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GAMBian children successfully treated with chloroquine can harbor and transmit Plasmodium falciparum gametocytes carrying resistance genes

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Abstract. Polymorphisms in two genes of Plasmodium falciparum (P. falciparum multidrug resistance 1 [pfmdr1] and P. falciparum chloroquine [CQ] resistance transporter [pfcrt]) are associated with CQ treatment failure. We found significant linkage disequilibrium between these loci among isolates from symptomatic Gambian children (P = 0.026) and strong selection for the resistance-associated alleles pfmdr1-86Tyr and pfcrt-76Thr in children with persistent or re-emerging P. falciparum trophozoites during post-treatment follow-up (P = 1.9 × 10^-7). Therefore, this genotype is characteristic of resistant infections among our study population. Since the long-term public health impact of parasites carrying such resistant genotypes depends upon their transmissibility, we examined the prevalence of pfmdr1-86Tyr and pfcrt-76Thr among Gambian children harboring sexual stage parasites during post-treatment follow-up. Gametocytes that emerged after successful treatment with CQ were significantly more likely to be of this genotype than were those emerging after other treatments (P = 4.83 × 10^-4), and were infective to Anopholes mosquitoes. Therapeutic success may thus be accompanied by public health failure as cured children pass resistance genes on to mosquitoes at an enhanced rate.

INTRODUCTION

In The Gambia, chloroquine (CQ) remains the recommended treatment for uncomplicated Plasmodium falciparum malaria. However, there is evidence of an increase in the prevalence of CQ-resistant parasites in the last decade.1-3 and in neighboring Senegal, an increase in the number of CQ-resistant infections has been associated with increased child mortality.4 It is crucial that the frequency of failures with CQ treatment is monitored carefully, since a change in malaria treatment policy may soon become necessary in The Gambia and other west African countries. Methods for the rapid identification of CQ-resistant parasites are essential for this monitoring at both the clinical and population levels.

The likely site of action of CQ is the parasite lysosome or food vacuole, in which the drug interferes with the detoxification of the products of hemoglobin digestion.5 Two proteins associated with the lysosomal membrane, Pgh1 and PFCRT, are thought to be important in this process, since polymorphisms in their respective genes are associated with CQ resistance in P. falciparum field isolates and laboratory lines. These genes are the P. falciparum multidrug resistance 1 (pfmdr1) gene on chromosome 5 of the parasite,6-8 and the P. falciparum chloroquine resistance transporter (pfcrt) gene on chromosome 7.9,10 To date, only a few studies of field isolates have examined both genes simultaneously. While pfcrt-76Thr was strongly associated with CQ resistance in each of these studies, the association between pfmdr1-86Tyr and treatment failure was not significant in all study populations,10-12 as had been previously been in studies of pfmdr1 in field populations outside west Africa.13-16 These data suggest that pfcrt mutations are the primary determinant of CQ resistance, but that other genotypic changes such as pfmdr1 mutations can be advantageous to the resistant parasite, perhaps essential, in some parasite populations.5 Therefore, in any analysis of CQ resistance at the population level, it is important to determine which alleles of which genes are associated with resistance in the particular malaria-endemic area being studied.

We are currently examining the effects of anti-malarial chemotherapy on the transmission of P. falciparum malaria in The Gambia. Among children who have been successfully treated with CQ, as shown by resolution of symptoms and the absence of trophozoites in thick blood films, a proportion nevertheless harbor transmissible gametocyte forms.17 Such cases are not considered treatment failures because gametocytes do not contribute to clinical malaria. Blood from these gametocyte-positive subjects is frequently capable of infecting Anopholes gambiae mosquitoes.18 It is not known whether these transmitted infections from children who have been successfully treated are composed of CQ-resistant gametocytes, and thus contribute to any increase in the prevalence of drug resistance in the total circulating parasite population in our study area.

In this study, the prevalence of pfmdr1-86 and pfcrt-76 genotypes among parasite isolates collected from Gambian children before and after treatment with CQ was investigated. The genotype pfmdr1-86Tyr/pfcrt-76Thr was significantly associated with treatment failure in our study population. We show that this same genotype was strongly selected in gametocytes emerging in children who had been successfully treated with CQ.

MATERIALS AND METHODS

Study subjects. The plan of the study is shown in Figure 1. Six hundred Gambian children with uncomplicated clinical falciparum malaria were enrolled in a controlled trial of antimalarial therapy as previously described.17 Full ethical approval for the trial was obtained from both the London School of Hygiene and Tropical Medicine ethical committee, and the joint Gambian Government/Medical Research Council ethics committee. Documented informed consent was obtained from parents or guardians.

To measure the baseline prevalence of the alleles of interest, 108 of the children were selected by taking approximately
every sixth child after ordering by parasite density at admission. This stratification was used because parasites possessing CQ-resistant genotypes may have intrinsically different patterns of growth to other parasites. This baseline group are referred to as the random sample. One hundred thirty-three of the 600 children, including 29 who were also in the random sample of 108, were assigned to the CQ treatment group, and received 10 mg of CQ base per kilogram of body weight for three days. Outcomes for all four treatment groups are evaluated elsewhere.\(^\text{17,18}\) Children were actively followed-up at 4, 7, 14, and 28 days after treatment when thick blood films and blood spots on glass fiber filters (Filtermat-A; Wallac Oy, Turku, Finland) were obtained.

A subject was deemed to have failed CQ treatment if \textit{P}. \textit{falciparum} trophozoites were present, with or without symptoms of clinical malaria, any time in the 28-day follow-up period. This definition is an adaptation of the World Health Organization definition of adequate clinical response, which requires that malaria patients remain parasite-free up to 14 days after treatment.\(^\text{19}\) A subject was considered gametocyte-negative if no \textit{P}. \textit{falciparum} gametocytes were observed per 1,000 leukocytes\(^\text{17}\) and trophozoite-negative if no ring-stage parasites were observed per 200 leukocytes.

Twenty-eight CQ-treated children who harbored \textit{P}. \textit{falciparum} trophozoites during parasitologic follow-up, with or without gametocytes, comprised the trophozoite-positive group. These were treatment failures by our definition and were first noted as parasite positive four days (\(n = 17\)), seven days (\(n = 5\)), 14 days (\(n = 2\)), or 28 days (\(n = 2\)) after treatment, and the blood spots used for analysis were obtained on these days. Three of the children sampled at day 4 were already gametocyte-positive at recruitment. Twenty-three children successfully treated with sulfadoxine/pyrimethamine (SP) (\(n = 19\)) or SP plus artemesunate (AS) (\(n = 4\)), and who became gametocyte positive four days (\(n = 15\)), seven days (\(n = 5\)), 10 days (\(n = 1\)), or 16 days (\(n = 1\)) after treatment comprised the non-CQ gametocyte group. None of these 23 children were gametocyte-positive at recruitment.

**Sampling of DNA.** The DNA was extracted from filter paper blood spots taken prior to treatment from the 108 subjects in the random sample using established methods.\(^\text{20}\) It was also extracted from blood spots of the 26 subjects in the gametocyte-positive group, the 28 subjects in the trophozoite-positive group, and the 23 subjects in the non-CQ gametocyte group. The yield of DNA was insufficient to permit amplification experiments for both \textit{pfdnrl} and \textit{pfcrt} in two cases from the gametocyte-positive group. None of these 23 children were gametocyte-positive at recruitment.

**Amplification of \textit{pfcrt} and \textit{pfdnrl} sequences.** The primary amplification reaction for the \textit{pfcrt} locus was performed using TCRP\(^\text{10}\) as the forward primer and either TCRP\(^\text{20}\) or AAP3
(5′-CGTAAATTATAAGCCACAAATAGG-3′) as the reverse primer. All secondary amplifications of pfcr were primed with TCRD2 and TCRD3 (5′-AGTTCTTGGC-TGGTAAATTTC-3′). The primary amplification for the pfmdr1 locus was performed using primers 5′-AGGTTGA-AAAGAGTTGAAC-3′ and 5′-ATGACACACAA-CATAAT-3′. The semi-nested secondary amplification was primed with the second of these primers, and 5′-TATTATCAGGAGGACATTA-3′. Successful amplification of pfcr was obtained for 98 of the 108 pre-treatment samples (Table 1). Of these DNA samples, 24 were either exhausted prior to performing amplification of the pfmdr1 locus, or did not generate any amplification products, leaving 74 isolates for which amplification of both genes was successful.

Twenty four of the 26 samples from the gametocyte-positive group were successfully amplified for both pfmdr1 and pfcr, as were all 28 samples from the trophozoite-positive group and all 23 samples from the non-CQ group.

Single-stranded oligonucleotide probing. The following oligonucleotide probes were used in hybridization experiments: \(\text{Pfmdr1-86Asn: 5′-AGA ACA TGA ATT TAG GTG-3′} \)

\(\text{Pfmdr1-86Tyr: 5′-AGA ACA TGT ATT TAG GTG-3′} \)

\(\text{Pfcr-76Lys: 5′-TAA TGA AAA TTT TTG-3′} \)

\(\text{Pfcr-76Thr: 5′-TAA TTG AAA CAA TTT TTG-3′} \)

The appropriate sequences for the pfcr probes were determined empirically by sequencing around codon 76 in individual plasmodid clones carrying the pfcr gene of resistant and sensitive isolates from our study area. Amplified products using either the pfcr or pfmdr1 primers were spotted onto nitrocellulose panels and hybridized with the appropriate oligonucleotide probes labeled with digoxygenin as previously described.20

Full details of the hybridization method (Alloueche A and others, unpublished data) will be published elsewhere.

Linkage disequilibrium estimates. The magnitude of disequilibrium in the pre-treatment population was estimated by the constants \(D^′\) and \(r^2\), which were calculated as previously described,7,22 using only those isolates in which a single allele was detectable at both loci (n = 50).

Statistical analyses. Data were analyzed using Stata version 6.0 software (Stata Corporation, College Station, TX) and Epi-Info version 6.04d (Centers for Disease Control and Prevention, Atlanta, GA). Associations between pairs of categorical variables were analyzed using the chi-square test.

RESULTS

Pre-treatment isolates. Allelic prevalences of pfmdr1 and pfcr among parasites from symptomatic infections in children were estimated by determining the genotype of 98 pre-treatment isolates (Table 1). As expected in our study area, in which multiple-genotype infections are common,22 31 children harbored two different alleles for pfcr and eight children harbored two different alleles for pfmdr1. Assuming that these prevalences approximate those in the whole population, they were used to test for any non-random association (linkage disequilibrium) between the pfcr and pfmdr1 genes (Table 2).

An association between the pfcr-76 and pfmdr1-86 loci was observed among the 50 infections with a single genotype at both loci, and this is likely to have occurred by chance \((\chi^2 = 3.7087, P = 0.054; \text{Table 2A})\). To include the 24 mixed infections in this analysis, each isolate was coded according to presence or absence of the respective resistance-associated alleles. This effectively treats mixed sensitive/resistant infections as resistant, reflecting the expected phenotype. A significant association between these two genes is again observed \((\chi^2 = 4.96, P = 0.026; \text{Table 2B})\).

Thus, these two loci are in linkage disequilibrium in our population. Linkage disequilibrium constants are as follows: \(D^′ = 0.27\) (95% confidence interval [CI] = 0.15–0.40), and \(r^2 = 0.074\) (95% CI = 0.0015–0.15) \((n = 50; \text{see Materials and Methods})\). Both \(D^′\) and \(r^2\) are greater than zero, even at the lower extremities of their respective confidence intervals, supporting the finding of linkage disequilibrium. Since these two genes reside on different chromosomes in the genome of \(P. falciparum\), this strong association is likely to be maintained by co-selection.

Post-treatment isolates. To determine if particular alleles of pfmdr1-86 and pfcr-76 are co-selected by CQ, we investigated whether changes in frequency of these alleles occurred after treatment. The prevalence of each of the four possible two-genre genotypes was determined for the trophozoite-positive group at the first post-treatment time point at which parasites were observed in each subject. The results are plotted as relative frequencies in Figure 2, alongside those calculated from the pre-treatment random sample. This shows that in the post-treatment group there is a strongly skewed distribution favoring the resistance-associated genotype \(\text{pfmdr1-86Tyr/pfcr-76Thr}\). The frequency distribution of genotypes was significantly different from the distribution observed in the pre-treatment random sample \((\chi^2 = 34.06, P = 1.9 \times 10^{-7})\), suggesting that treatment with

### Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequency</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pfcr codon 76 (n = 98)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys&lt;sup&gt;+&lt;/sup&gt;</td>
<td>49</td>
<td>50%</td>
</tr>
<tr>
<td>Lys&lt;sup&gt;+&lt;/sup&gt;/Thr&lt;sup&gt;+&lt;/sup&gt;</td>
<td>31</td>
<td>32%</td>
</tr>
<tr>
<td>Thr&lt;sup&gt;+&lt;/sup&gt;</td>
<td>18</td>
<td>18%</td>
</tr>
<tr>
<td>Asn&lt;sup&gt;+&lt;/sup&gt;</td>
<td>47</td>
<td>64%</td>
</tr>
<tr>
<td>Asn&lt;sup&gt;+&lt;/sup&gt;/Thr&lt;sup&gt;+&lt;/sup&gt;</td>
<td>8</td>
<td>11%</td>
</tr>
<tr>
<td>Tyr&lt;sup&gt;+&lt;/sup&gt;</td>
<td>19</td>
<td>26%</td>
</tr>
</tbody>
</table>

| Pfmdr1 codon 86 (n = 74) | | |
| Asn<sup>+</sup> | 47 | 64% |
| Asn<sup>+</sup>/Thr<sup>+</sup> | 8 | 11% |
| Tyr<sup>+</sup> | 19 | 26% |

<sup>*</sup>pfcrt = Plasmodium falciparum multidrug resistance 1; pfcr = P. falciparum chloroquine resistance transporter; † denotes a putative sensitive allele; ‡ denotes a putative resistant allele.

### Table 2

<table>
<thead>
<tr>
<th>Pre-treatment genotype</th>
<th>Pfcr-76Thr</th>
<th>Pfcr-76Thr</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pfmdr1-86Asn</td>
<td>30 (81%)</td>
<td>7 (19%)</td>
<td>37 (100%)</td>
</tr>
<tr>
<td>Pfmdr1-86Tyr</td>
<td>7 (54%)</td>
<td>6 (46%)</td>
<td>13 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td>37 (74%)</td>
<td>13 (26%)</td>
<td>50 (100%)</td>
</tr>
</tbody>
</table>

*For definitions of abbreviations, see Table 1. \(\chi^2 = 3.7087, P = 0.054\).
CQ had exerted selection for the resistance-associated genotype.

**New genotypes detected in follow-up.** To further examine this selection within individual infections, we compared pre-treatment and post-treatment genotypes from trophozoite-positive subjects for which we had the necessary paired DNA samples. Examining *pfmdr1* and *pfcrt* separately, we enumerated those children whose follow-up sample differed in genotype from their pre-treatment sample. Table 3 shows that among children who presented with trophozoites during follow-up, changes in parasite genotype were significantly more likely to be the gaining of Tyr at the *pfmdr1*-86 locus, and of Thr at the *pfcrt*-76 locus than the loss of either. These results demonstrate two things. First, within infected individuals, the use of CQ selects at both the *pfmdr1*-86 and *pfcrt*-76 loci in persisting or reappearing trophozoites. Second, many parasites detected during follow-up exhibited the resistant (TyrThr) genotype, despite its absence in the pre-treatment infection. These may have arisen from blood-stage parasites emerging from the liver after the pre-treatment DNA sample was obtained, particularly in the latter half of follow-up. Fourteen trophozoite-positive children who harboured both the *pfmdr1*-86Tyr and *pfcrt*-76Thr alleles were found in the first week of follow-up, and these are unlikely to be new infections. In six of these subjects, the resistant genotype was absent prior to treatment.

**Selection of resistant genotypes in gametocytes.** Having established that the two-gene genotype *pfmdr1*-86Tyr/*pfcrt*-76Thr is strongly associated with treatment failure in our study population, and that these two alleles are selected for in individuals who fail treatment with CQ, we sought to establish if such selection also occurs among children successfully treated with CQ who become gametocyte-positive during follow-up. To do this, we compared the prevalence of *pfmdr1*-86

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**Table 3**

Comparison of pre-treatment and follow-up parasites in individual trophozoite-positive children in which a change was observed* using the two-sided binomial test for paired observations†

<table>
<thead>
<tr>
<th>Allele</th>
<th>Observed change between pre-treatment and post-treatment‡</th>
<th>Number of observations</th>
<th>Binomial probability</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pfmdr1</em>-86Tyr (n = 22)</td>
<td>Absent → present</td>
<td>10</td>
<td>P = 0.002</td>
</tr>
<tr>
<td></td>
<td>Present → absent</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Pfcrt</em>-76Thr (n = 26)</td>
<td>Absent → present</td>
<td>12</td>
<td>P = 0.013</td>
</tr>
<tr>
<td></td>
<td>Present → absent</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

* Children from the trophozoite-positive group for whom both pre-treatment and post-treatment DNA samples were tested and found to differ.
† For definitions of abbreviations, see Table 1.
‡ These changes are not interpreted as representing new mutations occurring during the course of infection, but the replacement of one type of parasite by another. Mixed infections at either locus are included as in Table 2 and Figure 2.
and pfcr-76 genotypes in gametocyte-positive children from both the CQ-treated and non-CQ-treated groups. The results are presented in Figure 3.

The two-locus genotype prevalences of the two gametocyte-positive groups have distributions that are significantly different ($\chi^2 = 16.67, P = 8.27 \times 10^{-4}$). Whereas pfmdr1-86/pfcr-76 genotype prevalences in the non-CQ treated gametocyte-positive group were distributed similarly to that of the pre-treatment random sample ($\chi^2 = 3.97, P = 0.265$), that of the CQ-treated gametocyte-positive group differed significantly from this pattern ($\chi^2 = 16.71, P = 8.1 \times 10^{-4}$) and showed a strong skewing towards the pfmdr1-86Tyr/pfcr-76Thr resistance-associated genotype (Figure 3). Therefore, we conclude that gametocytes arising in children successfully treated with CQ are selected at these two resistance-associated loci.

**Infectivity to mosquitoes.** Fifteen of the CQ-treated gametocyte-positive isolates described in this report were also used in membrane-feeding experiments that have been previously described. Ten of these isolates were infective to An. gambiae mosquitoes, of which one had the pfmdr1-86/pfcr-76 genotype AsnS/LysS, one had the genotype TyrR/LysS, three had the genotype AsnS/ThrR, and five had the resistance-associated genotype TyrR/ThrR.

**DISCUSSION**

We have shown that treatment of Gambian children with CQ selects for *P. falciparum* carrying resistance-associated alleles at both pfmdr1-86 and pfcr-76 and that these two genes are in linkage disequilibrium in our study population. This agrees with the results of the report of Duraisingham and others, in which linkage between the cg2 locus (adjacent to pfcr on chromosome 7) and the pfmdr1 locus was demonstrated among a diverse collection of Gambian parasite isolates. Using previously published data, strong associations ($P < 0.01$) between pfmdr1 and pfcr have been reported in a Malian population, and can also be demonstrated among a large collection of isolates of diverse origin. Linkage disequilibrium between these genes has also been recently reported from Nigeria, Sudan, and Papua New Guinea. However, data from other parts of Africa and elsewhere suggest that this association is not universal among CQ-resistant parasites, and that mutations in pfmdr1 may not be important in some parasite populations in which resistance occurs.

We found that the two-locus genotype pfmdr1-86Tyr/pfcr-76Thr, which is strongly selected among persisting or recrudescent trophozoites, is significantly over-represented among gametocytes emerging after treatment with CQ, but not among gametocytes emerging after treatment with SP or SP/AS. This suggests that under CQ selection, these parasites are more likely to be transmitted than other genotypes, and that this is a mechanism by which the prevalence of such alleles may increase from generation to generation. The rate of this increase will be dependent upon the proportion of infected individuals using CQ, which determines the overall magnitude of drug pressure on the parasite population.

In 10 cases for pfmdr1-86Tyr and 12 cases for pfcr-76Thr, the resistant allele was not observed among the parasites present at the time of clinical presentation, but was first detected at the time that trophozoites appeared following drug treatment (Table 3). Such dramatic changes in parasite genotype within individual infections could occur in two ways. First, these children may have been infected with a new resistant parasite type subsequent to the infection that gave rise to the clinical attack. Alternatively, sub-populations of CQ-resistant trophozoites present at the time of treatment, but undetectable by the polymerase chain reaction (PCR), were able to expand in numbers following clearance of sensitive parasites.

**FIGURE 3.** Relative frequencies of *Plasmodium falciparum* multidrug resistance 1 (pfmdr1)-86 and *P. falciparum* chloroquine resistance transporter (pfcr)-76 genotypes in gametocyte-positive isolates from children treated with chloroquine (CQ), or with either sulfadoxine/pyrimethamine (SP) or SP/artesunate (SP/AS). All subjects shown were gametocyte-positive by microscopy, but trophozoite-negative, during follow-up. Non-CQ refers to study subjects who received either SP or SP/AS at the time of treatment. **P < 0.01.**
New infections emerging from the liver are generally considered a plausible source of late treatment failures in trials of malaria chemotherapy, and these can be identified with current genetic typing methods. However, except under very high intensities of transmission, early parasitologic treatment failures are unlikely to be caused by newly emergent infections. The intensity of transmission in The Gambia, as reflected by estimates of entomologic inoculation rates, is in the low-to-moderate range.27 Furthermore, since we have shown that only 23% of our subjects harbor both of the resistance-associated alleles (pfmdr1-86Tyr and pfcrt-76Thr) prior to treatment (Table 1), the majority of new emergents from the liver should be drug-sensitive parasites. These will not survive in the continuing presence of CQ, which has a terminal elimination half-life of 30-60 days.29 Among 14 parasitologic failures that occurred within seven days of treatment and that harbored both pfmdr1-86Tyr and pfcrt-76Thr, six were derived from pre-treatment infections in which at least one of these two alleles was not detectable. We favor the hypothesis that these failures are caused by undetectable resistant sub-populations present at the time of treatment that were selected by CQ. That these are able to expand to patency may indicate that density-dependent interactions are important in maintaining the balance among different genotypes present in an infection. Thus, when CQ removes all the sensitive parasite genotypes, low-density resistant genotypes, previously undetectable, expand into an essentially vacant niche. This may be exacerbated by the fact that during PCR amplification from a heterogeneous sample, abundant and rare templates are competing for the same oligonucleotide primers, resulting in only the abundant sequences being detected.

Treatment failures due to resistant parasites are classified according to the length of time that a treated person remains asexasitemic. Late parasitologic failures are designated RI and early failures are designated RII or RIII,19,29 but there is disagreement in the literature as to the relative contribution of newly emerged parasites and recrudescent parasites to treatment failure. First, we have shown using these markers that children showing an adequate clinical response to CQ nevertheless transmit CQ-resistant genotypes at a high frequency and thus can be considered public health failures. Second, an increasing prevalence of CQ resistance at the population level should correlate with increasing prevalence of the pfmdr1-86Tyr/pfcrt-76Thr genotype, at least within our study area. We are currently analyzing DNA samples from annual cross-sectional malaria surveys to test this assertion, and therefore to determine how useful these markers are for surveillance of changes in CQ resistance over time.

In the face of increasing resistance to first-line anti-malarial drugs in Africa, there is a concerted effort to introduce combination therapy for malaria.35,36 Whereas the therapeutic benefit of effective combination therapy resides in the prevention of further clinical episodes in the treated individual, any public health benefit will require that post-treatment transmission of drug-resistant parasites is reduced or prevented.27 Our finding that gametocytes emerging after treatment with CQ have undergone selection for resistance-associated alleles at the pfmdr1-86 and pfcrt-76 loci (Figure 3) is important in this regard, particularly since gametocyte carriers harboring this two-gene genotype are infectious to Anopheles mosquitoes. These results have implications for the long-term efficacy of combination therapy. We have recently shown using reverse transcriptase-PCR that low-density subpopulations of asexual parasites can escape short-acting drugs such as AS.22 Resistant parasites among them are then likely to recrudesce under the selective force of the second drug of the combination, and be transmitted to mosquitoes. We are investigating this possibility in the context of our ongoing combination therapy transmission trials in The Gambia.17

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