Prevalence of and Risk Factors Associated with Polymerase Chain Reaction-Determined \textit{Plasmodium falciparum} Positivity on Day 3 after Initiation of Artemether–Lumefantrine Treatment for Uncomplicated Malaria in Bagamoyo District, Tanzania

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Abstract. Prevalence of and risk factors associated with polymerase chain reaction (PCR)-determined \textit{Plasmodium falciparum} positivity were assessed on day 3 after initiation of treatment, pre-implementation and up to 8 years post-deployment of artemether–lumefantrine as first-line treatment for uncomplicated malaria in Bagamoyo district, Tanzania. Samples originated from previously reported trials conducted between 2006 and 2014. Cytochrome b-nested PCR was used to detect malaria parasites from blood samples collected on a filter paper on day 3. Chi-square and McNemar chi-squared tests, logistic regression models, and analysis of variance were used as appropriate. Primary outcome was based on the proportion of patients with day 3 PCR-determined \textit{P. falciparum} positivity. Overall, 256/584 (43.8%) of screened patients had day 3 PCR-determined positivity, whereas only 2/584 (0.3%) had microscopy-determined asexual parasitemia. Day 3 PCR-determined positivity increased from 28.0% (14/50) in 2006 to 74.2% (132/178) in 2014. When data were pooled, pretreatment microscopy-determined asexual parasitemia ≥ 100,000/µL, hemoglobin < 10 g/dL, age < 5 years, temperature ≥ 37.5°C, and year of study 2007–2008 and 2012–2013 were significantly associated with PCR-determined positivity on day 3. Significant increases in \textit{P. falciparum} multidrug resistance gene 1 N86 and \textit{P. falciparum} chloroquine resistant transporter K76 across years were not associated with PCR-determined positivity on day 3. No statistically significant association was observed between day 3 PCR-determined positivity and PCR-adjusted recrudescence. Day 3 PCR-determined \textit{P. falciparum} positivity remained common in patients treated before and after implementation of artemether–lumefantrine in Bagamoyo district, Tanzania. However, its presence was associated with pretreatment characteristics. Trials registration numbers: NCT00336375, ISRCTN69189899, NCT01998295, and NCT02090036.

INTRODUCTION

Artemisinin-based combination therapy (ACT) is the mainstream treatment for uncomplicated \textit{Plasmodium falciparum} malaria globally. 1 Artemisinin-based combination therapy consists of fast-acting artemisinin derivatives and a long-acting partner drug with a different mode of action. Initially, artemisinin rapidly clears a large number of asexual parasites before the long-acting partner drug eliminates the residual parasites. 2 For artemisinin-sensitive \textit{P. falciparum} parasites, microscopy-determined parasite clearance usually occurs within 48 hours in most of the patients treated with ACT. 3 Artemisinin-resistant \textit{P. falciparum} parasites, on the other hand, are phenotypically characterized by a slow clearance rate. 4 Microscopy-based \textit{P. falciparum} positivity rate on day 3 after initiation of ACT treatment is considered an important determinant of delayed parasite clearance, and if the positivity rate exceeds 10%, this is considered an alert for artemisinin resistance. 5 The resistance has been linked to polymorphisms in the \textit{Kelch} gene located on chromosome 13 (k13) of \textit{P. falciparum} parasites. 6

Artemisinin resistance has been reported from four countries in the Mekong region, Southeast Asia. 4, 7, 8 In Africa, despite recent case reports of suspected resistance, 9, 10 \textit{Pfk13} polymorphisms are rare and do not include those previously described and associated with delayed \textit{P. falciparum} clearance in Southeast Asia. 11, 12 Artemisinin-based combination therapy has also remained highly efficacious in Africa, with rapid microscopy-determined clearance of the asexual parasites within 48 hours after initiation of treatment. 13–15 However, few African studies have assessed the presence of residual polymerase chain reaction (PCR)-determined \textit{P. falciparum} positivity on day 3 after initiation of ACT treatment, 16, 17 a phenomenon that may indicate reduced susceptibility of \textit{P. falciparum} against artemisinin derivatives and thereby a potential early warning marker of artemisinin resistance. 16

Nonetheless, several pretreatment host and parasite characteristics including initial parasite density, age of the patient, and hemoglobin level influence parasite clearance after treatment with ACT. 18–20 Higher initial microscopy-determined asexual parasitemia (≥ 100,000 parasites/µL of blood) and anaemia, that is hemoglobin < 10 g/dL, are associated with slow parasite clearance. 19, 21 Conversely, in high malaria-endemic areas, malaria immunity increases with age, whereby children aged < 5 years have low acquired immunity against \textit{P. falciparum} infection and are, therefore, prone to slower parasite clearance than older individuals. 18 However, it is not
well understood whether these factors are responsible for the previously observed day 3 PCR-determined *P. falciparum* positivity in Africa, rather than reduced parasite susceptibility against artemisinins.16,17

Therefore, the aim of this study was to evaluate the prevalence of, and risk factors associated with, PCR-determined *P. falciparum* positivity on day 3 after artemether–lumefantrine treatment initiation in clinical trials conducted between 2006 and 2014, covering pre-implementation and 8 years post-implementation of artemether–lumefantrine as first-line treatment for uncomplicated *P. falciparum* malaria in Bagamoyo district, Tanzania.

**MATERIAL AND METHODS**

**Study area and design.** The blood samples used for PCR analyses in this report originated from four clinical trials conducted in Fukayosi and Yombo dispensaries in Bagamoyo district, Tanzania, between 2006 and 2014.22–24 The district has a tropical climatic condition, with annual rainfall averaging 1,100 mm. Malaria transmission is high and stable throughout the year, with a slight variation between rainfall and dry season.25 *Plasmodium falciparum* is the predominant malaria species and *Anopheles gambiae* sensu stricto is the main vector.26 Artemether–lumefantrine was adopted in Tanzania as a first-line treatment for uncomplicated *P. falciparum* malaria in November 2006. The use of long-lasting insecticide-treated mosquito nets is the major vector control method in the study area.27

This was a retrospective study designed to assess the prevalence of, and risk factors associated with, PCR-determined *P. falciparum* positivity on day 3 after initiation of artemether–lumefantrine treatment among patients with acute uncomplicated *P. falciparum* malaria before and after the implementation of artemether–lumefantrine treatment policy. Four clinical trials were included in the analysis: 1) an artemether–lumefantrine pharmacokinetics and pharmacodynamics trial, 2006;22 2) the supervised arm of an artemether–lumefantrine efficacy (supervised) versus effectiveness (unsupervised) trial, 2007–2008;23 3) an artemether–lumefantrine efficacy trial, 2012–2013,15 and 4) both arms of an artemether–lumefantrine versus artemether–lumefantrine plus 0.25 mg/kg single dose of primaquine efficacy and safety trial, 2014.24 The primaquine arm was included in the analysis because no statistically significant differences in microscopy-determined parasite clearance, prevalence of PCR-determined *P. falciparum* positivity on day 3, prevalence of *P. falciparum* multidrug resistance gene 1 (*Pfdmrt1*) N86 and *P. falciparum* chloroquine resistant transporter (*Pfcrt*) K76, and crude and PCR-adjusted cure rates were observed between the artemether–lumefantrine and artemether–lumefantrine plus primaquine arms.28

The 2006 trial was conducted in June, few months before deployment of artemether–lumefantrine in Bagamoyo district, whereas the 2007–2008, 2012–2013, and 2014 trials were conducted 1–2, 5–7, and 8 years, respectively, after wide scale use of artemether–lumefantrine as treatment policy in the study area.

**Study population, treatment, and procedures.** The study subjects were recruited from patients with symptoms and signs compatible with acute uncomplicated *P. falciparum* malaria attending the dispensaries as previously described.22–24

Patients with microscopically confirmed *P. falciparum* mono-infection and who fulfilled all the inclusion criteria and none of the exclusion criteria were enrolled and treated under supervision with standard weight-based six-dose artemether–lumefantrine regimen according to the national treatment guidelines in Tanzania. For patients allocated with artemether–lumefantrine plus primaquine treatment, an additional single 0.25 mg/kg bodyweight primaquine dose was administered concomitantly with the first artemether–lumefantrine dose.24 Patients were observed for 30 minutes after the intake of each drug dose; and treatment was readministered in case of vomiting within this period. Patients with fever greater than 38.5°C were treated with paracetamol.

Follow-up for clinical and laboratory assessments was performed on days 0, 1, 2, and 3, for the 2006 trial; 0, 1, 2, 3, 7, 14, 21, and 28 or any day of recurrent illness, for the 2014 trial; 0, 1, 2, 3, 7, 14, 21, 28, and 42, for the 2012–2013 trial; and 0, 1, 2, 3, 7, 14, 21, 28, 35, 42, 49, and 56 for the 2007–2008 trial. Every clinical assessment included the history of clinical symptoms, possible adverse events, concomitant drug consumption, clinical examination, and measurement of axillary temperature. Patients who missed a scheduled follow-up visit and who could not be found despite efforts to trace them at their homes were considered lost to follow-up and were consequently withdrawn.29 Patients with repetitive vomiting of the trial drug were managed according to national guidelines and followed up until recovery.27

Laboratory assessment involved collection of a finger-prick blood sample from patients in all four studies for hemoglobin quantification, thick blood films for the microscopic determination of the presence of *P. falciparum*, and parasite density thin films for the species identification for the 2012–2013 and 2014 cohorts, and 100 μL of blood on a filter paper (3MM Whatman) for molecular genotyping of asexual parasites.

Hemoglobin concentration was measured using a portable spectrophotometer HemoCue Hb 201+ (HemoCue AB, Angelholm, Sweden), as previously described.24 The blood films were stained using 10% giemsa and read by qualified microscopists, as previously described.22–24 The filter papers were labeled, air-dried at room temperature for 3–4 hours, packed in an individual plastic bag, and transported to Karolinska Institutet, Sweden, for molecular analysis.

**Molecular analysis.** Genomic DNA was extracted from dried blood spots collected on a filter paper on days 0, 3, and, when applicable, on the day of microscopy-determined recurrent *P. falciparum* infection during follow-up using ABI PRISM® 6100 Nucleic Acid Prep Station (Applied Biosystems, Fresno, CA) for 2006, and on day 0 and the day of recurrent infection for 2007–2008 samples, whereas, a 10% chelex-100 method was used for the day 3 samples for 2007–2008 trial, and days 0, 3, and the day of recurrent infection samples for the 2012–2013 and 2014 trials.22,30 The detection limit of the ABI PRISM 6100 extraction method has been estimated to be 200 parasites/μL and that of the chelex-100 method to be 2 parasites/μL.31

Genomic DNA extracted from filter papers collected on day 3 after artemether–lumefantrine treatment initiation from all four studies was used to detect PCR-determined *P. falciparum* positivity using cytochrome-b nested PCR, as previously described.32 Briefly, 5 μL of the extracted DNA was amplified with 0.1 μL of 100 pmol/μL of each primer, 2.5 μL of 2 mM dNTPs
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(Sigma Aldrich, MO), 2 μL of 25 mM MgCl₂, and 0.125 μL of 5 U/μL Taq DNA polymerase (Sigma Aldrich) and 10.175 μL of dH₂O, using the following cycling program: 3 minutes at 95°C, then 40 cycles of 15 seconds at 95°C, 60 seconds at 60°C, 70 seconds at 72°C, and a final extension of 5 minutes at 72°C. For the nested-PCR, 3 μL of primary PCR product was amplified with 0.05 μL of 100 pmol/μL of each primer, 2.5 μL of 2 mM dNTPs, 2 μL of 25 mM MgCl₂, and 0.125 μL of 5 U/μL Taq DNA polymerase and 12.175 μL of dH₂O, using the following cycling program: 3 minutes at 95°C, then 40 cycles of 15 seconds at 95°C, 60 seconds at 60°C, 60 seconds at 72°C, and a final extension of 5 minutes at 72°C. Pfmdr1 N86Y and Pfcr K76T were genotyped using nested PCR followed by restriction fragment length polymorphism using Apol restriction enzyme.²³

Blood samples from patients with recurrent infection during follow-up (2007–2008, 2012–2013, and 2014 trials) were further genotyped to differentiate recrudescence (treatment failure) from reinfection (new infection) by stepwise genotyping of P. falciparum block 3 of merozoite surface proteins (msp) 2, block 2 of msp 1, and region II (RII) of glutamate-rich protein (glurp).²³

The amplicons were loaded, respectively, on GelRed™ (Biotium, Inc., Hayward, CA) stained agarose, separated by electrophoresis, and visualized under Ultra Violet transillumination (Gel Doc™ System; Bio-Rad, Hercules, CA) using Image Lab™ software (Bio-Rad). For amplicon 1, 2, and glurp, alleles in each family were considered the same if fragments’ size was within 20 base pair intervals. Patients with microscopy-determined recurrent P. falciparum infection, but with negative PCR results, were considered to have uncertain PCR-adjusted outcome and were consequently excluded from the final analysis. Recrudescence was defined as the presence of at least one matching allelic band, and reinfection was defined as the absence of any matching allelic band at baseline and on the day of parasite recurrence.²³

**Study endpoints.** The primary outcome was the proportion of patients with PCR-determined P. falciparum positivity on day 3 after treatment initiation with artemether–lumefantrine between 2006 and 2014. Polymerase chain reaction–determined P. falciparum positivity was predefined as parasites that were detectable by cytochrome b–nested PCR. The secondary outcomes were as follows: prevalence of day 3 PCR-determined P. falciparum positivity across years between 2006 and 2014, the proportion of Pfmdr1 N86Y and Pfcr K76T on days 0 and 3, and their association with the presence of day 3 PCR-determined P. falciparum positivity across years; change in the proportion of Pfmdr1 N86Y and Pfcr K76T between days 0 and 3 across years; the association between baseline characteristics including age < 5 years, microscopy-determined pretreatment asexual parasite density (≥ 100,000/μL of blood), fever (≥ 37.5°C), anemia (hemoglobin <10 g/dL), Pfmdr1 N86 and Pfcr K76, and PCR-determined P. falciparum positivity on day 3; and the association between PCR-determined P. falciparum positivity on day 3 and crude recurrent infection, as well as PCR-adjusted recrudescence during follow-up after artemether–lumefantrine treatment.

**Ethical considerations.** Good Clinical Practice, Declaration of Helsinki, and applicable regulatory requirements in Tanzania were adhered to during the conduct of the trials. Approvals to conduct the respective trials were obtained from the Ethics Committees of the Muhimbili University of Health and Allied Sciences, National Institute for Medical Research, Tanzania Food and Drug Authority, and the Regional Ethics Committee, Stockholm, Sweden. Written informed consent was obtained from all patients and parents/guardians of patients aged < 18 years, before enrollment. The trials are registered with numbers: NCT00336375, ISRCTN69189899, NCT01998295, and NCT02090036.

**Statistical analysis.** Data were double entered and analyzed using R version 3.2.3 (R Foundation, Vienna, Austria). Independent proportions were compared using chi-squared test, whereas paired proportions were compared using McNemar chi-squared test. Age was negatively skewed and was, therefore, transformed using log. Trend of change in proportion of day 3 PCR-determined P. falciparum positivity, Pfmdr1 N86Y, and Pfcr K76T across years was assessed using logistic regression model. Univariate and multivariate logistic regression models were used to assess the association of pretreatment risk factors with both residual parasites and recurrent infection. The association between pretreatment characteristics and day 3 PCR-determined P. falciparum positivity was assessed both for each trial individually and when data from all trials were pooled. The 2006 trial had a follow-up of 3 days only, and hence excluded from the analysis to assess the association of day 3 PCR-determined P. falciparum positivity and recurrent infection, including both crude microscopy-based recurrent infection and PCR-adjusted recrudescence, during follow-up after artemether–lumefantrine treatment. Mean differences between groups were compared using the analysis of variance. Data were censored at the time of withdrawal in case of patient lost to follow-up, withdrawal of consent, and PCR-adjusted reinfection or uncertain PCR outcome. A statistical significance level was set at P ≤ 0.05.

**RESULTS**

**Characteristics of the study participants.** A total of 584 patients were screened for PCR-determined P. falciparum positivity on day 3, in which, 256 (43.8%) were PCR positive. Of the PCR-positive patients, only two (0.3%) had microscopy-determined asexual parasites on day 3. Pretreatment characteristics of the study participants are presented in Table 1. Participants in 2007–2008 were the youngest, had the lowest mean hemoglobin, and the highest microscopy-determined asexual mean parasite density, whereas in 2014 study, participants had the highest median age and hemoglobin levels, and the lowest mean parasite density. However, when a separate analysis was performed for children aged < 5 years, there was a slight change in the parameters, whereby children in 2006 had slightly lower mean age and hemoglobin concentration, whereas those in 2012–2013 had the highest microscopy-determined asexual parasite density (Table 1).

**Prevalence of PCR-determined P. falciparum positivity on day 3 across years** The prevalence of PCR-determined P. falciparum positivity on day 3 increased from 28.0% (14/50) in 2006 to 74.2% (132/178) in 2007–2008 and declined, thereafter, to 27.6% (60/217) in 2014, Figure 1A. Furthermore, Figure 1 shows that the prevalence of PCR-determined P. falciparum positivity on day 3 by study year in patients aged < 5 years (Figure 1B) was slightly higher but not statistically significantly different from that of the all ages (Figure 1A) and of those aged > 5 years (Figure 1C).
Prevalence of Pfmdr1 N86Y and Pfcr7 K76T on day 3. A total of 131/256 (51.2%) and 192/256 (75.0%) samples were PCR positive on day 3 for Pfmdr1 N86Y and Pfcr7 K76T, respectively. The prevalence of Pfmdr1 N86Y and Pfcr7 K76T during the study period is presented in Figure 2. The prevalence of Pfmdr1 N86 increased significantly from 7.7% (1/13) in 2006 to 57.6% (19/33) in 2007–2008, 72.9% (27/37) in 2008–2012, and 71.4% (35/49) in 2014 (odds ratio [OR] = 1.2, 95% CI: 1.09–1.33, P < 0.001), whereas that of Pfcr7 K76 increased significantly from 54.5% (6/11) in 2006 to 70.9% (66/93) in 2007–2008, 87.5% (28/32) in 2012–2013, and 91.1% (51/56) in 2014 (OR = 1.3, 95% CI: 1.11–1.44, P < 0.001).

Changes of Pfmdr1 N86Y and Pfcr7 K76T between pretreatment and on day 3 across years of study. There was no significant difference in the change of Pfmdr1 N86 to 86Y or N86Y, Pfmdr1 86Y to N86 or N86Y, and from Pfmdr1 N86Y to N86 or 86Y (McNemar χ² = 0.39, P = 0.5316) between pretreatment and day 3 across years of study. Similarly, the change of Pfcr7 K76 to 76T or K76T, Pfcr7 76T to K76 or K76T, and from Pfcr7 K76T to 76K or 76T between pretreatment and day 3 were not significantly different (McNemar χ² = 0.25, P = 0.6171).

Association between pretreatment characteristics and PCR-determined P. falciparum positivity on day 3. Results for logistic regression of univariate and multivariate analyses for association between pretreatment characteristics and day 3 PCR-determined P. falciparum positivity are presented in Table 2. The years of study 2007–2008 and 2012–2013 had significantly higher odds of PCR-determined P. falciparum positivity on day 3 with the OR of 6-folds (P < 0.001) and 3-folds (P = 0.017), respectively, as compared with data from 2006. Other predictors for PCR-determined P. falciparum positivity were microscopy-determined asexual parasite density ≥ 100,000/μL (P < 0.001), hemoglobin < 10 g/dL (P = 0.025), age < 5 years (P < 0.001), and fever (axillary temperature ≥ 37.5°C) at inclusion (P = 0.043). Both Pfmdr1 N86 (P = 0.71) and Pfcr7 K76 (P = 0.72) were not significantly associated with PCR-determined P. falciparum positivity on day 3.

Association between PCR-determined P. falciparum positivity on day 3 and recurrent infection. A total of 534 patients screened for PCR-determined P. falciparum positivity on day 3 were included in this analysis. Twenty-seven of 534 (5.1%) were either lost to follow-up or withdrew their consent between days 3 and 28. Of the remaining 507 patients, 44 (8.7%) had microscopy-determined recurrent infection, of whom seven (1.4%) had PCR-adjusted recrudescence. Of the 507 patients, 227 (44.8%) had PCR-determined P. falciparum positivity on day 3, of which 24 (10.6%) had microscopy-determined recurrent infection (χ² = 1.86, P = 0.173) and four
DISCUSSION

This study conducted in Bagamoyo district, Tanzania, where presently no data exist on presence of PfK13 polymorphisms associated with artemisinin resistance, showed that more than one-third of the patients tested positive for P. falciparum by PCR on day 3, whereas only two patients had day 3 microscopy-determined asexual parasites. Importantly, the prevalence of day 3 PCR-determined P. falciparum positivity across study years in patients aged < 5 years was slightly higher but not statistically significantly different from that of all ages and of those aged > 5 years. Parasite clearance following treatment is determined by many factors including the mode of action of the used antimalarial drug and pretreatment characteristics of both the parasite and host.17,18,21,35,36 The day 3 PCR-determined P. falciparum positivity observed in this study might have also been present during the chloroquine (CQ) and sulfadoxine–pyrimethamine era because of their modes of actions, resulting in a relatively slow parasite clearance rate as compared with artemisinin.35,36 In the present study, the day 3 PCR-determined P. falciparum positivity might represent a remaining fraction of the parasite biomass that was then probably cleared by lumefantrine, the long-acting partner drug.37 Our observations are substantiated by the presence of a significant proportion of patients in the 2006 trial with PCR-determined P. falciparum positivity on day 3, despite being treated for uncomplicated P. falciparum malaria 6 months before the implementation of artemether–lumefantrine policy in Bagamoyo district. However, a study in Kenya found an association of the presence of PCR-determined P. falciparum parasites on day 3 with parasite tolerance against ACT.16 Nevertheless, differences in parasite detection methods between the studies with the use of qPCR in the Kenyan study might explain the differences observed between these two studies.16 Further surveillance of PCR-determined P. falciparum positivity on day 3 to provide a better understanding of this phenomenon is, therefore, warranted. On the other hand, the proportion of PCR-determined P. falciparum positivity on day 3 increased between 2006 and 2007–2008, and, thereafter, declined toward 2014. Differences in methods used may have resulted in different findings between the individual trials in this report. However, with the exception of a different DNA extraction method used for the 2006 trial, the amount of blood collected, type and storage of a blotted filter paper, and amplification methods were the same for all the included trials. Therefore, it is not well understood why the 2007–2008 trial had a high proportion of day 3 PCR-determined P. falciparum positivity as compared with other trials included in the analysis.

After data were pooled, higher initial microscopy-determined asexual parasitemia (≥ 100,000/μL of blood), hemoglobin < 10 g/dL, age < 5 years, body temperature ≥ 37.5°C, and the year of study were found to be associated with the presence of PCR-determined P. falciparum positivity on day 3. However, when the respective trials were analyzed separately, the same factors were significantly associated with PCR positivity on day 3 in some trials, but not in others. Contrary to our findings, a study in Kenya did not observe any association of pretreatment characteristics with qPCR-determined parasite positivity on day 3.16 However, previous studies have shown pretreatment characteristics of both the host and parasite, including existing immunity, pretreatment parasitemia, parasite strains, parasite developmental stage, and parasite drug susceptibility, to affect microscopy-determined parasite clearance time.17,18,21,38,39

Plmdr1 N86 and Pfcrt K76 proportions increased significantly across years, in line with previous reports.40,41 However, the presence of Plmdr1 N86 and Pfcrt K76 alleles in this study was not associated with a statistically significant
increase in the presence of PCR-determined *P. falciparum* positivity on day 3. Furthermore, the changes between days 0 and 3 of *Pfmdr1* N86Y and *Pfcrt* K76T outcome were not significantly different across years. The absence of significant change of these alleles, particularly of *Pfmdr1* N86 and *Pfcrt* K76, between days 0 and 3 probably indicates that more time than 3 days is required for the parasite to be exposed to the drug for such selection to occur. Conversely, the relatively high proportions of *Pfmdr1* 86Y and *Pfcrt* 76T in 2006 probably reflect previous exposure of the parasite population to amodiaquine and CQ.

The PCR-determined *P. falciparum* positivity on day 3 was neither associated with the microscopy-determined recurrent infections nor the PCR-adjusted recrudescence during follow-up after artemether–lumefantrine treatment. Furthermore, a large majority (83.3%) of the recurrent infections were due to re-infection based on PCR-adjustment. Conversely, the relatively high proportions of *Pfmdr1* 86Y and *Pfcrt* 76T in 2006 probably reflect previous exposure of the parasite population to amodiaquine and CQ. The PCR-determined *P. falciparum* positivity on day 3 was neither associated with the microscopy-determined recurrent infections nor the PCR-adjusted recrudescence during follow-up after artemether–lumefantrine treatment. Furthermore, a large majority (83.3%) of the recurrent infections were due to re-infection based on PCR-adjustment. Conversely, the relatively high proportions of *Pfmdr1* 86Y and *Pfcrt* 76T in 2006 probably reflect previous exposure of the parasite population to amodiaquine and CQ.

The strength of this study is that it included four studies conducted over different years, before and up to 8 years after implementation of artemether–lumefantrine as first-line treatment of uncomplicated malaria in Bagamoyo district. However, study limitations include, for example, that no blood sampling was performed to assess whether the PCR-determined *P. falciparum* parasites were cleared during follow-up between days 4 and 7. Moreover, neither was any evaluation of in vitro responsiveness to artemether–lumefantrine carried out nor assessment of day 7 lumefantrine concentration levels.

**CONCLUSION**

Day 3 PCR-determined *P. falciparum* positivity remained common in patients treated with artemether–lumefantrine.

**TABLE 2**

Association between pretreatment characteristics and polymerase chain reaction–determined *Plasmodium falciparum* positivity on day 3

| Characteristic                           | Univariate analysis odds ratio (95% CI) | P-value | Multivariate analysis adjusted odds ratio (95% CI) | P-value |
|------------------------------------------|----------------------------------------|---------|--------------------------------------------------|---------|
| Year 2006                                | 1.0 (–)                                |         | 1.0 (–)                                           |         |
| 2007–2008                                | 7.4 (3.7–14.8)                         | < 0.001 | 6.3 (3.3–13.1)                                    | < 0.001 |
| 2012–2013                                | 1.4 (0.7–2.9)                          | 0.31    | 2.9 (1.2–7.0)                                     | 0.017   |
| 2014                                     | 0.9 (0.5–2.0)                          | 0.96    | 1.3 (0.6–2.8)                                     | 0.50    |
| Microscopy-determined parasite density   |                                        |         | 2.6 (1.6–4.0)                                     |         |
| ≥ 100,000/μL                             |                                        |         | 2.3 (1.4–3.8)                                     | < 0.001 |
| Hemoglobin level < 10 g/dL               | 2.4 (1.7–3.4)                          | < 0.001 | 1.5 (1.1–2.3)                                     | 0.025   |
| Age < 5 years                            | 3.8 (2.6–5.4)                          | < 0.001 | 3.1 (2.1–4.6)                                     | < 0.001 |
| Fever (≥ 37.5°C)                         | 1.7 (1.1–2.5)                          | 0.008   | 1.5 (1.0–2.3)                                     | 0.043   |
| *Pfmdr1* N86                             | 1.2 (0.8–1.9)                          | 0.403   | 1.1 (0.7–1.8)                                     | 0.71    |
| *Pfcrt* K76                              | 0.5 (0.3–1.2)                          | 0.113   | 0.9 (0.4–1.9)                                     | 0.72    |

**FIGURE 2.** Distribution of *Pfmdr1* N86Y (A) and *Pfcrt* K76T (B) on day 3 across years of study.
before and after implementation of ACT as policy in Bagamoyo district, Tanzania. However, its presence was associated with pretreatment characteristics including high pretreatment asexual parasitemia (≥ 100,000/μL of blood), anemia (hemoglobin < 10 g/dL), age < 5 years, documented fever at enrollment, and the year of study.

Received September 6, 2018. Accepted for publication December 16, 2018.

Acknowledgments: We would like to extend our profound gratitude to the patients and parents/guardians who participated in the study, as well as to the health-care workers at Fukayosi and Yombo dispensaries. We would also like to extend our gratitude to Anders Björkman for his immense support.

Financial support: Financial support came from the bilateral Sida grant: the Swedish International Development Agency (Bil-Tz 16/9875007059) and Swedish Research Council (2016-0577).

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