Epidemiology and Global Health
Medicine

Pharmacometric assessment of primaquine induced haemolysis in glucose-6-phosphate dehydrogenase deficiency

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Abstract

Background

Primaquine is the only widely available treatment to prevent relapses of *Plasmodium vivax* malaria, but is underused because of concerns over haemolysis in glucose-6-phosphate dehydrogenase deficient (G6PDd) individuals. G6PDd is common in malaria endemic areas but testing is often not available.

Methods

We conducted a pharmacometric study to characterise the relationship between primaquine dose and haemolysis in G6PDd. The aim was to explore shorter and safer pri-maquine radical cure regimens compared to those currently recommended, potentially obviating the need for G6PD testing. Hemizygous G6PDd healthy adult Thai male volunteers were admitted to the Hospital for Tropical Diseases in Bangkok. In Part 1, volunteers were given ascending dose primaquine regimens whereby daily doses were increased from 7.5 mg up to 45 mg over 15 to 20 days. In Part 2, a single primaquine 45 mg dose was given.

Results

24 volunteers were enrolled in Part 1, and 16 in Part 2 (13 participated in both studies). In three volunteers the ascending dose regimen was stopped because of primaquine related safety concerns (two had increased levels of transaminases, one haemolysis). Other-wise the ascending regimens were well tolerated with no drug-related serious adverse events. In Part 1, haemoglobin concentrations fell 3.7 g/dL (median; range: 2.1 to 5.9; relative fall of -26% [range: -15 to 40%]). Primaquine doses up to 0.87 mg/kg/day were tolerated subsequently without clinically significant further falls in haemoglobin. In Part 2, the haemoglobin concentrations fell by 1.7 g/dL (median; range -0.9 to 4.1; relative fall of 12% [range: 7 to 30%]). The ascending dose primaquine regimens gave 7 times more drug but resulted in only double the haemoglobin fall.

Conclusions and Interpretation

In patients with Southeast Asian G6PDd variants full radical cure treatment can be given in under three weeks compared with the current 8 week regimen.

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eLife assessment

This manuscript addresses an **important** question, that in countries endemic for *P. vivax* the need to administer a primaquine (PQ) course adequate to prevent relapse in G6PD deficient persons poses a real dilemma. On one hand PQ will cause haemolysis; on the other hand, without PQ the chance of relapse is very high. As a result, out of fear of severe haemolysis, PQ has been under-used. This manuscript is **convincing** that regimen (1) can be used successfully to deliver within 3 weeks, under hospital conditions, the dose of PQ required to prevent *P. vivax* relapse.

Background

Over the past 70 years primaquine has been the only drug widely available to prevent relapses of *Plasmodium vivax* and *P. ovale* malaria (radical cure). Primaquine has been given to hundreds of millions of patients in single doses to prevent *P. falciparum* transmission, in 5 to 14 day courses for radical cure of vivax and ovale malarias, and during mass treatments [1]. The main adverse effect of primaquine, and the other 8-aminoquinoline antimalarials, is dose-dependent oxidant haemolysis in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency [2, 3]. As G6PD deficiency is common in malaria endemic regions, primaquine is underused because reliable point-of-care testing for G6PD deficiency is usually not available [4] and prescribers are naturally reluctant to risk causing serious haemolysis. Relapses are a major cause of morbidity. They cause chronic anaemia [5], and are an important source of *P. vivax* transmission. The underuse of primaquine contributes to substantial morbidity and failure to control and eliminate vivax malaria in endemic areas.

G6PD deficiency is the most common red blood cell enzyme deficiency of humans [2]. G6PD deficiency is present mainly in malaria endemic or historically endemic regions [6]. Mutations in the *G6PD* gene on the X chromosome confer reduced enzyme stability. This results in impaired erythrocyte defences against oxidant stresses and thereby increases the risk of haemolysis of older G6PD depleted erythrocytes. More severe G6PD deficiency variants are associated with greater drug induced haemolysis as a broader erythrocyte age range is G6PD depleted [7]. Their substantial haemolytic risk means that the standard 7-14 day radical cure primaquine regimens are contraindicated in G6PD deficiency [8–11].

Seminal clinical investigations were conducted over 50 years ago in adults with the African A- variant of G6PD deficiency. These showed that oxidant haemolysis affected the older erythrocytes, and suggested a therapeutic strategy of controlled haemolysis which would limit the degree of anaemia by allowing time for the compensatory erythropoetic response [12, 13]. This was the underlying rationale for the once weekly primaquine regimen of 0.75 mg base/kg for 8 weeks currently recommended in patients with G6PD deficiency. However, the safety of this regimen in more severe G6PD deficiency variants was never established. Over the past fifty years there has been substantial variation in national policies and practices. Some countries (e.g. Iran, Myanmar), which did not have G6PD testing in endemic areas, have recommended the once weekly regimen as standard practice for all vivax malaria cases. Other countries have recommended giving standard courses of primaquine without testing, although this recommendation is often not followed. A recent small cohort study of the weekly primaquine regimen in 18 G6PD deficient (17 had the 871G>A [Viangchan] variant) and 57 G6PD normal adult vivax malaria patients in Cambodia suggested that single 45 mg doses may not be safe in the more severe G6PD deficiency variants [14]. A quarter of the G6PD deficient patients had a >25% fall in haemoglobin (compared to none in the G6PD normal group) and one patient required a blood transfusion (haemoglobin fell to 7.5 g/dL).

To develop an alternative shorter and potentially safer approach to primaquine dosing in G6PD deficiency, we conducted an adaptive pharmacometric study with the goal of characterising the dose-response relationship for primaquine induced haemolysis in G6PD deficient subjects.

Methods

Trial design

This was a two part study conducted in Bangkok of primaquine in hemizygote G6PD deficient male healthy volunteers. Part 1 evaluated ascending doses of primaquine and was adaptive. The primaquine regimen was titrated based on the observed incremental haemoglobin changes observed in the previous participants, continuous safety evaluation by the investigators, and a set of guiding pre-specified rules. This iterative adaptive approach accumulated information to refine the successive regimens. The primary consideration throughout the trial was participant safety. In Part 2 of the study, after a wash-out period of at least 6 months, a single 45 mg (base equivalent) primaquine dose was given, and the volunteers were monitored as in Part 1.

Ethics statement

The two parts of this study were approved as separate studies. Both parts were approved by the Faculty of Tropical Medicine's Ethics Committee (MUTM 2017-036-01 and MUTM 2021-031-02) and the Oxford Tropical Research Ethics Committee (OxTREC, number 48-16). The study protocols were pre-registered on the Thai Clinical Trial Registry (TCTR, numbers TCTR20170830002 and TCTR20220317004).

Study site and participants

The study took place in the Clinical Therapeutics Unit volunteer ward in the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. The recruitment and follow up periods were from November 2018 to October 2020 (Part 1) and June to September 2022 (Part 2). This coincided with the COVID-19 pandemic. A COVID-19 mitigation plan was implemented when local lockdown was lifted.

Healthy male volunteers were recruited if they were willing to comply with and complete the study protocol, had a G6PD enzyme activity <30% of the population median value determined by a validated quantitative spectrophotometric G6PD assay, a genotype confirmed G6PD variant (according to a previously published method [15]), and were aged between 18 and 65 years with a screening haemoglobin concentration >11 g/dL. Detailed exclusion criteria are provided in the Supplementary Materials.

Trial procedures

Enrollment and primaquine dosing

The risks and the rationale of the study were explained in detail to potential volunteers, who were enrolled if they gave fully informed written consent and agreed to the complete study procedures. It was explained that they could withdraw from the study at any time if they wished. Primaquine phosphate (Thailand Government Pharmaceutical Organization, Bangkok, Thailand) was provided as tablets containing either 5 or 15 mg primaquine base equivalent. A tablet cutter was used to split the tablets (smallest dose increment was 2.5 mg base equivalent). Primaquine was given orally following a standardised light snack and subjects were observed for the first 4 hours.

Part 1 We recruited in cohorts of five volunteers, with an interval of two weeks between cohorts to allow sufficient time to analyse the data and determine the next primaquine regimen. The overall goal was to increase the daily primaquine doses and cause gradual haemolysis which was offset by concomitant reticulocytosis, steadily reducing the age of the red cell population and thereby avoiding precipitous symptomatic falls in the haemoglobin concentration [12, 13]. The cumulative total primaquine dose given needed to be sufficient to provide a radical curative effect in the treatment of vivax malaria (i.e. between 5 and 7 mg base/kg). Careful monitoring was done throughout to assess the degree of haemolysis and adjust or stop the dosing as required. The dose regimens in this exploratory investigation (Part 1) were adapted as follows.

The primaquine regimen given to the first five volunteers consisted of four cycles of 5 days daily primaquine dosing (i.e. total 20 days). The ascending doses were 7.5, 15, 22.5 and 30 mg base equivalent, respectively. This initial dose regimen was chosen on the basis of a mathematical model of primaquine induced haemolysis in G6PD deficiency using data from malaria patients who had received single 45 mg primaquine doses [14, 16]. Subjects proceeded to the next higher dose cycle if they satisfied several prospectively defined safety criteria (Supplementary Figure S4). In this first round the total dose was 375 mg base equivalent. The results of the first five volunteers were reviewed and the dosing regimens adjusted. This iterative process of review and adjustment continued thereafter. The once daily dosing in each cycle was increased in increments of 2.5 mg or 7.5 mg (not adjusted for body weight). Once it became clear this rate of dose increase was generally well tolerated, the number of days per cycle was reduced to 3 or 4 days in subsequent subjects to test regimens of shorter duration with faster dose escalation. Subjects were reviewed clinically before each dose increase.

Part 2 In Part 2, a single dose of 45 mg primaquine base equivalent was administered, with similar careful monitoring in hospital for one week.

Monitoring procedures/evaluations

In both studies, volunteers were observed closely. In Part 1, all volunteers were admitted to the ward for 28 days with a subsequent follow-up visit on day 49. For Part 2, at least 6 months later, volunteers were re-admitted for 24 hours on day 0, reviewed daily until day 7, and then again on day 14.

At enrollment all volunteers underwent a detailed clinical examination. Thereafter, at each assessment volunteers were asked how they felt, had their vital signs measured, and two blood samples were taken for haemoglobin concentration (HemoCue®, Ängelholm, Sweden), and the average of the two was recorded. Blood methaemoglobin (%) was measured at least once daily using a Masimo Rad 57 oximeter®, and urine colour was recorded twice daily. Haemoglobinuria was assessed visually using a modified Hillmen score [17]. Wright-Giemsa stained and new methylene blue stained blood films were prepared for red cell morphology, and reticulocyte and Heinz body counts, respectively. In Part 1 these were done daily from day 0 (day of first primaquine dose) until day 20, and then on days 22, 24, 26, and 28, and finally on day 49. In Part 2 they were done at every visit. Other laboratory investigations included a full blood count (CBC) and reticulocyte count, plasma biochemistry (including LDH and haptoglobin), plasma haemoglobin, and plasma samples were taken for the measurement of primaquine and carboxyprimaquine concentrations. In Part 1 these were done at screening and then every 3-5 days (start of each new cycle, i.e. dosing increment); in Part 2 they were done at screening, days 3, 7 and 14.

Haemoglobin electrophoresis and genotyping of the common cytochrome P450 2D6 (CYP2D6) genotypes found in SE Asia was also performed. Presumptive alpha-thalassaemia (which is very common in Thailand) was defined as a mean cell volume less than 80 fl or a mean cell haemoglobin < 27 pg, and HbA2 ≤ 4.4%. Additional laboratory measurements were taken if unplanned dose adjustments were necessary, or there was a clinical indication.

Safety monitoring and stopping rules for Part 1

The pre-specified rules for adjusting primaquine dose regimens across cohorts in Part 1 are illustrated in Supplementary Figure S3, and for increasing primaquine doses for each enrolled subject are shown in Supplementary Figure S4. The overall aim was to titrate dosing in order to obtain small daily falls in haemoglobin of between 0.1 and 0.2 g/dL. For a given subject, primaquine doses were increased only if the haemoglobin concentration was >9 g/dL, was greater than 70% of baseline, the urine Hillmen score was ≤5, and the subject felt well and had no symptoms of anaemia.

We defined haemolysis which would result in stopping primaquine (study withdrawal), as any one of the following:

- >40% fall in haemoglobin from baseline;
- a haemoglobin below 8 g/dL (irrespective of symptoms);
- a fall in haemoglobin associated with clinically significant signs of haemolysis: jaundice, passing dark urine (Hillmen colour ≥6), evidence of acute renal injury (≥2 fold increase in serum creatinine from baseline), or hyperkalaemia (serum potassium >5.2 mmol/L).

Any individual whose laboratory tests met these criteria remained in hospital and was monitored closely until resolution of signs and symptoms, and haemoglobin concentrations had reached at least 10 g/dL. Blood transfusion was available at any time if needed if volunteers had a

symptomatic fall in haemoglobin to below 8g/dL.

An Independent Drug Safety Monitoring Board was established to review the data after each cohort had completed their follow up and gave feedback before the next cohort was allowed to proceed. For both studies, adverse events were recorded and graded using the Common Toxicity Criteria v 5.0 for grading adverse events.

Sample size

As this was an exploratory proof-of-concept, adaptive dose optimisation pharmacometric and safety study in healthy G6PD deficient males, there was no formal sample size calculation. We reasoned that if the tested primaquine regimens were well tolerated in 20 volunteers (i.e. 4 cohorts), this would provide preliminary evidence for the safety and the feasibility of this approach. In addition the rich longitudinal data could then be used to develop an intra-host model to design optimal ascending regimens [16].

Statistical analysis

All data analysis was done in R version 4.2.2. The baseline value for each continuous measurement was defined as the mean of the screening and day 0 measurement (if taken before 1 hour post first primaquine dose). Haemoglobin was measured using Haemocue® (daily, two samples) and using a laboratory processed complete blood count (CBC, every 4-5 days). The daily mean haemoglobin was calculated as the mean of the Haemocue® (itself the mean of the two values) and the haemoglobin concentration from the CBC (if no CBC was done then just the mean haemocue value). The baseline haemoglobin was then calculated as the mean of the daily values at screening and on day 0.

Data Sharing Statement

All analysis code and data are available via an accompanying github repository: *https://github.com/jwatowatson/Primaquine-Challenge*.

Results

Study population

Of 215 potential subjects (either identified through hospital records or screened at the walk-in clinic for G6PD deficiency), 27 male hemizygote G6PD deficient volunteers were enrolled to the two sub-studies between November 2018 and August 2022. In subjects who were interested in participating in the study, there were 2 screening failures (1 unidentified G6PD genotype and 1 elevated AST/ALT), see CONSORT diagrams in Supplementary Figures S1 and S2. The COVID-19 pandemic interrupted the end of Part 1 and delayed finishing the study by two years, resulting in a substantially longer interval between test regimens than planned. After the lockdowns, fewer than anticipated Part 1 participants could be recontacted for Part 2, so three additional volunteers were recruited. The volunteer baseline summary characteristics are shown in **Table 1**. All volunteers had low to unmeasurable G6PD enzyme activity. The most common G6PD deficiency genetic variant was Viangchan (871G>A, *n*=12), as it is in much of the eastern Greater Mekong subregion. This was followed by Canton (1376G>T, *n*=5) and Mahidol (487G>A, *n*=4). The majority of subjects had screening reticulocyte counts over 2%, i.e. above the normal range (17/24 in Part 1 and 5/16 in Part 2). Poor *CYP2D6* metaboliser genotype (homozygous *10) were identified in 8/27 (30%) of the volunteers.

	Part 1 - Ascending dose	Part 2 - Single 45 mg dose	Overall
n	24	16	27
Age (years)	32 (18 - 55)	34 (20-58)	32 (18-58)
Weight (kg)	64 (46 - 86)	64 (52-86)	64 (46-86)
G6PD genotype			
Viangchan (871G>A)	12	6	12
Mahidol (487G>A)	4	2	4
Canton (1376G>T)	4	3	5
Aures (143T>C)	1	1	1
Chinese-4 (392G>T)†	1	0	1
Orissa (131C>G)	1	1	1
Union (1360C>T)	1	2	2
Kaiping (1388G>A)	0	1	1
G6PD enzyme activity (U/g Hb)	0.15 (0-1.9)		
Haemoglobin (g/dL)	14.3 (11.8 - 15.8)	14.0 (12.3 - 15.9)	
Red cell count (x10^{12} per L)	4.9 (4.2-6.0)	5.1 (3.9-5.9)	
Reticulocyte count (%)	2.4 (1.1-4.0)	2.4 (1.0 - 2.9)	
Platelet count (x1000 per uL)	285 (190-424)	289 (174 - 412)	
Total white blood cell count (x1000 per uL)	6.6 (4.8-9.3)	6.6 (5.2-8.4)	
Blood methaemoglobin (%)	0.5 (0-1.5)	0.7 (0-1.4)	
AST (U/L)	23 (15-60)	21 (14-36)	
ALT (U/L)	26 (10-85)	22 (11-47)	
Plasma creatinine (mg/dL)	0.9 (0.8-1.1)	1.0 (0.7-1.1)	
Total bilirubin (mg/dL)	0.6 (0.3-1.6)	0.7 (0.3-1.3)	
Haptoglobin (g/L)	1.1 (0.5-1.7)	1.1 (0.5-1.7)	
CYP2D6 genotypes			
*10/*10	6	4	8
*2/*10	7	4	7
*1/*10	6	4	7
*1/*2	3	3	3
*1/*1	2	1	2
Haemoglobin E trait‡	7	4	7
Presumptive alpha-thalassaemia*	5	5	7

Table 1

Baseline characteristics of the healthy male volunteers. For the continuous variables we show the median (range). Of the 27 volunteers, 13 participated in both sub-studies. †also known as Quing Yan [18]. ‡haemoglobin typing done by electrophoresis in part 1 only. *see definition in Methods.

Ascending dose primaquine (Part 1)

Of 24 volunteers assigned ascending dose primaquine regimens, 23 were included in the analysis (Supplementary Figure S1). Volunteer number 18 was withdrawn from the study after 3 days of receiving 10 mg of primaquine daily because of severe back pain due to a MRI confirmed prolapsed intervertebral disc that improved with symptomatic treatment. He was not followed up and did not participate in Part 2; as this was considered unrelated to drug administration and the total primaquine dose was very low his data were excluded from the primary outcome analysis. There were no changes in his haemoglobin over the three days of primaquine dosing. The remaining 23 subjects in the primary analysis population received ascending primaquine dose regimens of between 11 and 20 days duration.

Safety concerns resulting in study withdrawal

There were no serious adverse effects or complications. In three subjects (13%), the ascending dose primaquine regimen was stopped because of safety concerns; one for excessive haemolysis and two because of abnormal liver function tests (elevated transaminases). After receiving 11 doses of a 16-day regimen, subject 11 (*G6PD* Union) reached a fractional haemoglobin fall from baseline of 39.5% (8.9 g/dL vs. 14.7 g/dL at baseline), associated with marked fatigue. This decrease met the stopping rule for study withdrawal (dose limiting toxicity, see supplementary Figure S4). He developed a substantial reticulocytosis (15%). Over the next five days, his haemoglobin remained at ~ 9 g/dL (nadir observed haemoglobin was 8.8 g/dL corresponding to a 40% decrease from baseline) and rose thereafter to 13.0 g/dL (day 28) and 14.9 g/dL by day 49 (Supplementary Figure S6). The haemoglobinuria Hillmen score peaked at 4 on day 10 and was 3 on day 12, dropping back to 1 on day 13 (Supplementary Figure S7).

Volunteer 7, who was receiving a 20-day regimen, developed an ALT of 207 U/L (>5 times ULN, grade 3) and AST of 89 U/L (>2 times ULN, grade 1) on day 16 and so primaquine was stopped (Supplementary Figure S8). There was no further increase in serum bilirubin and he remained asymptomatic. His haemoglobin was 11 g/dL at the time of withdrawal (baseline: 14.3 g/dL; 23% fall); he was subsequently lost to follow up. Volunteer 14 (16-day regimen) developed an ALT of 423 U/L (>10 ULN, grade 3) and AST 229 U/L (>5 times ULN, grade 3) on day 11. His haemoglobin was also 11 g/dL (baseline: 13.9; 21% fall). There was no further increase in serum bilirubin and he also remained asymptomatic. His ALT and AST then decreased, reaching 87 and 36 U/L by day 28, respectively (Supplementary Figure S7). All investigations for hepatitis viruses were negative. Inpatient liver ultrasound scans showed fatty liver in both volunteers.

Dose adjustments

In a further three subjects the intended ascending dose regimen was not completed as they had haemoglobin falls of 30 to 40% relative to baseline (Supplementary Figure S3). Subject 13 (16 day regimen assigned) did not escalate from 30 mg to 45 mg on day 15 but stayed at 30 mg until day 16 (33 and 34% fall from baseline haemoglobin on day 15 and 16 respectively). Subject 23 was given an additional day at 30 mg (day 12 as the day 11 haemoglobin gave a 32% fall from baseline), and subject 24 remained at 22.5 mg from day 7 to day 14 (instead of escalating to 30 and then 45 mg) as he had a fall in haemoglobin between 30 and 33%.

Haemolysis

The median absolute fall in haemoglobin from baseline was 3.7 g/dL (range: 2.1 to 5.9), corresponding to a median relative decrease of 26% (range: 15 to 40), **Figure 1** panels b and d. The median day of haemoglobin nadir was 16 days after starting primaquine (range: 11 to 20). There was substantial variation between individuals, including between those with the same G6PD geno-type. For example, volunteer 15 (Viangchan) received 6.8 mg/kg primaquine over 16 days and his haemoglobin dropped around 25% (baseline was 13.6 g/dL; nadir of 10.1 g/dL was

reached approximately by day 11); whereas volunteer 20 (also Viangchan) received 5.4 mg/kg over 15 days (slightly faster escalation using the same doses but he was 12 kg heavier), but his haemoglobin fell around 40% (baseline was 15.0 g/dL; nadir of 9.2 g/dL was reached by day 15), Supplementary Figure S5. None of the subjects had a fall of haemoglobin below 8 g/dL and none developed frank haemoglobinuria (Hillmen ≥6). Peak reticulocytosis occurred at approximately the same time as the haemoglobin nadir (day 16; range: 11 to 20), with a median peak reticulocyte count of 10.3% (range: 4.2 to 16.8), **Figure 1** panel c.

Part 2: single high-dose primaquine

Following a single 45 mg base equivalent dose of primaquine (mg/kg doses ranged from 0.52 to 0.87), there was a marked fall in haemoglobin concentrations reaching a median observed nadir on day 6 (range: day 4 to 7). The median total fall from baseline was -1.7 g/dL (range: -0.9 to -4.1), corresponding to a median relative decrease of -12% (range: -7 to -30%), see **Figure 2**. The largest daily falls in haemoglobin following the single dose were on day 3 (median fall in haemoglobin on day 3 was -0.73 g/dL, **Figure 3**). The reticulocyte response was characterised by a gradual rise with most volunteers having their observed peak proportions on day 7 (range day 4 to 14). The largest fall in haemoglobin, and greatest rise in reticulocyte count occurred in subject 13 who was *G6PD* Canton (absolute fall of -4.1 g/dL: baseline haemoglobin was 13.6 g/dL, their day 7 haemoglobin was 9.5 g/dL; relative fall of 30%). This subject did not participate in Part 1 so there are no ascending dose data with which to compare.

Comparison of ascending and single dose primaquine regimens

Figure 3 shows the maximum observed absolute and relative falls in haemoglobin as a function of the total primaquine dose across the two studies. Overall, the median fall in the single dose cohort was nearly half that of the median fall in the ascending dose group, whereas the median total dose in the single dose cohort was only 14% of the median total dose in the ascending dose cohort. Although the subjects receiving a single 45 mg dose had smaller absolute falls in haemoglobin, they experienced earlier and greater daily falls (bottom panels of **Figure 3**). The greatest median falls in haemoglobin in Part 1 were on days 6 and 7 (-0.55 and -0.57 g/dL, respectively), whereas the median fall on day 3 (day with the largest median fall) in the single dose cohort was -0.73 g/dL. Two subjects receiving the 45 mg single dose had daily haemoglobin falls of nearly 2 g/dL.

Haemolysis dose-response relationship

We summarised each ascending dose regimen in Part 1 by the cumulative dose of primaquine received by day 10. This summary exposure statistic was chosen following graphical visualisation of the haemoglobin data, which showed that the most substantial haemolysis had occurred by day 10 of the study (**Figure 1b**). In the 23 volunteers, the day 10 cumulative dose varied from 1.7 mg/kg to 3.5 mg/kg, with a median value of 2.6 mg/kg. The day 10 cumulative dose was predictive of both the maximum absolute fall in haemoglobin with respect to the baseline value (-1.2 g/dL fall per mg/kg increase [95%CI: -0.5 to -1.8]; p=0.001, r^2 = 0.40), and the maximum relative fall in haemoglobin with respect to the baseline value (-7.9% fall per mg/kg increase [95%CI: -4.4 to -11.3]; p=0.0002; r^2 = 0.49), but not of the average daily fall over days 5 to 10 (-0.06 g/dL per day [95%CI: -0.14 to 0.03]). **Figure 4** panels a-c show the dose-response data coloured by G6PD variant. For Part 2 the mg/kg dose was not associated significantly with either the absolute, relative or the mean daily falls in haemoglobin, although for all three outcomes the point estimates were in the expected direction (greater mg/kg dose resulting in larger falls), **Figure 2** panels d-f.

In an additional exploratory analysis, there was no evidence of substantial differences in haemolysis between the G6PD variants (although the sample size is very small). There was some evidence that subjects with higher baseline haemoglobin concentrations had larger relative falls.

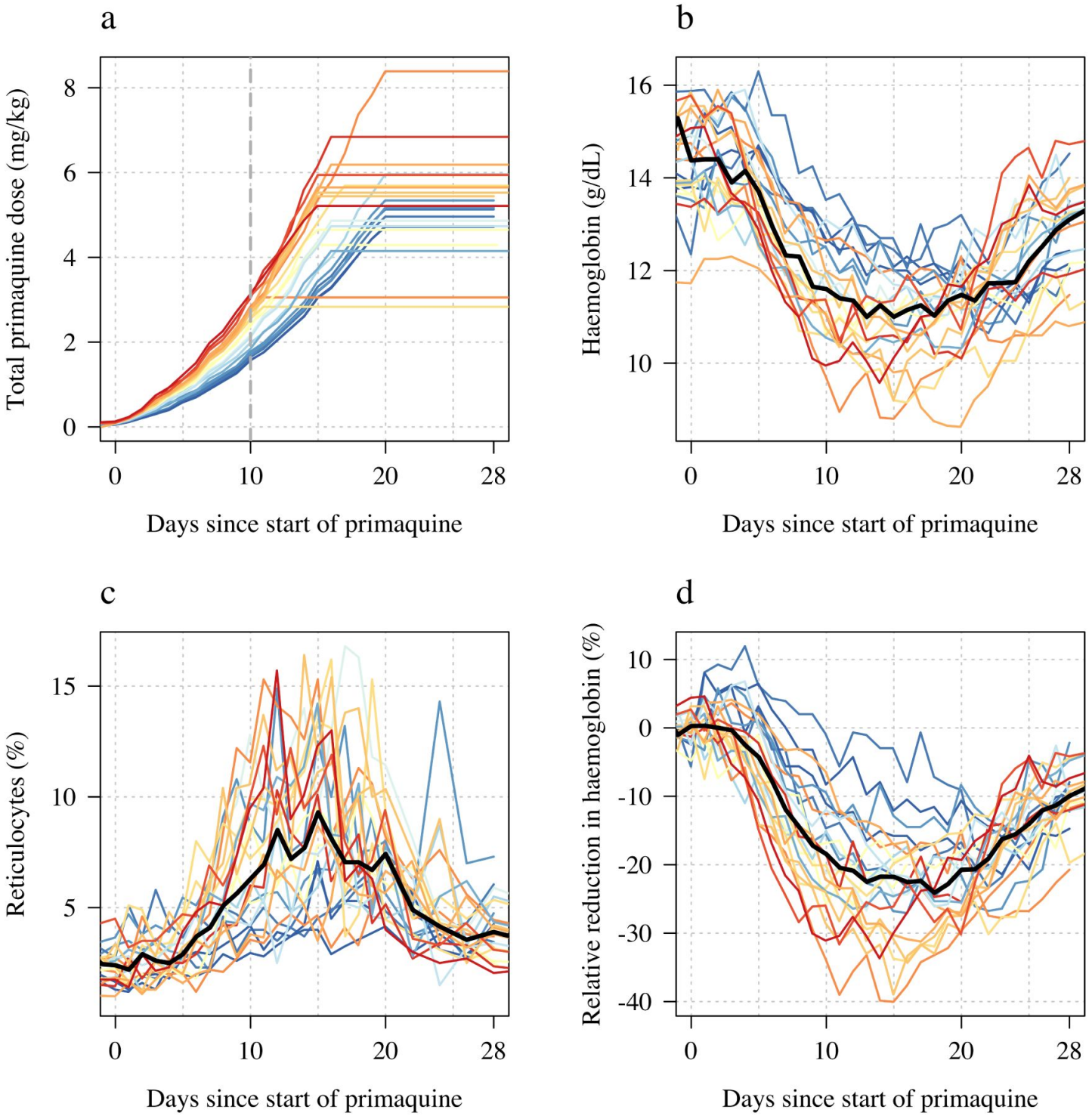

Figure 1

Ascending dose study in 23 male hemizygote G6PD deficient healthy volunteers included in the primary analysis. Colours from blue to red are in order of increasing day 10 total cumulative primaquine dose as shown in panel a (cumulative primaquine doses over time). Panels b & c show the absolute haemoglobin values and reticulocyte counts over time; panel d shows the relative change in haemoglobin over time. The thick black lines in panels b-d show the daily median values.

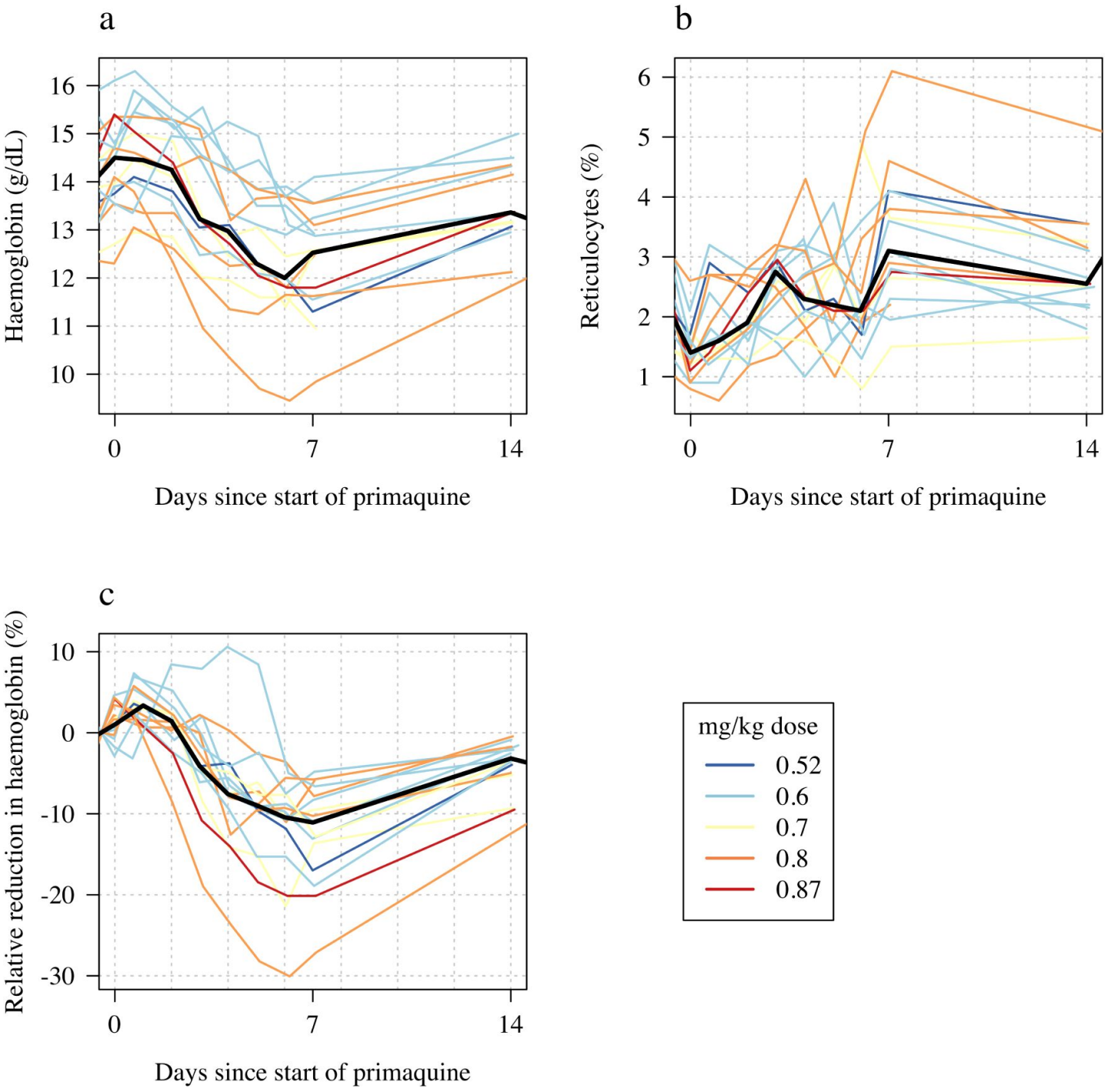

Figure 2

Haemolytic effect of a 45 mg single primaquine dose in 16 male hemizygote G6PD deficient healthy volunteers. Colours from blue to red are in order of increasing mg/kg dose. Panel a: absolute haemoglobin values; panel b: reticulocyte counts; panel c: relative change in haemoglobin from baseline. The thick black lines in panels a-c show the daily median values.

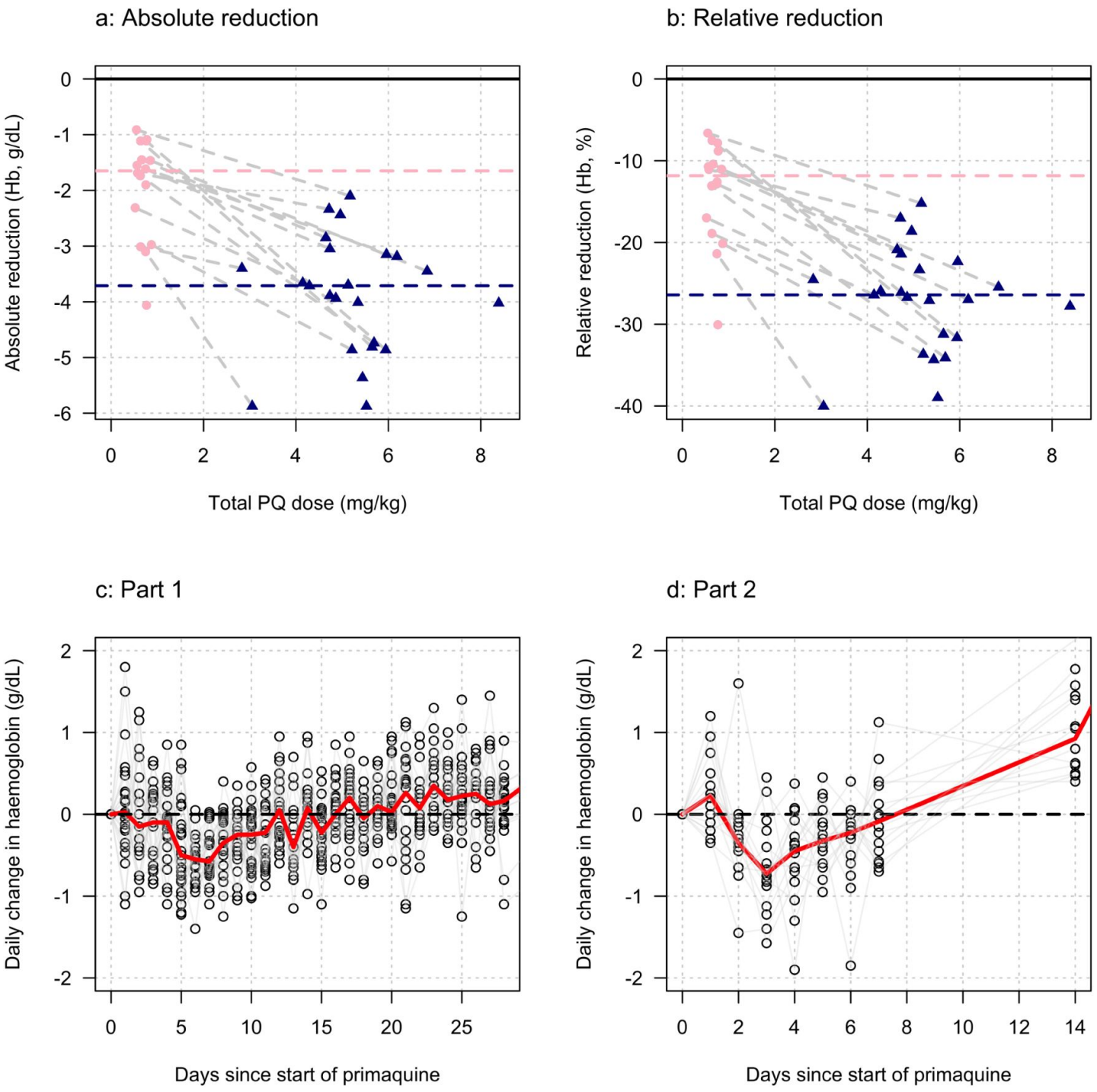

Figure 3

Comparing the haemolytic effect of ascending dose primaquine regimens (dark blue triangles) and single dose 45 mg primaquine (pink circles). The top panels show the relationship between the total cumulative dose of primaquine given to each subject in each study (x-axis) and the absolute fall in haemoglobin (left panel) or the relative fall (right panel). Subjects who participated in both parts are joined by the light grey dashed lines. The horizontal dashed lines show the median falls by sub-study. The bottom panels show the daily observed changes in haemoglobin (left: Part 1; right: Part 2), the red line shows the daily median change.

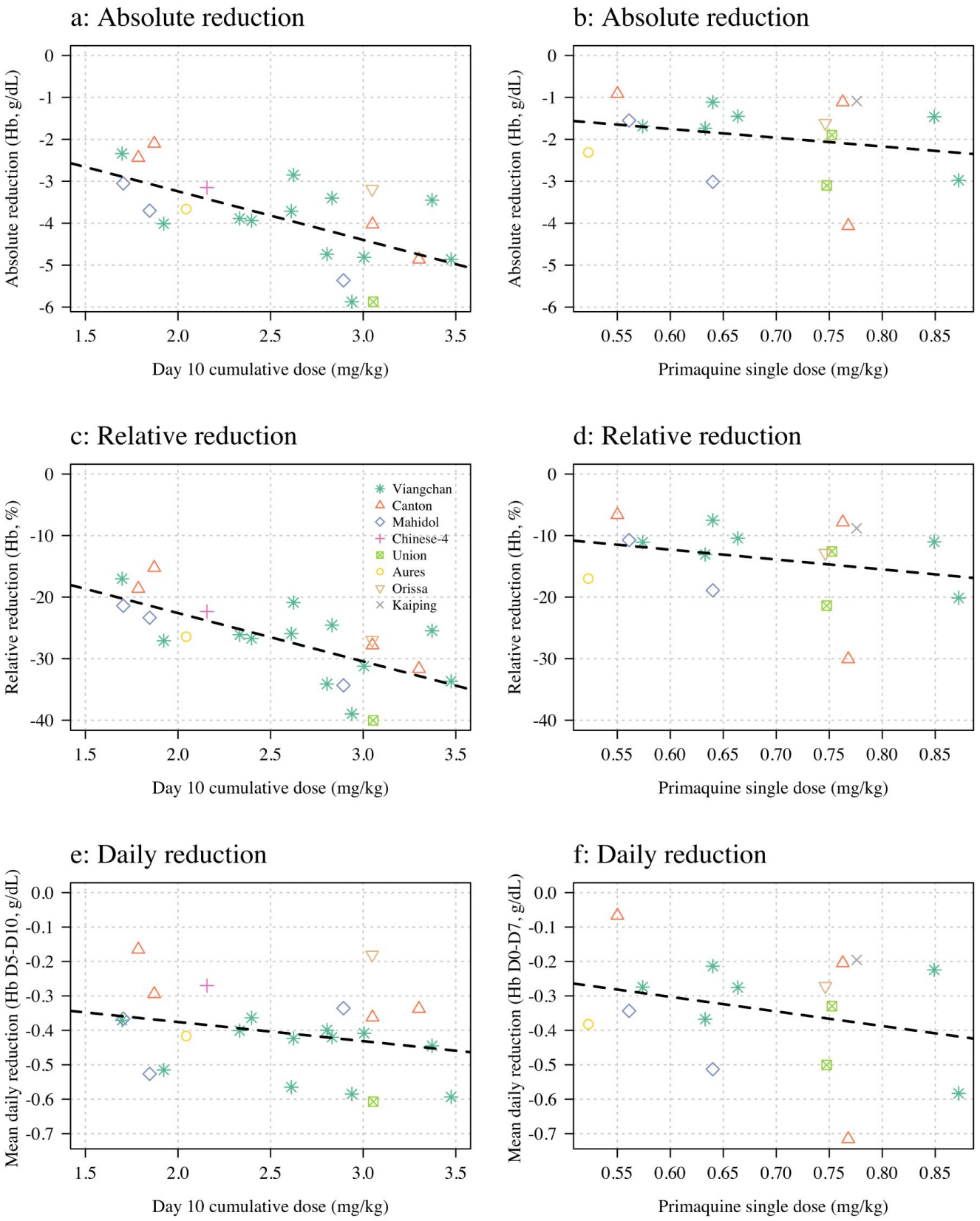

Figure 4

Haemolysis dose-response relationships (left column: Part 1; right column: Part 2). The dose exposure summary in Part 1 is the day 10 cumulative primaquine dose (*n*=23); for Part 2 it is the mg/kg single dose (*n*=16). Hb: haemoglobin.

There was no evidence for an association between the homozygous *10 *CYP2D6* genotype and haemolysis (i.e. no evidence that these subjects haemolysed less than the other subjects).

Effect on total bilirubin and LDH

The falls in haemoglobin were associated with predictable biochemical changes indicative of haemolysis. There were rises in plasma concentrations of the intraerythrocytic enzyme LDH and in total bilirubin reflecting haem metabolism. The more rapid fall in haemoglobin associated with the single 45 mg primaquine dose was associated with larger normalised rises in total bilirubin consistent with greater haemolysis (Supplementary Figure S12 and **Figure 5**).

Liver transaminases, creatinine, haptoglobin, and methaemoglobin

In both sub-studies there was no evidence for a relationship between primaquine dose (Part 1: cumulative day 10 total mg/kg dose; Part 2: single mg/kg dose) and maximum observed fold change in serum AST, ALT or creatinine, or the maximum observed absolute decrease in plasma haptoglobin (Supplementary Figures S10 and S11). There were no clinically significant rises in blood methaemoglobin in any of the study participants (**Figure 6** panels a-b). In part 1, the day 10 cumulative dose was negatively associated with the peak observed methaemoglobin (**Figure 6** panel c, p=0.02); a similar negative trend was also observed in part 2 (**Figure 6** panel d; p=0.06). There was no association between having a poor metaboliser CYP2D6 genotype (*10 homozygous versus other genotypes) and peak blood methaemoglobin concentration.

Discussion

Significant haemolysis from radical cure primaquine regimens cannot be avoided in G6PD deficiency, but it can be attenuated. Ascending dose primaquine regimens in G6PD deficient malaria patients exploit the same pharmacodynamic principle underling the current once weekly treatment recommendation [12]. They aim to provide controlled haemolysis while allowing for steady reconstitution of the red cell population with increasingly younger, and therefore “oxidant resistant” erythrocytes [13]. As G6PD testing is usually unavailable in malaria endemic areas the prescriber is faced with the therapeutic dilemma of either not giving the drug, and failing to prevent relapses of vivax or ovale malaria with their attendant morbidity, or giving it and causing iatrogenic haemolysis in G6PD deficient patients. The net result is that radical cure primaquine regimens are often not prescribed, even though they would be well tolerated and safe in the G6PD normal majority of patients. Primaquine underuse is a major contributor to global vivax malaria morbidity.

The currently recommended 8 week radical cure regimen for G6PD deficient patients attenuates the fall in haemoglobin, but it still risks significant haemolysis, particularly with the first 0.75 mg/kg dose. It has not been well evaluated in patients with severe G6PD deficiency variants who are at greatest risk, and it requires good adherence for 8 weeks. Failure to complete the eight week course, which is likely to be common, therefore incurs all the risk without the full benefit. In this study the single 45 mg dose resulted in a median fall in haemoglobin concentration of 1.7 g/dL, which was nearly half the median fall when using the full ascending dose regimen. In comparison, the ascending dose regimens gave a seven times higher total dose. These ascending dose primaquine regimens were relatively well tolerated in adult male volunteers with Southeast Asian variants of G6PD deficiency. Although these variants are generally regarded as moderate in severity, there is wide variation in the phenotype between variants and also between individuals with the same genotype. The ascending dose regimens are still associated with a significant risk but, in considering the radical cure treatment of vivax malaria, the risks of relapse, often on

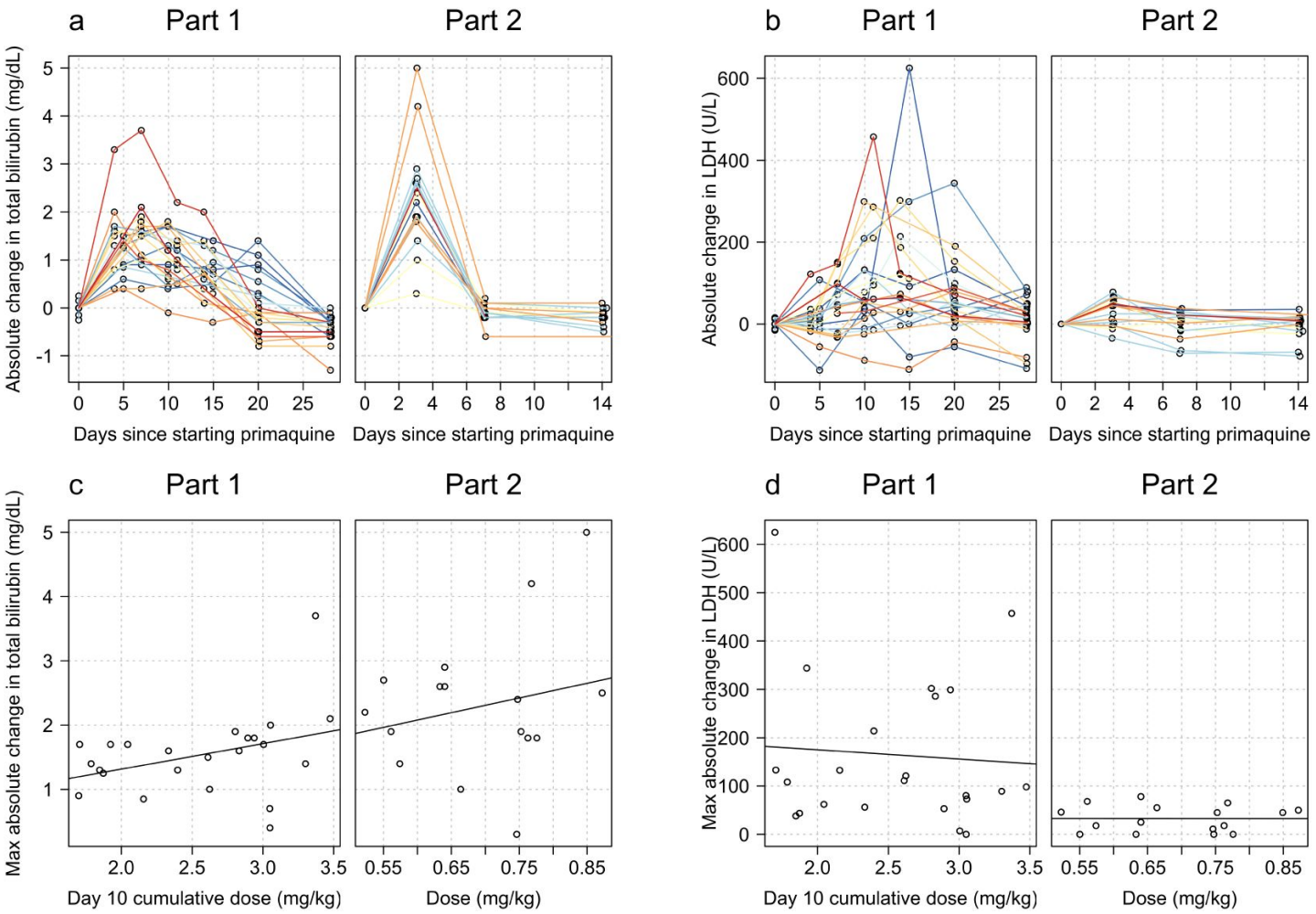

Figure 5

Relationship between primaquine dose (Part 1: day 10 cumulative mg/kg dose; Part 2: mg/kg single dose) and total bilirubin (panels a and c) and LDH (panels b and d). The top row show the normalised data; the bottom row the dose response relationship with the maximum normalised increases.

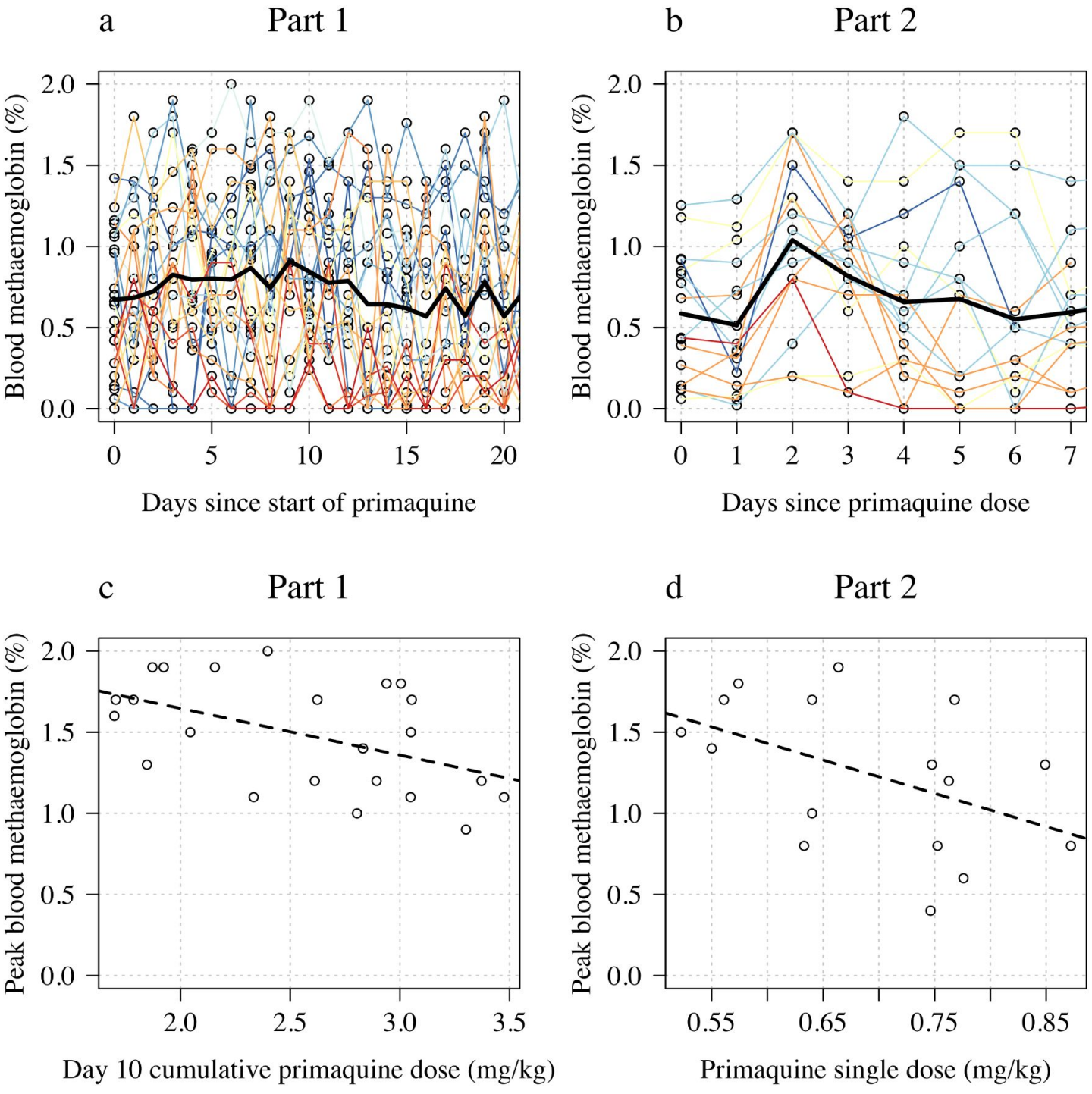

Figure 6

Changes in blood methaemoglobin concentration. Top row: individual data (left: Part 1; right: Part 2) with the daily mean value shown by the thick black line. Bottom row: relationship between dose (left: Part 1 summarised by the day 10 total dose; right: Part 2 summarised by the mg/kg dose) and peak observed blood methaemoglobin (%).

multiple occasions with consequent anaemia, must be balanced against the predictable haemolysis that will result from the oxidative effects of the 8-aminoquinoline radical cure regimen [5, 19]. In the Southeast Asian region approximately half the vivax malaria cases will be followed by at least one relapse if radical curative treatment is not given.

In this exploratory study, in which there were cautious stopping rules, 3 out of 23 (13%) of the volunteers did not complete the regimen because of drug toxicity; two because of hepatitis (a rare adverse effect of primaquine) [3], and the third because of significant haemolysis. Significant falls in haemoglobin were expected by design, but in none of the volunteers did haemoglobin concentrations fall below 8 g/dL. The extent of haemolysis varied substantially between volunteers, even within the same genotype. Higher baseline haemoglobin values tended to be followed by greater falls. Taken together with the high baseline reticulocytosis, this suggests that subjects with G6PD deficiency have variably shortened red cell survival, and thus variably sized populations of erythrocytes vulnerable to oxidant haemolysis. One of the important advantages of primaquine relative to its slowly eliminated analogue tafenoquine is that the treatment can be stopped as soon as there are signs or symptoms of haemolytic toxicity. Primaquine is eliminated rapidly ($t_{1/2}$ circa 5 hours). Despite use of primaquine for nearly 70 years and administration of hundreds of millions of treatments there have been very few reported deaths from haemolytic toxicity [3]. Very large mass treatments with primaquine have been used in malaria elimination campaigns involving millions of people in China, Nicaragua, Turkmenistan, Azerbaijan, Tajikistan, Afghanistan and North Korea [20, 21]. In the latter four countries an interrupted regimen, devised in the USSR, involved giving four days of primaquine, stopping for three, days and then completing a further ten days. Subjects with significant haemolysis could stop the drug at any time. Serious toxicity was very rare, despite high prevalences of G6PD deficiency in some of the regions.

In G6PD deficient patients with acute malaria preferential loss of older erythrocytes would ameliorate the adverse impact of oxidant drugs. On the other hand compensatory reticulocytosis may be inhibited. These differences are likely to be small as the illness usually resolves within a few days with effective treatment, while the ascending dose regimen would still be using the lowest dose. The main limitation of this approach is its complexity. This could be addressed by preparation of blister packed primaquine allowing easy dose transition. The G6PD deficiency genotypes studied here are representative of those present in the Southeast Asian region, and can be regarded as of moderate severity, with the African A-genotype (in which the currently recommended once weekly dosing regimen was developed) being at the less severe end of the spectrum, and the common Mediterranean G6PD genotype being at the more severe end. The safety of this regimen in patients with severe deficiency cannot be predicted based on these data. From a therapeutic perspective, as G6PD testing is usually not available, the individual patient risk assessment must take into account several factors including the prevalence of G6PD deficiency and its likely severity, the sex of the patient, the degree of anaemia, the probability of relapse, patient understanding of risks, and the likelihood and feasibility of accessing health care if there is severe haemolysis. Some G6PD deficiency variants protect against symptomatic vivax malaria but whether they affect the risk of relapse is not known [22].

In summary, shorter course ascending dose vivax malaria radical cure regimens in G6PD deficient subjects offer the prospect of an effective treatment which does not incur prohibitive haemolytic toxicity.

Data Availability

All data produced are available online at *https://github.com/jwatowatson/Primaquine-Challenge*

https://github.com/jwatowatson/Primaquine-Challenge

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Authorship contributions

SP helped plan and oversee the clinical study. PJ, BH, KP and SP recruited the volunteers and took clinical responsibility. KP and PL helped manage the volunteers and oversaw study procedures. CC and GB helped with genotyping, manual reticulocyte counts, and study design. BH and PL supervised the clinical study and measurements. KC oversaw laboratory sample preparations and storage. BT coordinated the clinical study with PJ and coordinated study administration. NPJD had overall responsibility for the research. JAW designed the analytical approach, managed the adaptive design and recommended dose adjustments, conducted the analysis, and wrote the first draft of the paper. NJW conceived the study, advised on dose adjustment and helped interpret the results. All authors contributed to the interpretation and writing of the manuscript.

Conflict of Interest Disclosures

The authors have no conflicts of interest.

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Public Review:

In countries endemic for P vivax the need to administer a primaquine (PQ) course adequate to prevent relapse in G6PD deficient persons poses a real dilemma. On one hand PQ will cause haemolysis; on the other hand, without PQ the chance of relapse is very high. As a result, out of fear of severe haemolysis, PQ has been under-used.

In view of the above, the Authors have investigated in well-informed volunteers, who were kept under close medical supervision in hospital throughout the study, two different schedules of PQ administration: (1) escalating doses (to a total of 5-7 mg/kg); (2) single 45 mg dose (0.75 mg/kg).

It is shown convincingly that regimen (1) can be used successfully to deliver within 3 weeks, under hospital conditions, the dose of PQ required to prevent P vivax relapse.

As expected, with both regimens acute haemolytic anaemia (AHA) developed in all cases. With regimen (2), not surprisingly, the fall in Hb was less, although it was abrupt. With regimen (1) the average fall in Hb was about 4 G. Only in one subject the fall in Hb mandated termination of the study.

Since the data from the Chicago group some sixty years ago, there has been no paper reporting a systematic daily analysis of AHA in so many closely monitored subjects with G6PD deficiency. The individual patient data in the Supplementary material are most informative and more than precious.

Having said this, I do have some general comments.
1. Through their remarkable Part 1 study, the Authors clearly wish to set the stage for a revision of the currently recommended PQ regimen for G6PD deficient patients. They have shown that 5-7 mg/kg can be administered within 3 weeks, whereas the currently recommended regimen provides 6 mg/kg over no less than 8 weeks.
2. Part 2 aims to show that, as was known already, even a single PQ dose of 0.75 mg/kg causes a significant degree of haemolysis: G6PD deficiency-related haemolysis is characteristically markedly dose-dependent. Although they do not state it explicitly in these words (I think they should), the Authors want to make it clear that the currently recommended regimen does cause AHA.
3. Regulatory agencies like to classify a drug regimen as either SAFE or NOT-SAFE; they also like to decide who is 'at risk' and who is 'not at risk'. A wealth of data, including those in this manuscript, show that it is not correct to say that a G6PD deficient person when taking PQ is at risk of haemolysis: he or she will definitely have haemolysis. As for SAFETY, it will depend on the clinical situation when PQ is started and on the severity of the AHA that will develop.

The above three issues are all present in the discussion, but I think they ought to be stated more clearly.

Finally, by the Authors' own statement on page 15, the main limitation is the complexity of this approach. The authors suggest that blister packed PQ may help; but to me the real complexity is managing patients in the field versus the painstaking hospital care in the hands of experts, of which volunteers in this study have had the benefit. It is not surprising that a fall in Hb of 4 g/dl is well tolerated by most non-anaemic men; but patients with P vivax in the field may often have mild to moderate to severe anaemia; and certainly they will not have their Hb, retics and bilirubin checked every day. In crude approximation, we are talking of a fall in Hb of 4 G with regimen (1), as against a fall in Hb of 2 G with regimen (2), that is part of the currently recommended regimen: it stands to reason that, in terms of safety, the latter is generally preferable (even though some degree of fall in Hb will recur with each weekly dose). In my view, these difficult points should be discussed deliberately.