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MECHANISMS OF RESISTANCE

Effect of Drug Pressure on Promoting the Emergence of Antimalarial-Resistant Parasites among Pregnant Women in Ghana

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ABSTRACT The continuous spread of antimalarial drug resistance is a threat to current chemotherapy efficacy. Therefore, characterizing the genetic diversity of drug resistance markers is needed to follow treatment effectiveness and further update control strategies. Here, we genotyped *Plasmodium falciparum* resistance gene markers associated with sulfadoxine-pyrimethamine (SP) and artemisinin-based combination therapy (ACT) in isolates from pregnant women in Ghana. The prevalence of the septuple **IRN**I-**A/FG**K**GS/T** *pfdhfr/pfdhps* haplotypes, including the *pfdhps* A581G and A613S/T mutations, was high at delivery among post-SP treatment isolates (18.2%) compared to those of first antenatal care (before initiation of intermittent preventive treatment of malaria in pregnancy with sulfadoxine-pyrimethamine [IPTp-SP]; 6.1%; $P = 0.03$). Regarding the *pfk13* marker gene, two nonsynonymous mutations (N458D and A481C) were detected at positions previously related to artemisinin resistance in isolates from Southeast Asia. These mutations were predicted *in silico* to alter the stability of the *pfk13* propeller-encoding domain. Overall, these findings highlight the need for intensified monitoring and surveillance of additional mutations associated with increased SP resistance as well as emergence of resistance against artemisinin derivatives.

KEYWORDS *Plasmodium falciparum*, *pfdhfr*, *pfdhps*, *pfk13*, sulfadoxine-pyrimethamine, artemisinin, drug resistance

In sub-Saharan Africa, *Plasmodium falciparum* infection represents one of the major causes of low birth weight (1–3). As such, expanded efforts to diminish the burden of the disease on affected populations have been increasingly prioritized over the last two decades (4). Intermittent preventive treatment of malaria in pregnancy (IPTp) with sulfadoxine-pyrimethamine (SP) has been the principal intervention toward that and has been successful across different transmission settings (5–7). However, growing concern over the rise and spread of SP resistance and its impact on the effectiveness of IPTp is rising, especially in eastern and southern Africa, where levels of parasite resistance to SP are high. Regardless, no alternative has been identified that effects positively on birth weight and is well tolerated (8–10). As such, the World Health Organization (WHO) updated its IPTp-SP policy from at least 2 doses to a monthly dose starting from the second trimester (11). This increase in SP pressure in pregnant women has likely favored the selection of parasites carrying mutant haplotypes in the *pfdhfr/*

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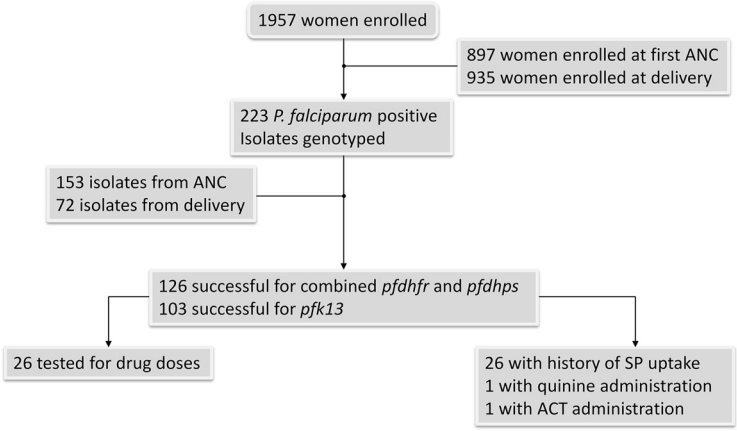

FIG 1 Study flow chart. ATS, acidic terminal segment of *Pf var* gene; *pfdhfr*, dihydrofolate reductase; *pfdhps*, dihydropterate synthase; *pfk13*, kelch13.

pfdhps genes that confer increased resistance to SP, for example, *pfdhps* K540E, A581G, and A613S/T mutations, which have been previously correlated with high levels of SP resistance (12). However, the prevalence of these mutations is lower in west and central Africa compared to eastern and southern Africa. Notwithstanding this, SP-resistant parasites are susceptible to artemisinin (ART)-based combination therapy (ACT), which is the first-line treatment in the management of uncomplicated malaria (13). However, since its inception, only a few studies have been conducted to determine the spread of resistance to these drugs in Ghana. In this study, we aimed to characterize the genetic diversity of SP and ART resistance markers among parasites from pregnant women in southern Ghana.

RESULTS

Study population characteristics. The general characteristics of pregnant women have been described elsewhere (14). In total, 223 isolates were evaluated, consisting of 151 isolates from pregnant women enrolled during their first antenatal clinic (ANC) visit and 72 isolates from pregnant women enrolled during delivery (Fig. 1). Of these, 68.6% (153/223), 63.2% (141/223), and 46.2% (103/223) of samples were successfully amplified, sequenced, and genotyped, respectively, for the *pfdhfr* and *pfdhps* genes and the *pfk13* propeller-encoding gene fragment. At ANC, 64.7% (99/153), 65.2% (92/141), and 50.5% (52/103) isolates were successfully genotyped, respectively, for *pfdhfr*, *pfdhps*, and *pfk13*. At delivery, 37.2% (57/153), 32.6% (46/141), and 49.5% (51/103) were also genotyped for *pfdhfr*, *pfdhps*, and *pfk13,* respectively.

Prevalence of *pfdhfr* and *pfdhps* mutations. Mutant *pfdhfr* alleles were predominant at codons 108 (S108N, 93.5%, 112/153), 59 (C59R, 90.8%, 111/153); and 51 (N51I, 85%, 130/153) (Table 1). The high prevalence of these mutant *pfdhfr* alleles was observed at both time points (Table 2). In the *pfdhps* gene associated with sulfadoxine resistance, the prevalence of mutant alleles was high at codons 436 (S436A/F, 95.0%, 134/141) and 437 (A437G, 96.4%, 133/141), but very low at codon 540 (K540E, 0.7%; 1/141). Mutations at codons 581 (A581G) and 613 (A613S/T) were, respectively, found in 12.8% (18/141) and 17.7% (25/141) of the isolates (Table 1). These proportions were similar at both time points (data not shown), except for mutant alleles at codon 581 (A581G), which were preferentially found in post-IPTp-SP treatment isolates (ratio of first ANC to delivery of 1:1.7) (Table 2).

Prevalence of *pfdhfr* and *pfdhps* haplotypes. Five and nine different haplotypes were detected for *pfdhfr* and *pfdhps* genes, respectively.

For *pfdhfr*, 6.5% (10/153) of isolates carried the wild-type (NCSI) haplotype, while 79.7% (96/153) carried the triple **IRN**I mutant haplotype. Double mutant haplotypes occurred at a low prevalence: 7.8% (12/153) and 2.0% (3/153) for N**RN**I and I**CN**I, respectively. A single mutant haplotype NC**N**I was observed once (0.7%; 1/153).

TABLE 1 Prevalence of *pfdhfr*, *pfdhps*, and *pfk13* SNPs among study isolates

Gene (total no.)	SNP	No. (%) of wild-type isolates	No. (%) of mutations	No. (%) of mixed-type isolates
pfdhfr (153)[a]	N51I	23 (15.0)	130 (85.0)	0 (0)
	C59R	14 (9.2)	111 (90.8)	0 (0)
	S108N	10 (6.5)	112 (93.5)	0 (0)
	I164L	153 (100)	0 (0)	0 (0)
pfdhps (141)[a]	S436A/F	2 (1.4)	134 (95.0)	5 (36)
	A437G	5 (3.6)	133 (96.4)	0 (0)
	K540E	140 (99.3)	1 (0.7)	0 (0)
	A581G	123 (87.2)	18 (12.8)	0 (0)
	A613S/T	116 (82.63)	25 (17.7)	0 (0)
pfk13 (103)[a]	E455G	101 (98.1)	2 (1.9)	
	N458D	101 (98.1)	2 (1.9)	
	A481C	102 (99.0)	1 (1.0)	
	T535A	102 (99.0)	1 (1.0)	
	Y616S	102 (99.0)	1 (1.0)	
	L618V	101 (98.1)	2 (1.9)	
	A621G	102 (99.0)	1 (1.0)	
	L663I	102 (99.0)	1 (1.0)	
	N672I	101 (98.1)	2 (1.9)	

[a]*pfdhfr*, dihydrofolate reductase; *pfdhps*, dihydropteroate synthase; *pfk13*, kelch13 propeller-encoding gene.

For *pfdhps*, two haplotypes predominated: the double mutant **A/FG**KAA and quadruple mutant **A/FG**K**GS/T** haplotypes that were present in 71.6% (101/141) and 9.9% (14/141) of isolates, respectively. Of the remaining isolates, 7.8% (11/141), 2.8% (4/141), and 0.7% (1/141) carried the triple mutant **A/FG**KA**S/T**, **A/FG**K**G**A, and **A/FGE**AA haplotypes, respectively. Overall, 2.1% (3/141) and 1.4% (2/141) of isolates carried a single mutant haplotype S**G**KAA or **A/F**AKAA, while 3.6% (5/141) isolates carried a mixed haplotype (Table 3).

Combined *pfdhfr-pfdhps* haplotypes. Of the successfully sequenced isolates, 126 were analyzed for combined *pfdhfr* and *pfdhps* haplotypes. Ten distinct haplotypes were observed. A quintuple mutant haplotype, consisting of triple **IRN**I and double **A/FG**KAA mutations, was the most common combined *pfdhfr*/*pfdhps* haplotype (63.5%; 80/126), followed by the septuple combined haplotype **IRN**I + **A/FG**K**GS/T** (10.3%; 13/126). The prevalence of other combined haplotypes was low. Only one isolate was wild type at both *pfdhfr* and *pfdhps* loci. The combined quintuple mutant haplotype was observed at similar proportions at both the first ANC (63.4%, 52/82) and delivery (63.6%, 28/44). However, there was a high prevalence of septuple mutant haplotypes observed at delivery (18.2%) compared to the first ANC (6.1%) ($P = 0.03$; chi-squared test) (Table 4).

Mutation frequency in the *pfk13* propeller-encoding domain. The *pfk13* propeller-encoding domain was sequenced for a total of 113 *P. falciparum* isolates from

TABLE 2 Distribution of *pfdhfr* and *pfdhps* at first ANC and delivery among study isolates

		Data from first ANC visit ($n = 99$)			Data from time of delivery ($n = 57$)		
Gene	SNP	No. (%) of wild-type isolates	No. (%) of mutations	No. (%) of mixed-type isolates	No. (%) of wild-type isolates	No. (%) of mutations	No. (%) of mixed-type isolates
pfdhfr[a]	N51I	15 (15.2)	82 (82.2)	0 (0)	8 (13.6)	48 (81.4)	0 (0)
	C59R	8 (8.1)	89 (89.9)	0 (0)	6 (10.2)	50 (84.8)	0 (0)
	S108N	5 (5.1)	92 (92.9)	0 (0)	5 (8.5)	51 (86.4)	0 (0)
pfdhps[a]	S436A/F	1 (1.0)	91 (91.9)	2 (2.0)	1 (1.8)	43 (75.4)	3 (5.3)
	A437G	2 (2.0)	90 (90.9)	0 (0)	0 (0.0)	46 (80.7)	0 (0)
	K540E	91 (91.9)	1 (1.0)	0 (0)	44 (77.2)	0 (0.0)	0 (0)
	A581G	83 (83.8)	9 (9.1)	0 (0)	35 (61.4)	9 (15.8)	0 (0)
	A613S/T	77 (77.8)	15 (15.2)	0 (0)	34 (59.6)	10 (17.5)	0 (0)

[a]*pfdhfr*, dihydrofolate reductase; *pfdhps*, dihydropteroate synthase.

TABLE 3 Prevalence of *pfdhfr* and *pfdhps* haplotypes

Gene (total no.)	Category	Haplotype	No. (%)
pfdhfr (153)[a]	Wild type	NCSI	10 (6.5)
	Single	NC**N**I	1 (0.7)
	Double	**I**C**N**I	3 (2.0)
		N**RN**I	12 (7.8)
	Triple	**IRN**I	96 (79.7)
pfdhps (141)[a]	Single	**A/F**AKAA	2 (1.4)
		S**G**KAA	3 (2.1)
	Double	**A/FG**KAA	101 (71.6)
	Triple	**A/FGE**AA	1 (0.7)
		A/FGK**G**A	4 (2.8)
		A/FGKA**S/T**	11 (7.8)
	Quadruple	**A/FG**K**GS/T**	14 (9.9)
	Mixed type	**S/A**AKAA	2 (1.4)
		S/AGKAA	3 (2.1)

[a]*pfdhfr*, dihydrofolate reductase; *pfdhps*, dihydropterate synthase.

both time points. Ten isolates failed to generate high-quality sequences and were discarded for further analysis. Among the 103 remaining sequences, 10 (9.7%) harbored nonsynonymous mutations only found in one (A481C, T535A, Y616S, A621G, L663I) or two (E455G, N458D, L618V, N672I) isolates. Interestingly, the nonsynonymous mutations N458D and A481C are related to positions at which ART resistance mutations (N458Y and A481V) were previously described in isolates from Southeast Asia (15). Another mutation (T535A) was observed at a position found mutated in Southeast Asia (T535M), although its relationship with ART resistance remains uncharacterized. The location of these *pfk13*-nonsynonymous mutations was spotted on the propeller tertiary structure (Fig. 2). Those were mainly located on the loops connecting blades (Table 5), except the mutation T535A, which was located on the inner strand (strand A) lining the central channel of the domain. Finally, the mutation N672I was localized at the shallow pocket, predicted as a putative propeller interaction surface (16).

The Missence 3D tool was used to predict structural damages caused by nonsynonymous mutations. None were predicted to be damaging for the propeller tertiary structure (Table 5). However, the DynaMut server predicted that five mutations may decrease the propeller domain stability, including mutations N458D and A481C.

Relationships between the number of IPTp doses taken, sulfadoxine-pyrimethamine plasma levels, and prevalence of *pfdhfr*/*pfdhps* mutations. Based on the declaration and verification on the ANC booklet, 9.1% (4/44) of pregnant women reported taking one dose of IPTp-SP, and 9.1% (4/44) reported taking two doses, while 63.6% (28/44) of pregnant women took three or more SP doses. Five pregnant women did not receive any SP during their pregnancy.

To explore the relationship between plasma SP levels and the prevalence of drug resistance mutations, we measured SP and ACT residual levels in peripheral plasma

TABLE 4 Combined *pfdhfr*/*pfdhps* haplotypes based on sample time point and study site

Gene (*n*)	Category	Haplotype	No. (%) at first ANC visit (total *n* = 82)	No. (%) at delivery (total *n* = 44)
pfdhfr/*pfdhps* (126)	Wild type	NCSI/SAKAA	1 (1.2)	0 (0.0)
	Double	NCSI-**A/FG**KAA	3 (3.7)	4 (9.1)
	Triple	NC**N**I-**A/FG**KAA	1 (1.2)	0 (0.0)
	Quadruple	I**CN**I-**A/FG**KAA	2 (2.4)	0 (0.0)
		N**RN**I-**A/FG**KAA	8 (9.8)	1 (2.3)
		IRNI-**A/F**AKAA	1 (1.2)	0 (0.0)
	Quintuple	**IRN**I-**A/FG**KAA	52 (63.4)	28 (63.6)
	Sextuple	**IRN**I-**A/FGE**AA	1 (1.2)	0 (0.0)
		IRNI-**A/FG**KA**S/T**	8 (9.8)	3 (6.8)
	Septuple	**IRN**I-**A/FG**K**GS/T**	5 (6.1)	8 (18.2)

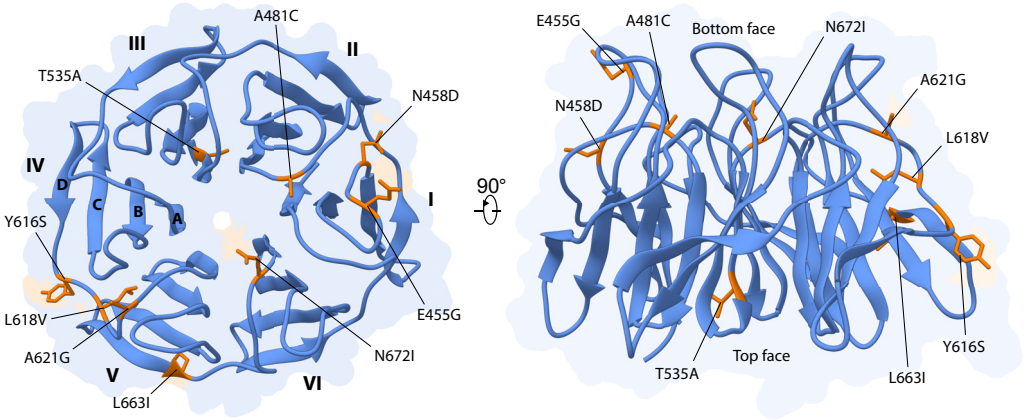

FIG 2 Location of amino acids associated with *pfk13* mutations observed in southern Ghana on the PfK13 propeller tertiary structure. The propeller domain is shown as cartoon from the bottom face (left) and from the side (right). Positions associated with *pfk13* mutations are colored in orange and shown as stick. On the left structure, blade number (I to VI) and strand label (A to D) are indicated.

at delivery. SP and ACT residual levels were measured in 31 plasma samples, with sulfadoxine metabolites being detected in 77.4% (24/31) of samples at a mean concentration of 3,467 ± 5,543.1 μg/ml, while pyrimethamine metabolites were detected in 16.1% (5/31) of samples at a mean concentration of 4.0 ± 10.2 ng/ml. Women in whom pyrimethamine was detected were also sulfadoxine positive; however, sulfadoxine was detected in more women. Therefore, we considered the presence of sulfadoxine as a proxy for SP in the analyses.

There was no association between the number of IPTp-SP doses taken and the frequency of observed mutant alleles or combined haplotypes among women at delivery. When the analysis was carried out with the SP level in the blood taken as a quantitative variable between women whose parasites were mutated or not, A581G and A613S/T were associated with increased SP levels (both $P = 0.05$; Welch *t* test). This significant difference was, however, lost when the *pfdhfr* and *pfdhps* haplotypes were combined. Finally, we were unable to determine an association between either ACT treatment or level of ACT metabolites and *pfk13* mutations because only one pregnant woman had taken ACT during pregnancy.

DISCUSSION

Gains achieved toward the fight against malaria are now dwindling, especially in sub-Saharan Africa, where most of the burden is felt. This is attributable to the high

TABLE 5 Prediction of structural damages and structure stability of the mutant propeller domains

Mutation	Blade[a]	Location[b]	State[c]	Result from Missence 3D	Result from DynaMut[d]
E455G	I	BC loop	Exposed	Not damaging	+0.140
N458D	I	BC loop	Exposed	Not damaging	−0.664
A481C	II	DA loop	Buried	Not damaging	−0.230
T535A	III	Strand A	Exposed	Not damaging	+0.116
Y616S	V	DA loop	Exposed	Not damaging	−3.063
L618V	V	DA loop	Buried	Not damaging	−0.865
A621G	V	DA loop	Exposed	Not damaging	−0.148
L663I	V	DA loop	Exposed	Not damaging	+0.073
N672I	VI	DA loop	Buried	Not damaging	+0.180

[a]Blade refers to the six Kelch-repeat motifs of the PfK13 propeller domain, labeled I to VI.
[b]Mutations are either located on strands (innermost, strand A; outermost, strand D) (Fig. 2). Each loop connects two strands; for example, the DA loop connects strand D with the strand A of the next blade.
[c]A position is supposed to be exposed to the solvent when it exhibited relative solvent accessibility ≥ 16%.
[d]Values correspond to the folding free energy (ΔΔG), expressed in kcal/mol. Negative and positive values suggest destabilizing and stabilizing effects, respectively.

transmission rate and the low coverage of intervention strategies. In regard to these, the WHO is now recommending an increased administration of SP as part of IPTp to correct the coverage deficit (11). Thus, understanding the effect of the increased drug pressure on parasite populations is crucial to the sustainability of the IPTp program, particularly in western Africa, where quintuple mutations (triple *pfdhfr* with double *pfdhps* mutations) are low (drugresistancemaps.org).

In this study, we characterized parasites obtained from pregnant women coming for their first contact with antenatal care and those of women at delivery who participated in ANCs and have been exposed to several doses of IPTp-SP. Ghana, like other African countries, has adopted and is implementing the new IPTp-SP policy, which advocates an increase in the number of treatment doses, and we have recently reported that this new policy was very well implemented in Ghana (14). This study is one of the first to assess the SP-resistant genetic variation among parasite populations in pregnant women in the context of increased IPTp-SP pressure. One noteworthy observation here is the high prevalence of highly mutated *pfdhfr/pfdhps* haplotypes observed among posttreatment isolates compared to the first ANC. The observation of a clear tendency toward the increase of highly mutated parasites in women still infected at delivery draws attention to a possible selection of these mutant parasites by treatment. Overall, the prevalence of mutant *pfdhfr* and *pfdhps* haplotypes conferring SP resistance was high among the parasite isolates in the study participants at both the first ANC visit and at delivery, respectively, with 63% and 64% of isolates carrying the quintuple *pfdhfr/pfdhps* mutant haplotype (**IRN**I**A/FG**KAA). This is in agreement with Mouchenhaupt et al. (17) and other studies in Africa (18–21) where the prevalence of the quadruple *pfdhfr/pfdhps* mutant haplotype (**IRN**I**A/FG**KAA) had been reported as very high with almost fixation of the triple *pfdhfr* mutant haplotype. This predominance of triple *pfdhfr* mutant parasites could be due to the transmission advantage that SP resistant parasites have since acquired in the context of continuous use of the drug (22). When looking specifically at the *pfdhps* gene, additional critical mutations capable of further impacting resistance to SP, like mutations at codons 581 (A581G) and 613 (A613S/T) of the *pfdhps* gene, have been observed at frequencies close to those reported among children in northern Ghana (23). However, in this study, we especially noted that these proportions slightly increased from 9% and 15% at first ANC (that is, before the initiation of IPTp-SP) to 16% and 18% at delivery, respectively. The fact that parasites carrying these mutations are found more in women at delivery with higher SP concentrations in their blood suggests that these parasites are better able to withstand these conditions and therefore to withstand SP treatment. When considering these observations, and adding to the fact that parasites carrying the K540E mutation are also present in the study area as recently reported in one isolate from Bioko Island in Equatorial Guinea (24), one may wonder whether the conditions necessary for the emergence of full resistance to SP in West Africa will not be gradually favored with the current IPTp-SP strategy. Whether the successful implementation of IPTp in Ghana raises fears in this regard remains a topical issue. These findings warn of the impact of increasing uptake of IPTp-SP on the rising level of resistance among circulating parasites if these few mutants come to disseminate and become the dominant population. In a meta-analysis study assessing IPTp-SP effectiveness, which includes 42,394 births, a mutation at codon 540 (K540E) was associated with a high prevalence of low birth weight (25). Therefore, it would certainly be necessary to study the impact on the pregnancy outcome of the *pfdhps* A581G and A613S/T mutant parasites that seem to be favored in the context of IPTp-SP.

The polymorphism data generated on the *pfk13* gene revealed two nonsynonymous mutations related to positions known to confer artemisinin resistance in Southeast Asia (26–29). However, the changes in amino acid observed differ from those described in Asia, and further investigations are needed to check whether they confer a survival advantage to parasites pressured by ART. Importantly, one Singaporean returning from Ghana presented with a *P. falciparum* infection exhibiting the N458D mutation and was not effectively cured by ACT treatment (30). These findings, in addition to those

reported by Ocan et al. from Uganda (31), Bayih et al. in northwest Ethiopia (32), and Ouattara et al. from Mali (33), are suggestive indications of possible ART resistance emergence in Africa. However, several studies have shown that increased parasite survival under ART pressure may also be caused by mutations at other loci such as *pfap2-mu*, *pfcoronin*, *falcipain 2a*, and *pfubp1* mutations (34–36) that were not investigated here.

In conclusion, this study shows the high prevalence of quintuple-*pfdhfr/pfdhps* mutations in parasite isolates from two Ghanaian study sites with the development of additional mutations that confer higher resistance to SP. The spread of these new mutations could be facilitated by the continued pressure of IPTp-SP and may impact negatively on its effectiveness. Thus, other interventions to better protect pregnancy from malaria infection and its damaging effects on mother and child should be evaluated now and not wait until the efficacy of SP is completely eroded. On the other hand, the description of mutations on ART resistance-related positions of the *pfk13* gene in this study also resonates as an alert for more surveillance on the emergence of resistance against ART derivatives, currently part of the recommended treatment of uncomplicated malaria cases during pregnancy.

MATERIALS AND METHODS

Ethics statement. Ethical clearance was obtained from the Institutional Ethics Review Committee of the Noguchi Memorial Institute for Medical Research (NMIMR) and the Ethical Review Committee of the Ghana Health Service. Written informed consent was obtained from each participant.

Study area and design. The study was a cross-sectional hospital-based survey conducted from December 2015 to May 2017 in two distinct communities in southern Ghana: Kpone on-Sea, a peri-urban community, and Maamobi, an urban community, both within the Greater Accra region in Ghana. Malaria transmission is perennial, with two peaks, one from April to July and the other from September to November. A two-parallel cross-sectional enrollment was carried out among pregnant women attending the first antenatal clinic (ANC) and at delivery. A detailed description of the study sites and design is reported elsewhere (14).

PCR assays for the detection and amplification of *pfdhfr*, *pfdhps*, and *pfk13* genes. DNA extraction was carried out on total blood from the peripheral circulation or placenta at delivery using the QIAamp DNA blood minikit (Qiagen, France) according to the manufacturer's recommendation. The presence of *P. falciparum* parasites was tested in duplicate by real-time quantitative PCR (qPCR) targeting the 18S rRNA of *P. falciparum* (37). Subsequently, *pfdhfr* and *pfdhps* genes were amplified by nested PCR, and the conditions for amplification were as previously described (38), while that of *pfk13* was amplified as previously described in reference 26. Mutations of the *pfdhfr*, *pfdhps*, and *pfk13* genes in the amplified nested PCR products were purified and detected subsequently by Sanger sequencing (GATC, Cologne, Germany). All sequences generated were analyzed with the Chromas software (Technelysium Pty Ltd.) and then aligned using MEGA 5.2 (39) and compared with reference genes of the *P. falciparum* 3D7 genome.

Effect(s) of *pfk13* nonsynonymous mutations on the structure and stability of the PfK13 propeller domain. The tools Missence3D (40) and DynaMut (41) were used to predict, respectively, the structural damages and structure stability alterations caused by amino acid changes in the propeller domain of the PfK13 protein. We used the X-ray crystallographic tertiary structure determined at a resolution of 1.5 Å (PDB ID 4YY8, chain A) (42). Beforehand, all the missing atoms were added with Swiss PDB Viewer (43).

Antimalarial drug level measurements. Plasma sulfadoxine and pyrimethamine levels were measured by liquid chromatography coupled to tandem mass spectrometry (TSQ Quantum Ultra; Thermo Fisher, France) as previously reported (44). Briefly, using Oase 96-well microplates (Waters, France), 100 μl of plasma was mixed with 300 μl of acetonitrile containing quinidine-d3 (50 ng/ml) as an internal standard. Phospholipids were eliminated by positive pressure (20 lb/in^2 during 1 min), and eluents were evaporated at room temperature. Dry residues were dissolved in 20 mM ammonium formate buffer with formic acid (0.5% vol/vol) before 10 μl was injected into the system. This was used to measure metabolites of sulfadoxine, pyrimethamine, amodiaquine, lumefantrine, primaquine, artemisinin, and quinine. Homemade and external controls obtained from the Worldwide Antimalarial Resistance Network (WWARN) were used as controls.

Statistical analysis. All data were analyzed with R programming. The chi-squared test was used to determine the association between the single nucleotide polymorphisms (SNPs) of the *pfdhfr* and *pfdhps* genes and the number of doses of SP taken. Association between mutations observed and the level of SP in the peripheral plasma were tested using the Welch *t* test or logistic regression where appropriate. *P* values of less than 0.05 indicated significance.

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We declare no conflicts of interest.

N.T.N., I.Q., M.F.O., K.A.K., and A.K.A. conceived and designed the study. B.T., N.C., and A.M. carried out the sample collections. B.T. performed the parasite genotyping. B.T. and P.H. carried out the SP level measurements. B.T., R.T., and B.A. performed the sequence analysis and statistical analysis. B.T. and R.C. investigated structural alterations induced by *pfk13* mutations. B.T., R.C., R.T., and N.T.N. wrote the paper. J.C. and P.D. reviewed the manuscript.

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