HIGH LEVEL OF SPECIFIC ANTI-*PLASMODIUM FALCIPARUM* MEROZOITE IgG1 ANTIBODIES IN RURAL ASYMPTOMATIC INDIVIDUALS OF DIENGA, SOUTH-EASTERN GABON

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Plasmodium falciparum merozoite antigens (PfMAgs) play an essential role in the development of immunity to malaria. Currently, *P. falciparum:* protein 113 (Pf 113), apical membrane antigen 1 (AMA1), erythrocyte binding antigens (EBA175), and reticulocyte binding protein homologue 5 (RH5) are among the most PfMAgs studied. A comparative analysis of naturally acquired antibodies against these antigens in children would increase our knowledge about the development of protective immunity.

Analysis of antibodies to Pf113, PfAMA1, PfEBA175, and PfRH5 was conducted in rural population during 2013 and 2014. Both prevalence and levels of total IgG anti-PfAMA1 were higher than that of IgG anti-PfEBA175, anti-PfRH5, and anti-Pf113. Seroconversion to PfAMA1 and PfEBA175 occurred moderately in young children and reached to the maximum in adolescent and in adults. High prevalence of IgG anti-Pf113 was observed in young children of 3 to 6 years old in 2013. The four antigens were recognized by IgG 1, 2, 3, and 4 antibodies from a large proportion of the subjects, and all of them induced high levels of specific IgG1 against PfAMA1, PfEBA175, fewer by Pf113 and PfRH5.

Many asymptomatic children had specific IgG1 recognizing multiple antigens, and these IgG1 antibodies could be associated with a reduced risk of developing malaria symptoms.

Keywords: Gabon rural area, *P. falciparum,* immune response, PfAMA1, Pf113, Pf EBA175, PfRh5, antibodies

Background

The merozoite is an erythrocyte stage and one of the few stages of the *Plasmodium* life cycle in which the parasite is extracellular and thus directly exposed to the host humoral immune system. The symptoms and pathology of malaria are caused by the intra-erythrocyte stages of the *Plasmodium* parasite life cycle. Most antigens are merozoite surface proteins which participate in receptor-ligand interactions occurring during the parasite's initial attachment to red blood cells (RBCs) [1–3]. Indeed, merozoites of *P. falciparum,* the most dangerous species, harbor a site that interacts with RBCs [4]; this step is crucial for the invasion and the development of the asexual intraerythrocytic cycle, hence causing the pathogenesis and symptoms of malaria [5–8]. Therefore, several vaccine candidates are known to target intraerythrocytic stages. The most studied of intraerythrocytic vaccine candidates are antigens such

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as PfEBAs (*P. falciparum* erythrocyte binding antigens) [9–12], AMA-1 (apical membrane antigen 1), MSPs (merozoite surface proteins) [13, 14], PfRH5 (*P. falciparum* reticulocyte binding protein homologue 5) [8, 15, 16], and, recently, Pf 113 (*P. falciparum* protein 113) [4, 17].

PfEBA175 is a 175 kDa sialic acid binding protein ligand known as erythrocyte binding antigen-175 [11, 12], and PfAMA1 presents a conserved hydrophobic cleft that interacts with rhoptry neck protein 2 (RON2) [18]. This interaction is essential to the formation of the junction, which commits the parasite to invade. Both PfAMA1 and RON2 are provided by the parasite to enable an active invasion mechanism [19]. Specifically, antibodies raised against PfAMA1 can inhibit invasion by binding to the hydrophobic cleft; thus, PfAMA1 is mostly seen as a viable vaccine target [20].

PfRH5 is essential for merozoite invasion of erythrocytes, and attempts to disrupt the gene encoding PfRH5 have failed to produce viable parasites [21, 22]. Moreover, antibodies rose in animals against either PfRH5 or its erythrocyte receptor inhibit parasite invasion into erythrocytes *in vitro* [16, 23]. Pfl13 is a *P. falciparum* protein predicted to be GPI-anchored that has been so far localized at the surface of merozoites, suggesting it could interact with the RBC surface during merozoite invasion [7, 24].

Pfl13, PfRH5, PfAMA1, and PfEBAs are all recognized by human sera from malaria endemic areas and are likely to be involved in the development of protective immunity against malaria [25–27]. Intensive studies on vaccine trials are ongoing, hoping that, by 2025, a 80% efficient vaccine could be developed and that it might last for 4 years, targeting different stages of life cycle, such as the pre-erythrocytic stage to prevent infection, and blood stages to reduce clinical disease or block transmission [28]. It is therefore important to further investigate the naturally acquired antibodies including symptomatic and asymptomatic individuals living in malaria endemic areas.

Many studies on the topic, comparing the responses to antigens have been performed in Kenya [29], Mali [25], and Papua New Guinea [26]. We know that, from one region to another, genetics can vary both in the parasite and in the host. Like RTS,S/AS01 most vaccines are mixtures of multiple antigens [30]. Effective immunity against *P. falciparum* malaria is a slow process, setting in after repeated exposure and protecting against the development of symptomatic and severe illness [31, 32].

Gabon, in Central Africa, is an area of high malaria transmission and one of the seven sub-Saharan countries where the third trial phase on the most advanced vaccine candidate RTS,S/AS01 was carried out [33]. However, only one study on humoral responses to PfRH5, Pfl13, and PfAMA1 antigens has been conducted in the country [34].

The aim of the present study was to measure and compare two periods (2013 and 2014) of naturally acquired antibodies specific for EBA peptide 4, PfRH5, PfAMA1, and Pfl13 in asymptomatic individuals living in Dienga, a south-east rural area of Gabon.

Materials and methods

Subjects and field methods

This study was conducted in Dienga, a rural area of south-east Gabon in the Ogooué-Lolo province. Dienga is a densely forested locality, situated near the Congo border with around 2500 inhabitants; malaria is highly endemic, and *P. falciparum* is predominant (80%) due to *Anopheles gambiae,* with an entomological infection rate of 100 infective bites per human per year [35]. Malaria seasonal peaks of transmission coincide with the rains, from February to June and from September to December. We enrolled 370 individuals over 3 years old in April 2013, who were followed during 15 months until June 2014. Initially, the study design was established to assess the new *P. falciparum* prevalence among asymptomatic carriers living in this village. Then, 216 samples obtained during the first field mission (April 2013) and 90 samples obtained the year after (March 2014) were used to scrutinize antibody response to four *P. falciparum* antigens.

Sample collection

Samples were collected from all individuals, and ~2000 μl of blood was drawn by venipuncture in 5-ml EDTA tubes for thick-film preparation and molecular diagnosis after the separation steps. Plasma was separated by centrifugation and cryopreserved at −80 °C.

Microscopy

Blood films were prepared in 2013 and 2014 as described [36]. Slides were stained with 10% Giemsa solution for 15 min and examined under a microscope. Samples were considered *Plasmodium*-positive if at least one parasite was seen in a ×100 oil-immersion field of a thick blood film (MI+). Readings were done by an experimented reader and verified by a second reader, with discrepancies resolved by a third reader. The entire slide was carefully scanned before being declared negative.

P. falciparum *DNA extraction and molecular diagnosis*

DNA extraction was performed using the D Neasy Blood & Tissue kit according to the manufacturer's procedure (QIAGEN, Hilden, Germany).

A polymerase chain reaction (PCR) was performed only on negative samples (MI−) to identify submicroscopic infection (SMI) using *P. falciparum STEVOR* gene amplification [37].

Antigen and antibody measurements

Synthetic peptides used as antigens were EBA-peptide 4 (aa 1062 1103: SNNEYKVNEREDERTLTKEYEDIVL-KSHMNRESDDGELYDEN) synthesized by Interactiva Biotechnology (Ulm, Germany), the recombinant protein Pf113 (PlasmoDB identification code: PfD7_1420700, UniProt accession number: Q8ILP3. Aa 730-KEKTSD-DATHKETQEKSDQE-750) from the Museum National d'Histoire Naturelle de Paris (France). The two recombinant proteins, *P. falciparum* reticulocyte binding homologue (PfRh)-5 and *P. falciparum* apical membrane antigen (PfAMA)-1 were provided by the Wellcome Trust Institute of London (United Kingdom).

Antibodies were measured by an enzyme-linked immunosorbent assay (ELISA) using 1.5 μg/ml of PfEBA peptide 4, PfPf113 peptide at 1.5 μg/ml, 1 μg/ml of PfAMA1, and 1.5 μl/g of PfRH5 peptide. All of these peptides were diluted in 1× PBS buffer pH 7.4. A 2000-fold diluted mouse anti-human IgG (Fc specific) conjugated to peroxidase (Sigma, St. Louis, MO, USA) was used, and bound enzyme was detected with tetra methyl benzidine (TMB) substrate (Prod Thermo-Scientific, USA); the absorbance was read at 405 nm as previously described [34, 38].

IgG subclass analysis was carried out using 50-fold diluted plasma, mouse anti-human IgG1, IgG2, IgG3, and IgG4 antibodies (codes: LMH 1013, 1022, 1032 and 1042; Caltag Laboratories, Burlingame, CA, USA) at a final concentration of 1, 0.25, 0.5, and 0.25 μg/ml, respectively, and a peroxidase-conjugated goat anti-mouse IgG at respective final concentrations of 2.5, 5, 1.3, and 2.5 μg/ml. Bound enzyme was detected as described above. Reference positive (a pool+ of 10 plasmas from infected individuals with at least 40,000 parasites/μl of blood of each subject) and negative (a pool− of 10 plasmas from Caucasian individuals, all negative for *Plasmodium* infection) control plasmas were included in each plate, and results were expressed in arbitrary units as previously described (AU) [27, 34, 35, 38].

Data processing and analysis

An electronic database was used for recording study information. Analyses were carried out with Epi-Info (version 5.04 dfr, ENSP-Epiconcept-InUS, 2001) and (STATA 14.0, 4905 Lakeway Drive College Station, Texas 77845, USA). A *p* value of <0.05 was considered statistically significant. Results are expressed as percentages (with 95% confidence intervals, CIs) and odds ratios (ORs, with 95% CIs). Conventional descriptive analysis consisted of proportion determination (for qualitative data), or mean ± standard deviation, median, and range (for quantitative data). χ^2 test for frequencies (proportions) and the Willcox test for means were used for comparative analyses.

Ethics statement

This study obtained ethical clearance from the "Comité National d'Ethique" (CNE) of Gabon, and it was registered under PROT N° 0018/2013/SG/CNE. It received also the agreement of the Public Health Minister, the Ogooué-Lolo province's Regional Health Director, and the Traditional Chiefs of Dienga.

The samples were anonymous, but age and geographic origin were retained. Written consent was also obtained from all individuals, and the parents or guardians answered for children younger than 16.

Results

Baseline demographic and total IgG antibodies status in 2013

In April 2013, 216 individuals were enrolled, including 119 males and 97 females. The study population was divided into five age groups: 27 children aged 3–6 years old, 72 children aged 7–10 years old, 47 children aged 11–14 years old, 20 adolescents aged 15–18 years old, and 50 adults over 18 years old. The diagnosis of *P. falciparum* infection (microscopy and PCR) has been previously reported [39]. Global *P. falciparum* infection (patent and submicroscopic infections) was observed in 104 individuals whereas 112 were non-infected *(Table 1)*. Of the 216 individuals analyzed, the overall prevalence rates of total IgG directed against Pf-113, PfAMA1, PfEBA175, and PfRH5 were respectively 89 (41.20%) [95% CI = 34.6–48.1], 144 (66.67%) [95% CI = 60.0–72.9], 109 (50.56%) [95% CI = 43.6–57.3], and 120 (55.56%) [95% CI = 48.7–62.3]. The prevalence rates of PfAMA1 and PfRH5 anti-IgG were higher than those of PfEBA175 and Pf113 anti-IgG *(Table 1)*.

Baseline demographic and total IgG antibodies status in 2014

Of the 216 individuals examined in 2013, 90 were analyzed again in 2014 (47 males and 43 females). These 90 subjects were composed of 11 children aged 3–6 years old, 26 aged 7–10 years, and 25 aged 11–14 years. The remaining individuals numbered 6 aged 15–18 years old and 22 adults over 18 years old. As in 2013, microscopy and PCR were carried out to detect *P. falciparum* microscopic and submicroscopic infections [39]. Sixty-three individuals were found to carry *P. falciparum* while 27 were non-infected during this field mission *(Table 2)*. However, 89 of these 90 subjects carried at least one *P. falciparum* infection during the follow-up.

The overall prevalence rates of total IgG directed against Pf113, PfAMA1, PfEBA175, and PfRH5 were respectively 42.20% [95% CI = 31.8–52.6], 72.20% [95%

Table 1. Baseline demographic and total IgG antibodies status in 2013. Seroprevalence of total IgG antibodies against Pf113, PfAMA1, PfEBA175, and PfRH5 antigens in all age groups and among *P. falciparum*-infected and non-infected individuals in 2013

Variables ($N = 216$)	Pf113	PfAMA1	PfEBA175	PfRH5	*p* value
	n (%)	*n* (%)	*n* (%)	*n* (%)	
IgG prevalence rates	89 (41.2)	144 (66.6)	109 (50.5)	120 (55.5)	<0.001
Sex					
Males ($n = 119$)	41 (34.45)	74 (62.18)	60 (50.42)	61 (51.26)	<0.001
Females ($n = 97$)	48 (49.48)	70 (72.16)	49 (50.51)	59 (60.82)	<0.001
Age group					
3–6 yrs. ($n = 27$)	16 (59.26)	13 (48.15)	5 (18.52)	9 (33.33)	0.003
7–10 yrs. ($n = 72$)	34 (47.22)	39 (54.17)	40 (55.56)	41 (56.94)	0.5
11–14 yrs. ($n = 47$)	17 (36.17)	35 (74.47)	20 (42.55)	28 (59.57)	<0.001
15–18 yrs. ($n = 20$)	3 (15.00)	17 (85.00)	8 (40.00)	9 (45.00)	<0.001
19+ yrs. ($n = 50$)	19 (38.00)	40 (80.00)	36 (72.00)	33 (66.00)	<0.001
Global infection					
Infected ($n = 104$)	55 (52.88)	44 (42.31)	74 (71.15)	57 (54.81)	<0.001
Non-infected ($n = 112$)	56 (50.00)	46 (41.07)	75 (66.96)	66 (58.93)	
MI					
Positive ($n = 60$)	27 (45.00)	30 (50.84)	39 (65.00)	32 (53.33)	<0.000
Negative ($n = 156$)	84 (53.84)	61 (39.10)	111 (71.15)	91 (58.33)	
PCR (SMI)					
Positive ($n = 44$)	28 (63.64)	18 (40.91)	35 (79.55)	25 (56.82)	<0.001
Negative ($n = 112$)	56 (50.00)	34 (30.36)	76 (67.86)	67 (59.82)	

N: number of subjects; *n* = number of positive subjects and seroprevalences in brackets; global infection: *P. falciparum* infections diagnosed by both microscopy and PCR; MI: *P. falciparum* infections detected only by microscopy; SMI: *P. falciparum* infections detected only by PCR (submicroscopic *P. falciparum* infections = SMI). The *p* values are χ^2 test comparing the seroprevalences between different antigen-specific IgG, according to age groups and infection status.
Abbreviations: IgG, immunoglobulin G; Pf113, *P. falciparum* protein 113; PfAMA1, *P. falciparum* apical membrane protein 1; PfEBA175, *P. falciparum* erythrocyte binding antigens-175; PfRH5, *P. falciparum* reticulocyte-binding protein homologue 5
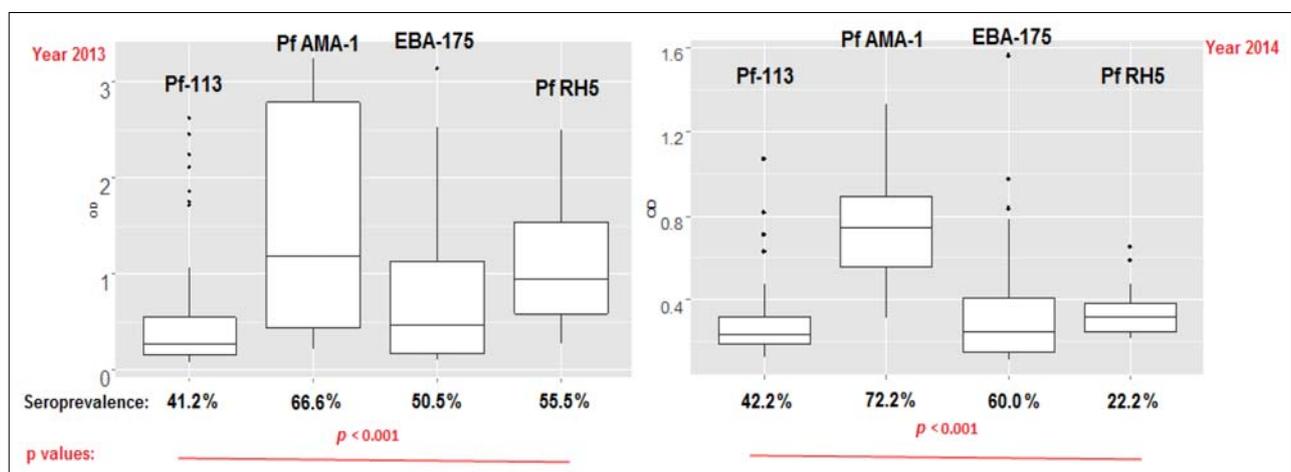
Fig. 1. Distribution of specific anti-merozoite IgG antibodies in 2013 and 2014. The box plots indicate interquartile range, central lines represent medians, whiskers indicate the 25th–75th percentiles, and dots are outliers. The differences between antigen specific-seroprevalence were assessed by the Chi-square test both in 2013 and 2014

Table 2. Baseline demographic and total IgG antibodies status in 2014. Seroprevalence of total IgG against Pf113, PfAMA1, PfEBA175, and PfRH5 antigens in all age groups and among *P. falciparum*-infected and non-infected individuals in 2014

Variables (N = 90)	Pf113	PfAMA1	PfEBA175	PfRH5	p value
	n (%)	n (%)	n (%)	n (%)	
IgG prevalence rates	38 (42.2)	65 (72.2)	54 (60.0)	20 (22.2)	<0.001
Sex					
Males (n = 47)	19 (40.4)	35 (74.5)	28 (59.6)	8 (17.0)	0.7
Females (n = 43)	19 (44.2)	30 (69.8)	26 (60.5)	12 (27.9)	
Age group					
3–6 yrs. (n = 11)	2 (18.18)	8 (72.72)	5 (45.45)	0 (0.00)	0.001
7–10 yrs. (n = 26)	10 (38.46)	18 (69.23)	14 (53.85)	7 (26.92)	0.01
11–14 yrs. (n = 25)	14 (56.00)	17 (68.00)	19 (76.00)	7 (28.00)	0.003
15–18 yrs. (n = 6)	3 (50.00)	5 (83.33)	5 (83.33)	2 (33.33)	0.18
19+ yrs. (n = 22)	9 (40.91)	17 (77.27)	11 (50.00)	4 (18.18)	0.001
Global infection					
Infected (n = 63)	20 (31.75)	39 (61.91)	27 (42.85)	15 (23.81)	<0.001
Non-infected (n = 27)	18 (66.67)	19 (70.37)	19 (70.37)	5 (18.52)	
MI					
Positive (n = 24)	9 (37.50)	17 (70.83)	13 (54.17)	8 (33.33)	<0.001
Negative (n = 66)	29 (43.94)	48 (72.73)	41 (62.12)	12 (18.18)	
PCR					
Positive (n = 39)	11 (28.21)	22 (56.41)	14 (35.89)	7 (17.95)	<0.001
Negative (n = 27)	18 (66.67)	19 (70.33)	19 (70.37)	5 (18.52)	

N: number of subjects; *n* = number of positive subjects and seroprevalence in brackets; global infection: *P. falciparum* infections diagnosed by both microscopy and PCR; MI: *P. falciparum* infections detected only by microscopy; SMI: *P. falciparum* infections detected only by PCR (submicroscopic *P. falciparum* infections = SMI). The *p* values are χ^2 test comparing the seroprevalence between different antigen-specific IgG, according to age groups and infection status.
Abbreviations: IgG, immunoglobulin G; Pf113, *P. falciparum* protein 113; PfAMA1, *P. falciparum* apical membrane protein 1; PfEBA175, *P. falciparum* erythrocyte binding antigens-175; PfRH5, *P. falciparum* reticulocyte-binding protein homologue 5

CI = 62.8–81.7], 60.0% [95% CI = 49.7–70.3], and 22.22% [95% CI = 13.5–30.9] *(Table 2)*.

Distribution of total IgG antibodies

In 2013, the prevalence rates of specific IgG antibodies directed against PfAMA1 were significantly higher than those directed against Pf113 ($p < 0.001$), PfEBA175 ($p = 0.01$), and PfRH5 ($p = 0.007$). A significant difference was also observed between PfRH5 and PfEBA175 ($p = 0.01$). Such a difference was not observed between the prevalence rates of anti-PfEBA175 IgG and Pf113 anti-IgG ($p = 0.421$), or between anti-PfRH5 IgG and anti-Pf113 IgG ($p = 0.338$) *(Fig. 1)*.

In 2014, the IgG antibodies against PfAMA1 were also more prevalent compared to those from other antigens, Pf113 ($p = 0.002$), PfEBA175 ($p = 0.004$), PfRH5 and ($p < 0.001$). PfEBA175 IgG antibodies were significantly higher than Pf-113 IgG antibodies ($p = 0.001$). No significant

difference was observed between PfRH5 and Pf-113 IgG antibodies ($p = 0.338$) *(Fig. 1)*.

When IgG antibody responses are compared between 2013 and 2014, the prevalence rates did not statistically vary for Pf113, PfAMA1, and PfEBA175 antigens, respectively ($p = 0.8$, $p = 0.8$, and $p = 0.3$). However, the prevalence rate of PfRH5 IgG antibodies was significantly higher in 2013 than in 2014 ($p < 0.001$) *(Fig. 1)*.

In 2013, quantitative analysis showed that median (P25–P75) optical density expressed as arbitrary unit (AU); 0.951 (0.128–2.556) for IgG anti-PfAMA1 was significantly higher than those of anti-Pf113, 0.062 (0.027–0.210), and PfEBA175, 0.188 (0.047–0.848) ($p = 0.006$ and $p = 0.01$, respectively). This is not the case for anti-PfRH5, 0.840 (0.493–1.637) ($p = 0.07$) *(Fig. 1)*.

In 2014, according to antibody levels, no significant difference was observed between all antigens *(Fig. 1)*. Total IgG antibody levels did not statistically vary between 2013 and 2014 for Pf113 [median (P25–P75)], 0.062 (0.027–0.210) and 0.137 (0.085–0.218) ($p = 0.4$), and for
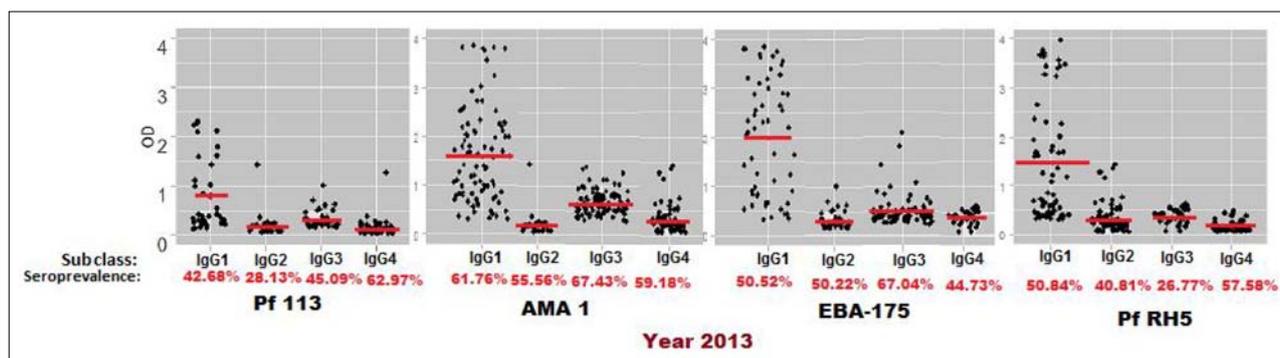
Fig. 2. Seroprevalence and level of anti-merozoite specific IgG subclasses in 2013. At the bottom, the seroprevalence of each IgG subclass directed against Pf113, PfEBA175, Pf AMA1 and PfRH5 in 2013 is indicated in Roman numerals and in percentage. The lines on the dot clouds represent the means and they were compared by the willcox test, whereas the comparison between IgG subclasses was done with the chi-square test

PfEBA175, 0.188 (0.047–0.848) and 0.188 (0.047–0.848) ($p = 1$), respectively, in 2013 and 2014. By contrast, these levels decreased for PfAMA1, from 0.951 (0.128–2.556) in 2013 to 0.612 (0.343–0.827) in 2014 ($p = 0.03$), and also for PfRH5, from 0.840 (0.493–1.637) to 0.123 (0.090–0.200), respectively, in 2013 and 2014 ($p < 0.001$) *(Fig. 1)*.

IgG subclass distribution

In 2013, the overall prevalence rates of specific IgG1, IgG2, IgG3, and IgG4 were, respectively, 42.68%, 28.13%, 45.09%, and 62.97% against Pf113; PfAMA1, 61.76%, 55.56%, 67.43%, and 59.18%; and PfEBA175, 50.52%, 50.22%, 67.04%, and 44.73% against PfRH5 50.84%, 40.81%, 26.77%, and 57.58%. This result showed a significant difference between IgG2 and IgG4 directed against Pf113 ($p < 0.001$), between IgG3 and IgG4 against PfRH5 ($p < 0.001$), between IgG1 and IgG3 anti-PfEBA175, between IgG3 and IgG2, and between IgG3 and IgG4 ($p < 0.01$ for all). There is no significant difference between all anti-PfAMA1 subclasses ($p = 0.05$ for all).

Quantitatively, the levels of IgG1 were higher than that of other subclasses directed against Pf113 [median (P25–P75)], 0.511 (0.262–1.240), when compared to IgG2

[0.353 (0.183–0.453)], IgG3 [0.452 (0.277–0.679)], and IgG4 [0.218 (0.122–0.333)] ($p = 0.03$); against PfRH5, 0.476 (0.313–0.943) compared to IgG2 [0.223 (0.098–0.373)], IgG3 [0.266 (0.103–0.298)], and IgG4 [0.178 (0.111–0.321)] ($p = 0.001$); PfAMA1, 0.820 (0.437–1.568), compared to IgG3 [0.439 (0.286–0.667)] ($p = 0.02$), IgG2 [0.242 (0.172–0.564)], and IgG4 [0.493 (0.234–0.648)]; PfEBA175, 0.511 (0.262–1.240), compared to IgG2 [0.242 (0.138–0.453)], IgG3 [0.303 (0.153–0.679)], and IgG4 [0.218 (0.122–0.333)] ($p = 0.01$) *(Fig. 2)*.

In 2014, the overall prevalence rates of specific IgG1, IgG2, IgG3, and IgG4 were 71.1%, 55.3%, 36.8%, and 76.3%, respectively, directed against Pf113; 30%, 30%, 60%, and 60% against PfRH5; 84.57%, 68.18%, 70.81%, and 72.22% against PfAMA1; and 66.69%, 56.55%, 55.64%, and 54.73% against Pf EBA175 *(Fig. 3)*.

A significant difference was observed between the seroprevalences of anti-Pf113 IgG4 and IgG1 ($p = 0.04$), IgG4 and IgG3 ($p < 0.001$), and also between IgG4 and IgG2 ($p = 0.03$). No statistical difference was found in anti-PfAMA1, anti-PfEBA175, and anti-PfRH5 subclasses prevalence rates.

The levels of IgG1 were also higher than that of other isotypes directed against Pf113 [median (P25–P75)], 0.473 (0.188–0.938), and PfAMA1, 2.015 (1.039–2.454), compared to other subclasses ($p < 0.05$). The levels of iso-
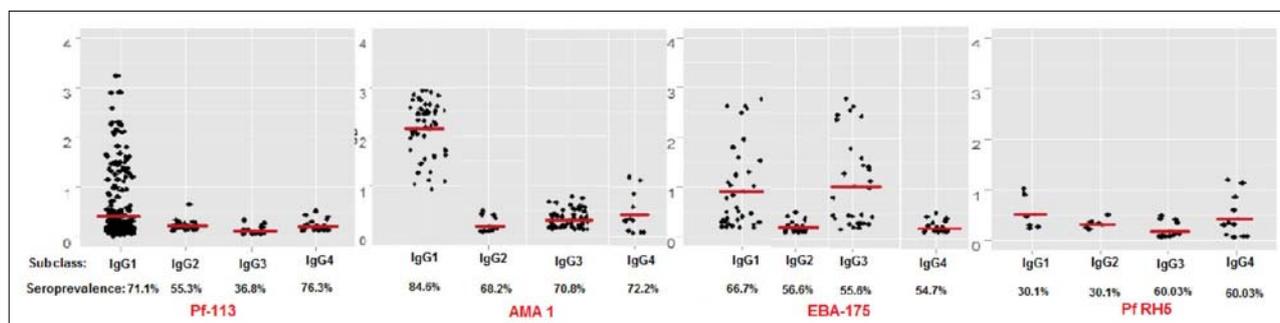
Fig. 3. Seroprevalence and level of anti-merozoite specific IgG subclasses in 2014. In 2014, the seroprevalence of IgG1, 2, 3 and 4 directed to Pf113, PfEBA175, Pf AMA1 and PfRH5 is showed in Roman numerals and in percentage at the bottom. The lines on the dot clouds represent the means which were compared by the willcox test. Chi-square test was used to compare IgG isotypes
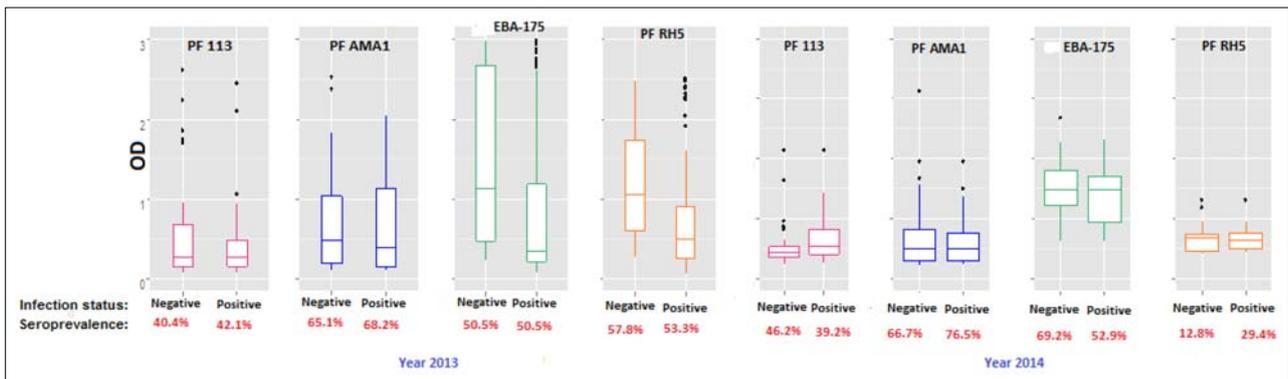
Fig. 4. Relationships between P. falciparum global infection and antibody response. The box plots indicate interquartile range, central lines represent medians, whiskers indicate the 25th–75th percentiles, and dots are outliers. The differences between antigen specific-seroprevalence were assessed by the Chi-square test both in 2013 and 2014. The *Negative box* express samples that not exhibited infection (nor by microscopy or PCR) and the *Positive box* express the global infection (microscopy and PCR)

types directed against PfEBA175 and PfRH5 were not significant different *(Fig. 3)*.

The prevalence rates of IgG1 anti-Pf113 and anti-PfAMA1 increased significantly from 2013 to 2014 ($p = 0.003$) and ($p < 0.001$), respectively. Many other antibodies did not show significant difference in their frequencies during the study period such as IgG2, -3, and -4 anti-Pf113 ($p = 0.3$, $p = 0.1$, and $p = 0.1$, respectively); IgG1 and IgG3 anti-PfEBA175 ($p = 0.07$ and $p = 0.1$, respectively); and IgG1, -3, and -4 anti-PfRH5 ($p = 0.08$, $p = 0.06$, and $p = 0.1$, respectively) *(Figs 2* and *3)*. Only the prevalence rate of specific IgG2 anti-PfRH5 decreased significantly in 2014 compared to 2013 ($p = 0.03$).

Relationships between P. falciparum *global infection (MI+ and SMI+) and total IgG responses*

There was no statistical difference between the prevalence rates of specific IgG in infected subjects compared to uninfected individuals, irrespective of the antigens used and the study period (2013 and 2014), except for Pf113 and PfEBA175 antigens. In 2014, the prevalence rates of these

two antigens among uninfected individuals were significantly higher than that in infected individuals ($p < 0.05$) *(Fig. 4)*.

The levels of total IgG antibodies directed against PfEBA175 and PfRH5 were found to be higher in uninfected subjects (*P. falciparum* negative) compared to infected ones (*P. falciparum* positive) in 2013 ($p < 0.001$ for both antigens) *(Fig. 4)*.

P. falciparum infection and total IgG antibody levels were correlated, even if this association was not statistically significant. However, harboring an infection increases 0.03-fold for the PfEBA175 antibody level (OR = 0.03, $p = 0.3$). Infection decreases −0.5-fold for the PfAMA1 antibody level, −0.2-fold for the PfRH5 antibody level, and −0.7 fold for the Pf113 antibody level (OR = −0.0915, $p = 0.2$; OR = −0.06, $p = 0.4$; and OR = −0.07, $p = 0.3$, respectively).

Relationships between P. falciparum *infections detected using microscope or by PCR and IgG responses*

The comparison of IgG prevalence rates and its levels among infected subjects detected using microscopes
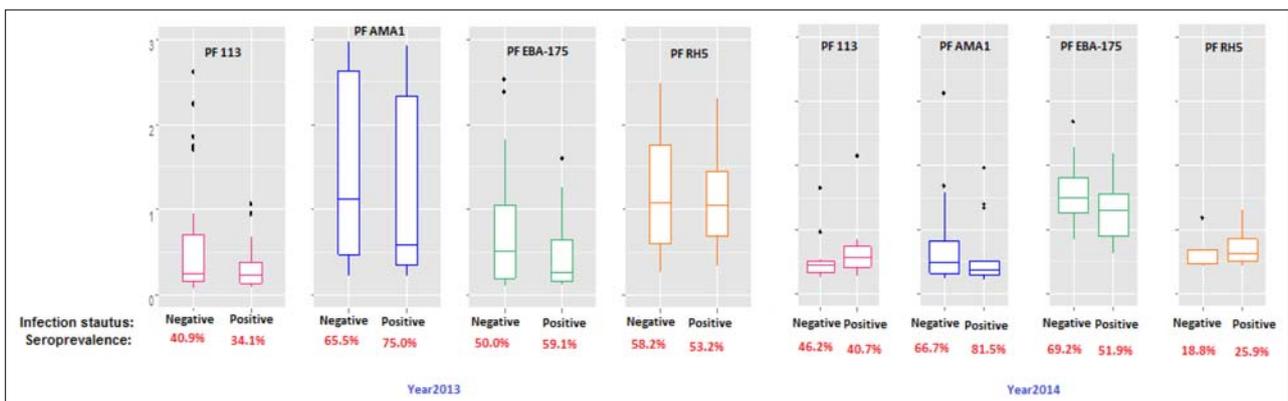
Fig. 5. Distribution of IgG in *P. falciparum*-positive microscopy subjects versus negative microscopy individuals. The box plots indicate interquartile range, central lines represent medians, whiskers indicate the 25th–75th percentiles, and dots are outliers. The differences between seroprevalence were assessed by the Chi-square test both in 2013 and 2014. The *Negative box* express samples that not exhibited infection by microscopy and the *Positive box* express the presence of an infection detected only by microscopy
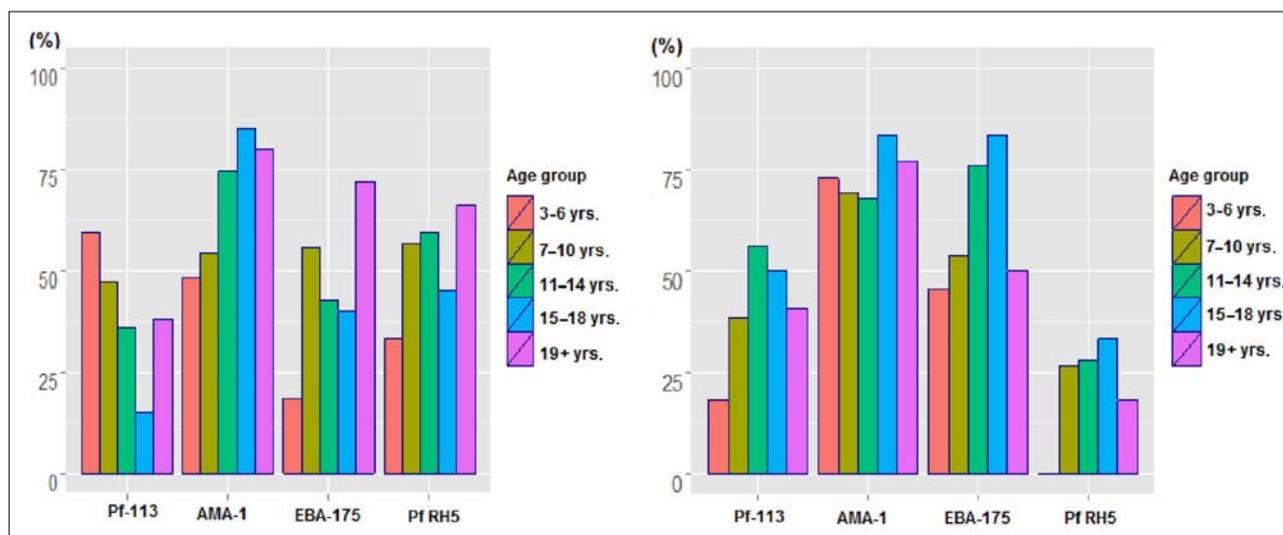
Fig. 6. Prevalence of anti-merozoite total IgG antibodies in each age group. Prevalence of total IgG antibodies for Pf-113, PfAMA1, PfEBA175 or PfRH5 across the indicated age groups

(MI+) versus (MI+) individuals did not show significant difference, whatever the antigens used and the study period (all *p* > 0.05); *(Fig. 5)*.

Harboring a submicroscopic *P. falciparum* infection (SMI) does not generally influence IgG antibody rates and levels in the study population (*p* > 0.05) between SMI versus uninfected individuals in 2013 *(Fig. 5)*. Nevertheless, in 2014, the prevalence rates of Pf-113 and Pf AMA1 anti-IgG were statistically higher in uninfected individuals than in infected subjects (*p* = 0.02 and *p* = 0.001). Moreover, in 2013, the level of PfAMA1 anti-IgG was significantly high in non-infected subjects [median (P25–P75)], 1.143 (0.442–1.844), compared to SMI individuals, 0.665 (0.112–1.218 with *p* = 0.001) *(Fig. 5)*.

Total IgG antibody responses and age of subjects

Overall, the prevalence rates of specific IgG increased with age for all antigens during the study period. However, in 2013, a high prevalence rate (59.26%) of IgG anti-Pf113 was observed in young children aged 3–6 years old which was not confirmed in 2014. The most specific IgG anti-PfAMA1 responses were obtained in adolescents aged 15–18 years old (85%), and the smallest were found in children aged 3–6 years old (48.1%) (*p* = 0.002). Individuals 19 years old and above had the most response for anti-PfEBA175 IgG with a prevalence of 72% in this age group. The prevalence rates of specific IgG anti-PfAMA1 and anti-PfEBA175 in children aged 3–6 years old tended to increase from 2013 to 2014 even if it was not significant *(Fig. 6)*.

Total IgG antibody prevalence rates and levels were both significantly correlated with age; irrespective of the antigens used and the study period. The associations are as follows: OR = 0.367, *p* < 0.001; OR = 0.252, *p* < 0.001; OR = 0.229, *p* < 0.001; and OR = 0.196, *p* < 0.001, for

Pf-113, AMA1, EBA-175, and PfRH5 antibodies, respectively.

Simple linear regression was adjusted for age, sex, and infection status. Age had a significant effect on antibody levels in all antigens compared to the sex and infection status. Analysis also showed an evolution of response with age whatever the antigen and period. Anti-PfAMA1 level was the one which most progressively increased with age, followed by anti-PfEBA175 and anti-PfRH5.

Antibody responses to multiple antigens in children

Globally, in 2014, 14.30% (10/70) of the 3- to 16-year-old individuals' plasmas recognized all the 4 antigens (Pfl13, PfEBA175, PfAMA1, and PfRH5) and 31.45% (22/70) recognized 3 of them. Twenty-five of the 70 plasma samples (35.71%) recognized 2 antigens. Finally, 2 children (2.86%) did not have any detectable IgG antibody directed against the four antigens during the study (data not shown).

Mixed antibody responders and fever

Among the 32 children with antibodies against at least 3 antigens at the same time, 4 (12.5%) had fever at the time of the sampling period. However, the levels of specific IgG PfAMA1 antibodies in these 4 children were slightly lower [mean OD (AU) ± SD: 0.478 (0.138)] than those of the other 28 children who did not have fever [mean OD (AU) ± SD: 0.603 (0.030)] (data not shown).

The evolution of antibodies

Among all the IgG responders, 6 (8.57%) individuals lost the antibodies which they had in 2013 the next year, in 2014 (data not shown).

Discussion

The naturally acquired antibodies to *P. falciparum* merozoite antigens are thought to play a key role in acquired immunity to malaria. Nevertheless, many studies on the protective effect of these anti-merozoite antibodies are sometimes conflicting [40]. Indeed, comparative measurement and analysis of such antibodies in an endemic population, especially in children, over different periods through longitudinal surveys, would increase our knowledge about the gradual development of specific protective immunity and unravel the immunological mechanisms of malaria, a puzzling phenomenon. Three recombinant antigens (PfAMA1, Pf113 and PfRH5) and one peptide (PfEBA175 peptide4) were used to determine the prevalence rates and levels of specific antibodies directed against these antigens in a rural Gabonese endemic population over two periods. This is the first study to compare the immune response between merozoite antigens in Dienga.

Most of our study population had IgG antibodies recognizing these merozoite antigens in 2013 as well as in 2014, therefore showing the involvement of these antigens in the humoral immune response during *P. falciparum* infection. The prevalence rates and levels of IgG antibodies directed against the PfAMA1 antigen in 2013 and 2014 were higher than the prevalence rates and levels of those directed against PfEBA175, PfRH5, and Pf113. This could be related to the immunogenicity of these antigens. Similar results were reported in Mali and Kenya where the IgG antibody reactivity to PfAMA1 was two times higher than the IgG antibody reactivity to PfRH5 [16, 25]. When IgG antibodies are compared between 2013 and 2014, only the prevalence rates of those directed against PfRH5 decreased significantly in 2014. By contrast, the prevalence rates of anti-Pf113, anti-PfAMA1, and anti-PfEBA175 IgG tended to increase between the 2 years without significant difference. The IgG prevalence rates in adults were high in 2013, and a year after, more than half of these enrolled adults were lost. This can explain the decrease of anti-PfRH5 IgG prevalence rates and the increase of anti-Pf113, anti-PfAMA1, and anti-PfEBA175 IgG prevalence rates in 2014. Moreover, our results showed that there were many IgG responders in children precisely in younger children aged 3–6 years old for Pf113, PfAMA1, and PfEBA175 antigens compared to the PfRH5 antigen for which no positive responder was observed in 2014.

According to the *P. falciparum* infection status, the prevalence rates of total IgG and its subclasses directed against Pf113 and PfEBA175 antigens were significantly higher in aparasitemic individuals than in parasitemic subjects *(Fig. 6)*. However, those directed against PfRH5 and PfAMA1 were higher in parasitemic than in aparasitemic subjects, whatever the study period. The levels of anti-EBA-175 and anti-PfRH5 IgG were higher in uninfected subjects than in infected individuals *(Fig. 6)*. Nevertheless, it has been documented that the prevalence rates as well as the levels of acquired antibodies can be influenced by parasite densities [41, 42]. This was not evi-

dently observed in our study and could be linked to the lack of children under 3 years old in our study population. It has also been shown that children below 5 years of age have common unstable immune responses associated with concurrent infections [43]. During the follow-up, all the enrolled subjects were infected at least one time by *P. falciparum* and some of them were infected twice or three times. Nonetheless, since monitoring was performed only six times, the possibility that many other infections were missed may exist. It is not worthy that almost all the enrolled subjects were asymptomatic carrier cases, i.e., individuals harboring parasites without clinical signs. These results showed that all the subjects have had at least one contact with the parasite which is generally polyclonal [44–46] and thus develop an immune response directed against a broad range of *P. falciparum* antigens including Pf113, PfRH5, PfEBA175, and PfAMA1 if they are not restricted. Therefore, the asymptomatic parasites may allow children to build their immune response and such parasites may also protect them from developing symptoms and severe disease [47, 48]. The difference in prevalence rates and/or the levels of IgG antibodies to these four antigens can be associated with seroconversion which occurs sometimes earlier in life depending on the antigens. Also, any significant variation in exposure to particular antigens can probably influence IgG antibody production. Of interest, ITNs were used by 33.47% of the inhabitants of Dienga, and this coverage was lower than the mean coverage observed in nationwide [39]. Finally, harboring a submicroscopic *P. falciparum* infection (SMI) does not influence IgG antibody rates and levels in the study population (SMI versus uninfected individuals) *(Fig. 5)*.

Overall, the prevalence rates and levels of specific IgG were both significantly correlated with age, whatever the antigens used and the study period. The IgG anti-Pf113 was highly prevalent in young children aged 3–6 years old in 2013, and a year after in 2014, this prevalence decreased drastically, perhaps associated with the antibody half-lives. In other words, the seroconversion of Pf113 takes place early in young children followed by an antibody decline in older children and adult individuals. Of interest, a study conducted by White et al. [49] suggested that antibodies from young African children have shorter half-lives than those of older ones. It has also been suggested that some antibodies to merozoite antigens are not boosted by increasing exposure to *P. falciparum* infection [50, 51]. Furthermore, the highest anti-PfAMA1 IgG responders were obtained with 15- to 18-year-old subjects (85%) and the smallest with 3- to 6-year-old children (48.1%). Individuals 19 years old and above were the most responders for anti-PfEBA175 IgG with a prevalence of 72%. The prevalence rates of anti-PfAMA1 and anti-PfEBA175 IgG in 3- to 6-year-old children tended to increase from 2013 to 2014 even it was not significant *(Fig. 6)*. Together, these results suggested that IgG antibodies to PfAMA1, PfEBA175, and Pf113 occur in children earlier than those directed against PfRH5. Seroconversion to PfAMA1 and to PfEBA175 occurred moderately in young children and

reached their maximum in adolescents and in adults without any evidence of age related decline in 2013 and also in 2014 (*Tables 1* and *2*).

All four antigens were specifically recognized by IgG1, IgG2, IgG3, and IgG4 antibodies from a large proportion of the subjects in 2013 and 2014. During the two periods of study, specific IgG4 directed against these antigens were relatively the most prevalent subclass followed by specific IgG1 and IgG3 antibodies notably those against PfAMA1, PfEBA175, and PfRH5 in 2013 and against Pf113, PfAMA1, and PfEBA175 in 2014. Also, the prevalence rates of IgG1 anti-Pf113 and anti-PfAMA1 increased significantly from 2013 to 2014, while the prevalence rates of IgG2 anti-PfRH5 decreased. These results show that *P. falciparum* infection can induce high prevalence rates of both cytophilic (IgG1 and IgG3) and non-cytophilic (IgG4) antibodies to the merozoite antigens and confirm our previous work in that a high proportion of individuals with plasma IgG4 antibodies to PfEBA175 peptide 4 were observed in Dienga [27]. The results also confirmed that a variety of *P. falciparum*-specific antigens predominantly induce IgG1 and IgG3 subclasses [26, 52–55], for example, PfAMA1 and PfEBA175 peptide 4 in the present study.

More interestingly, all our antigens Pf113, PfAMA1, PfEBA175 peptide 4, and PfRH5 induced high levels of specific IgG1 in 2013. This trend was also confirmed in 2014 by PfAMA1 and PfEBA175 peptide 4. On the contrary, the levels of specific IgG1 anti-Pf113 and anti-PfRH5 assessed in 2014 were higher than the levels of the other subclasses, although they did not show a significant difference. PfEBA175 peptide 4 induced high levels of IgG1 in 2013 and high levels of IgG3 only in 2014. The induction of IgG subclasses by malarial antigens is under genetic control and that was evidenced by many previous studies [56–60]. Moreover, several genes were identified to be associated with IgG or IgG subclass levels including hemoglobin β gene (HBB), IL4, TNF, and FCGR2A [61–65]. It was also demonstrated that IgG4 responses to merozoite antigens (PfAMA1 and PfMSP1, -2, and -3) had higher heritability than the other subclasses and therefore the most genetically regulated IgG subclasses [66]. Among all the IgG isotypes, the IgG4 antibody had the lowest levels in 2013 as well as in 2014 whatever the antigen used, even though its prevalence remained relatively high. Both IgG1 and IgG3 antibodies are cytophilic and protective against *P. falciparum,* whereas IgG4 is thought to block the protective mechanisms. However, protection against *P. falciparum* requires both the quality and quantity of the IgG responses to merozoite antigens [67] offering therefore an advantage to cytophilic antibodies over IgG4. Nevertheless, the aim of this study was not to investigate an association between antibody response and protection against malaria. However, during the follow-up, only 8.10% of febrile subjects and 91.90% of asymptomatic individuals were recorded. Interestingly, about 70% of these asymptomatic subjects had a high level of specific IgG1 antibodies, and we can only speculate that

this asymptomatic status at the time of sampling could be logically associated with IgG1 antibodies based on several facts. First, it has been shown that asymptomatic *P. falciparum* infections are associated with a reduced risk of malaria in high transmission areas [44, 45, 48, 68]. Second, Rono et al. demonstrated that children with high levels of IgG antibodies against individual malarial antigens had a lower risk of developing symptoms [69]. Furthermore, in our study, 57/70 children (81.42%) had specific IgG, particularly the IgG1 subclass recognizing at least 2 antigens. Nevertheless, 4 children with fever at the time of sampling were observed among those 32 children who had specific IgG antibodies recognizing at least 3 antigens at the same time. In these four febrile children, the levels of specific IgG antibodies to PfAMA1 were lower compared to those of children without fever in this group.

Of the 370 enrolled subjects, 6 (8.57%) lost their specific IgG antibodies at the end of the follow-up in 2014. Furthermore, during the whole study, 2 subjects (2.86%) did not have any detectable IgG antibody in their plasma samples to any of the four merozoite antigens. Together, these results can be associated with the decline of exposure which follows individual implementation of disease prevention measures and also the short half-life of anti-merozoite antibodies in absence of parasite exposure as described [70].

Conclusion

In conclusion, IgG from plasmas of most of the study population, including young children, recognized merozoite antigens (Pf113, PfAMA1, PfEBA175, and PfRH5). All the enrolled subjects were infected at least one time by *P. falciparum*. The antibody prevalence rates and levels increased with age and varied between the tested antigens. The high levels of specific IgG1 were induced by all the antigens in 2013 and in 2014 by PfAMA1 and PfEBA175. Many asymptomatic children had specific IgG1 recognizing multiple antigens, and these IgG1 antibodies could be associated with the reduced risk of developing malaria symptoms in children at the sampling time. We suggested that our results could be associated with the marked increase (near threefold) of *P. falciparum* infection in Dienga in 2013.

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Authors' contributions

Dr. Irene Pegha-Moukandja realized the field study, laboratory tests, data and statistical analysis, and wrote this article. Dr. Roméo-Karl Imboumy-Limoukou and Miss Nina Tchitoula-Makaya performed part of laboratory

tests. Jean Claude Biteghe-Bi-Essone conducted part of field study and laboratory tests. Augustin-Ghislain Mouinga-Ondembe and Dieudonne Nkoghe Mba contributed in reagents and laboratory facilities. Jean-Bernard Lekana-Douki supervised the field study and laboratory tests, and Fousseyni S. Touré Ndouo designed the study and wrote the article.

Conflict of interest

All authors have declared that they have no competing interests.

Acknowledgements

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