Prevalence of glucose 6-phosphate dehydrogenase deficiency in highly malaria-endemic municipalities in the Brazilian Amazon: A region-wide screening study

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Summary

Background Difficulties associated with the assessment of glucose-6-phosphate dehydrogenase deficiency (G6PDd), particularly in remote areas, hinders the safe use of 8-aminoquinolines such as primaquine (PQ) and tafenoquine against *Plasmodium vivax* malaria due to the risk of haemolysis.

Methods This cross-sectional study was conducted in 41 malaria-endemic municipalities of six states in the Brazilian Amazon, between 2014 and 2018. Male individuals were screened for G6PDd using the qualitative Fluorescent Spot Test using fingerpick-collected whole blood samples. Point and interval estimates of the G6PDd prevalence were calculated for each state. Deficient samples were genotyped for the most prevalent variants in the Amazon. Frequencies of *P. vivax* malaria recurrences were estimated for G6PDd and non-G6PDd patients.

Interpretation This is one of the largest surveys ever conducted in Latin America, covering the entire malaria endemic area in the Brazilian Amazon. These results indicate that an important proportion of the population is at risk of hemolysis if exposed to PQ and its congener drug tafenoquine. The adoption of G6PDd screening protocols is essential to ensure the safety of individuals treated with those drugs and should also be considered when implementing malaria elimination strategies.

Findings A total of 14,847 individuals were included, of which 5.6% presented G6PDd. The state of Acre had the highest G6PDd prevalence (8.3%), followed by Amapá (5.8%), Pará (5.7%), Rondônia (5.4%), Roraima (4.2%) and Amazonas (4.0%). From 828 genotyped samples, African A^+ (6.2%), African A^- (39.3%) and wild-type (non-African non-Mediterranean; 54.2%) variants were found. A greater proportion of malaria recurrences was found among G6PD deficient individuals [16.7% vs 4.1%, Risk ratio 3.52 (2.16–5.74) $p < 0.01$].

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Keywords: *Vivax* malaria; Primaquine; G6PD deficiency; Brazilian Amazon; Elimination; Hemolysis; Recurrences

Research in context

Evidence before the study

We searched through PubMed the following terms: 'G6PD deficiency' AND 'Amazon'. The search yielded a few results, mostly focused on the clinical impact of G6PD deficiency. Prevalence studies described local variants from the States of Acre and Amazonas in small cross-sectional surveys. A systematic review from 2014 described the African as the most prevalent variant in Latin America, in which the deficiency ranged between 4 and 10% in the Amazon.

Added value of the study

To our knowledge, this the largest prevalence survey ever conducted in Latin America, covering the entire malaria endemic area in Brazil. Our results revealed a 5.6% prevalence of G6PDd in the region and individuals with G6PDd had higher chances of recurrences, which is a new episode of malaria even without a new mosquito bite.

Implications of all the available evidence

In malaria endemic areas of Brazil, G6PDd screening should be coupled with malaria testing, to minimize the risk of PQ or tafenoquine-induced hemolysis in these populations and also to mitigate recurrences by providing safer radical cure alternatives, such as weekly PQ regimen.

Introduction

Primaquine (PQ) and tafenoquine (TQ), the only available drugs for radical cure of *Plasmodium vivax* malaria, are potentially harmful if administered to individuals with glucose-6-phosphate dehydrogenase deficiency (G6PDd).[1] Mutations in the G6PD gene can destabilize the enzyme leaving cells prone to damage caused by exogenous triggers that lead to acute hemolytic anemia (AHA).[2] Clinical symptoms vary from mild to life-threatening, depending largely on the G6PD variants involved and the dosage of the drug.[3,4]

In Brazil, patients with *P. vivax* malaria are prescribed chloroquine plus a short-course PQ treatment (0.5 mg/kg/day, 7 days).[5] In case of a new episode by day 60, they are prescribed with 0.5 mg/kg/day for 14 days. Currently, there are no measures to ensure the safe administration of PQ in this context, like G6PD screening and the use of the safer weekly PQ regime,[6] representing a challenge to current strategies for malaria control and elimination.[7,8] As a result of indiscriminate use of PQ, life-threatening anemia, acute renal failure, intensive care management, and death have been reported,[4,9,10] and costs associated with serious adverse events among G6PDd carriers have been estimated at US$ 5 million in the Brazilian Amazon, demonstrating the significant economic and social impact of the absence of a timely diagnosis of G6PDd.[11]

Despite the clinical and epidemiological importance of the interaction between G6PDd and malaria, the extent of its occurrence and the consequences for patients have been scarcely explored in populations living in the Brazilian Amazon.[12–15] A better understanding of the relative frequency of G6PD gene variants would support evaluations towards safe administration strategies and radical cure.[16,17] Therefore, this work aims to provide an accurate and comprehensive mapping of G6PD deficiency in malaria endemic areas in the Brazilian Amazon.

Methods

Study design and locations

This prevalence study was conducted in 41 municipalities in 6 Brazilian states: Acre, Amazonas, Amapá, Pará, Roraima and Rondônia from 2014 to 2018. Out of all malaria cases in Brazil, more than 99% occur in the Amazon region, of which about 89% are attributable to *P. vivax* infections.[18] Most of those cases (80%) are concentrated in municipalities within the states studied. The municipalities were selected because they had a higher Annual Parasitic Index[19] and/or due to their participation in the number of malaria cases in each state (Supplementary Table 1). Estimates of G6PDd prevalence were calculated for each of the states. This study was reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement (*Supplementary* STROBE Checklist).

Sample size calculation

Sample size was calculated to estimate G6PDd prevalence taking into consideration the following parameters: (i) population of each state; (ii) point estimate of G6PD deficiency obtained in preliminary studies in the state of Amazonas equivalent to 4.5% in males[13]; precision of ±1,0% for each state; and a 95% confidence level. Conservatively, it was decided to increase the sample size, using a design effect of 1.5 to mitigate heterogeneity between states. Thus, a sample of 2473 male

participants per state was estimated, leading to a total estimated sample of 14,838 participants.

Inclusion criteria

Male participants were included irrespective of age in municipalities with a high number of malaria cases, which would be the most benefitted, assuming the deleterious effects of 8-aminoquinoline associated hemolysis would cause the greatest impact in terms of morbidity and mortality.[3,4] Patients with signs of malaria (e.g. fever and/or chills) at the time of screening were excluded. Qualitative laboratory and point-of-care G6PD screening tests detect hemizygotes and homozygotes with high sensitivity and specificity,[20] however, female heterozygotes may express G6PD activity phenotypes ranging from fully deficient to completely normal as a consequence of mosaicism of their red blood cell populations.[21] This problem imposes uncertainty regarding a diagnostic reading of 'normal' (and eligibility for primaquine therapy) by those tests among females. Therefore, for this initial study, we opted for not including female participants, to certify an accurate prevalence calculation with the selected screening test.

Sample collection and G6PDd phenotype characterization

The World Health Organization (WHO) recommends the fluorescent spot test to be used for population screenings.[2,16,22,23] Field workers were trained for sample collection at a reference institution in Manaus, Brazil. Samples were collected through domicile visits (only one individual per household, the oldest if more than one present) and in places of great circulation within the peri urban areas of the municipalities. Initially, venous blood was collected by digital puncture and stored in 1 mL microcentrifuge tubes with EDTA and transported in cooled containers to perform the fluorescent spot test (FST) (R&D Diagnostic, Greece) in the closest associated laboratory, up to three hours after collection. Concomitantly, socio-epidemiological data and the history of the disease were collected through a standardized questionnaire.

Briefly, the spot test was performed using five microliters of blood that were mixed with 100 μL of reagents, incubated for 10 min at room temperature, spotted on filter paper and air dried. The spot tests were then visualized under UV light by a trained laboratory technician. Spots showing no fluorescence or light fluorescence were classified as G6PD deficient and strong fluorescence as normal.[24] Each result was read by two trained members, then photographed to be read by a third independent reviewer in Manaus. FST readers were blinded towards other results.

Approximately 500 µL of whole blood were stored at −20 °C and genotyping of deficient G6PDd samples was carried out at the Fundação de Medicina Tropical Dr Heitor Vieira Dourado, Manaus, Brazil.

DNA extraction and G6PD variant genotyping

Approximately 200 µL of whole blood from patients previously identified as G6PDd had their DNA extracted. DNA extraction from whole blood was carried out through the plate extraction method (Biopur Mini Cent). Genotyping was carried out using allelic discrimination assays, through the Taqman system for Single Nucleotide Polymorphisms - SNPs Genotyping.[25] The African (A^+ and A^-) and Mediterranean (B) variants of G6PDd, the most prevalent in Brazil,[14] were identified through the detection of the G202A, A376G, and C563T polymorphisms. Analyses were made on the software 7500 Fast Real-time polymerase chain reaction (RT-PCR) v2.3 (Applied Biosystems) per manufacturer's recommendations. For the QMAL assay, deficient samples were paired with normal samples (3:1 ratio) using age, sex and municipality.

Recurrences (new or relapsing infections)

P. vivax malaria index episodes and recurrences (new or relapsing infections could not be truly differentiated here) recorded in the National Malaria Epidemiological Surveillance Information System (SIVEP Malaria) between January 2011 and December 2020, were analyzed in this study. Participants with a positive thick blood smear (TBS) for *Plasmodium* spp. were treated according to the Brazilian Anti-Malarial Treatment Guidelines.[5] Patient's name, date of birth and mother's name were used for probabilistic linkage between G6PDd survey and SIVEP Malaria databases. All matches were double-checked and false matches were excluded. This inspection included visual confirmation of homonyms, possible siblings (twins), and duplicity.[26] We obtained a final selection of pairs identified as likely to be from the same patients by automatic verification, applying a probability threshold (probability >0.7) for all linkages. A final identification (ID) was created for all included participants.

Statistical analysis

The prevalence of G6PD deficiency and its variants were calculated as proportions, with 95% confidence interval. Data from SIVEP-malaria were imported, curated, and linked by fastLink 0.6.0 R software. Descriptive statistics were used for demographic variables. Fisher's exact or Chi-squared (X2) test were used to compare proportions of *P. vivax* recurrences in G6PD normal and deficient groups. Crude Relative Risk (RR) with its respective 95% Confidence Interval (95% CI) was determined in a univariate analysis. Logistic regression was used for the multivariate analyses and the adjusted RR (ARR) with 95% CI were also estimated. A

log binomial multivariate generalized linear regression was performed using an automated backward and forward stepwise estimation. All variables with an association at a significance level of $P < 0.20$ in the univariate analysis were included in the multivariate analysis. Statistical significance was considered if $P < 0.05$ in the Hosmer-Lemeshow goodness-of-fit test. A 2-tailed $P < 0.05$ was considered significant. The statistical analyses were carried out using R software (version 4.1.0), RStudio (version 1.4.17), and Stata v.13.0 (Stata Corp LP, College Station, TX).

Ethical clearance

This study was approved by the Ethics Review Board at *Fundação de Medicina Tropical Dr Heitor Vieira Dourado* (FMT-HVD) (CAEE: 8307814.7.0000.0005). All study participants (and their legal representatives, if applicable) provided written informed consent to participate.

Role of the funding source

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Results

Participants and G6PD status

A total of 14,847 samples were proportionally collected among the six states studied. From those, 828 (5.6%) had a G6PD deficient result. The state of Acre had the highest G6PDd prevalence (8.3%), followed by Amapá (5.8%), Pará (5.7%), Rondônia (5.4%), Roraima (4.2%) and Amazonas (4.0%) (Figure 1). G6PDd prevalence for each municipality is detailed in Supplementary Table 2 and Figure 1.

Most participants were aged between 20 and 40 years (46.5%), resided in urban areas (94.5%), were of mixed

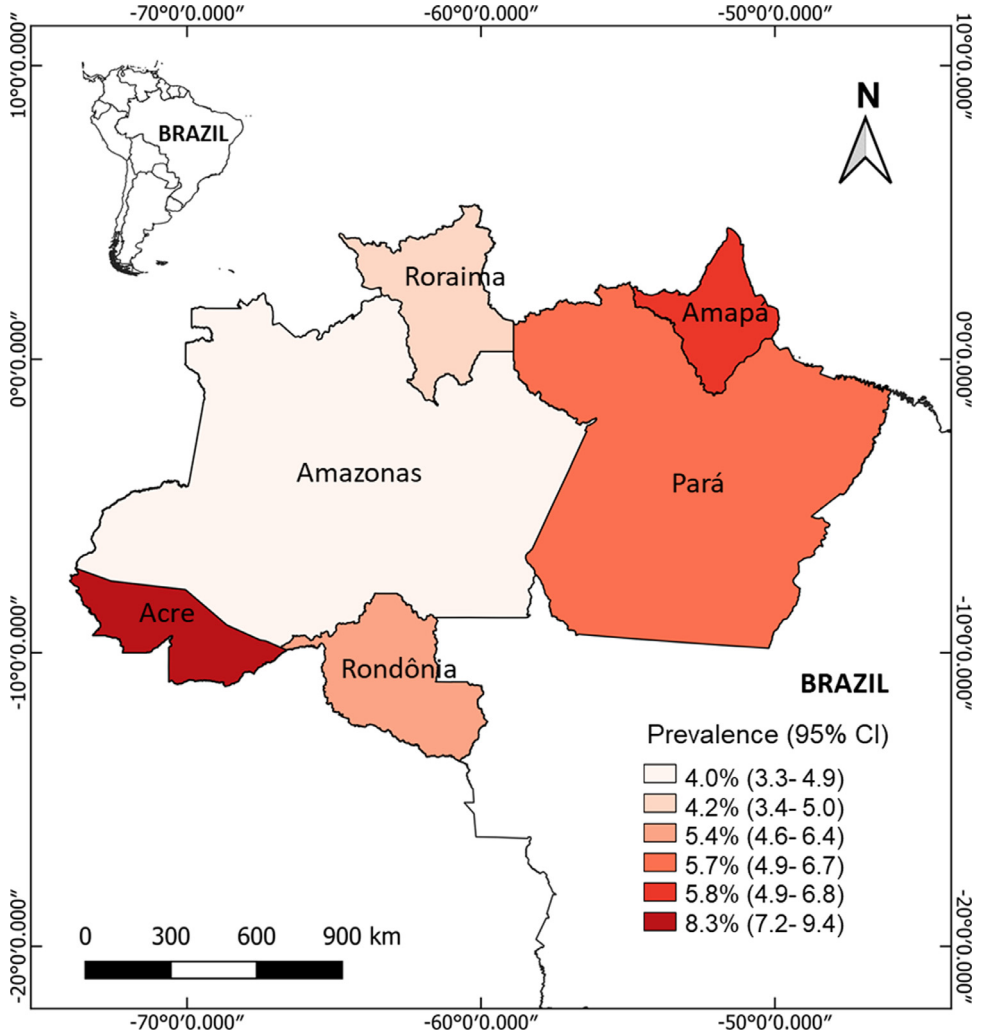

Figure 1. Prevalence of G6PD deficiency in six states in the Brazilian Amazon.

race (73.6%), and had a previous malaria infection (45.6%). All participant data are summarized in Table 1.

Among the deficient samples genotyped, the African A^- ($n = 325$; 39.3%) and A^+ ($n = 51$; 6.2%) variants were detected. Most samples (449; 54.4%) were classified as wild type (non-African non-Mediterranean). No Mediterranean variant was detected.

G6PD status and malaria recurrences

G6PDd participants had more recurrences compared to G6PDn individuals (14/828, 1.7% vs 31/14,019, 0.2%, respectively) in 180 days after the last malaria episode. Table 2 summarizes molecular data on malaria infection between groups at the time of G6PD testing.

G6PD deficiency was associated with a higher risk of recurrence [Risk ratio 3.52 (2.16–5.74) $p < 0.01$), with a higher proportion experiencing one or more recurrences compared to G6PD normal participants when using officially reported data on SIVEP-Malaria (Table 3).

Discussion

The study was designed to confirm that highly endemic municipalities also harbor a great incidence of G6PD

Variable	Total *n* = 14,847	G6PDn *n* = 14,019	G6PDd *n* = 828
State			
Acre	2473 (100%)	2268 (91.7%)	205 (8.3%)
Amazonas	2484 (100%)	2384 (96%)	100 (4%)
Amapá	2472 (100%)	2328 (94.2%)	144 (5.8%)
Pará	2472 (100%)	2330 (94.3%)	142 (5.7%)
Rondônia	2473 (100%)	2339 (94.6%)	134 (5.4%)
Roraima	2473 (100%)	2370 (95.8%)	103 (4.2%)
Age range			
0–10	268 (100%)	259 (96.6%)	9 (3.4%)
10–20	1331 (100%)	1259 (94.6%)	72 (5.4%)
20–40	6908 (100%)	6492 (94%)	416 (6%)
40–60	4922 (100%)	4662 (94.7%)	260 (5.3%)
>60	1418 (100%)	1347 (95%)	71 (5%)
Residency area			
Rural	821 (100%)	768 (93.5%)	53 (6.5%)
Urban	14,026 (100%)	13,251 (94.5%)	775 (5.5%)
Duration of residency, years			
≤10	4140 (100%)	3934 (95%)	206 (5%)
10-20	3412 (100%)	3237 (94.9%)	175 (5.1%)
20-30	3054 (100%)	2892 (94.7%)	162 (5.3%)
>30	4241 (100%)	3956 (93.3%)	285 (6.7%)
Mean (±SD)	24.0 (16.2)	23.9 (16.2)	26.1 (17.2)
Race			
White	1692 (100%)	1580 (93.4%)	112 (6.6%)
Mixed	10,921 (100%)	10,346 (94.7%)	575 (5.3%)
Black	2048 (100%)	1918 (93.7%)	130 (6.3%)
Indigenous	74 (100%)	73 (98.6%)	1 (1.4%)
Asian	112 (100%)	102 (91.1%)	10 (8.9%)
Education level			
Illiterate	476 (100%)	450 (94.5%)	26 (5.5%)
Primary school	9082 (100%)	8594 (94.6%)	488 (5.4%)
High school	4671 (100%)	4397 (94.1%)	274 (5.9%)
University	618 (100%)	578 (93.5%)	40 (6.5%)
Recurrence within 180 days*	45 (100%)	31 (68.9%)	14 (31.1%)
Number of recurrences within 180 days*			
2	34 (100%)	25 (73.5%)	9 (26.5%)
3	8 (100%)	5 (62.5%)	3 (37.5%)
4	2 (100%)	1 (50%)	1 (50%)

Table 1 *(Continued)*

Variable	Total *n* = 14,847	G6PDn *n* = 14,019	G6PDd *n* = 828
6	1 (100%)	0 (0%)	1 (100%)
Self-report of previous malaria	6766 (100%)	6314 (93.3%)	452 (6.7%)
Number of self-reported malaria episodes			
1	2206 (100%)	2074 (94.0%)	132 (6%)
2-5	2530 (100%)	2355 (93.1%)	175 (6.9%)
5-10	1049 (100%)	974 (92.9%)	75 (7.1%)
10-25	860 (100%)	798 (92.8%)	62 (7.2%)
>25	121 (100%)	113 (93.4%)	8 (6.6%)
Self-reported primaquine use (*n* = 6766)	5864 (100%)	5475 (93.4%)	389 (6.6%)
Dark urine or jaundice (*n* = 6766)	1694 (100%)	1587 (93.7%)	107 (6.3%)
History of blood transfusion (*n* = 6766)	153 (100%)	140 (91.5%)	13 (8.5%)
Malaria in the previous 15 days (*n* = 6766)	111 (100%)	102 (91.9%)	9 (8.1%)
Felt sick at the time of sample collection (*n* = 6766)	218 (100%)	199 (91.3%)	19 (8.7%)

***Table 1*: Baseline participant data.**
G6PDn/G6PDd, normal and deficient G6PD status, respectively, on fluorescent spot test. SD, standard deviation. Urban area defined as those surrounding the city centre. *Information from SIVEP-malaria.

deficient individuals and, therefore, it will support policy decisions to frame control programs and diagnosis routine in that specific population. Herein, the results from 14,847 samples collected in 41 malaria-endemic municipalities within the Brazilian Amazon region show an overall prevalence of G6PDd of 5.6%, highlighting the extent of the deficiency among different settings, highly endemic for malaria, in the Amazon. Furthermore, G6PDd participants had higher risk of recurrence compared to G6PDn participants. When exploring the global distribution and mapping of G6PDd, the Brazilian Amazon region stands out as one of the malaria-endemic areas providing fewer quality data, mostly derived from small studies. In this area, model-based estimates point to an allelic frequency of 3 to 7% G6PDd.[27] In other malaria-endemic countries of South America, G6PD variant distribution and prevalence are heterogeneous, reaching more than 10% in some areas.[14] Yet, most countries still have no policy requirement for G6PD deficiency screening before administration of drugs for radical cure.[8] Only one indigenous individual (self-reported) was found to have G6PD deficiency, corroborating previous reports on very low prevalence on this population.[14]

Interestingly, over half of deficient samples (54.9%) did not have genotyping confirmation of their variant to any of the three most common variants screened. Although possible FST test limitations and operational errors cannot be excluded,[23,24] the possibility of new circulating variants should not be disregarded. Considering the limited knowledge of the diversity of G6PDd variants in the Brazilian Amazon region and the representativeness of the sample of this study, it would be opportune and relevant to conduct whole genome sequencing of these samples (here defined as "*wild*") to precisely identify the G6PDd variants present in this population sample of the Brazilian Amazon region.

G6PD screening is not routinely performed in Brazil and PQ is systematically prescribed to all patients diagnosed with *P. vivax* malaria, except pregnant women

Variable	Total 3352, %	G6PDn 2524, %	G6PDd 828, %
QMAL assay (*n* = 3,352)			
Positive for *Plasmodium* spp.	60 (1.7%)	51 (2.0%)	9 (1.1%)
Malaria species (qPCR) (*n* = 60)			
P. vivax	32 (53.3%)	26 (51.0%)	6 (66.6%)
P. falciparum	17 (28.3%)	16 (31.4%)	1 (11.2%)
Plasmodium spp.	11 (18.4%)	9 (17.6%)	2 (22.2%)

***Table 2*: Malaria infection at G6PD testing.**
G6PDn/G6PDd, normal and deficient G6PD status, respectively.

	Deficient (*n* = 84)	Normal (*n* = 753)	RR (95% CI)	p
Unadjusted	14 (16.7%)	31 (4.1%)	3.52 (2.16-5.74)	<0.01
Adjusted*	-	-	3.59 (2.19-5.87)	<0.01

***Table 3*: Association between officially reported recurrences and G6PD status.**
* Adjusted by age and municipality Mean Annual Parasite Index. RR, risk ratio.

and children younger than 6 months.[5,8] Until recently, the national guidelines for the treatment of malaria had no specific treatment for G6PDd patients before it was updated in 2020 when the weekly PQ treatment was included.[5] The weekly PQ regimen (0.75 mg/kg/week for 8 weeks) is safer in such population by allowing hemoglobin recovery after the first doses.[28]The high rate of recurrence in G6PDd group can be ascertained due to treatment interruption led by the fear of hemolysis from previous malaria episodes and lack of suitable treatment. Cultural beliefs and traditional medicine also play an important role on treatment adherence in malaria endemic areas.[29,30] Emerging evidence shows that patients with acute malaria can have significantly higher G6PD activity than individuals without malaria.[31] Hence, risk estimates are possibly lower in malaria patients. Additionally, cytochrome P450 (CYP) polymorphisms can indirectly influence malaria recurrence in the Amazon by altering the rate at which the host metabolizes the drug.[32]

Tafenoquine, a single dose congener to PQ with a longer half-life,[33] was recently approved by Brazilian regulatory agencies. Its use in real life conditions requires quantitative G6PD screening because only individuals with enzymatic activity greater than 70% can safely receive the drug whilst qualitative tests do not reliably differentiate individuals with intermediate activity (30 to 70%). Thus, the operational challenges for its implementation need to be mitigated, given that once administered, its potential deleterious effects may not be reversed.[34] The Safeprim study in Brazil showed that health care professionals with no previous background on G6PDd, were able to correctly perform a qualitative test (CareStart^{TM}) and provide suitable treatment in the field after a single 4 h training session.[12] However, such qualitative testing platforms, including the FST, cannot be used to promote safe use of TQ due to the threshold level of enzyme deficiency detected (<30% of activity). The recent availability of reliable point-of-care quantitative screening tests at 30 and 70%, such as the Standard G6PD,[35,36] can overcome the limitations of qualitative testing platforms and have moved to operational studies in Brazil.[37] This way, individuals with activity over 70% can safely receive TQ.[33]

This study had limitations. The FST is a more complex test compared to other rapid diagnostic platforms, requiring specialized equipment and preventing field testing thus requiring samples to be sent back to local labs for testing. Subjectivity to test interpretation may also have played a role, however we used a careful approach with two blinded technicians and an external confirmatory opinion to mitigate any interpretation bias. Due to the limitations of qualitative testing, only males were included and hence the absence of data from females may influence the prevalence estimates. The mosaicism in the female population renders G6PD deficiency diagnosis challenging compared to males, leaving them at risk of clinically relevant hemolysis if misdiagnosed.[38] Quantitative screening is advised for prevalence studies involving females for better discrimination. Genotyping only comprised the most commons variants previously reported in the Amazon, but the presence of new variants warrants further investigation. It is possible that there was a distortion in the prevalence obtained due to geographical clustering, because the municipalities with the highest incidence of malaria were purposely selected for the survey. However, this sampling process was chosen to ensure that the prevalence of G6PDd obtained can be strategically used to shape control programs in priority areas. No hemoglobin measures were conducted to help extrapolate spurious FST results. Caution should be taken when interpreting secondary data analysis.

In conclusion, these results indicate that an important proportion of the population of the Brazilian Amazon is at risk of hemolysis due to the indiscriminate use of PQ. The adoption of a routine G6PDd screening is essential to ensure the safety of individuals taking PQ and should be considered when implementing malaria elimination strategies. Greater caution applies to heterozygous females, especially in tafenoquine rollout studies.

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Data sharing

Full data set and dictionary are available as supplementary material.

Editorial note

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Declaration of interests

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.lana.2022.100273.

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