Author's response to reviews

Title: Tolerability and safety of weekly primaquine against relapse of Plasmodium vivax in glucose-6-phosphate dehydrogenase deficient and normal Cambodians.

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Author’s response to reviews: see over
**Responses to the reviewers.**

**Wuelton Monteiro.**

We have responded to most of the suggestions for minor comments.

The references have been added, except the one from Cuba which I cannot obtain and which would be difficult to interpret by a non Spanish speaker.

We have not added data about tafenoquine in the Introduction as few relevant data are currently in the public domain as peer reviewed publications. However, we have added a note in the Discussion regarding the safety implications for tafenoquine which is being developed for restricted use.

The key to the safe use of primaquine and, in the future, tafenoquine for antirelapse treatment is the accurate diagnosis of G6PDd and identifying those with more severe G6PDd. Indeed, tafenoquine registration trials are excluding patients with enzyme activities < 70% of the population median (NCT02216123)[29]. In our setting, a G6PDd patient who would be misclassified as G6PDn and who would receive the appropriate primaquine antirelapse dose of 0.5 mg/kg/d (30 mg in an adult) would probably suffer severe AHA [12] [17] [23] [30].

Patients were admitted for the first 72 hours – this was already in the Methods section.

**Alan Magill**

We take a more optimistic view regarding what the data show. We feel that they show there is risk in prescribing the current dose of weekly primaquine and that this risk was transient in those G6PDd patients who had substantial fractional drops in haemoglobin. We do not know the benefits of the weekly regimen Cambodia which is why we call for piloting a strategy of G6PDd testing using the G6PD rapid diagnostic test, managing patients according to the result, and adequate follow up to assess its efficacy and safety.

At the start of the study, we wanted to know if the weekly regimen could be given without the need to pretest for G6PDd i.e. whether its tolerability would be acceptable in our setting. The small numbers in the study make it very difficult to say whether it is safe at a population level. We cannot now change the aim of the study retrospectively. Never the less, we have clarified this sentence to read:

*Given this fear and the paucity of data on 0.75 mg/kg of weekly primaquine, we assessed the tolerability of this primaquine regimen in Cambodian G6PD variants to ascertain whether weekly primaquine could be given without testing for G6PDd.*

We agree that Viangchan is the dominant variant in Cambodia, as has been reported by others using much larger sample sizes.
The thrust of this paper was its public health implications for Cambodia and other counties with similar variants. We are planning a second paper that will examine in more detail the haemoglobin dynamics.

Major comments

1. We have noted the criticism and have tightened up the paper accordingly. We only managed to recruit 18 patients and most of them had G6PD activities < 1 U/g Hb. The enzyme activity range is smaller compared to previous studies so most of our patients were at the severe end of the G6PD spectrum.

Everett treated healthy Airmen with very healthy starting haemoglobin concentrations; they did not have malaria and were given 15 mg of daily primaquine, half the recommended dose for "tropical" vivax. Although they are not representative of patients we see in Cambodia, the data generated are still useful.

We have clarified this in the Discussion:

In our setting, a G6PDd patient who would be misclassified as G6PDn and who would receive the appropriate primaquine antirelapse dose of 0.5 mg/kg/d (30 mg in an adult) would probably suffer severe AHA [12] [17] [23] [30]. Currently in Cambodia, G6PDd testing is laboratory-based but the wider availability of a promising and robust point-of-care rapid diagnostic test (RDT) [31] that is capable of detecting patients with G6PD enzyme activities < 3.6 U/gHb (i.e. < 30% of the Cambodian median) would open up the option of G6PDd testing by village malaria workers (VMWs), referring the RDT diagnosed G6PDd patients for medical supervision and treating the other patients in the community. Such a strategy should be piloted to assess its feasibility, VMW acceptability, cost, efficacy and safety.

2. First line of the Discussion.

We have amended the sentence as follows:

These results preclude the use of unsupervised weekly primaquine in settings where severe G6PDd is present and mandate prior testing for G6PDd.

We think this clarifies our position.

3. Definition of G6PDd

We agree that the current system (classes I to V) for classifying definition of G6PDd is not standardised. Nevertheless, we have used it so that readers can compare more easily our findings with those of others. We would be happy to drop the class I to V classification in this paper, if the reviewers feel strongly about this.

Interestingly, the G6PD Evidence Review Group of the WHO is now talking in terms of classifying individuals as G6PD deficient, G6PD intermediate, and G6PD normal. This is better. In Table 1, G6PDd
and G6PD normals is based on the genotype. It so happens that the G6PD activities were all low even in the three heterozygote females.

We have amended the paragraph on Definitions as follows:

**G6PD enzyme activity and G6PD status**

G6PD enzyme activity was classified I to V according to the measured G6PD activity expressed as a proportion of population median [21]. G6PD status was determined by the results of G6PD genotype as either wild type, G6PDd hemizygote male, G6PDd homozygous female or G6PDd heterozygous female.

For all G6PD deficient patients, DNA was extracted from the buffy coat using the QIAamp DNA Blood Mini Kit (Qiagen, Courtaboeuf, France), according to the manufacturer’s instructions. DNA was used to detect the most frequent mutations in the glucose-6-phosphate dehydrogenase gene by a PCR/sequencing approach [21]: (i) in exon 6 for the Mahidol (487G>A), Mediterranean (563C>T) and Coimbra (592C>T) variants, (ii) exon 9 for the Viangchan (871G>A), Chinese-5 (1024C>T) variants, (iii) exon 11 for the Union, (1360C>T) variant, and (iv) exon 12 for the Canton (1376G>T) variant [21].

We do not share the view that severe G6PD Viangchan patients cannot take this regimen, 17/18 patients with Hbs > 10 g/dL tolerated it well despite substantial drops in Hb in some of them. However, we must be cautious in extrapolating these results more widely. This explains our call for a pilot implementation and more data in patients with Hb concentrations < 8 g/dL. We have acknowledged that the collected are from essentially one G6PDd variant and have flagged this as a study limitation.

One patient was transfused and his decline in Hb may well have been related to taking cimetidine and ciprofloxacin; this was not declared when he was enrolled. However, we still consider this a risk because in real life patients will be on other drugs and have warned clinicians to be wary of concomitant drugs and primaquine.

We have underlined in the Limitations part of the Discussion the severe nature of the G6PD we treated:

This study had limitations. The total number of G6PDd patients was only 18 who mostly had the Viangchan variant; their measured enzyme activities were low (median < 1 U/g Hb), placing them at the severe end of the G6PD spectrum.

With the amended first paragraph of the Discussion:

These results preclude the use of unsupervised weekly primaquine in settings where severe G6PDd is present and mandate prior testing for G6PDd.

and an amended Conclusion:

This is the first study to evaluate weekly primaquine in vivax infected patients with low/very low G6PD enzyme activities. In our setting, primaquine as antirelapse treatment should not be given without
knowing the G6PD status of patients and should be given under medical supervision to those found to be G6PDd.

we believe that the tone of the paper has shifted in accordance with the reviewer’s comments regarding severe G6PDd.

4. Trial profile

We have clarified how patients were recruited in the Methods section and added a foot note to Figure 2. Because the sample size requirement for G6PD normals was met in Pailin, we only recruited patients at the other two sites if the FST result showed they were G6PDd.

Figure 1. Trial profile*.

* G6PD status was determined initially using the fluorescent spot test (FST). At Anlong Venh and Veal Veng, only FST diagnosed G6PDd patients were recruited. Final G6PD status shown here is based on G6PD enzyme activity and G6PD genotype.

5. Aspirin

We have removed the sentence regarding aspirin. We agree with the general comments made about drugs with alleged haemolytic potential in G6PDd. Interestingly, aspirin may be unsuitable in congenital non spherotic haemolytic anaemia (Beutler 1998). Data form Hong Kong (n=1) found a reduced red cell half life following a large dose of aspirin (Chan et al BMJ 1975 2 1227).

Minor comments

We have added in the name and country of the pregnancy tests used (Biotest™, Selangor, Malaysia).

We have clarified the reading of the urine.

The WHO Cambodia office sent Holey-Cotec DHAPP for analysis as part of routine testing before Sigma Tau DHAPP became available in Cambodia. Primaquine was not sent for QC prestudy. The Thai GPO follows Thai GMP and so the product is assumed to be of high quality. We did not send primaquine for prestudy testing.
We used dihydroartemisinin piperazine (DHAPP) that was produced by Holley-Cotec, Beijing, under the brand name Duo-Cotecxin™. Before being distributed in the health system, samples from new batches of Duo-Cotecxin™ were sent for analysis to an independent laboratory by the WHO Cambodia office and found to be satisfactory. DHAPP was dosed................. Primaquine was obtained from the Government Pharmaceutical Organisation, Thailand, and was not sent for analysis.

The endpoints we chose were discussed and agreed upon by the research team. This has been added in the paper.

The primary outcome was patients completing eight primaquine doses i.e. not having primaquine stopped because of primaquine toxicity which was defined, by research team consensus, as any one of: This paper has increased our knowledge of haemolysis and we would use different endpoints in a future study. Moreover, we see the possible development of haematological “danger signs” that would trigger closer follow up / clinical care / stopping primaquine. This is something we will discuss in the next paper.

We used the NIH DAIDS toxicity table 2004. We have added 2004 in brackets.

We agree there are genotype phenotype discrepancies that could result from acute haemolysis; the latter would tend to increase the G6PD activity at presentation and decline subsequently with disease recovery. We did not experience this. Rather we had to tackle the issue of low G6PD enzyme activities and wild type PCR results. We have been back to the database and clarified this paragraph. There were 4 such patients. Three had later G6PD results that were much higher than baseline and one patient had no later results. The G6PD activity was measured several days after the blood was taken and so the G6PD activity is likely to have declined. We feel these baseline results are not reliable so have excluded them from Table 2.

Four PCR-determined G6PD wild type patients had low G6PD enzyme activities that were probably due to delayed measurement; in three patients, the baseline values were inconsistent with later G6PD activity values and in one there were no other G6PD activity values. All values have been excluded from Table 2.

Regarding the G6PDd patients, there were two missing G6PD values on Day 0, not four. This has been corrected. The missing values were excluded from Table 2 but we substituted G6PD enzyme values measured on D7 or D56 to be able to categorise them using the class I to V system. In our small series we did not see much change in G6PD activity over time (for the next paper) so we feel justified in substituting these later values purely for the sake of classifying them.
Two G6PDd had missing baseline enzyme activity values \uline{also excluded from Table 2} but were classified by class using post D0 G6PD enzyme activity results. Of the 18 G6PDd patients, 13 were class II (1 - < 10% population median of 12 U/g Hb) and five were class III (≥ 10 - 60%) G6PDd.

We have not shown a line on the Figure as we now prefer the new WHO way of thinking of G6PD. It is not as straightforward as a straight line.

We have not added a Figure of enzyme activity and the fractional change on D7 because the association was weak - the coefficient of variation is about 8%: \underline{and was associated weakly with G6PD enzyme activity at baseline (p=0.013), for a coefficient of variation of ~8%}.

We have also amended slightly the statistics paragraph:

\underline{The relationship between the fractional fall in Hb on D7 vs. baseline and: (i) the mg/kg dose of primaquine was assessed by Spearman rho test (skewed data), and (ii) the baseline G6PD enzyme activity by Pearson’s correlation coefficient (transforming the G6PD data to become normally distributed).}

We have removed Table 5.

We have clarified the sentence regarding low dose primaquine for transmission blocking. We have also added the Ashley review of primaquine safety. The WHO 2015 update on low dose PQ is very similar to the paper by White et al, so we have kept the latter.

\underline{Other points}

The cure rate of chloroquine resistant \underline{P. vivax} is indeed increased by primaquine but we chose to leave this out.

In our study, giving the primaquine with the first dose of ACT was well tolerated. We await the results of other studies before we can comment with confidence on Dr. Clyde’s thoughts. However, he may have made this suggestion because chloroquine has a greater tendency to cause vomiting than DHAPP.

This is taken from Dr. Clyde’s review:

\textit{It is important to consider at what stage during the course of the malaria primaquine should be administered. If possible, it should not be given during the acute stage of the disease. Apart from its inducing haemolysis, it adds to the intolerance of chloroquine and other blood schizontocides and may thus produce vomiting of both drugs. Also, it may have an immunosuppressive effect. A course of}
Primaquine is best commenced, therefore, as soon as the severe symptoms begin to subside; in vivax malaria this is usually the day following completion of the course of chloroquine. However, under field conditions, where the patient is available for treatment only during the acute phase of illness, primaquine should be given immediately and continued for as long as possible.

The point about not giving primaquine during the acute stage is not referenced.

Interestingly the patient with the highest reticulocyte count on Day 0 had the lowest G6PD enzyme activity. However, there was no correlation between the two in our small sample.

We are in agreement with the last comments. Fever is a well known causes of haemolysis in G6PDd and some thalassaemias. The taking of concomitant drugs is a risk that needs to be considered with primaquine and tafenoquine when these drugs are deployed.

We have made a few small changes to the text; all are highlighted in yellow.