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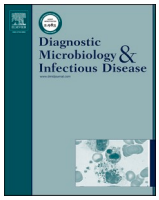

Original Article

Polymorphisms of the *CYP2D6* gene and its relationship with *Plasmodium vivax* relapses after chloroquine-primaquine treatment in Turbo, Colombia

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ABSTRACT

Background: *Plasmodium vivax* relapse due to hypnozoites represents a significant mechanism for parasite persistence in the population. Primaquine (PQ), the drug of choice for eliminating hypnozoites, requires metabolic activation by Cytochrome P450-2D6 (CYP2D6). Genetic variations in *CYP2D6* can alter PQ metabolism, potentially increasing the risk of relapses. This study aimed to determine *CYP2D6* polymorphisms in subjects with *Plasmodium vivax* under supervised chloroquine-primaquine treatment and explore their association with relapses and PQ plasma levels.

Methods: *CYP2D6* phenotypes and genotypes were successfully determined for 71 out of 78 patients included in the study. Nine polymorphisms (SNPs and indels) and gene copy number variation were analyzed. The association between the *CYP2D6* phenotype, *P. vivax* relapse over six months follow-up, and PQ plasma levels were explored.

Results: Most diplotypes (81.7 %) were associated with normal (gNM-F) and ultrarapid (gUM) CYP2D6 metabolizers, while 18.3 % were associated with poor (gPM) and intermediate (gIM and gNM-S) metabolizers. The median plasma PQ concentration on day 2 was higher in impaired CYP2D6 activity group (poor/intermediate) compared normal metabolizers (normal/ultrarapid) (660.4 ng/ml vs 313.5 ng/ml; effect size r -0.51, 95 % CI -0.82 to 0.03). No significant difference was found in the hazard ratio (HR) of relapse between impaired and normal CYP2D6 activity (adjusted HR: 1.45; 95 % CI: 0.39–5.39).

Conclusion: Impaired *CYP2D6* activity phenotypes were frequent in individuals infected with *P. vivax* from an endemic region of Colombia. Further research is essential to elucidate the relationship between these phenotypes and *P. vivax* relapses, as suggested by this exploratory study.

1. Introduction

Malaria still affects the poorest and most vulnerable communities in tropical regions. In the Americas, *Plasmodium vivax* accounts for 75 % of cases [1]. In 2023, Colombia reported 102,457 malaria cases, with 98.3 % being uncomplicated and *P. vivax* responsible for 63 % [2].

Eliminating *P. vivax* in endemic areas is challenging due to its ability to form hypnozoites in the liver, leading to relapses [3]. There is no method to distinguish relapses from recrudescence or re-infection. Radical cure, which targets hepatic hypnozoites, requires drugs like primaquine (PQ) and tafenoquine (TQ) [4].

In Colombia, the Ministry of Health recommends treating *P. vivax* with 25 mg/kg of chloroquine (CQ) for 3 days and 3.5 mg/Kg of PQ for 14 days [5]. Although this treatment is effective [6–9], PQ has a 24.1 %

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cumulative incidence to first recurrence within six months, with at least 50 % being relapses [8]. Understanding PQ efficacy is crucial for preventing relapses and eliminating *P. vivax*.

The enzyme CYP2D6 is key in metabolizing drugs like PQ and TQ [10,11]. The most probable mechanism of action for PQ involves the generation of reactive oxygen species through hydroxylated metabolites, which are mainly produced by CYP2D6 [12].

Its gene is highly polymorphic, with variants associated with enzyme activity, allowing subject classification into poor (gPM), intermediate (gIM/gNM-S), normal (gNM-F), or ultrarapid (gUM) metabolizers [13]. Studies have linked impaired CYP2D6 activity to *P. vivax* relapses [14]. Individuals with *P. vivax* who experienced relapses tended to exhibit impaired phenotype, also associated with higher plasma PQ levels, slower clearance, and increased bioavailability compared to a no-relapses group [14].

Baird *et al.* (2018) identified higher *P. vivax* relapse risk in individuals with impaired CYP2D6 activity [29]. Silvino *et al.* (2016; 2020) found that subjects with impaired CYP2D6 phenotypes had higher risk of multiple recurrence episodes [15,16]. Brasil et al. 2018 reported higher recurrence incidence in individuals treated with CQ-PQ, who exhibited reduced CYP2D6 activity phenotypes [17]. This association may be influenced by the role of CYP2D6 in PQ biotransformation.

In Colombia few studies have been conducted on CYP2D6 phenotypes in non-malaria endemic areas, showing impaired CYP2D6 activity ranging from 6.6 % to 34.4 % [18,19]. The potential association between impaired CYP2D6 phenotype and *P. vivax* relapses has not been explored.

This study aims to describe, for the first time in Colombia, the frequency of CYP2D6 polymorphisms in the malaria-endemic region of Turbo, Antioquia, where 96 % of cases are caused by *P. vivax* [2], and to examine the relationship between impaired CYP2D6 activity, PQ plasma levels, and *P vivax* relapses.

2. Methodology

2.1. Study design

This observational study uses data and blood samples from a previous investigation on *P. vivax* recurrences conducted between 2012 and 2013 in Turbo, Colombia involving 78 participants. The study found a 24.1 % cumulative incidence of the first *P. vivax* recurrence (21/87) within six months of treatment initiation [8]. Among those with recurrences, 33 % (7/21) had three or more episodes. Participants received the standard treatment in Colombia, which consisted of (CQ at 25 mg/kg over 3 days and PQ at 3.5 mg/kg over 14 days), administered under supervision.

A recurrence was defined as a positive thick blood smear for *P. vivax* between days 29 and 180 post-treatment, with or without symptoms. To identify relapses, multilocus genotyping of day zero and recurrence samples were compared, and a criteria based on Pmatch estimation within genetic clusters was applied [8]. In the previous study, 13.8 % (12/87) of participants had at least one relapse.

In the current study, participants with relapses ($n = 12$) and without relapses ($n = 66$) from the previous study were selected. Eight single nucleotide polymorphisms (SNPs) and one deletion in the *CYP2D6* gene were analyzed, and gene copy number was determined. The enzyme metabolizer phenotype was predicted for each participant, and plasma levels of PQ on day 2 post-treatment were measured. This study aimed to explored the association between the predicted CYP2D6 phenotype, PQ plasma levels and *P. vivax* relapses in the study sample.

2.2. Study location

Participants were recruited in Turbo, a municipality in Antioquia, Colombia (Fig. 1), with a population of approximately 135,967 inhabitants, 61 % living in rural areas [20]. Turbo faces a persistent challenge with *P. vivax* malaria. In 2013, the annual parasite index was 2.4 cases per 1,000 inhabitants, increasing to 6.1 cases per 1,000 in 2023 [21]. Consequently, *P. vivax* remains high, representing approximately 96 % of the current cases [21].

Fig. 1. Map of the study area. It shows the municipality of Turbo highlighted in red (top right). Study participants were recruited from Turbo Hospital and three peripheral health centers: El Dos, Tres, and Currulao.

2.3. Eligibility criteria

Participants were eligible for this study based on the following criteria: having received complete and supervised treatment with CQ and PQ, having completed follow-up up to day 180, and having a dry blood sample available. Participants with recurrences classified as re-infections were excluded.

2.4. Laboratory procedures

2.4.1. DNA extraction and quantification

DNA was extracted from whole blood samples on filter paper using the QIAmp DNA Mini Kit (QIAGEN), according to the manufacturer's instructions. The DNA was quantified with a Qubit 3.0 Spectrophotometer (Life Technologies - Invitrogen-Thermo Fisher Scientific) and concentrated at 45°C using the Concentrator Plus/Vacufuge Plus. The final DNA concentration was adjusted to above 10 ng/µl, as per a prior study [16].

2.4.2. Genotyping of CYP2D6 gene polymorphisms

Genotyping of *CYP2D6 gene* polymorphism was performed by quantitative PCR (qPCR) using specific hydrolysis probes (TaqMan SNP genotyping assays; Thermo Fisher Scientific) [16]. Nine polymorphisms, previously identified in Latin American populations as affecting enzyme functionality, were selected: G-1584C [rs1080985], C100T [rs1065852], C1022T [rs28371706], G1847A [rs3892097], G2851T [rs16947], G2989A [rs28371725], G3184A [rs59421388], G4181C [rs1135840], and 2615_2617delAAG [rs5030656]. Amplification reactions were performed in 384-well plates using the ViiA7 Real-Time PCR System (Thermo Fisher Scientific) [16]., with results analyzed by QuantStudio Real-Time PCR software (Thermo Fisher Scientific) [16].

2.4.3. Copy number assay

To determine the *CYP2D6* copy number, qPCR reactions were performed in triplicate on 384-well plates, with a total reaction volume of 10 µL. Each reaction contained 5.0 µL of TaqPath® Universal PCR Mastermix (Thermo Fisher Scientific), 0.5 µL of the Hs00010001_cn assay (Thermo Fisher Scientific), 0.5 µL of the human RNase P reference gene (Thermo Fisher Scientific), 3.0 µL of nuclease-free water, and 1.0 µL of DNA (≈10 ng/µL).

The reactions were run on the ViiA7 Real-Time PCR System, and the data were analyzed using CopyCaller® v.2.0 [16].

2.5. Prediction of star alleles/haplotypes and classification of CYP2D6 phenotypes

CYP2D6 haplotypes were inferred using PHASE v.2.1 [22]. Allele designations, allele functions, and translation of diplotypes into phenotypes were assigned according to the PharmVar database (https://www.pharmvar.org/gene/CYP2D6) and the 2019 update by the Clinical Pharmacogenetics Implementation Consortium (CPIC) and the Dutch Pharmacogenetics Working Group (DPWG) [13,23–25].

2.6. Plasma quantification of primaquine

Plasma concentrations of PQ were available for 24 participants on day 2 of follow-up after treatment initiation. These data were obtained from the previous study using high-performance liquid chromatography (HPLC) with a diode array detector (DAD), following a standardized protocol developed by the Malaria Group at the University of Antioquia [26].

2.7. Statistical analysis

To explore the association between relapse and impaired CYP2D6 activity, participants were grouped based on their phenotypic categories: AS <1.25 (gPM, gIM, and gNM-S) and AS ≥1.25 (gNM-F and gUM). A Cox regression model was applied to estimate crude and adjusted Hazard Ratios (HRs) and their 95 % confidence intervals for the first relapse episode, comparing impaired activity (poor/intermediate metabolism) to normal activity (normal/increased metabolism). The adjusted HR included time of residence as a covariate, as it was previously associated with recurrence [8]. Other variables were not considered due to the limited number of events.

The medians of PQ level in relapse and non-relapses groups were compared using the Wilcoxon-Mann-Whitney test. The effect size (*r*) was calculated to assess the standardized magnitude of the difference between groups, classified as very small (≥ 0.05 to <0.1), small (≥ 0.1 to < 0.2), medium (≥ 0.2 to < 0.3), large (≥ 0.3 to < 0.4), and very large (≥ 0.4) [27].

3. Results

3.1. Demographic, epidemiological, and parasitological characteristics of participants with Plasmodium vivax infection

The main characteristics of the participants included in this study are presented in Table 1. Among all participants, 12 (15.4 %) experienced relapses during the 6-month follow-up period. The first relapse occurred between days 51 and 110; 7 had one relapse, 4 had two relapses, and 1 participant experienced three relapses.

3.2. Frequency of polymorphisms (SNPs) evaluated in the CYP2D6 gene in participants with Plasmodium vivax infection

The frequencies of the eight selected SNPs and one deletion in the *CYP2D6* gene evaluated in the 78 participants of the study are summarized in Table 2. The occurrence of both homozygous and heterozygous mutated genotypes for each SNP varied between 0 % and 41 %. Among the SNPs, G4180C had the highest frequency of homozygous and heterozygous mutations (69 %), followed by C2850T (57.7 %) and C-1584 G (39.8 %). The SNPs C100T (21.8 %) and G1846A (19.2 %) exhibited intermediate frequencies of mutated genotypes. On the other hand, the SNPs G2988A, G3183A and the 2615_2617delAAG deletion were less common, each of them with a frequency of 6.4 %, 6.4 % and 3.9 % respectively.

Table 1
Characteristics of the participants in this study. The main characteristics of the participants included in this study are presented. Among all participants, 12 (15.4 %) experienced relapses during the 6-month follow-up period. The first relapse occurred between days 51 and 110; 7 had one relapse, 4 had two relapses, and 1 participant experienced three relapses.

Parameter	Relapse, *n* = 12	No Relapse, *n* = 66	Total *n* = 78
Age in years; Me (IQR)	**23.5 [17.5 - 32.0]**	**29 [15.7 - 42.5]**	**29.0 [16.0 - 39.0]**
Male; n (%)	**8 (66.7 %)**	**37 (56.1 %)**	**45 (57.7 %)**
Symptomatic malaria in the last year (self-reported); n (%)	**4 (33.3 %)**	**14 (21.2 %)**	**18 (23.1 %)**
Last malaria episode (self-reported between participants with a history of malaria); n (%)			
1 - 6 months	**4 (57.1 %)**	**13 (33.3 %)**	**17 (36.9 %)**
6 - 12 months	**0 (0.0 %)**	**1 (2.6 %)**	**1 (2.2 %)**
> 12 months	**3 (42.9 %)**	**25 (64.1 %)**	**28 (60.9 %)**
Years of residency in an endemic region; Me (IQR)	**4.50 [1.75 - 13.5]**	**4.00 [1.00 - 12.0]**	**4.00 [1.00 - 12.0]**
Chloroquine dosage in mg/Kg; Me (IQR)	**24.3 [22.3 - 25.6]**	**23.9 [22.1 - 26.4]**	**24.1 [22.1 - 26.3]**
Primaquine dosage in mg/Kg; Me (IQR)	**3.60 [3.35 - 3.76]**	**3.70 [3.49 - 4.05]**	**3.68 [3.45 - 4.04]**
Parasitemia day 0; Me (IQR) [parasites/µl])	**6860 [4210-7550]**	**4940 [2270-9480]**	**5040 [2740-9310]**

Me = Median, IQR = Interquartile Range.

3.3. Frequency of CYP2D6 star alleles identified in the study participants

Based on the *PharmVar* database, ten star alleles/haplotypes were identified in the study participants, with frequencies ranging from 0.6 % to 44.2 % Table 3. Four individuals (5.1 %) had nucleotide combinations that did not allow allele identification. Among the identified alleles, those linked to normal CYP2D6 activity (*1, *2, *34, *39) were most frequent (77.5 %), while variants associated with decreased or null activity accounted for 19.9 %. The most common of these was *4 (9.6 %), associated with null activity.

3.4. Determination of CYP2D6 diplotypes, gene copy number and association with enzyme phenotype in participants with Plasmodium vivax infection

The Table 4 presents the frequency of *CYP2D6* diplotypes in 74 of the participants, where it was possible to obtain this data. In total, 18 diplotypes were identified, with frequencies ranging from 1.3 % to 27.0 % in the study participants. It was not possible to establish the diplotype in four participants (5.1 %) for whom one allele was not identified. Regarding the number of copies, multiplication of the gene was found in seven participants (9.9 %). In three of them, it was not possible to establish the total score because the alleles had different scores associated. The activity score (AS) for subjects is represented in the table with one copy of each allele, as it was not possible to determine which allele had multicopies for most ultrarapid metabolizer (gUM) individuals.

Table 2
Frequency of polymorphisms (SNPs) evaluated in the CYP2D6 gene in participants with P. vivax malaria residing in Turbo. The frequencies of the eight selected SNPs and one deletion in the CYP2D6 gene evaluated in the 78 participants of the study are summarized. The occurrence of both homozygous and heterozygous mutated genotypes for each SNP varied between 0 % and 41 %. Among the SNPs, G4180C had the highest frequency of homozygous and heterozygous mutations (69 %), followed by C2850T (57.7 %) and C-1584 G (39.8 %). The SNPs C100T (21.8 %) and G1846A (19.2 %) exhibited intermediate frequencies of mutated genotypes. On the other hand, the SNPs G2988A, G3183A and the 2615_2617delAAG deletion were less common, each of them with a frequency of 6.4 %, 6.4 % and 3.9 % respectively.

Frequencies of the CYP2D6 genotypes n (%)		
SNPs	Genotypes	n (%)
C −1584G	CC	47 (60.2)
	CG	24 (30.8)
	GG	7 (9)
C100T	CC	61 (78.2)
	CT	14 (18)
	TT	3 (3.8)
C1023T	CC	72 (92.3)
	CT	5 (6.4)
	TT	1 (1.3)
G1846A	GG	63 (80.8)
	GA	13 (16.6)
	AA	2 (2.6)
G2850T	CC	33 (42.3)
	CT	26 (33.3)
	TT	19 (24.4)
G2988A	GG	73 (93.6)
	GA	5 (6.4)
	AA	0 (0)
G3183A	GG	73 (93.6)
	GA	5 (6.4)
	AA	0 (0)
G4180C	GG	24 (31)
	GC	32 (41)
	CC	22 (28)
2615_2617delAAG	AAG	75 (96.1)
	delAAG/AAG	2 (2.6)
	delAAG/delAAG	1 (1.3)

Table 3
Frequency of CYP2D6 alleles identified in the study participants. The frequencies of the identified CYP2D6 alleles/haplotypes (*1, *2, *4, *5, *9, *10, *17, *29, *34, *39 and *41) in the study population are presented. The frequencies ranged from 0.6 % to 44.2 %. For four individuals (5.1 %), the combinations of nucleotides did not allow identification of one allele. Among the identified star alleles, those associated with normal CYP2D6 enzymatic activity (*1, *2, *34 and *39) had the higher frequencies in the population (77.5 %). Variants associated with decreased or null enzymatic activity had an overall frequency of 19.9 %. Among these, the most frequent star alleles were *4 (9.6 %), associated with null activity.

CYP2D6 alleles	Activity Score	SNPs associated	Enzymatic Activity	n (%)
*4	0	G1847A, C100T, 4181G>C	Null	15 (9.6)
*5	0	CYP2D6 full gene deletion	Null	2 (1.3)
*9	0.25	2615_2617delAAG	Decreased	3 (1.9)
*17	0.5	C1023T, G2851T, G4181C	Decreased	2 (1.3)
*29	0.5	G2851T, G4181C, 3183G>A	Decreased	4 (2.6)
*41	0.25	G2851T, G2988A, G4181C	Decreased	5 (3.2)
*1	1	G-1584C	Normal	69 (44.2)
*2	1	G-1584C, G2851T, G4181C	Normal	49 (31.4)
*39	1	G4181C	Normal	1 (0.6)
*34	2	G2851T	Normal	2 (1.3)

Table 4
Frequency of CYP2D6 metabolizing diplotypes in the study participants. The frequency of CYP2D6 diplotypes in 74 participants is presented, where this data could be obtained. A total of 18 diplotypes were identified, with frequencies ranging from 1.3 % to 27.0 % among the study participants. It was not possible to determine the diplotype for four participants (5.1 %) for whom one allele was not identified. Regarding copy number, gene duplication was found in seven participants (9.9 %). In three of these cases, it was not possible to determine the total score because the alleles had different associated scores.

CYP2D6 diplotypes	Genetic determination	Activity Score*	CYP2D6 phenotypes	Total n = 74, n (%)
*4/*4	Homozygous	0	gPM	2 (2.7)
*4/*5	Heterozygous	0	gPM	1 (1.3)
*4/*9	Heterozygous	0.25	gIM	1 (1.3)
*2/*4	Heterozygous	1	gNM-S	4 (5.4)
*1/*4	Heterozygous	1	gNM-S	5 (6.8)
*1/*5	Heterozygous	1	gNM-S	1 (1.3)
*9/*9	Homozygous	0.5	gNM-S	1 (1.3)
*2/*41	Heterozygous	1.25	gNM-F	3 (4.0)
*1/*41	Heterozygous	1.25	gNM-F	2 (2.7)
*2/*17	Heterozygous	1.5	gNM-F	1 (1.3)
*2/*29	Heterozygous	1.5	gNM-F	2 (2.7)
*1/*29	Heterozygous	1.5	gNM-F	1 (1.3)
*1/*1	Homozygous	2	gNM-F	20 (27.0)
*1/*2	Heterozygous	2	gNM-F	17 (23.0)
*2/*2	Homozygous	2	gNM-F	10 (13.5)
*1/*39	Heterozygous	2	gNM-F	1 (1.3)
*1/*34	Heterozygous	2	gNM-F	1 (1.3)
*2/*34	Heterozygous	2	gNM-F	1 (1.3)

CYP2D6 gene phenotypes were represented as poor metabolizer (gPM), intermediate metabolizer (gIM/gNM-S), normal metabolizer (gNM-F), ultrarapid metabolizer (gUM).

3.5. Association between CYP2D6 metabolizing phenotypes and relapses of P. vivax infection

In Table 5, the descriptive analysis of the phenotypic frequencies of CYP2D6 is presented in relation to the relapse of *P. vivax* infection over a six-month follow-up period. Among the 71 individuals analyzed, 18.3 % ($n = 13$) belonged to the group with diplotypes associated with impaired CYP2D6 enzyme activity with an AS < 1.25. The frequency of these phenotypes, associated with enzymatic dysfunction, was similar in those

Table 5
Frequency of CYP2D6 metabolizing phenotypes in participants with *P. vivax* infection according to the state of relapses during six-months follow-up. The descriptive analysis of the phenotypic frequencies of CYP2D6 is presented in relation to the relapse of P. vivax infection over a six-month follow-up period. Among the 71 individuals analyzed, 18.3 % ($n = 13$) belonged to the group with diplotypes associated with a poor (gPM) or intermediate (gIM and gNM-S) metabolizer phenotype of the CYP2D6 enzyme with an AS < 1.25. The frequency of these phenotypes, associated with enzymatic dysfunction, was similar in those with and without relapse (25 % vs 17 %).

Phenotypes of CYP2D6	Enzymatic activity	Activity Score	Relapses n (%)	Non-Relapses n (%)	Total n = 71, n (%)
gPM	Poor	0	0 (0 %)	3 (5.1 %)	3 (4.2 %)
gIM	Intermediate	0.25 – 0.75	0 (0 %)	2 (3.4 %)	1 (1.4 %)
gNM-S	Intermediate	1	3 (25 %)	5 (8.5 %)	9 (12.7 %)
gNM-F	Normal	1.25 – 2.25	9 (75 %)	46 (78 %)	55 (77.5 %)
gUM	Increased	> 2.25	0 (0 %)	3 (5.1 %)	3 (4.2 %)
		Total	**12 (100 %)**	**59 (100 %)**	**71 (100 %)**

CYP2D6 gene phenotypes were represented as poor metabolizer (gPM), intermediate metabolizer (gIM/gNM-S), normal metabolizer (gNM-F), increased or ultrafast metabolizer (gUM).

with and without relapse (25 % vs 17 %).

The Fig. 2 shows the survival curve for the first relapse episode over six-month, based on metabolizer phenotype. Cox regression analysis showed no significant difference in instantaneous risk (Hazard ratio or HR) of relapse between impaired and normal CYP2D6 metabolizers (Adjusted HR 1.45, 95 % CI 0.39-5.39, adjusted for residence time).

Regarding the number of relapses, seven participants had one relapse, four had two and one had three relapses over a six-month follow-up; the frequencies of impaired CYP2D6 metabolizer phenotype were 14.3 % ($n = 1$), 25 % ($n = 1$) and 100 % ($n = 1$) respectively.

3.6. Plasma levels of primaquine and CYP2D6 phenotypes

Plasma PQ levels were measured in 24 individuals, with the CYP2D6 metabolizer phenotype identified in 22 (4 with relapse,18 without relapse) (Supplementary Table 1). The median plasma PQ concentration on day 2 was higher in impaired CYP2D6 metabolizers (660.4 ng/ml, IQR: 363.5 ng/ml - 832.6 ng/ml, geomean 594.2) compared normal/increased metabolizer (313.5 ng/ml, IQR: 229.7 ng/ml - 556.5 ng/ml, geomean 322.9), but the difference was not statistically significant (Wilcoxon-Mann-Whitney test, p-value = 0.09, effect size r −0.51, 95 % CI −0.82 to 0.03).

In an exploratory manner, PQ levels on day 2 (median 548.0 ng/mL IQR 321.6 – 775.4; geomean 460.1 ng/mL) were higher in the relapse group, compared to those in the group without relapse (median 313.5 ng/mL IQR 224.5 – 556.5; geomean 298.35 ng/mL), with no statistical difference (Wilcoxon, p-value = 0.2155, effect size −0.33; 95 % CI −0.70, 0.18).

4. Discussion

This pioneering study in Colombia explores the association between *CYP2D6* gene polymorphisms and *P. vivax* relapses. The frequencies of the analyzed SNPs showed significant variability, similar to findings in Brazil [15–17] and other regions [28]. This variation likely reflects the genetic diversity of Ibero-American populations, shaped by the admixture of local Amerindian, European, and African ancestries. While most participants self-identified as mestizo, a notable proportion identified as Afro-Colombian, white, or indigenous, which contributes to the genetic diversity observed.

The study confirms the polymorphic nature of the *CYP2D6* gene, offering valuable insights into its allelic distribution in Colombian population. The methodology enabled allele identification in 93,6 % of participants, providing a cost-effective alternative to sequencing. Despite over 100 known CYP2D6 variants, focusing on eight SNPs and one deletion gave a robust characterization of our study population, identifying alleles associated to deficient enzyme. Other studies have tested comparable number of polymorphisms [14–16,17,29].

The frequencies of the identified star alleles ranged from 0.6 % to 44.2 %. Of the ten star alleles detected, 19.9 % were associated with null or decreased enzyme activity. The most common allele was the **4* (9.6 %), followed by **5* (1.3 %) associated with null activity, and **41* (3.2 %), **29* (2.6 %), **9* (1.9 %) and **17* (1.3 %), which were associated with decreased enzymatic activity. These allele frequencies were observed in a population from Turbo. They were like the frequencies found in other Latin American countries, such as Brazil, Argentina, and in previous studies conducted in Colombia [18,19,28]. The set of SNPs evaluated in this study included some already identified in similar populations and some known to be associated with decreased enzymatic function [16,18,19].

The most frequent diplotypes associated with decreased enzymatic function were **1/*4* (6.8 %), **2/*4* (5.4 %), and **4/*4* (2.7 %). A previous study by Sarmiento et al. in subjects from Bogotá, Colombia reported higher frequencies for impaired CYP2D6 phenotypes (34.3 %) with diplotypes **1/*4* (11.4 %), **2/*4* (3.1 %), and **4/*4* (4.1 %) [18]. Although the results are similar, the differences in diplotype frequencies could be attributed to heterogeneity in analyzed populations [18,19]. Additionally, a 2018 study by G. Naranjo *et al.* reported a null phenotype frequency of around 10.2 % in countries such as Mexico, Brazil, Argentina, Costa Rica, Peru, and Colombia [28].

Phenotypic classification in this study followed the most recent guidelines from the *Clinical Pharmacogenetics Implementation Consortium (CPIC)* and Dutch Pharmacogenetics Working Group (DPWG) [13]. This classification introduced a new threshold implies that the AS of 1.25 is now the threshold to call impaired CYP2D6 activity phenotypes (AS <1.25), which includes 0 (poor metabolizers) and 0.25 / 0.5 / 0.75 / 1 (intermediate), and normal CYP2D6 activity (AS ≥ 1.25), which includes 1.25 / 1.5 / 2 / 2.25 (normal metabolizers) and >2.25 (ultrafast) [13]. Depending on the threshold criterion used to classify as impaired enzyme activity, the comparability of CYP2D6 phenotypes among studies may be affected [14,16,17]. In this study, we chose to use the most recent AS classification in order to take a conservative approach

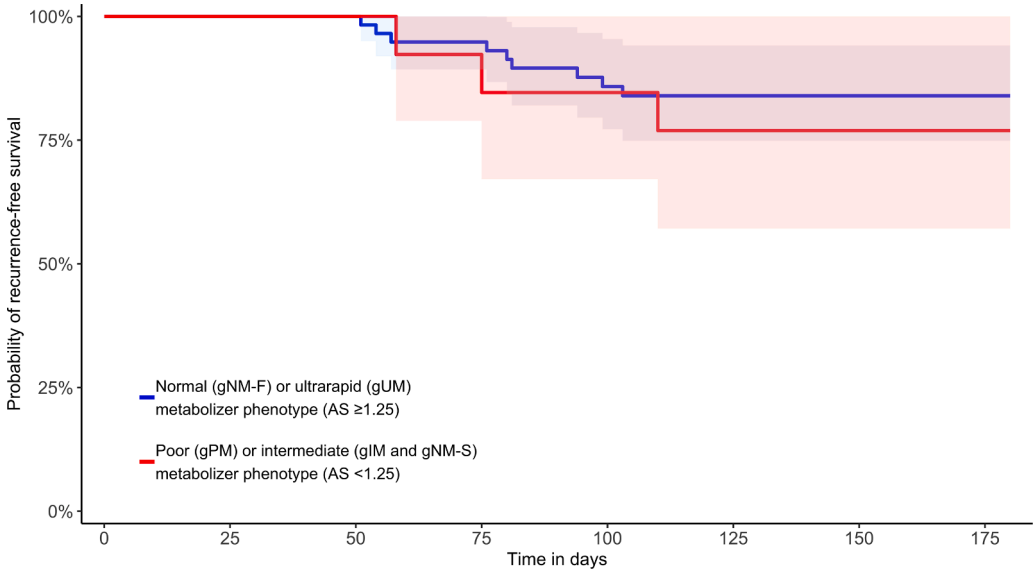

Fig. 2. Risk of relapses in individuals with P. vivax infection and treated with CQ-PQ, in relation to CYP2D6 metabolic activity phenotype. Blue line: normal/increased metabolizer group. Red line: poor/intermediate metabolizers.

and avoid biasing the results in favor of the hypothesis suggesting an association between impaired CYP2D6 activity and *P. vivax* relapse. Recognizing that results of this study are merely exploratory, we observed 3 patients with AS = 1.25, with two of them having high PQ levels (like those classified as impaired phenotype, by the AS classification) (Table S1).

Impaired CYP2D6 activity have been associated with elevated PQ blood levels, as the drug remains unmetabolized in individuals with deficient enzyme activity [29]. Unfortunately, due to resource limitations it was only possible to analyze the samples from day 2 for PQ and it was not possible to quantify the carboxyPQ. Considering that PQ measurement was carried out independently of the outcome evaluation (relapses) and was quantified with the same procedure for all samples, we consider that the data is useful as a proxy for the metabolism of the PQ in the study participants at a time point where it is expected to reach its maximum blood concentration. A trend toward higher PQ levels was observed in impaired CYP2D6 metabolizers compared to normal metabolizers (660.4 ng/ml *vs* 313.5 ng/ml), similar to findings by Salazar et al. 2024 [30]. Although our findings are limited by the small sample size, an important size effect is observed [27]. The results support the hypothesis of the association between relapse and poor PQ metabolism, since here we also found that participants with relapse have higher plasma levels of PQ, possibly related to the impaired CYP2D6 activity. Based upon this exploratory data and event rates in this place and time, >2000 participants are necessary for statistical significance.

The findings support the hypothesis that individuals with impaired CYP2D6 activity may maintain higher concentrations of unmetabolized PQ in the blood. Although the current study was exploratory, this study highlights the need for further research with larger sample sizes to confirm this relationship. It is also important to consider alternative metabolic pathways, such as the Monoamina Oxidase A (MAO-A) pathway, which may compensate for CYP2D6 deficiencies and maintain normal PQ levels in individuals with impaired CYP2D6 activity phenotypes [31].

It is important to highlight that TQ, a recent treatment alternative for the radical cure of *P. vivax*, also requires CYP2D6 enzyme activity for effective metabolism [32]. Screening for impaired CYP2D6 metabolic activity in populations from malaria-endemic areas should be considered when implementing TQ to ensure its efficacy.

Although this study could not definitively establish a link between impaired CYP2D6 phenotypes and *P. vivax* relapse, previous research has indicated a higher risk of relapse and recurrence in individuals with an impaired CYP2D6 activity [15–17,29]. Differences in sample size, study design, outcome evaluation (single *vs* multiple recurrences), and phenotypic classification may explain the lack of a clear association in this study. We do not rule out the possibility that other differences in ancestral genetic makeup of human populations could explain the lack of concordance with previous reports. Participants in this study come from areas with high genetic mix of Afro-descendant populations, which could differ from populations included in the Brazilian Amazon studies. On the other hand, *P. vivax* strains could also have different levels of tolerance to PQ depending on their geographic origin and favor the occurrence of relapses by mechanisms independent of impaired CYP2D6 activity, as discussed by Nanhua Chen *et al.* (2019) [34]. On the other hand, evaluating relapse as an outcome is challenging, as it can be influenced by transmission dynamics and acquired immunity [33]. All these factors may have limited the comparisons made in this study.

This study provides valuable insights into the genetic characteristics of *CYP2D6* in *P. vivax* infections and its association with relapses. However, further research with a more representative sample from Colombia and additional drug measurement at multiple time points is necessary to confirm these findings and guide malaria control and elimination efforts.

In conclusion, CYP2D6 genetic variations associated with impaired activity phenotypes are common among individuals with *P. vivax* in an endemic area of Colombia. Considering that radical cure treatments for *P. vivax* requires the use of PQ or TQ, further studies are essential to explore the implications of these genetic alterations on response to treatment.

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Ethical approval

The base study from 2012 was approved by the Ethics Committee of the Faculty of Medicine at the University of Antioquia, Colombia. All participants provided written informed consent, which included approval for the use of their samples and information in future studies [8]. This study was approved on February 4, 2021, by the Ethics Committee of the Institute of Medical Research at the University of Antioquia, Colombia (Act No 001).

Sequence information

Not applicable.

CRediT authorship contribution statement

Veronica Sierra-Cifuentes: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Lina Zuluaga-Idárraga:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Daniel Aguirre-Acevedo:** Writing – review & editing, Formal analysis. **Maria Carolina Silva de Barros Puça:** Writing – review & editing, Validation, Methodology, Formal analysis. **Tais Nobrega de Sousa:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation. **Tatiana M. Lopera-Mesa:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.diagmicrobio.2025.117013.

Data availability

All datasets can be provided upon request to tmaria.lopera@udea.edu.co.

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