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Persistent detection of *Plasmodium falciparum*, *P. malariae*, *P. ovale curtisi* and *P. ovale wallikeri* after ACT treatment of asymptomatic Ghanaian school-children

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A B S T R A C T

Two hundred and seventy four asymptomatic Ghanaian school-children aged 5 to 17 years were screened for malaria parasites by examination of blood films. One hundred and fifty five microscopically-positive individuals were treated with dihydroartemisinin-piperaquine and followed for 3 weeks. Retrospective species-specific PCR of all 274 screened samples identified an additional 60 children with sub-patent parasitaemia, and a substantial proportion of co-infections with *Plasmodium malariae*, *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri*. One hundred individuals harboured at least one non-falciparum parasite species. Using standard double-read microscopy, the 21-day efficacy of treatment against *Plasmodium falciparum* was 91.4% among the 117 children seen at all 5 visits. Using nested PCR to test 152 visit 5 blood samples, 22 were found to be parasite-positive. Twenty individuals harboured *P. falciparum*, four harboured *P. ovale* spp. and two *P. malariae*, with four of these 22 isolates being mixed species infections. The persistent detection of low density *Plasmodium sp.* infections following antimalarial treatment suggests these may be a hitherto unrecognised obstacle to malaria elimination.

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1. Introduction

Studies of malaria in sub-Saharan Africa have been focused on a single parasite species, *Plasmodium falciparum*, because of its predominance among clinical cases, and its association with severe disease and mortality. Three other species are widespread in Africa but generally thought to be uncommon among clinical malaria cases: *Plasmodium malariae*, *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* (Collins and Jeffery, 2007; Sutherland et al., 2010). However recent cross-sectional population-based studies in Malawi, Uganda, Equatorial Guinea and Angola, using PCR for parasite detection and species discrimination, have shown that these three species are found in between 1% and 17% of tested individuals, regardless of whether malaria symptoms were present (Bruce et al., 2008; Oguike et al., 2011; Fançony et al., 2012). In each of these studies, the majority of these occurrences were as co-infections with *P. falciparum*, and we therefore surmise that, in Africa, *P. malariae* and *P. ovale* spp. will frequently encounter

control measures and case management strategies designed specifically for falciparum malaria.

Infections with non-falciparum malaria parasites have been adequately treated with chloroquine for many decades (Lalloo et al., 2007). However, in most African settings, species-level diagnosis is not available for people suffering from malaria (or suspected malaria), and so virtually all patients are treated for *P. falciparum* and are thus likely to receive artemisinin-combination therapy (ACT) where these drugs are available. Unfortunately, there are few *in vivo* data available to indicate how well non-falciparum species respond to ACT. Clinical trials commonly exclude mixed infections at enrolment and molecular characterisation of treated parasite isolates is not commonplace. Partially based on evidence for good efficacy of ACT against *Plasmodium vivax* in Asia (Kolaczinski et al., 2007; Douglas et al., 2010), it is expected that ACT will be very effective against *P. malariae*, *P. o. curtisi* and *P. o. wallikeri*. Recent evidence from Gabon would support this view (Mombo-Ngoma et al., 2012). As current research is now illuminating the moderately high prevalence of these species across Africa, it is important to assess the response of non-falciparum parasites to commonly used antimalarial drugs, and in particular to ACT.

As part of a study of *P. falciparum* gametocyte carriage and associated antibody responses, asymptomatic Ghanaian school

children with parasitaemia were enrolled in a longitudinal study. Microscopy-confirmed *P. falciparum* malaria infections were treated with a full course of the ACT dihydroartemisinin-piperaquine (DP). As an ancillary analysis, we tested for the presence of all *Plasmodium* species by PCR, both prior to and 3 weeks after treatment, and estimated drug efficacy against sub-patent parasitaemia.

2. Materials & methods

2.1. Study area

The study was conducted during the months of October–December 2010, in Pokukrom in the Ahafo Ano South District of the Ashanti region, which lies in the tropical rainforest ecological zone of Ghana. The mean monthly temperature ranges between 18 and 38 °C and the average rainfall in the neighbouring area of Kintampo is 1250 mm per annum, occurring mainly between May and October each year (Owusu-Agyei et al., 2009). The district is considered endemic for malaria and a parasite carriage rate of 65% has previously been recorded (H. Tagbor, unpublished data). Transmission of malaria in this area is perennial but peaks during the rainy season. Malaria is predominantly caused by *P. falciparum* (Asante et al., 2011) and no published studies have looked at the prevalence of the other malaria species in the district. The main vectors are *Anopheles gambiae* and *Anopheles funestus*; no current estimates of entomologic inoculation rate are available for Ahafo Ano District, but in Kintampo this is estimated to be 250 infectious bites per year (Owusu-Agyei et al., 2009). Laboratory support and microscopy facilities were provided at the Kwame Nkrumah University of Science and Technology (KNUST), Department of Biology, Kumasi. The study protocols were approved by the Ghana Health Service Ethics Committee (proposal # GHS-ERC-08/7/10) and the Ethics Committee of the London School of Hygiene and Tropical Medicine (proposal # 5775). Individual informed consent was obtained from the children and their parents/guardians before enrolment. Meetings were also held in the community and schools to explain the objectives of the study and to seek the consent of the community leaders and education authorities.

2.2. Participants and sample collection

Asymptomatic school children of Pokukrom Methodist primary between the ages of 5 and 17 years were screened for asexual malaria parasites in finger-prick peripheral blood. For each sample a rapid immunochromatographic point-of-care test (RDT) for antigenaemia was carried out (Malaria Pf rapid test, Shenyang LTH Technology Development Company, Beijing, China), blood smears made for microscopy, and approximately 10–50 μL of blood spotted directly from the child's finger onto Whatman grade 3 filter paper (Whatman, Maidstone, UK). Microscope slides were dried in the field and taken to KNUST for staining and examination. Filter paper blood spots were allowed to dry for 2–3 min and then transported in separate unsealed plastic bags together with a desiccant. Upon reaching the lab the filter papers were dried in an air-conditioned room for 24 h before freezing at −80 °C until processing for parasite DNA extraction was carried out.

All children were clinically examined by a trained nurse to ensure that no child had symptoms suggestive of malaria. Any child with a positive RDT and symptoms suggestive of malaria at visit 1 was immediately treated with DP as described below; these symptomatic cases were not enrolled and do not contribute to the current study on asymptomatic parasite carriage. The medical history and other information of each child were recorded in a case report form. School children who participated in the screening process were monitored daily for malaria symptoms by a nurse for one week at school. Children were advised to seek medical treatment at a designated health facility if they were to experience any symptoms related to malaria outside of school hours. The slides were read at KNUST by experienced microscopists and at the second visit of the study team to the participating school, 7 days after the first visit when samples were taken, all asymptomatic children with confirmed parasitaemia (sexual or asexual or both) at the time of visit 1 were enrolled. Inclusion criteria were: a recorded axillary body temperature of less than 37.5 °C, no history of fever in the previous 48 h and microscopically confirmed parasitaemia caused by *P. falciparum* with or without the presence of other *Plasmodium* species. All enrollees were treated with a standard regimen of DP, comprising 3 daily doses each of 1–3 tablets, depending on the weight and age, of P-ALAXIN (Bliss GVS Pharma LTD, Mumbai, India), containing 40 mg dihydroartemisinin (DHA) and 320 mg piperaquine phosphate per tablet. The study medicines were sourced from the Komfo Anokye Teaching Hospital in Kumasi. A sample of two tablets was tested for quality using an established method (Ioset and Kaur, 2009), and found to contain 98% of the stated amount of DHA and 101% of the stated amount of piperaquine. The medicinal quality of this product was therefore within the accepted tolerance limits of 95–105%, as set by the US Pharmacopeia (2012).

Each daily dose of the fixed-combination was administered under observation in the classroom, or if falling on a weekend, in the child's home, under supervision by teachers or study staff members. Children with symptomatic malaria or with evidence of any other chronic or acute illness were excluded from the study. Enrolled children were followed up for repeat finger-prick blood samples weekly for 4 weeks after the first blood sample was taken; as treatment was given on the second visit, each child was thus seen three further times after treatment. During this period the participants had access to free anti-malarial treatment and clinical care if required. The longitudinal study was designed to permit testing for plasma antibodies against *P. falciparum* gametocyte antigens in children with asymptomatic *P. falciparum* infections cleared by DP. Parasitological findings reported here were ancillary to the main study.

2.3. Microscopy or examination of blood smears

Thick and thin blood smears were stained with 10% Giemsa after fixing thin smears with methanol. Asexual parasite densities were determined by counting against 200 leukocytes, and converting to parasites per μL by assuming a standard leukocyte count of 8000/μL (Greenwood and Armstrong, 1991). At least 100 high power fields were examined before a thick smear was declared negative. Each slide was read independently by two experienced readers, and the geometric mean reading of the two replicates used for analysis. A third reader read the slide in cases where the parasite density estimates of the first two readers differed by more than 10%. Sexual parasite rate and density were determined by counting against 500 leukocytes and converted to parasites per μL as for asexual parasites (Drakeley et al., 2004).

2.4. Species identification by nested PCR

DNA was extracted from filter paper blood spots from visits 1 and 5 respectively in a 96-well plate using Chelex resin as previously described (Oguike et al., 2011). *Plasmodium* species were identified by nested PCR amplification of the small sub-unit ribosomal genes as previously described (Snounou et al., 1993) with the following second round PCR primers and amplicon sizes:

P. falciparum primers:	rfal1 + rfal2	205 bp product
P. malariae primers:	rmal1 + rmal2	144 bp product
P. vivax primers:	rviv1 + rviv2	120 bp product
P. ovale primers:	PovaFWD + RVScommon	375 bp product

Newly designed *P. ovale* spp. primers were deployed because the original pair of primers described by Snounou et al. (1993) do not amplify *P. o. wallikeri.*

| PovaFWD: | 5′-CTGTTCTTTGCATTCCTTATGC-3′ |
| RVS common: | 5′-GTATCTGATCGTCTTCACTCCC-3′ |

For the second round amplification, 1 µl of first round PCR product was amplified in a 20 µl reaction containing 10 µl of HotStar Taq master mix (Qiagen, UK) and 0.2 µM of each primer. PCR products were resolved on 2% agarose gels.

2.5. Discrimination between P. o. curtisi and P. o. wallikeri

P. o. curtisi and *P. o. wallikeri* were distinguished from each other by nested PCR amplification of the gene coding for tryptophan-rich antigen, and fractionation on agarose gels, or by direct sequencing of *pog3p* and *porbp2* amplicons as previously described (Oguike et al., 2011).

2.6. Statistical analysis

Associations between binary variables were tested using the *chi*-squared statistic, with Fisher's exact correction for comparisons in which any expected value was less than 6. Estimation of summary statistics and all tests of association were performed in STATA software (Stata 12.0, Statacorp, Texas, US).

## 3. Results

3.1. Parasite carriage by microscopy

Single-read microscopy of all 274 children immediately following screening identified 165 with positive blood films (geometric mean density 1.87 parasites μL^{-1} peripheral blood, range 0–428 μL^{-1}). Consent for inclusion in the longitudinal study was obtained from parents/guardians for 155 of these children. Definitive double-read microscopy carried out after completion of the study found an additional 16 children positive for *P. falciparum* trophozoites that had not been enrolled in the longitudinal study. Twenty-one of the enrolled children (13.6%) carried patent gametocytes of *P. falciparum*, 14 of whom carried concurrent asexual parasites. Further, the microscopists reported that the blood films of a small number of children with visible *P. falciparum* trophozoites also harboured blood-stage parasites of either *P. malariae* (24 children) or *P. ovale* spp. (3 children) (Fig. 1A).

At visit 2 (7 days after the first visit), DP treatment was given under observation to all 155 enrolled participants and a further blood film and filter paper collected. Double-read microscopy found that 55 of the 155 enrolled children no longer harboured patent asexual parasites, and that two of seven children who at visit 1 had circulating gametocytes only now harboured detectable asexual parasites of *P. falciparum*. These data show that a number of children in the study harboured fluctuating parasite densities very close to the limit of detection of microscopy, and hence parasite detection was often discordant between the two visits, despite

A. **Microscopic prevalence of *Plasmodium* spp. at visits 2, 3, 4 and 5.**
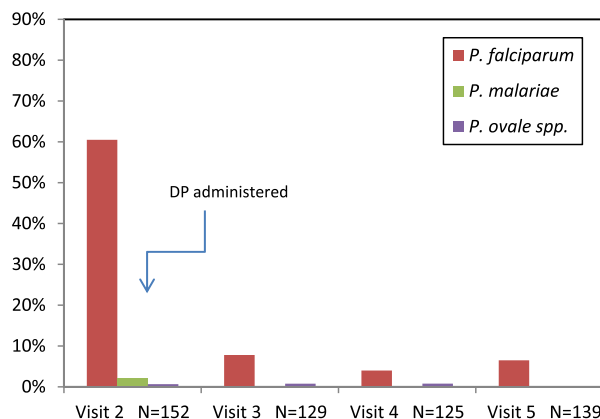
B. **PCR prevalence of *Plasmodium* spp. at visits 1 and 5.**
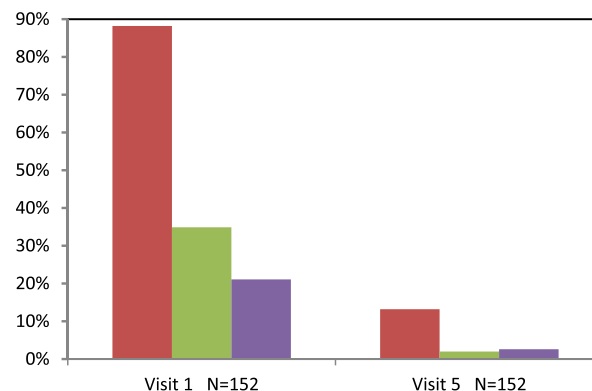
Fig. 1. Prevalence of *Plasmodium* spp. by microscopy (Panel A) and species-specific rRNA SSU nested PCR (Panel B). All children in the longitudinal study were identified as positive for *P. falciparum* at visit 1. Some children missed follow-up on visits 2–5 due to absence from school on the day. Only parasitological results from children in the longitudinal study are shown. DP: dihydroartemisinin-piperaquine.

Table 1
Mixed parasite species carriage detected by PCR at enrolment and 3 weeks post-treatment Data on parasite carriage is presented as a binary code for each of the three species (with all ovale infections considered together), where "0" denotes the absence of a species, and "1" denotes its presence by nested PCR. Only children with PCR data indicating the presence of at least one species are shown at each timepoint.

Pf/Pm/Po PCR code	Visit 1 (N = 270)		Visit 5: 3 weeks post-treatment (N = 152)	
	Freq.	Percent	Freq.	Percent
000	55	20.4	130	85.5
001	4	1.5	2	1.3
010	3	1.1	0	0.0
011	1	0.4	0	0.0
100	115	42.6	16	10.5
101	20	7.4	1	0.7
110	53	19.6	2	1.3
111	19	7.0	1	0.7
Total	270	100.0	152	100

Pf: *Plasmodium falciparum*; Pm: *P. malariae*; Po: *P. ovale* spp.

no treatment having been administered at the time of taking the second blood sample.

3.2. Species-specific PCR to detect parasite carriage prior to treatment

As expected, retrospective PCR analysis of DNA extracted from all 274 blood spots collected at visit 1 found 45 additional sub-patent *P. falciparum* infections. It was also shown that a high proportion of enrolled individuals were found to carry non-falciparum parasite species. A further 15 children previously parasite-negative by microscopy harboured *P. malariae*, and 8 were PCR positive for *P. ovale* spp., many children harbouring multi-species infections (Table 1). In total, 215 individuals out of 270 evaluable PCR tests (79.6%) were parasite positive for at least one species. Whereas 115 children among the 215 who were parasite positive were found to harbour *P. falciparum* alone, eight individuals were infected only with non-falciparum species, and 92 with *P. falciparum* and at least one of the other species. These findings are summarised using a simple binary species code in Table 1. The overall prevalence of *P. malariae* in all children tested was 28% (76 children), and that of *P. ovale* spp. was 16.3% (44 children). Taking the median age of 10 years as a cut-off, evidence was found that PCR-detected carriage was more common in the older group for both *P. falciparum* (OR: 2.21; 95% CI: 1.18–4.25; $P = 0.0082$) and *P. malariae* (OR: 2.07; 95% CI: 1.17–3.69; $P = 0.0077$), but not for *P. ovale* spp. (OR: 0.860; 95% CI: 0.422–1.73; $P = 0.651$). Further, the occurrence of either or both non-falciparum species at visit 1 was much more likely in children who were also positive for *P. falciparum* by PCR (odds ratio OR: 5.50; 95% CI: 2.43–14.0; $P < 0.001$). This relationship with *P. falciparum* carriage was also seen for *P. malariae* or *P. ovale* spp. when considered separately (OR: 7.87; 95% CI: 2.73–30.8; $P < 0.001$ and OR: 2.69; 95% CI: 0.990–9.14; $P = 0.040$, respectively). Thus 67 of the 155 children treated with DP in this study and followed up also harboured PCR-confirmed non-falciparum malaria infections, which were thus inadvertently exposed to one of the currently recommended ACT regimens.

P. o. curtisi and *P. o. wallikeri* are indistinguishable by microscopy, and so PCR amplification of the *potra* and *pog3p* genes was performed to discriminate which species were present in the 44 children identified by nested PCR as carrying *P. ovale* spp. (Oguike et al., 2011). Electrophoretic fractionation of *potra* amplification products, or direct sequencing of *pog3p* amplicons was able to identify *P. o. curtisi* in 27 individuals and *P. o. wallikeri* in 7 individuals. (In the remaining 10 children there was insufficient material to make a species-level identification.) Two children appeared to harbour both species by the *potra* assay. As individuals with both

species present simultaneously have only recently been described (Fançony et al., 2012; Fuehrer et al., 2012), we investigated these two children in more detail, repeating the original tests, and using other discriminatory loci (Sutherland et al., 2010). The presence of both *P. o. curtisi* and *P. o. wallikeri* sequences at each of the loci *potra*, *pog3p*, and *porbp2* was confirmed in both individuals, as sequences identical to those described for both species in previous studies were found (Sutherland et al., 2010; Oguike et al., 2011). Thus we conclude that each of these children harboured both parasite species that cause ovale malaria, supporting the recent demonstration that these related species are able to infect a single host simultaneously.

3.3. Parasite carriage following DHA-piperaquine

Of 155 children microscopy positive on visit 1, treated with DP on visit 2 and followed up, four remained positive for *P. falciparum* trophozoites by microscopy at visit 5 (Fig. 1A). Species-specific PCR gave four different species codes for these individuals (Table 1): 100, 110, 111 and 001. Thus, in one case, the microscopists identified *P. ovale* spp. parasites as a recurrent *P. falciparum* infection. Nested PCR testing of all samples at visit 5 identified 22 parasite positive individuals, a prevalence of recurrent parasitaemia 3 weeks after treatment of 14.5%. Unexpectedly, in only 16 of these individuals was *P. falciparum* the sole parasite species detected by nested PCR (Table 1; Fig. 1B). Thus post-treatment parasitaemia with non-falciparum malaria was detected in 6 individuals, and of these four were positive for this same species prior to treatment, two children each for *P. ovale* and *P. malariae*. The proportion of post-treatment infections positive for DNA from *P. ovale* spp. was 18%, which is similar to the baseline proportion of 20.5%. Two of these were confirmed as *P. o. curtisi* by genotyping at both *potra* and *pog3p* loci, whereas insufficient material was available to definitively identify the remaining two *P. ovale* spp. infections. DNA from *P. malariae* was detected in 13.6% of post-treatment infections, compared to 35.4% of infections prior to treatment.

4. Discussion

This study examines, in asymptomatic Ghanaian school children, the effect of ACT against the three less-common parasite species which infect people in sub-Saharan Africa: *P. malariae*, *P. o. curtisi* and *P. o. wallikeri*. These species are generally considered sensitive to standard antimalarial drugs including chloroquine (Collins and Jeffery, 2007; Mueller et al., 2007), yet we have shown using PCR-based detection that a small number of these infections recurred 3 weeks after treatment of asymptomatic parasite-positive individuals. As DP is known to provide good post-treatment prophylaxis (Four ABC Study Group, 2011), this finding was unexpected. In addition, we provide confirmation, using a multi-gene typing approach, that *P. o. curtisi* and *P. o. wallikeri* can be found simultaneously in a single individual, as two of our school children were found to harbour both ovale parasite types prior to treatment. Taking these results together with recent findings from Bangladesh and Angola (Fançony et al., 2012; Fuehrer et al., 2012), the weight of evidence is now firmly against our previously stated hypothesis that human blood groups may restrict *P. o. curtisi* and *P. o. wallikeri* to different subsets of sympatric human communities (Sutherland et al., 2010).

An interesting finding was that, among the 274 children originally screened, *P. malariae* and *P. falciparum*, but not *P. ovale* spp., were detected significantly more often in children 10 years and over. This is likely to be related to fewer episodes of febrile illness (whether malaria or not) in the older children and thus less frequent use of antimalarial drugs. If this suggestion is true, the absence of such an age relationship for *P. ovale* spp. can be

attributed to the ability of *P. o. curtisi* and *P. o. wallikeri* to relapse from dormant liver-stage hypnozoites once antimalarial drugs have cleared from the host bloodstream (Nolder, Oguike & Sutherland, unpublished data). Further, *P. malariae* and *P. ovale* spp. were significantly more commonly detected by PCR in individuals that were also PCR-positive for *P. falciparum*. Whereas hitherto unknown biological mechanisms may be responsible for this phenomenon, a simple and parsimonious explanation is that all three parasites are circulating in the same *Anopheles* mosquito populations, and thus children more exposed to *P. falciparum* infection because they live near mosquito habitat, have an unscreened house or do not use a bednet will also be more often exposed to the other *Plasmodium* species. Identification of *Plasmodium* species in blood-fed anophelines from this area would help to test this explanation. Further detailed studies of the associations between age, multi-species parasite carriage, quality of housing, exposure to biting mosquitoes and patterns of community drug use in African endemic areas are clearly needed.

The presence of PCR-detectable parasitaemia 3 weeks after ACT treatment in some of the study children is of concern. However, *in vivo* antimalarial drug efficacy cannot be meaningfully estimated from treatment outcomes in asymptomatic infections, and PCR positivity is not (yet) validated as a primary endpoint for clinical trials of antimalarial therapy. Bearing in mind these caveats, we nevertheless expected a higher rate of complete parasite clearance among the asymptomatic cases of *Plasmodium* spp. infection in our study. As piperaquine is considered to have a prophylactic effect lasting more than 3 weeks (d'Alessandro, 2009; Four ABC Study Group, 2011), emergent blood-stage infections from recent mosquito bites should also have been prevented. The observation that DNA from non-falciparum parasite species can be detected within 3 weeks of treatment with an effective ACT in school children was particularly unexpected, suggesting continuing asexual parasitaemia of *P. malariae* and *P. ovale* spp. in these individuals. There are a few possible alternative hypotheses to explain our findings of persistent sub-patent parasitaemia. Firstly, DNA from dead malaria parasites may persist post-treatment, either in circulating soluble form or in phagocytes that have engulfed parasites, and deliver a "false-positive" signal in diagnostic PCR tests (Sutherland and Hallett, 2009). However, there is no direct evidence to support this, and persistent DNA from dead parasites is unlikely to remain detectable after 3 weeks, particularly with the low starting parasite densities in our study subjects. A second plausible explanation is that the DNA we have detected is only from the gametocytes (sexual stages) of these species. It is known for *P. falciparum* that gametocyte carriage after DP treatment is more common than for other regimens such as artemether-lumefantrine (Four ABC Study Group, 2011), and that gametocytes of this species remain circulating in a proportion of ACT-treated malaria patients long after apparent clearance of asexual stages (Targett et al., 2001). Gametocytes of *P. malariae* and *P. ovale* spp. circulate in the peripheral blood of infected people at the same time as asexual stage parasites, and it is generally assumed that they have only a short lifespan *in vivo*. However, knowledge of the patterns of gametocyte carriage for these species is mostly drawn from malaria in travellers, experimental infections in naive volunteers and from studies of therapeutic malaria induced in syphilis patients in the mid 20th century (Garnham, 1966). This knowledge is therefore unlikely to accurately reflect the actual patterns in endemic country populations. A full understanding of the effect of treatment on the gametocytes of *P. malariae* and *P. ovale* spp. can only be gained once new methods of detection for these sexual stages are developed.

Given that the arguments above may not explain our results, the apparent "failure" of ACT to clear *P. malariae* and *P. ovale* spp. in Ghanaian school children requires further consideration. *P. ovale*

spp. are known to relapse from dormant liver-stages analogous to the hypnozoites of *P. vivax* and *Plasmodium cynomolgi* (Garnham, 1948; Shortt and Garnham, 1948) and both ovale species are known to cause relapse clinical malaria months or years after exposure to infected mosquitoes (Sutherland et al., 2010). There is thus reason to think that *P. ovale* spp. relapses may follow treatment of *P. falciparum* infections, as is described for *P. vivax* in Asia (Douglas et al., 2011). However, given the long half-life of piperaquine (d'Alessandro, 2009; Four ABC Study Group, 2011) and the relatively short period of follow-up in the present study (3 weeks) a relapse emergence from the liver would have been expected to encounter reasonable plasma levels of piperaquine and thus fail to reach detectable levels (even by PCR) in this short time. Thus there may be some other intrinsic survival mechanism that *P. ovale* spp. can deploy to evade drug clearance. *P. malariae* on the other hand is thought to lack the ability to relapse from dormant liver stages (Shortt and Garnham, 1948; Ciuca et al., 1964; Collins and Jeffery, 2007). The 72 h (quartan) intra-erythrocytic cycle time may make this species particularly well suited to evading the effect of most artemisinin regimens, as each dose generates only a short pulse of active plasma DHA which is very rapidly cleared. *P. malariae* infections may therefore be more likely to leave a few survivors after each dose of artemisinin is administered. There are concerns that the current amount of DHA used in the fixed combination DP as given to children results in under-dosing (d'Alessandro, 2009; Tarning et al., 2012), and longer dosage regimens of ACT may be required for complete clearance of *P. malariae* infections. In addition, this parasite is well known to possess a capacity for dormancy, lasting decades in some well documented cases (Vinetz et al., 1998). Although the mechanism or host tissue reservoirs are unknown, it is plausible that this same ability may assist in evasion of antimalarial therapy in this species.

Equally of concern is the persistent detection of *P. falciparum* at 3 weeks in approximately 15% of DP-treated individuals in this study. By microscopy, 4 individuals were recorded as positive for *P. falciparum* asexual parasites 3 weeks after treatment, whereas PCR identified 10 individuals positive for this species, some of which may be carrying only gametocytes. *A priori*, it could be assumed that asymptomatic infections with *P. falciparum*, at low parasite densities, would be easier to clear with efficacious antimalarial regimens. On the contrary, we find a number of PCR-detectable infections only 3 weeks after DP. A possible explanation for this is that in chronic, asymptomatic infections malaria parasites are in a steady-state relationship with the host, and do not elicit immune responses that are capable of participating in drug-induced parasite clearance; thus drug efficacy may be partially compromised against such infections. In contrast, symptomatic infections are by definition immunogenic, and thus the outcome of treatment is the sum effect of drug and both innate and acquired immune responses (Djimdé et al., 2003; Diallo et al., 2007). A potential mechanism for such a phenomenon is the expression, by parasites in a chronic infection, of surface antigen PfEMP1 variants that have weak adhesive properties and are poorly immunogenic (Jensen et al., 2004; Bull et al., 2005). This would explain a relative lack of immune clearance in comparison to symptomatic infections, but does not explain how these parasites evade drug-induced killing.

5. Conclusions

This report raises two areas of concern for programmes seeking to eliminate malaria in sub-Saharan Africa. Firstly, co-infecting non-falciparum species were found by PCR to be common in asymptomatic individuals with patent *P. falciparum* parasitaemia, and the deployment of an ACT thought efficacious against all three

species failed to completely clear *P. malariae* and *P. ovale* spp. in approximately 10% of individuals over 3 weeks of follow-up. The reason for persistent detection of parasites is unknown. Secondly, although ACT clearance of asymptomatic *P. falciparum* infections was efficacious using a microscopy endpoint, PCR-detectable parasites were identified within 21 days of treatment. The role of the immune system in assisting drug-induced parasite clearance may be compromised in chronic low-grade infections of this parasite.

Acknowledgements

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