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BRIEF REPORT

Estimation of the Antirelapse Efficacy of Tafenoquine, Using *Plasmodium vivax* Genotyping

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Prevention of relapse of *Plasmodium vivax* infection is a key treatment goal in malaria. Use of *P. vivax* genotyping in a multicenter, double-blind, randomized, placebo-controlled phase 2b study in Peru, India, Thailand, and Brazil allowed determination of genetically heterologous or homologous *P. vivax* infection recurrence following receipt of chloroquine plus one of 4 doses of tafenoquine (50, 100, 300, or 600 mg) or chloroquine plus primaquine, compared with receipt of chloroquine alone. The antihypnozoite efficacy of tafenoquine was evident as a reduction in homologous recurrences of *P. vivax* infection as drug doses were increased. No clear dose-response pattern was evident for heterologous recurrences of *P. vivax* infection. Rates of homologous recurrence of *P. vivax* infection appear to be clinically useful for comparing drug efficacy for the prevention of *P. vivax* infection relapse.

Clinical Trials Registration. NCT01376167.

Keywords. tafenoquine; *Plasmodium vivax*; genotyping; antihypnozoite; efficacy.

One third of the world's population is at risk for *Plasmodium vivax* malaria [1]. Periodic reactivation of a dormant liver stage, the hypnozoite, causes repeated clinical relapses of a single *P. vivax* infection (hereafter referred to as "*P. vivax* relapse"). Prevention of *P. vivax* relapses is a key treatment goal in malaria because of their significant morbidity and economic burden [2]. Also, as the hypnozoite reservoir maintains the potential for further transmission, relapse prevention is essential for effective malaria control and eradication.

Tafenoquine is an 8-aminoquinoline in development as a single-dose treatment for the prevention of *P. vivax* relapse. Evaluating drugs to prevent *P. vivax* relapse is challenging because recurrent *P. vivax* infection (hereafter, "*P. vivax* recurrence") cannot be easily resolved into recrudescence (ie, failure to treat the initial infection), reinfection (ie, new infection), or relapse (ie, hypnozoite reactivation). Recently, evaluation techniques for *P. vivax* genotype markers have been developed, based on capillary electrophoresis [3, 4] and amplicon deep sequencing [5]. Although distinguishing between the causes of parasite recurrence remains problematic, genotyping improves the evaluation of drug efficacy, compared with microscopy alone [3–5].

This study reports *P. vivax* genotyping data from a multicenter, double-blind, randomized, placebo-controlled phase 2b tafenoquine dose-selection study (DETECTIVE; clinical trials registration NCT01376167), conducted across 7 sites in Peru, India, Thailand, and Brazil [6]. Treatment with the antihypnozoite drugs tafenoquine (50, 100, 300, and 600 mg) and primaquine, in combination with chloroquine, were compared to treatment with chloroquine alone, to determine their effect on the frequency of genetically heterologous or homologous *P. vivax* recurrences.

METHODS

This clinical trial conformed to good clinical practice, the Declaration of Helsinki (2000), and all applicable regulatory requirements. Protocol approval was obtained from each study site's ethics committee or institutional review board. Written informed consent was obtained from subjects or from parents or guardians of subjects combined with the subject's assent. Full study methods have been reported previously [6].

Eligible patients were of either sex, aged ≥16 years, and had microscopically confirmed *P. vivax* monoinfection (parasite density, >100 to <100 000 parasites/μL blood). Major exclusion criteria were recent antimalarial treatment, severe malaria, concurrent illness, pregnancy/lactation, and glucose-6-phosphate dehydrogenase enzyme activity of <70% of the derived site population reference value, owing to the hemolysis risk with 8-aminoquinolines.

All enrolled patients received once-daily oral chloroquine on day 1 (600 mg), day 2 (600 mg), and day 3 (300 mg). Patients were randomly assigned to one of 6 treatment groups: single-dose oral tafenoquine on day 1 or 2 at doses of 50 mg

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(n = 55), 100 mg (n = 57), 300 mg (n = 57), or 600 mg (n = 56); oral primaquine 15 mg for 14 days (n = 50); or chloroquine alone (n = 54).

Patients were followed up for 180 days. Giemsa-stained thick blood smears for parasite assessments were performed at screening; after treatment on day 1 and days 2 and 3 twice daily every 6–12 hours until parasite clearance; and on days 8, 15, 22, 29, 60, 90, 120, and 180 or when the patient presented with malaria symptoms.

In the case of *P. vivax* recurrence, parasite genomic DNA was amplified from blood specimens collected using the dried blood spot technique on FTA Classic Cards (Whatman, Germany) according to the manufacturer's protocol (BD08). Genotyping was performed using published methods [3]. Primary multiplex polymerase chain reaction (PCR) used published primer sequences for 3 discriminatory markers: merozoite surface protein 1 (*msp1*F3) and microsatellites MS16 and *Pv3.27* [3]. Fifty microliters of primary PCR master mix contained 1× Phusion HF Buffer (New England Biolabs), 1.4 mM dNTPs (Invitrogen, Germany), 0.25 μM of each primer (Eurofin MWG Operon, Germany), 1 unit of Phusion High-Fidelity Polymerase (New England Biolabs), and 1 processed punch (3 mm) from FTA cards. From the primary multiplex PCR, 0.5 μL of PCR product was used as a template for nested PCR amplification of *msp1*F3, MS16, and *Pv3.27*, using previously published primers, reaction mix, and thermocycling conditions [3]. PCR products were checked for quality and to estimate the product amount, using agarose-gel electrophoresis. Failed samples not yielding PCR products were repeated 3 times. Capillary electrophoresis was conducted to determine amplicon sizes [3]. Samples were analyzed by ABI3130*xl,* using GS500LIZ as size standard (Applied Biosystems), and data were analyzed using GeneMapper, version 3.7 (Applied Biosystems). Alleles were grouped by size into 3–base pair bins for coding regions and by repeat length for microsatellites (3 base pairs for MS16 and 4 base pairs for *Pv3.27*). If recurrent infections contained identical alleles for all 3 markers versus the enrollment sample, circumsporozoite protein (*Pvcsp*) and apical membrane antigen 1 (*Pvama1*) loci were sequenced [7, 8]. Quality control was performed on 20 sample pairs at the Institute of Tropical Medicine, Antwerp.

Recurrent infections that differed by at least 1 marker from pretreatment infections were defined as heterologous; all others were considered homologous. A post hoc Kaplan–Meier analysis (log-rank test) estimated the risk of homologous or heterologous recurrence for chloroquine, primaquine, low-dose tafenoquine (50 or 100 mg), and high-dose tafenoquine (300 or 600 mg), with censoring at the time of the event for recurrent infections not determined as homologous or heterologous, infections in patients who were lost to follow-up, or patients with no recurrence.

Virtual heterozygosity was calculated using the equation $[n/(n-1)] \times [1 - \Sigma\, p_i^2]$, where n is the number of samples analyzed and p_i is the frequency of the allele i [4]. Mean multiplicity of infection (MOI) was estimated for all markers independently by dividing the total number of *P. vivax* clones detected by the number of samples that were PCR positive for the parasite.

RESULTS

Of 329 patients recruited to the study and treated, 100 (30.4%) presented with *P. vivax* recurrence. Five patients had missing samples for PCR; thus, 95 of 329 patients (28.9%) had recurrences classified as heterologous or homologous (Figure 1*A*). In Peru, 60 of 136 patients had recurrences (30 heterologous and 30 homologous); in Thailand, 18 of 99 (6 and 12, respectively); in Brazil, 16 of 37 (2 and 14, respectively); and in India, 1 of 57 (0 and 1, respectively). In the Kaplan–Meier analysis, 25 patients were censored at their last visit, owing to missing efficacy outcomes for day 180.

Heterologous recurrences could not be used to distinguish between tafenoquine doses (Figure 1*B*). Kaplan–Meier estimates indicated that there was no statistically significant difference in heterologous recurrence rate between high-dose and low-dose tafenoquine (6.7% and 15.2%, respectively; treatment difference, −8.5% [95% confidence interval {CI}, −17.9% to 1.0%]; $P = .069$). Only high-dose tafenoquine produced a statistically significant decrease in the heterologous recurrence rate, compared with chloroquine (treatment difference, −27.5% [95% CI, −47.4% to −7.6%]; $P < .001$), although heterologous recurrences tended to decrease with the 8-aminoquinolines. Heterologous recurrences in the chloroquine group mostly occurred between days 35 and 50 and were relatively delayed with tafenoquine.

Both tafenoquine and primaquine have activity against blood-stage infection, and the long half-life of tafenoquine may have reduced heterologous recurrences by decreasing the parasite reinfection rate. However, reinfection is thought to be infrequent in areas of low endemicity, as in this study, and there were no treatment failures in the chloroquine group before day 28. All subjects were provided with insecticide-treated bed nets, although their use was not audited. Thus, prevention of heterologous recurrence could be explained as a combination of reinfection prophylaxis and the antihypnozoite effect of high-dose tafenoquine in this setting.

In contrast to the heterologous recurrences, homologous recurrences showed a dose-response for tafenoquine (Figure 1*C*). Kaplan–Meier analysis indicated that the rate of homologous recurrence was just 2.9% with high-dose tafenoquine, compared with 31.2% with low-dose tafenoquine (treatment difference, −28.3% [95% CI, −37.9% to −18.7%]; $P < .001$). Homologous recurrences occurred in 39.4% of patients in the chloroquine group and were significantly reduced with high-dose tafenoquine (treatment difference, −36.5% [95% CI, −51.8% to

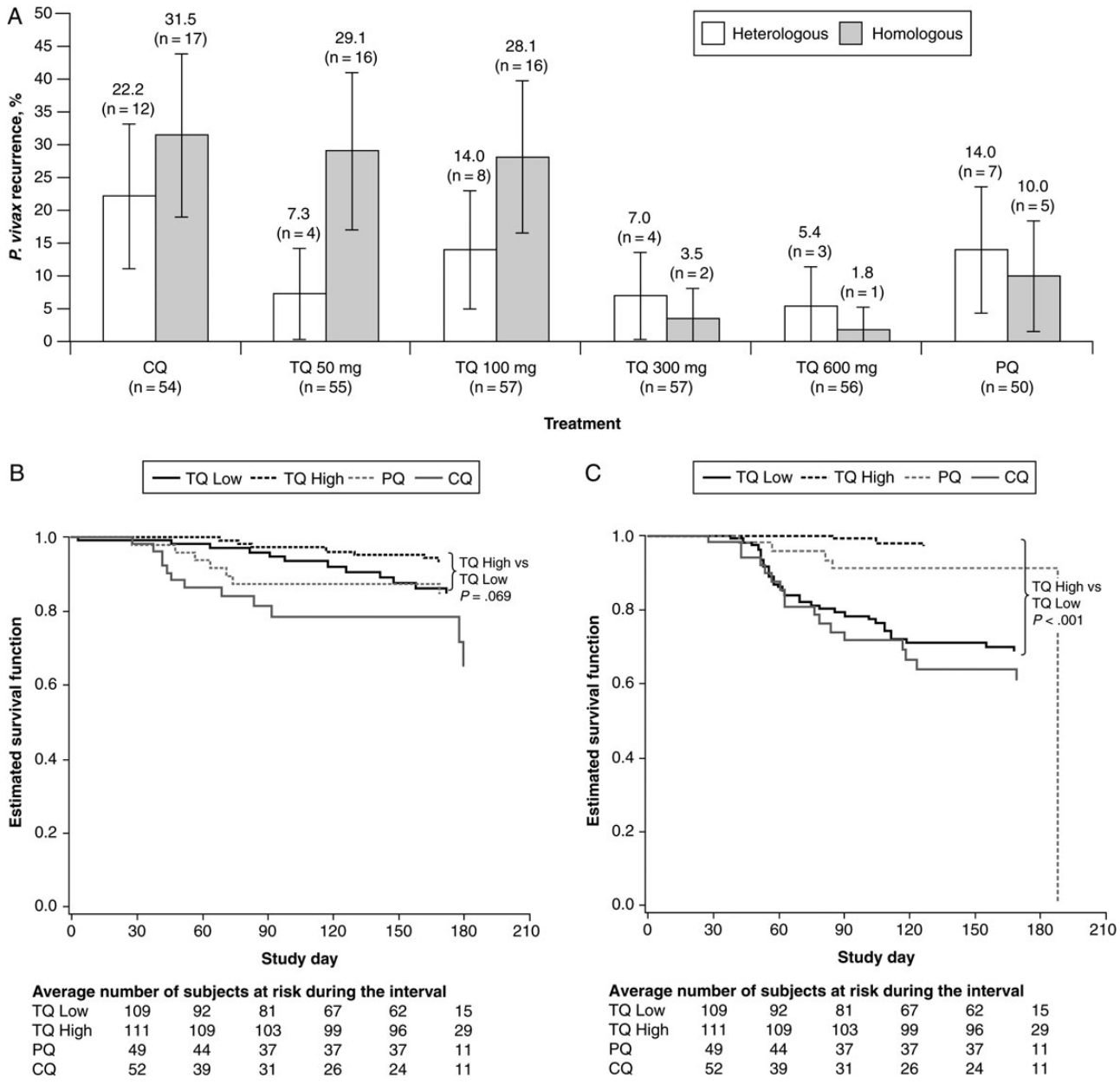

Figure 1. *Plasmodium vivax* infection recurrence (hereafter, "*P. vivax* recurrence"). *A*, Frequency of genetically heterologous and homologous *P. vivax* recurrence following treatment with chloroquine (CQ), with or without one of 4 doses of tafenoquine (TQ), or with CQ and primaquine (PQ). *B*, Kaplan–Meier analysis of heterologous *P. vivax* recurrence following treatment with CQ, with or without high-dose (300 or 600 mg) or low-dose (50 or 100 mg) TQ, or with CQ and PQ. *C*, Kaplan–Meier analysis of homologous *P. vivax* recurrence following treatment with CQ, with or without high-dose (300 or 600 mg) or low-dose (50 or 100 mg) TQ, or with CQ and PQ. Abbreviations: CQ, chloroquine; PQ, primaquine; TQ, tafenoquine.

−21.2%]; $P < .001$) and primaquine (treatment difference, −30.5% [95% CI, −47.6% to −13.3%]; $P = .001$). The effect of low-dose tafenoquine was similar to that of chloroquine alone (treatment difference, −8.2% [95% CI, −25.7% to 9.3%]; $P = .394$).

The observed dose-response for tafenoquine for prevention of homologous recurrence would be expected if an antihypnozoite effect of these drug regimens could be detected. This strongly suggests that, in areas of low endemicity, homologous recurrences mainly represent relapses.

There were 3 homologous recurrences with high-dose tafenoquine (days 85 and 127 in Brazil and day 105 in Thailand) and 5 in the primaquine group (days 42–189 in Peru). These homologous recurrences could be attributed to a drug failure against hypnozoites. There is some clinical evidence for primaquine that associates 2D6 drug metabolism to antihypnozoite drug failure

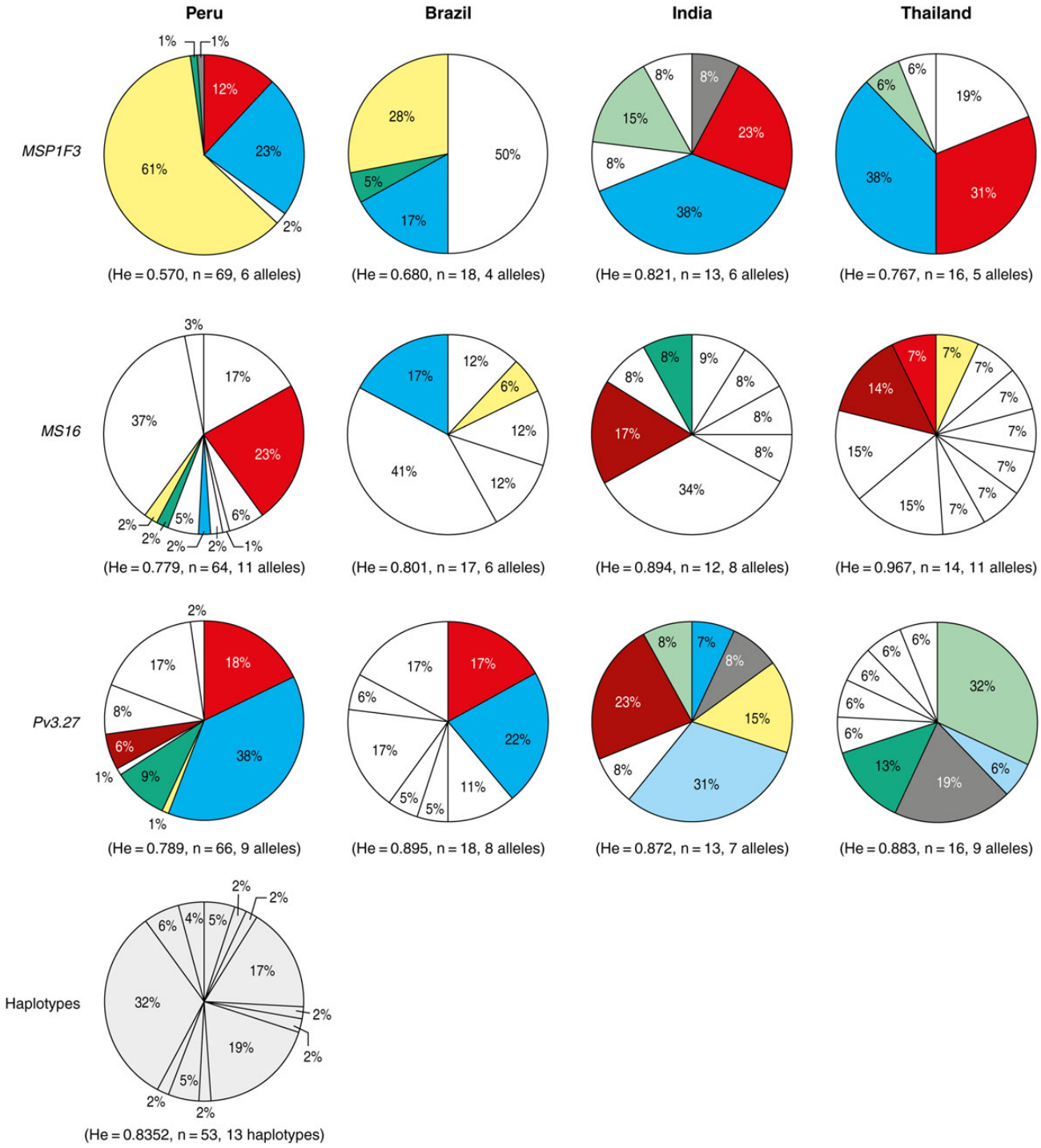

Figure 2. *Plasmodium vivax* genotype diversity. Pie charts represent allelic frequencies of different-sized fragments for each of 3 potential genetic markers among *P. vivax*–positive samples at study sites in Peru (n = 199), Brazil (n = 53), India (n = 38), and Thailand (n = 46), indicating that heterogeneity was generally low. Colored sections denote the frequencies of identical alleles in each marker in different sites. White sections denote the frequencies of identical alleles that occurred only at the specified site. The lower chart represents the discernible haplotypes for the Peru site. All sites had only unique haplotypes. Abbreviation: He, virtual heterozygosity index.

[9]. However, recent analysis of clinical data and prospective preclinical data indicate that this may not be the case for tafenoquine (St. Jean, unpublished data). Alternatively, if parasite genetic diversity was low, failures could represent homologous reinfection.

To understand the likelihood of homologous recurrences being caused by relapse versus reinfection, parasite genetic diversity was examined. Heterogeneity indices for the microsatellite markers were variable between countries (Figure 2) and

generally consistent with previous reports [10–12]. Genetic diversity was mostly low (heterozygosity, 0.59–0.96), with 4–11 different circulating strains. Thus, it is possible that a proportion of homologous recurrences could be caused by reinfection. Alternatively, for a minority of variants, greater sensitivity of genetic testing may be required to distinguish heterology.

As another indicator of low endemicity in the study areas, a low mean MOI (±SD) was observed for Brazil (1.06 ± 0.246) and Peru (1.11 ± 0.318). In areas of high endemicity, much higher MOI values have been observed (eg, 2.7 in Papua New Guinea) [4]. The mean MOI estimates (±SD) for Thailand (1.77 ± 1.07) and India (1.50 ± 0.704) were intermediate and consistent with previous reports [11, 13].

Notably, in only 4 of 95 paired samples did subsequent sequencing of *ama1* or *csp* loci result in a difference between the pretreatment sample and recurrent sample in cases where *msp1*F3, MS16, and *Pv3.27* were identical.

DISCUSSION

In this placebo-controlled trial, the effect of antihypnozoite drug pressure with primaquine or 4 different doses of tafenoquine on the genotypes of *P. vivax* recurrences was determined. This novel study indicates that it is feasible, within a clinical trial, to further analyze antihypnozoite efficacy by *P. vivax* genotyping with a precision that has relevance for regulatory assessment. Although data interpretation is not straightforward, the antihypnozoite drug efficacy of tafenoquine was evident as a dose-dependent reduction in homologous recurrences. No clear dose-response pattern was evident for heterologous recurrence. Thus, we conclude that the reduction in homologous recurrences with high-dose tafenoquine is a consequence of its antihypnozoite effect, resulting in almost complete suppression of *P. vivax* relapse. At a population level, therefore, homologous recurrence rates appear to be a useful estimate of antihypnozoite drug efficacy in *P. vivax* relapse prevention.

A limitation of this analysis is that only pretreatment samples from patients with recurrences were genotyped. Thus, the true *P. vivax* population diversity was likely underestimated. Genotyping of all baseline samples would more accurately reflect the parasite genetic diversity in the population. Also, this study was conducted in regions of low *P. vivax* endemicity; the MOI was low, and polyclonal infections were uncommon. Consequently, our findings cannot be extrapolated to areas of high endemicity, where individuals may carry hypnozoites from several parasite strains, any of which could cause a relapse. Thus, relapses would be more likely to be heterologous to the initial infection than in the current study. Importantly, hypnozoites are an important source of *P. vivax* infection even in areas of high endemicity. For example, in Papua New Guinea, treating children with artesunate-primaquine reduced their *P. vivax* infection risk by 49% at 3 months after treatment, compared with artesunate alone [14]. In comparative trials, by determining the proportion of heterologous versus homologous recurrences, genotyping can distinguish between recrudescence and reinfection, although in areas of low parasite genetic diversity there may be some overlap. However, genotyping cannot distinguish between recrudescence and relapse should samples be homologous. Recrudescence is caused by the failure of chloroquine and will usually occur within 15 to 28 days of treatment. There was only 1 failure before day 21 in this study, and this was a heterologous recurrence, suggesting that all failures were caused by either reinfection or relapse.

Tafenoquine is currently being evaluated in phase 3 studies. At the 300-mg clinical dose, there was a 9-fold reduction in homologous recurrence, compared with chloroquine, representing a clinically important impact on *P. vivax* relapse prevention; heterologous recurrences were also reduced 3-fold. Overall, the profound clinical impact of tafenoquine at preventing *P. vivax* recurrence has potentially important health and economic benefits.

In conclusion, analysis of the frequency of homologous and heterologous recurrences allowed estimation of tafenoquine's antihypnozoite efficacy against *P. vivax*. This study supports high-resolution *P. vivax* genotyping in drug trials aiming to eliminate *P. vivax* hypnozoites, although confirmatory studies are required.

Notes

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All authors critically reviewed this manuscript and approved the final version for submission. H.-P. B. and R. W. conducted the genotyping studies and developed the genotyping protocols. J. A. G., N. C., R. R., S. K., M. V. L., A. L.-C., and S. D. were involved in development of the DETECTIVE study protocol. R. R., S. K., M. V. L., and A. L.-C. were principal investigators and, along with L. O., involved in data acquisition. S. D. and J. A. G. provided study oversight. H.-P. B., R. W., J. A. G., J. P. R., G. K., L. O., and N. C. contributed to the analysis and interpretation of the data.

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References

1. Battle KE, Gething PW, Elyazar IR, et al. The global public health significance of *Plasmodium vivax*. Adv Parasitol **2012**; 80:1–111.
2. Campo B, Vandal O, Wesche DL, Burrows JN. Killing the hypnozoite—drug discovery approaches to prevent relapse in *Plasmodium vivax*. Pathog Glob Health **2015**; 109:107–22.

3. Koepfli C, Mueller I, Marfurt J, et al. Evaluation of *Plasmodium vivax* genotyping markers for molecular monitoring in clinical trials. J Infect Dis **2009**; 199:1074–80.
4. Koepfli C, Ross A, Kiniboro B, et al. Multiplicity and diversity of *Plasmodium vivax* infections in a highly endemic region in Papua New Guinea. PLoS Negl Trop Dis **2011**; 5:e1424.
5. Lin JT, Hathaway NJ, Saunders DL, et al. Using amplicon deep sequencing to detect genetic signatures of *Plasmodium vivax* relapse. J Infect Dis **2015**; 212:999–1008.
6. Llanos-Cuentas A, Lacerda MV, Rueangweerayut R, et al. Tafenoquine plus chloroquine for the treatment and relapse prevention of *Plasmodium vivax* malaria (DETECTIVE): a multicentre, double-blind, randomised, phase 2b dose-selection study. Lancet **2014**; 383:1049–58.
7. Mendes C, Dias F, Figueiredo J, et al. Duffy negative antigen is no longer a barrier to *Plasmodium vivax*–molecular evidences from the African West Coast (Angola and Equatorial Guinea). PLoS Negl Trop Dis **2011**; 5:e1192.
8. Ord RL, Tami A, Sutherland CJ. ama1 genes of sympatric *Plasmodium vivax* and *P. falciparum* from Venezuela differ significantly in genetic diversity and recombination frequency. PLoS One **2008**; 3:e3366.
9. Bennett JW, Pybus BS, Yadava A, et al. Primaquine failure and cytochrome P-450 2D6 in *Plasmodium vivax* malaria. N Engl J Med **2013**; 369:1381–2.
10. Batista CL, Barbosa S, Da Silva Bastos M, Viana SA, Ferreira MU. Genetic diversity of *Plasmodium vivax* over time and space: a community-based study in rural Amazonia. Parasitology **2014**:1–11.
11. Thanapongpichat S, McGready R, Luxemburger C, et al. Microsatellite genotyping of *Plasmodium vivax* infections and their relapses in pregnant and non-pregnant patients on the Thai-Myanmar border. Malar J **2013**; 12:275.
12. Van den Eede P, Van der Auwera G, Delgado C, et al. Multilocus genotyping reveals high heterogeneity and strong local population structure of the *Plasmodium vivax* population in the Peruvian Amazon. Malar J **2010**; 9:151.
13. Dhangadamajhi G, Rout BK, Kar SK, Ranjit MR. Genetic diversity of *Plasmodium vivax* in a hyperendemic area predominated by *Plasmodium falciparum*; a preliminary study. Trop Biomed **2010**; 27:578–84.
14. Betuela I, Rosanas-Urgell A, Kiniboro B, et al. Relapses contribute significantly to the risk of *Plasmodium vivax* infection and disease in Papua New Guinean children 1–5 years of age. J Infect Dis **2012**; 206:1771–80.