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Influence of *CYP2D6*, *CYP3A4* and *CYP2C19* Genotypes on Recurrence of *Plasmodium vivax*

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Background: The influence of the CYPs (cytochrome P-450) in the success of antimalarial therapy remains uncertain. In this study, the association of *CYP2D6, CYP2C19* and *CYP3A4* polymorphisms and predicted phenotypes with malaria recurrence was investigated.

Methods: After diagnosis of vivax malaria, individuals treated at a reference center in Manaus were followed up for 180 days. Patients were separated into two groups: a recurrence group and a non-recurrence group. Genotyping of *CYP2D6, CYP2C19* and *CYP3A4* was performed using a TaqMan™ assay and real-time PCR.

Findings: The frequencies of decreased-function and normal-function alleles and phenotypes for all CYPs were similar between the groups, except for the *CYP2D6**2xN allele (p=0.047) and the *CYP2D6* gUM phenotype (p=0.057), which were more frequent in individuals without recurrence. Despite this, the *CYP2D6*, *CYP2C19* and *CYP3A4* genotypes had no association with an increased risk of recurrence. CYPs polymorphisms also had no influence in parasite clearance, neither in the time nor the number of recurrence episodes. MAIN

Conclusion: This prospective cohort study demonstrated that *CYP2D6*, *CYP2C19* and *CYP3A4* polymorphisms have no influence on malaria recurrence. Nonetheless, our findings suggest that the *CYP2D6* predicted ultrarapid phenotype was less susceptible to recurrence, and that patients with the *CYP2D6* gUM phenotype are less susceptible to primaquine failure. Additional investigation of pharmacogenetics and pharmacokinetics are needed before implementing CYP analysis to better orientate individualized radical treatment of vivax malaria in reference centers that treat patients with multiple recurrences.

Keywords: ***Plasmodium vivax*, malaria, primaquine, recurrence, CYP450**

INTRODUCTION

Malaria is a globally distributed infectious disease and, in 2020, 241 million cases and 627 thousand deaths from malaria were reported worldwide (1). In Brazil, for the same period, approximately 145,188 cases and 42 deaths were recorded, of these, 82.5% were caused by *Plasmodium vivax* (1). *P. vivax* has certain characteristics, such as its ability to remain latent in the liver in the form of hypnozoites that can cause relapses when reactivated, that are an obstacle for its elimination (2, 3).

Primaquine (PQ), a hypnozoiticidal drug, needs to be biotransformed into an active metabolite in order to exert its antimalarial effect (4). Biotransformation occurs through two main pathways: cytochrome P-450 (CYPs) and monoamine oxidase (MAO-A) (5). Biotransformation mediated by CYPs is attributed to the enzymes CYP2C19, CYP2D6 and CYP3A4 (6). The MAO-A pathway generates aldehyde derivatives, such as carboxyprimaquine, a predominant but inert metabolite (6, 7).

The contribution of the human host's genetics to the results of antimalarial treatment has been demonstrated in recent studies (8–11). Bennet et al. (8) reported therapeutic failure of PQ in the treatment of vivax malaria, which was attributed to the presence of polymorphism in *CYP2D6*, thus resulting in decreased biotransformation of the drug and, consequently, low levels of the active metabolites. Baird et al. (9) showed that decreased CYP2D6 activity was associated with an increased risk of therapeutic failure, which suggests a relation between *CYP2D6* with PQ biotransformation and an increased risk of relapse. The relation between the therapeutic failure of PQ and the presence of polymorphism in *CYP2D6* has also been reported in Brazil (10–12).

Pybus et al. (6) demonstrated that MAO-A and CYP2D6 are responsible for 93% of PQ metabolites and that only traces levels of oxidated and dimethylated metabolites are generated by CYP3A4 and CYP2C19. Ariffin et al. (13) associated the presence of the *CYP2C19*2* allele with the decrease in PQ biotransformation into carboxyprimaquine (13). This null activity allele has also been related to the decrease in biotransformation of other antimalarial drugs (7, 14, 15). Furthermore, *CYP3A4* has a role in the biotransformation of PQ, but no clear genotype-phenotype association for this enzyme, and the effect of its variants remains controversial (6, 16–18).

Polymorphic variants of *CYP2C19*, *CYP2D6* and *CYP3A4* have been reported in populations in Brazilian Amazon, as well as incidences of *P. vivax* malaria infection and recurrent infections by the parasite. Genetic variability of these CYPs is likely to have an impact on the clinical response of patients, and the detection of genotypes related to PQ metabolism could guide individualized treatment protocols in the future in patients with multiple recurrences. As such, this study aimed to investigate the frequency of genotypes and phenotypes of *CYP2D6*, *CYP3A4* and *CYP2C19* and their association with recurrence of *P. vivax* in patients from the Brazilian Amazon.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the Ethics Review Board of Fundação de Medicina Tropical Dr Heitor Vieira Dourado (FMT-HVD) (CAAE 0002.0.114.000-11 and 44605015.4.0000.0005). The individuals invited to participate were informed about the objectives of the study and signed an informed consent form. In the case of individuals under 18 years old, an assent form was signed by the parents or a legal representative.

Selection of Patients

This study was conducted at FMT-HVD, a reference center for infeccious diseases in Manaus, Brazil, in the periods from 2012 to 2014 and 2016 to 2017. The study included individuals of either gender, aged 6 months or older, with a bodyweight of greater than 5 kg, blood parasite density from 250 to 100,000 parasites/mL and axillary temperature of 37.5°C or history of fever in the last 48 hours. Use of antimalarial drugs in the previous 30 days, refusal to be followed up, pregnancy, or any clinical complication were considered to be non-inclusion criteria.

All individuals were treated with 25 mg/kg of chloroquine phosphate (CQ) for 3 days (10 mg/kg on day 1 and 7.5 mg/kg on days 2 and 3). A dose of 0.5 mg/kg/day of PQ was administered for 7 days, together with CQ or on the 42nd day. Clinical and laboratory tests were performed, and interviews and sample collection were done on D1, D2, D3, D4, D7, D14, D28 and D42 of follow-up. If there were any extra days of follow-up, the same sample collection procedures were performed.

This study was carried out using convenience sampling from other previous follow-ups. The individuals were alocated in two groups: a recurrence group (patients with at least one episode of recurrence in the last 180 days) and a non-recurrence group (those who had no reports of malaria episode in the last 180 days). The dates of the recurrence episodes were obtained during the follow-up or by passive detection *via* the national SIVEP-Malaria system (Malaria Epidemiological Surveillance System), which is the official malaria epidemiological surveillance system in Brazil.

Malaria Diagnosis

Asexual parasitemia and gametocytemia, as well as clearance of parasitemia, were determined using optical microscopy. All diagnostics were performed by an experienced microscopist using parasite counts per 500 leukocytes.

Laboratory Procedures

Genomic DNA was purifed from whole blood samples using the QIAmp® Blood Mini kit (Qiagen, Hilden, Germany). The single-nucleotide polymorphisms (SNPs) that were genotyped were chosen according to their functional importance and their frequency in the Brazilian population. We selected eleven polymorphisms in *CYP2D6* (2549delA [rs35742686], 100C>T [rs1065852], 1846G>A [rs3892097], 4180G>C [rs1135840], 2988G>A [rs28371725], 3183G>A [rs59421388], 1584C>G [rs1080985], 1023C>t [rs28371706], 2615_2617delAAG [rs5030656], 31G>A [rs769258], 2850C>T [rs16947]); one polymorphism in *CYP2C19* (681G>A [rs4244285]); and one polymorphism in *CYP3A4* (-392A>G [rs2740574]). The choice of CYP2D6, CYP2C19 and CYP3A4 genes and polymorphisms was based on data from previous work with populations from the Amazon region, which dealt with the influence of CYP450 gene

polymorphisms on the response to antimalarials (11, 19, 20), as well as the frequencies of the alleles already described in the Brazilian population (21).

The analysis was performed *via* an allelic discrimination assay using TaqMan™ probes and a real-time PCR System (7500 Fast, Applied Biosystems Foster City, CA). *CYP2D6* haplotypes were inferred using the HaploStats package (version 1.7.7) implemented in the R platform (www.r-project.org). The haplotypes identified were compared to the Human Cytochrome P450 (CYP) Allele Nomenclature Database, for star (*) allele designation. The wild-type allele (*1) was determined when there was no mutated allele (13).

The determination of the predicted *CYP2D6* phenotype was performed according to the activity score system (AS). Alleles were grouped according to their perceived functionality: zero, null-function alleles (*4,*4xN,*5); 0.5, decreased-function alleles (*9, *17, *29, *41) and 0.25 (*10); 1, normal-function alleles (*1, *2, *35, *39); and 2 or more, increased-function alleles (*1xN, *2xN), depending on the number of copies (CNV). The predicted phenotype was obtained from the sum of diplotype activity scores. For heterozygous individuals with variations in the number of copies, this was accounted for in the activity score calculation, even when it was not possible to attribute the multiplication with complete certainty to one of the alleles of these individuals. Individuals with AS = 0 and AS >2.5 were designated as genetically poor and ultrarapid metabolizers (gPM and gUM), respectively. On the other hand, individuals with AS = 0.25-1 and AS = 1.25-2.25 were designated as genetically intermediate and normal metabolizers (gIM and gNM), respectively (22).

The predicted *CYP2C19* phenotype was classified as a normal metabolizer (*1/*1), intermediate metabolizer (*1/*2) or poor metabolizer (*2/*2) (23). For *CYP3A4*, the presence or absence of the *CYP3A4*1B* was analyzed, without the inference of the phenotype, since there are no guidelines for its prediction.

Statistical Analysis

Difference in frequency values of the star alleles of *CYP2D6*, *CYP2C19* and *CYP3A4* were assessed using the chi-square test. To evaluate the influence of the genotype in the recurrence and clearance time, the survival analysis method was applied through Kaplan-Meier curves and the Wilcoxon-Breslow-Gehan test of equality. Poisson regression was used to assess the effect of genotype in the number of recurrence episodes. The association between the genetic polymorphisms of *CYP2D6*, *CYP3A4* and *CYP2C19* and the clinical response of patients who were treated with PQ was assessed using a multiple log-binomial generalized linear regression model. Analyses were performed using the software Stata v. 13.

RESULTS

Population Study

A total of 311 patients diagnosed with *P. vivax* malaria were selected for the study, of which 102 presented recurrence and 209 had no recurrence. Of all the 311 patients recruited for the study, 256 samples were genotyped for *CYP2D6*, 309 samples for *CYP3A4* and 303 samples for *CYP2C19*. Genotyping of the 3 CYPs was achieved for 249 samples (**Figure 1**).

The baseline characteristics of the participants are shown in **Table 1**. Between the two groups, gender distribution was similar. The mean age for patients with recurrence and without recurrence was 40 and 36.7 years, respectively (p=0.036). For malaria recurrence episodes, 80.4% had only one recurrence episode, which occurred between 61-120 days (56.9%) after the initial malaria episode, with a mean time of 85.5 days (95%CI 78.4-92.6).

The asexual parasite clearance and gametocyte clearance analysis was performed with 147 individuals that took PQ on day 1 of their treatment. The clearance of asexual parasitemia on D1, D3 and D7 was similar between patients with and without recurrence (p>0.05). On D2, the clearance occurred in 72.9% of patients with recurrence and 56.0% of patients without recurrence (p=0.05). For gametocyte clearance, there was no significant difference between the groups according the clearance day (p>0.05).

Allele Frequencies of *CYP2D6, CYP2C19* and *CYP3A4* and Predicted *CYP2D6* and *CYP2C19* Phenotypes

The allele frequency distribution of *CYP2D6*, *CYP2C19* and *CYP3A4* and of predicted *CYP2D6* and *CYP2C19* phenotypes are presented in **Table 2**. The star allele frequencies of *CYP2D6*, *CYP2C19* and *CYP3A4* were similar between the groups (p>0.05). The *CYP2C19*2* null function allele was present in 9.8% and 10.5% of the patients with and without recurrence, respectively (p=0.924). The *CYP3A4 *1B* allele had similar frequency in both groups (p=0.841) (**Table 2**). The proportions of *CYP2D6* null function alleles (*4 and *5), decreased function alleles (*9, *10, *17, *29 and *41) and normal function alleles (*1, *2, *35 and *39) were similar between the groups (p>0.05). After the analysis of CNV, it was found that 6.3% of individuals genotyped for *CYP2D6* had multiplications and, of the 32 alleles of these individuals, it was possible to attribute the multiplications to only 11 alleles of homozygous individuals. *CYP2D6* ultrarapid alleles (*1xN and *2xN) were observed only in patients without recurrence (p=0.018).

The frequency of the predicted *CYP2D6* phenotype was not different between the groups (p=0.372). The most frequent phenotype in both groups was the normal metabolizer phenotype (p=0.656). Although the ultrarapid alleles were found only in individuals without recurrence, the calculation of the activity score revealed the occurrence of the ultrarapid phenotype in patients with recurrence and without recurrence, 2.3% and 8.3% respectively (p=0.057) (**Table 2**).

The frequency of the predicted *CYP2C19* phenotype was similar in both groups (p=0.873). The frequency of gPM was 2.0% and 1.5% in individuals with recurrence and without recurrence, respectively (p=0.750). The frequency of gIM was 15.8% in the recurrent group and 17.8% in non-recurrent group (p=0.667).

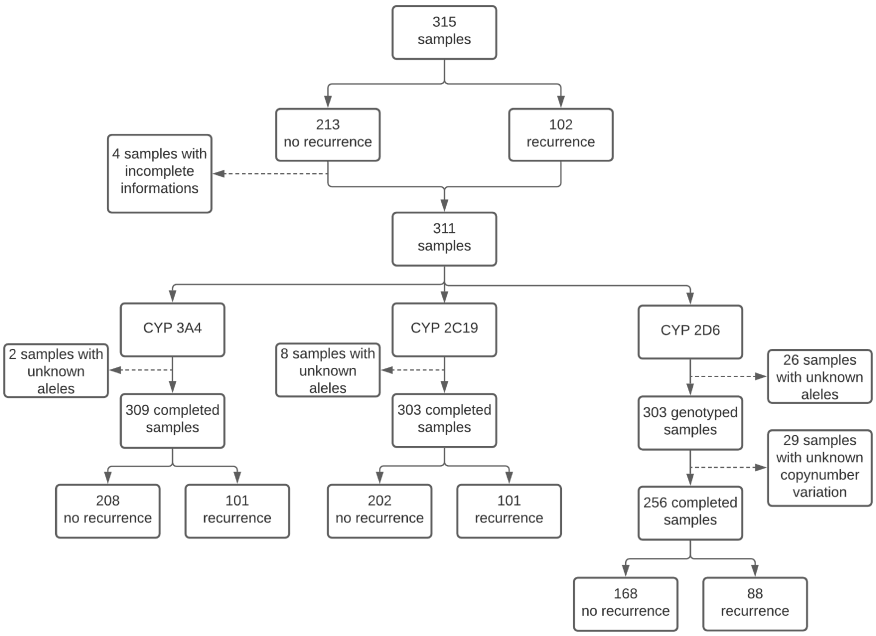

FIGURE 1 | Flowchart of processed samples.

Predicted *CYP2D6* and *CYP2C19* Phenotypes and *CYP3A4* Mutant Allele Versus Asexual Parasitemia Clearance and Gametocytemia Clearance

The predicted *CYP2D6* phenotype did not differ in frequency between the days of asexual parasite clearance (p=0.066). However, even with no statistical significance, it was observed that asexual parasitemia clearance occurred earlier in individuals with the ultrarapid metabolizer phenotype (p=0.108). There was no difference in the asexual parasitemia clearance according to the predicted *CYP2C19* phenotype and *CYP3A4*1B* (p>0.05) (**Table 3**).

The Kaplan-Meier analysis of the time to asexual parasitemia clearance was not significant for the predicted *CYP2D6* and *CYP2C19* phenotype and the presence *CYP3A4*1B* (p>0.05) (**Figure 2**).

No association was found between gametocytes clearance and predicted *CYP2D6* and *CYP2C19* phenotype and the presence of *CYP3A4*1B* (p=0.576, p=0.676 and p=0.535, respectively) (**Figure 3** and **Table 3**).

TABLE 1 | Baseline characteristics of individuals involved in this study.

		Total (311)*	Recurrence (102)*	No recurrence (209)*	p value
Age		37.8 (0.89; 36.1–39.5)	40 (1.36; 37.4–42.7)	36.7 (1.12; 34.5–38.9)	0.036
Gender	Male	210 (67.5%)	72 (72.5%)	136 (65.1%)	0.186
	Female	101 (32.5%)	28 (27.5%)	72 (34.9%)	
Malaria recurrence episodes	1	–	82 (80.4%)	–	–
	2	–	18 (17.6%)	–	
	3	–	1 (1.0%)	–	
	4	–	1 (1.0%)	–	
Time to first recurrence (days)	<60	–	24 (23.5%)	–	–
	61-120	–	58 (56.9%)	–	
	121-180	–	20 (19.6%)	–	
Parasite clearance day	1	9 (6.5%)	2 (4.2%)	7 (7.7%)	0.159
	2	86 (61.9%)	32 (72.9%)	51 (56.0%)	
	3	29 (20.8%)	9 (18.7%)	20 (22.0%)	
	7	15 (10.8%)	2 (4.2%)	13 (14.3%)	
Gametocyte clearance day	1	24 (17.3%)	8 (16.7%)	16 (17.6%)	0.452
	2	83 (59.7%)	32 (66.7%)	51 (56.0%)	
	3	24 (17.3%)	7 (14.6%)	17 (18.7%)	
	7	8 (5.7%)	1 (2.1%)	7 (7.7%)	

**n (%) or mean (± standard deviation; CI 95%).*

TABLE 2 | Allele frequency of CYP2D6, CYP2C19 and CYP3A4 and predicted CYP2D6 and CYP2C19 phenotypes.

Gene	Allele	Total (311)		Recurrence (85)		No recurrence (226)		p value
			Frequency (%)	n	Frequency (%)	n	Frequency (%)	
	***1**	234	37.6	77	37.7	154	36.8	0.827
	***2**	191	30.7	63	30.8	128	30.6	0.947
	***4**	55	8.8	22	10.8	33	7.9	0.233
	***5**	37	5.9	12	5.9	25	6.0	0.961
CYP2D6	***9**	6	1.0	1	0.5	5	1.2	0.398
	***10**	14	2.2	7	3.4	7	1.7	0.166
	***17**	13	2.1	3	1.5	10	2.4	0.451
	***29**	7	1.3	2	1.0	5	1.2	0.811
	***35**	6	0.9	2	1.0	4	0.9	0.978
	***39**	14	2.2	7	3.5	7	1.7	0.166
	***41**	16	2.6	4	1.9	12	2.9	0.501
	***1x**	3	0.5	0	0	3	0.7	0.225
	***2x**	8	1.3	0	0	8	1.9	0.047
	ND	21	3.4	4	1.9	17	4.1	0.172
Predicted phenotype CYP2D6	**gPM**	5	1.9	2	2.3	3	1.8	0.789
	gIM	71	2.8	26	2.9	45	26.8	0.639
	gNM	164	64.1	58	66.0	106	63.2	0.656
	gUM	16	6.2	2	2.3	14	8.3	0.057
	***1**	544	87.5	182	89.2	362	86.6	0.356
CYP2c19	***2**	62	10	20	9.8	42	10.5	0.924
	ND	16	2.6	2	1.0	14	3.3	0.080
Predicted phenotype CYP2C19	**gPM**	5	1.6	2	2.0	3	1.5	0.750
	gIM	52	17.2	4	15.8	44	17.8	0.667
	gNM	246	81.2	83	82.2	163	80.7	0.755
	***1**	527	84.7	172	84.3	355	84.9	0.970
cyp3A4	***1B**	91	14.6	30	14.7	61	14.6	0.841
	ND	4	0.6	2	9.8	2	4.8	0.462

**PM, poor metabolizer; IM, intermediate metabolizer; NM, normal metabolizer; UM, ultrarapid metabolizer; ND, Not determined.*

Predicted *CYP2D6* and *CYP2C19* Phenotypes and *CYP3A4* Mutant Allele Versus Malaria Recurrence Episodes

The number of malaria recurrence episodes did not differ for the predicted phenotype of the *CYP2D6*, *CYP2C19* and *CYP3A4* allele (p>0.05). Additionally, there were no significant differences in the occurrence of one or more malaria episodes (*CYP2D6* p=0.455, *CYP2C19* p=0.832 and *CYP3A4* p=0.437).

Predicted *CYP2D6* and *CYP2C19* Phenotypes and *CYP3A4* Mutant Allele Versus Time to First Recurrence

For *CYP2D6* and *CYP2C19*, the time until the first recurrence episode was similar between the two groups (poor metabolizer + intermediate metabolizer x normal metabolizer) (p=0.868 and p=0.916). For *CYP3A4*, the length of time did not differ with the presence of *CYP3A4*1B* (p=0.847) (**Figure 4**).

TABLE 3 | Predicted phenotype of CYP2D6, CYP2C19 and *CYP3A4* allele x parasite clearance day.

		Day 1		Day 2		Day 3		Day 7			
		PC** frequency (%)	GC** frequency (%)	PC** frequency (%)	GC** frequency (%)	PC** frequency (%)	GC** frequency (%)	PC** frequency (%)	GC** frequency (%)	PC** p value	GC** p value
Predicted phenotype CYP2D6*	**gPM**	2 (2.0)	1 (25.0)	2 (50.0)	2 (50.0)	0 (0.0)	1 (25.0)	1 (25.0)	0 (0.0)	0.285	0.881
	gIM	2 (5.0)	3 (7.0)	26 (68.0)	30 (78.0)	10 (26.0)	5 (13.0)	0 (0.0)	0 (0.0)	0.080	0.031
	gNM	5 (5.0)	18 (2.1)	51 (60.0)	46 (54.0)	17 (20.0)	14 (16%)	12 (14.1)	7 (8.0)	0.193	0.060
	gUM	1 (5.0)	1 (5.0)	1 (5.0)	1 (5.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.108	0.649
	Total	9 (7.0)	23 (17.0)	80 (62.0)	79 (61.0)	27 (20.0)	20 (15.0)	13 (10.0)	7 (5.0)	0.066	0.273
Predicted phenotype CYP2C19*	**gPM**	0 (0.0)	0 (0.0)	1 (1.0)	1 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.886	0.882
	gIM	2 (10.0)	3 (15.0)	10 (50.0)	11 (55.0)	4 (20.0)	5 (25.0)	4 (20.0)	1 (5.0)	0.457	0.778
	gNM	7 (6.0)	20 (17.0)	72 (62.0)	70 (60.0)	25 (21.0)	18 (15.0)	11 (9.0)	7 (6.0)	0.546	0.827
	Total	9 (6.0)	23 (16.0)	83 (61.0)	82 (60.0)	29 (21.0)	23 (17.0)	15 (11.0)	8 (5.0)	0.785	0.943
CYP3A4	***1**	16 (88.0)	39 (81.0)	136 (79.0	134 (80.0)	50 (86.0)	42 (87.0)	25 (83.0)	12 (75.0)		
	***1B**	2 (3.0)	9 (18.0)	36 (20.0)	32 (19.0)	8 (13.0)	6 (12.0)	5 (16.0)	4 (25.0)	0.521	0.644
	Total	18 (6.0)	48 (17.0)	172 (61.0)	166 (59.0)	58 (20.0)	48 (17.0)	30 (10.0)	16 (5.0)		

**PM, poor metabolizer; IM, intermediate metabolizer; NM, normal metabolizer; UM, ultrarapid metabolizer.*

***PC, parasite clearance; GC, gametocyte clearance.*

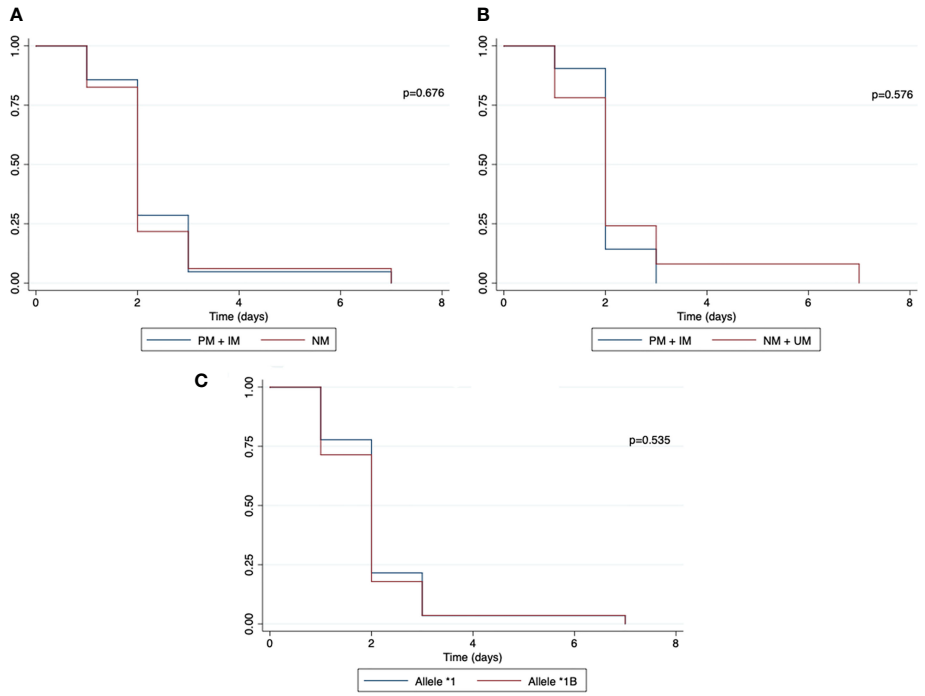

FIGURE 2 | Malaria parasite clearance between individuals with different CYP2D6 and CYP2C19 phenotypes. Kaplan–Meier curve shows malaria parasite clearance time between individuals with normal and reduced predicted CYP2D6 and CYP2C19 activity phenotype and normal and mutated CYP3A4 allele. **(A)** CYP2D6 phenotype; **(B)** CYP2C19 phenotype; **(C)** CYP3A4 allele; PM, poor metabolizer; IM, intermediate metabolizer; NM, normal metabolizer; UM, ultrarapid metabolizer. The symbol * refers to the star allele.

Influence of Predicted *CYP2C19* and *CYP2D6* Phenotype, *CYP3A4* Mutant Allele and Combined *CYP2C19+CYP2D6* on the Occurrence of Recurrence

The relative risk (RR) was measured to evaluate the influence of predicted *CYP2C19* and *CYP2D6* phenotype, *CYP3A4*1B* and combined *CYP2C19+CYP2D6* in the clinical response of patients treated with PQ (**Table 4**). The RR of recurrence with classification *CYP2C19* gPM or gIM was 0.93 (95%CI, 0.61-1.42; p=0.758) when compared with a classification of normal metabolizer. For *CYP2D6*, the RR with decreased predicted phenotype (gPM+gIM) was 1.10 (95%CI, 0.77-1.58; p=0.585) in comparison with normal and ultrarapid metabolizers. There was no association between recurrence and *CYP3A4*1B* (95%CI, 0.73-1.38; p=0.951).

Finally, the association between *CYP2C19+CYP2D6* and recurrence was also measured. The presence of one or two gPM or gIM alleles in any of the CYPs was not associated with risk of recurrence (p=0.972).

DISCUSSION

This study is the first to simultaneously evaluate the influence of *CYP2D6*, *CYP2C19* and *CYP3A4* genetic polymorphisms in relation to the recurrence of *P. vivax* in the Brazilian Amazon. Since some patients followed up in this study come from urban areas, where the vector mosquito is absent, the cause of these recurrences could be attributed to *P. vivax* relapses (24). This causes problems in the elimination of malaria, since these individuals can directly contribute to the maintenance of local transmission. In addition, hypnozoites are difficult to eradicate, as they can only be eliminated by treatment with primaquine, which may have its effectiveness reduced due to altered metabolism of *CYP2D6* (6, 8) With the constant decline in malaria transmission in some areas of the Brazilian Amazon (25, 26), protocols for personalized therapy could become more viable and their implementation could be possible in referral centers.

In this study, after genotyping of 11 SNPs, no significant difference was found between *CYP2D6* allele frequencies and patients with or without recurrence (p=0.646). In addition, increased function alleles were only observed in patients without recurrence (p=0.018). In general, the frequency of these alleles is low and since the majority of the studies are conducted to evaluate the association of impaired genotypes and PQ failure, this is the first study to report a high frequency of *CYP2D6* multiplication in individuals without recurrence.

Furthermore, the gUM phenotype presented a higher frequency in those without recurrence (p=0.057). Some studies have shown that the presence of the gUM phenotype is related to the high risk of toxicity of the prodrug codeine, and a high risk of failure of tricyclic antidepressants (27, 28). However, for individuals with the gUM and gNM phenotype, it is expected

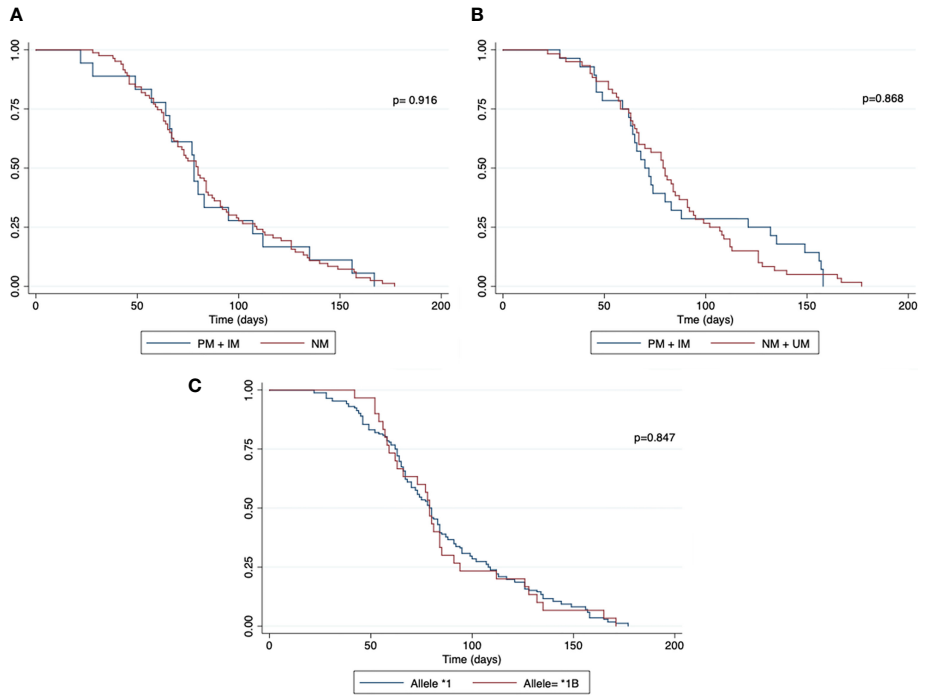

FIGURE 3 | Malaria gametocyte clearance between individuals with different CYP2D6 and CYP2C19 phenotypes. Kaplan–Meier curve shows malaria gametocyte clearance time between individuals with normal and reduced CYP2D6 and CYP2C19 activity phenotype and normal and mutated CYP3A4 allele. **(A)** CYP2D6 phenotype; **(B)** CYP2C19 phenotype; **(C)** CYP3A4 allele; PM, poor metabolizer; IM, intermediate metabolizer; NM, normal metabolizer; UM, ultrarapid metabolizer. The symbol * refers to the star allele.

that a standard dose of tamoxifen should achieve the therapeutic effect in breast cancer treatment (29). For PQ, it is supposed that individuals with the gUM phenotype would exhibit the fastest metabolism through the *CYP2D6* pathway. Gonçalves et al. (30) evaluated the effect of the *CYP2D6* activity score (AS) on PQ plasma concentration over time after a single dose of PQ, and showed that the prodrug concentration decreased faster in children with AS=3 than those with AS ≤ 2. Moreover, the clinical effect of the gUM phenotype is still unknown (5). In our study, the frequencies of the *CYP2D6* gPM, gIM and gNM phenotypes were similar between those with or without recurrence, similar to what has previously been observed in the Brazilian Amazon region (11).

The influence of decreased *CYP2D6* phenotypes (PM+IM) in the recurrence was not associated with high risk of recurrence. No significant effect of predicted *CYP2D6* phenotype in recurrence episodes was reported in Australia and Thailand (31, 32). However, the effect of the *CYP2D6* impaired phenotype and the increased risk of PQ failure has been demonstrated in other studies, thus suggesting that PQ metabolism is dependent on CYP2D6 (8, 9, 11, 33).

The Brazilian Amazon is a region that is endemic for malaria; therefore, there is a possibility that part of the unidentified recurrences could be reinfections and relapses due to primaquine failure. This could be the cause of the disagreements between the results of this study and other previously published studies (8–11). Furthermore, there are differences regarding the system for *CYP2D6* phenotype prediction from the genotype; specifically, regarding the activity index and the cut-off points used to predict CYP2D6 activity levels (22, 34). Some studies have already reported inhibition of *CYP2D6* with the use of chloroquine, which would affect the actual phenotype of the enzyme and the response to primaquine (35, 36).

In this study, we did not find any association between the *CYP2D6* predicted phenotype and gametocytemia or asexual parasitemia clearance. A recent study in the Amazon region also did not show any association between the presence of polymorphisms in CYP genes and early asexual clearance of *P. vivax* (37). However, Pett et al. (38) demonstrated the effect of the *CYP2D6* gPM/gIM phenotype in *P. falciparum* gametocyte clearance, and it was also demonstrated that the biotransformation of PQ is not a condition for the eradication of the blood stages (asexual and sexual) of *P. berghei* (39). The *CYP2D6* predicted phenotype was also not associated with the number of recurrence episodes and neither with the time to the first episode of recurrence, similar to previously observed (11, 33).

In our study, the frequency of this allele is in agreement with what is already known for northern Brazilians (40, 41) (**Table 2**). Even though the PM phenotype was more often observed in individuals with recurrence, the frequencies of PM and IM phenotypes were similar between the groups. Furthermore, this study demonstrated that there is no association between the *CYP2C19* polymorphism and the recurrence episodes neither with asexual parasitemia clearance nor gametocyte clearance. Pybus et al. (6) showed that CYP2C19 plays a role in PQ

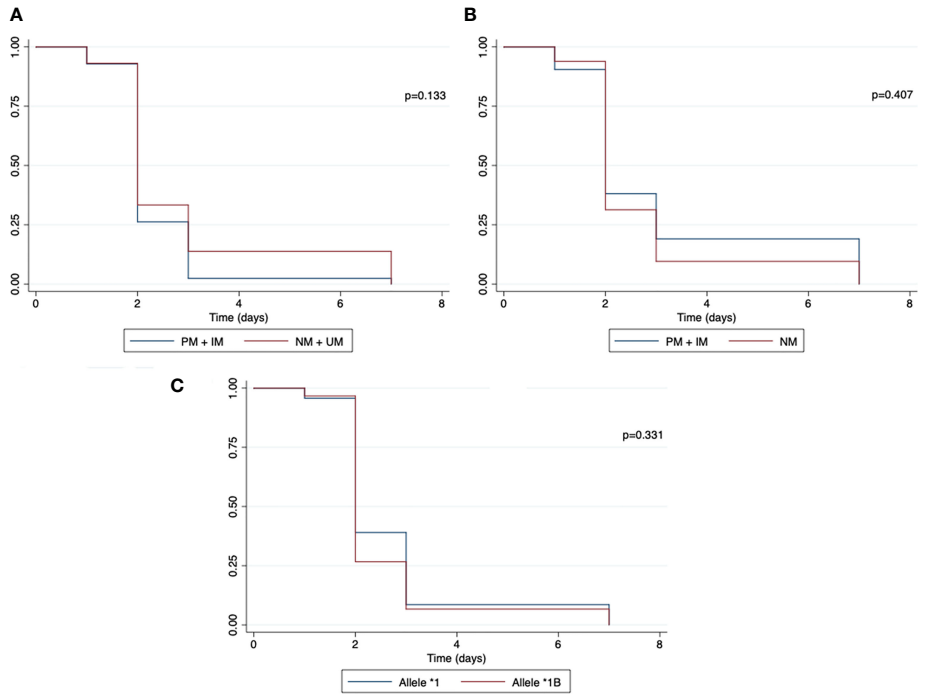

FIGURE 4 | Kaplan–Meier curve for malaria recurrence time between individuals with normal and reduced CYP2D6 and CYP2C19 activity phenotype and normal and mutated CYP3A4 allele. **(A)** CYP2D6 phenotype; **(B)** CYP2C19 phenotype; **(C)** CYP3A4 allele; PM, poor metabolizer; IM, intermediate metabolizer; NM, normal metabolizer. The symbol * refers to the star allele.

metabolism, although the influence of this CYP in the production of PQ's hemolytic and/or therapeutic metabolite is not clear.

For *CYP3A4*1B*, no statistical difference was found in the allele frequency between the individuals with or without recurrence (p=0.762). Several studies have been conducted to explain the importance of polymorphisms in the *CYP3A4* genotype–phenotype relationship, but the functional effect of the polymorphism remains contentious (15, 42). Although CYP3A4 is known to have a role in PQ biotransformation (6), we could not show the effect of *CYP3A4*1B* in the recurrence episodes in individuals treated with PQ, neither in the asexual parasitemia clearance nor in gametocyte clearance. A previous study conducted in the Amazon also showed no association between this allele and the recurrence episodes nor between the presence of *CYP3A4*1B* and the clearance time (37).

Because PQ is not metabolized by only one enzyme (6, 12), a multigenic analysis might be more suitable for determining the effect of the *CYP450* polymorphism in PQ failure. In our study, we found a single individual representing the *CYP2D6* gPM (**4/*4*) + *CYP2C19* gIM *(*1/*2*) that had had 2 recurrence episodes. Despite this, a *CYP2D6* + *CYP2C19* analysis was not associated with a higher risk of recurrence.

This genotype-based prediction assumes that the genotyping precisely corresponds the metabolic activity (42, 43). However, the prediction of phenotype from the genotype has not been correlated with complete certainty, since the gene splicing, SNPs, epigenetics, microRNA, transcription regulation and multiple gene copies are factors that can modify the enzymatic activity (44–47). Furthermore, phenoconversion may occur, whereby a genotypic gNM can be converted into a transient phenotypic IM or PM, which is mainly caused by extrinsic factors, such as drug-drug interactions and some pro-inflammatory cytokines in the inflammatory process (43, 44, 48).

This study had some limitations. The phenotype was predicted by the genotype. Recently, Baird et al. (9) showed a highly significant correlation between the genotype-determined activity score and the measured phenotypes when using dextromethorphan

TABLE 4 | Relative risk for recurrence associated with CYP2C19 and CYP2D6 phenotype, CYP3A4 genotype and combined CYP2C19+CYP2D6.

	Recurrence (n)	No recurrence (n)	RR (95%CI)	p value
CYP2C19 decreased predicted phenotype	18	39	0.93 (0.61-1.42)	0.758
CYP2D6 decreased predicted phenotype	28	48	1.10 (0.77-1.58)	0.585
CYP3A4 mutated genotype	61	30	1.01 (0.73-1.38)	0.951
Combined decreased predicted phenotype CYP2C19+CYP2D6	39	73	0.99 (0.70-1.39)	0.972

as a probe drug. Primaquine metabolites were not assessed to confirm the impact of CYP2D6 in the pharmacokinetics of the drug. Drug-drug interactions and the presence of other inflammatory conditions were not evaluated. It was not possible to certify factors related to the quality of the CQ and PQ drugs administered to patients and PQ administration was not supervised. The low sample size for individuals with recurrence found here is in agreement with other studies (9, 11). Moreover, data regarding recurrence episodes were accessed using the SIVEP-Malaria platform, which is susceptible to underreporting issues.

In this study, it was not possible to establish an association between *CYP2D6*, *CYP3A4*, and *CYP2C19* decreased metabolizing genotypes and recurrence of *P. vivax*. The investigation of other causes of recurrence for these individuals is necessary since it is a region where there is a high risk of re-infection and many asymptomatic infections (49).

CONCLUSION

This prospective cohort study demonstrated no influence of *CYP2D6*, *CYP2C19* and *CYP3A4* polymorphisms on malaria recurrence. Despite this, our findings suggest that the *CYP2D6* predicted ultrarapid phenotype was less susceptible to recurrence (p=0.057). Future studies are warranted in order to understand the association of PQ and *CYP450* considering factors such as the metabolite concentration, the drug–drug interactions and the presence of some inflammatory conditions, and these may guide the individualized radical treatment of vivax malaria in reference centers that treat patients with multiple recurrences.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Fundacao de Medicina Tropical Dr. Heitor Vieira Dourado. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: GM, WM, ML, VS, and JC. Sample processing: JC and YS. Performed the experiments: JC, AA and LB. Data entry and analyses: JC, MR, VS, and AA. Wrote the paper: JC, GM, YS, LB, VS, FR-S, WM, and ML. All authors read and approved the final manuscript.

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