COMPARATIVE ANTIBODY RESPONSES AGAINST THREE ANTIMALARIAL VACCINE CANDIDATE ANTIGENS FROM URBAN AND RURAL EXPOSED INDIVIDUALS IN GABON

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The analysis of immune responses in diverse malaria endemic regions provides more information to understand the host's immune response to *Plasmodium falciparum.* Several plasmodial antigens have been reported as targets of human immunity. PfAMA1 is one of most studied vaccine candidates; PfRH5 and Pf113 are new promising vaccine candidates. The aim of this study was to evaluate humoral response against these three antigens among children of Lastourville (rural area) and Franceville (urban area). Malaria was diagnosed using rapid diagnosis tests. Plasma samples were tested against these antigens by enzyme-linked immunosorbent assay (ELISA). We found that malaria prevalence was five times higher in the rural area than in the urban area ($p < 0.0001$). The anti-PfAMA1 and PfRh5 response levels were significantly higher in Lastourville than in Franceville ($p < 0.0001$; $p = 0.005$). The anti-AMA1 response was higher than the anti-Pf113 response, which in turn was higher than the anti-PfRh5 response in both sites. Anti-PfAMA1 levels were significantly higher in infected children than those in uninfected children ($p = 0.001$) in Franceville. Anti-Pf113 and anti-PfRh5 antibody levels were lowest in children presenting severe malarial anemia. These three antigens are targets of immunity in Gabon. Further studies on the role of Pf113 in antimalarial protection against severe anemia are needed.

Keywords: P. falciparum, antibodies, vaccine candidates, Pf AMA1, Pf113, PfRh5, Gabon

Introduction

Plasmodium falciparum is responsible for most of the malaria-related deaths and accounts for more than 25% of children deaths in Africa [1]. The clinical manifestations of malaria are due to the repeated cycles of replication of the asexual parasite *P. falciparum* in the host's red blood cells. Antigens presented by the asexual parasite stages in the bloodstream are critical in the development of protective immunity to the disease. In the course of malarial infection, red blood cells (RBC) are invaded by the merozoite during a very short moment through a complex multistep process. It begins with an initial attachment of the merozoite to the RBC surface via several protein–protein interactions, followed by an apical reorientation of the merozoite, a tight junction formation between the parasite and the host cell, and the final entry of the merozoite into the RBC [2–5]. Hence, any intervention that could block

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this multistep process could lead to the control of malaria parasite replication in RBC. The passive transfer of immunoglobulins from immune adults into *P. falciparum*-infected individuals has provided strong evidences that antibodies (Abs) play an important role in mediating protective immunity [6]. This indicates that the induction of appropriate antibody responses could be an important element in finding a way for an efficient vaccine strategy. Thus, the identification of *P. falciparum* antigens containing epitopes that are targets of naturally acquired immunity is important for the design of a vaccine. The *Plasmodium* parasite genome encodes over 5,000 proteins, a mere handful of which have been identified as candidate vaccine components [7]. More than 40 merozoite proteins involved in invasion have been identified, most of which have been shown to be targets of acquired immunity. Several of these are in early-stage clinical evaluation, making this an exciting time for the field. Among these antigens are PfAMA1, PfRh5, and Pf113.

The *P. falciparum* apical membrane antigen 1 (AMA1) is a membrane protein present in most apicomplexan parasites including all the *Plasmodium* species sequenced to date, *Toxoplasma gondii,* and *Babesia divergens* [8, 9]. PfAMA1 is a structurally conserved type I integral membrane protein (622 amino acids closely related to chimpanzee malaria, *Plasmodium reichenowi*) [10]. PfAMA1 is an important target and leading vaccine candidate which is presently being tested in clinical trials [11]. Antibodies to PfAMA1 are found in most people exposed to malaria, with the prevalence of antibody positivity increasing with age [12], and antibodies to PfAMA1 have been associated with reduced risk of clinical malaria in prospective studies [13, 14].

P. falciparum reticulocyte-binding protein homolog 5 (PfRH5) is a member of the family of PfRh invasion ligand recently identified among merozoite protein that is located in the rhoptries [15], secreted onto the merozoite surface prior to RBC invasion, and binds basigin, a RBC protein [16]. In contrast with other malarial antigens involved in invasion, PfRh5 exhibits a limited genetic diversity. *In vitro* studies have identified PfRH5 as the highest priority target in the blood-stage malaria vaccine field over the last decade [17].

The *P. falciparum* 113 antigen (Pf113) is thought to be located at the *P. falciparum* merozoite surface, a favorable location to interfere with the erythrocyte surface during RBC invasion [18]. The malarial adhesins and adhesin-like proteins predictor (MAAP) classifies Pf113 as an adhesin together with other well-characterized GPI-anchored RBC-binding proteins like MSP1, MSP2, and MSP10 [18]. Furthermore, it has been associated with protection from symptomatic malaria in Papua New Guinea (PNG) and Kenya [19]. Moreover, cumulative responses to combinations comprising 5 of the 10 top-ranked antigens, including Pf113, were associated with 100% protection against clinical episodes of malaria [20], suggesting that Pf113 is a promising candidate in the malaria vaccine development pipeline.

Gabon is an endemic area in which the malaria burden fluctuates according to the living area. After the implementation of artemisinin-based combination therapies (ACTs) in 2005, a decrease of malaria prevalence was observed, but for the past few years, a recrudescence appeared in the urban areas of Franceville and Libreville. However, prevalence did not change in rural areas [21], suggesting different profiles of epidemiology. It has been reported that ACT interventions induced a loss of acquired immunity [22] which could explain why differential epidemiology profiles exist between rural and urban areas.

Franceville is the third largest town in Gabon, with a good economic development. In 2011, a study estimated malaria prevalence among the febrile children at 17.9% [23], and a more recent study estimated the prevalence of malaria at 22.1% in febrile children [24].

The city of Lastourville had an estimated population of 8531 inhabitants in 2006. The city has a poor economic development, few industries are present. Lastourville is surrounded by forest and crossed by several rivers, among which the main river of Gabon: the river Ogooué. Malaria transmission is high year-round in Lastourville; a recent study estimated the prevalence of *Plasmodium* infections at 79.5% in children aged 6 to 168 months [24].

The main objective of the present study was to evaluate the naturally acquired antibody responses to three recombinant proteins of *P. falciparum* (Pf113, PfAMA1, and PfRh5) among *P. falciparum*-infected malaria subjects in a rural area versus an urban area in Gabon.

Materials and methods

Study area and population

This study was conducted at the Amissa Bongo regional hospital of Franceville and at the medical center of Lastourville.

Franceville is an urban region of south-east Gabon (1° 37′ 15″ S, 13° 34′ 58″ E) of 56,002 inhabitants (estimated in 2010), and Lastourville is a rural region of eastern central Gabon (0° 49′ S, 12° 42′ E), the capital of the Mulundu department. The city of Lastourville had an estimated population of 8531 inhabitants in 2006. During 2015, children reporting to the pediatric ward aged 0 to 156 months were included in the study.

Blood samples

Venous blood (2.0–5.0 ml) was collected into ethylenediaminetetraacetic acid (EDTA)-containing vacutainer tubes. Of the 210 children included in this study, 104 were from Franceville and 106 were from Lastourville. Blood smears were stained with Giemsa according to the Lambaréné method for microscopic *P. falciparum* identification and quantification [25]. All slides were examined by two well-trained

microscopists from the Centre International de Recherches Médicales de Franceville and two well-trained microscopists from the Centre Médical de Lastourville. Blood samples were centrifuged, and plasma samples were aliquoted and stored at −80 °C until use.

Antigens

The *P. falciparum* apical membrane antigen (PfAMA1), *P. falciparum* reticulocyte-binding homologue (PfRh5), and *P. falciparum* 113 (Pfl13) were evaluated in this study. PfAMA1 and PfRh5 were provided by the Welcome Trust Institute of London. Recombinant *P. falciparum* proteins were expressed by transient transfection of HEK293 cells from the 3D7 strain using expression plasmids described in Crosnier et al. [26]. Recombinant PfRH5 was processed when expressed in HEK293 media supplemented with fetal calf serum; this processing was reduced by using HEK293 cells adapted to serum-free media and was prevented, when necessary, by the addition of 2 to 10 µg/ml aprotinin. Briefly, all plasmids were chemically synthesized using codons optimized for expression in human cells, potential N-linked glycosylation sequons were mutated, and a C-terminal rat Cd4d3+4 tag was appended. The monomeric "bait" proteins were enzymatically mono-biotinylated by cotransfection with a plasmid encoding a secreted BirA enzyme as described in Bushell et al. [27]. Proteins were purified from supernatants using an ÄKTA Express or ÄKTA pure (GE Healthcare).

A recombinant form of Pfl13 was produced in the course of this study. Briefly, a 616 amino acid domain of the Pfl13 protein devoid of GPI anchor signal (D283-E899) was chosen to be expressed. The nucleotide sequence encoding for this Pfl13 domain was amplified by PCR by using total FcB1 genomic DNA prepared as previously described, as template [28]. The PCR product was subcloned downstream of the *trc* promoter in plasmid pTrCHis2B, with in frame fusion to a C-terminal poly-Histidine (6xHis) tag for rapid purification affinity chromatography on His GraviTrap column and detection with an anti-His antibody (Sigma-Aldrich). This Pfl13 recombinant domain was expressed in *Escherichia coli (E. coli)* Rosetta 2 DE3, purified from supernatants on HisTrap HP columns using an ÄKTA pure (GE Healthcare). Purified recombinant Pfl13 was migrated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred on nitrocellulose membranes for immunoblot staining, using anti-His tag antibodies (Sigma-Aldrich, St. Louis, USA).

Measurement of antibody levels

Enzyme-linked immunosorbent assay (ELISA) was performed to evaluate levels of malaria-specific antibodies from the patients' plasma. Immunosorbent plates (Nunc-MaxiSorp®, Thermo Scientific) were coated with each antigen (5 ng/µl for PfAMA1, 20 ng/µl for PfRh5, or 15 ng/µl for Pfl13), diluted in phosphate-buffered saline (PBS) in coating buffer overnight at 4 °C or 3 h at 37 °C, and then washed four times with PBS (pH 7.4) containing 0.05% Tween 20 (PBS-T). The plates were blocked with PBS containing 3% skim-milk for 1 h at 37 °C and washed four times with PBS-T. Plasma samples were diluted 1:100 in PBS containing 3% skim-milk, added in triplicate to microtiter plates (100 µl/well), and incubated at 37 °C for 2 h. The plates were then washed four times in PBS-T and incubated with peroxidase-conjugated mouse anti-human antibodies (Sigma-Aldrich, St. Louis, USA) diluted to 1:15,000 in PBS with 1% skim-milk for 1 h at room temperature. The plates were washed four times with a PBS-T washing solution. Tetramethylbenzidine substrate (TMB) solution (Thermo-Scientific, USA) was added to each well and incubated for 30 min. The reaction was stopped with 25 µl of 1 M sulfuric acid (H_2SO_4) per well. The plates were read at 450 nm with microplate reader (Sunrise™, Tecan). During the analyses, a pool of ten (10) sera from non-exposed donors, negative for *P. falciparum* infections, was used as negative controls and the washing solution PBST was used as a blank control. Microtiter plates were read using Sunrise™, Tecan at 450 nm. Test was considered positive if the sample optical density (OD) mean was $\geq OD_{450}$ mean + 3 SD of negative controls (non-exposed donors). Antibody levels were expressed in arbitrary units (AU), where an AU of 0 is defined as the mean optical density (OD) value plus 3 standard deviations for ten malaria-naive European donors. Individuals with antigen specific AU ≤ 0 or AU > 0 were defined as non-responders or responders to that antigen, respectively. Each sample was tested in triplicate.

Statistical analysis

To compare multiple groups of data, the non-parametric Kruskal–Wallis test was used. The Mann–Whitney *U* non-parametric test was used to test for differences in variables between two groups. The association of antibody levels with malaria protection was evaluated by logistic regression. Thus, the analysis was performed including age and antibodies showing an association or trend toward association with different groups in univariate analysis. $p < 0.05$ was considered as indicating a significant association. All statistical analyses were performed using SPSS version 17.0 for windows (SPSS Inc., Chicago, USA).

Ethic statements

The parents or legal guardians gave their written informed consent before each child's enrollment in the study, which was approved by the Gabonese National Research Ethics Committee. This study was authorized by the Ministry of Health and has received the agreement of the National Ethics Committee on Research (authorization number: PROT N°0023/2013/SG/CNE).

Results

Clinical and biological characteristics of the study population

Table 1 summarizes the main characteristics of all children recruited for this study. A total of 210 children aged between 6 and 156 months were included: 71 children had *P. falciparum* infection (12 [11.5%] from Franceville and 59 [55.7%] from Lastourville), and 139 were free of malaria parasites (92 from Franceville and 47 from Lastourville).

In the rural area, mean parasitemia was 4529 ± 515 parasites/µl. Infected children were older (65.17 ± 5.3 months) than uninfected children (48.3 ± 5.9 months, $p = 0.012$). Infected children had significant lower hemoglobin levels (9.14 ± 0.25 g/dl) than uninfected children (10.5 ± 0.24 g/dl), $p < 0.0001$. Red blood cell [(3.88 ± 0.12) · 10^6 cells/mm^3] and platelet [(1.54 ± 0.14) · 10^5 cells/mm^3] counts were also significantly lower in infect-

ed children than in uninfected children [(4.6 ± 0.14) · 10^6 cells/mm^3 and (3.17 ± 0.2) · 10^5 cells/mm^3, respectively, $p < 0.00001$]. There was no significant difference in the white blood cells counts between infected children [(8.95 ± 0.62) · 10^3 cells/mm^3] and uninfected children [(10.1 ± 1.22) · 10^3 cells/mm^3, $p = 0.38$].

However, in the urban region of Franceville, high levels of parasite density were found (mean of $57,727 \pm 31,103$ parasites/µl), infected children were also older (70.15 ± 10.56 months) than uninfected children (46.7 ± 4.6 months, $p = 0.016$). Infected children had significantly lower white blood cell [(6.75 ± 0.75) · 10^3 cells/mm^3] and platelet [(2.04 ± 0.35) · 10^5 cells/mm^3] counts than uninfected children [(10.7 ± 0.86) · 10^3 cells/mm^3, (4.24 ± 0.77) · 10^5 cells/mm^3, $p = 0.036$ and $p = 0.015$, respectively]. There was no difference regarding hemoglobin levels and red blood cell counts between the two clinical groups. The parasite densities found in Franceville ($57,727 \pm 31,103$ parasites/µl) were stronger than in Lastourville (4529 ± 515 parasites/µl, $p = < 0.00001$, *Table 1*).

Table 1. Demographical and hematological parameters of included patients (mean ± SD)

	Lastourville children			Franceville children		
	Infected ($n = 59$)	Uninfected ($n = 47$)	p	Infected ($n = 12$)	Uninfected ($n = 92$)	p
Age (months)	65.17 ± 5.3	48.36 ± 5.9	0.012	70.15 ± 10.56	46.71 ± 4.59	0.016
Haemoglobin (g/dl)	9.14 ± 0.25	10.5 ± 0.24	<0.0001	11.58 ± 0.4	11.35 ± 0.3	0.9
Red blood cells (10^6 cells/mm3)	3.88 ± 0.12	4.6 ± 0.14	<0.00001	3.97 ± 0.15	3.86 ± 0.12	0.6
White blood cells (10^3 cells/mm^3)	8.95 ± 0.62	10.1 ± 1.22	0.38	6.75 ± 0.75	10.7 ± 0.86	0.036
Platelets (10^5 cells/mm^3)	1.54 ± 0.14	3.17 ± 0.2	<0.000001	2.04 ± 0.35	4.24 ± 0.77	0.015
Parasitemia (parasites/µl)	4529 ± 515			57727 ± 31103		

Age, leucocytes counts, hemoglobin concentrations, and parasite densities in uninfected and infected children. *Plasmodium falciparum*-exposed children negative for parasites in thick blood smears for *P. falciparum* were defined as uninfected children. SD = standard deviation

Table 2. Distribution of malaria-specific PfAMA-1, Pf113, and PfRh5 antibodies according to age in Franceville and Lastourville children

	ALMA-1			Pf113			Rh5		
	Franceville	Lastourville	p	Franceville	Lastourville	p	Franceville	Lastourville	p
0–6 months	0.17 ± 0.13	1.47 ± 0.64	0.048	0.12 ± 0.06	0.38 ± 0.21	n.s.	0.1 ± 0.1	0.45 ± 0.43	n.s.
7–24 months	0.14 ± 0.006	0.37 ± 0.17	n.s.	0.072 ± 0.015	0.068 ± 0.023	n.s.	0.051 ± 0.023	0.061 ± 0.024	n.s.
25–60 months	0.31 ± 0.14	0.56 ± 0.16	n.s.	0.042 ± 0.012	0.065 ± 0.015	n.s.	0.045 ± 0.026	0.1 ± 0.025	n.s.
61–108 months	0.24 ± 0.12	0.88 ± 0.22	0.004	0.09 ± 0.029	0.19 ± 0.048	n.s.	0.01 ± 0.006	0.13 ± 0.049	0.006
109–156 months	0.58 ± 0.26	1.04 ± 0.35	n.s.	0.056 ± 0.024	0.12 ± 0.09	n.s.	0.21 ± 0.08	0.058 ± 0.04	n.s.

n.s.: not significant

Anti-PfAMA1, Pf113, and PfRh5 IgG responses according to living area and age in children

Antibody responses against PfAMA1, Pf113, and PfRh5 were assessed in the plasma samples from Lastourville and Franceville children. When analyzing the data between the two areas, the Lastourville children had higher levels of both anti-PfAMA1 (0.67 ± 0.1 AU) and anti-PfRh5 (0.1 ± 0.02 AU) antibodies compared to the Franceville children (0.27 ± 0.06 AU for PfAMA1, 0.065 ± 0.02 AU for PfRh5; $p < 0.0001$ and $p = 0.006$, respectively; *Fig. 1A* and *C*). However, no such difference was seen for anti-Pf113 between the two living areas ($p > 0.05$, *Fig. 1B*). When comparing infected and uninfected children from Lastourville, there was no significant difference for the three antigens between these groups (*Fig. 2A, B,* and *C*). However, in Franceville, infected children had significant higher levels of anti-PfAMA1 antibodies (0.72 ± 0.3 AU) compared to uninfected Franceville children (0.21 ± 0.05 AU; $p = 0.001$, *Fig. 2D*). No such difference was seen for anti-Pf113 and anti-PfRh5 antibody levels between these groups in the Franceville population (*Fig. 2E* and *F*).

When analyzing the data according to age categories, the Lastourville children aged 0 to 6 months exhibited significant higher titers of anti-PfAMA1 antibodies (1.47 ± 0.64 AU) as compared to the Franceville children of the same age group (0.17 ± 0.13 AU; $p = 0.048$, *Table 2*). The same differences were also found when comparing the children aged 61 to 108 months from the two localities for anti-PfAMA1 antibodies (0.88 ± 0.22 AU for Lastourville, 0.24 ± 0.12 AU for Franceville, $p = 0.004$) and for anti-PfRh5 antibodies (0.13 ± 0.049 AU for Lastourville, 0.01 ± 0.006 AU for Franceville; $p = 0.006$, *Table 2*). We used logistic regression to assess the associations between anti-PfAMA1, Pf113, PfRh5 specific antibody titers, age, and clinical malaria. Anti-PfAMA1 ($p = 0.017$) and age ($p = 0.007$) were associated with protection of malaria. An increase in anti-PfAMA1 levels was associated with a 0.1-fold (CI 0.02–0.16) decrease in the risk of malaria (data not showed).

When comparing children from Lastourville, anti-PfAMA1 and anti-Pf113 responses were significantly different between age groups ($p = 0.016$ and 0.024, respectively, *Fig. 3A* and *3B*). The highest responses against PfAMA1 (1.47 ± 0.6 AU) and Pf113 (0.38 ± 0.2 AU) were found in children aged under 6 months and then it decreased in children aged from 7 to 24 months (anti-PfAMA1 = 0.37 ± 0.17 AU and anti-Pf113 = 0.07 ± 0.02 AU; $p = 0.03$ and $p = 0.013$, respectively). When comparing different age groups together for the response to the same antigen, the children aged under 6 months had higher anti-Pf113 titers (0.38 ± 0.2 AU) compared to the children aged 25–
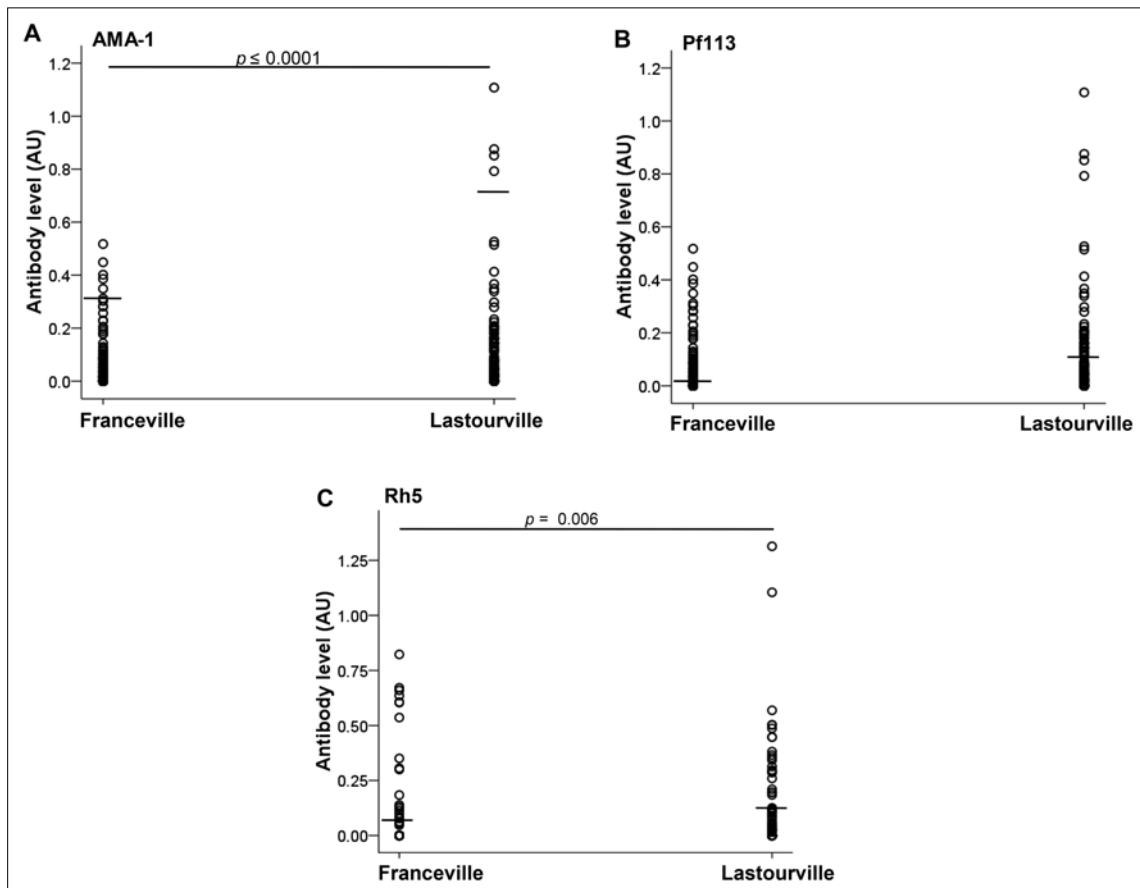

Fig. 1. Malaria-specific PfAMA-1, Pf113, and PfRh5 antibodies in Franceville and Lastourville children. Blood plasma levels from 210 children were analyzed for malaria-specific Pf AMA-1 (A), Pf113 (B), and PfRh5 (C) antibodies. The children were subdivided according to the life area
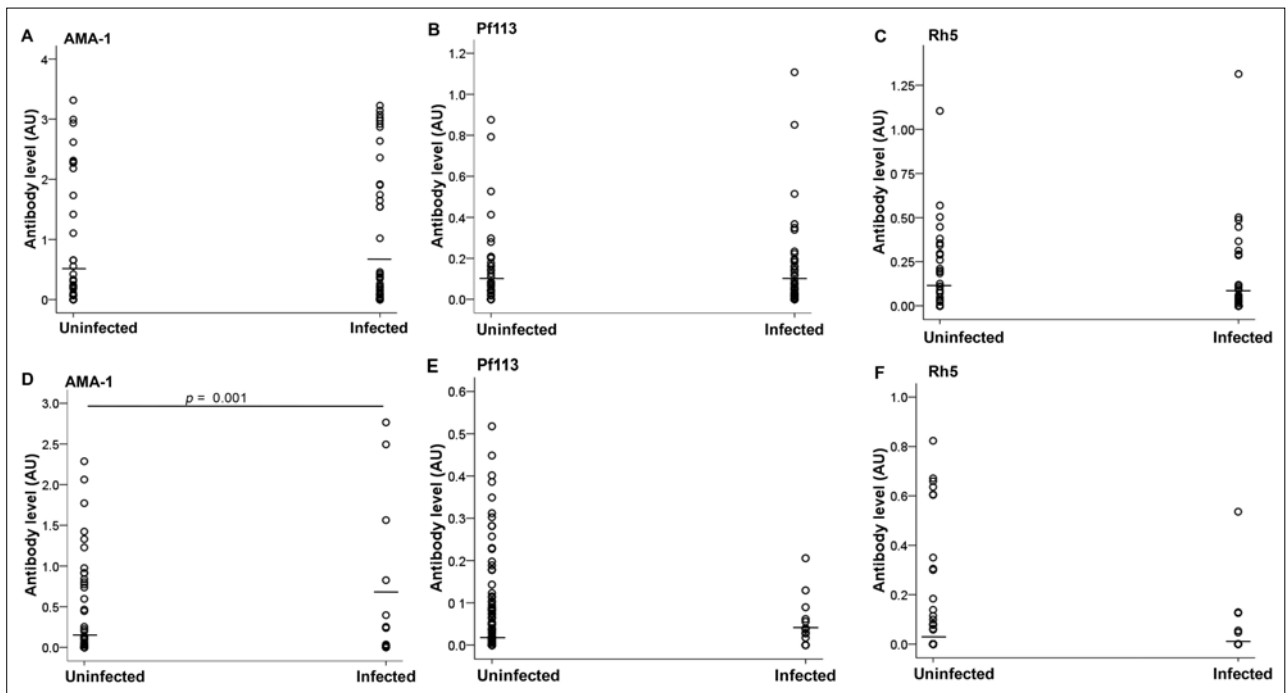
Fig. 2. Distribution of malaria-specific PfAMA-1, Pf113, and PfRh5 antibodies in response to *Plasmodium falciparum* exposition in Franceville and Lastourville children. Plasma concentrations of anti-PfAMA-1 (A), anti-Pf113 (B), and anti-PfRh5 (C) in Lastourville children and anti-PfAMA-1 (D), anti-Pf113 (E), and anti-PfRh5 (F) in Franceville children were quantified in *P. falciparum*-uninfected and -infected children
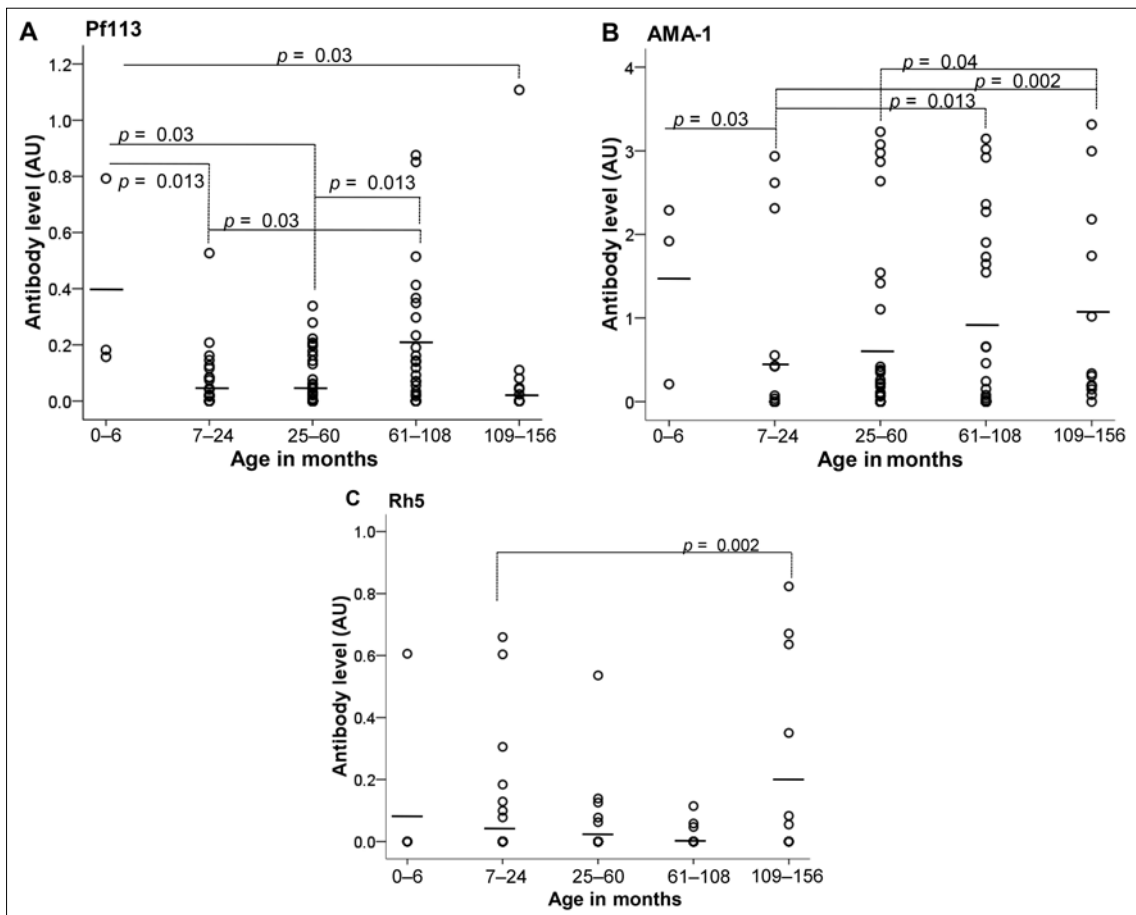

Fig. 3. Levels of malaria-specific PfAMA-1, Pf113, and PfRh5 antibodies in Franceville and Lastourville children according to age. Plasma concentrations of anti-Pf113 (A) and anti-AMA-1 (B) in Lastourville children and anti-Rh5 (C) in Franceville children were quantified. *P. falciparum*-exposed infants negative for parasites in blood smears for *P. falciparum* were uninfected children

60 months (0.065 ± 0.014 AU) and those aged 109–156 months (0.12 ± 0.09 AU), *p* = 0.03 *(Fig. 3A)*. However, higher anti-Pf113 titers were observed among children aged to 61–108 months (0.19 ± 0.047 AU) as compared to children aged to 7–25 months (0.067 ± 0.02 AU, *p* = 0.03). The same difference was also seen when comparing the children aged to 61–108 months with the children aged to 25–60 months (*p* = 0.013). When the anti-PfAMA1 IgG response was compared, children exhibited an age-dependent increase of anti-PfAMA1 titers after 24 months of age *(Fig. 3B)*. Significant differences were seen between age groups of 7–24 (0.37 ± 0.17 AU), 25–60 (0.56 ± 0.16 AU), 61–108 (0.88 ± 0.22 AU), and 109–156 (1.042 ± 0.35 AU) months, with *p* = 0.013 for 7–24 vs. 61–108 months,

tibodies. There was no significant difference between anti-PfAMA1 and anti-Pf113 responses, according to the parasite density. We also compared antibody levels according to parasite densities in Franceville. This analysis revealed no significant difference of antibody levels for the three *P. falciparum* antigens in all parasites density groups *(Fig. 4)*.

We then analyzed antibody levels in children with unanemic malaria (*n* = 18), mild malarial anemia (*n* = 30), and severe malarial anemia (*n* = 5). Although the stratification into clinical groups did not show differences of antibody responses against the three tested *P. falciparum* malaria antigens in Lastourville, the comparison between the separate groups revealed that children with unanemic
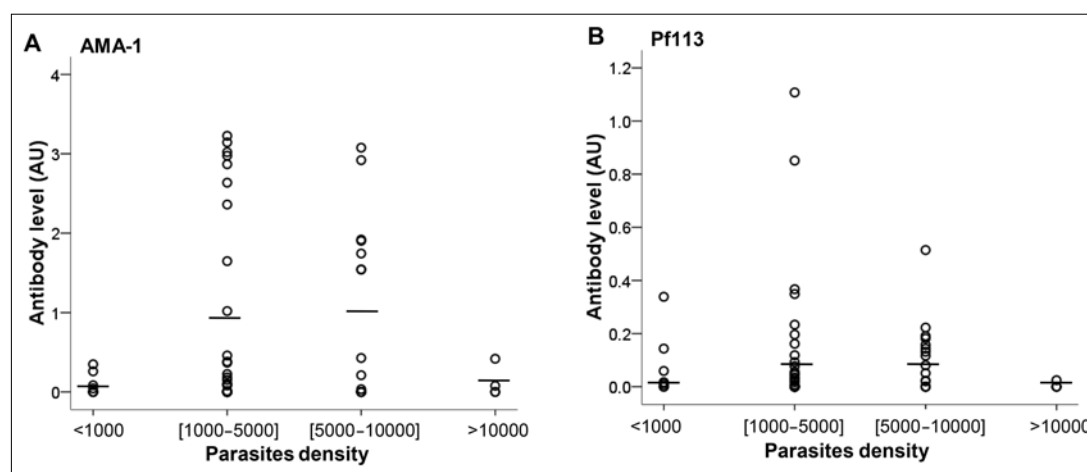

Fig. 4. Distribution of antibodies (anti-PfAMA-1 and anti-Pf113) concentrations according to the parasitemia. Plasma concentrations of malaria-specific antibodies were quantified in *P. falciparum*-infected Lastourville children. *P. falciparum*-infected children were subdivided into four groups based upon the parasite density. The very low density group was characterized by parasite density <1000 parasites/µl of blood. The low density group was defined by parasite density included between 1000 and 5000 parasites/µl. Medium and high density groups were defined by parasite density included between 5000 and 10000 parasites/µl and >10,000 parasites/µl of blood, respectively

0.002 for 7–24 vs. 109–156 months, and 0.04 for 25–60 vs. 109–156 months, respectively, *p* < 0.04. Anti-PfRh5 levels did not show a significant difference between all the age groups (*p* > 0.05; data not showed). We simultaneously tested all factors (anti-PfAMA1, Pf113, PfRh5, and age) for association with clinical malaria. The multivariate analysis detected no association between antibody levels and malaria protection. In Franceville, there were no statistically significant differences for any of the antigens tested between all the groups. However, the group aged 109 to 156 months exhibited significant higher anti-PfRh5 titers (0.22 ± 0.09 AU) than those of the 7 to 24 months age group (0.054 ± 0.03 AU; *p* = 0.02, *Fig. 3C*).

Anti-PfAMA1, Pf113, and PfRh5 antibody responses according to parasitemia and clinical groups in children

Infected children from Lastourville were investigated regarding *P. falciparum* density to determine the association between parasitemia and the production of an-
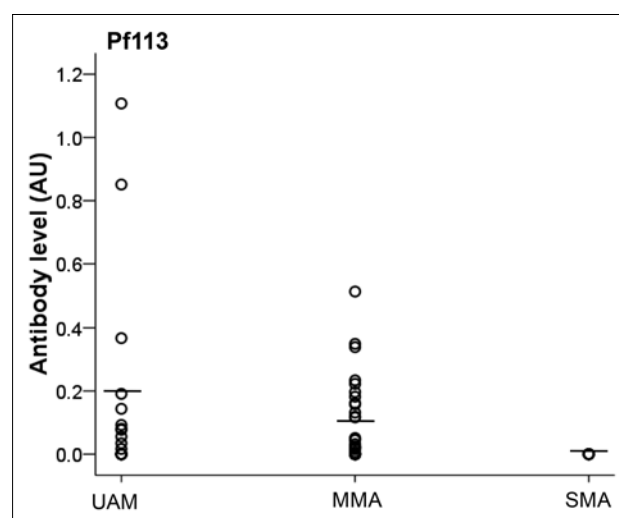

Fig. 5. Malaria-specific antibody levels according to malaria anemia status. Plasma concentrations of anti-Pf113 antibodies were quantified in *P. falciparum*-infected Lastourville children. Children were subdivided into severe malaria anemia (SMA: Hb < 5.0 g/dl), mild malaria anemia (MMA: 5 ≤ Hb < 11 g/dl) and unanemial malaria (UAM: Hb ≥ 11 g/dl)

malaria (0.17 ± 0.07 AU) and mild malaria anemia (0.1 ± 0.02 AU) exhibited higher anti-Pf113 titers than those with severe malaria anemia ($p = 0.03$ and $p = 0.004$, respectively, *Fig. 5*). No significant difference in the antibody levels for anti-PfAMA1 and anti-PfRh5 was observed between the clinical groups. In Franceville, only children with unanemic malaria were observed.

Discussion

The prevalence of malarial infection was significantly higher in the rural area (Lastourville) than in the urban area (Franceville), this result being probably due to a difference in urbanization level, equipment, housing, and access to treatment or education between both cities, consistent with previous data comparing prevalence of malaria in different areas of Gabon [29]. We observed that the mean age of infected children was significantly higher than the mean age of uninfected children. It has been demonstrated in Gabon that the mean age of children developing malaria has increased significantly and that children over 5 years old are now those who have the greatest risk to contract malaria [23, 29]. This could be the consequence of a better protection of children under five by the antimalarial programs in Gabon.

Although the etiology of anemia in tropical areas is multifactorial, *P. falciparum* malaria is commonly associated with anemia in children living in holoendemic malaria areas [30]. In this context, we found that hemoglobin levels were lower in infected Gabonese children, which is consistent with data from Kenya, where severe anemia was associated with parasitemia in 85% of the admissions [31]. The same result was obtained in a recent study from Nigeria [32]. Hemolysis may be due to destruction of the parasitized cells during schizogony and erythrophagocytosis in the spleen [33]. Another mechanism involved in malaria anemia is the transfer of parasite antigens such as RSP2 to uninfected red blood cells and the elimination of the erythroid lineage in the spleen and in some immune phagocytosis [34, 35].

In areas with intense *P. falciparum* transmission such as Gabon, clinical immunity to malaria and development of *P. falciparum*-specific antibodies are acquired gradually over the years, following repeated infections [36], but the acquisition of host immunity could vary according to the living areas. Data showed that children living in Lastourville, where the malaria prevalence was highest, had significant higher levels of anti-PfAMA1 and anti-PfRh5 antibodies compared to children living in Franceville; this is consistent with a study from Uganda [37]. This could be due to the frequency of contacts with the parasite leading to a better immunity boost [6]. It has been shown that naturally acquired immunoglobulin G (IgG) specific for PfRH5 and PfAMA1 increased with age and was boosted by *P. falciparum* transmission [19, 38]. Our findings suggest that antibody responses to PfAMA1 and PfRh5 appeared to best reflect the intensity of transmis-

sion and may be the best indicator for malaria surveillance, a result also supported by a previous study [37]. However, no such difference was seen for Pf113 in both sites, so the antibody response against Pf113 could be unrelated to the number of contacts with the *P. falciparum* parasite. An interpretation is that the acquisition of anti-Pf113 immunity would require fewer contacts with the parasite and would persist without any need for repeated stimulations.

Data showed antibody response classification depending on antigens. Anti-PfAMA1 response was higher than the anti-Pf113 response, which was itself higher than the anti-PfRh5 response, in terms of antibody levels. Such an observation is consistent with a recent study from Kenya and Mali, where it was observed that the IgG reactivity to PfRH5 was approximately 2 orders of magnitude lower than the IgG reactivity to PfAMA1 [17, 38]. This study shows new information regarding the Pf113 response which is established as an intermediary between the PfAMA1 and PfRh5 responses.

We confirm that the anti-PfAMA1 response was higher in infected children compared to those without *P. falciparum* infection; this is consistent with previous immuno-epidemiological studies [37, 38]. Anti-PfAMA1 response and age of children were also associated with protection against malaria. In previous studies, vaccination with PfAMA1 has been shown to elicit antibody responses that give good protection against homologous parasite challenges in a number of rodent and primate models [39–46].

The presence of higher levels of anti-PfAMA1 and anti-Pf113 antibodies in children under 6 months could be explained by IgG transfer across the maternal placenta and breastfeeding [47, 48].

From 7 to 24 months of age, anti-PfAMA1 levels increased with age in the area of Lastourville; this confirms the findings reported in the population of Nagongera in Uganda [37] and Kalifabougou in Mali [38].

No association between parasitemia and the production of antibodies could be established in both sites, even though the levels of antibodies were high. This supports the idea that the function of antibodies, and not just their presence, is important for protection against malaria as established by Reddy et al. [49].

The lowest level of anti-Pf113 antibody was found in children with severe malarial anemia contrary to the levels in children without anemia or mild malarial anemia, suggesting that anti-Pf113 antibodies could induce a protection against severe malarial anemia. We hypothesize that anti-Pf113 antibodies may not protect against *Plasmodium* parasite proliferation but rather against malarial anemia. Other studies reported that high levels of anti-MSP1 IgG1 antibodies are associated with protection against malaria attacks [50]. A more recent study from Ghana revealed that the risk of clinical malaria decreased with increasing antibody levels against GLURP R2, MSP3, AMA1, MSP1, and EBA175 [51]. However, the number of children with severe anemia was too low to draw conclusions; further

studies are needed to confirm that anti-Pfl13 antibodies are associated with protection against anemia.

Conclusion

This study provides the first evidences for naturally acquired immunity to PfAMA1, Pfl13, and PfRh5 antimalarial antigen vaccine candidates, in populations exposed to malaria transmission in Gabon. We established a classification of immunogenicity to these three antigens: PfAMA1 is the best, followed by Pfl13, and the last being PfRh5. Antibody levels to PfAMA1 and PfRh5 appeared to be the best parameters reflecting transmission intensity and may be the best indicators for malaria surveillance. The anti-Pfl13 and anti-PfRh5 responses could be associated with protection against anemia, but further studies are needed.

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