCl2. The composition of this kit was a pre-mix for the reaction mixture.

For the primary PCR, the pair of primers used for the amplification of the *pfK13 gene* were K13_PCR_F (5'CGGAGTGACCAAATCTGGGA / K13_PCR_R (5'GGGAATCTGGTGGTAACAGC).

Primary PCR of this gene was performed in a reaction volume of 25 µL containing: 0.625 µL of each primer, 3 µL of plasmodial DNA, 5 µL of Taq polymerase and 15.75 µL of milliQ water. The mixture was then placed in a PTC-100TM thermal cycler (Eppendorf Mastercycler, PTC-100 Peltier Thermal Cycler), programmed as follows: An initial denaturation at 95°C for 15 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, hybridization at 58°C for 2 minutes and extension at 72°C for 2 minutes. Finally, a terminal extension at 72°C for 10 minutes.

The second PCR was performed on the amplification products of the primary PCR in a reaction volume of 50 µl containing: 1.25 µL of each primer, 5 µL of amplification product (ampliate) from the first PCR, 5 µL of Taq polymerase, and 37.5 µl of milliQ water. The primer pairs used for the secondary PCR, were. K13_N1_F (5'GCCAAGCTGCCATTCATTTG / K13_N1_R (5'GCCTTGTT GAAAGAAGCAGA).

Detection and analysis of PCR products

The amplification products were migrated onto a 1.5% (w/v) agarose gel containing Ethidium Bromide (BET). After migration, the gel was recovered and observed under a UV lamp using the

UV transluminator (Gel DocTM EZ Imager). The presence or absence of bands was used to judge the efficiency of the PCR.

Sequencing Amplification

In this work, the amplified DNA fragments (*pfK13* gene) were sequenced using the Sanger method by Eurofins MWG operon (Cochin Sequencing Platform). Samples were provided to the platform in a microplate (Greiner Bio-one-652270B) with a deposit slip that was sent to the platform's email address. A reaction medium was prepared for the nested PCR sense primer (sequencing primer) from the amplification products.

In each well of the microplate, a volume of 13 µL of amplification product was added to 2 µL of sequencing primer at 10 µM. The wells containing the sequencing reaction medium were sealed with cap strips (4titude-044737) before covering the entire surface of the microplate with adhesive film (AmpliSeal, Greiner Bio-one-676040). This microplate containing our samples was provided to the platform for sequencing.

After the sequencing reaction, the received DNA sequences were recovered in fasta form. For this work, these are the sequences corresponding to the *pfK13* gene of the collected isolates. The use of BioEdit software allowed to analyze the sequences for possible mutations. Indeed, the loci of interest, i.e. codons at position 476, 493, 539, 543, 561 and 580 of the PFK13 protein or nucleotides at position 1428, 1479, 1617, 1623, 1683 and 1740 of the *pfK13* gene, were identified and analyzed after a parallel alignment of two or more DNA sequences including the reference sequence of the *pfK13* gene, maximizing the number of identical nucleotides or residues, while minimizing the number of mismatches and gaps.

Statistical analysis of the data

Data were collected on a standard questionnaire that was tested and validated. They were then entered and analyzed on R statistical software; version 3.2.2 (Core Team, 2013). The χ^2 test for comparison of three means was used to compare the prevalences of the M476I, Y493H, R539T, I543T, R561H, and C580Y mutations of the *pfK13* gene.

The χ^2 test was used to determine whether the molecular marker prevalences can be considered all equal (null hypothesis H0) or whether at least two prevalences are different (alternative hypothesis Ha). A statistical difference and/or association was considered significant if p of the χ^2 test <0.05.

Results and Discussion

Profile of patients and selected isolates

A total of 186 persons infected with *Plasmodium falciparum* were selected for this study, 114 (61.3%) of whom were female and 72 (38.7%) male. The age of the patients ranged from 2 to 62 years with an average age in Anonkoua-Kouté, Port-bouët and Ayamé of 16.60, 16.69 and 15.84 years respectively. Thus, 186 blood samples were collected from all three study sites (Table 1).

Sequencing results

For all three study sites, 186 DNA fragments from patient isolates were successfully sequenced.

Molecular analysis of the 186 fragments showed that the number of successfully sequenced DNA fragments varied according to the presence of codons of interest on the fragment obtained.

Thus, for these 186 successfully sequenced DNA fragments, 158 (84.94%), 170 (91.4%), 178 (95.7%), 186 (100%), 154 (82.8%) and 176 (94.62%) DNA fragments were successfully sequenced for nucleotides at positions 1479, 1617, 1629, 1740, 1428, and 1683, which correspond to the amino acids at which the Tyr-493-Ile, Arg-539-Thr, Ile-543-Thr, Cys-580-Tyr, Met-476-Ile, and Arg-561-His mutations are observed respectively (Table 2).

Sequencing of the DNA region leading to the Cys-580-Tyr mutation was performed more successfully (186/186; i.e., 100%).

Polymorphism of the *pfk13 propeller* gene in the study sites

Prevalence of individual alleles of the pfk13 propeller gene and molecular analysis of genotypes

For all three study sites, our results indicate that the prevalences of isolates carrying the His-493 (1.26%), Thr-539 (3.52%) and Ile-476 (1, 29%) are very low (less than 5%) compared to those of the wild-type isolates Tyr-493 (92.40%), Arg-539 (94.11%), Ile-543 (94.38%), Cys-580 (91.39%), Met-476 (89.61%) and Arg-561 (88.63%) of the *pfK13 propeller* gene. (Table 3).

The Thr-543, Tyr-580 and His-561 mutations were not observed. However, isolates carrying the Cys-561 mutation (7.95%) were observed.

Very low prevalences (less than 5%) of other mutations in the *pfk13 propeller* gene were also recorded (Table 3).

Molecular analysis showed that the YRICMR allelic form (susceptible haplotype) was predominant in the isolates with a prevalence of 77.42% compared to 7.53%, 9.68%, 3.22% and 2.15% prevalences respectively for the single mutant, double mutant, triple mutant and quadruple mutant genotypes observed (Table 4).

Prevalence of mutations in the *pfk13 propeller* gene in Anonkoua-Kouté, Port-Bouët and Ayamé

Our results show that the Ile-543-Thr, Cys-580-Tyr and Arg-561-His mutations were not observed in Anonkoua-Kouté, Port-Bouët and Ayamé while the Tyr-493-His, Arg-539-Thr and Met-476-Ile mutations were observed at very low proportions (less than 5%) in these three localities. None of the Tyr-493-His, Arg-539-Thr, Ile-543-Thr, Cys-580-Tyr, Met-476-Ile and Arg-561-His mutations were observed in Port-Bouët.

Table.1 Samples used for molecular analysis of artemisinin chemoresistance.

Sites	Collection periodin 2015	Age groups (years)	Averageage (years)	Conffetiscollected
Anonkoua-kouté	February - March	2 à 53	16,60	60
Port - Bouët	April - May - July	2 à 62	16,69	64
Ayamé	June - July - August	2 à 55	15,84	62
Total				**186**

Table.2 Summary of DNA sequencing of isolates according to mutations

Sequenced fragments	Mutations	Successful n (%)	Failures n (%)
pfk13 **(N = 186)**	Tyr-493-**His**	158 (84,95)	28 (15,05)
	Arg-539-**Thr**	170 (91,4)	16 (8,6)
	Ile-543-**Thr**	178 (95,7)	8 (4,3)
	Cys-580-Tyr	**186** (100)	**0** (0)
	Met-476-**Ile**	154 (82,8)	32 (17,2)
	Arg-561-**His**	176 (94,62)	10 (5,38)

Table.3 Prevalence of individual alleles of the pfk13 porpeller gene at the study sites.

			Study sites (N=186)
Codons	**Strain and mutations observed**		**Numbers (%)**
K13_493 (n = 158)	**Wild**	**Tyr-493**	**146 (92,40)**
	Mutants	**His-493**	**2 (1,26)**
		Pro-493	2 (1,26)
		Phe-493	6 (3,79)
		Cys-493	2 (1,26)
K13_539 (n = 170)	**Wild**	**Arg-539**	**160 (94,11)**
	Mutants	**Thr-539**	**6 (3,52)**
		Gly-539	2 (1,17)
		Pro-539	2 (1,17)
K13_543 (n = 178)	**Wild**	**Ile-543**	**168 (94,38)**
	Mutants	**Thr-543**	**0,00**
		Met-543	4 (2,24)
		Phe-543	4 (2,24)
		Ser-543	2 (1,12)
K13_580 (n = 186)	**Wild**	**Cys-580**	**170 (91,39)**
	Mutants	**Tyr-580**	**0,00**
		Ser-580	6 (3,22)
		Pro-580	6 (3,22)
		Gly-580	2 (1,07)
		Arg-580	2 (1,07)
K13_476 (n = 154)	**Wild**	**Met-476**	**138 (89,61)**
	Mutants	**Ile-476**	**2 (1,29)**
		Gly-476	4 (2,59)
		Lys-476	2 (1,07)
		Arg-476	4 (2,59)
		Thr-476	2 (1,29)
		Ser-476	2 (1,29)
R561H (n = 176)	**Wild**	**Arg-561**	**156 (88,63)**
	Mutants	**His-561**	**0,00**
		Cys-561	**14 (7,95)**
		Trp-561	2 (1,13)
		Ser-561	2 (1,13)
		Leu-561	2 (1,13)

Notes: "N" represents the total number of isolates successfully sequenced at the three sites. "n" represents the number of isolates for which codons of interest (493, 539, 543, 580, 478, and 561) or nucleotides at positions 1479, 1617, 1629, 1740, 1428, 1683, of the sequence were located.

Table.4 Prevalence of genotypes corresponding to pfk13propeller in the three sites.

							Blood (N=186)	
Haplotype	**Y493H**	**R539T**	**I543T**	**C580Y**	**M476I**	**R561H**	**n**	**proportion**
Wild	**Y**	**R**	**I**	**C**	**M**	**R**	**144**	**77,42**
Single Mutants							**14**	**7,53**
	Y	R	I	C	**K**	R	2	
	Y	R	I	C	**R**	R	2	
	Y	R	I	C	**T**	R	2	
	Y	R	I	C	M	**C**	2	
	C	R	I	C	M	R	2	
	Y	R	I	**G**	M	R	2	
	Y	R	I	**S**	M	R	2	
Double Mutants							**18**	**9,68**
	Y	R	I	C	**G**	**C**	2	
	Y	**G**	I	**S**	M	R	2	
	P	R	I	C	M	**C**	2	
	Y	R	**M**	C	M	**C**	2	
	Y	R	**F**	**P**	M	R	2	
	F	**T**	I	C	M	R	2	
	F	R	I	C	**R**	R	2	
	Y	R	I	C	**S**	**C**	2	
	Y	R	I	**R**	M	**L**	2	
Triple Mutants							**6**	**3,22**
	H	R	I	C	**I**	**C**	2	
	Y	R	**M**	**P**	M	**W**	2	
	Y	**P**	**F**	**F**	M	R	2	
Quadruple Mutants							**4**	**2,15**
	Y	**T**	**S**	**P**	M	**S**	2	
	F	**T**	I	C	**G**	**C**	2	

Notes: A capital letter in the "genotypes" column represents the single-letter code for the amino acids. The amino acids resulting from the mutation are underlined and in bold. The prevalences determined correspond to the number of observations over the number of hits per gene.

Table.5 Prevalence of wild alleles of the pfK13 propeller gene in Anonkoua-Kouté, Port-Bouët and Ayamé

Codon K13_493 (N=158)							
Alleles	**Anonkoua-Kouté** (n=46)		**Port-Bouët** (n=56)		**Ayamé** (n=56)		**p of the χ^2 test**
	Numbers	(%)	Numbers	(%)	Numbers	(%)	
Tyr-493	42	91,3	54	96,43	50	89,29	0,342
His-493	**2**	**4,35**	**0**	**0**	**0**	**0**	**0,085**
Other	2	4,35	2	3,57	6	10,71	0,242
Codon K13_539 (N=170)							
Alleles	**Anonkoua-Kouté** (n=54)		**Port-Bouët** (n=60)		**Ayamé** (n=56)		**p of the χ^2 test**
	Numbers	(%)	Numbers	(%)	Numbers	(%)	
Arg-539	52	96,30	58	96,67	54	96,43	0,994
Thr-539	**0**	**0**	**0**	**0**	**2**	**3,57**	**0,127**
Other	2	3,70	2	3,33	0	0	0,362
Codon K13_543 (N=178)							
Alleles	**Anonkoua-Kouté** (n=60)		**Port-Bouët** (n=64)		**Ayamé** (n=54)		**p of the χ^2 test**
	Numbers	(%)	Numbers	(%)	Numbers	(%)	
Ile-543	58	96,7	62	96,87	54	100	0,409
Thr-543	**0**	**0**	**0**	**0**	**0**	**0**	**NA**
Other	2	3,33	2	3,13	0	0	0,409
Codon K13_580 (N=186)							
Alleles	**Anonkoua-Kouté** (n=60)		**Port-Bouët** (n=64)		**Ayamé** (n=62)		**p of the χ^2 test**
	Numbers	(%)	Numbers	(%)	Numbers	(%)	
Cys-580	56	93,33	60	93,75	56	92,32	0,731
Tyr-580	**0**	**0**	**0**	**0**	**0**	**0**	**NA**
Other	4	6,67	4	6,25	6	9,68	0,731
Codon K13_476 (N=154)							
Alleles	**Anonkoua-Kouté** (n=46)		**Port-Bouët** (n=54)		**Ayamé** (n=54)		**p of the χ^2 test**
	Numbers	(%)	Numbers	(%)	Numbers	(%)	
Met-476	38	82,61	52	96,30	48	88,90	0,080
Ile-476	2	4,35	0	0	0	0	0,092
Other	6	13,04	2	3,70	6	11,10	0,219
Codon K13_561 (N=176)							
Alleles	**Anonkoua-Kouté** (n=60)		**Port-Bouët** (n=58)		**Ayamé** (n=58)		**p of the χ^2 test**
	Numbers	(%)	Numbers	(%)	Numbers	(%)	
Arg-561	50	83,33	56	96,55	50	86,20	0,060
His-561	**0**	**0**	**0**	**0**	**0**	**0**	**NA**
Other	10	16,67	2	3,45	8	13,8	0,060

Notes: "N" represents the total number of successfully sequenced isolates per study site. "n" represents the number of isolates successfully sequenced for codons K13_493, K13_539, K13_543, K13_580, K13_476 and K13_561. The list of other mutants can be found in Appendix 1.

The analysis also showed high prevalences of wild strains Tyr-493 (91.3%, 96.43% and 89.29%), Arg-539 (96.3%, 93.67% and 96.43%), Ile-543 (93.33%, 90, 63% and 100%), Cys-580 (93.33%, 90.63% and 90.82%) and Met-476 (82.61%, 96.3% and 88.90%) and Arg-561 (83.33%, 96.55% and 86.20%) in Anonkoua-Kouté, Port-Bouët and Ayamé respectively. No significant difference was observed between the frequencies of wild strains in these three localities ($p > 0.05$). Table 5.

In Côte d'Ivoire, ACTs have been recommended as first- and second-line treatment for uncomplicated malaria since 2005, and the efficacy of these combinations remains high as reported by Touré *et al.*, (Touré *et al.*, 2018; Touré *et al.*, 2020). The use of ACTs increased in the country after the adoption of their free distribution to children under five. Therefore, drug pressure due to uncontrolled use (prescription or self-medication) of ACTs might have selected resistant parasites over time. Also, it is important to monitor the possible appearance of a population of parasites resistant to ACTs.

This study analyzed the polymorphism related to resistance to ACTs in three localities in southern Côte d'Ivoire. It made it possible to determine the prevalences of the YRICMR sensitive haplotype of the *pfK13 propeller* gene and those of its main alleles carrying the mutations Tyr-493-His (His-493), Arg-539-Thr (Thr-539), Ile- 543-Thr (Thr-543), Cys-580-Tyr (Tyr-580), Met-476-Ile (Ile-476) and Arg-561-His (His-561) whose implication in resistance to derivatives of artemisinin in Southeast Asia has been described (Ariey *et al.*, 2014). For all three study sites, the analysis showed that the YRICMR allelic form (susceptible haplotype) was predominant in the blood isolates, with a prevalence of 78.49%.

The results also indicate prevalences of 1.26%, 3.52% and 1.29% respectively for the mutant alleles His-493, Thr-539 and Ile-476 against 92.40% (His-493), 94, 11% (Thr-539), 94.38% (Thr-543), 91.39% (Tyr-580), 89.61% (Ile-476) and 88.63% (His-561) for wild alleles. No mutations in the Ile-543, Cys-580 and His-561 alleles were observed at the three study sites. Overall, our results indicate very low prevalences of mutations associated with resistance to artemisinin derivatives. These low proportions of known mutations in the *pfK13 propeller* gene are in favor of the effectiveness of ACTs in Côte d'Ivoire. This is in accordance with the studies updating the efficacy data of Artesunate-Amodiaquine (ASAQ) and Artemether-Lumefantrine (AL) carried out in Côte d'Ivoire and which showed good efficacy of these ACTs (Yavo *et al.*, 2015; Abibatou *et al.*, 2018; Touré *et al.*, 2018, 2015; Touré *et al.*, 2020, Offianan *et al.*, 2015, 2020).

In addition, molecular studies conducted in Côte d'Ivoire and other African countries have shown the absence of the known mutations in the *kelch 13* (Pfk-13) gene associated with artemisinin resistance (Kamau *et al.*, 2015, Taylor *et al.*, 2015, Pembe *et al.*, 2018; Umar *et al.*, 2020; Uwimana *et al.*, 2021), suggesting that artemisinins are still effective. Moreover, the His-561 mutation first associated with a delay in parasite clearance in Southeast Asia (Ashley *et al.*, 2014; Phyo *et al.*, 2016), and very recently identified at a prevalence ranging from up to 13% in some districts of Rwanda (Uwimana *et al.*, 2020; Bergmann *et al.*, 2021; Uwimana *et al.*, 2021) was not found in isolates from our three study sites.

More careful monitoring of the evolution of these resistance markers is needed in malaria-endemic regions of Africa and particularly in Côte d'Ivoire in order to anticipate countermeasures. Although this work gives us hope for the effectiveness of ACTs in Côte d'Ivoire, it has limitations. Indeed, for monitoring chemoresistance to artemisinin derivatives, we did not perform *in vitro* culture like the RSA (Ring-stage Survival Assay) and we did not do the sequencing of the complementary DNA strand of our amplification products. This limitation of the work led us not to associate the observed mutations (prevalences below 5) with resistance to artemisinin derivatives in the three study sites. Moreover, the financial means did not allow us to use many more samples for the realization of this work. This limit does not allow us to conclude on

the situation of resistance of *P. falciparum* to artemisinin derivatives at the national level.

In the context of our study, we cannot conclude that the observed mutations are associated with resistance to artemisinin derivatives. However, these results deserve to be confirmed by sequencing of the complementary DNA strand and also by in vitro tests such as the RSA (Ring-stage Survival Assay).

The main objective of this work was to analyze the polymorphism of the *pfK13 propeller* gene, a molecular marker of *Plasmodium falciparum* chemoresistance to artemisinin derivatives. The analysis indicated a predominance of the YRICMR allelic form representing a susceptible haplotype. In addition, very low prevalences of alleles of the *K13 propeller* gene carrying mutations associated with resistance to artemisinin derivatives in Southeast Asia were observed in the three study sites (Anonkoua-kouté, Port-Bouët and Ayamé). However, these mutations cannot be correlated to *P. falciparum* resistance to artemisinin derivatives without further analysis including complementary DNA strand sequencing and RSA (Ring-stage Survival Assay). Thus, more than a decade after the adoption of ACTs in Côte d'Ivoire, these combinations are still effective in the three localities (Anonkoua-Kouté, Port-Bouët and Ayamé) of southern Côte d'Ivoire.

Ethical Considerations

The study was conducted in accordance with the Declaration of Helsinki and approval was received from the National Committee on Ethics and Research (CNER) of the Ministry of Health and AIDS of Côte d'Ivoire. After appropriate information and explanation, written consent was obtained from the adult participants and parents or legal guardians of all children who wished to participate in the study prior to sampling.

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