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MECHANISMS OF RESISTANCE

Influence of *CYP2C8*, *CYP3A4*, and *CYP3A5* Host Genotypes on Early Recurrence of *Plasmodium vivax*

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ABSTRACT Cytochrome P450 (CYP) enzymes are involved in the biotransformation of chloroquine (CQ), but the role of the different profiles of metabolism of this drug in relation to *Plasmodium vivax* recurrences has not been properly investigated. To investigate the influence of the CYP genotypes associated with CQ metabolism on the rates of *P. vivax* early recurrences, a case-control study was carried out. The cases included patients presenting with an early recurrence (CQ-recurrent individuals), defined as a recurrence during the first 28 days after initial infection and plasma concentrations of CQ plus desethylchloroquine (DCQ; the major CQ metabolite) higher than 100 ng/ml. A control group with no parasite recurrence over the follow-up (the CQ-responsive group) was also included. CQ and DCQ plasma levels were measured on day 28. CQ-metabolizing CYP (*CYP2C8, CYP3A4*, and *CYP3A5*) genotypes were determined by real-time PCR. An *ex vivo* study was conducted to verify the efficacy of CQ and DCQ against *P. vivax* isolates. The frequency of alleles associated with normal and slow metabolism was similar between the cases and the controls for the *CYP2C8* (odds ratio [OR] = 1.45, 95% confidence interval [CI] = 0.51 to 4.14, $P = 0.570$), *CYP3A4* (OR = 2.38, 95% CI = 0.92 to 6.19, $P = 0.105$), and *CYP3A5* (OR = 4.17, 95% CI = 0.79 to 22.04, $P = 1.038$) genes. DCQ levels were higher than CQ levels, regardless of the genotype. Regarding the DCQ/CQ ratio, there was no difference between groups or between those patients who had a normal genotype and those patients who had a mutant genotype. DCQ and CQ showed similar efficacy *ex vivo*. CYP genotypes had no influence on early recurrence rates. The similar efficacy of CQ and DCQ *ex vivo* could explain the absence of therapeutic failure, despite the presence of alleles associated with slow metabolism.

KEYWORDS CYP450, *Plasmodium vivax*, chloroquine, early recurrence, malaria

Malaria remains an important public health problem worldwide. In 2018, there were 219 million cases, and 435,000 deaths were caused by malaria (1). In Brazil, the Amazon region is the main area of transmission, and *Plasmodium vivax* accounts for 88.4% of the reported cases in the country (2). An important obstacle to *P. vivax* malaria elimination in areas where *P. vivax* is endemic stems from the frequent recurrences caused by this parasite. These recurrences are characterized as a relapse when they are caused by activation of hypnozoites in the liver; as a reinfection if parasitemia returns due to a new infected mosquito bite; and as a recrudescence when there is an early return of asexual parasitemia despite adequate levels of chloroquine (CQ) and the

Citation Almeida ACG, Puça MCB, Figueiredo EFG, Barbosa LR, Salazar YEAR, Silva EL, Brito MAM, Siqueira AM, Vieira JLF, Lacerda MVG, Monteiro WM, Melo GC. 2020. Influence of *CYP2C8*, *CYP3A4*, and *CYP3A5* host genotypes on early recurrence of *Plasmodium vivax*. Antimicrob Agents Chemother 64:e02125-19. https://doi.org/10.1128/AAC.02125-19.

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Received 22 October 2019
Returned for modification 4 January 2020
Accepted 25 April 2020
Accepted manuscript posted online 4 May 2020
Published 23 June 2020

metabolite desethylchloroquine (DCQ) in host plasma, which often indicates the presence of drug-resistant parasites, leading to therapeutic failures (3).

CQ undergoes hepatic biotransformation through the N-dealkylation pathway into two main metabolites: DCQ and bisdesethylchloroquine (BDCQ). DCQ is the major CQ metabolite, with the concentrations detected in plasma ranging from 20% to 50% of those of CQ (4). In contrast, BDCQ plasma or blood concentrations never reach more than 10% to 15% of CQ levels (5). The antimalarial action of DCQ, evaluated only for *Plasmodium falciparum*, was as active as that of CQ against a CQ-sensitive strain, but DCQ was significantly less active than the parent compound against a CQ-resistant strain (6) For *P. vivax*, in *in vivo* studies to assess resistance to CQ, only CQ and DCQ levels are generally measured (7, 8), since they are the major CQ metabolites (4).

P. vivax resistance to CQ has been reported, with a high prevalence of resistance being found in Indonesia and Papua New Guinea (9). In the Brazilian Amazon, CQ resistance rates range from 5% to 11% *in vivo* (8, 10, 11) and 9.8% to 10.7% *ex vivo* (12, 13). Although the mechanisms that lead to *P. vivax* resistance to CQ are not well understood, some studies have shown an association between gene expression and variations in the copy number of the *pvcrt-o* and *pvmdr-1* genes and resistance to CQ (7, 14). CQ remains at therapeutic levels against *P. vivax* until 35 days after starting treatment (3, 15). After this period, with decreasing plasma levels of CQ and DCQ, the return of parasitemia is due to reinfection or relapse (9). Several studies used day 28 as the cutoff point to assess possible therapeutic failures by CQ (7, 8, 10, 11, 16), following the recommendation of the World Health Organization for monitoring the effectiveness of antimalarials (17).

Recent studies have demonstrated the importance of host genetics in antimalarial treatment outcomes (18–21), based on single-nucleotide polymorphisms (SNPs) detected in genes encoding drug-metabolizing enzymes (22). The presence of certain genotypes related to the metabolism of these enzymes may be associated with an increase in drug metabolism rates, generating adverse effects, and an increase in the elimination rate or a decrease in the rate of metabolism may lead to therapeutic failure (20, 22, 23).

The biotransformation of primaquine (PQ), mediated by cytochrome P450 (CYP) enzymes, is attributed to the *CYP2D6*, *CYP3A4*, *CYP1A2*, and *CYP2C19* enzymes (22, 24). Therapeutic failures in the treatment of *P. vivax* malaria with PQ are generally attributed to the presence of *CYP2D6* polymorphisms (19), a relationship that has also been reported in Brazil (21, 25). *CYP2C8*, *CYP3A4*, and *CYP3A5* were reported to be the major enzymes involved in the formation of DCQ from CQ (26). An effect of the *CYP2C8*2/*3/*4* gene on the gametocyte clearance rate in patients with malaria undergoing CQ and PQ treatment has been reported (27).

Pharmacogenetics has gained great importance over the last few years, since it can enable patients to received personalized drug therapy for various diseases (28). However, the frequency of CYP alleles associated with the slow metabolism of CQ in individuals from the Brazilian Amazon has not been fully studied, in particular, whether the presence of these alleles influences early recurrence. In addition, there is a paucity of studies to understand which molecule (CQ or DCQ) has the best antimalarial action on *P. vivax* and whether a profile of low drug metabolism contributes to increased early recurrence rates.

This study aimed to investigate the frequency of genotypes associated with slow CQ metabolism for the main metabolizing CYPs in patients from the Brazilian Amazon and verify the influence of these alleles in the early recurrence of *P. vivax* malaria.

RESULTS

Allele frequencies of CYPs associated with CQ metabolism. Twenty-six cases (CQ-recurrent individuals) and 99 controls (CQ-responsive individuals) were included in this study. The clinical and laboratory characteristics of the patients included in this study are presented in Table 1. All individuals with CQ-recurrent *P. vivax* malaria had a

TABLE 1 Demographic and parasitological baseline data for individuals involved in this study[a]

Characteristic	Value for the following group:		*P* value[b]
	CQ-recurrent	CQ-responsive	
No. of subjects	26	99	
Mean (95% CI) age (yr)	34.69 (27.59–41.78)	35.21 (31.94–38.47)	0.8872
No. (%) of male subjects	19 (73.1)	73 (73.7)	1.0000
Mean no. of parasites/μl (95% CI) on day 0	2,618.82 (1,359.20–3,878.43)	2,961.6 (2,297.90–3,625.29)	0.6368
Mean no. of gametocytes/μl (95% CI) on day 0	45.26 (24.89–65.62)	75.3 (39.12–111.47)	0.4060

[a]Recurrent indicates a new malaria episode by day 28, and responsive indicates no malaria episode by day 28.
[b]*P* values were determined by the χ^2 test, *t* test, or Fisher's exact test.

positive quantitative PCR result for *P. vivax*, and their mean blood levels of CQ plus DCQ were greater than 100 ng/ml at day 28.

The allele frequencies of *CYP2C8* ($P = 0.3196$), *CYP3A4* ($P = 0.0916$), and *CYP3A5* ($P = 0.1064$) were similar in the cases and the controls. Most individuals carried alleles associated with normal enzyme activity (**1* or **1A*). Alleles associated with slow enzyme activity were found in both cases and controls (Table 2).

The most frequent diplotypes for *CYP2C8* were **1/*1* (76.2% of cases and 82.8% of controls) and **1/*3* (15.3% of cases and 10.1% of controls). For *CYP3A4*, the most frequent diplotypes were **1A/*1A* (65.3% of cases and 81.2% of controls) and **1A/*1B* (30.7% of cases and 16.1% of controls). For *CYP3A5*, **1/*1* (88.4% of cases and 96.9% of controls) was the most frequent diplotype, *1/*3* was found in both cases (7.6%) and controls (2.0%), and **1/*6* was found in both cases (3.8%) and controls (1.0%). The frequency of diplotypes associated with normal and slow metabolism was similar between cases and controls for the *CYP2C8* (odds ratio [OR] = 1.45, 95% confidence interval [CI] = 0.51 to 4.14, $P = 0.570$), *CYP3A4* (OR = 2.38, 95% CI = 0.92 to 6.19, $P = 0.105$), and *CYP3A5* (OR = 4.17, 95% CI = 0.79 to 22.04, $P = 1.038$) genes.

Genotypes and *P. vivax* malaria recurrences. In addition to following up the patients until day 28, we also investigated the occurrence of vivax malaria recurrences in patients for up to 1 year by passive case detection using the SIVEP-Malaria platform (the Brazilian official malaria epidemiological surveillance information system). When considering individuals with alleles of *CYP2C8*, *CYP3A4*, and *CYP3A5* associated with normal and slow metabolism, no significant differences in the recurrence rates between the cases and the controls were found over the 1-year follow-up (Table 3).

Drug levels and CQ-metabolizing CYP genotypes. When we compared the CQ and DCQ concentrations between the cases and the controls, no significant differences were found. Furthermore, no significant differences in the mean concentrations of CQ and DCQ were found between individuals carrying alleles associated with slow metabolism (Table 3). The DCQ concentration was higher than the CQ concentration irrespective of genotype, indicating that most individuals had a drug biotransformation ability, despite the mutated genotypes found (for cases with *CYP2C8*1*, the DCQ

TABLE 2 Chloroquine-metabolizing CYP allele frequency

Gene	Allele	CQ-recurrent group (n = 52)		CQ-responsive group (n = 198)		*P* value[a]
		No. of chromosomes	Frequency	No. of chromosomes	Frequency	
CYP2C8	**1*	46	0.8846	179	0.9040	0.6137
	**2*	0	0.0000	6	0.0303	0.3493
	**3*	4	0.0769	11	0.0556	0.5225
	**4*	2	0.0385	2	0.0101	0.192
CYP3A4	**1A*	42	0.8077	178	0.8990	0.0916
	**1B*	10	0.1923	20	0.1010	
CYP3A5	**1A*	49	0.9423	195	0.9848	0.1064
	**3*	2	0.0385	2	0.0101	0.192
	**6*	1	0.0192	1	0.0051	0.3734

[a]*P* values were determined by the χ^2 test.

TABLE 3 Parasitemia clearance, drug levels, and malaria recurrence patterns between different genotype groups

Gene, patient group, and genotype	No. of patients	No. (%) of patients with asexual parasitemia clearance on[a]:				Mean (95% CI) drug level (ng/ml) at day 28[b]		No. (%) of patients with the following no. of malaria episodes up to 1 yr[a,c]:		
		Day 1	Day 2	Day 3	Day 7	CQ	DCQ	0	1	>1
CYP2C8										
CQ-recurrent										
**1*	20	0 (0)	11 (55)	4 (20)	5 (25)	63.8 (49.33–78.26)	108.4 (89.68–127.11)	8 (40)	9 (45)	3 (15)
**2/*3/*4* allele carriers	6	1 (16.7)	2 (33.3)	0 (0)	3 (50)	42.2 (17.37–67.02)	130.6 (57.82–203.37)	2 (33.3)	3 (50)	1 (16.7)
P value		0.2308	0.6447	0.5425	0.3301	0.1293	0.3266	1.0000	1.0000	1.0000
CQ-responsive										
**1*	82	3 (3.7)	36 (43.9)	30 (36.6)	13 (15.8)	43.4 (37.85–48.94)	93.3 (68.17–118.42)	59 (71.9)	14 (17.1)	9 (11)
**2/*3/*4* allele carriers	17	1 (5.9)	9 (52.9)	5 (29.4)	2 (11.8)	64.1 (23.29–104.90)	79.9 (40.23–119.56)	10 (58.9)	4 (23.5)	3 (17.6)
P value		0.5354	0.5956	0.7813	1.0000	0.0528	0.6459	0.3843	0.5047	0.4277
CYP3A4										
CQ-recurrent										
**1A*	17	1 (5.9)	7 (41.2)	3 (17.6)	6 (35.3)	62.06 (46.61–77.50)	121.93 (94.73–149.12)	5 (29.4)	10 (58.8)	2 (11.8)
**1B* allele carriers	9	0 (0)	6 (66.7)	1 (11.1)	2 (22.2)	55 (30.04–79.95)	96.66 (75.71–117.60)	5 (55.6)	2 (22.2)	2 (22.2)
P value		0.4375	0.411	1.0000	0.6673	0.5842	0.1948	0.2341	0.11	0.5906
CQ-responsive										
**1A*	81	4 (4.9)	37 (45.7)	27 (33.3)	13 (16.1)	48.68 (39.82–57.53)	100.48 (74.65–126.30)	56 (69.2)	15 (18.5)	10 (12.3)
**1B* allele carriers	18	0 (0)	8 (44.4)	8 (44.4)	2 (11.1)	39.25 (18.81–59.68)	48.52 (29.23–67.80)	13 (72.2)	3 (16.7)	2 (11.1)
P value		1.0000	1.0000	0.4195	0.732	0.3706	0.0662	1.0000	1.0000	1.0000
CYP3A5										
CQ-recurrent										
**1A*	23	1 (4.4)	10 (43.5)	4 (17.4)	8 (34.7)	61.63 (47.95–75.30)	113 (91.80–134.19)	7 (30.4)	12 (52.2)	4 (17.4)
**3/*6* allele carriers	3	0 (0)	3 (100)	0 (0)	0 (0)	44 (6.91–81.08)	111.66 (53.18–170.13)	3 100	0 (0)	0 (0)
P value		1.0000	0.22	1.0000	0.5292	0.3570	0.9637	0.0462	0.2246	1.0000
CQ-responsive										
**1A*	96	4 (4.2)	42 (43.8)	35 (36.5)	15 (15.5)	47.5 (39.29–55.70)	92.46 (70.20–114.71)	67 (69.8)	17 (17.7)	12 (12.5)
**3/*6* allele carriers	3	0 (0)	3 (100)	0 (0)	0 (0)	29.92 (42.81–102.65)	45.25 (71.03–161.53)	2 (66.7)	1 (33.3)	0 (0)
P value		1.0000	0.0905	0.5501	1.0000	0.4584	0.4615	1.0000	0.456	1.0000

[a]*P* values were determined by the χ^2test.
[b]*P* values were determined by the *t* test or Fisher's exact test.
[c]Determined by use of the SIVEP-Malaria platform.

concentration was 108.4 nM [95% CI = 89.7 to 127.1 nM]; for cases with *CYP2C8*2/*3/*4*, the DCQ concentration was 130.6 nM [95% CI = 57.8 to 203.4 nM]; for controls with *CYP2C8*1*, the DCQ concentration was 93.3 nM [95% CI = 68.2 to 118.4 nM]; and for controls with *CYP2C8*2/*3/*4*, the DCQ concentration was 79.9 nM [95% CI = 40.2 to 119.6 nM]).

The possibility that the genotype influenced the metabolite/drug ratio was analyzed. However, there was no significant difference in the DCQ/CQ ratio between individuals carrying alleles associated with the normal metabolism and the slow metabolism of *CYP2C8* and *CYP3A5*. In the control group, the metabolite/drug ratio was higher in individuals carrying alleles associated with normal *CYP3A4* metabolism than in individuals with a mutated genotype (Fig. 1). For *CYP3A4*, a significant difference was observed (for cases with *CYP3A4*1A* the mean DCQ/CQ was 1.76 and for cases with *CYP3A4*1B* the mean DCQ/CQ was 1.55 [$P = 0.629$]; for controls with *CYP3A4*1A* the mean DCQ/CQ was 1.89 and for controls with *CYP3A4*1B* the mean DCQ/CQ was 1.21 [$P = 0.0347$]). No difference in CQ and DCQ levels (metabolite/drug ratio) was found between cases and controls for *CYP2C8* and *CYP3A5* ($P > 0.05$) (Fig. 1).

Clearance of *P. vivax* parasitemia and CQ-metabolizing CYP alleles. Most individuals had clearance of the asexual parasitemia on day 2 (58/125; 46.4%) or day 3 (39/125; 31.2%). Late clearance (from days 4 to 7) was recorded for 23 patients (18.4%). The slow clearance of asexual parasitemia did not predict early recurrences (OR = 2.49, 95% CI = 0.92 to 6.75, $P = 0.088$). Genotypes associated with normal and slow metabolism were found in these patients at a similar frequency (Table 3), which suggests that the presence of a mutated genotype is not associated with the clearance time for *P. vivax*. There was also no difference regarding the mean asexual parasitemia on days 1, 2, 3, and 7 between patients with alleles associated with normal activity and patients with alleles associated with low activity for all the enzymes studied (Fig. 2). Only 1

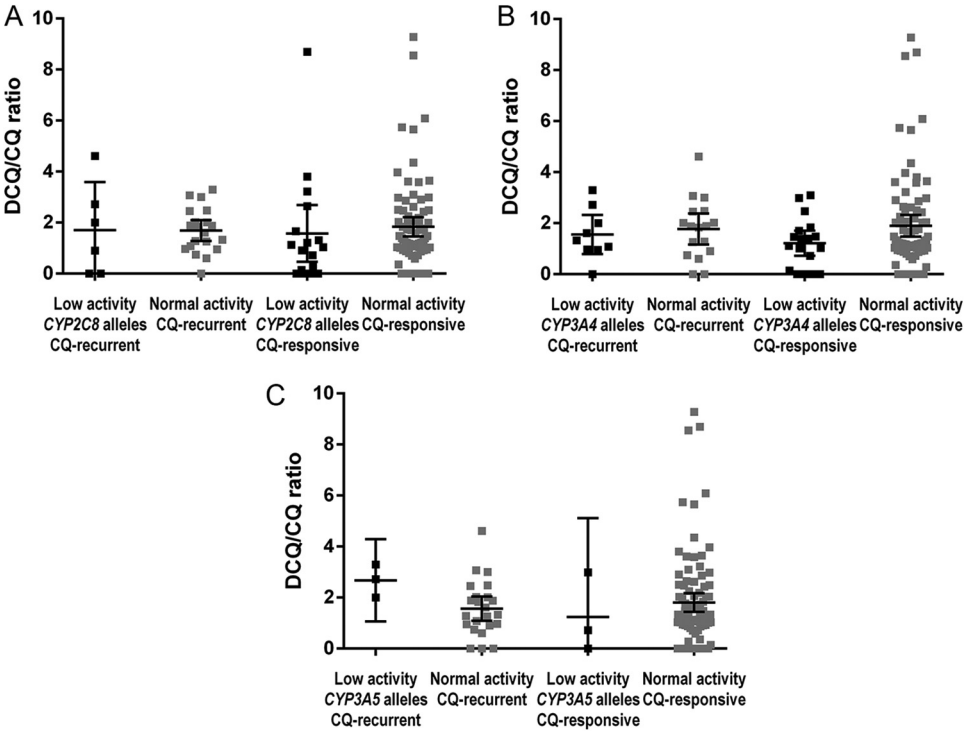

FIG 1 Desethylchloroquine/chloroquine ratios of the different genotype groups. (A and C) No significant difference in the metabolite/drug ratios was found between patients with different genotypes (associated with low and normal enzyme activity) for patients with a *P. vivax* early recurrence (CQ-recurrent) and CQ-responsive patients with the *CYP2C8* (A) and *CYP3A5* (C) genotypes. (B) The ratio was higher among CQ-responsive patients with the genotype associated with normal metabolism. Values were obtained by an unpaired *t* test.

patient, belonging to the control group (1.01%, 1/99), showed gametocyte clearance on day 7. No association between late gametocyte clearance (days 3 to 7) and genotypes associated with the slow metabolism of CYP was observed in the cases ($P = 1.000$) or the controls ($P = 0.259$).

***P. vivax ex vivo* assay.** Twenty-four malaria patients not included in the cases or controls were enrolled in the *ex vivo* assays to evaluate the efficacy of CQ and DCQ against *P. vivax*. None of these isolates was resistant to CQ. The mean 50% inhibitory concentration (IC_{50}) of CQ was 11.67 nM (95% CI = 6.520 to 16.82 nM), and the mean IC_{50} of DCQ was 8.95 nM (95% CI = 4.25 to 13.65 nM) (Fig. 3). None of these 24 patients presented with an early recurrence.

DISCUSSION

This is the first study to investigate the role of CYP genotypes in the early recurrence of *P. vivax* malaria in samples from the Brazilian Amazon and the first to provide results regarding an evaluation of the sensitivity of *P. vivax* isolates to CQ and DCQ.

The frequency of allelic gene variants has already been used to predict the activity of drug-metabolizing enzymes (29). In Brazil, the frequency of these variants for cytochrome P450 enzymes has been described (30–33), but there is still little information regarding the influence of these genotypes on the failure of antimalarial therapy, including CQ therapy. Studies of *P. falciparum* have demonstrated the importance of pharmacogenetics in the elimination of this parasite (34, 35).

For the *CYP2C8* gene, variant **2* is more frequent in Africans (11 to 17%) and *CYP2C8*3* is more frequent in Caucasians (15%) (36). The frequency of *CYP2C8*4* is higher in European populations (4 to 7%) (37). For *CYP3A4*, the frequency of the **1B* allele is variable, ranging from 3.6% among white Americans to 76% among Africans (38). For *CYP3A5*, the highest frequencies of **3* are among Asians (60 to 75%) and Caucasians (85 to 95%); **6* is present in Africans (22%) and rare in Caucasians and Asians

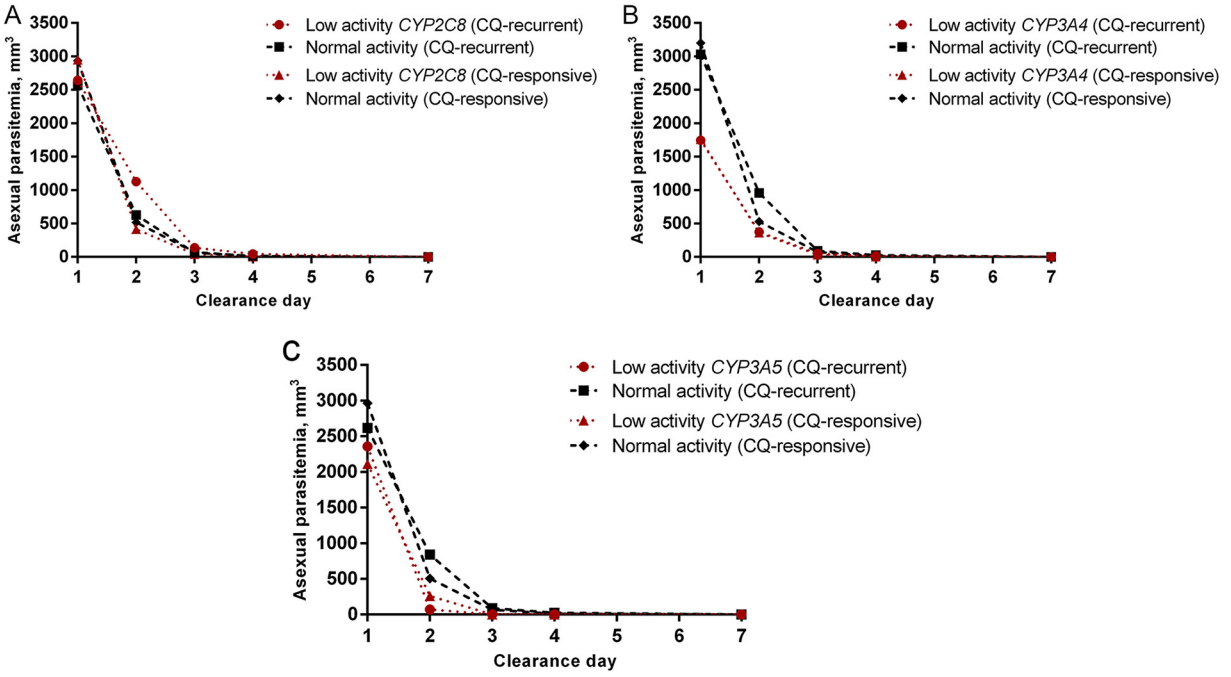

FIG 2 Asexual parasitemia clearance between patients with genotypes with low and normal activity. The mean levels of parasitemia were evaluated at visits on days 1, 2, 3, 4, and 7. There was no significant difference in the clearance day and asexual parasitemia between patients in the CQ-recurrent and CQ-responsive groups with genotypes associated with low and normal enzyme activity for *CYP2C8* (A), *CYP3A4* (B), and *CYP3A5* (C). Values were obtained by a paired *t* test.

(22). The frequency of mutated alleles for *CYP2C8*, *CYP3A4*, and *CYP3A5**6 found in our study is in agreement with that found in other studies of Brazilian populations (27, 30, 33). Only the frequency of the *CYP3A5*3* variant was found to be lower.

The *CYP2C8*2* variant is associated with a 50% reduction in metabolic activity and the *CYP2C8*3* variant is associated with an 85% reduction in normal enzyme activity compared to the activity of the wild-type allele, which was found in a study with paclitaxel and arachidonic acid drugs *in vitro* (36, 39). In Burkina Faso and Ghana, the role of *CYP2C8* variants in the response to amodiaquine and the correlation between *CYP2C8*2* and low levels of enzyme metabolism have been investigated, confirming their influence on drug metabolism (40, 41). However, they did not demonstrate an

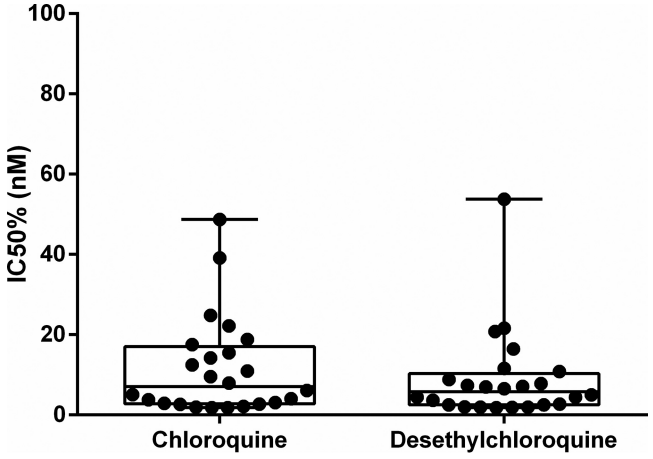

FIG 3 *Ex vivo* efficacy of chloroquine and desethylchloroquine against *P. vivax* isolates. The drug and metabolite showed similar efficacy against isolates in patients from the Brazilian Amazon. Values were obtained by a paired *t* test.

association between alleles associated with low enzyme activity and the treatment outcome.

Studies conducted in Papua New Guinea and Thailand described the risk of *P. vivax* recurrence in individuals with late parasitemia clearance (41, 42). In our study, the clearance of asexual parasitemia at day 7 and gametocytemia after day 3 was not a predictor of an early recurrence by *P. vivax* and was not associated with the presence of mutated CYP genotypes. A recent study in Brazil found an association between *CYP2C8*2*3/*4* and the gametocyte clearance rate in patients with malaria undergoing CQ/PQ treatment. From the baseline to the first day of treatment, individuals homozygous for wild-type *CYP2C8* achieved a greater gametocyte reduction ($P = 0.007$) than individuals without this genotype (27). There was no difference in CQ and DCQ levels between patients with normal genotypes and patients with mutated genotypes or in the frequency of these *CYP2C8* alleles between cases and controls.

The *CYP3A5*3* variant is related to decreased enzyme activity, and *CYP3A5*6* and *CYP3A5*7* are null alleles that cause enzyme absence (29). Kim et al. (26) demonstrated a role for *CYP3A5* in CQ metabolism, but another study showed a smaller role for this enzyme in the formation of DCQ *in vitro* (42). A recent study demonstrated that polymorphisms of *CYP3A5* and *CYP3A4* did not show any significant association with the blood levels of hydroxychloroquine and desethylhydroxychloroquine in patients with systemic lupus erythematosus (43). In our study, in the control group, the metabolite/drug ratio was higher in individuals carrying alleles associated with normal *CYP3A4* metabolism than in individuals with mutated genotypes. However, studies conducted so far have not clarified whether the occurrence of SNPs alters the metabolism of the drug (22).

In our study, we found no association between mutated genotypes and changes in CQ and DCQ levels. Although other studies have reported a role for the alleles associated with slow metabolism (*CYP2C8*, *CYP3A4*, and *CYP3A5*) in the levels of drugs for clinical use (37, 44, 45), evidence of the influence of these alleles on the enzyme phenotype is still limited (46). It is known that there may be a failure in the cytochrome P450 genotype/phenotype correlation, with about 50% of errors in phenotype prediction being described. Factors such as gene splicing, transcriptional regulation, and the influence of microRNAs, in addition to other existing SNPs, may play a role in the final enzyme activity phenotype (47). These studies suggest that genotype information alone may not be sufficient to replace phenotype measurements, in this case, measurements of drug levels. In addition, the expression and activity of CYP enzymes can be affected by the inflammatory response triggered by the infection, as has already been demonstrated for *P. falciparum* malaria with *CYP1A2* (48, 49) and *CYP3A* (50).

In vivo resistance to CQ signifies a persistence of *P. vivax* asexual stages, despite the presence of adequate levels of CQ plus DCQ (a concentration higher than 100 ng/dl) (16). For *ex vivo* conditions, resistance occurs when IC_{50} values greater than 100 nM CQ are obtained after 42 h of *P. vivax* culture (13, 51).

It is known that DCQ's action is similar to that of CQ and that it is effective against avian malaria, but little is known about its action in human malaria (52). According to Fu et al. (6), DCQ is as effective as CQ against sensitive isolates of *P. falciparum*, but its efficacy against resistant isolates is significantly reduced. *In vivo* sensitivity trials with CQ and DCQ showed that DCQ was more effective and that a combination of drugs had better potential than monotherapy with just CQ. For resistant isolates, better effectiveness was reported using CQ monotherapy, and the combination of drugs was shown to be better than when CQ or DCQ was used alone (53).

A limitation of this study was that the possibility of recurrent parasitemia prior to day 28 in the presence of normally lethal CQ blood levels could not be excluded, since there may have been a relapse of the CQ-recurrent parasite after clearance of the original CQ-responsive parasitemia; however, the frequency of this event is not expected to vary between different CYP genotypes. Unfortunately, the *ex vivo* and *in vivo* studies were not performed with the same samples of *P. vivax*. According to our experience, the level of parasitemia on the recrudescent day (DR) is often very low and

less than 50% of parasites are at the ring stage of development, making the experiment unfeasible on DR (54), but patients in the *ex vivo* study were followed up for 28 days, and none showed a recurrence during this period. The sample size used in the *ex vivo* study is not adequate to confirm CQ resistance rates.

The study had a small sample size for the CQ-recurrent group; however, the frequency of recurrence was in agreement with that reported in previous studies (8, 10, 11). No genetic marker analysis for differentiating relapse, resistance, or reinfection was performed, as some researchers have stated that there are no ideal genetic markers for these (55, 56).

Our results demonstrate that CQ and DCQ had similar efficacy against *P. vivax* isolates from the Amazon and can explain why alleles associated with low enzyme activity found in patients in the control group did not necessarily lead to the failure of CQ treatment and an early recurrence. The absence of drug metabolism problems, even in the occurrence of SNPs in CYP genes in the control group, indicates that these genetic host characteristics had little influence on the early recurrence caused by *P. vivax*.

MATERIALS AND METHODS

Ethics statement. The study was approved by the Ethics Review Board of the Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD; approval number 343/2009). Participants were instructed regarding the objectives of the study and signed an informed consent form. In the case of minors, the consent form was signed by the parents or legal guardians. Patients diagnosed with malaria were treated according to Brazilian Ministry of Health guidelines (57).

Study site. The study was carried out between 2012 and 2014 at FMT-HVD, an infectious disease referral center located in Manaus, western Brazilian Amazon. *Ex vivo* studies were carried out on samples obtained from 2017 to 2018.

Selection of patients. The study included patients of either sex who were infected with *P. vivax* malaria; aged 6 months to 60 years; and had a body weight of greater than 5 kg, a blood parasite density of from 250 to 100,000 parasites/ml, and an axillary temperature of 37.5°C or a history of fever in the last 48 h. Exclusion criteria were the use of antimalarials in the previous 30 days, refusal to be followed up for 28 days, and any clinical complication (58). All patients received supervised treatment with 25 mg/kg of body weight of chloroquine phosphate over a 3-day period (10 mg/kg on day 1 and 7.5 mg/kg on days 2 and 3). PQ was prescribed for a 7-day period at a dosage of 0.5 mg/kg per day, starting only at the end of the follow-up or on the day of recurrence. Patients who vomited the first dose within 30 min after drug ingestion were retreated with the same dose. Clinical and laboratory tests were performed, and interviews and sample collection were done on follow-up days 1, 2, 3, 4, 7, 14, and 28. If there were any extra days of follow-up, the same sample collection procedures were performed.

This study was conducted by using a convenience sampling from previous patient follow-ups. The enrolled patients were divided into cases (CQ-recurrent patients), when the patients presented with a *P. vivax* early recurrence, in which parasites returned during the first 28 days after initial infection, and plasma levels of CQ plus DCQ were higher than 100 ng/ml, and controls (CQ-responsive patients), a group that consisted of patients with no parasitemia recurrence during the 28 days of follow-up.

***P. vivax* malaria diagnosis.** Asexual and sexual parasitemia at day 1 and the clearance of asexual parasitemia were determined by microscopy by two experienced microscopists, using parasite counts per 500 leukocytes. Patients were actively followed up for 28 days. After day 28, recurrences were detected by passive case detection by use of the SIVEP-Malaria system, the official malaria epidemiological surveillance system used in Brazil.

Genotyping of CQ CYPs. DNA was purified from whole-blood samples using a QIAamp DNA minikit (Qiagen, USA). We genotyped three single-nucleotide polymorphisms (SNPs) in *CYP2C8* (A805T [rs11572103], C792G [rs1058930], G416A [rs11572080]), one SNP in *CYP3A4* (A392G [rs2740574]), and two SNPs in *CYP3A5* (G14690A [rs10264272], A6986G [rs776746]). Analyses were assessed using 7500 Fast real-time PCR software (version 2.3; Applied Biosystems). Amplification reactions and cycling parameters were determined according to the manufacturer's protocols.

CQ and DCQ levels. Three 100-μl aliquots of the sample collected on day 28 of follow-up were used for measuring CQ and DCQ levels. Analysis was assessed by high-performance liquid chromatography (HPLC), as previously described (59).

***P. vivax ex vivo* culture.** *Plasmodium* isolates were collected between August 2017 and June 2018 from FMT-HVD patients. Patients were recruited if they presented with a monoinfection with *P. vivax*, a parasitemia of $\geq$10,000 parasites/ml, and a majority (>50%) of parasites at the ring stage of development. Four milliliters of whole blood was collected by venipuncture. After removal of leukocytes by using CF11 cellulose (Sigma-Aldrich), infected red blood cells (IRBC) were used for *ex vivo* drug susceptibility testing.

The CQ and DCQ susceptibilities of the *P. vivax* isolates were measured using a protocol modified from the WHO microtest (60). Two hundred microliters of a 2% hematocrit blood medium mixture (BMM), which consisted of McCoy's 5A medium plus 20% type AB human serum, was added to each well of predosed drug plates containing 11 serial concentrations (2-fold dilutions) of the antimalarials (1.95 to 1,000 ng/ml) CQ diphosphate and DCQ, and each concentration of drug was tested in quadruplicate. A

candle jar was used to mature the parasites at 37.5°C for 48 h. Incubation was stopped when ≥40% of the ring-stage parasites in the drug-free control well had reached the mature schizont stage.

Thick blood smears were made from each well and then stained with 5% Giemsa solution and examined microscopically. The number of schizonts per 200 asexual-stage parasites was determined for each drug concentration and normalized to the value for the control well.

Statistical analyses. Allele and genotype frequencies were estimated by gene counting, and haplotype frequencies and linkage disequilibrium were estimated with Haploview software. Fisher's exact test or the χ^2 test was performed to compare the *CYP2C8*/*CYP3A4*/*CYP3A5* alleles and the genotype frequencies between cases and controls. The odds ratios (OR) with their respective 95% confidence intervals (95% CI) were determined to verify the risk of CQ recurrence, depending on the diplotypes found, and to relate the late clearance of parasitemia and cases. In the *ex vivo* essay, the percentage of mature schizonts at each drug concentration was estimated for 200 asexual-stage parasites, the results were entered in the online IC Estimator software to calculate the IC_{50} of each sample by nonlinear regression analysis and a *t* test of the average comparisons, and graph construction was performed. A *P* value of <0.05 was considered significant in all analyses. Analysis was performed using GraphPad Prism software.

ACKNOWLEDGMENTS

We thank all participants who collaborated with this study and Monica Costa (head of the Malaria Department at FMT-HVD).

We thank CNPq for funding our research and CAPES for awarding scholarships. W.M.M. and M.V.G.L. are CNPq fellows. We thank FAPEAM for providing financial support for publication via PAPAC Edital no. 005/2019.

Conceived and designed the experiments: G.C.M., W.M.M., M.V.G.L., and A.M.S. Sample processing: A.C.G.A., M.C.B.P., E.F.G.F., Y.E.A.R.S., and E.L.S. Performed the experiments: A.C.G.A., M.C.B.P., E.F.G.F., L.R.B., and J.L.F.V. Data entry and analyses: A.C.G.A. and M.C.B.P. Wrote the paper: A.C.G.A., M.C.B.P., G.C.M., Y.E.A.R.S., L.R.B., and M.A.M.B. All authors read and approved the final manuscript.

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