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Plasmodium falciparum Polymorphisms Associated with *Ex Vivo* Drug Susceptibility and Clinical Effectiveness of Artemisinin-Based Combination Therapies in Benin

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Artemisinin-based combination therapies (ACTs) are the main option to treat malaria, and their efficacy and susceptibility must be closely monitored to avoid resistance. We assessed the association of *Plasmodium falciparum* polymorphisms and *ex vivo* drug susceptibility with clinical effectiveness. Patients enrolled in an effectiveness trial comparing artemether-lumefantrine ($n = 96$), fixed-dose artesunate-amodiaquine ($n = 96$), and sulfadoxine-pyrimethamine ($n = 48$) for the treatment of uncomplicated malaria 2007 in Benin were assessed. *pfcrt*, *pfmdr1*, *pfmrp1*, *pfdhfr*, and *pfdhps* polymorphisms were analyzed pretreatment and in recurrent infections. Drug susceptibility was determined in fresh baseline isolates by *Plasmodium* lactate dehydrogenase enzyme-linked immunosorbent assay (ELISA). A majority had 50% inhibitory concentration (IC_{50}) estimates (the concentration required for 50% growth inhibition) lower than those of the 3D7 reference clone for desethylamodiaquine, lumefantrine, mefloquine, and quinine and was considered to be susceptible, while dihydroartemisinin and pyrimethamine IC_{50}s were higher. No association was found between susceptibility to the ACT compounds and treatment outcome. Selection was observed for the *pfmdr1* N86 allele in artemether-lumefantrine recrudescences (recurring infections) (4/7 [57.1%] versus 36/195 [18.5%]), and of the opposite allele, 86Y, in artesunate-amodiaquine reinfections (new infections) (20/22 [90.9%] versus 137/195 [70.3%]) compared to baseline infections. The importance of *pfmdr1* N86 in lumefantrine tolerance was emphasized by its association with elevated lumefantrine IC_{50}s. Genetic linkage between N86 and Y184 was observed, which together with the low frequency of 1246Y may explain regional differences in selection of *pfmdr1* loci. Selection of opposite alleles in artemether-lumefantrine and artesunate-amodiaquine recurrent infections supports the strategy of multiple first-line treatment. Surveillance based on clinical, *ex vivo*, molecular, and pharmacological data is warranted.

Half of the world's population lives at risk for malaria, and more than half a million people die from the disease every year (1). An effective treatment is essential for malaria control, and the emergence and spread of chloroquine resistance have contributed significantly to malaria-attributed mortality (2, 3). Spread of antimalarial drug resistance in most areas where *Plasmodium falciparum* is endemic prompted the World Health Organization to recommend the use of artemisinin-based combination therapies (ACTs), which have now been adopted by most countries where *P. falciparum* malaria is endemic as a first-line treatment for uncomplicated malaria (1). Artemether-lumefantrine (AL) and artesunate-amodiaquine (ASAQ) are the ACTs most used in Africa. For intermittent preventive treatment (IPT) of pregnant women the antifolate sulfadoxine-pyrimethamine (SP) is the main present option (1). In Southeast Asia, resistance to artemisinin derivatives has been suggested to emerge, associated with delayed parasite clearance (4–6). Emergence and spread of resistance to artemisinins and the partner drugs would raise a serious problem for malaria control and need to be closely monitored. *P. falciparum* drug resistance is assessed by clinical efficacy/effectiveness trials, by *in vitro* susceptibility assays, and by the use of molecular markers associated with parasite drug resistance (7). The relevance of *in vitro* susceptibility testing for artemisinin derivatives is being discussed (8, 9). However, it is important for susceptibility testing of ACT partner drugs and for detecting true drug resistance to avoid confounding host factors. The molecular mechanisms of artemisinin resistance have not been elucidated yet, but a region in chromosome 13 has been identified that may be associated with the delayed parasite clearance phenotype (10).

Decreased sensitivity or parasite tolerance to artemisinin partner drugs has been observed in Africa, where single nucleotide polymorphisms (SNPs) in *pfcrt*, *pfmdr1*, and *pfmrp1* have been selected in recurrent infections after AL and ASAQ treatments (11–18). The involvement of these genes in partner drug tolerance is supported by the association of *pfcrt* and *pfmdr1* with variation in *in vitro* susceptibility to lumefantrine (LUM) and monodeseth-

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ylamodiaquine (DEAQ), the active metabolite of amodiaquine (AQ) (17, 19, 20).

Molecular characterization of the *P. falciparum* altered response to ACTs as described above has mainly been assessed in East Africa, and only a few studies have been performed in West Africa, showing selection patterns similar to those seen in East Africa (21–23). As East and West Africa may differ in terms of drug use and parasite genetics (24), it is of importance to further describe the basis of drug tolerance and resistance also in West Africa.

We performed a comprehensive study in Benin to characterize the genetic bases of the *P. falciparum* response to antimalarial drugs, and the *ex vivo* drug susceptibility, in an ACT effectiveness study performed before ACT implementation. To our knowledge, this is the first study exploring the association of *P. falciparum* alleles and *ex vivo* susceptibility with *in vivo* efficacy of the currently used ACT and SP in Benin.

MATERIALS AND METHODS

Clinical trial and blood sampling. The effectiveness of SP and unsupervised AL and ASAQ fixed-dose formulation for the treatment of uncomplicated malaria was compared during a randomized effectiveness noninferiority trial including 240 children, described elsewhere (25). Briefly, *P. falciparum* isolates were collected from children <5 years old between May and November 2007 in Allada and Sekou, southern Benin. Blood sampling for *ex vivo* drug susceptibility testing was collected in EDTA tubes at inclusion and in case of treatment failure. For molecular analysis, blood was spotted on filter paper at inclusion, on days 3, 7, 14, 21, 28, 35, and 42 during follow-up, and in case of infection recurrence. Recurrent infections were classified as recrudescences (reappearances) or reinfections (new infections) based on *msp1* and *msp2* analyses, as previously described (25). Concentrations of lumefantrine or DEAQ in blood on day 3 were determined using high-pressure liquid chromatography with electrochemical (DEAQ) or UV (LUM) detection from blood samples collected on Whatman 3MM filter paper (25). Both drug assays had a lower limit of quantification of 0.02 μg/ml.

***Ex vivo* drug susceptibility assay.** DEAQ was provided by SAPEC (Barbengo, Switzerland), and LUM was provided by Novartis Pharma (Basel, Switzerland). Quinine (QN), mefloquine (MQ), dihydroartemisinin (DHA), and pyrimethamine (PYR) were obtained from Sigma-Aldrich Company (St. Louis, MO). Chloroquine (CQ) was also tested, but the results were inconclusive due to technical difficulties. Antimalarial drug stock solutions were made in the appropriate solvent, while dilutions were made in water, except for lumefantrine, which was diluted in ethanol. The dilutions were distributed and dried in 96-well tissue culture plates in the following range of concentrations: QN in ethanol, 25 to 3,200 nM; MQ in methanol, 3.12 to 400 nM; DHA in water, 0.25 to 64 nM; PYR in ethanol, 50 to 40,000 nM; DEAQ in water, 7.5 to 1,920 nM; and LUM in ethanol, 1.25 to 320 nM. The reference laboratory *P. falciparum* clones 3D7 (Africa) and W2 (Indochina) were used to control each batch of plates. For each drug tested, three control wells were drug free, and each concentration was studied in duplicate or triplicate. Parasitized blood samples were washed and cultured in drug-coated plates for 42 h before freezing, as described previously (26, 27). Briefly, after washes, blood samples were resuspended in 1.5% hematocrit in RPMI 1640 (Gibco, Invitrogen Life Technologies, Auckland, New Zealand) supplemented with 25 mM HEPES (Invitrogen, Cergy Pontoise, France), 25 mM $NaHCO_3$ (Sigma-Aldrich), and 10% human serum (Abcys Biowest, Paris, France). PYR susceptibility was assessed in RPMI SP 241 medium (Gibco BRL, Paisley, United Kingdom) with a low concentration of folic acid and *p*-aminobenzoic acid. For initial parasitemias of >1%, a dilution was made by adding uninfected O^+ group erythrocytes to obtain 0.5% to 1% parasite density. The plates were incubated for 42 h at 37°C with 5% CO_2 and then frozen at −20°C. The *in vitro* assay output was determined by *Plasmodium* lactate dehydrogenase (PLDH) production with a commercial enzyme-linked immunosorbent assay (ELISA)-malaria antigen test (DiaMed AG, Cressier sur Morat, Switzerland), as previously described (27). Optical density was measured with a spectrophotometer (E960; Fisher Bioblock Scientific, Illkirch, France). Fifty percent inhibitory concentrations (IC_{50}s) with their 95% confidence intervals (CIs) were calculated with ICEstimator software (http://www.antimalarial-icestimator.net) (28).

DNA extraction. DNA was extracted from blood spots on filter paper using the QIAamp DNA blood minikit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for dried blood spots.

***pfmrp1* SNPs.** *pfmrp1* single nucleotide polymorphisms (SNPs) in the I876V and K1466R codons were analyzed by pyrosequencing. Details regarding the pyrosequencing method (29) and the specific PCR amplification and pyrosequencing of *pfmrp1* SNPs in the I876V and K1466R codons have been previously described (30). Briefly, extracted DNA was amplified in a nested PCR using GoTaq polymerase (Promega, Madison, WI). Primers used in amplification and pyrosequencing reactions are shown in Table 1. In the nested PCR, one of the primers was biotinylated, allowing the purification of specific single-strand products with streptavidin Sepharose beads (Amersham Bioscience, Little Chalfont, United Kingdom) for the pyrosequencing reaction. The reagents used for pyrosequencing were provided by Biotage (Uppsala, Sweden). Nucleotide dispensation order was GATACTGAT and CGACGATGT for I876V and K1466R, respectively. The definition for a mixed-genotype infection was a pyrosequencing result between 10% and 90% for both genotypes, and for a single genotype (pure) infection, it was above 90%. For *pfmrp1* I876V, the results were adjusted against a standard curve derived from different proportions of mixes of the reference laboratory strains 3D7 and W2.

***pfcrt* and *pfmdr1* SNPs.** The *pfcrt* K76T and *pfmdr1* N86Y and Y184F codons were simultaneously amplified in a multiplex PCR. Two separated nested PCRs were performed: one duplex for determining the genotype at both *pfcrt* 76 and *pfmdr1* 86 and one simplex for *pfmdr1* 184. Primers are shown in Table 1. All PCRs were performed with 200 μM deoxynucleoside triphosphates (dNTPs) and 0.5 U of GoTaq polymerase (Promega). The PCR master mix contained various concentrations of $MgCl_2$ and primers according to the reaction: first multiplex PCR, 3.5 mM $MgCl_2$ and 10 nM for each of primers CRTP1 and CRTP2 (31) and A1 and A3 (32); nested PCR for SNPs at codons *pfcrt* 76 and *pfmdr1* 86, 3.5 mM $MgCl_2$ and 300 nM for primers CRTD1 and CRTD2 and 100 nM for primers A2 and A4 (31, 32); nested PCR for SNPs at codon *pfmdr1* 184, 2 mM $MgCl_2$ and 100 nM for primers A2 and A4. The nested amplification product sizes were 145 bp and 560 bp for *pfcrt* and *pfmdr1*, respectively.

Genotyping of the *pfmdr1* D1246Y codon was derived from conditions previously published (32). The first PCR master mix contained 3.5 mM $MgCl_2$ and 100 nM (each) primers O1 and O2. The seminested PCR mixture contained 3 mM $MgCl_2$ and 250 nM primers O2 and 1246f. The amplification product was 344 bp.

***pfdhfr* SNPs.** The N51I, C59R, S108N, and I164L codons were analyzed as described previously (33), with minor modifications. The first PCR master mix contained 3 mM $MgCl_2$ and 100 nM (each) primers AMP1F and AMP2R. The nested PCRs were all performed with 3 mM $MgCl_2$ but differed in the concentration of primers (100 nM primers AMP3F and dhfrR4 for SNPs at codons 51 and 108 and 300 nM for primers F [34] and dhfrR2 for SNPs at codon 59 or dhfr_164_r and F/ [34] for SNPs at codon 164). The results were products of 376 bp for the dhfrR4-AMP3F primer pair, 189 bp for dhfrR2-F, and 168 bp for dhfr_164_r-F/.

***pfdhps* SNPs.** Genotyping of the *pfdhps* A437G and K540E codons was carried out as described by Duraisingh et al. (34). The mixtures for both the first and the nested PCRs contained 2 mM $MgCl_2$ and 100 nM (each) primers R2 and R/ (first) and K and K/ (nest). The result was a product of 438 bp.

RFLP. The genotype of each SNP (except *pfmrp1*) was determined by restriction fragment length polymorphisms (RFLP) of nested PCR products using appropriate restriction enzymes purchased from New England BioLabs (Ipswich, MA). Four microliters of the nested PCR product was

TABLE 1 Primers used in PCR, sequencing, and pyrosequencing reactions

Gene	Reaction or locus	Primer name	Primer sequence (5′ to 3′)[b]
pfdhfr	Outer	AMP1F	TTTATATTTTCTCCTTTTTA
		AMP2R	CATTTTATTATTCGTTTTCT
	51 and 108	AMP3F	TGATGGAACAAGTCTGCGAC
		dhfrR4	ATAACATTTATCCTATTGCTTAAAGGTT
	59	F	GAAATGTAATTCCCTAGATATGgAATATT
		dhfrR2	TTTGGAATGCTTTCCCAG
	164	dhfr_164_r	CCTTTAAGCAATAGGATAAATGTTATATTG
		F/	AAATTCTTGATAAACAACGGAACCTttTA
pfcrt	Outer	CRTP1	CCGTTAATAATAAATACACGCAG
		CRTP2	CGGATGTTACAAAACTATAGTTACC
	76	CRTD1	TGTGCTCATGTGTTTAAACTT
		CRTD2	CAAAACTATAGTTACCAATTTTG
pfmdr1	Outer	A1	TGTTGAAAGATGGGTAAAGAGCAGAAAGAG
		A3	TACTTTCTTATTACATATGACACCACAAACA
	86 and 184	A2	GTCAAACGTGCATTTTTTATTAATGACCATTTA
		A4	AAAGATGGTAACCTCAGTATCAAAGAAGAG
	Outer	O1	AGAAGATTATTTCTGTAATTTGATACAAAAAGC
		O2	ATGATTCGATAAATTCATCTATAGCAGCAA
	1246[a]	1246f	ATGATCACATTATATTAAAAAATGATATGACAAAT
pfdhps	Outer	R2	AACCTAAACGTGCTGTTCAA
		R/	AATTGTGTGATTTGTCCACAA
	437 and 540	K	TGCTAGTGTTATAGATATAGGatGAGcATC
		K/	CTATAACGAGGTATTgCATTTAATgCAAGAA
pfmrp1	876 outer	A2626G PS First fw	AATATTCCATTCAATGAAAATTAC
		A2626G PS First rev	CAACGTACTTTTATTCATTGAGA
	876 nest	A2626G PS Nest fw	Biotin-TATTCCATTCAATGAAAATTACCT
		A2626G PS Nest rev	TATGGAAGGATCTAAAGATGTAAA
	876 seq	A2626G PS Seq rev	GGAAGGATCTAAAGATGTAA
	1466 outer	A4397G PS First fw	AATAAAGAACATTCAGACACAAT
		A4397G PS First rev	TGATTTTCCTACTATCCCAATT
	1466 nest	A4397G PS Nest fw	TGGATACTGTATATCGTTTTCTGC
		A4397G PS Nest rev	Biotin-CCCAATTTTTTGATTTTTTAAAGC
	1466 seq	A4397G PS Seq fw	TGATTATACTCACATAGAAA

[a] Primer pair O2-1246f was used for the seminested PCR.
[b] Lowercase letters indicate variant nucleotides.

incubated with restriction enzymes in a total volume of 20 μl according to the manufacturer's instructions. The products obtained from the duplex PCR with the D1-D2 and A2-A4 primer pairs were digested with ApoI for SNPs at the *pfcrt* K76 and *pfmdr1* N86 codons. For SNPs at the *pfmdr1* 184F and 1246Y codons, the PCR products obtained with the A2-A4 and O2-1246f primer pairs were digested with DraI and EcoRV, respectively. For the *pfdhfr* gene, the PCR product obtained with the AMP3F-dhfrR4 primer pair was digested with AluI and *Tsp*509I for SNPs at the *pfdhfr* S108 and N51 codons, respectively, the one obtained with dhfrR2-F was digested with *Xmn*I for SNP at the *pfdhfr* 59R codon, and the one obtained with the dhfr_164_r-F/ primer pair was digested with DraI for SNP at the *pfdhfr* 164L codon. For the *pfdhps* gene, the PCR product was digested with AvaII and FokI for SNPs at the *pfdhps* 437G and 540E codons, respectively. The products of digestion were resolved on 2 to 2.5% agarose gels (Invitrogen, Life Technologies, Carlsbad, CA). DNAs from 6 laboratory *P. falciparum* strains (3D7, HB3, 7G8, W2, Dd2, and FCR3) were used as controls for the PCR and the digestion.

pfcrt, *pfmdr1*, and *pfmrp1* SNPs were genotyped in the samples of all three treatment arms, while *pfdhfr* and *pfdhps* loci were tested only in the SP arm.

Statistical analysis. In the molecular analysis, patients were excluded from the pretreatment population due to missing blood samples or filter papers, infection with other *Plasmodium* species, or failure to meet the inclusion criteria. Additional patients were excluded from the molecular selection analysis of recurrent infections because of wrong dosage, intake of other antimalarial drugs during the follow-up, and early treatment failure. Additional exclusion criteria for the molecular prediction analysis included loss to follow-up, consent withdrawal, and adverse events (Fig. 1). The correlation between *ex vivo* drug susceptibilities was established by a correlation matrix with Bonferroni adjustment to calculated significance levels. The data were log transformed since they were not normally distributed according to the Shapiro-Wilk W test. Multiple linear regression analysis was used to estimate the relations between drugs. Reported *P* values are two tailed. Fisher's two-tailed test was used to evaluate the difference in genotype frequencies between pretreatment samples and recrudescences and reinfections. Mixed-genotype infections were analyzed together with the nonselected genotypes against the selected genotype, as previously suggested (35). To evaluate an association between the *pfmdr1* N86 and Y184 genotypes, observed and expected frequencies were compared with the χ^2 test. Mixed-genotype infections were removed from this analysis. The association between alleles and *ex vivo* susceptibility was determined with the Mann-Whitney test. Mixed-genotype infections were excluded from the Mann-Whitney analysis. The association of *pfmdr1* haplotypes and *ex vivo* susceptibility was assessed with the

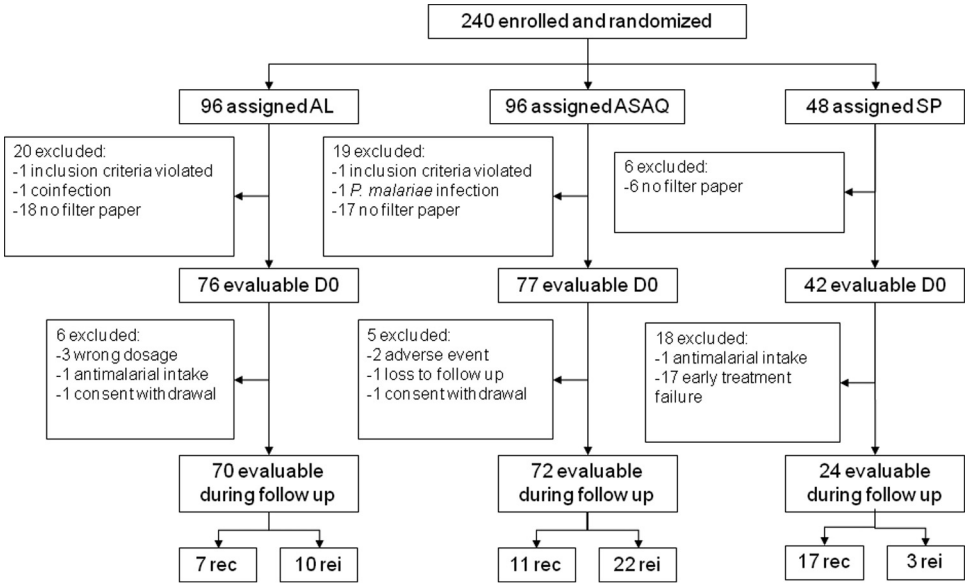

FIG 1 Flowchart describing excluded and evaluable patients for molecular analysis pretreatment (D0) and during the follow-up for recredescences (rec) and reinfections (rei).

Kruskal-Wallis test. Significant associations were further tested *post hoc* by the Mann-Whitney test. The relation between genotype at the baseline and *in vivo* treatment outcome was assessed by the χ^2 test. The association between *pfmdr1* 86 genotype and drug concentration was assessed with the Mann-Whitney test. Statistical significance for all of described tests was defined as a P value of <0.05. Fisher's exact two-tailed test and the χ^2 test were performed with GraphPad QuickCalcs (GraphPad Software Inc., San Diego, CA) and the Mann-Whitney and Kruskal-Wallis tests with VassarStats (http://vassarstats.net/).

RESULTS

***P. falciparum in vitro* drug susceptibility at baseline.** Minimal blood volume for isolates to be tested *ex vivo* was obtained for 118 children before treatment (day 0). Results from the PLDH ELISA *ex vivo* drug susceptibility assay were available for 58 to 101 isolates depending on the drug tested (Table 2), resulting in a mean of 71.5% successful assays. For these isolates, the geometric mean IC_{50}s of DEAQ, LUM, MQ, and QN were similar to or lower than the corresponding IC_{50} for 3D7 (considered to be drug susceptible), and a majority of the isolates had IC_{50} estimates lower than that of 3D7 (Table 2). The geometric mean PYR IC_{50} of the tested isolates was considerably higher (3,577 nM) than that of 3D7 (876.9 nM), and 52/58 (89.7%) isolates had higher individual IC_{50}s than 3D7. Susceptibility to DHA was also reduced in the isolates (3.1 nM) compared to that of 3D7 (1.9 nM), and 73/95 (76.8%) isolates had higher IC_{50}s than 3D7. Susceptibilities to MQ and LUM were strongly correlated (Table 3), while an inverse correlation was observed between IC_{50}s to MQ and DEAQ. DHA was correlated with QN and PYR. In univariate analysis, there was a positive correlation between DEAQ, QN, and PYR susceptibilities that was confirmed in multivariate analysis (data not shown).

Relation between *in vitro* drug susceptibility at baseline and treatment outcome. No relation was found between day 42 treatment outcome and day 0 IC_{50} estimates for DHA, LUM, and DEAQ (Mann-Whitney U test, $P > 0.05$), as shown in Table 4. PYR IC_{50}s were high in isolates from patients with *in vivo* failure to SP. However, only one isolate from a patient successfully treated with SP could be analyzed.

***P. falciparum* genetic diversity.** Genotyping was successful in *pfcrt* and *pfmdr1* loci in all of the 195 available baseline samples included in the study and in *pfmrp1* loci in 183/195 (94%) samples. *pfdhr* and *pfdhps* loci were successfully assessed in samples from all SP patients. At baseline, the frequencies of alleles previously associated with resistance, *pfcrt* 76T (chloroquine resistance) and the *pfdhfr* triple mutant 108N 51I 59R and *pfdhps* 437G (SP resistance), were close to 100% in the parasite population. The *pfmrp1* mutation 876V was almost absent. No deviation from the wild-type genotype was identified in *pfdhfr* codon 164 or *pfdhps*

TABLE 2 *Ex vivo* drug susceptibilities of field isolates from Benin and reference clones

Drug	No. of isolates tested	Geometric mean IC_{50} (nM)	95% confidence interval (nM)	3D7 geometric mean IC_{50} (nM)	W2 geometric mean IC_{50} (nM)	% of isolates (no./total) with a higher IC_{50} than 3D7
DEAQ	101	19.6	6.1–62.5	20.6	79.5	34.6 (35/101)
LUM	90	9.3	1.9–49.6	23.2	29.9	8.9 (8/90)
DHA	95	3.1	0.7–12.8	1.9	1.2	76.8 (73/95)
MQ	79	9.8	2.1–45.4	49.9	45.7	3.8 (3/79)
QN	83	71.2	23.0–220.6	129.9	528.7	15.7 (13/83)
PYR	58	3,577	194–66,057	876.9	ND[a]	89.7 (52/58)

[a] ND, not done.

TABLE 3 Correlations between *ex vivo* drug susceptibilities

Drug	Correlation				
	DEAQ	LUM	DHA	MQ	QN
LUM	0.121				
DHA	0.176	0.153			
MQ	−0.243[a]	0.613[a]	−0.019		
QN	0.289[a]	0.096	0.393[a]	−0.076	
PYR	0.391[a]	0.090	0.402[a]	−0.172	0.373[a]

[a] Statistical significance ($P < 0.05$) with Bonferroni adjustment.

TABLE 4 Relation between *ex vivo* drug susceptibility at baseline and treatment outcome at day 42

Ex vivo agent tested pretreatment	Treatment	Treatment success		Recrudescence[a]	
		No. of tested *ex vivo* isolates	$IC_{50} \pm SE$ (nM)	No. of tested *ex vivo* isolates	$IC_{50} \pm SE$ (nM)
DEAQ	ASAQ	27	21.3 ± 2.7	8	31.8 ± 9.3
LUM	AL	32	15.1 ± 2.1	2	9.6 ± 3.4
DHA	ASAQ or AL	56	4.2 ± 0.4	9	3.6 ± 0.7
PYR	SP	1	1,001	13	7,390 ± 2,340

[a] Recrudescence defined by *msp1* and *msp2* analyses.

codon 540 (Table 5). Consequently, these nonpolymorphic loci were not informative in further assessments of *P. falciparum* polymorphisms with *in vivo* and *ex vivo* drug susceptibilities. *pfmdr1* loci were found to be polymorphic, with mutations 86Y, 184F, and 1246Y identified in 81.6%, 79.5%, and 11.8% of the patients, respectively. The mutant allele *pfmrp1* 1466R was found in 13.2% (Table 5). An association between the *pfmdr1* 86 and 184 genotypes was found ($\chi^2 = 13.82$; $P = 0.0032$) where N86 plus Y184 was observed in a higher frequency (11.9%) than expected (4.5%) based on individual alleles, as well as 86Y plus 184F (observed, 88.9%; expected, 62.2%); N86 plus 184F (observed, 9.5%; expected, 16.3%) and 86Y plus Y184 (observed, 12.5%; expected, 17%) were observed in lower frequencies than expected (Table 6).

Selection of polymorphisms in recurrent infections. To determine if any of the studied SNPs was under selection, the allele

TABLE 5 Number and frequency of *pfcrt*, *pfmdr1*, *pfmrp1*, *pfdhfr*, and *pfdhps* genotypes in *P. falciparum* infections pretreatment and after treatment with ASAQ, AL, and SP

			Posttreatment[a]											
			ASAQ				AL				SP			
	Pretreatment		Recrudescences		Reinfections		Recrudescences		Reinfections		Recrudescences		Reinfections	
Genotype	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
pfcrt 76														
K	4	2.1	1	9.1	1	4.8	0	0.0	0	0.0				
K/T	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0				
T	191	97.9	10	90.9	20	95.2	7	100.0	10	100.0				
pfmdr1 86														
N	36	18.5	1	9.1	1	4.5	4[b]	57.1	2	20.0				
N/Y	22	11.3	2	18.2	1	4.5	1	14.3	0	0.0				
Y	137	70.3	8	72.7	20[b]	90.9	2	28.6	8	80.0				
pfmdr1 184														
Y	40	20.5	2	18.2	3	14.3	3	42.9	2	20.0				
Y/F	9	4.6	0	0.0	0	0.0	1	14.3	1	10.0				
F	146	74.9	9	81.8	18	85.7	3	42.9	7	70.0				
pfmdr1 1246														
D	172	88.2	11	100.0	19	86.4	7	100.0	10	100.0				
D/Y	11	5.6	0	0.0	2	9.1	0	0.0	0	0.0				
Y	12	6.2	0	0.0	1	4.5	0	0.0	0	0.0				
pfmrp1 876														
I	178	97.3	11	100.0	20	90.9	7	100.0	9	90.0	16	94.1	3	100.0
I/V	2	1.1	0	0.0	1	4.5	0	0.0	0	0.0	0	0.0	0	0.0
V	3	1.6	0	0.0	1	4.5	0	0.0	1	10.0	1	5.9	0	0.0
pfmrp1 1466														
K	159	86.9	10	90.9	18	81.8	6	85.7	8	80.0	13	76.5	3	100.0
K/R	10	5.5	1	9.1	2	9.1	1	14.3	0	0.0	1	5.9	0	0.0
R	14	7.7	0	0.0	2	9.1	0	0.0	2	20.0	3	17.6	0	0.0
pfdhfr														
Triple mutant 108N 51I 59R	41	100.0									17	100.0	3	100.0
164L	41	0.0									17	0.0	3	0.0
pfdhps														
437G	39	95.1									17	100.0	3	100.0
540E	41	0.0									17	0.0	3	0.0

[a] Recrudescences (reappearances) and reinfections (new infections) were defined by *msp1* and *msp2* analyses.
[b] $P < 0.05$.

TABLE 6 Frequency of *pfmdr1* 86 plus 184 haplotypes and the association with *ex vivo* drug susceptibility (IC_{50} in nM) at baseline[a]

pfmdr1 86 plus 184 haplotype[b]	Frequency		DEAQ		LUM		DHA		MQ		QN		PYR	
	No.	%	No.	Mean ± SEM	No.	Mean ± SEM	No.	Mean ± SEM	No.	Mean ± SEM	No.	Mean ± SEM	No.	Mean ± SEM
N + F	16	9.5	13	20.3 ± 3,6	13	21.1 ± 5.1* A	12	4.21 ± 1.1	12	29.6 ± 6.1*** C	10	86.8 ± 14.2	5	12,447.6 ± 4,457.4
N + Y	20	11.9	11	23.8 ± 4.4	12	14.6 ± 2.4* B	12	4.0 ± 0.8	12	17.0 ± 3.7* D	11	109.9 ± 16.5* E	6	9,676.9 ± 3,483.2
Y + F	111	88.9	55	23.2 ± 2.2	48	9.4 ± 1.2* AB	50	4.0 ± 0.4	42	8.7 ± 0.9*** C/* D	43	75.6 ± 6.4* EF	32	7,001.3 ± 1,408.8
Y + Y	21	12.5	7	25.1 ± 5.9	6	10.7 ± 1.9	8	3.3 ± 0.6	4	9.7 ± 1.3	6	111.6 ± 10.6* F	5	15,243 ± 5,894.3

[a] *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Difference in the *ex vivo* drug sensitivity between the haplotypes was assessed by Kruskal-Wallis followed by the Mann-Whitney U test to distinguish between which two haplotypes there was a difference. The same letter is placed by haplotype pairs between which there was a significant difference.
[b] Samples containing mixed alleles in either or both loci were excluded from the analysis.

prevalence was compared between baseline infections and recrudescences and reinfections. The frequency of the selected genotype was compared with the frequency of the deselected genotype together with the mixed genotype. The numbers of recrudescences and reinfections distinguished by *msp1* and *msp2* analyses were 11 and 22 in the ASAQ arm, 7 and 10 in the AL arm, and 17 and 3 in the SP arm, respectively. The molecular analyses of recurrent infections were successful for all samples except for one reinfection in the ASAQ arm. As shown in Table 5, we observed a statistically significant selection of the *pfmdr1* N86 allele in AL recrudescences (57.1% versus 18.5%; $P = 0.030$) and of the opposite allele, 86Y, in ASAQ reinfections (90.9% versus 70.3%; $P = 0.044$). It was assessed whether the genotype of baseline isolates could predict treatment outcome in the different treatment groups. Although *pfmdr1* N86 and *pfmdr1* 86Y isolates were shown to be selected in recurrent infections in the AL and ASAQ arms, respectively, the presence of these mutations in baseline isolates did not predict the occurrence of treatment failure.

Blood drug concentrations at day 3 and reinfection. It was assessed whether the *pfmdr1* 86 genotype in reinfections was associated with drug concentration at day 3 and the day of reinfection. In the AL arm, the two reinfections with organisms carrying N86 were associated with significantly higher blood LUM concentrations (mean, 3.36 ± 0.68 μg/ml; $P = 0.04$) and were mainly reinfecting earlier (day 28) than those carrying 86Y (mean, 0.74 ± 0.12 μg/ml) that were reinfecting later (day 35). In the ASAQ arm, there were no differences between reinfections with organisms carrying N86 and 86Y: the two groups had the same mean blood DEAQ concentration and day of reinfection.

Association between polymorphisms and *ex vivo* drug susceptibility. Association between the IC_{50}s to DEAQ, LUM, DHA, MQ, QN, and PYR and *P. falciparum* polymorphisms was assessed in the baseline samples. The loci *pfmdr1* 86, 184, and 1246 and *pfmrp1* 1466 were included in the analyses (Table 7), while *pfcrt* 76, *pfmrp1* 876, and *pfdhfr* and *pfdhps* loci could not be evaluated because of limited genetic variation in the samples with available IC_{50} estimates.

We found an association between both the *pfmdr1* allele N86 ($P = 0.0039$) and *pfmdr1* Y184 ($P = 0.0466$) and decreased *ex vivo* susceptibility to LUM. *pfmdr1* N86 was strongly associated with decreased MQ susceptibility ($P < 0.0001$). *pfmdr1* Y184 ($P = 0.0105$) and 1246Y ($P = 0.0332$) were associated with decreased QN susceptibility (Table 7). Evaluation of the *pfmdr1* 86 plus 184 haplotype showed a decreased susceptibility to LUM in parasites with N86 plus 184F ($P = 0.011$) and N86 plus Y184 ($P = 0.044$) compared to 86Y plus 184F (Table 6). A similar association was seen for MQ. While there was no significant difference between N86 plus 184F and N86 plus Y184 parasite susceptibilities to LUM ($P = 0.81$), N86 plus 184F was associated with a slightly higher IC_{50} to MQ than was N86 plus Y184 ($P = 0.111$), although statistical significance was not reached. N86 plus Y184 ($P = 0.038$) and 86Y plus Y184 ($P = 0.017$) haplotypes were associated with decreased QN susceptibility in comparison to that of the 86Y plus 184F haplotype.

TABLE 7 Association between *P. falciparum* polymorphisms and *ex vivo* drug susceptibility (IC_{50} in nM) at baseline[a]

Locus	Allele[b]	DEAQ		LUM		DHA		MQ		QN		PYR	
		No.	IC_{50}, nM (mean ± SEM)	No.	IC_{50}, nM (mean ± SEM)	No.	IC_{50}, nM (mean ± SEM)	No.	IC_{50}, nM (mean ± SEM)	No.	IC_{50}, nM (mean ± SEM)	No.	IC_{50}, nM (mean ± SEM)
pfmdr1 86	N	24	21.9 ± 2.8	25	18.0 ± 2.9**	24	4.1 ± 0.7	24	23.3 ± 3.7***	21	98.9 ± 11.0	11	9894.0 ± 2542.3
	Y	63	24.0 ± 2.1	54	9.5 ± 1.1	59	3.8 ± 0.3	46	8.7 ± 0.8	49	78.2 ± 6.0	37	8115.1 ± 1490.0
pfmdr1 184	Y	20	24.0 ± 3.1	20	15.2 ± 2.2*	69	4.0 ± 0.3	18	14.8 ± 2.6	19	106.2 ± 10.9*	13	10512.7 ± 2940.4
	F	77	22.1 ± 1.7	67	12.0 ± 1.5	22	3.7 ± 0.5	60	13.1 ± 1.7	61	76.4 ± 5.6	43	6904.9 ± 1174.4
pfmdr1 1246	D	91	23.0 ± 1.9	83	13.0 ± 1.3	84	4.0 ± 0.3	73	13.8 ± 1.6	75	80.8 ± 5.4*	44	6584.9 ± 1167.2
	Y	5	27.3 ± 8.4	6	10.7 ± 1.9	6	3.1 ± 0.8	4	9.7 ± 1.3	5	117.9 ± 10.3	4	17069.2 ± 7235.2
pfmrp1 1466	K	79	23.6 ± 2.1	71	12.4 ± 1.3	71	3.9 ± 0.3	63	13.9 ± 1.8	63	83.2 ± 6.1	47	7637.4 ± 1146.2
	R	7	21.8 ± 7.8	7	16.2 ± 5.5	8	4.5 ± 1.8	4	11.6 ± 2.8	8	66.8 ± 9.7	2	1169.0 ± 1070.4

[a] *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Difference in the *vivo* drug sensitivity between the two alleles was assessed using the Mann-Whitney U test.
[b] Samples containing mixed alleles in the specific locus were excluded from the analysis.

DISCUSSION

P. falciparum polymorphisms in *pfcrt*, *pfmdr1*, *pfmrp1*, *dhfr*, and *dhps* in conjunction with *ex vivo* drug susceptibility and with AL, ASAQ, and SP treatment effectiveness was assessed in southern Benin. Although AL was adopted in 2004 in Benin to replace CQ as first-line therapy for uncomplicated malaria, there was an insufficient supply and when the clinical trial was done, in 2007, AL still was not widely available (25). SP, previously used as a second-line treatment, was reserved for IPT of pregnant women. Resistance to CQ and SP *in vivo* was high in Benin previous to this trial (36). The history of drug usage and treatment efficacy is reflected in the parasite genetic diversity in the area. The CQ resistance-associated allele, *pfcrt* 76T (31), was close to fixation in the baseline parasite population, and *pfmdr1* 86Y, related to CQ resistance (37), was present at a high prevalence. The triple *dhfr* haplotype (108N, 51I, and 59R) and *dhps* 437G, associated with a higher risk of clinical SP failure (38), had increased from a previously high frequency (39) to 100% or almost 100% in this study. The extensive spread of SP resistance was confirmed by *ex vivo* susceptibility testing, in which the geometric mean IC_{50} of the tested isolates was considerably higher than that of 3D7. A vast majority of baseline isolates had IC_{50} estimates of LUM, MQ, and QN lower than those of 3D7, suggesting that these isolates are sensitive to the drugs. However, 36% of the isolates had higher DEAQ IC_{50} estimates than 3D7, which may represent a decreased sensitivity to DEAQ in these isolates. Decreased sensitivity to DEAQ may depend on the widespread resistance to CQ in the country, since a strong positive correlation has been shown between *ex vivo* susceptibilities to DEAQ and CQ (40, 41).

A majority of isolates had a DHA IC_{50} higher than that of 3D7. Current *in vitro* methodology was developed to assess the growth of long-acting drugs that are mainly effective on schizont stages and may not be adapted for the rapidly acting artemisinins that act on ring stages, as well as mature stages (42). A new method assessing *in vitro* susceptibility to artemisinins by pulsing ring stage parasites with DHA has demonstrated differences between isolates from regions with different levels of artemisinin resistance in Cambodia. The difference was not observed with the classical [^{3}H]hypoxanthine uptake inhibition assay (43). As methods taking into account artemisinin-related phenomena such as parasite clearance (A. Ndour, presented at the ASTMH 60th annual meeting, Philadelphia, PA, 4 to 8 December 2011) and ring stage survival (43) are being evaluated, DHA susceptibility measured by classical *in vitro* methods, as in this study, is difficult to interpret. It would be of interest to follow up on changes in DHA susceptibility after the implementation of ACT.

Ex vivo susceptibilities to MQ and LUM were strongly and positively correlated, probably as a consequence of the association of *pfmdr1* N86 with decreased susceptibility to both MQ and LUM. These results are consistent with previous studies showing the correlation of MQ and LUM *ex vivo* susceptibilities (44, 45) and reduced MQ and LUM susceptibility in isolates with *pfmdr1* N86 (19, 46). Decreased susceptibility to LUM was also associated with *pfmdr1* Y184, inconsistent with the selection of *pfmdr1* 184F after AL treatment (12, 14, 16, 47). This may be a consequence of the genetic linkage observed between the *pfmdr1* alleles N86 and Y184 in this study. N86 was strongly associated with decreased susceptibility to LUM and is probably more important for the phenotype than the 184 genotype. This is supported by the haplotype analyses showing decreased LUM susceptibility of both N86 plus Y184 and N86 plus 184F haplotypes and no difference between the two haplotypes. The association between Y184 and decreased susceptibility to LUM may therefore be due to a higher proportion of N86 in isolates with Y184 than in those carrying 184F. Genetic linkage between N86 and Y184 warrants further investigation, as it may explain the regional differences in the selection of recurrent infections by AL treatment, where *pfmdr1* N86 and Y184 were selected in Burkina Faso (23), while N86 and 184F have been selected in East Africa (12, 14, 16, 47).

In the ASAQ arm, reinfecting parasites carrying *pfmdr1* 86Y were selected. Reinfections represented the majority of the recurrent infections after ASAQ treatment. Subtherapeutic levels of long-half-life drugs can select for tolerant or resistant reinfecting parasites by suppressing the growth of sensitive parasites for weeks or months after treatment (48–50). DEAQ is likely to exert selection pressure on reinfections, as the metabolism of AQ to DEAQ is rapid and DEAQ has a longer half-life. Selection of *pfmdr1* 86Y by ASAQ has been observed in Mali in West Africa (21) and in East Africa (13–15). In East Africa, the *pfmdr1* haplotype (86Y, Y184, and 1246Y) was observed to be selected. In our study, 1246Y was present at a low frequency in the baseline infections (11.8%) compared to populations in East Africa (22 to 83%) (13–15) and Y184 was associated with N86, explaining why this *pfmdr1* haplotype was rare in Benin (9%) and was less likely to be selected posttreatment. The difference in frequency of 1246Y and the *pfmdr1* haplotype between East and West Africa was further underlined in a study assessing the genetic diversity of resistance-associated polymorphisms in travelers (24), providing a rationale for the differences in selection patterns between regions.

After AL treatment, the opposite allele, *pfmdr1* N86, was selected in recrudescent parasites. Selection of this allele after AL treatment has been observed in East (12, 14, 16, 17) and West (22, 23) Africa, mainly in reinfections. In this study, selection of reinfections was not observed. However, the *pfmdr1* 86 locus was still important for reinfections, since it was demonstrated that the reinfecting organisms carrying *pfmdr1* N86 were exposed to significantly higher concentrations of LUM at day 3 and occurred earlier, suggesting that they could sustain higher LUM concentrations than parasites with 86Y. A limitation in this study was that only two reinfecting parasites carried N86. However, the importance of considering the pharmacology when studying parasite genetics in clinical trials is supported by a similar observation by Malmberg and colleagues that reinfecting parasites carrying N86 sustain 12-fold-higher LUM concentrations than 86Y parasites, made by a novel model based on day 7 concentrations (51). In this study, the clinical relevance of the *pfmdr1* N86 allele in LUM was supported by its association with decreased *ex vivo* susceptibility to LUM, as also seen in Kenyan isolates (19).

The samples in this study were collected from an effectiveness trial, which may blur the selection analyses since all patients did not receive a complete treatment. Although adherence, based on drug blister recovery and drug level measurements, was suboptimal in some patients, there was no association with treatment outcome (25), so we consider that the recurrent infections were under drug pressure, resulting in the observed selection.

In transfection studies, *pfmdr1* SNPs including 1246Y have been implicated in QN resistance (52). Here we show an association between *pfmdr1* Y184 and 1246Y, respectively, and reduced *ex vivo* susceptibility to QN. Studies on the influence of *pfmdr1* on

quinine *in vitro* susceptibility have had inconsistent outcomes (53), maybe as a consequence of differences in the parasite genetic background. Further variation in susceptibility to QN may be explained by the Na^+/H^+ exchanger (*pfnhe-1*) that was demonstrated to be involved in QN resistance (54).

More reinfections were observed in the ASAQ arm than in the AL arm. A higher proportion of patients had undetectable levels of DEAQ than LUM on day 7 (tested in a subset of the patients), and it was suggested that the difference in the posttreatment prophylaxis between the two treatments could be due to fast elimination of AQ metabolites (25). An additional factor favoring reinfections following ASAQ treatment could be that the alleles selected by ASAQ (*pfcrt* 76T and *pfmdr1* 86Y) were present at high prevalence in the parasite population of the area, while the alleles that allow reinfections to survive AL treatment (*pfcrt* K76 and *pfmdr1* N86) were present at low frequency at the baseline. Furthermore, a slight decrease in *ex vivo* susceptibility to DEAQ was observed, while the parasite population seems to be sensitive to LUM.

No association was observed between *ex vivo* susceptibility of pretreatment parasites to DHA, DEAQ, LUM, and PYR, at the baseline and outcome in the respective treatment arms. The number of patients in each treatment arm was low, especially in the SP arm, and drug sensitivity data were available for less than half of the patients, so the study may not have had enough power to detect *ex vivo* susceptibility differences between treatment successes and failures. This may account for the lack of an association. The relation between AQ *in vivo* efficacy and *in vitro* DEAQ susceptibility may be confounded by interindividual variations in pharmacokinetic factors, such as the metabolism of AQ to DEAQ by CYP2C8 (55, 56), and the complex dynamics between AQ, DEAQ, and possibly other metabolites (57). *In vitro* susceptibility to artemisinins and *in vivo* efficacy of artesunate have been associated (5), but inconsistently (4).

This study assessed parasite drug sensitivity in Benin before the introduction of ACTs as a first-line antimalarial treatment. It shows *ex vivo* susceptibility to LUM and a slight decrease in DEAQ susceptibility and a phenotypic and genetic parasite profile associated with chloroquine and/or SP resistance. Selection of specific alleles in recurrent infections after ACT treatment confirms the tolerance of certain parasite strains to the partner drugs, as in East Africa. The results from the molecular and *in vitro* analysis underline the importance of performing these studies in different regions because of regional parasite genetic differences between West and East Africa. Selection of opposite alleles among recurrent parasites following AL or ASAQ treatment emphasizes the benefits of the strategy of multiple first-line treatments (58) where drug pressure for both ACTs would be reduced because of inversed tolerance and resistance mechanisms. ACT including new partner drugs with potentially different mechanisms of resistance, such as piperaquine or pyronaridine, should be encouraged to increase the options. For a comprehensive picture of drug resistance emergence and spread, it is important to continue clinical, *ex vivo*, molecular, and pharmacological surveillance after ACT implementation.

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