Comparing drug regimens for clearance of malaria parasites in asymptomatic adults using PCR in Kilifi County, Kenya: an open-label randomised controlled clinical trial (MalPaC)

[version 1; peer review: 2 approved with reservations]

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Abstract
Background: To restrict trial endpoints to infections acquired after vaccination in Phase IIb trials of candidate malaria vaccines, participants are treated with anti-malarial drugs to clear existing infections. Anti-malarial drugs with a long half-life may inhibit the acquisition of new infections. This study evaluated the effects of three anti-malarial drug regimens on the clearance of existing infections and acquisition of new infections.

Methods: An open-label randomised controlled trial (MalPaC) was conducted between November 2013 and February 2014. Ninety adults were randomised 1:1:1 to receive one of three treatments: atovaquone/proguanil and artesunate (AP+AS); artesunate (AS); or sulphadoxine-pyrimethamine, artesunate, and primaquine (SP+AS+PQ). Parasite monitoring was determined over 84-day follow-up by assessing Plasmodium falciparum positivity by 18s qPCR, live and sexual stage parasites by RT-PCR, and recrudescence of infections by msp2 genotyping.

Results: At enrolment, parasite prevalence by qPCR was 44% (40/90, day 0), which fell to 10% (9/90, day 16), then rose to almost the initial rates by day 84 (39%, 35/90). Individuals treated with AS and...
SP+AS+PQ were more likely to have higher qPCR positive rates compared to participants treated with AP+AS in the immediate post-treatment phase (days 16-28) (OR=7.7 [95%CI 4.6-12.8] p<0.0005 and OR=4.2 [95%CI 2.6-6.8] p=0.0005, respectively). In the immediate post-treatment phase, qPCR positivity was less likely associated with evidence of live parasites and gametocytæmia. Prevalence of “old”, “new” or “undetectable” infections did not differ significantly over time or drug regimen. However, participants on the AP+AS drug regimen were less likely to have parasite infection recrudescence compared to participants treated with AS and SP+AS+PQ.

**Conclusion:** Falciparum DNA remained detectable by PCR post-treatment with incomplete parasite clearance regardless of drug regimen. Though AP+AS drug regimen may also have partially suppressed the acquisition of new infections during post-treatment follow-up.

Trial registration: Pan African Clinical Trials Registry, 22nd of August 2013, PACTR201309000625311.

**Keywords**
Malaria, Parasite clearance, PCR monitoring, Atovaquone/proguanil, Artesunate, Primaquine, Sulphadoxine-pyrimethamine, antimalarials

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Introduction

*Plasmodium falciparum* malaria is an important cause of morbidity and mortality in endemic populations in Africa. Interventions including insecticide treated mosquito nets (ITN) and anti-malarial drugs have reduced mortality globally; however, more interventions are required for elimination and/or eradication. A vaccine is urgently needed, and several candidates are undergoing testing. Prior to large scale field trials, Phase II studies are undertaken to obtain preliminary evidence of efficacy. These can include controlled human malaria infections or randomized field trials including adults or children. Adults in endemic regions have immunity to clinical malaria, and hence trials involving adults normally use the acquisition of infection as an endpoint. In these trials, anti-malarial drugs are used prior to surveillance for malaria infection to differentiate between new infections and sustained infections acquired before vaccination, particularly for pre-erythrocytic vaccines which can only act against new infections acquired after vaccination and not old infection acquired prior to vaccination. The ideal anti-malarial drug(s) for this purpose should be able to clear parasites but not inhibit the acquisition of new infections during follow-up as this would undermine the ability to detect vaccine efficacy.

Clearance of parasite post-treatment can be assessed using PCR, microscopy, or rapid diagnostic tests (RDTs). PCR is more sensitive at detecting malaria parasites and increases the power of studies to detect endpoints. PCR can detect parasites at low density, detecting parasites as low as 2 to 5 parasites/µl of blood depending on the volume of blood used for PCR extraction, whereas microscopy and RDTs can detect parasite densities of above 50 parasites/µl. Furthermore, PCR based genotyping of *P. falciparum* parasites is also used to differentiate new from recrudescence infections.

Different antimalarial drugs have been used for clearing parasitaemia prior to surveillance in previous Phase IIb trials for malaria vaccines. Sulphadoxine-pyrimethamine (SP) was given to participants to clear blood stage *P. falciparum* infections two weeks before administering the third dose of the RTS,S/AS02 vaccine in a study in The Gambia to test the efficacy of RTS,S/AS02. The acquisition of infection(s) was then monitored by blood film microscopy. In another study in The Gambia, participants, who were randomized to receive either DNA ME-TRAP followed by MVA ME-TRAP or rabies vaccine (control group), were given a dose of SP two weeks before the last vaccination, with follow-up by microscopy. Furthermore, another trial using PCR monitoring for malaria parasites rather than microscopy, parasite clearance was achieved using Lap/Dap (a combination of chlorproguanil and dapsone), artesunate and primaquine were used. Lap/Dap has since been withdrawn from public use because of its strong association with haemolytic anaemia. Further studies in Kenya with PCR monitoring have used atovaquone/proguanil with artesunate. However, there are no studies comparing the different antimalarial drugs in clearing parasites in the context of vaccine trials and their impact on the acquisition of new infections post-vaccination. In this study, we compared the clearance of PCR-detectable malaria parasites in adult participants in a Phase IIb clinical trial following the administration of three different drug regimens.

Methods

Trial registration

The trial was registered on the Pan African Clinical Trials Registry on the 22nd of August 2013. Trial identifier number: PACTR201309000625311.

Ethical statement

The study was approved by the Kenya Medical Research Institute Scientific and Ethics Review Unit (KEMRI-SERU, SSC 2565) and the University of Oxford Tropical Ethics Research Committee (OxTREC, 1034–13). The trial was also approved by the Kenyan regulatory authority, Pharmacy and Poisons Board (ECCT/13/08/02). Informed consent was sought at the study dispensers by study clinicians with the assistance of experienced field workers. Potential volunteers received verbal explanations and study information sheets that were translated in relevant local languages and discussed any concerns that required clarifications with investigators. The volunteers were asked to provide written informed consent before being recruited to participate in the study. Volunteers who were unable to read and write were asked to thumb print the consent form if they wished to consent, and this was counter-signed by an independent witness. Personal information was handled with utmost confidentiality and documents secured in the Clinical Trials Facility. Personal information was not entered into the electronic database.

Study site

This study was carried out in Junju, Kilifi County in Kenya, an area of moderate to high transmission with estimates of 22 infective bites/person/year between November 2013 to February 2014. ITN use in the study area increased from 55.9% in 2009 to a peak of 82.6% in 2013 partly due to a mass distribution of ITNs in the same year.

Inclusion and exclusion criteria

Local administrative and community leaders were first sensitized about the project. Public meetings were then arranged in the study villages to inform the communities of the study. These meetings were facilitated by the investigators. Field workers helped to identify potential volunteers where they conducted one-on-one meetings with them to ensure understanding of the information sheet and the consent form. Individuals who then felt the trial was appropriate for them to participate in were invited to attend a formal screening visit in the study dispensary while the study clinicians sought their informed consent with the help of experienced field workers. Residents of the study area aged between 18 and 50 years, who consented to participate in the study, were included in the study. Upon consenting, volunteers were screened for any clinically significant acute and chronic illnesses by physical examination and laboratory tests. Volunteers were excluded if they had: (1) significant medical conditions or low levels of haemoglobin (less than 11.3 g/dl for men and less than 10g/dl for women); (2) blood transfusion within a month preceding enrolment; and (3) enrolled in
another clinical trial under 12 weeks preceding enrolment in this study. In addition, female volunteers were screened for pregnancy on recruitment and on the first day of drug administration. Pregnant and lactating women and those unwilling to use contraception for the duration of drug treatment were excluded from the study. Recruitment and screening of volunteers was continued until 90 eligible volunteers were identified.

**Sample size considerations**

In a previous study, 60% of adults were PCR positive prior to drug treatment. At 7 days after treatment 30% were positive and only 5% subsequently had high-level positive PCR results.

According to the primary objective, we needed to detect the difference between 30% positivity at 7 days and lower levels, which would ideally be 1 to 2%. Using STATA software version 12\(^{22}\) for a comparison of proportions at \(p=0.05\) and \(power=0.8\), this suggests a sample size of 30 subjects per group.

To detect the difference between 5% late high-level positivity and our estimated proportion in the absence of any long-term drug effect, at 40%. Using STATA software version 12\(^{22}\) for a comparison of proportions at \(p=0.05\) and \(power=0.8\), this suggests a sample size of 20 subjects per group.

Since the former calculation required a larger sample size, a sample size of 30 per study group was used.

**Study design**

This was an open-label, single site, parallel, randomised controlled trial involving adults.

**Randomisation.** Ninety healthy asymptomatic adult participants were recruited to participate in the study and randomized by an independent statistician to three different groups in the ratio 1:1:1 to receive one of the following drug regimens: Group 1: atovaquone/proguanil (4 tablets each of 250mg/100mg taken once daily for 3 days) and artesunate (200mg once daily for 3 days) (AP+AS); Group 2: artesunate (400mg on day 1 and then 200mg once daily for 6 days) (AS); or Group 3: sulphadoxine-pyrimethamine (3 tablets 500mg/25mg taken once on day 1), artesunate (200mg once daily for 3 days) (AS), and primaquine (1 dose of 15mg taken once on day 1) (SP+AS+PQ).

Randomization was carried out after screening had been done and participant eligibility has been confirmed. A two stage randomization process was carried out. 20 G6PD deficient participants were first randomised to Group 1 and 2 by simple randomization. The remaining 70 (without G6PD deficiency) were randomly assigned in a ratio of 2 to 3 to group (1 and 2) and group 3 respectively by simple randomisation. This was to avoid the potential for haemolytic anaemia if a G6PD deficient volunteer is given primaquine (Group 3).

The random allocation sequence was generated by an independent statistician using STATA version 13, and this allocation was indicated on a card that was sealed inside an opaque envelope with the study ID number of eligible participants written on it. Inside the envelopes were cards printed with the study ID corresponding to what had been written on the envelope and treatment allocation stuck on them. Participants were enrolled by the study clinicians after they did a final eligibility check, after which they handed over the patient case report form (CRF; Extended data\(^{23}\)) and sealed randomization envelope (with a study number corresponding to the study ID number on the CRF) to the study nurse to administer the randomised treatment. Drug administration commenced on day 0. The laboratory scientists were blinded to the drug allocation until the end of the study, but the clinical investigators, participants and field teams were not blinded.

**Malaria parasite monitoring.** Malaria parasite monitoring was done by PCR for markers for asexual and sexual parasites. This was done at screening for eligibility followed by a total of 25 time points (2,249 samples) after enrolment into the study. In the first 3 weeks after enrolment, venous blood samples in ethylenediaminetetraacetic acid (EDTA) were taken and tested by PCR three times per week, subsequently, samples were taken for PCR twice per week for 3 weeks and then once every week for another 6 weeks. For qPCR analysis, 0.5ml of whole blood was collected. For RT-PCR parasite monitoring, 100 µL of whole blood samples and the appropriate amount of trizol was added and the sample kept at −80 °C until analysed.

**qPCR for detection P. falciparum parasites.** DNA was extracted from 500 µL of whole blood after white cell depletion using a previously described method of filtering\(^{6}\). The DNA was eluted in 50 µL, from which 15 µL of DNA (5 µL run in triplicate) were amplified by quantitative PCR using a TaqMan assay for *P. falciparum* multicycle 18S ribosomal RNA gene\(^{6,21}\). Quantification was on an Applied Biosystems 7500 Real-Time PCR System with quantification by Applied Biosystems 7500 software v2.0.6. Samples were analyzed in singlet wells. Three negative control wells (non-template control) and 7 serial dilutions of DNA extracted from *in vitro* parasite cultures were included as standards on each plate in triplicate as described previously\(^{8}\). Plates failing quality control standards were repeated. This was done for all the 2,249 samples across all 25 time points.

**RT-PCR for live parasites and sexual parasite markers.** A subset of samples were analysed for asexual and sexual parasites by RT-PCR. To understand the reasons for persistent DNA positivity at low parasite densities, the time points with low qPCR parasitaemia (≤100 parasites/µl) were particularly selected. Baseline samples (day 0 samples) for comparison were needed, and thus included for all respective participants meeting the criteria of low parasitaemia. In addition, the respective time points with the highest qPCR parasitaemia for the respective participants were included in the analysis with two other randomly selected time points of qPCR positivity per participant.

RT-PCR for *P. falciparum* DNA using a previously described method of filtering\(^8\). The DNA was eluted in 50 µL, from which 15 µL of DNA (5 µL run in triplicate) were amplified by quantitative PCR using a TaqMan assay for *P. falciparum* multicycle 18S ribosomal RNA gene\(^{6,21}\). Quantification was on an Applied Biosystems 7500 Real-Time PCR System with quantification by Applied Biosystems 7500 software v2.0.6. Samples were analyzed in singlet wells. Three negative control wells (non-template control) and 7 serial dilutions of DNA extracted from *in vitro* parasite cultures were included as standards on each plate in triplicate as described previously\(^8\). Plates failing quality control standards were repeated. This was done for all the 2,249 samples across all 25 time points.

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by SuperScript VILO cDNA Synthesis Kit (Invitrogen). For *P. falciparum*, multiplicity 18S ribosomal RNA gene or Pf25 gene was amplified by RT-PCR reactions to detect gametocytes. The reaction mixture contained 1 µl Superscript 2 reaction buffer, 0.2 µl RNaseOUT, 1 µl dNTP mix (10 mM each), 1 µl 10X PCR buffer (Applied Biosystem), 1 µl Taq DNA polymerase (Applied Biosystem), 1 µl of primer (either target or housekeeping) and 5 µl of cDNA sample was used to amplify target sequences by reverse transcriptase PCR. All RT-PCR reactions were run on an ABI 7500 Real-Time PCR system (Applied Biosystem). Target primer sequences were assayed alongside housekeeping gene primers. Positive and negative control samples were included in all RT-PCR reactions. For positive control, asexual parasites of 3D7 strain were either cultured up to gametocyte stage as described elsewhere or up to asexual stages. RNA was extracted from day 14 mature gametocytes and cDNA synthesized using the above-mentioned kits and included in the gametocyte detection assays. Uninfected red blood cells were also taken through the same process and used as a negative control. Non-template control was also included as a control.

**Msp2 genotyping.** *Msp2* genotyping was performed to differentiate between old (i.e. recrudescent from pre-treatment samples) and new infections for *P. falciparum* infections within individuals over time for time points with qPCR parasite positivity. The qPCR positive DNA samples were analyzed by a previously described nested PCR method in which fluorescently labeled primers specific to the two allelic types (FC27 and IC/3D7) were used in the nested reaction, followed by fragment sizing by multi-coloured capillary electrophoresis in a DNA sequencer.

**Endpoints.** The primary endpoint was to compare drug regimens for the first episode of *P. falciparum*, defined as two or more consecutive blood samples confirmed positive by PCR for *P. falciparum*. The secondary endpoints were: 1) To determine the percentage of PCR results that are confirmed by RNA positive results indicating live *P. falciparum* parasites; 2) To compare the percentage of positive results after day 7 after drug treatment to examine clearance rates and longitudinal patterns of PCR positivity to distinguish re-infection from failure to clear parasites.

**Statistical analysis**

STATA software version 15 was used to perform statistical analysis. Clearance rates following the three drug regimens were compared to determine the optimal drug regimen. These were expressed as counts and as percentages of samples that were PCR positive at various time points following treatment. The Lowess curve was used to show the trend in malaria infections. Concordance between various malaria parasite monitoring strategies was assessed using the chi-square test. The relationship between demographic characteristics of study participants and PCR positivity was assessed using Logistic regression. A multilevel logistic regression model was used to adjust for the non-independence of repeated measures per individual over time.

Classification of infections was done using *msp2* genotyping. Two genotypes were considered different if they were of different allelic types (FC27 or IC/3D7) or if their fragment length differed by more than 3 base pairs within the same allelic type. An infection was classified as “old” if its genotype was first detected before visit day 7 or “new” if its genotype was not seen before visit day 7. A genotype was only considered once per individual for classification as “new” or “old” on first identification after day 7 (e.g. a genotype detected on days 13, 15 and 17 was only considered on day 13). An infection was classified as undetected if the PCR sample was positive, but the genotyping failed. The Kruskal-Wallis equality of populations test was used to compare differences in prevalence of the type of infection.

**Results**

**Demographic characteristics**

In total 167 participants were recruited and screened for eligibility and enrolment into the study. 90 participants met the eligibility criteria, were enrolled and randomised into one of the drug regimen groups in the study (Figure 1). Treatment groups were balanced for gender, age and bed net use (Table 1).

**Parasite prevalence following treatment**

There was 99.9% completion rate with 89 participants completing all 25 study visits and only one participant not attending the last visit on day 84. A total of 2,249 samples for the 25 timepoints were collected and analysed for the presence or absence of malaria parasites by qPCR.

In total, 44.4% (40/90) participants were positive for *P. falciparum* specific 18s qPCR on enrolment (day 0) and the positivity rate fell to 24.4% (24/90) participants and 16.7% (15/90) participants by day 9 and day 14, respectively. Rates then fell further by day 16 to 10.0% (9/90) participants. The prevalence rose to 16.7% (15/90) participants by day 18 and 39.3% (35/90) at the end of monitoring (day 84) (Table 2). When aggregated by time period, there was an overall reduction in the qPCR positive rate for malaria parasites from 32.7% (147 out of 450 samples) during the drug treatment period (between day 0 and 7) to 20.4% (55/270) in the immediate post-treatment period (i.e. between day 9 and day 14) and to 14.1% (76/540) during post-treatment follow-up (between visit days 16 and 28) (Figure 2).

During post-treatment follow-up (between days 16 and 28), the highest positivity rate was in those who received AS at 22.8% (41/180), while the lowest was in those who received AP+ AS drug regimen at the rate of 7.2% (13/180). The trend in malaria infections by drug regimen was evident as shown by the Lowess curve (Figure 3).

**Predictors of qPCR positivity.** To assess the determinants of parasite positivity by qPCR including day, drug treatment, age, and bed net use in the reference category for drug treatment, a multivariable logistic model was used. Compared with AP+AS, participants treated with AS were more likely to be parasite qPCR positive during post-treatment follow-up (between day 16 and 28, OR=7.7 (95%CI 4.6-12.8) p<0.0005) and in the subsequent follow-up period (between day 31 and 56,
Figure 1. Study participants flow diagram. AP+AS, atovaquone/proguanil and artesunate; AS, artesunate; SP+AS+PQ, sulphadoxine-pyrimethamine, artesunate and Primaquine; N, count.

Table 1. Demographic characteristics of the study participants.

<table>
<thead>
<tr>
<th></th>
<th>AP+AS</th>
<th>AS</th>
<th>SP+AS+PQ</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male, N (%)</td>
<td>14 (46.7)</td>
<td>13 (43.3)</td>
<td>19 (63.3)</td>
<td>46 (51.1)</td>
</tr>
<tr>
<td>Female, N (%)</td>
<td>16 (53.3)</td>
<td>17 (56.7)</td>
<td>11 (36.7)</td>
<td>44 (48.9)</td>
</tr>
<tr>
<td><strong>Age (years) Mean (SEM)</strong></td>
<td>30.5 (1.51)</td>
<td>30.9 (1.53)</td>
<td>30.7 (1.52)</td>
<td>30.7 (0.87)</td>
</tr>
<tr>
<td><strong>Bed net use, N (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No bed net use</td>
<td>10 (33.3)</td>
<td>4 (13.3)</td>
<td>9 (30.0)</td>
<td>23 (25.6)</td>
</tr>
<tr>
<td>Treated bed net with no holes</td>
<td>8 (26.7)</td>
<td>16 (53.3)</td>
<td>10 (33.3)</td>
<td>34 (37.8)</td>
</tr>
<tr>
<td>Treated bed net with holes</td>
<td>10 (33.3)</td>
<td>6 (20.0)</td>
<td>9 (30.0)</td>
<td>25 (27.8)</td>
</tr>
<tr>
<td>Untreated bed net</td>
<td>2 (6.7)</td>
<td>4 (13.3)</td>
<td>2 (6.7)</td>
<td>8 (8.9)</td>
</tr>
<tr>
<td>Total</td>
<td>30 (100.0)</td>
<td>30 (100.0)</td>
<td>30 (100.0)</td>
<td>90 (100.0)</td>
</tr>
</tbody>
</table>

OR=2.1 (95%CI 1.2-3.5) p=0.011). Similarly, those who were treated with SP+AS+PQ were more likely to be parasite qPCR positive over the same time periods respectively: between visit days 16 and 28 (OR=4.2 (95%CI 2.6-6.8) p<0.0005) and between days 31 and 56 (OR=2.8 (95%CI 2.0-4.1) p<0.0005).

Bed net use was significantly associated with lower likelihood of qPCR positivity (Figure 4).

The impact of clustering by visit day and volunteer was examined using a) a two-level hierarchical model and b) using
Table 2. The prevalence of positive qPCR participants by drug regimen and visit days.

<table>
<thead>
<tr>
<th>Visit day</th>
<th>AP+AS (n=30)</th>
<th>AS (n=30)</th>
<th>SP+AS+PQ (n=30)</th>
<th>Overall (n=90)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>17 (56.7%)</td>
<td>12 (40.0%)</td>
<td>11 (36.7%)</td>
<td>40 (44.4%)</td>
</tr>
<tr>
<td>1</td>
<td>14 (46.7%)</td>
<td>10 (33.3%)</td>
<td>10 (33.3%)</td>
<td>34 (37.9%)</td>
</tr>
<tr>
<td>2</td>
<td>14 (46.7%)</td>
<td>9 (30.0%)</td>
<td>7 (23.3%)</td>
<td>30 (33.3%)</td>
</tr>
<tr>
<td>4</td>
<td>11 (36.7%)</td>
<td>6 (20.0%)</td>
<td>5 (16.7%)</td>
<td>22 (24.4%)</td>
</tr>
<tr>
<td>7</td>
<td>10 (33.3%)</td>
<td>6 (20.5%)</td>
<td>5 (16.7%)</td>
<td>21 (23.3%)</td>
</tr>
<tr>
<td>9</td>
<td>10 (33.3%)</td>
<td>8 (26.7%)</td>
<td>4 (13.3%)</td>
<td>22 (24.4%)</td>
</tr>
<tr>
<td>11</td>
<td>6 (20.0%)</td>
<td>4 (13.3%)</td>
<td>8 (26.7%)</td>
<td>18 (20.0%)</td>
</tr>
<tr>
<td>14</td>
<td>6 (20.0%)</td>
<td>5 (16.7%)</td>
<td>4 (13.3%)</td>
<td>15 (16.7%)</td>
</tr>
<tr>
<td>16</td>
<td>3 (10.0%)</td>
<td>4 (13.3%)</td>
<td>2 (6.7%)</td>
<td>9 (10.0%)</td>
</tr>
<tr>
<td>18</td>
<td>3 (10.0%)</td>
<td>6 (20.0%)</td>
<td>6 (20.0%)</td>
<td>15 (16.7%)</td>
</tr>
<tr>
<td>21</td>
<td>2 (6.7%)</td>
<td>7 (23.3%)</td>
<td>4 (13.3%)</td>
<td>13 (14.4%)</td>
</tr>
<tr>
<td>23</td>
<td>2 (6.7%)</td>
<td>7 (23.3%)</td>
<td>2 (6.7%)</td>
<td>11 (12.2%)</td>
</tr>
<tr>
<td>25</td>
<td>1 (3.3%)</td>
<td>8 (26.7%)</td>
<td>4 (13.3%)</td>
<td>13 (14.4%)</td>
</tr>
<tr>
<td>28</td>
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<td>56</td>
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</tr>
<tr>
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<td>10 (33.3%)</td>
<td>12 (40.0%)</td>
<td>33 (36.7%)</td>
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<td>84</td>
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<td>11 (36.7%)</td>
<td>11 (37.9%)</td>
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<tr>
<td>Total</td>
<td>203 (27.1%)</td>
<td>194 (25.9%)</td>
<td>178 (23.7%)</td>
<td>575 (25.6%)</td>
</tr>
</tbody>
</table>

AP+AS, atovaquone/proguanil and artesunate; AS, artesunate; SP+AS+PQ, sulphadoxine-pyrimethamine, artesunate and primaquine; N, count.

Figure 2. Prevalence of positive qPCR samples by drug regimen and aggregated visit days. Samples were analysed for asexual parasite positivity (18s DNA qPCR assay) for each participant over time enrolled in the three different drug regimen groups. Percent prevalence of parasite positivity is shown over aggregated visit periods. AP+AS, atovaquone/proguanil and artesunate; AS, artesunate; SP+AS+PQ, sulphadoxine-pyrimethamine, artesunate and primaquine.
Figure 3. Trend in qPCR parasite densities over time. Samples were analysed for *P. falciparum* positivity by 18s DNA qPCR assay for each participant over time enrolled in the three different drug regimen groups. Log transformed parasite densities (parasites/µL) with Lowess curve (red line) to show trend over time. Each individual circle represents a participant at each time point. AP+AS, atovaquone/proguanil and artesunate; AS, artesunate; SP+AS+PQ, sulphadoxine-pyrimethamine, artesunate and primaquine.

Clustering by visit day (i.e. the Huber White sandwich robust variance estimator). Similar results were observed in both these models (Extended data: Supplementary Tables 1 and 2).

Molecular characterization of malaria parasites

To determine if the infections detected after drug treatment were gametocyte positive (*Pfs25* RNA as the representative marker) or non-viable parasite material without active transcription (18s RNA as the representative marker), RT-PCR analysis was conducted on a subset of samples. 475 samples (26% of total representing 32 individuals) were analysed by RT-PCR after RNA extraction and cDNA synthesis.

Overall, 56.4% of the samples (348/617) within the subset assessed had concordant results for both 18s DNA and 18s RNA, while 6.32% (39/617) were negative for both (Figure 5A). 30.6% were 18s DNA positive and 18s RNA negative (i.e. implying non-viable parasites) while 6.7% (41/617) were 18s DNA negative but 18s RNA positive (implying low-levels of viable parasites). The concordance varied over time (Figure 5A). During the immediate post-treatment and follow-up period (between days 7 and 28), 18s DNA positive but 18s RNA negative parasites were more frequently observed after day 28 (χ² = 36.4, p < 0.0005 for the comparison of visit days 7 to 28 versus after day 28) (Figure 5A).

Concordance between qPCR and gametocyte RT-PCR positivity also varied over time (Figure 5B). Prior to treatment, 3.4% (34/211) of samples were *Pfs25* RNA positive. During the post-treatment period (between days 9 and 28), 9.4% (28/297) of samples were *Pfs25* RNA positive, and after day 28 12.6% (63/501) of samples were *Pfs25* RNA positive (Figures 5B and 5C).

Association between msp2 genotyping outcome and malaria parasite density

Genotyping was performed to differentiate where pre-treatment infection was not cleared by antimalarial treatment (i.e. “old” infection) from where participants were re-infected with new infections (i.e. “new” infection). Given the number of samples with either low density parasitaemia or different variants present where genotypes were undetected, the power
Figure 4. Determinants of qPCR positivity. Determinants of *P. falciparum* 18s DNA positivity were assessed using a multivariable logistic model. AP+AS, atovaquone/proguanil and artesunate; AS, artesunate; SP+AS+PQ, sulphadoxine-pyrimethamine, artesunate and primaquine.

Figure 5. Concordance between molecular markers of parasitaemia. Samples were analysed for asexual parasite positivity (18s DNA qPCR) and a subset of these for gametocyte positivity (Pfs25 RT-PCR) and whether they represented live parasites (18s RT-PCR) using cDNA. (a) correlation analysis between 18s DNA and 18s RNA; (b) correlation between 18s DNA and Pfs25 RNA; and (c) correlation between 18s RNA and Pfs25 RNA.
of analysis was moderate. It was therefore necessary to aggregate samples by time interval to test for significant variation by drug regimen for old versus new infections. The prevalence of “old”; “new”; or “undetected” genotypes did not differ significantly according to period of visit or drug regimen (Table 3).

Higher levels of parasite density were associated with success in msp2 genotyping while genotyping was highly likely to fail or undetectable if there were low levels of parasite densities (p=0.0001). Msp2 genotyping failure or undetectability was rare above a threshold of 1000 parasites per µl (Table 4; Figure 6).

**Discussion**

In this study, overall parasite positive rates among study participants with one of the three drug regimens (AP+AS, or AS, or SP+AS+PQ), declined from 32.7% qPCR positivity in the treatment period to 20.4% immediately post-treatment period to 14.1% decrease in post-treatment follow-up.

RNA markers specific to gametocytes (i.e. Pfs25) and non-specific RNA markers to detect all viable parasites (i.e. 18s) were used to determine if the persistent parasite DNA detection despite anti-malarial drug treatment reflected viable parasite or gametocyte. Parasite DNA was less often associated with signals of parasite viability (i.e. 18s RNA) in the post-treatment period compared with pre-treatment or later time periods. Furthermore, Pfs25 positivity was infrequent following drug treatment. Taken together, these findings suggest that gametocyteaemia was not a common cause for persistent qPCR parasite positivity after treatment, and furthermore that the detection of parasite DNA was not always linked to viable parasites,

<table>
<thead>
<tr>
<th>Table 3. Association between type of infection and drug regimen.</th>
</tr>
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<tbody>
<tr>
<td><strong>Old infections</strong></td>
</tr>
<tr>
<td>Visit day</td>
</tr>
<tr>
<td>9–28</td>
</tr>
<tr>
<td>31–84</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Pearson χ² = 3.74 P-value = 0.15</td>
</tr>
<tr>
<td><strong>New infections</strong></td>
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<tr>
<td>Visit day</td>
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<tr>
<td>9–28</td>
</tr>
<tr>
<td>31–84</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Pearson χ² = 1.74 P-value = 0.42</td>
</tr>
<tr>
<td><strong>Undetected</strong></td>
</tr>
<tr>
<td>Visit day</td>
</tr>
<tr>
<td>9–28</td>
</tr>
<tr>
<td>31–84</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Pearson χ² = 1.14 P-value = 0.57</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 4. Association between msp2 genotyping outcome and malaria parasite density.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parasites/µl of blood (PCR)</strong></td>
</tr>
<tr>
<td>0–10</td>
</tr>
<tr>
<td>10–10⁰</td>
</tr>
<tr>
<td>10⁰–10¹</td>
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<tr>
<td>10¹–10²</td>
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<tr>
<td>10²–10³</td>
</tr>
<tr>
<td>10³–10⁴</td>
</tr>
<tr>
<td>10⁴–10⁵</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>
raising the possibility that DNA detection methods might be positive even in the absence of live parasites. These findings are consistent with a study involving travellers treated for malaria in Sweden who had no risk of reinfection where parasite DNA was detectable by species-species qPCR up to the day 42 after treatment. The persistence of pre-vaccination DNA positivity complicates the use of PCR detectable infections as an endpoint for vaccine efficacy as it has the potential to result in an underestimation of vaccine efficacy. This is particularly important in case of pre-erythrocytic vaccines where the vaccine cannot conceivably protect against pre-vaccination blood-stage parasitaemia. Where an effect of a pre-erythrocytic vaccination is seen in a randomized trial this may be taken as evidence of vaccine effect, but where no effect is seen one cannot rule out that this may be the result of incomplete pre-vaccination asexual parasitaemia clearance and suppression of new infections in both controls and vaccines.

Compared with AP+AS, participants who were treated with AS or SP+AS+PQ were more likely to be qPCR parasite positive during the post-treatment follow-up period. Since parasite rates immediately post-treatment (between days 9 and 14) were similar for all three drug regimens, we conclude that the difference between the post-treatment follow-up period (days 16 and 28) are on balance more likely to be due to variations in reinfection rates between the drug regimens rather than variations in clearance rates. However, many samples were positive for low parasite densities, and it was often not possible to genotype the infections in order to distinguish recrudescence from reinfection to confirm this. Furthermore, although these differences were statistically significant, there is still significant DNA positivity after treatment with AP+AS, hence even this drug regimen is far from ideal for Phase IIb studies with PCR positivity as an endpoint.

It is unlikely that artesunate tolerance or resistance is relevant to these findings since the genetic polymorphisms associated with artesunate resistance have not been found in surveys in Coastal Kenya. The long-term suppression of reinfection would be consistent with the previously described long-lasting effect of atovaquone. The associations between qPCR positivity and msp2 genotyping outcome provides a measure to what level of parasite density one is able to resolve parasite msp2 genotypes.

Studies using DNA-based qPCR positivity for malaria parasites as an endpoint for vaccine efficacy have been done in non-endemic areas such as the Netherlands, and The Gambia where malaria is more markedly seasonal than in Kenya, and it is possible that this form of surveillance is easier to interpret in settings where a long dry season results in less frequent asexual parasite positivity prior to the start of monitoring.

**Limitations**
The sample size was limited to 30 participants per drug regimen. This limits power, particularly when infections are divided into new infection versus recurrence of previous genotypes. Genotyping undetectability was common at low parasite densities,
making it difficult to determine if the infection detected post-treatment was new or old. The study was undertaken in a single site and a single transmission season. It is possible that delayed parasite clearance would be less problematic in a site where transmission is minimal outside a malaria season that occurs after randomization, where fewer participants would have high density parasitaemia at the start of monitoring.

Conclusion
There was an overall significant reduction in the prevalence of malaria parasites by qPCR between day 9 and day 14 following treatment of the study participants with either of the three drug regimens, though it was surprising that substantial levels of qPCR parasite positivity were seen after day 9, and these were often due to parasite genotypes that had been present prior to treatment. Although AP+AS was less prone to this problem than the other regimens examined, it also may have inhibited the acquisition of new infections. More robust assessments of vaccine efficacy in small-scale Phase IIb trials may be obtained in controlled human malaria infection studies where the exposure is experimentally controlled and volunteers can be selected for falciparum DNA-based PCR monitoring prior to exposure to infection.

Data availability
Underlying data
Harvard Dataverse: Replication Data for: Comparing Drug Regimens for Clearance of Malaria Parasites in Asymptomatic Adults using PCR in Kilifi County, Kenya: an open-label randomised controlled clinical trial (MalPaC), https://doi.org/10.7910/DVN/HJHSIC.

This project contains the following underlying data:
- MALPAC_merged_20190114_unblinded.tab
- NKhaemba_ParasiteClearance_Codebook.pdf
- NKhaemba_ParasiteClearance_doFile.do
- NKhaemba_ParasiteClearance_Readme.txt

Extended data

This project contains the following extended data:
- Supplementary table 1: Determinants of PCR positivity using multilevel logistic regression
- Supplementary table 2: Determinants of PCR positivity using logistic regression

Reporting guidelines
Harvard Dataverse: Consort checklist for: ‘Comparing Drug Regimens for Clearance of Malaria Parasites in Asymptomatic Adults using PCR in Kilifi County, Kenya: an open-label randomised controlled clinical trial (MalPaC)’. https://doi.org/10.7910/DVN/CVZMNV.

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Acknowledgements
We thank all the study participants. Also like to acknowledge Moses Ngari the study independent statistician; John Fikiri who was the study data clerk; the field workers (who were overseen by Elias Pembe and the late Anthony Mbaru); Chrispinah Kaulu the study nurse; and the Junju dispensary county staff. This work was supported through the DELTAS Africa Initiative [DEL-15-003]. The DELTAS Africa Initiative is an independent funding scheme of the African Academy of Sciences (AAS)’s Alliance for Accelerating Excellence in Science in Africa (AESA) and supported by the New Partnership for Africa’s Development Planning and Coordinating Agency (NEPAD Agency) with funding from the Wellcome Trust and the UK government. The views expressed in this publication are those of the author(s) and not necessarily those of AAS, NEPAD Agency, Wellcome Trust or the UK government. This paper is published with permission from the Director of KEMRI.

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2011.


parasite density. PubMed Abstract | Publisher Full Text

dielectrophoretic and magnetophoretic methods. malaria diagnostic techniques: a review of the approaches with focus on PubMed Abstract | Publisher Full Text

methods (microscopy, RDT, and PCR) for the detection of malaria parasites in representative samples from Equatorial Guinea. methods (microscopy, RDT, and PCR) for the detection of malaria parasites in representative samples from Equatorial Guinea. PubMed Abstract | Publisher Full Text

The development of malaria diagnostic techniques: a review of the approaches with focus on PubMed Abstract | Publisher Full Text

diagnostic methods (microscopy, RDT, and PCR) for the detection of malaria parasites in representative samples from Equatorial Guinea. Malar J. 2018; 17(1): 333. PubMed Abstract | Publisher Full Text


The authors compared three drug regimens for eradication of asymptomatic malaria: AS+AP, AS, and SP+AS+PQ. This is an interesting study with rich data using a sensitive diagnostic tool (PCR) in asymptomatic adults on the Kenyan coast. PCR is also used in a sophisticated manner, to distinguish live from dead parasites, and gametocytes from other stages. There was a high prevalence of parasitemia at baseline (40%) that decreased temporarily, presumably due to reinfections in this zone of high endemicity.

Simple randomization of the 90 participants was not done; rather, 20 patients with G6PD deficiency were justifiably excluded from group 3 for safety reasons (which included primaquine). This detail could be included in the trial flow diagram since allocation was not truly random.

Because G6PD deficiency is X-linked, it is more common in males. Since 20 trial participants had G6PD deficiency and were deliberately not randomized to group 3 (SP+AS+PQ), we would expect a lower number of males in the SP+AS+PQ group, yet there were 63% males in this group (compared to 43-47% in the other groups, Table 1). This is puzzling. Can the authors provide the sex of the patients with G6PD deficiency?

The statistical analysis of the data requires additional attention.

This is a longitudinal cohort, with repeated measurements over time on the same patient. The data points are not independent, and require an approach (such as linear mixed effects models) to account for non-independence of data from repeated measurements on the same patient. The non-independence of the data does violate assumptions of the multivariable logistic model, and this is therefore not a valid analytic approach. STATA software can certainly account for clustering of data (repeated measures) and an analysis accounting for patient as a random effect would not be difficult. The results of the current regression model without accounting for repeated measures are not valid.

In addition to representing the data by multiple cross-sectional comparisons (percentage positive
at each time point), it would be important to look at the data longitudinally using the patient as the unit of analysis. Among the patients who tested positive on day 0 (17, 12 and 11, in groups 1, 2, and 3, respectively, denominator) how many were still positive on days 1, 2, 4, 7, etc. with a parasite of the same genotype (“old”). This is the clearance of pre-existing parasitemia prior to treatment, and may be a marker of drug effectiveness. It could be expressed as a clearance time, for example.

Then, the incidence of new infections (PCR positive in a previously negative participant, or infection with a different genotype) should be given over time (e.g., Kaplan-Meier curve). This is the rate of new infections and may be a function of drug half-life.

The current analysis does not clearly separate the "old" and "new" infections using the patient as the unit of analysis. Table 3 is particularly confusing in this respect and it is difficult to interpret the numbers and percentages. For "old infections; the denominator should be 17,12, and 11 in groups 1, 2, and 3, for example). If in fact 5/17 patients treated with AS+AP were still positive for the same genotype of parasite at day 31-84, this is a surprising result, and a very high failure rate for medications that have a reported efficacy >90% individually for the treatment of symptomatic infection. I suspect these are not "old" infections but reinfections. The timecourse for these 5 patients should be examined in detail: did the PCR become negative, then come back (possible recrudescent infection)? Was the genotyping insensitive in determining differences in parasite strain (e.g., reinfection incorrectly determined to be "old" infection because small differences in strain were not picked up)?

Successful msp2 genotyping was associated with higher parasite density, but this is hardly a surprising result and I’m not sure this warrants an entire Table (Table 4) and Figure (Figure 6). The presence of higher levels of template will increase the likelihood of successful amplification and sequencing (a sentence would suffice).

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.
Reviewer Expertise: Pediatric infectious diseases, global health

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 15 April 2020

https://doi.org/10.21956/wellcomeopenres.17120.r38410

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Ingrid Felger
Swiss Tropical and Public Health Institute (Swiss TPH), Basel, Switzerland

The manuscript of Khaemba et al. addresses antimalarial treatment strategies for use in participants of Phase IIb malaria vaccine trials, where participants are followed-up by molecular monitoring to assess pilot vaccine efficacy. This topic is not novel, as molecular monitoring has been implemented in field trials for many years. This manuscript is nevertheless very interesting and worth publishing, as it presents rich data from a drug trial comparing three drug regimens, and results from this drug trial may well be utilized for the design of pilot efficacy vaccine trials. The rationale, objectives and conclusions of this study require clarification. This manuscript needs substantial revision.

The major problem with this manuscript is that the stated objective is not aligned with the actual study design. The presented data derives from a comparative trial of the antimalarial combinations with a drug trial-specific study designed. Post-treatment parasite clearance and reinfections were investigated by qPCR, genotyping and RT-PCR for sexual parasites. How the drug trial data presented relates to the setting of a vaccine trial is not obvious at first glance and seems far-fetched. Instead, the investigations presented here seems to focus primarily on a thorough analysis of low level residual DNA persisting post-treatment, following earlier observations of one of the authors (Ref 29). The methodology is well aligned with research on persisting residual DNA, a question which could certainly stand on its own.

A shortfall of this manuscript is that the work presented does not reflect the principle study design of vaccine trials. If molecular monitoring would be performed in a vaccine trial, qPCR prevalence or number of new clones per follow-up period per participant would be compared between both arms of a vaccine trial and residual DNA persistence would apply equally to both arms. Moreover, if the objective of the here presented drug trial would have been investigation of pre-treatment options for a vaccine trial, the rationale for including an SP treatment arm seems obscure and needs clarification. Why would a long-acting drug (SP) be selected to achieve parasite clearance at baseline of a Phase IIb vaccine trial, where pilot vaccine efficacy is to be measured? The prophylactic effect of SP is well known, and the actual study design chosen seems not to be in line with the authors' suggestion of a short acting drug for Phase IIb vaccine trials. Also unclear: if
a vaccine trial design involves treatment at baseline and thus a potentially very long (in case of SP) prophylactic time period preventing new infections, what is the rationale for a follow up period of only 84 days. Is this follow-up period sufficient for assessing pilot efficacy of a blood stage vaccine for example? What are the requirements for a trial site's transmission intensity to achieve this? Instead of focusing specifically on vaccine trials, the trial presented here has a study design and sampling strategy adequate for measuring clearance rates in a drug trial (frequent bleeds in the initial phase), an outcome not relevant for vaccines. Thus, the design and stated purpose of this study seem unrelated.

While authors clearly state the primary and secondary outcome for their drug trial, they fail to clarify the actual primary and secondary outcomes in a vaccine trial. The range of possible molecular outcomes is neither reviewed nor discussed. Instead, this drug trial report presents a number of molecular assays but it is unclear what their role would be in monitoring vaccine efficacy? Missing in the manuscript is the information on which specific methodology the authors propose for molecular monitoring a Phase IIb vaccine trial. In their manuscript authors should add a clarification of relevant methodological issues, such as primary outcome in a Phase IIb vaccine trial of the question whether an outcome “positivity by qPCR” should be based on analysis of high volume of blood, as presented in the methodology section? Furthermore, is it necessary or helpful to track gametocytes in a vaccine trial? Should genotyping of new infections be performed in a vaccine trial, e.g. with incidence of new infections (genotypes) as outcome? (For an overview of outcome considered previously see Felger et al. 2003 Molecular monitoring in malaria vaccine trials. Trends in Parasitology). A lot of data and comprehensive analyses are presented in this manuscript, but the translation of these analyses to a vaccine trial context is not sufficiently clear.

Title: The title of the manuscript is misleading and not aligned with the vaccine trial topic of the introduction and discussion. The title refers to a drug efficacy study, reporting “Comparing drug regimens for clearance of malaria parasites in asymptomatic adults using PCR....“ and does not indicate a vaccine trial-related content.

Abstract: The conclusion of the abstract should be clarified and aligned with the study objectives: what is the outcome of this study regarding molecular outcomes in a vaccine trial? Do authors conclude that molecular monitoring in a Phase IIb vaccine trial is not feasible?

Methods and Results sections:
Overall, this study was conducted very thoroughly, the data were well presented and the manuscript was written clearly. The molecular methods used were adequate. The description of the experimental part would benefit from more detail, e.g. the efforts made regarding contamination control, a crucial part in highly sensitive parasite detection, e.g. the number of negative controls performed in DNA or RNA extraction and in each PCRs/RT-PCR experiments, or were the melt curves of amplicons inspected in each experiment to detect false positivity by primer dimers (for the SYBR Green–based assays). In line with this, some results from repeated extractions and amplifications performed in a subset of all samples should be included and presented (reproducibility). The reason for taking extra care, when using RNA-based detection methods, is the following: Performance of 18S rRNA RT-PCR is technically tricky owing to a great potential for contamination deriving from massive amounts of 18S rRNA templates per cell. Thus, aerosols from a single sample of high or moderate parasite density can cause contamination in some of the negative samples handled in parallel. Several authors of malaria molecular-diagnostics papers discussed this issue.
From a technical point of view, the data on discordance between positivity by 18S rDNA versus 18S rRNA is interesting. Firstly, the proportion of 18S rDNA+/18S rRNA- samples seems very high (Figure 5A). As the authors have used an adequate protocol (Trizol) for RNA sampling, 18S rRNA-based detection would be expected to be much more sensitive than 18S rDNA-based detection, owing to the huge difference in template abundance. It would be interesting to know, whether any of these 18S RNA negative samples were positive by pfs25 RT-PCR? To a limited degree, fluctuations in positivity (owing to stochastic template distribution) must be expected when aiming at detection of extremely low parasite densities. To understand the extent of such fluctuations, repeated analyses of samples with discrepant results are crucial. Was reproducibility of test results assessed?

The question arises how important is - in the context of vaccine field trial - the optimized detection sensitivity used here (using high volume of 0.5 ml whole blood and white blood cell depletion) that can detect ultra-low parasitemias, but comes at the cost of limitations in reproducibility of test results (through template fluctuation) and detection of irrelevant residual DNA? These issues should be discussed in detail.

Discussion section:
Authors should discuss the SP drug resistance situation in Kenya as well as SP half-life. SP has a pronounced prophylactic effect, however, this paper reported that SP+AS+PQ treated individuals were more likely to be qPCR parasite positive during the post-treatment follow-up period. How is this explained?

The discussion states that “these findings suggest that gametocytemia was not a common cause for persistent qPCR parasite positivity after treatment”. It would be important to add a statement explaining that gametocyte detection was performed in 100 µl whole blood samples, whereas DNA-based positivity was assessed in a larger blood volume of 0.5 ml. Because gametocytes are generally much less frequent in an infection compared to asexual parasites, the blood volume used for gametocyte detection matters in a crucial way. Upscaling DNA-based sensitivity but not RNA-based gametocyte detection, could have led to an underestimation of the contribution of surviving gametocytes as source of residual DNA and thus could challenge the authors’ conclusion. Figure 5B indicates that indeed in some individuals gametocytes have survived treatment.

In their discussion authors argue that qPCR positivity as an endpoint is “complicated by persistence of pre-vaccination DNA positivity“ and that even their best performing drug combination AP+AS showed “still significant DNA positivity after treatment, hence even this drug regimen is far from ideal for Phase IIb studies“. Whatever the reasons for persisting residual DNA are, the authors have made every effort to detect it. This upscale of sensitivity comes at a cost in diagnostic robustness (potentially enhancing contamination, undetected gametocytes, loss in reproducibility/template fluctuations). In analogy to the current WHO/MMV 2008 recommendations on recrudescence typing procedures, which suggest to perform genotyping for differentiation of recrudescence/new infection only, if a recurrent infection was positive by light microscopy, one could argue that a less sensitive methods may well be sufficient to measure outcomes also in a vaccine trial. An intermediate sensitivity is reached when amplifying length-polymorphic genotyping markers, which would permit to detect an infection and at the same time genotype each infection occurring during follow-up. As this distinguishes new infections from
those present at baseline, pre-treating all study participants at baseline would become redundant and the incidence of new infections could be considered as a trial outcome. Avoiding pre-treatment would permit an unbiased assessment of a vaccine in a host naturally infected with long-term asymptomatic infections (see Genton et al. 2002, JID). Phase IIb field trials are highly informative and should not be replaced, as the authors conclude: “More robust assessments of vaccine efficacy in small-scale Phase IIb trials may be obtained in controlled human malaria infection studies\textsuperscript{35} where the exposure is experimentally controlled and volunteers can be selected for falciparum DNA-based PCR monitoring prior to exposure to infection.” In my view this conclusion is not justified, as it is based on technical shortfalls or artefacts, caused mainly by maximizing diagnostic sensitivity. Sensitivity to detect new infections is not crucial if longitudinal follow-up bleeds are performed in short intervals, as an infection below the detection limit at a given time point likely will be identified in the subsequent follow-up sample.

The valuable data of this paper could led the way to establish alternative protocols, e.g., thorough assessment of the required test sensitivity, promotion of less sensitive genotyping approaches to establish the force of infection/number of new clones over the follow-up period in a Phase IIb vaccine trial.

Is the work clearly and accurately presented and does it cite the current literature? Yes

Is the study design appropriate and is the work technically sound? Partly

Are sufficient details of methods and analysis provided to allow replication by others? Yes

If applicable, is the statistical analysis and its interpretation appropriate? Yes

Are all the source data underlying the results available to ensure full reproducibility? Yes

Are the conclusions drawn adequately supported by the results? Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Molecular epidemiology and molecular diagnostics with focus on malaria parasites; molecular monitoring in drug and vaccine trials

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.