The effect of primaquine on gametocyte development and clearance in the treatment of uncomplicated falciparum malaria with dihydroartemisinin-piperaquine in South Sumatra, Western Indonesia: an open label randomized controlled trial

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40-word summary
A 2-arm randomized controlled trial conducted in Indonesia showing that the addition of a single dose of primaquine (0.75 mg/kg) shortens the infectivity period of malaria patients treated with dihydroartemisinin-piperaquine with normal glucose-6-phosphate dehydrogenase (G6PD) enzyme levels.

ABSTRACT

Background. Artemisinin-based combination therapy (ACT) is very effective in clearing asexual stages of malaria and reduces gametocytemia, but may not affect mature gametocytes. Primaquine is the only commercially available drug that eliminates mature gametocytes.

Methods and objectives. We conducted a two-arm open-label randomized controlled trial to evaluate the efficacy of single dose primaquine (0.75 mg/kg) following treatment with dihydroartemisinin-piperaquine on *P. falciparum*'s gametocytemia, in Indonesia. Patients with symptomatic uncomplicated falciparum malaria, normal glucose-6-phosphate dehydrogenase (G6PD) enzyme levels, aged ≥5 years and hemoglobin levels ≥8 g/dL were assigned by computerized-generating sequence to receive either a standard 3-day course dihydroartemisinin-piperaquine alone (n=178) or combined with a single dose of primaquine on Day-3 (n=171). Patients were seen on days 1, 2, 3, 7 and then weekly for 42 days to assess the presence of gametocytes and asexual parasites by microscopy. Survival analysis was stratified by the presence of gametocytes on Day-3.

Results. DHP prevented development of gametocytes in 277 patients without gametocytes on Day-3. In the gametocytemic patients (n=72), primaquine was associated with faster clearance of gametocytes (HR=2.42, 95% CI 1.39- 4.19, *P*= 0.002) and reduced gametocyte densities (geometric mean area-under-the-curve 157 vs 330, *P*= 0.018). The Day-42 cure rate of asexual stages in the DHP-PQ and DHP-alone arms were: PCR-unadjusted: 98.7% vs 99.4% respectively; PCR-adjusted:100% for both. Primaquine was well tolerated.

Conclusion. Addition of a single dose of primaquine shortens the infectivity period of DHP treated patients with acute uncomplicated malaria and should be considered in low transmission regions that aim to control and ultimately eliminate falciparum malaria.

Clinical Trial Registration. NCT01392014.

INTRODUCTION

The World Health Organization (WHO) recommends that a single dose of primaquine be added to standard blood schizonticidal therapy of *P. falciparum* malaria because of its gametocidal effect and potential transmission blocking activity [1]. Primaquine is a member of the 8-aminoquinolines approved by the US-based FDA in 1952 and one of the older antimalarials still in widespread use. It's use has received renewed attention with the increased interest in malaria elimination [2]. However, the use of primaquine to reduce malaria transmission is controversial. Although it only adds $0.05 to the cost of therapy [3], primaquine can cause severe and occasionally life-threatening hemolysis in patients with severe glucose-6-phosphate dehydrogenase (G6PD) deficiency. Moreover, the artemisinins themselves, which are part of artemisinin based combination therapy (ACT), are very efficient in inhibiting the growth of asexual stages; thereby, indirectly inhibiting the development of *P. falciparum* gametocytes by reducing the asexual stage progenitors [4, 5]. Furthermore, numerous in vitro studies suggest that artemisinin derivatives may directly inhibit the development of young *P. falciparum* gametocytes into mature gametocytes [6, 7]. These later stage gametocytes, circulate in the peripheral circulation for several weeks to allow ingestion by the mosquito vector [8]. Hence, anti-malarial drug treatments that can reduce the development of mature gametocytes may play a key role in the elimination of malaria transmission. However, some studies suggest that artemisinins alone or in combination with other drugs merely reduce the number of immature gametocytes and are not effective in eradicating all mature gametocytes; consequently, ACT treated patients may remain infectious for 2 to 4 weeks [9-14]. Thus, additional drugs such as PQ, that act

directly against mature gametocytes may be beneficial to reduce or eliminate transmission in communities [1, 15].

In Indonesia, dihydroartemisinin-piperaquine (DHP) has been the first-line treatment for both uncomplicated *P. falciparum* and *P. vivax* infections since 2010. The risk of *P. falciparum* gametocytemia is higher after treatment with DHP than with other ACTs, possibly reflecting the relatively lower dose of artemisinins in DHP[16]. The national policy also includes the use of a single dose of 0.75 mg/kg primaquine in addition to DHP, however, with the exception of the bigger health centers and hospitals, screening for G6PD-deficiency is usually unavailable. Further, the effectiveness of primaquine in reducing gametocytes has never been evaluated in DHP-treated patients in Indonesia. The present trial was designed to determine the risk-benefit profile of a single dose of primaquine when given directly following treatment with DHP.

MATERIALS AND METHODS

Study site

The study was conducted between December 2008 and March 2010 at Hanura Primary Health Center, Padang Cermin district, Lampung province (105°45'-103°48'E and 3°45'-6°45'S) located at the southern end of Sumatra. The health center was the only place in the immediate area providing artemisinin-based combination drugs for antimalarial treatment. Nearby abandoned shrimp cultivation ponds, provide larval habitat for *Anopheles sundaicus,* an efficient and important malaria vector in the region [17]. Seasonal malaria transmission peaks following the rainy season between September and April. At the time of the study, artesunate-amodiaquine with a single dose of primaquine

was the first-line therapy for patients with *P. falciparum* infection. High-grade chloroquine resistance to both *P. falciparum* and *P. vivax* is well-established in the area [18]. Community based surveys showed that the study area had low malaria endemicity with a malaria prevalence of 1.8% across all age groups at the time of this study (Sutanto *et al*, unpublished).

Participants

This was a single center open-label two-arm randomized controlled superiority trial in patients with fever or a history of fever within the past 24 hour with microscopically confirmed *P. falciparum* infections, regardless of the presence of gametocyte stages. Inclusion criteria consisted of: 1) parasite density≥ 1,000/µL;2) age ≥5 years; 3) normal glucose-6-phosphate dehydrogenase (G6PD) enzyme levels based on a qualitative test (Trinity Biotech no 203, USA); 4) hemoglobin level ≥ 8 gr/dL (Hemocue®Hb 201+); 5) negative pregnancy test (assessed by HCG urine test) or not breastfeeding; 6) no signs of severe malnutrition; 7) no other chronic diseases; 8) no history of allergy to the study drugs; 9) ability to return for 42-day-follow up. A history of previous antimalarial drug use was not an exclusion criterion. The protocol was approved by the Ethical Committee of the Faculty of Medicine, University of Indonesia, Jakarta.

Procedures

Subjects received a standard 3-day course of DHP (fixed-dose tablets of 40 mg dihydroartemisinin and 320 mg piperaquine; D-ARTEPP®, Guilin Pharmaceutical Co. , Ltd, China) with (DHP-PQ) and without PQ (DHP-alone). The daily DHP regimen was based on weight (≥41 kg: 3 tablets; 31-40kg: 2 tablets; 18-30kg: 1 tablet). The total dose of dihydroartemisinin ranged from 4-10.9 mg/kg and piperaquine 32-87.3 mg/kg. In

the DHP-PQ arm, a single dose of primaquine (PQ, 15 mg base, PT Phapros TbK, Jakarta, Indonesia) was given on day 3 to achieve a dose of 0.75 mg/kg, rounded to the nearest half tablet. The mean dose was 0.74 mg/kg (range 0.5 to 0.94 mg/kg). Modeling has suggested that the greatest impact of primaquine on gametocytes can be achieved by administrating primaquine on day 8 after the start of symptoms [19]. However, self-treatment and corresponding delay in seeking care from health centers is common in Indonesia such that many patients attend clinic several days after the onset of symptoms. We chose to administer primaquine on day 3 to maximize the delay of primaquine administration while minimizing the impact on compliance.

All treatment doses were given as directly observed therapy by the health-care attendants. If the patient vomited within 30 minutes, the same dose was repeated. If drug vomiting re-occurred the subject was excluded. Before taking the tablets, each subject took biscuits provided by the study. Both manufacturers provided certificates of analysis for the batches used in the study, and fulfilled standards for Good Manufacturing Practices in China and Indonesia, respectively.

Subjects with fever (≥ 38.5^{o}C using tympanic membrane measurement) received paracetamol. Subjects were requested to return to the health center on Days 1, 2, 3, 7, 14, 21, 28, 35 & 42 or any other day in between if they felt ill. At each scheduled visit (except on Day 1) a finger-prick blood sample was collected for malaria blood smears and dried blood blots for parasite genotyping (100 µL of blood on Whatman FTA filter paper). Patients found to be parasitemic on day-7 or later were treated with quinine (3 x 10 mg/kg/day for 7 days). Compliance or outcome following treatment with quinine was not monitored.

Thick and thin blood smears were stained with 3% Giemsa solution for 40 minutes. The number of asexual parasites was counted against 200 leukocytes and expressed per microliter assuming a leukocyte count of 8,000 /µL blood. If fewer than 10 parasites were detected in the first 200 leukocytes, counting was continued against 500 leukocytes. If no parasites were detected, 100 fields were examined before a smear was declared negative. The counting of sexual stage parasites was always against 500 leukocytes. Blood smears were read independently by 2 laboratory technicians, in the field (unblinded to the treatment allocation) and in the central laboratory in Jakarta (blinded). In case of disagreement, a third reader (blinded) reread the slide and final results were based on the third reader. Parasite densities were determined using the average parasite counts from both technicians. Hemoglobin levels were assessed on Days 0, 7 and 42 using a Hemocue (Hb 201+, Angelholm, Sweden). G6PD deficiency was defined using qualitative assays based on the fluorescent spot test (Trinity Biotech, USA, cat no 203-A).

Molecular analysis to distinguish reinfections from recrudescences of *P. falciparum* was performed at the Eijkman Institute, Jakarta, by genotyping GLURP, MSP2, and MSP1 in paired samples of treatment failures[20, 21].

Randomization and masking

A computerized randomization sequence was prepared in advance. Block sizes of four were used to ensure equal distribution across the study arms by time. Allocation concealment was achieved by using pre-numbered opaque and sealed envelopes. Eligible subjects were randomized sequentially by drawing successive envelopes according to their order on the patient's list at the health care.

Outcomes and sample size

The main objective was to determine the effect of the standard single dose of primaquine in addition to the completed 3-day treatment with DHP on gametocyte carriage. This was done by comparing the overall risk of gametocyte carriages on day-7 and then weekly till day-42, to measure the combined effect on gametocyte development and clearance (primary endpoint). This 2-arm trial required 165 patients per arm to detect a 7% absolute reduction in the prevalence of gametocytes following the week after treatment with primaquine from 9% in DHP-only arm to 2% in the DHP+PQ arm with 80% power and a two-sided alpha of 0.05. The anticipated gametocyte prevalence of 9% was based on the Day-7 prevalence in an earlier study with DHP (Sutanto *et al*, unpublished observations). We planned to recruit 200 patients per arm, to allow for 17.5% loss to follow-up, but recruitment was stopped earlier because the success rate of follow-up was higher than anticipated.

We also assessed the impact on gametocytes using the following secondary endpoints: a) the gametocyte clearance rates by day-42 in patients with gametocytes on day 3, b) the incidence of gametocyte development by day-42 in patients who were gametocyte free on the day 3, and c) gametocyte densities between days 3 and 42 inclusive. Other endpoints included the effect of primaquine on recurrence of the asexual stages of *P.falciparum*, PCR-adjusted and unadjusted for reinfections.

Adverse events were evaluated using a questionnaire prompting for symptoms of headache, weakness, nausea, vomit, abdominal pain, diarrhea, itching and paresthesia.

Statistical analysis

Modified Intention-To-Treat (mITT) analysis of the effect of primaquine was used and the evaluable population included all patients that were seen on Day-3. Patients who withdrew, were lost to follow up or had protocol deviations before day 3 were excluded from the analysis; however they were included if these events occurred after day 3.

Data were analyzed using STATA v10.0. Differences in gametocyte prevalence were compared using the prevalence ratio (PR) and corresponding 95% confidence interval (CI), along with P-values using Chi-square test or Fisher Exact test. The therapeutic response to treatment was assessed by calculating the cumulative risks of recurrence of asexual parasites (unadjusted and PCR-adjusted for reinfections by genotyping) and compared by survival analysis, using the Kaplan-Meier product limit formula[22]. In the PCR-unadjusted analysis, recurrences were treated as treatment failures and all other events (withdrawal, protocol deviations, occurrence of *P. vivax*) resulted in censoring at the time of that event, or at the time of their last follow-up visit in case of loss to follow-up. A similar strategy was used for the PCR-adjusted analysis except that patients with new *P. falciparum* infections were censored at the time of parasite reappearance[22]. Unadjusted Kaplan Meier survival analysis was also used to compare the cumulative risk of gametocyte development in the gametocyte free population on day 3, and to measure the risk of persistence of gametocytes or appearance of new gametocytes in those gametocytemic on day 3. Similar criteria for censoring were used as for the unadjusted analysis of the asexual stages.The difference in area-under-the-curve (AUC) of log transformed gametocyte density starting from Day-3 to Day-42 was compared using the T-test. Gametocyte densities are presented as the geometric mean (95% CI) and the

difference compared by geometric mean ratio (95% CI). Adverse events on Day-7 were evaluated among patients without the sign or symptom on Day-3 and differences between the two groups compared by the prevalence ratio. Mean hemoglobin levels on days 7 and 42 were analyzed with a generalized linear model, adjusted for the baseline hemoglobin levels on Day-0 and results expressed as mean difference.

RESULTS

Evaluable population

A total of 374 individuals were randomized; 186 received DHP+PQ and 188 DHP-alone. Of the 374, 25 (6.6%) were excluded from analysis (15 and 10 subjects from DHP+PQ and DHP-alone groups, respectively (Figure 1). Thus, 171 subjects in the DHP+PQ arm and 178 subjects in the DHP-alone arm contributed to the mITT analysis of which 41 and 31 had gametocytes on Day-3 respectively. Baseline characteristics on Day-3 were comparable (Table 1). During the follow up period, 9 and 11 subjects from DHP+PQ and DHP-alone groups were censored (7 took prohibited drugs, 10 moved from the study area, 3 withdrew from the study).

Gametocyte responses

The prevalence of gametocytemia was slightly, but not significantly higher among the DHP-PQ group on Day-3 just prior to the administration of primaquine (PR=1.38, 95% CI 0.91-2.09, P= 0.13). By Day-7 (4 days after PQ administration), the ratio had changed to PR=0.93 (95% CI 0.54-1.58, P= 0.78), and by Day-14 the prevalence of gametocytes in primaquine group was 16.6-fold lower than in the DHP-alone arm (PR=0.06, 95% CI 0.01-0.47, P<0.0001) (Figure 2). No-one developed microscopically patent gametocytes

among the patients who were gametocyte free on Day-3. Among those who were gametocytemic on Day-3, patients in the DHP-alone group remained gametocytemic for longer: HR 2.42 (95% CI1.39-4.19, *P*=0.002, Figure 3). The geometric mean gametocyte densities were lower in the primaquine group (157/µl, 95% CI 111-222 vs 330/µL, 95% CI 197-554; geometric mean ratio 0.47, 95% CI 0.26-0.88, *P*= 0.018)

Therapeutic response on asexual stages by Day-42

Primaquine did not affect asexual stages of *P. falciparum*. The PCR-unadjusted recurrence rates were 1.3% (2 subjects) and 0.6% (1 subject) in the DHP-PQ and DHP-alone groups (HR=0.87, 95%CI 0.39-1.97, *P*= 0.913). Molecular genotyping suggested that all 3 recurrences were due to re-infections.

Hemoglobin measurements and adverse events

Hemoglobin levels were much the same between the two study arms: Day-0: Mean difference (MD) (95% CI) -0.09 g/dL (-0.54, 0.36); Day-7: -0.02 g/dL (-0.43, 0.39); Day-42: -0.03 g/dL (-0.39, 0.33). The main complaints reported on day-7 were headache, fatigue and weakness, nausea and vomiting, abdominal pain, diarrhea, pruritus and parasthesia, with similar numbers in both arms (Table 2).

DISCUSSION

DHP effectively treated falciparum malaria and successfully prevented the appearance of new gametocytes. The addition of a single dose of primaquine reduced gametocyte densities and enhanced gametocyte clearance and shortened the median clearance time by about 1 week resulting in overall lower gametocyte carriage. By day-14, gametocytes were undetectable in all but one patient (0.6%) with primaquine compared to 9.6% (17

patients) without. Primaquine had no additional effect against asexual blood stages of *P. falciparum*. These results are consisted with previous reports in symptomatic malaria patients treated with artemisinins or ACTs showing that a single dose of primaquine suppresses and quickly eliminates persistent mature blood-stage gametocytemia that were not affected by the artemisinins[12, 23-24].

Concerns exist about the potential for a one-off dose of primaquine to induce hemolysis, especially in glucose-6-phosphate dehydrogenase (G6PD) deficient individuals[25]. A recent study from Tanzania suggested that co-administration of artemisinins with primaquine may be associated with mild asymptomatic haemolysis and transient reductions in Hb concentrations even in individuals with the wild-type genotype[26]. This study used genotyping and screened for the most common mutation associated with G6PD deficiency in Tanzania (G6PD A). Since 140 mutations are known to be potentially associated with G6PD deficiency one possible explanation suggested by the authors is that patients with less common mutations causing G6PD deficiency may have been misclassified. Functional screening test based on enzyme activity that measures the NADPH production capacity of G6PD, such as used in our study, do not have this limitation. We found that mean hemoglobin concentrations by day 7 and 42 were similar in both groups in this selected sample. However, the sample size was too small to draw more definitive conclusions about the safety of a single dose of primaquine as the study was not designed with safety as primary endpoint and excluded patients with G6PD deficiency.

There is typically a 7 to 15 days delay after the initial acute attack for mature *P. falciparum* gametocytes to become apparent. The timing of the single dose of

primaquine is therefore important, especially because with a half-life of about 8 hours primaquine is effective only for a few days. In Thailand, gametocytemia peaked 3 days after start of treatment with artemisinins and in about of quarter of gametocytemic patients they emerged after treatment[27]. The median duration of the history of fever prior to treatment was 5 days, consistent with models by Lawpoolsri *et al* predicting a maximum gametocytemia 8 days after the onset of symptoms[19]. In Indonesia, similar delays in seeking care are common. We therefore provided primaquine the day after the last dose of DHP to maximize the delay of primaquine administration.

There are several study limitations: This was an unblinded study, so all parties involved were potentially aware of the treatment allocation. Second, we omitted 14 patients from the analysis because 7 randomized to DHP-only were accidentally provided primaquine. The allocation sequence concealment was good, and there was no indication that this was due to preferential treatment by study-staff. Third, our study reflects the impact on microscopically detectable gametocytemia and does not exclude the persistence of sub-patent gametocytemia detectable by PCR. Our results however are consistent with one previous treatment trial in Tanzania suggesting a marked benefit of primaquine in clearing submicroscopic gametocytes in symptomatic children receiving sulphadoxine-pyrimethamine-artsunate [24]. By contrast, another trial in Sudan that aimed to eliminate submicroscopic gametocytes in asymptomatic carriers during the dry season found no effect of primaquine[28]. They hypothesized that ACTs alone are sufficient to clear gametocytes in asymptomatic carriers of sub-microscopic infections, but that primaquine is required in patients with acute malaria with higher parasite densities.

Although haphazardly implemented, the use of single dose primaquine to reduce malaria transmission of falciparum malaria is widely recommended. As countries move from malaria control to elimination a better understanding of the risks and benefits of adding single-dose primaquine to ACTs, has become increasingly important[23]. Because a relatively high proportion of patients had detectable gametocytes on enrolment (21%), sick malaria patients constitute a significant source of transmissible malaria in this low transmission areas. The addition of primaquine could thus have a major effect on malaria transmission from treated patients by shortening the period of infectivity, consistent with previous observations in Myanmar[23] and other studies looking at the added value of primaquine when provided in addition to ACTs or artemisinins[24]. Combined, these findings support recommendations that in settings where screening for G6PD deficiency is feasible, a single gametocidal dose of primaquine should be added to ACTs in low-transmission regions that aim to control and ultimately eliminate falciparum malaria. Further research is needed of primaquine as adjunctive gametocidal therapy under more programmatic conditions where routine screening for G6PD may not be feasible, including dose-finding studies to determine the minimal safe and effective dose.

NOTES

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Conflicts of interest.

All authors declare that they have no conflict of interests.

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Table 1. Baseline characteristic of study subjects.

Variables	Total		Without gametocyte		With gametocyte	
	DHP + PQ	DHP	DHP + PQ	DHP	DHP + PQ	DHP
Total subjects	171	178	130	147	41	31
Male/Female	107/64	107/71	83/47	86/61	24/17	21/10
Age (years), median (IQR)	20 (11–31)	17 (10–30)	23 (12-35)	19 (11-31)	12 (8-23)	13 (9-24)
Body weight (kg), median (IQR)	45.0 (25.0–55.0)	45.0 (24.0–55.0)	48.5 (33.0-58.0)	45.0 (24.0-55.0)	28.0 (18.0-48.5)	29.0 (20.0-50.0)
Body temperature (^{o}C), mean (SD)	35.7 (0.7)	35.7 (0.6)	35.7 (0.7)	35.7 (0.6)	35.7 (0.7)	35.8 (0.7)
Fever on Day-3 (≥ 37.5^{o}C), No. (%)	0 (0%)	1 (0.6%)	0 (0%)	1 (0.7%)	0 (0%)	0 (0%)
History of duration of illness (days), median (IQR)	3 (2-5)	3 (2–5)	3 (2-4)	3 (2-4)	7 (4-7)	3 (2-7)
Hemoglobin (gr/dL), mean (SD) on Day-0	13.3 (2.15)	13.3 (2.08)	13.8 (1.93)	13.7 (1.87)	11.5 (1.81)	11.2 (1.77)
Density of asexual stage (per µL), mean (SD) on Day-3	NF	NF	NF	NF	NF	NF
Gametocytemia on Day-3, No. (%)	41 (24.0%)	31 (17.4%)	0 (0%)	0 (0%)	41 (100%)	31 (100%)
Gametocyte density(per µL), median (IQR) on Day-3	128 (52-260)	196 (88 -552)	NF	NF	128 (52-260)	196 (88 -552)
History of self-treatment prior to health center, No. (%)	39 (22.8%)	33 (18.5%)	30 (23.1%)	21 (14.3%)	9 (22.0%)	12 (38.7%)

Abbreviation: NF= not found, IQR= interquartile range, SD=standard deviation.

Table 2. Tolerance of DHP+PQ versus DHP-alone by Day-7

Adverse events	DHP + PQ No. (%)	DHP-alone No. (%)	RR (95% CI)	*P* value
Headache	13/97 (13.4%)	18/96 (18.8%)	0.71 (0.37-1.38)	0.312
Fatigue	13/93 (14.0%)	11/96 (11.5%)	1.22 (0.58-2.58)	0.603
Nausea	1/139 (0.7%)	4/150 (2.7%)	0.27 (0.03-2.40)	0.205
Vomiting	1/163 (0.6%)	1/168 (0.6%)	1.03 (0.07-16.34)	1.000
Abdominal pain	4/156 (2.6%)	4/170 (2.4%)	1.09 (0.28-4.28)	1.000
Diarrhoea	1/163 (0.6%)	3/172 (1.7%)	0.35 (0.04-3.35)	0.623
Pruritus	0/170 (0.0%)	0/177 (0.0%)	--	--
Paresthesia	2/161 (1.2%)	2/170 (1.2%)	1.06 (0.15-7.41)	1.000

Evaluable population includes only patients without the symptoms on Day 3.

Figure 1. Trial profile

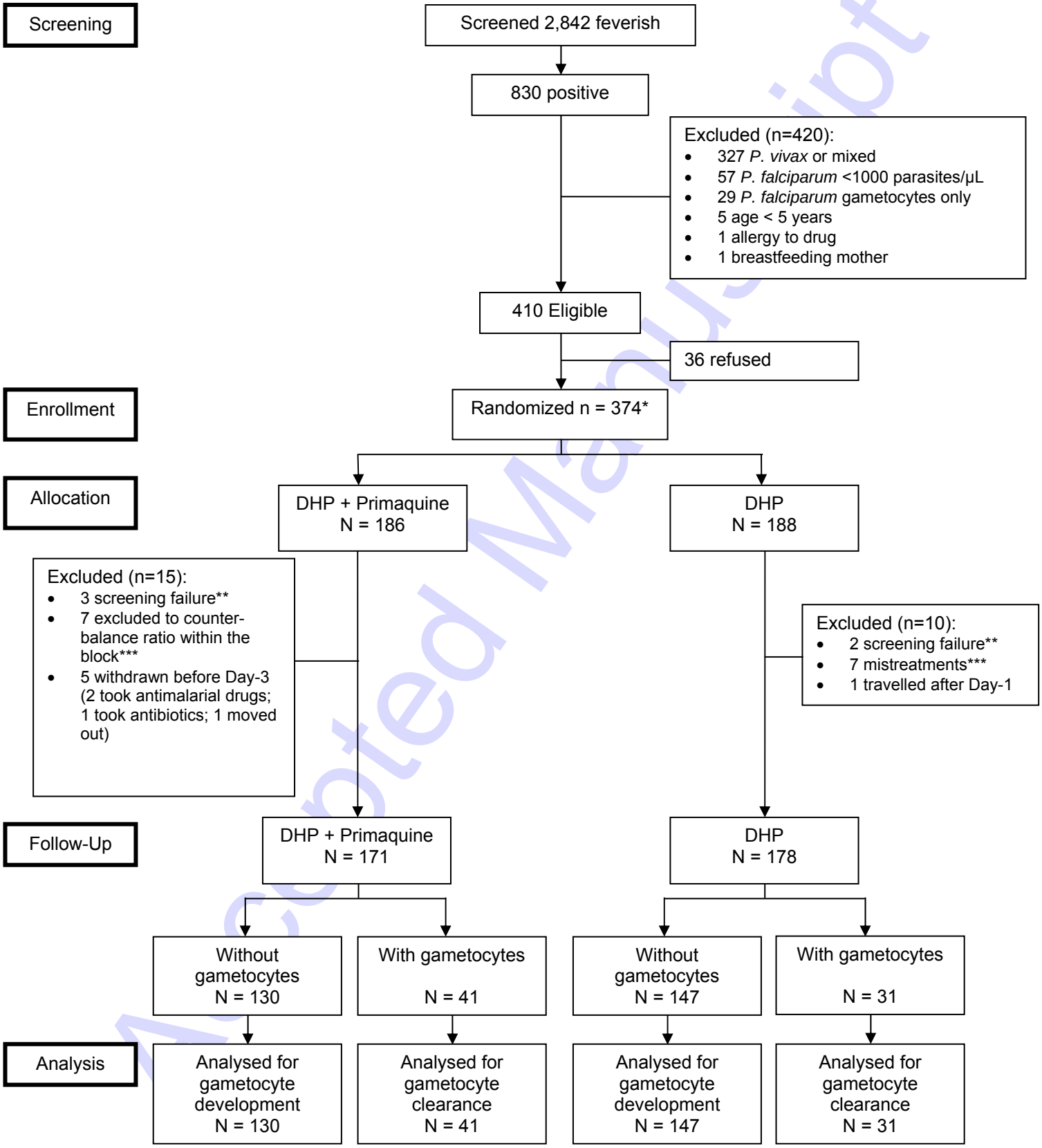


Figure 1 notes:

* All 374 patients who provided informed consent and were screened for G6PD deficiency were found to have normal levels of enzyme activity.

**Five subjects were screening failures: 4 had mixed infections with *P. vivax* and 1 was infected by *P. vivax* instead of *P. falciparum*.

*** Seven individuals allocated to the DHP-alone arm were accidently given the DHA-PQ regimen due to human error. In order to maintain the balance within the block randomization, seven subjects randomised to DHP+PQ within the same blocks of 4 were also excluded.

Figure 2.Gametocyte prevalence and prevalence ratio of the two regimens (black box=DHP+PQ and white box= DHP-alone) during 42 days follow up period.

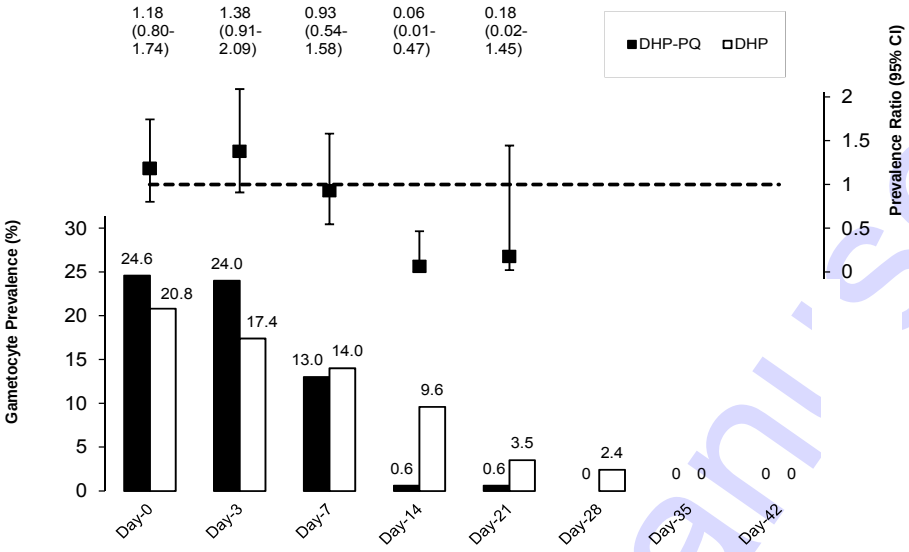
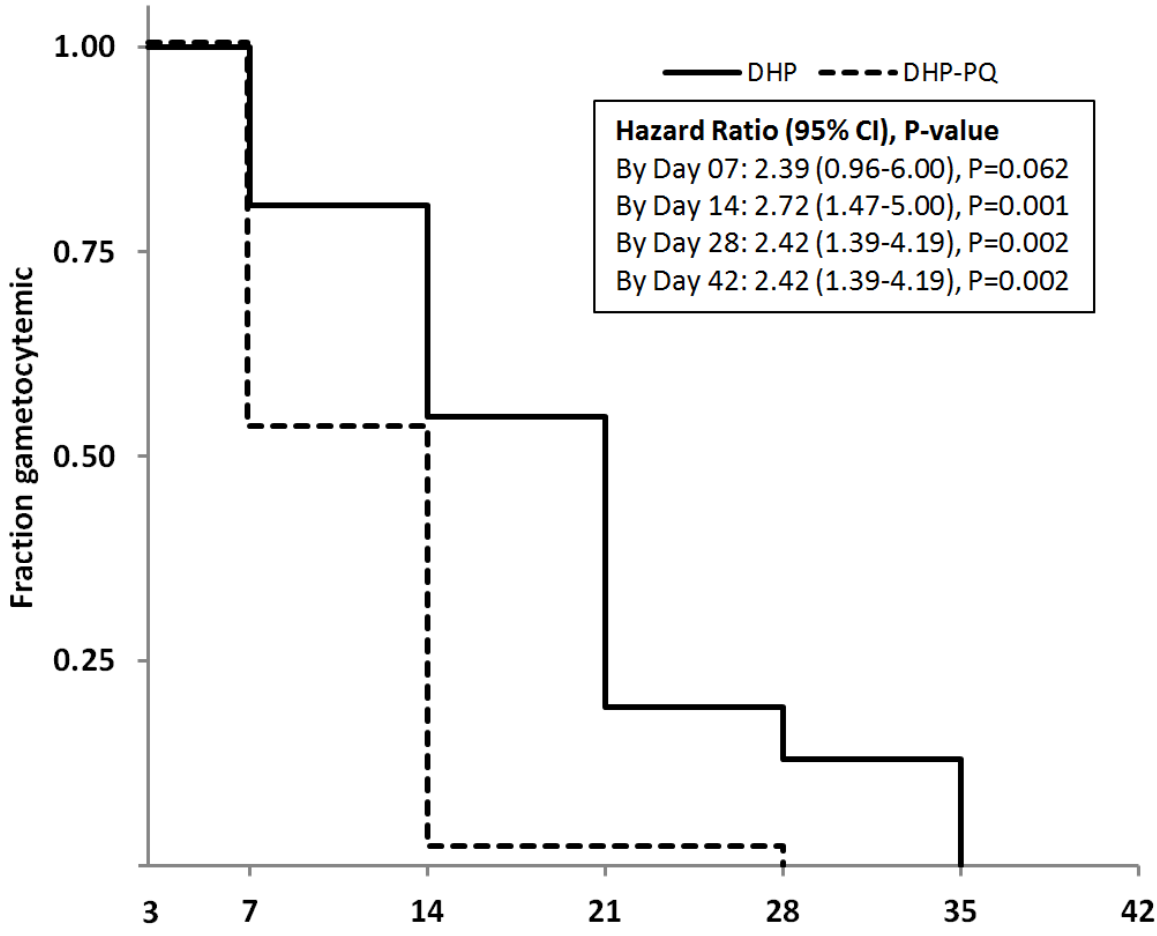


Figure 3.Kaplan Meier Survival curves of gametocyte clearance by treatment regimen



Number at risk	Day-3	Day-7	Day-14	Day-21	Day-28	Day-35	Day-42
DHP-PQ	41	22	1	1	0	0	0
DHP	31	25	17	6	4	0	0