Parasite Epidemiology and Control 19 (2022) e00276

Contents lists available at ScienceDirect

Parasite Epidemiology and Control

journal homepage: www.elsevier.com/locate/parepi

Parasite clearance dynamics in children hospitalised with severe malaria in the Ho Teaching Hospital, Volta Region, Ghana

Laura Paris [a], Richmond G. Tackie [b], Khalid B. Beshir [a], John Tampuori [c], Gordon A. Awandare [d], Fred N. Binka [e], Britta C. Urban [f], Bismarck Dinko [b,*], Colin J. Sutherland [a]

[a] Department of Infection Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK
[b] Department of Biomedical Sciences, School of Basic and Biomedical Sciences, University of Health and Allied Sciences, Ho, Ghana
[c] Department of Urology, Ho Teaching Hospital, Ho, Ghana
[d] West African Centre for Cell Biology of Infectious Pathogens, Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon, Accra, Ghana
[e] Department of Epidemiology and Biostatistics, School of Public Health, University of Health and Allied Sciences, Ho, Ghana
[f] Department of Tropical Disease Biology, Faculty of Biological Sciences, Liverpool School of Tropical Medicine, Liverpool, UK

ARTICLE INFO

Keywords:
Severe malaria
Artemisinin-based combination therapy
Parasite clearance

ABSTRACT

Background: Over 90% of severe malaria (SM) cases occur in African children. Parenteral artesunate is currently the recommended treatment for SM. Studies of parasite clearance in paediatric SM cases are needed for assessment of therapeutic outcomes but are lacking in Africa.
Methods: Severe malaria patients were recruited in the children's emergency ward at Ho Teaching Hospital, Ghana, in 2018. Blood samples were taken upon admission, every 24 h for 3 days and 1 week after treatment, and DNA extracted. Parasitaemia and parasite densities were performed by microscopy at enrolment and the follow-up days wherever possible. Relative parasite density was measured at each timepoint by duplex qPCR and parameters of parasite clearance estimated.
Results: Of 25 evaluable SM patients, clearance of qPCR-detectable parasites occurred within 48 h for 17 patients, but three out of the remaining eight were still qPCR-positive on day 3. Increased time to parasite clearance was seen in children ≥5 years old, those with lower haemoglobin levels and those with a high number of previous malaria diagnoses, but these associations were not statistically significant.
Conclusion: We examined parasite clearance dynamics among paediatric cases of SM. Our observations suggest that daily sampling for qPCR estimation of *P. falciparum* peripheral density is a useful method for assessing treatment response in hospitalised SM cases. The study demonstrated varied parasite clearance response, thus illuminating the complex nature of the mechanism in this important patient group, and further investigations utilizing larger sample sizes are needed to confirm our findings.

* Corresponding author at: Department of Biomedical Sciences, School of Basic and Biomedical Sciences, University of Health and Allied Sciences, PMB 31, Ho, Volta Region, Ghana.
E-mail address: bdinko@uhas.edu.gh (B. Dinko).

https://doi.org/10.1016/j.parepi.2022.e00276
Received 30 November 2021; Received in revised form 7 August 2022; Accepted 30 September 2022
Available online 10 October 2022

1. Introduction

Malaria is a major life-threatening parasitic infectious disease that continues to impair the wellbeing of people in sub-Saharan Africa, with children and pregnant women bearing the major burden of morbidity (WHO, 2021). Severe malaria (SM), characterized by a range of complications (Mousa et al., 2020), requires hospitalization and careful patient management. According to the WHO (2021), 228 million malaria cases and 405,000 deaths worldwide were reported in 2018, with >90% of both cases and deaths occurring in sub-Saharan Africa. An accurate estimate of the proportion of cases with SM is difficult because malaria is most prevalent in poor and rural communities where methods of disease identification, documentation and reporting are the weakest (WHO, 2021). The global annual incidence of SM, last estimated in 2014, was approximately 2 million, with over 90% of all cases occurring due to *Plasmodium falciparum* infections in African children (WHO, 2014). In countries with moderate to high malaria endemicity such as those in West Africa, children under 5 years account for most of the SM cases, presenting with complications including hyperparasitaemia, cerebral malaria (CM) and severe malaria anaemia (SMA) (Meerman et al., 2005). Therefore, in clinical practice it is vital that these patients are assessed and given prompt treatment.

Artemisinin-based combination therapy (ACT) is recommended for the management of uncomplicated malaria in African countries, whereas parenteral artesunate monotherapy is the recommended regimen for hospitalised SM cases (Dondorp et al., 2010). Reduced susceptibility to artemisinin in *P. falciparum* manifests as slower parasite clearance *in vivo*, commonly measured as parasite clearance half-life in patients treated with artesunate monotherapy and monitored by microscopy of closely-spaced (6–12 h apart) blood samples (Ashley et al., 2014) or by qPCR estimation of parasite density in daily finger-prick blood samples (Beshir et al., 2021). The emergence in the Mekong countries of *P. falciparum* parasites displaying reduced susceptibility threatens ACT treatment efficacy (Noedl et al., 2008; Dondorp et al., 2009; Ashley et al., 2014; Spring et al., 2015). Polymorphisms in the propeller domain of a *kelch* gene on chromosome 13 are thought to be the major determinants of artemisinin resistance in the Mekong countries (Ariey et al., 2014; Takala-Harrison et al., 2015). Recently, a *P. falciparum* K13 R561H (*pfk13* R561H) mutation was detected in Rwandan isolates and associated with day 3 parasite positivity after ACT, providing evidence of *de novo* emergence of this genotype (Uwimana et al., 2020). In Northern Uganda, an association was found between parasite clearance half-life with both A675V or C469Y mutations indicating emergence of artemisinin resistance in Africa (Balikagala et al., 2021). Therefore, there are concerns that sustained use of ACT will favour spread of these parasites within Africa (Conrad et al., 2014; Taylor et al., 2015; Sutherland et al., 2017: Uwimana et al., 2020; Balikagala et al., 2021).

There have been some reports of delayed parasite clearance in Africa. The distributions observed do not yet support a widespread selective sweep for an ACT-resistant phenotype, but does warrant continued surveillance (Beshir et al., 2013, 2021; Taylor et al., 2015; Lu et al., 2017; Sutherland et al., 2017). Recent *in vitro* studies have greatly expanded our understanding of reduced artemisinin susceptibility in *P. falciparum* and identified important K13-independent resistance phenotypes mediated *in vitro* by variants of other cellular proteins, including Coronin, trafficking adaptin subunit AP-2μ and ubiquitin binding protein 1 (UBP-1) (Sutherland et al., 2020).

In the African setting, with large paediatric patient population, and in remote areas of Asia qPCR estimation of changes in parasite density in daily blood samples at 24 h, 48 h and 72 h provides an alternative to the frequent blood sampling deployed by studies in the Mekong countries (Beshir et al., 2010, 2013, 2021; Lubis et al., 2020). Declining ACT efficacy would not only jeopardize malaria eradication but may also result in a surge in African childhood morbidity and mortality rates. This might lead to an increase in severe malaria incidence, as is thought to have occurred after mass failure of chloroquine efficacy in the 1980s and 1990s (Trape et al., 1998). Few published studies on parasite clearance in African SM patients exists, and these deploy frequent blood sampling and parasite density estimation by microscopy as in the Mekong studies described above (Byakika-Kibwika et al., 2018). The qPCR-based protocol, requiring only once-daily blood sampling (Beshir et al., 2010), has not been evaluated as a means to monitor treatment outcomes in paediatric SM patients in African hospitals.

In this study we assessed parasite clearance dynamics in Ghanaian children with severe malaria admitted as in-patients to a regional teaching hospital using an established multiplex qPCR assay.

2. Materials and methods

2.1. Study area

The study was carried out in the Ho municipality of the Volta Region, located in the Eastern part of Ghana. The samples were collected at the Ho Teaching Hospital in urban Ho, and the laboratory procedures were carried out in the Laboratory for Infectious and Tropical Diseases, Department of Biomedical Sciences, School of Basic and Biomedical Sciences, University of Health and Allied Sciences, Ho (UHAS). qPCR analysis was performed in the Department of Infection Biology, London School of Hygiene & Tropical Medicine. Southern Ghana is characterized by two distinct malaria transmission seasons: a major rainy season occurring from April to July and a less intense rainy season from September to November, with malaria cases beginning to rise a month after the start of the rains (Mba and Aboh, 2007). Transmission during the rainy season is considered moderate to high in this region, and *P. falciparum* accounts for 85–90% of all infections with *P. malariae* (<10%) and *P. ovale* (<1%) also present (Dinko et al., 2013).

2.2. Patients and study design

2.2.1. Study participants and design

Study participants were children between 1 and 14 years at the children's ward (CW) of the Ho Teaching Hospital. Patients were recruited post-admission under a sub-study renewal of the project protocol "Immunobiology of Sexual Stage Antigens of the *Plasmodium falciparum* Parasites", approved by the Ghana Health Service Ethical Review Committee (GHS-ERC: 03/09/2016) and the LSHTM Research Ethics Committee (#14967). Consent was obtained for each individual from parents or guardians, as set out in the main protocol. The study was conducted in accordance with the ethical standards of the Helsinki Declaration (2008) of the World Medical Association. The main inclusion criteria were signs and symptoms of severe malaria such as repeated convulsions, persistent fever, anaemia, high parasitaemia and the exclusion criterion was any other severe disease.

2.2.2. Sample collection, treatment and follow-up

Samples were collected in June to August 2018, during the latter part of the major rainy season, Febrile children admitted to the ward with suspected malaria were first screened for *P. falciparum* parasites by finger-prick peripheral blood using a *P. falciparum*-specific RDT (Malaria Pf rapid test, Shenyang LTH Technology Development Company, Beijing, China). A dried blood spot (DBS) was also obtained on Whatman grade 3 filter paper (Whatman, Maidstone, UK) from the same finger-prick and blood smears made to confirm the presence of parasites by microscopy. Venous blood (2-4mL) was collected into EDTA tubes for preparation of comparative and comfirmatory blood smears and haemaglobin (Hb) estimation using the Sysmex haematology analyzer (Sysmex, UK). DBS were transferred to the Laboratory for Infectious and Tropical Diseases in UHAS in individually-sealed Ziplock bags and air-dried for 24 h before storage at −80 °C. Recruitment and sampling procedures are summarised in Fig. 1.

Children testing positive for malaria and requiring hospitalization for SM received parenteral artesunate at the time of admission for a minimum of 24 (h) followed by the locally recommended oral ACT artemether-lumefantrine (AL) for a full course of 6 doses over three days as recommended (Ministry of Health Ghana, 2014). Follow-up blood samples were taken at 24 h, 48 h and 72 h post-treatment and, following discharge, patients were invited to return for review 7 days after commencing treatment. On each follow up day a finger-prick blood spot was collected onto filter paper as previously described by trained hospital staff following local hospital guidelines and national protocols. The study team had no influence over clinical management of patients, and no clinical follow-up was carried out beyond the 7 day timepoint. Decisions to hospitalize children were made solely by hospital clinical staff, and in-patients were managed following established guidelines (WHO, 2014). Our participants therefore received close to standard in-patient care for children with SM at Ho Teaching Hospital, the only difference being collection of daily finger-prick samples and a day 7 follow-up at the hospital. Routine clinical data including peripheral haemoglobin levels were provided by the hospital clinicians.

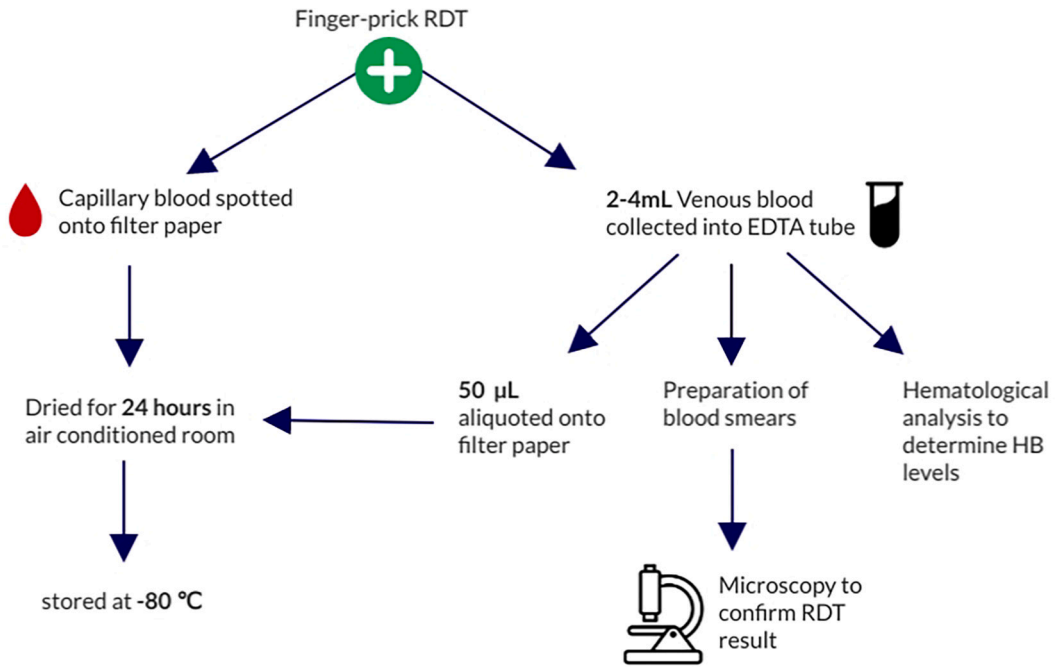

Fig. 1. Sampling procedures for recruited children at the Children's Emergency Ward.
Sample flow for collection of dried blood spots on filter paper from children admitted for severe malaria.

2.3. Microscopy

Thick and thin blood smears were stained in 10% Giemsa, after fixing the thin smear in methanol, and these were examined under high powered microscope, and detected parasites were counted. All initial parasite counts were performed by hospital laboratory staff as previously described (Dinko et al., 2013, 2018). Each slide was read independently by two experienced microscopists, and the geometric mean reading of the two replicates used for analysis. Independent microscopists read the slide in cases where the parasite counts estimates of the first two readers differed by >10%, and the third reading was used in computing the geometric mean in place of the one with bigger difference. Sexual parasite rate and density were determined as described (Dinko et al., 2013).

2.4. DNA extraction and parasite clearance time qPCR (PCT qPCR) assay

DNA extraction from DBS was carried out as previously described (Robinson et al., 2018; Robinson et al., 2019). Relative parasite densities in patients pre- and post- treatment were determined using an established method (Beshir et al., 2010). Briefly, quantitative PCR with a pan-genus apicoplast genome target (*pgmet*) was used to provide relative quantification of parasite DNA in each blood sample against a human gene signal (β-tubulin). Relative parasite density from samples collected on days 1 to 3 was then normalised against the relative parasite density of the day 0 sample. qPCR reactions for each sample were run in duplicate alongside a calibrator, the WHO international standard for *P. falciparum* DNA, as previously described (Robinson et al., 2019). This method has an estimated limit of detection of 0.05 parasites per μL of whole blood (Beshir et al., 2013, 2021; Lubis et al., 2020).

2.5. Data and statistical analysis

Parasite clearance curves for each patient were used to estimate relative parasite density data and estimates of parasite reduction ratio (PRR) at 24 and 48 h after treatment by standard methods as described (Beshir et al., 2010, 2013; Robinson et al., 2019). The chi-squared distribution was used to test for statistical associations between pairs of binary variables, and odds ratios (OR) calculated. The variables evaluated for association with parasite clearance (in days) included history of malaria episodes, haemoglobin levels at presentation and age of participants.

3. Results

3.1. Patient characteristics

A total of 33 patients with SM who tested positive for *P. falciparum* parasites by RDT and microscopy were enrolled in the study, of which 25 provided at least 2 evaluable samples, pre- and post-treatment, for qPCR analysis. Of the 25 enrollees, 13 of them were female, and the pre-treatment (day 0) asexual parasite density by microscopy ranged from 771 to 32,800 parasites/μl. Each in-patient was followed up 24 h, 48 h and 72 h post-treatment, and 9 returned for review 7 days after commencement of treatment. Enrolment characteristics are presented in Table 1. None of the SM patient had taken antimalarials or traditional herbal medicine within the 2 weeks prior to hospital admission. All 25 patients were initially treated with intravenous artesunate, 14 of which were switched to oral ACT, intra-muscular artemether or artesunate suppository as antimalarial medication after 24 h (Table 2). By the 3rd day of admission 88% of the children had their symptoms resolved and were discharged to complete a standard 3-day, 6-dose course of oral AL at home.

Table 1
Patient characteristics at clinical presentation and treatment received, $N = 25$.

Characteristic	Category	Number (%)
Gender		
	Female	13 (50%)
Age		
	<2 y	1 (4%)
	2 y to <5 y	12 (48%
	≥5 y	12 (48%)
Age range		1-12y
Mean age		4y 8 months
Initial treatment		
	parenteral artesunate	25 (100%
Subsequent treatment		
	artemether-lumefantrine	13 (52%)
	artesunate suppository	1 (4%)
	not recorded	11 (44%)
Previous malaria diagnoses1		
	0	10 (40%)
	1	3 (12%)
	2	7 (28%)
	3–7	5 (20%)

Table 2
Presentation parasite density and parasite reduction ratios following treatment in 25 hospitalised children with SM.

ID	Age yrs	Enrolment parasites/μL[#]	Hb	Parasite clearance[*]	prev. Malaria diagnoses	PRR_{24}[**]	PRR_{48}[**]	parasites at day 7
SM004	3	6123	–	day 1	2	100,000		ND[***]
SM007	7	–	14.5	day 1	2	100,000		ND
SM037	2	–	11.9	day 1	0	100,000		ND
SM038	4	8800	11.4	day 1	0	100,000		ND
SM041	12	–	6.8	day 1	1	100,000		YES
SM047	2	–	10.9	day 1	1	100,000		NO
SM049	6	32,800	4.48	day 1	0	100,000		NO
SM006	2	4167	–	day 2	1	44.24	100,000	ND
SM008	2	771	10.8	day 2	2	26.36	100,000	ND
SM036	2	–	12.2	day 2	0	0.06	100,000	ND
SM043	5	21,600	–	day 2[*]	3	526.32	100,000	ND
SM044	4	–	–	day 2	2	4	100,000	YES
SM051	5	–	8.6	day 2	7	2500	100,000	NO
SM052	8	–	–	day 2	0	9.85	100,000	YES
SM053	2	29,600	9	day 2	0	65.79	100,000	ND
SM054	5	–	–	day 2	3	68.97	100,000	NO
SM056	10	28,400	–	day 2	0	42.73	100,000	ND
SM001	1	39,326	9.7	day 3	0	4.48	434.78	ND
SM003	3	1449	7	day 3	2	21.55	35.97	ND
SM005	5	1980	9.7	day 3	2	6.84	1666.67	ND
SM048	7	1280	3.81	day 3	3	24.75	4117.65	NO
SM050	8	–	5.1	day 3	6	0.6	1.61	ND
SM042	5	16,400	10.1	Not cleared	2	49.26	2500	NO
SM045	4	–	10.2	Not cleared	0	4.16	28.74	NO
SM046	2	–	10.2	Not cleared	0	0.54	0.66	ND

[#] Dash indicates standard microscopy was not performed at initial presentation, in most cases because very sick patients were immediately given presumptive parenteral treatment. Parasitaemia was confirmed later at the time of Hb estimation, as a qualitative check only.

[*] "Parasite clearance" refers to the day of collection of the first qPCR-negative blood sample. One individual (SM043) with parasite clearance stated as "Day 2" did not have follow-up at day 3 due to discharge from the hospital, but the 48 h blood sample was subsequently found to be qPCR positive.

[**] To avoid infinity values in the parasite reduction ratio (PRR), full clearance at 24 or 48 h was arbitrarily designated as a PRR of 100,000.

[***] ND means test not done on day 7 for parasite detection.

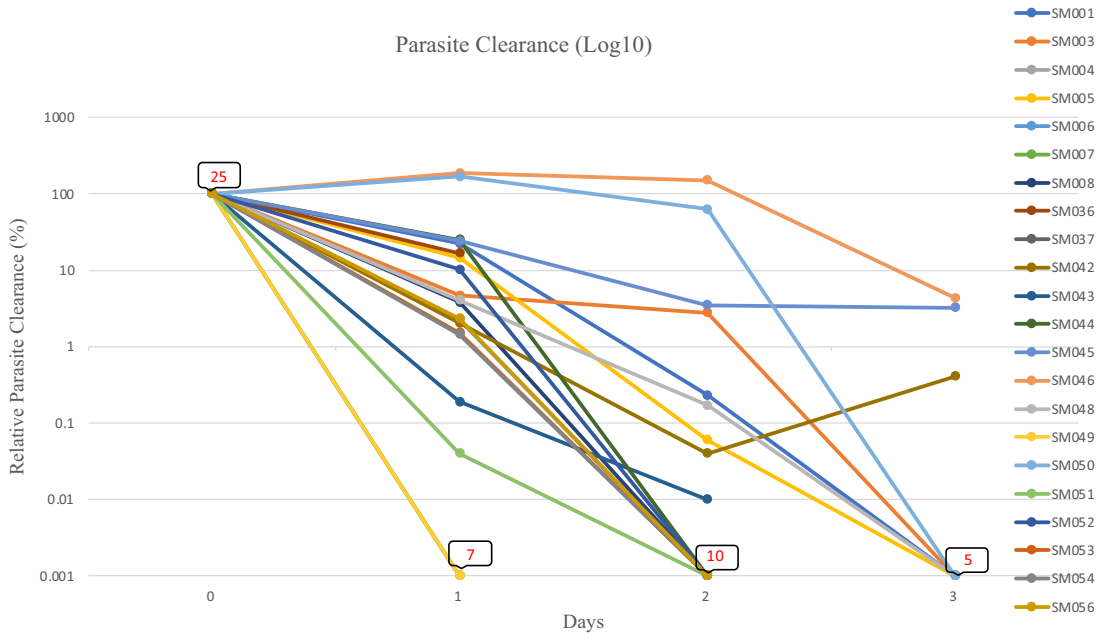

Fig. 2. Plot of estimated relative parasite clearance over time in treated SM patients.
Relative parasite densities compared to pre-treatment were estimated from daily qPCR estimates for each evaluable participant ($N = 25$). Y-axis is plotted on a log-scale. SM042, SM045 and SM046 did not clear qPCR-detectable parasitaemia by day 3. The number of children examined (day 0 $n = 25$) and cleared parasites on the follow-up days (day 1 $n = 5$, day 2 $n = 10$ and day 3 $n = 6$) are shown.

3.2. Estimation of parasite clearance time by qPCR

Twenty-five patients were confirmed positive for *P. falciparum* by PCR and provided at least one post-treatment blood sample for assessment of parasite clearance dynamics and thus contributed to the analysis presented (Table 2). These patients had a relative parasite density during follow-up ranging from 0.01% to 186% as determined by qPCR (Fig. 2). Parasite clearance varied among the patients, with 7 children clearing (qPCR negative) within 24 h of treatment, 10 clearing within 48 h and 5 children clearing detectable parasites by 72 h. Three patients still had detectable parasites on day 3. In the 9 patients followed up a week after treatment 4 children had detectable parasite DNA in their blood, 1 of which was a patient that had not cleared by day 3. The parasite reduction ratio on day 1 and day 2 (PRR_{24} and PRR_{48}) varied among SM patients as shown on Table 2.

3.3. Microscopic assessment of gametocyte carriage

No gametocytes were detected on blood smears using microscopy from day zero to 7 as described (Dinko et al., 2013), and no asexual parasites were observed in day 7 blood films from any of the SM patients.

3.4. Association of parasite clearance dynamics with patient characteristics

Given the sample size and small number of infections persisting beyond 48 h, the study was insufficiently powered to identify risk factors associated with slow parasite clearance. There was no evidence that children <5 years were likely to clear parasites slowly, since five of the ten children with qPCR positive results at 48 h post treatment were ≥ 5 years. The data suggest that a history of high malaria exposure rendered our patients less likely to clear in 48 h, with those children with 2 or more previous episodes of malaria (12/25) more likely to remain parasitaemia at 48 h than those with one or no previous episodes (13/25) (OR 3.33, 95% CI 0.46–27.5; $P = 0.16$). The ten children with parasitaemia persisting at 48 h were more likely to have Hb at enrolment under 10 g/dL, but this was not statistically significant (OR 2.5; 95% CI 1.31–4.79; Fisher's exact $P = 0.32$).

4. Discussion

This study shows that parasite clearance dynamics varied considerably among SM cases, and that 10 of 25 evaluable children failed to clear qPCR-detectable parasitaemia by 48 h. Further, of the nine children who were available for day 7 post-treatment follow-up, four had detectable *Plasmodium* DNA in their peripheral blood. Our proof-of-principle study was insufficiently powered to evaluate risk factors for slow clearance in this important patient group, but our *post hoc* exploratory analyses suggest that low Hb levels at presentation and a history of 2 or more previous episodes of clinical malaria might be associated with impaired parasite clearance after i.v. artesunate and ACT treatment. These factors should be rigorously assessed in larger studies.

Delayed parasite clearance after treatment, which has been associated with reduced parasite susceptibility to artemisinin *in vitro* mediated by certain *P. falciparum* gene variants (Ariey et al., 2014; Sutherland et al., 2020), may be an early indicator of loss of efficacy of artemisinin-based therapies. This would pose a particularly serious threat to the management of paediatric SM, the patient group which account for the majority of malaria mortality world-wide. Therefore, the ability to monitor clearance dynamics in this group with a simple daily venous blood sampling onto filter paper would be a useful addition to routine practice in tertiary hospital settings throughout sub-Saharan Africa. Platforms for *post hoc* qPCR analysis are now available in many African countries, and likely to become standard equipment in tertiary hospital laboratories following the SARS-CoV-2 pandemic. Systematic collection of such data would also provide something of an early warning system for any evidence of falling effectiveness in ACT treatment of uncomplicated outpatient malaria in primary health care settings, which is heavily reliant on the same artemisinin-based antimalarial combination drugs. Gametocytes persisting after therapeutic clearance of asexual stages of *P. falciparum* can also generate a qPCR signal. A study using microscopy to examine gametocyte clearance in both uncomplicated malaria and SM patients showed that although all participants were negative for asexual parasites on day 7 post-treatment with ACT, 13.1% and 8.2% were positive for gametocytes, respectively (Tangpukdee et al., 2008). We have previously demonstrated that a proportion of Kenyan children treated with ACT for uncomplicated *P. falciparum* malaria with persisting *P. falciparum* DNA by qPCR were gametocyte-positive at day 7, but this only accounted for a subset of persisting infections (Beshir et al., 2013). Future studies should include collection of RNA and RT-PCR detection of gametocytes, as we have previously described for patients attending hospitals in the Volta region (Dinko et al., 2018).

Two of the patients from the group who cleared qPCR-detectable parasitaemia by day 3 were known to be affected by sickle cell anaemia (SCA) (genotype SS). Homozygote SS patients with SCA experience fewer malaria episodes than other children, but are not protected from malaria as in heterozygous Ss patients. It is therefore thought that malaria infections might compound the severity of anaemia in homozygous SCA patients (Makani et al., 2010). Furthermore, SCA patients with SM often present with hyposplenism and impaired splenic function which might reduce effective parasite clearance (Luzzatto, 2012).

The simple qPCR sampling protocol presented here provides a useful platform for further investigation into the unique biological mechanisms occurring within this specific, highly vulnerable patient group when hospitalised with malaria parasite infections. Our data provide a useful proof-of-principle that this protocol could be effectively applied in larger studies. A weakness of our study is the passive nature of our sample set; the research team did not play a part in routine care, treatment decisions or hospital discharge decisions, and this should be addressed in larger studies.

5. Conclusion

The observation of variability in the parasite clearance dynamics among this small cohort of paediatric SM cases highlights a need to more closely evaluate parasitological recovery and its influence on patient prognosis. Our simple daily sampling protocol provides a basis for further investigation with a larger number of cases to better understand parasite clearance trends in SM patients, as well as the risk factors contributing to sub-optimal treatment response.

Authors' contributions

LP, KBB, CJS and BD designed the study protocol; LP, RT, JT and BD carried out the clinic liaison and sample collection; FNB, GAA and BCU contributed to study design, assisted with IRB submissions and provided academic support and supervision; LP, KBB and CJS performed the qPCR studies, and analysis and interpretation of these data. LP, BD and CJS drafted the manuscript; all authors read and approved the manuscript.

BD and CJS are guarantors of the paper.

Ethical approval

Patients were recruited under a sub-study renewal of the project protocol "Immunobiology of Sexual Stage Antigens of the *Plasmodium falciparum* Parasites", approved by the Ghana Health Service Ethical Review Committee (GHS-ERC: 03/09/2016) and the LSHTM Research Ethics Committee (#14967). Written informed consent was obtained for each individual, as set out in the main protocol. The study was conducted in accordance with the ethical standards of the Helsinki Declaration (2008) of the World Medical Association.

Funding

BD is supported by a Wellcome Trust Training Fellowship in Public Health and Tropical Medicine (110090/Z/15/Z).

CJS is supported by Public Health England and the UK Medical Research Council (Grant Ref: MR/T016124/1).

Data availability statement

The data underlying this article are available in the article.

Declaration of Competing Interest

The authors have no conflicts to declare.

Acknowledgements

We thank study participants, parents, caregivers and guardians for their co-operation and support. We would like to thank the authorities of the Ho Teaching Hospitals and the respective departmental health delivery staff for their support: Dr. Lord Mensah, Mr. Prosper Mensah and Edem Tsigbey. We are grateful for the expert assistance of Rupert Delimini, Comfort Agyare-Kwabi and Eli Osabutey.

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