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EPIDEMIOLOGY AND SURVEILLANCE

Monitoring the Efficacy of Chloroquine-Primaquine Therapy for Uncomplicated *Plasmodium vivax* Malaria in the Main Transmission Hot Spot of Brazil

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ABSTRACT Emerging *Plasmodium vivax* resistance to chloroquine (CQ) may undermine malaria elimination efforts in South America. CQ-resistant *P. vivax* has been found in the major port city of Manaus but not in the main malaria hot spots across the Amazon Basin of Brazil, where CQ is routinely coadministered with primaquine (PQ) for radical cure of vivax malaria. Here we randomly assigned 204 uncomplicated vivax malaria patients from Juruá Valley, northwestern Brazil, to receive either sequential (arm 1) or concomitant (arm 2) CQ-PQ treatment. Because PQ may synergize the blood schizontocidal effect of CQ and mask low-level CQ resistance, we monitored CQ-only efficacy in arm 1 subjects, who had PQ administered only at the end of the 28-day follow-up. We found adequate clinical and parasitological responses in all subjects assigned to arm 2. However, 2.2% of arm 1 patients had microscopy-detected parasite recrudescences at day 28. When PCR-detected parasitemias at day 28 were considered, response rates decreased to 92.1% and 98.8% in arms 1 and 2, respectively. Therapeutic CQ levels were documented in 6 of 8 recurrences, consistent with true CQ resistance *in vivo*. In contrast, *ex vivo* assays provided no evidence of CQ resistance in 49 local *P. vivax* isolates analyzed. CQ-PQ coadministration was not found to potentiate the antirelapse efficacy of PQ over 180 days of surveillance; however, we suggest that larger studies are needed to examine whether and how CQ-PQ interactions, e.g., CQ-mediated inhibition of PQ metabolism, modulate radical cure efficacy in different *P. vivax*-infected populations. (This study has been registered at ClinicalTrials.gov under identifier NCT02691910.)

KEYWORDS Amazon, Brazil, *Plasmodium vivax*, chloroquine, efficacy, malaria, primaquine

The emergence of *Plasmodium vivax* resistance to chloroquine (CQ), a safe, inexpensive, and fast-acting antimalarial drug in clinical use since 1946, may undermine current efforts to control and eventually eliminate malaria worldwide (1). CQ resistance *in vivo* is defined by parasitological failure, namely, persistence beyond day 3 of treatment or a reappearance of asexual blood stages, over 28 days of follow-up, despite therapeutic (>100 ng/ml) blood levels of CQ and its main active metabolite, desethylchloroquine (DCQ) (2). By applying this definition, *P. vivax* CQ resistance has been confirmed across most of the tropical world, reaching an alarming prevalence in Indonesia, East Timor, and Papua New Guinea (3). However, *in vivo* tests of drug resistance may be affected by factors such as previous or concomitant antimalarial use and naturally acquired antiparasite immunity. Alternatively, *P. vivax* drug susceptibility can be monitored using short-term *ex vivo* schizont maturation tests in the presence of

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increasing drug concentrations, but the use of these assays has been limited until recently by the lack of practical and standardized protocols (4, 5). Moreover, results may be affected by previous or concomitant antimalarial use, patterns of parasite synchronicity in clinical samples, and time delays in processing field-collected samples (6).

With nearly 130,000 laboratory-confirmed infections, Brazil contributed 39% of all malaria cases reported in the Americas in 2016. *Plasmodium vivax* currently accounts for 88% of the malaria burden in Brazil (7). Whether CQ resistance significantly contributes to persisting vivax malaria transmission across the Brazilian Amazon remains undetermined. *P. vivax* resistance to CQ has been documented in the major Amazonian port city of Manaus, in northwestern Brazil, with parasitological failure rates of 6.4% to 10.1% over 28 days of follow-up in patients treated with CQ alone (8, 9), compared with 5.2% failures in patients treated according to the current malaria therapy guidelines in Brazil with concomitant CQ and primaquine (PQ) (10). Moreover, *P. vivax* resistance to CQ has been occasionally described in countries sharing borders with Brazil, such as Bolivia (11), Peru, and Guyana (12). However, recrudescences at day 28 were not observed following CQ-PQ treatment of vivax malaria in other settings of endemicity in Brazil (13), including Juruá Valley, the region with the highest malaria rates in this country (14).

The reasons why *P. vivax* resistance to CQ remains infrequently reported in the Americas are open to speculation (12). One hypothesis is that CQ resistance *in vivo* may have been obscured by PQ coadministration to eradicate *P. vivax* hypnozoites, the dormant liver stages that may eventually cause relapses. Radical cure of vivax malaria, i.e., eradication of both the parasite's blood stages and hepatic hypnozoites, requires PQ, the only widely available hypnozoitocidal drug, which also appears to synergize the blood schizontocidal effect of CQ (15). Accordingly, PQ significantly reduces the risk of parasitological failure in CQ-treated uncomplicated vivax malaria patients, suggesting that coadministration of both drugs may clear CQ-resistant parasites and lead to an overestimation of CQ efficacy in clinical trials. Therefore, properly monitoring the clinical efficacy of CQ requires PQ administration to be withheld until day 28 (3).

Concomitant CQ-PQ use poses additional challenges for monitoring treatment outcomes. On the one hand, small clinical trials in the 1950s showed that volunteers treated with PQ plus quinine (QN) or CQ and not exposed to a risk of reinfection had a greatly reduced *P. vivax* relapse risk, compared with those given PQ alone (16, 17). These results were interpreted as evidence that concomitant use of QN and CQ might potentiate the hypnozoitocidal effect of PQ (17, 18). On the other hand, an opposite effect of CQ and QN might also be expected. PQ is an inactive prodrug that requires biotransformation for antirelapse activity, which appears to involve hydroxylated metabolites generated by the cytochrome P450 (CYP) isoenzyme CYP2D6 (19). Accordingly, subjects with low-activity CYP2D6 variants are at an increased risk of *P. vivax* relapses following supervised PQ treatment (20). Significantly, blood schizontocidal antimalarials that are often coadministered with PQ, including CQ, QN, and artemisinin derivatives, can inhibit CYP2D6 activity and decrease the generation of pharmacologically active PQ metabolites (19). This may increase the risk of relapses despite PQ treatment, especially in patients carrying low-activity CYP2D6 variants.

Here we combine *in vitro*, *ex vivo*, and molecular approaches to characterize levels of *P. vivax* CQ resistance in Juruá Valley, a region that contributes 20% of the total malaria burden of Brazil. We show that the CQ-PQ combination remains efficacious *in vivo* against *P. vivax* blood stages, with a 100% adequate clinical and parasitological response (ACPR) over 28 days of follow-up as assessed by microscopy, although 2.2% of the patients treated with CQ alone had parasite recrudescences. Results from *ex vivo* assays are also consistent with a low frequency of CQ resistance in this setting of South America, with 50% inhibitory concentrations (IC_{50}) ranging between 4.1 and 63.3 nM in 49 local *P. vivax* isolates analyzed. Interestingly, CQ-PQ coadministration did not appear to significantly increase the antirelapse efficacy of PQ, and the time of the first recurrent vivax malaria episode after treatment was not significantly affected by the patients' CYP2D6 genotype.

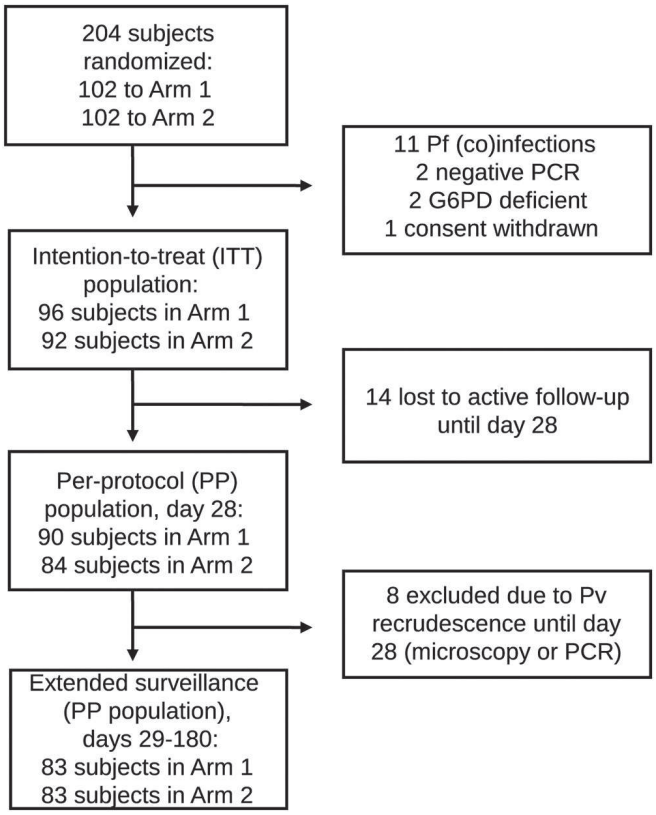

FIG 1 Study flow diagram. Between June 2014 and July 2015, 204 patients with uncomplicated single-species *P. vivax* (Pv) infection were recruited at three health centers in the urban area of Mâncio Lima and randomly allocated to either of the two treatments. Reasons for exclusion or censure and the number of subjects analyzed per treatment arm in the intention-to-treat and per-protocol populations (up to day 28) and in the extended follow-up (6 months) of the PP population are indicated. Pf, *P. falciparum*.

RESULTS

Study population and adverse effects. Of 204 patients randomly allocated to either of the two treatments (102 to each arm), 16 subjects (7.8%), 6 in arm 1 and 10 in arm 2, were excluded because of protocol violations, mostly due to species misdiagnosis at admission (Fig. 1). The baseline characteristics of the intention-to-treat (ITT) population ($n = 188$), shown in Table 1, were very similar between treatment arms. The study drugs were well tolerated, and no study withdrawal was attributable to adverse events related to study drugs. The most common adverse events reported by the ITT population between days 1 and 3 are listed in Table S1 in the supplemental material, with similar frequencies between treatment groups. A single study participant reported generalized pruritus that may be associated with CQ use. Note that the proportions of study participants with anemia diagnosed at days 1, 2, 3, 7, and 28 were similar between treatment arms (Table 2), although only arm 2 subjects had been exposed to the risk of PQ-induced hemolysis.

Treatment efficacy over 28 days. We failed to obtain complete 28-day follow-up data for 14 subjects (7.4%), 6 in arm 1 and 8 in arm 2, who left the study after 1 to 21 days of follow-up. The per-protocol (PP) analysis therefore comprised 174 subjects (92.6% of those enrolled), 90 in arm 1 and 84 in arm 2 (Fig. 1). We found microscopy-based ACPR rates at day 28 of 97.8% (95% confidence interval [CI], 91.4% to 99.6%) for the sequential CQ-PQ regimen and 100.0% (95% CI, 94.5 to 100.0%) for the concomitant CQ-PQ regimen (Table 2). Note that although no statistically significant difference in ACPR was found between arms ($P = 0.498$ by Fisher's exact test), the study was underpowered for such a comparison. No study participant had patent *P. vivax* parasitemia observed between days 7 and 21.

TABLE 1 Baseline characteristics of the intention-to-treat study population according to treatment arm, either sequential or concomitant chloroquine plus primaquine

Variable	Value for treatment regimen group	
	Sequential CQ-PQ (arm 1) (n = 96)	Concomitant CQ-PQ (arm 2) (n = 92)
Mean age (yr) (range)	27.8 (5–63)	25.7 (7–60)
No. of male subjects/no. of female subjects	55/41	62/32
Mean wt (kg) (range)	58.8 (20.3–101.0)	57.8 (20.0–94.0)
No. (%) of individuals with temp > 37.5°C	40 (42.1)	42 (46.7)
No. (%) of subjects with reported sign or symptom in the past 48 h		
Fever	91 (94.8)	87 (94.6)
Headache	87 (90.6)	84 (91.3)
Chills	64 (66.7)	54 (58.7)
Dizziness	58 (60.4)	56 (60.9)
Malaise	72 (75.0)	67 (72.8)
Arthralgia	69 (71.9)	51 (55.4)
Myalgia	65 (67.7)	51 (55.4)
Low back pain	60 (62.5)	41 (44.6)
Abdominal pain	18 (18.8)	11 (12.0)
Appetite loss	55 (57.3)	57 (62.0)
Vomiting	30 (31.3)	22 (23.4)
Diarrhea	3 (3.1)	5 (5.4)
Tinnitus	6 (6.3)	6 (6.5)
Blurred vision	8 (8.3)	6 (6.5)
Dark urine	13 (13.5)	9 (9.6)
Mean no. of days ill prior to enrollment (range)	2.7 (1–7)	2.7 (1–8)
Mean hemoglobin level (g/100 ml) (range)	12.5 (7.1–16.3)	12.7 (8.8–17.2)
Mean no. (%) of subjects with anemia	31 (32.3)	34 (37.0)
Mean platelet count (10^9/liter) (range) (n = 160)	155.8 (35.0–367.0) (n = 88)	152.9 (39.0–319.0) (n = 72)
No. (%) of subjects with thrombocytopenia	45 (51.1) (n = 88)	33 (45.8) (n = 72)
No. (%) of subjects with predicted CYP2D6 activity phenotype (n = 175)	88	87
Poor metabolizer	5 (5.7)	4 (4.6)
Intermediate metabolizer	4 (4.5)	4 (4.6)
Normal-slow metabolizer	18 (20.5)	27 (31.0)
Normal-fast metabolizer	56 (63.6)	50 (57.5)
Ultrarapid metabolizer	5 (5.7)	2 (2.3)
Parasitemia (no. of asexual forms/μl)		
Mean (range)	3,160.1 (87–25,000)	3,332.8 (60–24,383)
Median	2,228	2,761
No. (%) of subjects with gametocyte carriage detected by PCR (n = 187)	72 (75.1) (n = 96)	59 (64.8) (n = 91)

Parasite and fever clearance times. Only 3 patients (3.4%) in arm 1 and 1 patient (1.1%) in arm 2 remained slide positive by day 3. Of those patients, none had parasite recurrence detected by microscopy at day 28, suggesting that microscopy-detected parasitemia at day 3 did not predict treatment failure. Parasite clearance times (PCTs) were similar between arms (mean, 2.5 versus 1.9 days) (Table 2) but longer than those recently reported for concomitant CQ-PQ treatment along the Thai-Myanmar border (1.0 days [95% CI, 0.5 to 1.7 days]), an area where *P. vivax* CQ resistance remains infrequent (21). Interestingly, the mean PCT decreased significantly with age among our patients treated with CQ alone, ranging from 3.9 days (95% CI, 1.4 to 6.4 days) in those aged <16 years to 1.8 days (95% CI, 1.5 to 2.1 days) in those aged >30 years (P = 0.031 by a Mantel-Cox log rank test). These results are consistent with an age-dependent contribution of acquired immunity to parasite clearance (22). The mean fever clearance times (FCTs) did not vary significantly according to treatment arm (1.5 versus 1.8 days) (Table 2) or age but were slightly longer than those reported for concomitant CQ-PQ treatment along the Thai-Myanmar border (1.2 days [95% CI, 0.7 to 1.5 days]) (21).

Residual parasite DNA and gametocyte carriage detected by molecular methods. The proportions of subjects with residual *P. vivax* DNA (21.7% of those in arm 1

TABLE 2 Trial outcomes over 28 days of follow-up and prevalence of parasitemia, anemia, and thrombocytopenia at selected time points[a]

Outcome	No. of individuals tested	Value for treatment regimen		*P* value[b]
		Sequential CQ-PQ (arm 1)	Concomitant CQ-PQ (arm 2)	
ACPR rate (%) over 28 days (95% CI)	174	97.8 (91.4–99.6) ($n = 90$)	100.0 (94.5–100.0) ($n = 84$)	0.498[c]
Mean parasite clearance time (days) (95% CI)	188	2.54 (1.73–3.35) ($n = 96$)	1.89 (1.71–2.05) ($n = 92$)	0.482[d]
Mean parasite DNA clearance time (days) (95% CI)	176	3.26 (2.84–3.69) ($n = 88$)	2.77 (2.41–3.13) ($n = 88$)	0.063[d]
Mean fever clearance time (days) (95% CI)	179	1.51 (1.39–1.62) ($n = 91$)	1.78 (1.47–2.07) ($n = 88$)	0.086[d]
No. (%) of subjects with asexual parasite prevalence detected by microscopy				
Day 1	182	62 (66.7) ($n = 93$)	61 (68.5) ($n = 89$)	0.874[c]
Day 2	180	13 (14.3) ($n = 91$)	13 (14.6) ($n = 89$)	1.000[c]
Day 3	180	3 (3.4) ($n = 92$)	1 (1.1) ($n = 88$)	0.621[c]
No. (%) of subjects with parasite DNA carriage determined by PCR				
Day 1	182	84 (90.3) ($n = 93$)	78 (87.6) ($n = 89$)	0.639[c]
Day 2	181	52 (56.5) ($n = 92$)	33 (37.1) ($n = 89$)	0.011[c]
Day 3	178	20 (21.7) ($n = 92$)	11 (12.8) ($n = 86$)	0.166[c]
Day 28	173	7 (7.9) ($n = 89$)	1 (1.1) ($n = 84$)	0.065[c]
No. (%) of subjects with gametocyte-specific transcript carriage on day 3	167	19 (11.5) ($n = 87$)	3 (3.8) ($n = 80$)	0.088[c]
No. (%) of subjects with anemia				
Day 1	181	43 (46.2) ($n = 93$)	43 (48.9) ($n = 88$)	0.767[c]
Day 2	181	46 (50.0) ($n = 92$)	45 (50.6) ($n = 89$)	1.000[c]
Day 3	180	37 (40.2) ($n = 92$)	44 (50.0) ($n = 88$)	0.231[c]
Day 7	166	34 (37.0) ($n = 92$)	28 (37.8) ($n = 74$)	1.000[c]
Day 28	163	24 (27.6) ($n = 87$)	28 (36.8) ($n = 76$)	0.240[c]
No. (%) of subjects with thrombocytopenia on day 28	156	9 (13.2) ($n = 68$)	5 (7.7) ($n = 65$)	0.340[c]

[a]ACPR, adequate clinical and parasitological response, defined as the absence of asexual blood-stage parasites detected by microscopy by day 28, regardless of axillary temperature, with no evidence of earlier treatment failure.
[b]Statistical comparisons of response rates, clearance times, and prevalences are presented for exploratory purposes, but this study was not originally designed and powered to detect significant differences between arms.
[c]By Fisher's exact test.
[d]By a Mantel-Cox log rank test.

and 12.8% in arm 2) and gametocyte-specific transcripts (11.5% of those in arm 1 and 3.8% in arm 2) detected by molecular methods at day 3 were substantially higher than those in subjects with patent parasitemia detected by microscopy (Table 2). In fact, study subjects took 28% to 46% longer to clear parasite DNA, compared with the time required to become negative by microscopy. Differences between arms did not reach statistical significance, but overall parasite DNA and gametocyte-specific transcripts tended to take longer to clear in arm 1 subjects. None of the subjects with PCR-detected parasite DNA (pDNA) at day 3 remained positive at day 7. However, seven study participants in arm 1 (7.9%) and one in arm 2 (1.1%; 95% CI, 0.6% to 7.4%) were again PCR positive at day 28 (Table 2) but were negative by both microscopy and PCR between days 7 and 21. These findings are consistent with late *P. vivax* recrudescences; most of these recrudescences were subpatent and asymptomatic at the time of detection (Table 3). ACPR rates recalculated considering PCR results would decrease to 92.1% (95% CI, 83.9% to 96.5%) in arm 1 and 98.8% (95% CI, 92.6% to 99.9%) in arm 2. Overall, subjects with residual *P. vivax* DNA detected at day 3, suggestive of delayed parasite clearance, were more likely to have parasitemia detected by microscopy or PCR at day 28 ($P < 0.0001$ for both comparisons, by a McNemar test). However, only two (both in arm 1) of the eight patients who were PCR positive at day 28 had *P. vivax* DNA detected at day 3. Moreover, only one of the two subjects with parasitemia detected by microscopy was PCR positive at day 3. These findings suggest that PCR-detected

TABLE 3 Characteristics of the study participants with day 28 parasitemia detected by microscopy or PCR

Patient ID	Sex/age (yr)	Trial arm	Day 28 parasitemia (no. of parasites/μl blood) determined by:		Day 28 sign(s) and/or symptom(s) reported	Day 28 CQ/DCQ level (ng/ml)[a]	Status of paired genotypes[b]
			Microscopy	PCR			
1/4	Male/13	1	Negative	25	None	331.5	Identical[c]
1/27	Female/36	1	Negative	4	None	119.3	Identical[c]
1/28	Female/24	1	Negative	3	None	980.9	Identical[d]
1/55	Female/10	1	83	22	Malaise, anorexia, headache, and arthralgia	108.5	Identical[c]
1/58	Female/12	1	Negative	99	None	97.7	Identical[c]
1/66	Male/13	1	Negative	167	None	387.9	Identical[c]
1/74	Female/14	1	2,942	1,573	Chills, headache, myalgia, arthralgia, and low-back pain	140.9	Identical[c]
2/70	Male/12	2	Negative	3	None	78.3	Identical[c]

[a]Therapeutic whole-blood levels (>100 ng/ml) are underlined.
[b]Comparison of multilocus genotypes obtained with six microsatellite loci from parasite samples collected from the same subject at day 0 (pretreatment) and day 28 (day of recurrence).
[c]Identical predominant or only alleles at all 6 microsatellite loci that were successfully amplified from paired samples.
[d]Identical predominant alleles or only alleles at 5 microsatellite loci; amplification of one marker from the day 28 sample failed.

parasitemia at day 3 is a poorly sensitive predictor of day 28 positivity by any method. All but one of the eight subjects with PCR-detected recrudescences were younger than the average study participant (26.8 years), putatively because of more-effective immune-mediated parasite clearance in older subjects (22).

Molecular characterization of day 28 recrudescences. Characteristics of the study participants with evidence of day 28 recurrence are shown in Table 3. Note that day 28 parasites were genetically identical to those recovered at day 0 in all patients evaluated, if we consider both major and minor alleles in multiple-clone infections. These findings are consistent with recrudescences or early relapses of the same *P. vivax* strain. However, two pairs of samples from different patients (patient 1/27 versus 1/28 and patient 1/55 versus 1/58) also shared identical genotypes (Table S2), suggesting that our genotyping approach may not distinguish between genetically similar but clonally unrelated isolates. Only two subjects with patent parasitemia at day 28, both of them with infection confirmed by both microscopy and PCR, had malaria-related symptoms. All asymptomatic day 28 parasitemias that were missed by onsite microscopy but later confirmed by PCR were left untreated because the results of molecular diagnosis were not available before the end of the study. Six study participants (including both slide-positive subjects) had therapeutic CQ/DCQ levels at the time of the recrudescence (Table 3), consistent with strictly defined resistance to CQ characterized *in vivo* (2).

Primaquine timing, CYP2D6 activity, and *Plasmodium vivax* recurrences over 180 days. After 6 months of extended surveillance of the PP population, 49 patients had experienced at least one recurrent episode of *P. vivax* malaria determined by microscopy (21 out of 83 in arm 1 and 28 out of 83 in arm 2) (Fig. S2). These recurrences may be due to late recrudescences, relapses, or new infections. The median times to the first recurrent vivax malaria episode, confirmed by microscopy between days 29 and 180 of successful CQ therapy, were 157 days (95% CI, 148 to 166 days) in arm 1 and 148 days (95% CI, 137 to 158 days) in arm 2 ($P = 0.213$ by a Mantel-Cox log rank test). Cox proportional-hazards models adjusted for age and sex showed no significant difference between arms in the prospective risk of vivax malaria recurrence (Table 4). In fact, age and sex were the only independent predictors of risk of recurrence in the adjusted Cox model. Female sex was associated with a reduced risk of recurrence (hazard ratio [HR] = 0.493 [95% CI, 0.257 to 0.944]; $P = 0.033$), while an age of between 21 and 40 years was associated with an increased risk (HR = 2.667 [95% CI, 1.087 to 6.543]; $P = 0.032$) in the fully adjusted model. These findings most likely reflect the contribution of new infections to parasite recurrences during follow-up in young male adults, since they constitute the population stratum at the highest risk for malaria in the study site (R. M. Corder and M. U. Ferreira, unpublished results).

TABLE 4 Association between timing of primaquine use relative to chloroquine administration and prospective risk of *Plasmodium vivax* recurrence over 180 days in the per-protocol study population

	Concomitant CQ-PQ (arm 2) vs sequential CQ-PQ (arm 1)		
Cox model	Hazard ratio	95% confidence interval	*P* value
Unadjusted	1.421	0.807–2.502	0.224
Adjusted for age	1.493	0.847–2.634	0.166
Adjusted for gender	1.395	0.792–2.457	0.250
Adjusted for CYP2D6 activity	1.254	0.703–2.237	0.443
Adjusted for age and gender	1.379	0.779–2.439	0.270
Adjusted for age, gender, and CYP2D6 activity	1.248	0.698–2.231	0.322

Interestingly, low CYP2D6 activity (activity score [AS] of ≤1.0) was not associated with a shorter time to recurrence in the fully adjusted model (HR = 1.092 [95% CI, 0.548 to 2.038]; $P = 0.782$). Overall, we diagnosed 20 *P. vivax* recurrences in 15 out of 54 subjects with low CYP2D6 activity (AS of ≤1.0), who contributed 9,147 person-days of follow-up (incidence density, 2.9/1,000 person-days [95% CI, 1.3 to 3.4/1,000 person-days]), and 48 recurrences in 32 out of 102 subjects with normal or high CYP2D6 activity (AS of >1.0), who contributed 16,229 person-days of follow-up (incidence density, 3.0/1,000 person-days [95% CI, 2.2 to 3.9/1,000 person-days]). The incidence of *P. vivax* recurrences over the extended 180-day surveillance period did not vary according to CYP2D6 activity ($P = 0.311$ by Fisher's exact test).

***Ex vivo* monitoring of chloroquine resistance.** Forty of 128 (31.2%) cryopreserved *P. vivax* isolates from study participants met the parasite density and synchronicity thresholds after thawing and achieved >40% maturation to schizonts within 44 h of culture. Nine additional *P. vivax* samples meeting these criteria, collected in Mâncio Lima between 2016 and 2017, were tested, giving a total of 49 local isolates successfully monitored for *ex vivo* CQ resistance. The median IC_{50} for CQ was 17.4 nM (interquartile range, 8.9 to 31.9 nM), comparable to estimates from recent studies in other sites across South America (Table 5), with individual IC_{50} values ranging between 4.1 and 63.3 nM (Fig. S3). Similar results were seen when the analysis was limited to isolates from the 40 participants in the clinical trial (26 allocated to treatment arm 1 and 14 allocated to arm 2), with a median IC_{50} of 19.1 nM (interquartile range, 9.6 to 34.6 nM). IC_{50} values of >100 nM, suggestive of CQ resistance (23), were not observed in this and another recent study in Porto Velho, Brazil (24), but had previously been found in approximately 10% of *P. vivax* isolates from Urabá, Colombia (25), and Manaus, Brazil (26, 27) (Table 5). Day 0 parasites from patient 1/4, who had a PCR-confirmed parasite recrudescence at day 28 (Table 3), were fully sensitive to CQ (IC_{50} estimate, 14.1 nM). Nevertheless, we had no cryopreserved sample of recrudescent parasites for *ex vivo* confirmation of CQ resistance. We hypothesize that the CQ-resistant parasites that emerged in patient 1/4 at day 28 represented a minor subpopulation in the pretreatment sample tested for CQ resistance that was later selected under CQ pressure. Since microsatellite genotyping

TABLE 5 Comparison of *ex vivo* chloroquine resistance data currently available for *Plasmodium vivax* isolates from South America

Reference	Yr of collection	Site, country	No. of samples	IC_{50} for chloroquine (nM) estimated by *P. vivax* schizont maturation test			% of isolates with IC_{50} > 100 nM (95% CI)
				Geometric mean	Median	Range	
27	2004–2007	Manaus, Brazil	132[a,b]	24.1		5–729	9.8 (5.6–16.6)
26	2007–2008	Manaus, Brazil	112[a,b]			~8–500	10.7 (5.9–18.3)
25	2010–2012	Urabá, Colombia	30[b,c]	23.3		2.5–1,109.0	13.3 (4.4–31.6)
24	2012–2013	Porto Velho, Brazil	32[b,c]		32	3–69	0.0 (0.0–13.3)
Present study	2014–2017	Mâncio Lima, Brazil	49[c,d]	15.8	17.4	4.1–63.3	0.0 (0.0–9.1)

[a]Parasite density assessed by double-site enzyme-linked immunodetection (DELI) test.
[b]Assays carried out with fresh isolates.
[c]Parasite density and staging assessed by microscopy.
[d]Assays carried out with cryopreserved isolates. Forty of 128 (31.2%) cryopreserved *P. vivax* isolates from participants in the clinical trial had IC_{50} estimates determined; a further 9 isolates from subjects who did not participate in the clinical trial but were living in the same area were successfully tested, giving a total of 49 local samples analyzed.

did not show genetic differences between pretreatment and recrudescent parasites, we further hypothesize that the major CQ-sensitive and the minor CQ-resistant parasite populations present in day 0 samples were closely related genetically (although not identical) albeit phenotypically distinct.

DISCUSSION

CQ remains the first-line treatment for uncomplicated *P. vivax* malaria in Brazil (28), but declining efficacy has been repeatedly characterized in the large port city of Manaus, using both *in vivo* (8–10) and *ex vivo* (26, 27) assays. However, it remains undetermined whether CQ resistance has spread to other sites across the Amazon Basin and represents a major challenge for current malaria elimination in this country and its neighbors (12, 29).

Here we show that CQ resistance is present but remains infrequent in Juruá Valley, the main malaria hot spot of Brazil. We and others (14) found an ACPR in all patients treated with the currently recommended CQ-PQ combination and assessed by conventional microscopy. We and others (24) found no evidence of CQ resistance from *ex vivo* sensitivity assays with *P. vivax* isolates from sites other than Manaus, further suggesting that this phenotype remains, at least in Brazil, mostly confined to its original epicenter. However, two (2.2%) of our patients treated with CQ alone had patent parasitemia at day 28 of follow-up, showing that *in vivo* CQ resistance in Juruá Valley may be found when the confounding effect of concomitant PQ administration is removed. These data are consistent with the notion that PQ synergizes the blood schizontocidal effect of CQ against parasites with low-grade resistance (15). Accordingly, a meta-analysis of pooled individual patients' data has shown a 90% reduction in recurrence rates over 42 days among patients given a CQ-PQ combination, compared with CQ alone (30). Moreover, in our study, CQ-PQ coadministration appeared to favor faster parasite clearance, with a lower positivity rate by PCR and a lower frequency of gametocyte-specific transcript carriage at day 3, than with CQ alone, although these differences did not reach statistical significance.

Interestingly, CQ treatment failure was more frequently detected when PCR was used to diagnose recurring parasitemias. ACPR rates over 28 days decreased to 92.1% and 98.8% in our patients treated with CQ alone and CQ-PQ, respectively, when PCR-detected *P. vivax* infections at day 28 were also considered. We suggest that low-density recrudescences of drug-resistant *P. vivax* subpopulations occur following CQ treatment in areas with low-grade CQ resistance but may be entirely missed by routine surveillance. We argue that these low-density parasitemias may remain subpatent until day 28 of follow-up, leading to overestimated cure rates in microscopy-based studies with relatively short follow-up. We suggest that at least some of these subpatent parasitemias detected only by PCR on day 28 might have later been detected by standard microscopy in a clinical trial with an extended duration. Indeed, most CQ failures in a recent trial in Manaus, Brazil, were seen between days 29 and 42 after treatment and would have been missed in a 28-day trial (9). Whether monitoring CQ responses requires an extended follow-up is open to debate, and further data on CQ levels beyond day 28 of standard CQ regimens are urgently needed to inform clinicians and policy makers.

Parasite clearance delayed beyond day 3 was recently shown to be significantly associated with a higher risk of *P. vivax* recurrence at day 28 (30). Here we confirm that subjects with parasitemia detected by PCR (but not by microscopy) at day 3 were more likely to have day 28 recrudescence detected by either microscopy or PCR. Nevertheless, not all subjects with treatment failure by day 28 had delayed parasite clearance characterized in our and other studies (30), indicating that a short, 3-day follow-up of CQ-treated subjects would be unable to predict treatment failure by day 28 with acceptable sensitivity.

We further examined whether CQ coadministration greatly increased the antirelapse efficacy of PQ, as previously suggested (17). We acknowledge that this study was underpowered for detecting small differences between treatment arms, but available

data suggested an overwhelming potentiation of PQ by concomitant CQ or QN use (16–18). Alving and colleagues, for example, randomized 57 adult vivax malaria patients to either arm 1, with 2 g/day of QN for 14 days followed by 15 mg/day of PQ for 14 days starting on day 28; arm 2, with 2 g/day of QN concomitantly with 15 mg/day of PQ, both for 14 days; or arm 3, with 1,000 mg of CQ over 2 days concomitantly with 15 mg/day of PQ, the latter for 14 days ($n = 19$ in each arm). After 12 months of follow-up, 15 of 19 (79%) patients in arm 1 had relapsed, compared with only 1 of 19 (5%) in arm 2 and 5 of 19 (26%) in arm 3 (17). The vast majority of symptomatic relapses in malaria-free areas of Brazil were seen within 4 to 5 months after treatment (31), suggesting that our extended 180-day surveillance would be able to capture most of them. Surprisingly, the time to the first slide-positive vivax malaria recurrence was actually slightly shorter, although not significantly, among subjects given CQ and PQ concomitantly (mean, 148 days), than for those on the sequential CQ-PQ regimen (mean, 158 days). Survival analysis further indicated that the risk of recurrence was not modulated by patients' CYP2D6 polymorphisms that decrease PQ metabolism. These findings must not be overinterpreted but indicate that further studies are required to examine, in patient populations with various CYP2D6 activity levels, the opposing effects of CQ coadministration on PQ efficacy. A delicate balance between CQ- or QN-mediated potentiation of PQ activity (18) and CQ- or QN-dependent inhibition of CYP2D6-mediated generation of active PQ metabolites (19), which may be more pronounced in individuals with low-activity CYP2D6 variants, will determine the outcome of antirelapse treatment. We suggest that the timing of PQ administration (and possibly of tafenoquine as well) relative to CQ requires further optimization in order to increase the efficacy of radical-cure regimens in vivax malaria.

The main limitation of this and other recent studies that examined the efficacy of two or more regimens for vivax malaria in Brazil (13) is their limited power for detecting differences in comparisons between study arms. Some significant differences, however, were found between treatment groups and may stimulate further investigation, especially into CQ-PQ interactions and risk of relapses following concomitant versus sequential CQ-PQ administration. Our current understanding of the CQ- or QN-mediated potentiation of PQ effects actually came from rather small studies (16–18). Moreover, we genotyped a limited number of CYP2D6 polymorphisms. These were the alleles that had been previously described in Brazilian populations, but we may have missed undescribed polymorphisms associated with major phenotypic expression. A further potential limitation is that we enrolled patients with low-grade parasitemias (<250 asexual blood-stage parasites/μl) at admission, which may reduce our ability to detect and quantify declining parasite densities following CQ treatment. We argue that subjects with low-grade parasitemias constitute a large proportion of patients who seek treatment in most sites of endemicity in Brazil, especially in urban areas, given the easy and rapid access to free diagnosis and treatment in a widespread network of government-run malaria clinics (29). Therefore, these are the real-life patients treated with CQ-PQ regimens whose efficacy must be evaluated. Moreover, we argue that PCR-based diagnosis has substantially increased our ability to detect and quantify residual posttreatment parasites in the present study, even when pretreatment parasite loads were low.

We conclude that *P. vivax* resistance to CQ has emerged in the main site in Brazil where malaria is endemic, underscoring the need for continuous monitoring in this and neighboring countries. The value of including a CQ-only treatment arm in clinical trials is confirmed by our data, further indicating that trials with concomitant CQ-PQ administration may have underestimated CQ resistance rates in this setting (14) and potentially other settings in South America (12). Whether *ex vivo* assays play a major role in routine surveillance remains to be further evaluated, given the relatively small proportion of field-collected parasite isolates that meet all criteria for *in vitro* testing. Finally, the "potentiation" versus "CYP2D6-mediated metabolism suppression" dilemma regarding the concomitant administration of CQ and PQ calls for further evaluation of

different PQ timings, relative to CQ and other schizontocidal drugs (including artemisinin derivatives), in patients with various CYP2D6 activity levels.

MATERIALS AND METHODS

Study site and design. The study site, Mâncio Lima (07°36′ 51″S, 72°53′ 45″W), is situated in the upper Juruá Valley, next to the border with Peru (see Fig. S1 in the supplemental material). Juruá Valley is unique in that a large proportion of malaria infections are reportedly acquired in urban settings, up to 45% in Mâncio Lima, compared with the country's average of 17% in 2013 (32). Fish farming ponds that opened over the past 2 decades are now the main larval habitats for malaria vectors across this town (33). With 17,910 inhabitants and 9,278 slide-confirmed malaria cases notified in 2017, the municipality of Mâncio Lima currently has the highest annual parasite incidence (API) in Brazil (518.0 malaria cases per 1,000 inhabitants [Ministry of Health of Brazil, unpublished data]).

We carried out an open-label randomized clinical trial to monitor the efficacy of CQ alone and the CQ-PQ combination in the treatment of uncomplicated *P. vivax* malaria (ClinicalTrials.gov registration number NCT02691910). The primary objective was to assess, over 28 days of follow-up, the efficacy of CQ alone ("sequential CQ-PQ" [arm 1], with PQ withheld until the end of follow-up) and the CQ-PQ combination ("concomitant CQ-PQ" [arm 2]) as schizontocidal therapies for uncomplicated vivax malaria. The secondary objective was to assess the efficacy of concomitant and sequential CQ-PQ regimens as radical cures for uncomplicated vivax malaria over an extended, 6-month follow-up. From June 2014 through July 2015, 204 patients with uncomplicated single-species *P. vivax* infection were randomly allocated to either of the two treatments (102 to each arm). Note that study participants in arm 1 received PQ at a time when CQ blood levels were expected to be decreasing and less likely to severely inhibit CYP2D6-mediated metabolism. The study protocol followed the Pan American Health Organization (PAHO) recommendations for *P. vivax* drug efficacy trials (34). We used the exact binomial approach to calculate sample size (35), assuming an ACPR rate of 95% at day 28 (10) and a study dropout rate of 15%, with a final enrollment target of 102 subjects per arm. The study was not originally designed and powered to compare ACPR rates between treatment arms.

Study subjects. Eligible subjects were vivax malaria patients of either sex, aged between 5 and 70 years, with fever (axillary temperature of ≥37.5°C) or history of fever in the past 48 h, attending the three government-run malaria clinics in the town of Mâncio Lima. Because over 95% of the study participants were born in the municipality of Mâncio Lima, we consider age a proxy of the duration of past exposure to malaria. Only subjects living in the urban or nearby periurban areas of Mâncio Lima were enrolled, since directly observed therapy and 28-day follow-up would be impractical for study participants living in remote rural sites. All study subjects had to have a *P. vivax* single-species infection confirmed by both microscopy and PCR, but no minimal parasite density was set because subjects with <250 asexual parasites/μl of blood (the recommended parasite density threshold for enrollment according to the PAHO protocol) represent a large proportion of the actual population of patients seeking malaria treatment in Brazil. Given that diagnosis and treatment are provided at no cost in a vast network of malaria clinics, over two-thirds of malaria episodes in Juruá Valley are treated within 48 h after the onset of clinical symptoms, and vivax malaria patients rarely have relatively high-grade parasitemias. Exclusion criteria were severe or complicated malaria, pregnancy or lactation, severe malnutrition (weight-for-age z-scores less than or equal to −3), severe anemia (hemoglobin level of <8.0 g/100 ml), glucose-6-phosphate dehydrogenase (G6PD) deficiency, history of a serious chronic condition (including cardiovascular and psychiatric disorders, liver cirrhosis, chronic renal failure, and HIV/AIDS), antimalarial use in the preceding 2 weeks, and known hypersensitivity or allergy to study drugs. Women of childbearing age (11 to 45 years) were tested for pregnancy before enrollment. Prospective participants were tested for G6PD deficiency using a rapid chromatographic test (BinaxNow G6PD; Alere, Waltham, MA) approved for diagnostic use in Brazil (36); G6PD-deficient subjects were excluded before randomization. A 40-ml pretreatment venous blood sample was collected for (i) molecular confirmation of single-species malaria diagnosis and *P. vivax* gametocyte detection, (ii) determination of hemoglobin levels and platelet counts using an Abx Micro 60 automated cell counter (Horiba, Montpellier, France), (iii) CYP2D6 genotyping (see below), and (iv) leukocyte depletion with BioR 01 Plus filters (Fresenius Kabi, Bad Homburg, Germany), as described previously (37), followed by parasite cryopreservation in liquid nitrogen for *ex vivo* CQ resistance monitoring (see below).

Treatment and follow-up over 28 days. Study drugs, all supplied by Farmanguinhos (Rio de Janeiro, Brazil), were CQ (150-mg tablets) and PQ (15-mg or 5-mg tablets). CQ was administered orally over three consecutive days (days 0, 1, and 2), under direct observation by a study nurse, with a total dose of 25 mg/kg of body weight (28). PQ (total dose of 3.5 mg/kg) was administered under direct observation over 7 days, starting either on day 0 (arm 2) or on day 29 (arm 1). Patients vomiting within 30 min of CQ or PQ administration were given another dose. Follow-up visits were made by a study nurse at the patients' homes on days 1, 2, 3, 7, 14, 21, and 28. Patients were also advised to return to the health care facility where they had been enrolled whenever they felt sick, passed dark urine, or had abdominal cramps (signs of PQ toxicity), for clinical and laboratory assessment between the scheduled home visits. Venous blood was collected at each home visit and whenever patients returned to health care facilities and examined for malaria parasites. Patients with recurrent *P. vivax* parasitemia detected by microscopy within 28 days of follow-up were retreated with the standard concomitant CQ-PQ regimen (28) and closely monitored for clinical and parasitological responses.

Passive surveillance over 6 months. No home visit with blood sample collection was routinely scheduled after day 28. Recurrent parasitemias between days 29 and 180 (due to either late recrudescences, relapses, or new infections) were diagnosed through passive surveillance (i.e., we did not actively

search for parasitemic study participants after day 28 but instead collected data on malaria episodes diagnosed among them). To this end, study participants were instructed to visit government-run malaria outposts whenever malaria-related signs and symptoms returned. Information on laboratory-confirmed malaria episodes diagnosed in the study population up to 6 months after treatment was retrieved from the Malaria Epidemiological Surveillance and Information System (SIVEP) electronic database of the Ministry of Health of Brazil. Malaria is a notifiable disease in Brazil; diagnosis and treatment are not offered by local private clinics, and antimalarials cannot be purchased in drugstores. We therefore assume that virtually all malaria episodes in study participants over this period were treated in public facilities and notified to the Ministry of Health. We further assume that a negligible proportion of study participants may have moved away from the study site and had repeated malaria episodes diagnosed outside the municipalities of Mâncio Lima and Cruzeiro do Sul (the nearest city), for which we had complete malaria case records for the study period. Indeed, we found the vast majority of study participants during a population census carried out in urban Mâncio Lima by our field team between November 2015 and April 2016 (A. Pincelli, R. M. Corder, and M. U. Ferreira, unpublished data). Recurrent vivax malaria episodes during the extended follow-up period were treated with the standard concomitant CQ-PQ regimen; incident falciparum malaria was treated with a 3-day course of artemether (2 to 4 mg/kg/day) plus lumefantrine (12 to 24 mg/kg/day) (28).

Laboratory diagnosis of malaria. At least 200 thick smear fields were examined on-site by an experienced microscopist, at a ×1,000 magnification, and revised by an expert microscopist, blind to the initial reading, before a slide was declared negative. Whenever discrepancies between readings were found, a third microscopist, blind to both readings, provided the definitive diagnosis. Parasite densities were estimated by the expert microscopist by counting the number of asexual blood-stage parasites against 200 leukocytes, assuming 6,000 leukocytes/μl of blood. We used 200-μl aliquots of venous blood samples collected at each home visit to isolate parasite DNA, using QIAamp DNA blood kits (Qiagen, Hilden, Germany), for confirmatory molecular diagnosis of malaria by quantitative real-time PCR targeting a species-specific 100-bp fragment of the *Plasmodium falciparum* and *P. vivax* 18S rRNA genes. We used a Step One Plus real-time PCR system (Applied Biosystems, Foster City, CA) for PCR amplification as described previously (38). The detection threshold of this diagnostic PCR is approximately 3 parasites/μl of blood. No-template controls, containing all reagents for amplification except for the DNA template, were run for every PCR microplate. Parasite density estimates obtained by PCR and expert microscopy were strongly correlated (Spearman correlation coefficient [r_s] = 0.675; $P < 0.0001$).

RT-PCR for *P. vivax* gametocyte transcripts. We used 200-μl venous blood aliquots cryopreserved in liquid nitrogen for RNA isolation with the QIAamp viral RNA minikit (Qiagen). Eluted RNA was treated with RNase-free DNase (Fermentas) for removal of residual genomic DNA from templates for cDNA synthesis. SYBR green-based reverse transcriptase PCR (RT-PCR) was used to amplify 267-bp transcripts of the gametocyte-specific *pvs25* gene as described previously (39). The following negative controls were used: (i) to control for genomic DNA contamination, a RT-minus control (containing all reagents for reverse transcription except for RT) was run for each RNA sample, and (ii) to control for reagent contamination, no-template controls (containing all reagents for reverse transcription except for the RNA template) were run for every PCR microplate. As a control for cDNA template integrity, for each sample, we amplified the 18S rRNA gene of *P. vivax* as described above, since this gene is expressed by all parasite blood stages. RNA isolation and cDNA synthesis were repeated whenever amplification of the 18S rRNA control product failed. If no 18S rRNA control product was obtained after the second amplification attempt, with the new RNA template, the sample was excluded from analysis. As a positive control for *pvs25* gene amplification, genomic *P. vivax* DNA templates were run for every PCR microplate.

Parasite genotyping. Genotyping was carried out to determine whether parasitemia reappearing at day 28 was due to a recrudescence of the original parasite strain circulating on day 0. We used PCR to genotype six highly polymorphic single-copy markers: one variable domain of the merozoite surface protein 1 gene (*Msp1F1*) and five microsatellite DNA markers, namely, *Pv3.27*, *MS3*, *MS6*, *MS9*, and *MS16*. PCR products were amplified and analyzed by capillary electrophoresis on an ABI 3500 automated DNA sequencer (Applied Biosystems) essentially as described previously (40, 41); their lengths (in base pairs) and relative abundances (peak heights in electropherograms) were determined using GeneMapper 4.1 software (Applied Biosystems). The minimal detectable peak height was set to 200 arbitrary fluorescence units. Because stutter bands may occasionally be observed in microsatellite genotyping, we scored two alleles at a locus only when the minor peak was >33% of the height of the predominant peak. Multilocus genotypes were defined as unique combinations of alleles at each locus analyzed; samples were considered to harbor identical parasites when they had exactly the same predominant (or only) genotype.

CYP2D6 genotyping. Over 100 CYP2D6 variant alleles have been defined by the cytochrome P450 nomenclature committee (http://www.cypalleles.ki.se/cyp2d6.htm), consisting of single-nucleotide polymorphisms (SNPs), small insertions and deletions, and copy number variations (CNVs) arising from deletion or duplications of the entire gene. We genotyped eight single-nucleotide polymorphisms and one trinucleotide deletion at the *CYP2D6* locus that are commonly found in Brazil (42–45): −1584C>G (rs1080985), 100C>T (rs1065852), 1023C>T (rs28371706), 1846G>A (rs3892097), 2615–2617delAAG (rs5030656), 2850C>T (rs16947), 2988G>A (rs28371725), 3183G>A (rs59421388), and 4180G>C (rs1135840). To this end, we used CYP2D6 TaqMan SNP genotyping assays (Applied Biosystems) with specific hydrolysis probes (44). Amplification and fluorescence detection were carried out using the ViiA 7 real-time PCR system (Applied Biosystems). We further used the Hs00010001_cn copy number TaqMan assay (Applied Biosystems), which targets exon 9, to determine CNVs at the *CYP2D6* locus in our study participants. Amplification reactions were carried out on a ViiA 7 real-time PCR system (Applied

Biosystems) as described previously (44). Study participants were grouped according to predicted CYP2D6 phenotypes following the activity score (AS) model (46), which assigns activity values (0.0, 0.5, or 1.0) to each allele and defines the AS of a genotype as the sum of these values for each allele, which may exceed 2 due to allele CNV. Subjects were classified as poor metabolizers (gPM) (AS = 0), intermediate metabolizers (gIM) (AS = 0.5), normal-slow metabolizers (gNM-S) (AS = 1), normal-fast metabolizers (gNM-F) (AS = 1.5 to 2.0), and ultrarapid metabolizers (gUM) (AS > 2).

Outcome measures and data analysis. The intention-to-treat (ITT) study population included all enrolled patients who received at least one treatment dose, whereas the per-protocol (PP) population included only patients who completed the full supervised treatment course and attended all scheduled visits, with outcome data for the primary efficacy endpoint (Fig. 1). The primary outcome was ACPR, defined as the absence of asexual blood-stage parasites detected by microscopy in the PP population by day 28, regardless of axillary temperature, with no evidence of earlier treatment failure (47). The secondary endpoint was the time of the first recurrent vivax malaria episode confirmed by microscopy, among study subjects who had no parasite recrudescence diagnosed by day 28, over the extended follow-up between days 29 and 180 of CQ therapy. We additionally considered the following endpoints over the 28-day follow-up of the ITT population: (i) proportions of patients with blood-stage parasites (detected by microscopy or PCR) and gametocytes (detected by RT-PCR) at selected time points, (ii) parasite clearance time (PCT) (time from the first CQ dose to the first microscopically negative slide), (iii) parasite DNA clearance time (pDNA CT) (time from the first CQ dose to the first quantitative PCR [qPCR]-negative sample), and (iv) fever clearance time (FCT) (time from the first CQ dose to the first normal temperature reading, among those who were febrile at admission). Parasitological failure was defined as the persistence or reappearance of *P. vivax* asexual blood stages between days 7 and 28 in the presence of therapeutic levels of CQ and DCQ. Whole-blood CQ and DCQ concentrations were measured, under contract, at the Analytical Service of the London School of Hygiene and Tropical Medicine, United Kingdom; high-performance liquid chromatography coupled with a photodiode array detector was used.

Proportions are given with 95% confidence intervals (CIs) calculated with Wilson's continuity correction, whenever appropriate, and were compared with Fisher's exact tests (2-by-2 tables), McNemar tests (for repeated samples from the same subjects), or χ^2 tests (2-by-*n* tables). Continuous variables were compared with Mann-Whitney U tests. The time to the first vivax malaria recurrence, PCT, pDNA CT, and FCT were estimated with Kaplan-Meier survival analysis and compared with Mantel-Cox log rank tests. We used Cox proportional-hazards models to compare hazard ratios (HRs) for the time to the first vivax malaria recurrence in subjects given sequential CQ-PQ (arm 1) and in those given concomitant CQ-PQ (arm 2) while adjusting for subjects' age (stratified as <13, 13 to 20, 21 to 40, and >40 years), gender, and CYP2D6 activity level. CYP2D6 activity scores calculated according to the AS model were entered in Cox proportional-hazards models as a dichotomous variable, either an AS of ≤1.0 (gPM, gIM, and gNM-S) or an AS of >1.0 (gNM-F and gUM). Separate Cox models were run with additional adjustment for total PQ dose (milligrams per kilogram), with quite similar results (data not shown). Analyses were done using SPSS version 17.0 (SPSS, Chicago, IL) and STATA 14.0 (STATA, College Station, TX), with statistical significance set at the 5% level.

***Ex vivo* CQ sensitivity assay.** Leukocyte-depleted, pretreatment blood samples cryopreserved in liquid nitrogen were carefully thawed as described previously (48). Red blood cells were resuspended in McCoy's 5A medium supplemented with glucose (0.5%, wt/vol), HEPES (25 mM), hypoxanthine (0.005%, wt/vol), and 25% heat-inactivated human serum matching the parasite donor's blood type, to a final hematocrit of 2%. CQ sensitivity was evaluated using a standard 44-h schizont maturation inhibition assay in 96-well flat-bottomed microplates maintained in a gas chamber at 37°C with controlled O_2 and CO_2 levels. We tested pretreatment *P. vivax*-infected blood samples from study participants (collected between 2014 and 2015) and nine additional cryopreserved *P. vivax* isolates from urban Mâncio Lima collected between 2015 and 2017, all of them with >1,000 parasites/μl of blood and >50% ring stages at the time of thawing (6). Test microplates had the following concentrations of CQ diphosphate salt (catalog number C6628; Sigma-Aldrich, St. Louis, MO): 3,200 nM, 1,600 nM, 800 nM, 400 nM, 200 nM, 100 nM, 50 nM, 25 nM, 12.5 nM, 6.2 nM, and 3.1 nM. The assay was carried out as previously described (6). Parasite counts and staging were assessed by microscopy, and mature schizonts were defined as those with ≥4 nuclei. IC_{50} values were estimated using ICEstimator 1.2 software (http://www.antimalarial-icestimator.net/index.htm). There is no consensus regarding the IC_{50} value indicative of CQ resistance in *P. vivax*, but here we adopt the tentative cutoff value of 100 nM (23).

Ethical approval. The institutional review board of the Institute of Biomedical Sciences, University of São Paulo, approved the study protocol (1169/CEPSH, 2014). Written informed consent was obtained from all study participants or their parents or guardians; assent was obtained from children aged less than 18 years.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01965-18.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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