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Recrudescent *Plasmodium falciparum* infections in children in an endemic area following artemisinin-based combination treatments: Implications for disease control

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ABSTRACT

Objective: To evaluate the features and risk factors associated with recrudescent infections that arose following artemisinin-based combination drug treatment of the primary infections. **Methods:** The clinical features and risk factors associated with subsequent recrudescence of primary *Plasmodium falciparum* infections were evaluated in 37 of 877 children following artesunate or artemisinin-based combination treatments (ACTs). Recrudescence was determined by polymerase chain reaction. **Results:** Compared to children with sensitive infections, children with recrudescent infections had significantly higher gametocytaemia and proportion with parasitaemia >50 000/ μ L. Compared with primary infections, recrudescent infections that arose from primary infections were accompanied by significantly fewer symptoms, lower body temperatures and asexual parasitaemias. In age- and gender- matched children with and without recrudescence, declines in parasitaemias following treatment were monoexponential but elimination half-life of parasitaemia was significantly longer in children with recrudescence. In a multiple regression model, at enrolment, 3 factors were independent risk factors for subsequent recrudescence of primary infections: parasitaemia $\geqslant$50 000/ μ L [adjusted odds ratio (AOR)=2.63, 95% confidence interval (*CI*): 1.17-5.90, *P*=0.018], parasite clearance time $\geqslant$2 days (AOR=2.47, 95% *CI*: 1.24-4.90, *P*=0.04) and treatment with artesunate compared with ACTs (AOR=2.35, 95% *CI*: 1.08-5.12, *P*=0.03). **Conclusions:** Recrudescent infections following artesunate or ACTs differ significantly from the primary infections from which they arose and have implications for malaria control efforts in Sub-Saharan Africa where ACTs are now first-line treatments.

1. Introduction

With increasing adoption and use as first-line antimalarials globally, it is conceivable that increasing parasite populations will be exposed to artemisinin-based combination drugs. This raises the possibility that parasites with reduced susceptibilities to one or both components of artemisinin-based combination treatments (ACTs) will be encountered in some parasite populations. Two of the earliest manifestations, amongst others, of reduced responses to antimalarials *in vivo* in *Plasmodium falciparum (P. falciparum)* are delay in parasite clearance and recrudescence following initial clearance of the primary infections[1,2].

In Southeast Asia where the risk of reinfection after therapy is low, recrudescence after artesunate monotherapy is related to heavy parasitaemias but not inherent resistance in parasite population[3]. In addition, heavy parasitaemias and delay in parasite clearance (>2 days) are associated with *in vivo* reduced susceptibilities to artemisinins[2]. In Nigeria, ACTs were adopted as first-line treatments in 2004-2005[4,5]. Despite five years into adoption in an area of *in vitro* reduced susceptibility of *P. falciparum* to artemisinin[6], the parent drug from which currently available artemisinins are derived, it is unclear what factors are associated with the recrudescence of infections following ACTs. Such information may assist with community control of infections in endemic settings.

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Therefore, the present study was aimed to evaluate the clinical and parasitological features and the risk factors associated with polymerase chain reaction (PCR)-determined recrudescent infections that arose following artemisinin-based combination drug treatment of the primary infections in children in an endemic area of Southwestern Nigeria and to examine whether the recrudescent infections that arose following ACTs treatment of the primary infections confer a survival and propagation advantage on the recrudescent parasites.

2. Materials and methods

2.1. Patients

The study took place at the University College Hospital in Ibadan, Southwestern Nigeria where malaria is hyper-endemic[7]. The subjects were 37 of 877 children who presented between January 2006 and June 2010 with an acute, symptomatic, uncomplicated *P. falciparum* infection that subsequently recrudesced after the administration of ACTs.

In general, to be enrolled, the children had to be aged 0.5-15.0 years, and had symptoms compatible with acute *P. falciparum* malaria *i.e.* with fever or history of fever in the 24-48 h preceding presentation and a mono *P. falciparum* parasitaemia >2 000 asexual forms /μL of blood. Those who had taken antimalarial drugs in the 2 weeks preceding presentation or who had a concomitant illness, such as sickle-cell anaemia or severe or complicated malaria[8] were excluded. A written informed consent was obtained from the parents or guardians of the children. The study received ethical approval from the local ethics committee.

Before enrolment, a medical history was obtained from an accompanying parent or guardian, and each child was physically examined. Body weight and oral temperature were recorded, and thick and thin smears were prepared from finger-prick samples of blood. These smears were Giemsa-stained for parasite identification and quantification of any peripheral parasitaemia.

2.2. Drug treatment

The primary infection in each child was treated with any of the following ACTs: artesunate-mefloquine (n=174), artesunate-amodiaquine (n=312) or artemether-lumefantrine (n=271) for 3 days, or artesunate alone for 7 days (n=120). The details of the procedures and treatment regimens have been reported elsewhere[5,9-13].

2.3. Follow-up

Clinical observations were recorded daily for 8 days from the first day of treatment (day 0) until day 7 and then on day 14, 21, 28, 35, and 42. Thick and thin blood smears, for quantification of parasitaemia, were prepared at the same times. At each follow-up, the guardians or parents (and when possible, the children were actively questioned, using standard questionnaire), and children were examined for the presence of any adverse reactions to ACTs.

2.4. Assessment of parasitaemia and haematocrit estimation

Thick and thin blood films prepared from a finger prick were Giemsa-stained and were examined by light microscopy under an oil-immersion objective, at ×1 000 magnification, by two independent assessors. Parasitaemia in thick films was estimated by counting asexual parasites relative to 500 leukocytes, or 500 asexual forms, whichever occurred first. From this figure, the parasite density was calculated assuming a leukocyte count of 6 000/μL of blood. Gametocytes were also counted in thick blood films against 1 000 leukocytes assuming an average leukocyte count of 6 000/μL of blood. Capillary blood collected before and during follow-up was used to measure packed cell volume (PCV) or haematocrit. Haematocrits were measured using a microhaematocrit tube and microcentrifuge (Hawksley, Lancing, UK). Routine hematocrit was done on days 0-7, 14, 21, 28, 35 and 42.

2.5. Evaluation of responses

Parasite clearance time was defined as the time elapsing between drug administration and absence of detectable parasitaemia for at least 2 days. Fever clearance time was defined as the time from drug administration until the body temperature fell to or below 37.4 ℃ and remained so for 2 days. Response to drug treatment was also classified according to a modified version of the WHO 14-day *in vivo* clinical classification system[14]. Because all patients were not febrile at enrolment, a temperature < 37.5 ℃ was not an exclusion criterion for enrolment. The modification also involved a follow up for 42 days in this area of intense transmission. The clinical classification system consisted of the following categories of response: adequate clinical and parasitological response (ACPR), late parasitological failure (LPF), late clinical failure (LCF) and early treatment failure (ETF).

2.6. Diagnosis of recrudescence

An infection was considered recrudescent if parasitaemia was cleared before day 7 but re-appeared before day 42 and was confirmed by PCR. Briefly, blood was spotted on filter papers on day 0, 3, 7, 14, 21, 28, 35 and 42 and at the time of treatment failure for parasite genotyping. PCR genotyping was done immediately after completion of each study. Paired primary and post-treatment parasites were analysed using parasite loci that exhibit repeated numbers of polymorphisms to distinguish true treatment failures

from new infections. Block 2 of merozoite surface protein-1 (MSP-1) and the block 3 of merozoite surface protein-2 (MSP-2) and region II of glutamine-rich protein (GLURP) were amplified by two rounds of PCR using primers and amplification conditions described previously[15-17]. 10 μL of the nested PCR products were resolved by electrophoresis on a 2% agarose gel and sized against a 100-base pair molecular weight marker (New England Biolabs, Beverly, MA). The banding pattern of the post-treatment parasites was compared with matched primary parasites in each of the patients who had parasitaemia after treatment. Post-treatment and primary infection parasites showing at least one pair of identical bands between primary and post-treatment parasites were considered as true treatment failure, while non-identity in banding patterns with even one marker was considered as newly acquired infections.

2.7. Kinetics of parasitemia

In 13 children with recrudescent infections and in 13 age- and gender- matched children with sensitive infections, follow-up with clinical and parasitological evaluation was done at the following times: 0, 1, 2, 4, 8, 16, 24 h and daily on day 2-7, 14, 21, 28, 35 and 42. The kinetics of the time course of the parasitaemia was estimated by using a non-compartmental model as previously described[18].

2.8. Statistical analysis

Data were analysed using version 6 of the Epi-Info software and the statistical program SPSS for Windows version 10.01. Proportions were compared by calculating with Yates' correction. Normally distributed, continuous data were compared by Student's *t*-tests and analysis of variance (ANOVA). Data not conforming to a normal distribution were compared by the Mann-Whitney *U*-test and the Kruskal-Wallis test (or by Wilcoxon rank sum test). The cumulative risk of parasite recrudescence was calculated by survival using the Kaplan-Meier method. A multiple logistic regression model was used to test the association between recrudescence (yes or no following treatment) and factors that were significant at univariate analysis: parasite density, parasite clearance time, and treatment with artesunate compared to ACTs. Because the study was conducted over a period of 4 years, time in years since the commencement of study was included as a covariate in the model. *P*-values of <0.05 were taken to indicate significant differences.

3. Results

3.1. Recrudescent rates after administration of ACTs

During the study period January 2006 to June 2010, 877 children were treated with artesunate or ACTs. PCR confirmed recrudescence occurred in 10 of 120, 15 of 271, 10 of 312, and 2 of 174 children treated with artesunate, artemether-lumefantrine, artesunate-amodiaquine, and artesunate-mefloquine, respectively (Figure 1). The proportion of children in which the primary infections subsequently recrudesced was significantly higher in artesunate-treated children (χ^2=11.04, *df*=3, *P*=0.01). The risk of recrudescence was significantly higher following artesunate treatment (log rank statistic=12.18, *P*=0.007) (Figure 2). Recrudescence occurred on days 14 (two children), 21 (seven), 28 (eleven), 31-35 (ten) and 42 (seven). Median time to recrudescence was 28 days.

Because of the relatively small number of recrudescent infections in each treatment regimens, the recrudescent infections were combined for subsequent analysis.

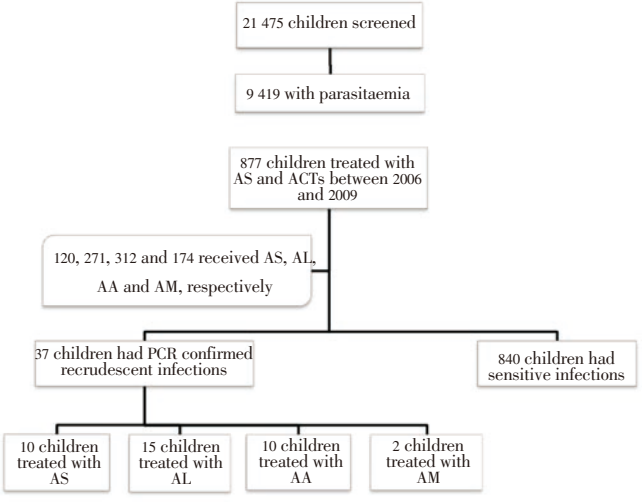

Figure 1. Study profile.
AL: artemether-lumefantrine; AA: amodiaquine-artesunate; AS: artesunate; AM: artesunate-mefloquine.

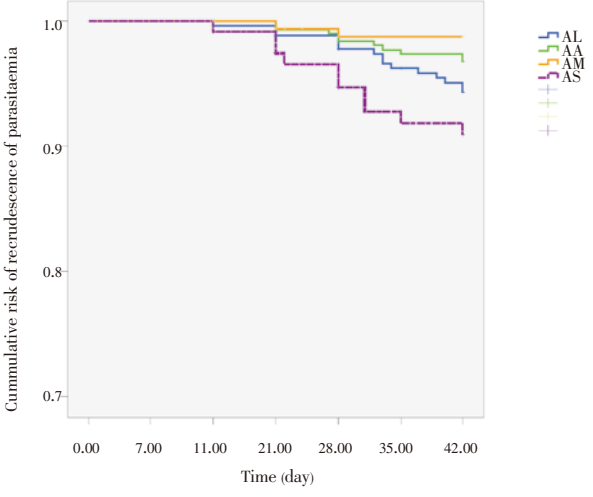

Figure 2. Kaplan-Meier plot (survival curve) of the cumulative risk of recrudescence of parasitemia after treatment with artemisinin based drus (log rank statistic=12.18, *P*=0.007).
AL: artemether-lumefantrine; AA: amodiaquine-artesunate; AS: artesunate; AM: artesunate-mefloquine.

Table 1
Demographic and clinical features of children with or without subsequent recrudescence enrolled in the study.

Variable	Sensitive (*n*=840)	Recrudescent (*n*=37)	*P*-value
Age	6.61±3.01	7.26±2.85	0.20
Range	0.42-15.00	0.07-13.00	-
< 5 years	251	8	0.26
Weight (kg)	18.54±6.49	20.16±8.00	0.14
Range	6-59	6-46	-
Duration (day)	2.74±1.19	3.06±1.45	0.15
Range	1-10	2-7	-
Haematocrit (%)	31.47±5.06 (*n*=694)	30.13±4.42 (*n*=24)	0.15
Range	21-38	17-38	-
No. with <30%	207	11	0.52
Temperature (℃)	38.34±1.14	38.34±1.00	0.98
Range	35.80-41.10	36.60-40.50	-
No. with > 40℃	2	1	-
GMPD (/μL blood)	54 298	68 273	0.44
Range	2 024-1 183 145	15 000-1 125 000	-
No. with > 250 000	1	2	-
> 50 000	464	28	0.016
GMGD (/μL blood)	24 (*n*=54)	96 (*n*=4)	0.04
Range	6-450	18-396	-
FCT (day)	1.13±0.40	1.0	0.1
Range	1-4 (*n*=630)	1 (*n*=23)	-
PCT (day)	1.17±0.61	1.37±0.62	0.06
Range	0.08-4.00	0.33-2.00	-
ACPR	846	-	-
ETF	-	-	-
LPF	-	9	-
LCF	-	21	-

GMPD: geometric mean parasite density; GMGD: geometric mean gametocyte density; ACPR: adequate clinical and parasitological response; ETF: early treatment failure; LPF: late parasitological failure; LCF: late clinical failure.

3.2. Comparison of children with and without subsequent recrudescence of primary infections

Enrolment characteristics of the children in which the primary infections subsequently recrudesced and those in which the primary infection did not recrudesce were summarized in Table 1. Children in which the primary infections subsequently recrudesced had significantly higher proportion of children with a parasitaemia >50 000/μL of blood and a significantly higher gametocytaemia than children in which there was no subsequent recrudescence of the primary infections.

3.3. Clinical features of primary and recrudescent infections

In 9 children, no symptoms were present at recrudescence. The frequencies of symptoms on presentation and other clinical and parasitological parameters of the primary infections and during the recrudescent infections that emerged after ACTs of the primary infections were summarized in Table 2. Compared with the primary, the recrudescent infections were accompanied by significantly fewer symptoms, lower body temperatures, lower densities of parasitaemia, and significantly higher gametocytaemia: parasitaemia ratio. The prevalences of gametocytaemia in the primary and recrudescent infections were similar (11% *vs* 8%) (Table 2).

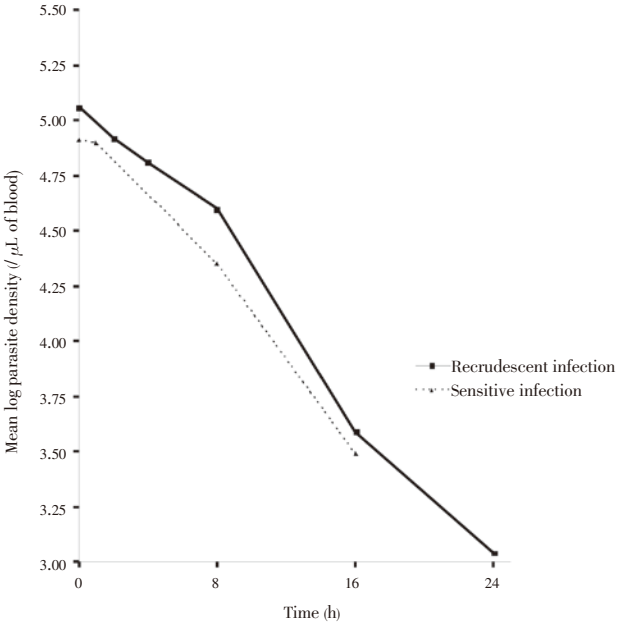

Figure 3. Semi-log plots of parasitemia versus time in children with recrudescent (solid line) and in age and gender matched children with sensitive infections (broken line) after treatment of *P. falciparum* infections with artemisinin-based combination drugs.

Table 2
Frequencies of symptoms and other parameters at enrolment (during the primary infection) and recrudescence among the 37 malarious children.

Variables	Primary infection	Recrudescent infection	*P*-value
Fever	29	21	0.015 0
Headache	21	6	0.000 2
Abdominal pain	18	4	0.000 4
Vomiting	9	7	0.770 0
Cough	5	2	0.420 0*
Anorexia	6	5	1.000 0
Haematocrit (%)	32.7±5.7	34.1±3.2	0.98
Range	17-38	27-36	-
No. with <30%			
Temperature (℃)	38.3±0.9	37.6±1.4	0. 020 0
Range	36.6-39.8	35.9-40.5	-
No. with >40 ℃	0	2	-
GMPD (/μL blood)	68 273	28 604	0. 045 0
Range	10 781-1 125 000	845-132 030	-
No. with >50 000	28	5	<0.001 0
No. with gametocytaemia	2	3	1.000 0
GMGD (/μL blood)	20	176	-
GMPD: GMGD	0.000 029	0.008 7	<0.001 0

GMPD: geometric mean parasite density; GMGD: geometric mean gametocyte density.

Table 3
Associated and independent risk factors, at enrolment for subsequent recrudescence of a primary infection following treatment with artemisinin-based combination drugs.

Variable		Total No. of children	No. with recrudescence	OR (95% *CI*)	*P*-value	AOR (95% *CI*)	*P*-value
Age (year)	< 5	259	8	1	0.26	-	-
	⩾ 5	608	29	1.57 (0.70-3.48)			
Gender	Male	439	17	1	0. 60	-	-
	Female	437	20	1.19 (0.61-2.30)			
PCV (%)	< 30	218	11	1	0. 52	-	-
	⩾ 30	500	14	1.27 (0.60-2.71)	0. 47	-	-
Duration of illness (day)	⩽ 3	144	7	1			
	> 3	692	25	1.36 (0.57-3.21)			
Fever*	Present	646	24	1	0.60	-	-
	Absent	199	9	1.22 (0.56-2.68)			
Parasite density (/μL of blood)	< 50 000	378	9	1	0.016	1	0.018
	⩾ 50 000	492	28	2.47 (1.15-5.30)		2.63 (1.17-5.90)	
Parasite clearance time (day)	< 2	638	18	1	0.001	1	0.04
	⩾ 2	235	18	2.85 (1.46-5.59)		2. 47 (1.24-4.90)	
Year of enrollment	Before 2008	380	15	1	0.74	-	-
	2008 and after	400	22	0.89 (0.45-1.74)			
Drug treatment	AM	174	2	1		1	
	AS	120	10	2.48 (1.16-5.26)	0.015	2.35 (1.08-5.12)	0.03
	AA	312	10	0.66 (0.32-1.38)	0.26	-	-
	AL	271	15	1. 55 (0.79-3.04)	0.19	-	-

* Temperature >37.4 ℃; AL: artemether-lumefantrine; AA: amodiaquine-artesunate; AS: artesunate; AM: artesunate-mefloquine.

3.4. Kinetics of parasitaemia

Data for evaluation of the kinetics of parasitaemia were available in 26 children: 13 children with recrudescence of the primary infections and age and gender matched 13 children without recrudescence. The mean age and weight of these children at enrolment were (7.4±3.1) years, (22.6±9.5) kg, respectively. Enrolment geometric mean parasite densities were 114 882 and 80 049 per μL of blood, respectively in children with and without recrudescence of their infections (P=0.55). Overall, there was monoexponential decline of the parasitemia with estimated mean half-life ($t_{1/2el}$) of (1.64±0.18) h (SEM) and (0.86±0.11) h (SEM), respectively in children with and without recrudescence of the primary infections (Figure 3). The difference between the mean values was significant (P=0.002).

3.5. Factors associated with subsequent recrudescence of a primary infection

Factors, at enrolment, that are associated with subsequent recrudescence of a primary infection were presented in Table 3. A parasitaemia ≥50 000/μL of blood, and a parasite clearance time ≥2 days were related to subsequent recrudescence of a primary infection. Age, gender, duration of illness, haematocrit value, or year of enrolment were not related to subsequent recrudescence of a primary infection (Table 3). Using artesunate-mefloquine as the reference artemisinin drug, artesunate had a greater proclivity than ACTs to cause a subsequent recrudescence of a primary infection (Table 3 and Figure 2).

3.6. Risk factors for subsequent recrudescence of a primary infection

In the multivariable analysis, a parasitaemia ≥50 000/μL of blood, a parasite clearance time ≥2 days, and treatment with artesunate were found to be independent risk factors for subsequent recrudescence of a primary infection (Table 3).

4. Discussion

In this study, 4.2% of patients recrudesced during the 42-day observation period. Recrudescence was significantly higher with artesunate monotherapy (9%). However, irrespective of ACTs, the recrudescent rates after treatments were similar but the rate was the lowest with artesunate-mefloquine; perhaps reflecting the relatively long half-life of mefloquine of 15-33 days compared with the other partner drugs. The relatively high recrudescent rate with artesunate monotherapy is of concern in this endemic area where primary reduced *in vitro* susceptibility of *P. falciparum* isolates to artemisinin was described about 20 years ago[6] and artesunate alone is readily available over the counter. However, the rate is considerably lower than 31% reported in an area of lesser intensity of transmission in Thailand[3] where there is no innate reduced *in vitro* susceptibility in *P. falciparum* isolates. Previous studies showed that recrudescent rates following ACTs are generally low and <5%-10% [13,19,20] but are much higher in Southeast Asia, particularly to artesunate-mefloquine[21,22]. However, that recrudescence was detected in 50% of the children with recrudescence 28 days after commencement of treatment justifies prolongation of follow-up beyond 28 days in children treated with ACTs in endemic areas of Africa. Thus it is likely that in many endemic settings in Africa, recrudescent rates after ACTs based on 28 days of follow-up may have been underestimated. With a median time to recrudescence of 28 days, it is conceivable that as sensitivity becomes progressively reduced to the currently available ACTs, median time to recrudescence will become progressively shorter.

The proportion of children with a parasitaemia >50 000/μL of blood on the day of admission of patients in which primary infections subsequently recrudesced was significantly higher than those in which the primary infection did not recrudesce, suggesting higher parasite burdens in the former. These data are consistent with previous studies on artemisinin drugs[3] which showed that patients in which the primary infection subsequently recrudesced tended to have significantly higher parasite burdens than in those in which the primary infections did not recrudesced.

In Nigeria, ACTs have become effective first-line treatments producing a parasitological efficacy >96% in children with uncomplicated infections even after 5 years of adoption[13]. Although recrudescent rates are low, the recrudescent infections that emerged after ACTs of the primary infections have failed are clinically considerably different from the primary infections. The significant modifications observed include a propensity to produce fewer symptoms and signs of infections and a lower parasitaemia. These data are consistent with previous studies on chloroquine[23-25], sulphadoxine-pyrimethamine[26], and amodiaquine[17] which showed that recrudescent infections that emerged after the primary infections have failed are much different clinically and parasitologically from the primary infections. These differences may significantly affect the ability of a health care worker to make a prompt diagnosis of recrudescence and therefore institute appropriate treatment of the recrudescent infections. Approximately a quarter of children were asymptomatic at the time of recrudescence. As those who are free of symptoms will not seek treatment, the days of asymptomatic gametocytaemia that often follow a recrudescence will favour the transmission of the gametocytes with reduced drug sensitivities.

It is intriguing that at enrolment, estimated parasite elimination half life was approximately two folds and significantly higher in children in which the primary infections subsequently recrudesced than in those in which there was no recrudescence. There are three possible explanations, amongst others for the observation: 1) Sequestered parasite burden may be considerably higher in children with recrudescence. However, peripheral parasitaemias, which may reflect parasite load, were

insignificantly different in age and gender-matched children. 2) Antimalarial drug concentration profiles over time may differ. However, drug concentrations were not measured. Since impaired absorption may occur in malaria, the extent of impairment of drug absorption may differ considerably. 3) The parasites may have differing *in vitro* susceptibility profiles. However, *in vitro* susceptibility profiles were not done in the present study. In Thailand, in patients with recrudescent infections following artesunate treatment, *in vitro* susceptibility profiles to dihydroartemisinin, the metabolite to which artesunate is rapidly converted, was thought not to be contributory to the recrudescence of infection[3]. In other studies, for example in Cambodia, elevated IC_{50} to artesunate and the partner drug, mefloquine are associated with increased risk of recrudescence to artesunate-mefloquine[21].

Despite approximately 4% recrudescent rates in patients treated with ACTs[13,21], very few studies have examined the risk factors associated with recrudescence in such patients in Africa. In this study, at presentation, heavy parasite burdens and delay in parasite clearance were independent risk factors for the subsequent recrudescence of a primary infection. As young children have relatively lower degree of antimalarial immunity than the older children, it was surprising that age was not associated with recrudescence in an area of innate reduced susceptibility to artemisinin[6]. The association of subsequent risk of recrudescence with high enrolment parasite burden is in agreement with finding in patients in areas of low intensity of transmission in Southeast Asia[3,21], and suggests that despite regional difference in transmission intensities, some of the risk factors associated with recrudescence are remarkably similar.

The pharmacodynamic hallmark of artesunate and ACTs is the rapid reduction of parasite numbers by approximately 10^4 per asexual cycle[27-35], resulting in parasite clearance time of usually $\leqslant$2 days. This rapid effect may be impacted by parasite burden[2,36]. Thus, it seems likely that the large parasite burdens and the subsequently relatively longer parasite clearance time should be independent risk factors for subsequent recrudescence of a primary infection. These data support recent studies in which both parasite burden and delay in parasite clearance are associated with *in vivo* treatment failure of artemisinin drugs[2,19,21-23].

All the possible risk factors for subsequent recrudescence of a primary infection following treatment with ACTs were not captured in this study. *In vitro* sensitivity data of *P. falciparum* isolates, drug concentrations and the molecular genotypes including the *Pfmdr 1* alleles, for example, were not evaluated. Nevertheless, a relatively large number of children with recrudescent infections were evaluated during the 4-years study period. With a significantly higher pre-treatment gametocytaemia and significantly higher gametocytaemia: parasitaemia ratio following recrudescence, it will be necessary to explore whether gametocytes arising from recrudescent infections following ACTs are more infectious to the mosquito than those from non-recrudescent infections as has been shown for chloroquine[23].

There are limitations of the present study: the number of children with recrudescent infection is small and there was slow accrual confirming high efficacy of ACTs in the first five years of adoption as first-line antimalarials in this endemic area of Africa[13]; *in-vitro* sensitivity profiles of the primary and the recrudescent parasites and drug levels were not measured. However, the data accrued form the baseline data against which the future behaviour of the recrudescent infections may be measured in this endemic area.

In conclusion, fewer clinical symptoms and signs and long elimination half life of parasitaemia are features, and young age, high parasite burdens, and delay in parasite clearance are risk factors for subsequent recrudescence of a primary infection following ACTs. These have implications particularly with the spread of drug-resistant infections and for malaria control efforts in sub-Saharan Africa where ACTs are now first-line treatments.

Conflict of interest statement

We declare that we have no conflict of interest.

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