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Antibody and *P. falciparum* Parasites Profiles during Clinical Malaria Episodes Following Artemisinin-Based Combination Therapy in Burkina Faso

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Abstract

Background: Artemisinin-based Combination Therapies (ACTS) are the first recommended drug for uncomplicated malaria treatment in many endemic countries. They are responsible for rapid parasites clearance and in reducing fever. Artemisinin has been found to have an immunosuppressive effect in animal's models. In the present study, we assessed the effect of ACTs on malaria antigens specific antibodies production during subsequent malaria episodes in a population living in malaria hyperendemic area.

Methods: In 2012, 371 patients with, presenting uncomplicated clinical malaria aged over 6 months and adults were recruited and allocated to receive ACTs and follow up for 2 years. Antibodies titers against three *P. falciparum* blood stage malaria vaccine candidates (MSP3, GLURP R0, and GLURP R2) were measured by ELISA during subsequent malaria episodes.

Results: Antibody concentration increased during subsequent malaria episodes for GLURP R0, and this was statistically significant. IgG to all tested antigens increased with age and this trend was maintained over all episodes.

Conclusion: Asexual *P. falciparum* densities were showing different trends and immune responses against certain erythrocytic antigens were boosted during subsequent malaria episodes.

Keywords: *P. falciparum*; Malaria; Parasites

Introduction

Malaria led to 219 million clinical malaria cases and 445 000 deaths all over the world in [1]. During the same period, 4784 151 and 677 350 uncomplicated clinical malaria cases were reported in children under 5 years and in pregnant women respectively in Burkina Faso. These data highlight the fact that malaria is still a public health problem in countries in sub-Saharan African. The control or elimination of malaria is problematic due to emerging parasite resistance to existing drugs and the resistance of malaria vectors to insecticides. For many decades, the treatment of uncomplicated malaria relied on chloroquine as the first-line drug. At the end of years the 90s, the unprecedented level of resistance of *Plasmodium falciparum* led to the introduction of Artemisinin-Based Combination Therapies (ACT) in many endemic countries. These treatments combine rapid acting artemisinin or one of its derivatives (i.e. artesunate, artemether, dihydroartemisinin) with another more slowly eliminated anti-malarial (e.g. amodiaquine, mefloquine, piperaquine) [2]. For *P. falciparum* the use of two or more drugs with different modes of action in combination has been recommended to provide adequate cure rate and delay the development of resistance. Many studies had proven the efficacy of ACTs on parasite and fever clearance. To assess the safety and efficacy of the repetitive use of ACTs, a study has been designed to compare the incidence rate of uncomplicated malaria episode in children and adults treated repeatedly with ACT therapy over a period of 2 years. In a three-arm study, Pyronaridine Artesunate (PA) and Dihydroartemisinin-Piperaquine (DHA-PQ) were compared to either Artesunate-Amodiaquine (ASAQ) or Artemether-Lumefantrine (AL) (depending on the site location). The study was designed as a comparative, randomized, multi center, open-label longitudinal clinical study to assess the safety and efficacy of repeated ACT therapy over a period of 2 years in uncomplicated *P. falciparum* malaria in children and adults [3-11].

In the sub-cohort conducted by Centre National de Recherche et de Formation sur le Paludisme (CNRFP) (Ouagadougou/Burkina Faso) carried out at the Banfora trial site and at Niangoloko sites, we profiled the IgG antibody responses to malaria-specific antigens, Merozoite Surface Protein 3 (MSP3), Glutamate Rich Protein (R0 and R2) [12-14] and measured *P. falciparum* asexual stage parasites by light microscopy at enrolment at each subsequent malaria episode.

Materials and Methods

Study area

This study was carried out at the Banfora and Niangoloko District trial site, amongst the most humid areas of Burkina Faso with annual rainfall above 900 mm per year. Both sites are located 30 km apart and 500 km from Ouagadougou, the capital of Burkina Faso. These two sites were chosen to evaluate the malaria incidence rate as well as the efficacy and safety of Pyronaridine Artesunate (PA), Dihydroartemisinin-Piperaquine (DHA-PQ) compared to Artesunate-Amodiaquine (ASAQ) when used repeatedly for consecutive clinical malaria episodes. Burkina Faso is an endemic malaria country characterized by a dry and rainy season, with stable, seasonal malaria. Peak transmission is observed during the rainy season between May and November (in Banfora and Niangologo). *P. falciparum* is the predominant malaria parasite, and the main vectors are *Anopheles gambiae* and *Anopheles funestus* [15].

Study design and population

The study volunteers were drawn from a cohort study carried out at the villages nears to the Banfora Regional Hospital trial site and at Niangoloko Medical Center. The cohort study was a comparative, randomized, multi-center, open-label longitudinal clinical study to assess the safety and efficacy of repeated ACT therapy over a period of 2 years in uncomplicated malaria in children and adults in Burkina Faso, Guinea and Mali. In this 3 arms study, PA and DHA-PQ were compared to either ASAQ. Patients were followed for 2 years starting from the first enrolment with the randomized study drug. For each treatment period, patients were followed for 42 days primarily for safety. For each treatment arm, the same ACT (PA, DHA-PQ or comparator) was given unless one of the reasons for the use of an alternative was met. Samples from a sub-cohort were selectively collected from the two sites to assess the antibody responses and the *P. falciparum* parasitemia asexual in subjects with uncomplicated malaria and treated with the study ACTs.

Prior to any study procedures, the study protocol including the sub-cohort study was approved by the Institutional Review Board of Centre National de Recherche et de Formation sur le Paludisme (Comité institutional de Bioéthique du Centre National de Recherche et de Formation sur le Paludisme-Ouagadougou-Burkina Faso) and Ministry of Health ethical committee for biomedical research.

Blood sampling

For immunological assessment, 4 mL of venous blood was collected from study volunteers at each malaria episode and the separated sera were stored at-40°C prior to antibody measurement. Finger prick blood was also collected at each episode to prepare thick and thin blood films.

Laboratory methods

Malaria diagnosis: Thick and thin blood smears were prepared and stained with 6% Giemsa and read by experienced microscopists for malaria diagnosis. The number of malaria parasites of each *Plasmodium* species and stage was recorded. Each slide was read by two independent technicians and the final result was the average of the two readings. A 100% of the qualitative agreement for the diagnosis was required for each slide between readers, and 30% difference for quantitative diagnosis was accepted between the two readers. A third reading was performed in case of significant discrepancy between the two readers. The number of parasites per µl of blood was calculated according to the leukocyte count obtained after the full blood count for each slide collected during the malaria transmission season. A slide was declared negative if no parasite was seen after 200 HPF (High Power Field) were examined.

Antibodies measurement: The levels of antibodies (total IgG) to the three malaria antigens (MSP3, GLURP R0, and GLURP R2) were measured using Enzyme-Linked Immunosorbent Assays (ELISA). Briefly, 96-well micro-ELISA plates (NUNC F96 Maxisorb, Roskilde, Denmark) were coated with each antigen at 0.5 µg/ml in Phosphate Buffered Saline (PBS) and incubated overnight at 4°C. After blocking at 37°C for one hour with blocking buffer (0.1%Tween20+5% non-fat skimmed milk in PBS), the plates were washed with washing buffer (NaCl+0.1% Tween20 in PBS). 50 µl/well of plasma samples diluted at 1/200 in serum dilution buffer (0.1% Tween20+2.5% milk in PBS) was added to the plates and incubated for 2 hours at 37°C. In each plate, positive and negative control plasma pools were added, as well as two blank wells with only PBS-Tween20 (1%) added. The plates were then washed, and 50 µl per well of peroxidase-conjugated goat anti-human IgG (1:80000), diluted in dilution buffer (0.1% Tween20+2.5% milk in PBS was added (Caltag, Camarillo, CA 93012 USA). After washing, the plates were developed with Diethanolamine Substrate buffer 1X+4-Nitrophenyl phosphate disodium hexahydrate tablet substrate and reactions stopped after 30 min by adding 50 µl of 0.2 M of sulfuric acid per well. Antibody levels, measured as Optical Density (O.D.) were determined using a Biotek Lx808 microplate reader (Winooski, Vermont 05404-0998 USA) at 450 nm with a reference at 620 nm. The OD values of the test-samples were converted into Arbitrary Units (AU) by means of extrapolation from a standard curve in each plate, obtained from 12 serial dilutions of both pools of negative and positive hyperimmune sera. Positive control plasma was obtained from positive Bukinabè adults above 20 years living in malaria hyper-endemic area, and negative control plasma was from Danish individuals never exposed to malaria from the Statens Serum Institute (Copenhagen, Denmark).

Statistical analysis: Data were double entered with Epi Info and analyzed with R. The study population was categorized by age groups to compare their differences in parasitological and immunological variables. Volunteers were categorized according to the number of uncomplicated malaria episodes. The geometric mean of *P. falciparum* parasitemia (sexual and asexual stages) was calculated for each episode group. The means of IgG responses were compared between the first episode and subsequent episodes. Statistical X^2 test was used to compare antibodies production. A value of $p<0.05$ was considered statistically significant.

Results

Study population characteristic

A total of 371 volunteers were enrolled in this immunological study, 162 were less than 5 years old, 124 were aged between 5 and 10 years and 85 were above 10 years old. The mean age was 8.8 years old in volunteers with one malaria episode. During the follow up 162, 94, 44, 42 and 29 volunteers did one, two, three, four and five malaria episodes respectively. Mean age decrease with the number of repetitive episodes. The younger patients experienced a high number of repetitive malaria episodes (Table 1).

Number of episode	Volunteers number	Mean age
01	162	8.8 (3.6-11.3)
02	94	7.7 (3.3-9.3)
03	44	6.8 (3.5-9.9)
04	42	6.3 (3.4-8.3)
05	29	5.1 (3.4-7.5)

Table 1: Population characteristics.

Relationship with age and number of malaria episode

Table 2 summarizes the number of uncomplicated malaria episodes by age category (<5 years; 5 to 10 years and >10 years). We observe that among our study participants below 5 years 39.5% (64/162) had one malaria episode. While in the same age group 24.1% (39/162) had two episodes, 15.4% (25/162), and 11.1% (18/162) had respectively four and five episodes. Children between 5 and 10 years of age, 41.1%, 27.4% (34/124), and 15.3% (19/124) did respectively 1, 2 and 3 episodes. The number of volunteers in this age group also decreased with an increasing number of the episode and 7.25% of this age group did 5 episodes. More than half of patients (55.3%; 47/85) above 10 years had only one episode. In this age group, the number of subjects also decreased with an increasing number of the episode. 7.1% of them did four episodes and only 2.3% (2/85) did five malaria episodes. In volunteers aged more than 10 years, the majority of volunteers did one episode comparatively to subjects aged less than five years (Table 2).

Number of episode	Volunteers number by age group			Total
	<5y	5-10 y	≥ 10y	
01	64	51	47	162
02	39	34	21	94
03	16	19	9	44
04	25	11	6	42
05	18	9	2	29
Total	162	124	85	372

Table 2: Volunteers by age groupe during subsequent episodes.

Parasitaemia evolution during subsequent episodes

At enrollment, the inclusion criteria for parasitemia was ≥ 1000 asexual stages of *P. falciparum*/µL of blood. The lowest geometric mean of parasitemia was recorded at the first malaria episode and the highest parasitemia was reported among the patients who had 3 episodes (Table 3). A decrease in the geometric mean of *P. falciparum* asexual stages was observed in subjects with 5 episodes.

Number of episode	Volunteers Number	Mean of parasitaemia (Tf) /microliter (95% CI)
01	162	6010 (4178-8647)
02	94	9208 (5972-14198)
03	44	20705 (10909-39299)
04	42	19976 (10635-37524)
05	29	7374 (3061-17766)

Table 3: Geometric mean of asexual stage (Tf) density during malaria subsequent episodes.

Antibodies level, age, and subsequent episodes

IgG to all tested antigens increased with age and this trend was maintained over all episodes this trend was maintained. Volunteers above 10 years had more antibodies than those below 10 years. Antibody responses were highest to GLURP R2, and lowest to MSP3 (Table 4).

Number of episodes	Age group	Mean IgG-MSP3 (95% CI) AU	Mean IgG-R0 (95% CI) AU	Mean IgG-R2 (95% CI) AU
1	<5 y	0.8 (0.6-1.1)	2.5 (1.9-3.4)	7.1 (7.4-9.3)
	5-10 y	0.7 (0.5-1.1)	3.5 (2.5-5.0)	6.1 (4.4-8.5)
	>10 y	2.2 (1.5-3.3)	7.0 (4.8-10.4)	11.2 (8.5-14.8)
2	<5 y	0.6 (0.4-0.8)	4 (3-5.3)	6.7 (4.5-9.9)
	5-10 y	0.8 (0.5-1.1)	6.3 (4.8-8.4)	8.2 (6.1-11.0)
	>10 y	2.7 (1.5-4.8)	10 (6.8-14.5)	20.3 (12.3-33.4)
3	<5 y	0.4 (0.3-0.7)	2.5 (2-3.2)	7.3 (4.8-11.0)
	5-10 y	0.6 (0.5-0.9)	5.2 (3.8-7.1)	8.5 (6.4-11.4)
	>10 y	2.6 (1.4-4.9)	17 (12.3-23.4)	28 (17.6-44.5)
4	<5 y	0.6 (0.5-0.7)	4 (3.3-4.7)	5.1 (4-6.5)
	5-10 y	0.9 (0.5-1.5)	7.5 (5-11.2)	9 (5.6-14.4)
	>10 y	2.2 (1.3-3.6)	12.6 (7.4-21.7)	32.0 (21.1-48.4)
5	<5 y	0.4 (0.3-0.6)	6.7 (4.8-9.4)	4.2 (3.1-5.6)
	5-10 y	1.1 (0.7-1.8)	10.0 (6.4-15.7)	9.3 (5.6-15.5)
	>10 y	3.5 (2.4-5.0)	18.9 (9.8-36.3)	23.9 (14.7-38.7)

Table 4: Geometric mean of antibodies production during subsequent episode based on age.

GLURP R0 concentration doubled between episode 1 (3.8 AU) and episode 2 (5.89 AU) (p=0.01). Antibody concentration increased during subsequent malaria episodes for GLURP R0, and this was statistically significant starting from 3.8 AU at episode 1 to 8.83 AU at episode 5 (Table 5). IgG against GLURP R2 also increased with subsequent episodes and this was statistically significant between episode 1 (7.7 AU) and episode 3 (10.4 AU) (p=0.04) (Table 4). However, no changes in MSP3 IgG were observed between subsequent episodes (Table 5).

Antigens	Number of episodes	Mean IgG (95% CI) AU	Mean IgG-E1 AU	p-value
Glurp Ro	2	5.9 (4.9-7.1)	3.8 (2.6-4.9)	0.001
	3	5.2 (4.2-6.4)		0.03
	4	6.1 (5.0-7.4)		0.0008
	5	8.8 (6.9-11.4)		3.9 10-7
Glurp R2	2	9.6 (7.7-11.9)	7.7 (5.8-9.6)	0.13
	3	10.4 (8.3-13.1)		0.041
	4	8.7 (6.9-10.9)		0.41
	5	6.9 (5.3-9.0)		0.49
MSP3	2	0.9 (0.7-1.2)	1.1 (0.9-1.4)	0.5
	3	0.8 (0.6-1)		0.18
	4	0.8 (0.7-1.1)		0.16
	5	0.8 (0.6-1.0)		0.114

Table 5: Geometric mean of IgG against tested antigens during malaria subsequent episodes.

Treatments arms during subsequent episodes

In total 150 volunteers were treated with ASAQ, 101 with DHA-PQ and 120 with PA. In ASAQ treated patients 52% (78/150) had one episode, 24.6% (37/150) had two episodes. The percentage of volunteer experiencing repeated episodes decreased with each treatment, and only 5.3% (15/150) of ASAQ treated patients had 5 episodes. The same trend was observed with DHA-PQ and Pyramax treated patients (38.6% at one episode, 24.7% at two episodes, 6.9% at five episodes, 37.9% at one episode, 26.6% at two episodes and 11.6% at five episodes respectively). While comparing ASAQ to DHA-PQ or ASAQ to PA the number of patients with at least four uncomplicated malaria episodes increased in PA and DHA-PQ arms compare to ASAQ arm (Table 6).

Number of episodes	Volunteers treated with ASAQ	Volunteers treated with DHA-PQ	Volunteers treated with PA
1	78	39	45
2	37	25	32
3	16	15	13
4	11	15	16
5	8	7	14
Total	**150**	**101**	**120**

Table 6: Volunteers and their treatment during the subsequent episode.

Antibodies responses during subsequent episodes and treatment arms

Among the three treatment arms and malaria episodes, no change was observed for MSP3 antibody responses. However, in the ASAQ treatment arm, the antibody responses against GLURP R0 and R2 were increasing with the number of malaria episodes. The antibody arbitrary units in participants with 5 episodes were approximately the double of that measured at children of 1 episode. A similar trend was observed for R0 in PA arm. R2 antibody responses in DHA-PQ and PA arms were waning; no clear trends were observed (Table 7).

Treatment	Number of episodes	Abs to MSP3 (95% CI) AU	Abs to R0 (95% CI) AU	Abs to R2 (95% CI) AU
ASAQ	01	1.0 (0.7-1.4)	3.7 (2.7-5)	7.3 (5.6-9.5)
	02	(0.8-1.6)	7 (5.1-9.5)	12.1 (8.4-17.2)
	03	0.6 (0.4-0.8)	4.5 (3.2-6.3)	6.0 (4.1-8.8)
	04	1.5 (0.8-2.8)	10.6 (7.1-16)	10.7 (6.3-18.1)
	05	1.2 (0.7-2.1)	9.5 (6-15)	12 (7.618.7)
p		0.5	0.1	0.4
DHA-PQ	01	1.3 (0.7-2.1)	4.1 (2.7-6.2)	7.7 (5.6-10.6)
	02	1.0 (0.6-1.8)	5.1 (3.7-6.9)	9.2 (6-14.5)
	03	1.0 (0.6-1.7)	6.8 (4.6-10.2)	15.3 (10.4-22.6)
	04	0.6 (0.5-0.9)	4.1 (3-5.6)	7.1 (5.1-10)
	05	0.5 (0.3-0.7)	5.9 (3.8-9.3)	4.3 (2.4-7.6)
p		0.007	0.6	0.5
PA	01	1.0 (0.7-1.4)	3.8 (2.6-5.4)	8.5 (6.1-11.8)
	02	0.7 (0.5-1.1)	5.5 (4-7.5)	7.5 (5.3-10.8)
	03	0. 8 (0.5-1.2)	4.6 (3.2-6.5)	13 (8.8-19.2)
	04	0.8 (0.5-1.)	6 (4.5-8)	9.2 (6.3-13.3)
	05	0.8 (0.6-1.2)	10.3 (7-15.4)	6.5 (4.4-9.5)
p		0.5	0.07	0.8

Table 7: Relationship between treatment and subsequent episodes.

Parasitaemia evolution according to treatment during subsequent episodes

In patients treated with ASAQ, the lowest geometric mean of parasitemia was observed at the first malaria episode and the highest parasitemia was reported among the patients who had 3 episodes (Table 8). A decrease in the geometric mean of *P. falciparum* asexual stages was observed in subjects with 4 and 5 episodes. However in patients treated with PA, parasitemia increase from to one at four malaria episodes but not significantly. In patients treated with DHA-PQ, parasitemia increased from significantly to one to at five malaria episodes with a borderline significance (p=0.05).

	Number of episodes	Treatment with ASAQ	Treatment with DHA-PQ	Treatment with PA
Parasitaemia/microliter (95% CI)	1	5967 (3551-10027)	4944 (2123-11513)	7207 (3707-14009)
	2	10665 (5420-20986)	7999 (3601-17766)	8671 (3669-20488)
	3	44907 (28589-70538)	14586 (4053-52491)	11961 (2284-62622)
	4	33546 (8325-135176)	10448 (2560-42640)	25681 (13048-50543)
	5	21473 (5248-87860)	21855 (4130-115655)	2325 (585-9239)
	P	0.4	0.05	0.8

Table 8: Parasitaemia according to treatment and subsequent episodes.

Discussion and Conclusion

In the present paper, the profile of total IgG responses against three blood stage antigens (MSP3, GLURP-R0, and GLURP-R2) and asexual *P. falciparum* parasite density was investigated. The patients who presenting uncomplicated malaria patients were enrolled from a randomized clinical trial with treated with the same ACTs for the first and the subsequent malaria episode for a period of two years. The South-Western of Burkina Faso which is characterized as malaria stable and hyperendemic area was the study site. The malaria transmission in that part of Burkina Faso is longer and intense compare to the central part of the country where malaria transmission is described as less long. During the follow-up period, the maximum number of malaria episodes in the sub-cohort reached 5 while more than half of enrolled volunteers experienced at least two episodes. These data confirm that malaria is still a public health problem in Burkina Faso [16]. Looking at the distribution of the subsequent malaria episodes, younger patients were still suffering the highest burden of malaria [17,18]. Older patients experienced fewer malaria episodes when compared to children. These findings are consistent with previous observations that long term exposure leads to the acquisition of immunological responses which help to control malaria parasites load when they are infected and prevent them of developing clinical malaria [17,19,20]. Despite the treatment with ACTs a substantial number of study participants experienced up to 5 episodes, above half of them were among less than 5 years old children. These observations raise a few issues: 1) those experiencing many episodes may be more exposed to mosquitoes bites and then to infection and clinical attacks compare to their counterparts; 2) treatment may increase susceptibility to malaria infection and clinical malaria episodes as reported by Ouédraogo et al., in Burkina Faso [21] and 3) susceptibility may increase because of the carriage of some genetics factors [22] (not assessed in this study) or socio-cultural behaviors The parasite density was high in participants who experienced more than one episode confirming the hypothesis that parasite cure with ACTs may induce the susceptibility to malaria [23,24]. This could potentially be due to the ACT treatment eliminating parasites quickly and preventing the development of naturally acquired immunity [25].

The kinetics of antibody responses during the subsequent malaria episodes throughout the study has different profiles. As the number of episodes increased, IgG to R0 also increased, while no change was observed for MSP3. IgG to R2 increased between one to three episodes and started declining at four and five episodes. Similarly, antibody responses to *P. falciparum* erythrocytic antigens declined after treatment in children treated with Seasonal Malaria Chemoprophylaxis (SMC) [23] while no significant modification in antibody levels was observed in the first 2 years of life [26]. It clearly appears that the subsequent malaria episodes had a boosting effect on IgG responses to R2 antigens. Each episode is a booster to the pre-existing antibody responses to R2. Within each subsequent malaria episodes, we observed a parallel increase of antibody responses with age and a subsequent number of episodes for all antigens tested. Similar findings were reported earlier by Nebie et al. [27] in central Burkina Faso where levels of IgG to MSP3 and GLURP increased with increasing age, most steeply in the case of R0 [27]. There is no clear evidence from the data generated that ACTs boosted or reduced the antibody responses. A control group with untreated asymptomatic volunteers would be needed to determine the effect of treatment on antibody development. In a previous study, it was reported that treatment acts in synergy with pre-existing antibody to clear malaria parasites [28-30]. Artemisinin derivatives were reported in the mouse model to have an immunosuppressive action by affecting inflammation and autoimmunity [31]. In this study, inflammatory and immunoregulatory functions were not assessed so the immunosuppressive effect of artemisinin could not be determined.

In conclusion, our study has reported multiple episodes of malaria mainly in the younger less immune enrolled participants. Asexual *P. falciparum* loads were showing different trends and immune responses against certain erythrocytic antigens were boosted during subsequent malaria episodes.

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