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IgG antibody response against *Plasmodium falciparum* aminopeptidase 1 antigen in Gabonese children living in Makokou and Franceville

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Summary

The search for novel chemical classes of anti-malarial compounds to cope with the current state of chemoresistance of malaria parasites has led to the identification of *Plasmodium falciparum* aminopeptidase 1 (PfA-M1) as a new therapeutic target. PfA-M1, known to be involved in the hemoglobin digestion cascade which helps to provide most of the amino acids necessary to the parasite's metabolism, is currently considered as a promising target for anti-malarial chemotherapy. However, its immunogenic properties have not yet been tested in the Gabonese population. In Gabon, the prevalence of malaria remains three times higher in semi-urban areas (60·12%) than in urban areas (17·06%). We show that malaria-specific PfA-M1 antibodies are present in children and increase with the level of infection. Children living in semi-urban areas have higher anti-PfA-M1 antibody titers (0·14 ± 0·02 AU) than those living in urban areas (0·08 ± 0·02 AU, $P = 0·03$), and their antibody titers increase with age ($P < 0·0001$). Moreover, anti-PfA-M1 antibody titers decrease in children with hyperparasitemia (0·027 ± 0·055 AU) but they remain high in children with low parasite density (0·21 ± 0·034 AU, $P = 0·034$). In conclusion, our results suggest that malaria-specific PfA-M1 antibodies may play an important role in the immune response of the host against *P. falciparum* in Gabonese children. Further studies on the role of PfA-M1 during anemia are needed.

Keywords: antibody, Gabon, humoral immunity, malaria infection, *Plasmodium falciparum* aminopeptidase 1 (PfA-M1)

Introduction

Despite recent improvements in reducing the burden of malaria in some endemic areas of seasonal or sporadic transmission [1], malaria is still a major public health problem and remains one of the most important causes of morbidity and mortality in many sub-Saharan African countries. According to the World Health Organization, approximately 216 million people were infected and 445 000 deaths due to malaria occurred worldwide in 2016 [2]. In addition, more than 90% of the deaths due to malaria in sub-Saharan Africa occur in children aged under 5 years, with *Plasmodium falciparum* being the most virulent species. Indeed, 285 000 deaths due to malaria were recorded in this population in 2016 [2].

Intensified malaria control strategies have resulted in a decline in deaths by malaria in recent years; therefore, many countries are turning to malaria eradication programs [3,4]. In order to lead an effective eradication of malaria transmission a combination of control strategies is necessary, which includes the use of rapid diagnostic tests for all

suspected cases of malaria, artemisinin-based combination therapy, insecticide-treated nets, indoor residual sprays, intermittent preventive treatment for pregnant women [4–6] and the development of an effective malaria vaccine.

The use of passive transfer of immunoglobulin from immune African adults to treat children infected with *P. falciparum* and with severe malaria provides evidence that antibodies are important mediators in the protective response to malaria [7]. The induction of this protective humoral immune response is an essential element which supports the efforts of the malaria research community towards the development of an effective vaccine against malaria. Thus, several parasite antigens were identified as targets of natural immunity, among which a vast majority are expressed during the blood stage. Indeed, vaccines targeting antigens expressed during the blood stage have been evaluated for their ability to prevent the disease [8–11]. Many studies suggest that protection against malaria depends on high concentrations of antibodies [12–16]. Previous studies have shown that high levels of antibodies to apical membrane antigen 1 (AMA-1), merozoite surface protein 1 (MSP1–19) and MSP2 are significantly associated with protection against clinical malaria in older children [13]. The anti-erythrocyte-binding antigen 140 (EBA140), EBA175 and EBA181 responses were associated most particularly with protection against clinical malaria in older children [14], but were also associated with a higher risk of clinical malaria in young children. In young children who acquire immunity, higher levels of antibodies to *P. falciparum* merozoite antigens are not protective but rather predictive of an increased risk of malaria development. Also, the anti-MSP2, AMA-1, EBA175, EBA140 and EBA181 responses were significantly associated with an increased risk of malaria in young children. Interestingly, the responses to MSP1-19 were not significantly associated with this risk [17]. Similarly, anti-glutamate-rich protein region 2 (GLURP-R2) antibodies were associated with reduced risk of symptomatic malaria infection in African children [18]. However, although antibodies to merozoite antigens are considered important targets of protective antibodies, epidemiological evidence of the protective effect of naturally acquired anti-merozoite responses is not specific, and studies in different cohorts or areas of transmission sometimes provide conflicting results [19]. There could be many reasons for these inconsistencies. In malaria-endemic areas the rate at which natural immunity develops is dependent on age, intensity, stability of exposure, endemicity of malaria and clinical incidence [19]. Other considerations in immuno-epidemiological studies, such as recognition of multiple antigens in combination rather than just a single antigen, are probably required for protection [20,21]. Also, although merozoite antigens have been of particular interest in the search for vaccine candidates, it would be useful to consider the immunogenic potential of other parasite antigens such as proteins involved in hemoglobin breakdown during the parasite's erythrocyte cycle. Very little attention has been paid to assess the immunogenic potential of the proteins involved in hemoglobin breakdown during the parasite's erythrocyte cycle. During the erythrocytic replication cycle, *P. falciparum* endocytoses and catabolizes the soluble erythrocytic proteins hemoglobin [22,23]. Hemoglobin catabolism provides the amino acids necessary for protein synthesis in the general metabolism [24]. This process of liberation of free amino acids requires the implication of two aminopeptidases, among which is *P. falciparum* aminopeptidase 1 (PfA-M1) [25–27]. PfA-M1, also called *P. falciparum* M1-family alanyl aminopeptidase (PfA-M1/M1AAP), a validated target for anti-malarial drug development [28], is a neutral zinc-aminopeptidase belonging to the M1-aminopeptidase family [27,29]. Encoded by the PF3D7_1311800.1 gene located in chromosome 13 [30], PfA-M1 is composed of 1085 amino acids, and its first 30 amino acids form a signal peptide [26,27,30]. Biochemical and enzymatic studies using the purified native enzyme have revealed that this protein of approximately 126 kDa exists in three major maturation forms of approximately 120, 96 and 68 kDa named p120, p96 and p68 [25,27,31–33]. The p35 C-terminal domain remains associated with p68 to form the p68/p35 enzyme complex, and a recombinant form of the enzyme corresponding to monomeric p96 has been produced and studied [31,32,34,35]. In addition to its key role in hemoglobin degradation, PfA-M1 could have a possible additional role in late stages of parasite development. Indeed, the treatments by a T5 inhibitor specific for PfA-M1-induced morphological alterations, notably in both trophozoite and schizont stage parasites of the *P. falciparum* FcB1 strain [31]. Thus, the gathering of evidence on the biological functions of PfA-M1, particularly its involvement in the breakdown of hemoglobin but also its potential role in the late stages of parasite development, suggests that this protein could be a potential vaccine candidate in malaria vaccine development.

Gabon is a hyperendemic country where malaria transmission is perennial. The implementation of new anti-malarial policies since 2005 has led to a decline in the malaria burden in urban areas, but was followed by a recrudescence a few years later. However, no change in the prevalence of infection has been observed in rural areas [36–39]. Gabonese children, like the majority of children living in endemic sub-Saharan Africa countries, are most at risk of clinical and severe malaria, probably because of their lack of protective immunity to malaria [40]. The acquisition of protective immunity to malaria depends on the age and level of parasite exposure. Thus, after repeated exposure to malaria parasites, individuals develop effective immunity to control parasitemia and

prevent severe complications [41]. Our previous studies, comparing the humoral response against plasmodial antigens among Gabonese children from urban and rural areas, support this idea by revealing high levels of immunoglobulin (Ig)G antibodies against Pf113 antigen in children from rural areas and more exposed to plasmodial infection. [42,43]. Unlike IgG, IgM responses are not influenced by either clinical or parasitological status in malaria in Gabonese children [44]. Also, in this study we evaluated the humoral IgG immune response against the PfA-M1 antigen in Gabonese children with *P. falciparum* clinical malaria.

Materials and methods

Study area and participants

This study was conducted at a medical center and the Hôpital Régional Omar Bongo Ondimba (Omar Bongo Ondimba Regional Hospital) in Makokou and the Hôpital de l'Amitié Sino-Gabonaise (Chinese–Gabonese Friendship Hospital) in Franceville. Makokou is the administrative capital of the Ogooué-Ivindo Province and a semi-urban area in North-Eastern Gabon (0°33′33″ N and 12°50′48″ E). Franceville is the administrative capital of the Haut-Ogooué Province and an urban area in South-Eastern Gabon (1°37′15″ S and 13°34′58″ E) (Fig. 1). From November 2015 to February 2016, 684 children aged 0–15 years who came to the pediatric ward and presented a febrile syndrome or a history of fever during the previous 48 h were included in the longitudinal study. Patients with malaria were characterized by the presence of *P. falciparum* infection confirmed by a positive rapid diagnostic test and light microscopy. The enrollment exclusion criteria were a temperature of ≤ 37·5°C, lack of written informed consent, signs of other affections without fever or history of fever and an age above 16 years. The children were classified according to their hemoglobin (Hb) level: unanemic malaria (UAM: Hb > 10 g/dL; n = 31), mild malaria anemia (MMA: 5 ≤ Hb ≤ 10 g/dL; n = 101) and severe malaria anemia (SMA: Hb < 5 g/dL; n = 19). Written informed consent was obtained from the parents or the legal guardians of the children. This study was approved by the Gabonese National Research Ethics Committee (PROT no. 0023/2013/SG/CNE).

Blood samples

Venous blood samples (2·0–5·0 ml) were collected using aseptic techniques into ethylenediamine tetraacetic acid (EDTA) vacutainer tubes. Of the 684 children included in this study, only 405 were randomly selected for the total anti-PfA-M1 IgG assay (235 from Makokou and 170 from Franceville). Blood smears were stained with Giemsa,

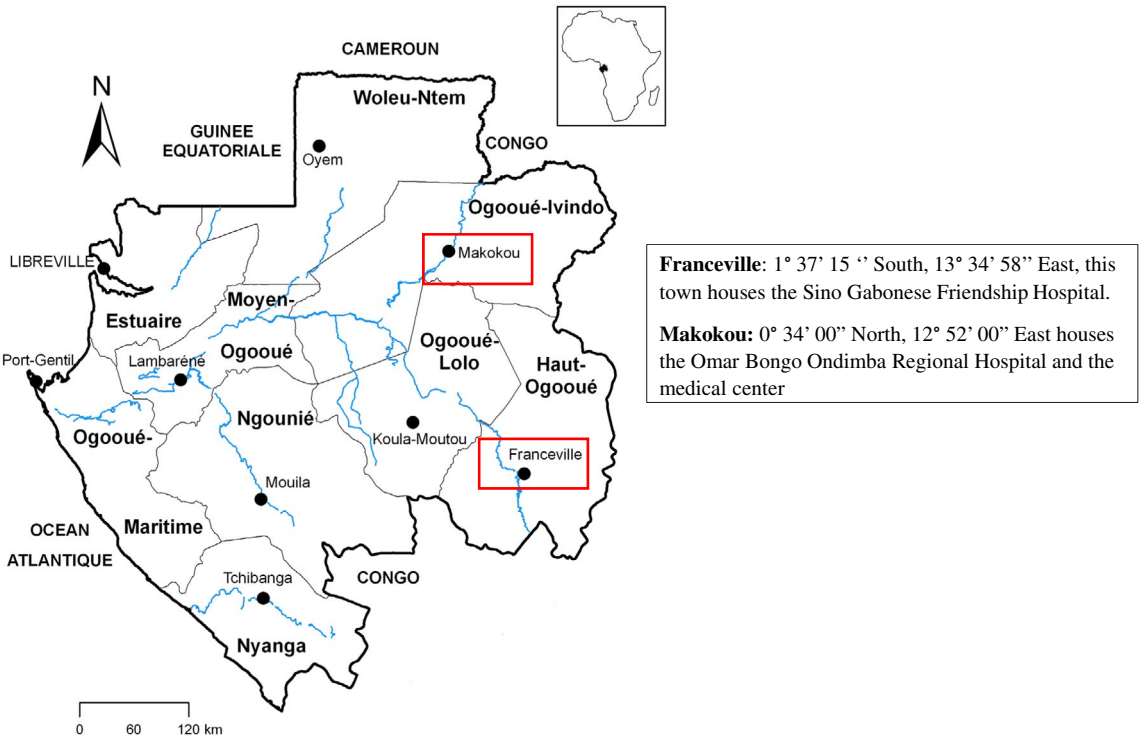

Fig. 1. Map of Gabon indicating the geographical location of the study sites (source: Laboratoire National de Cartographie, 1983).

according to the Lambaréné method for microscopic *P. falciparum* identification and quantification [45]. All slides were examined by two well-trained microscopists from the Medical Center and Regional Hospital in Makokou, and two experienced microscopists from the Centre International de Recherches Médicales de Franceville (CIRMF, International Medical Research Center of Franceville). Hemoglobin, red blood cells, white blood cells and platelets were measured with an automated device (Beckman Coulter AcT diff2; Beckman Coulter Corporation, Miami, FL, USA). Plasma was separated by centrifugation, aliquoted and stored at −80°C until use.

Antigen

PfA-M1 (FcB1 strain, which is identical to that of the 3D7 strain) was provided by the MCAM UMR 7245 as a recombinant protein from position 192 to 1085 of the native enzyme. The expression and purification of this recombinant protein have been described previously [31,46]. Briefly, the synthetic gene (Genecust, Ellange, Luxembourg) encoding residues 192–1085 of the native PfA-M1 (PlasmoDB PF3D7_1311800) was cloned into the pET45b (Novagen; Merck KGaA, Darmstadt, Germany) vector which appended a N-terminal hexahistidine tag (*Knp*I and *Sal*l sites). This PfA-M1 recombinant protein was expressed in *Escherichia coli* Rosetta 2 (DE3) bacteria (Novagen), purified from clarified lysates on a HisTrap column (GE Healthcare, Chicago, IL, USA). Protein was finally purified from eluted fraction using an Äkta purifier chromatography system (GE Healthcare).

Measurement of antibody levels

The IgG responses against PfA-M1 were assessed by an enzyme-linked immunosorbent assay (ELISA) technique. Maxisorp microtiter plates (Nunc-MaxiSorp®; Thermo Scientific, Fremont, CA, USA) were coated with 10 ng/µl antigen in phosphate-buffered saline (PBS) at 4°C overnight and washed four times in PBS containing 0·05% Tween 20 (PBS-Tween 20). The plates were blocked with PBS-3% skimmed milk for 1 h at 37°C and washed four times with PBS-Tween 20. Plasma samples at 1 : 500 in PBS-1% skimmed milk were added in duplicate (100 µl/well) and incubated at 37°C for 1 h 30 min. After washing the unbound antibody four times with PBS-Tween 20, the plates were incubated for 1 h 30 min at 37°C with peroxidase-conjugated mouse anti-human IgG (Sigma-Aldrich, St Louis, USA) at 1 : 10000 dilution in PBS-1% skimmed milk. The plates were washed four times with PBS-Tween 20 and a tetramethylbenzidine substrate (TMB) solution (Thermo Scientific) was added and incubated for 30 min in the dark at room temperature. The reaction was stopped with 50 µl of 1 M sulfuric acid (H_2SO_4) per well. The plates were read at 450 nm with a reference at 620 nm in microplate reader (Sunrise™; Tecan, Männedorf, Zürich, Switzerland). Standardization of the assays was achieved using positive control plasma pools on each plate. Positive control plasmas were obtained from positive Gabonese adults living in malaria hyperendemic areas, and negative controls were European (never exposed to malaria) plasma samples. Background values (wells with no plasma) were deducted from the mean of each sample, and the cut-off threshold for positivity was determined as the mean plus 3 standard deviations (s.d.) from the 10 naive plasma samples included in each assay. All samples were tested in duplicate and results were expressed in arbitrary units.

Statistical analysis

The data collected were entered into an Excel spreadsheet and analyzed using the IBM® SPSS Statistics version 21.0 (SPSS Inc., Chicago, IL, USA). Pearson's χ^2 for categorical variables, Student's *t*-test and analysis of variance (ANOVA) for the comparison of group means were used; to compare multiple groups of data, the non-parametric Kruskal–Wallis test was used; and the Mann–Whitney *U*-non-parametric test was used for pairwise comparisons.

Table 1. Demographical and hematological parameters of the patients included in the study (mean ± s.d.)

	Makokou children			Franceville children		
	Infected (n = 309)	Uninfected (n = 205)	P	Infected (n = 29)	Uninfected (n = 141)	P
Age (months)	44·31 ± 2	35·05 ± 2·56	0·004	77·24 ± 7·75	55·8 ± 3·48	0·012
Hemoglobin (g/dL)	7·41 ± 0·12	9·01 ± 0·14	< 0·00001	9·2 ± 0·35	9·14 ± 0·21	0·9
Red blood cells (10^6 cells/mm^3)	3·32 ± 0·06	4·15 ± 0·06	< 0·00001	3·9 ± 0·12	3·93 ± 0·29	0·4
White blood cells (10^3 cells/mm^3)	10·62 ± 0·36	12·62 ± 0·53	0·001	6·5 ± 0·59	7·25 ± 0·39	0·36
Platelets (10^3 cells/mm^3)	209·61 ± 6·72	357·25 ± 10·72	< 0·0000001	171·96 ± 19·32	327·38 ± 16·77	< 0·0001
Parasitemia (parasites/µl)	44804 ± 4076					

Age, leukocyte counts, hemoglobin concentrations and parasite densities in infected and uninfected children. *Plasmodium falciparum*-exposed children who tested negative for parasites in thick blood smears and negative in rapid detection test kits for *P. falciparum* were defined as uninfected children. Statistical significance was calculated using a one-way analysis of variance (ANOVA) test; s.d. = standard deviation.

Correlation was performed using dot-plot and Spearman's rank non-parametric test. The statistical significance was set at $P < 0{\cdot}05$. In order to evaluate the variation of the anti-PfA-M1 humoral response, the samples were subdivided according to infection, age, type of malaria anemia and parasitemia.

Results

Clinical and biological characteristics of the study population

Table 1 summarizes the main characteristics of all the children who participated in this study. A total of 684 children aged from 6 to 180 months were included, among whom 338 had *P. falciparum* infection [309 (60·12%) from Makokou and 29 (17·06%) from Franceville, and 346 were uninfected by the malaria parasites (205 from Makokou and 141 from Franceville)].

In the semi-urban area of Makokou, mean parasitemia was 44 804 ± 4076 parasites/µl. Infected children were older (44·31 ± 2 months) than uninfected children (35·05 ± 2·56 months; $P = 0{\cdot}004$). Infected children had significantly lower hemoglobin levels (7·41 ± 0·12 g/dL) than uninfected children (9·01 ± 0·14 g/dL, $P < 0{\cdot}0001$). Red blood cell ($3{\cdot}32 \pm 0{\cdot}06 \times 10^6$ cells/mm^3), white blood cell ($10{\cdot}62 \pm 0{\cdot}36 \times 10^3$ cells/mm^3) and platelet ($20{\cdot}9{\cdot}61 \pm 6{\cdot}72 \times 10^3$ cells/mm^3) counts were also significantly lower in infected children than in uninfected children ($4{\cdot}15 \pm 0{\cdot}06 \times 10^6$ cells/mm^3, $12{\cdot}62 \pm 0{\cdot}53 \times 10^3$ cells/mm^3 and $357{\cdot}25 \pm 10{\cdot}72 \times 10^3$ cells/mm^3, $P < 0{\cdot}0001$, 0·001 and < 0·0001, respectively).

In the urban region of Franceville, infected children were also older (77·24 ± 7·75 months) than uninfected children (55·8 ± 3·48 months, $P = 0{\cdot}012$). Infected children had significantly lower levels of platelet ($171{\cdot}96 \pm 19{\cdot}32 \times 10^3$ cells/mm^3) counts than uninfected children ($327{\cdot}38 \pm 16{\cdot}77 \times 10^3$ cells/mm^3, $P < 0{\cdot}0001$). White blood cell counts were lower in infected children ($6{\cdot}5 \pm 0{\cdot}59 \times 10^3$ cells/mm^3) than in uninfected children ($7{\cdot}25 \pm 0{\cdot}39 \times 10^3$ cells/mm^3), but the difference was not statistically significant. In addition, there was no difference regarding hemoglobin levels and red blood cell counts between the two groups.

Anti-PfA-M1 IgG response according to the living area and age of children

Malaria-specific PfA-M1 antibodies were assessed in the plasma samples from 405 children (235 from Makokou and 170 from Franceville). Data analysis between the two areas showed that the children from Makokou had significantly higher levels of anti-PfA-M1 antibodies (0·14 ± 0·02 AU) compared to children from Franceville (0·08 ± 0·02 AU, $P = 0{\cdot}03$; Fig. 2). When comparing infected and uninfected children from Makokou, infected children had significantly higher levels of anti-PfA-M1 antibodies (0·2 ± 0·027 AU) than uninfected children (0·08 ± 0·023 AU; $P = 0{\cdot}004$, Fig. 3). In Franceville, infected children also had significantly higher levels of anti-PfA-M1 antibodies (0·21 ± 0·05 AU) than uninfected children (0·05 ± 0·02 AU, $P = 0{\cdot}003$, Fig. 3).

When analyzing the data according to age categories between the two areas, the children from Makokou aged 49–96 months had significantly higher titers of anti-PfA-M1 antibodies (0·213 ± 0·035 AU) than the children from Franceville of the same age group (0·107 ± 0·046 AU; $P = 0{\cdot}006$). The same difference was also observed when comparing children aged 97–132 months between the two localities (0·403 ± 0·078 AU for Makokou and 0·126 ± 0·064 AU for Franceville; $P = 0{\cdot}012$). No difference was observed when comparing children aged 0–6, 7–48 or 133–180 months between the two areas.

When comparing children from Makokou, the anti-PfA-M1 response was significantly different between age groups ($P < 0{\cdot}0001$, Fig. 4). Anti-PfA-M1 response increased with age until 132 months. Strong responses against PfA-M1 were observed in children aged 49–96 (0·2 ± 0·035 AU), 97–132 (0·4 ± 0·08 AU) and 133–180 months (0·42 ± 0·13 AU). However, low responses were observed in children younger than 49 months (0·045 ± 0·06 AU for 0–6 and 0·068 ± 0·02 AU for 7–48 months). When comparing different age groups, the children younger than 6 months had significantly lower antibody titers compared to children aged 49–96 ($P = 0{\cdot}03$), 97–132 months ($P = 0{\cdot}003$) and children aged 133–180 months ($P = 0{\cdot}02$). The same differences were observed between the children aged 7–48 months and the children aged 49–96 ($P = 0{\cdot}0001$), 97–132 ($P = 0{\cdot}0001$) and 133–180 months ($P = 0{\cdot}003$). Similarly, a significant difference in antibody levels was observed between children aged 49–96 months (0·02 ± 0·035 AU) and those aged 97–132 months (0·4 ± 0·08 AU, $P = 0{\cdot}02$, Fig. 4a).

However, the comparison of antibody titers for the children from Franceville showed no significant difference between age groups (Fig. 4b).

Association between anti-PfA-M1 antibody response, parasitemia and age

Because parasite density could influence antibody production during infection, we analyzed whether anti-PfA-M1 antibody levels correlated with parasitemia density in infected children from the semi-urban area of Makokou. This analysis revealed no significant association between antibody level and parasite density. Nevertheless, a suggestive correlation has been observed between antibody level and parasitemia in these children ($R = 0{\cdot}2$, $P = 0{\cdot}06$) (Fig. 4a). Thus, an association is observed between anti-PfA-M1 antibody levels and age in these children ($R = 0{\cdot}4$, $P < 0{\cdot}0001$). No association was observed between

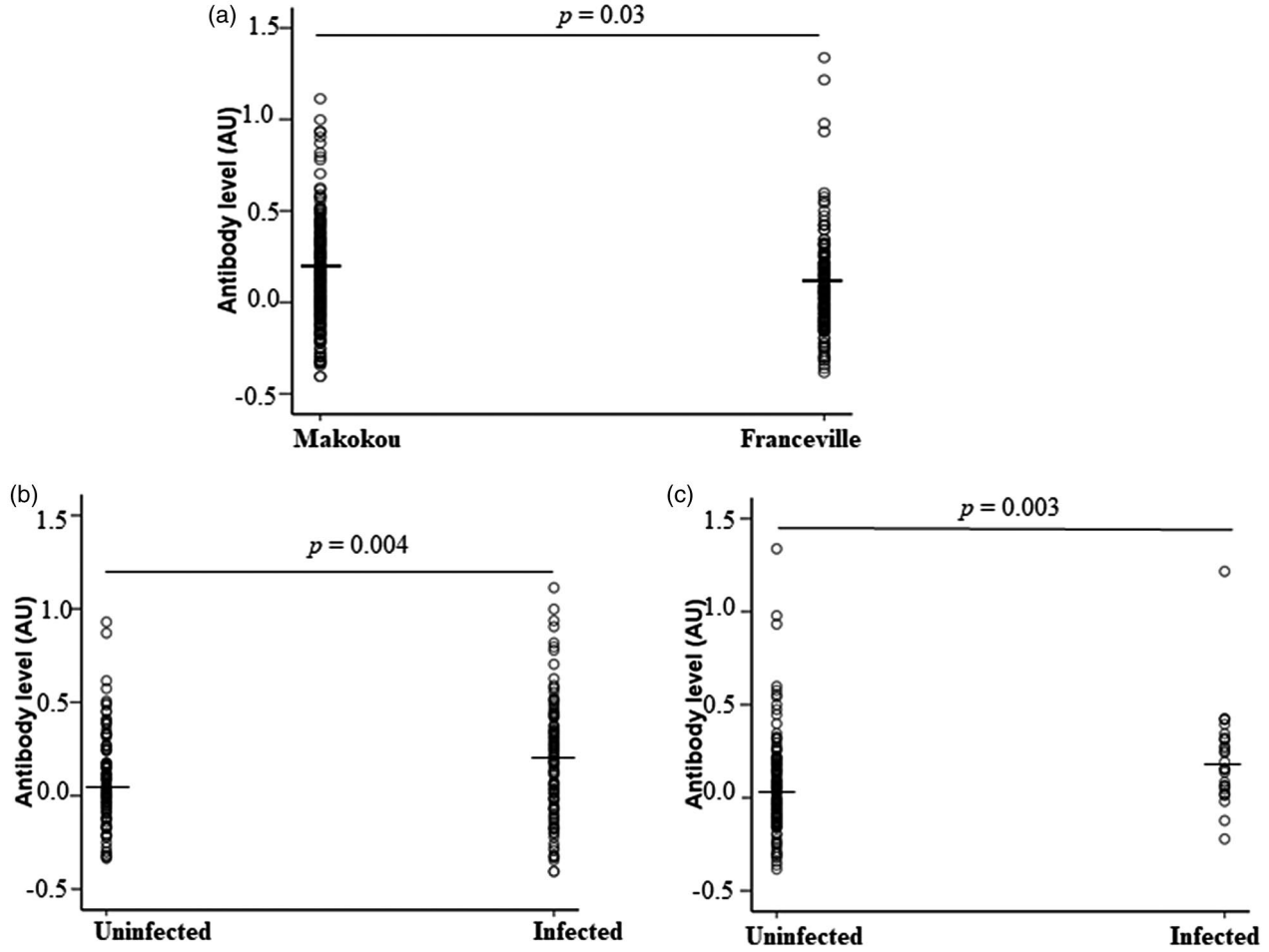

Fig. 2. Malaria-specific anti-*Plasmodium falciparum* aminopeptidase 1 (PfA-M1) antibody levels in response to *P. falciparum* exposition in children from Makokou and Franceville. Malaria-specific PfA-M1 antibodies in plasma from Makokou and Franceville children were assayed by enzyme-linked immunosorbent assay (ELISA). (a) PfA-M1 antibody levels in 405 plasma from these children were assayed. All the children were subdivided according to the living area. Plasma from 235 children from Makokou and 170 children from Franceville were analyzed in technical duplicate. The mean antibody concentrations for children from Makokou and Franceville are indicated on the figure. Plasma concentrations of anti-PfA-M1 in children from (b) Makokou and (c) Franceville were quantified in *Plasmodium*-uninfected and -infected children. *P. falciparum*-exposed children who tested negative for parasites in rapid detection test kits and blood smears were defined as uninfected children. (b) Plasma from 125 uninfected and 110 infected children from Makokou were analyzed in technical duplicate. (c) Plasma from 141 uninfected and 29 infected children from Franceville were analyzed in technical duplicate. The mean antibody concentrations for uninfected and infected children from Makokou and Franceville are indicated on the figure. Each circle represents the mean level of PfA-M1 antibody concentration in each child. Statistical significance was calculated using pairwise comparisons using the Mann–Whitney *U*-test.

parasitemia and age (Table 2). To evaluate the change in levels of antibody production as a function of the parasite load, infected children from the semi-urban area of Makokou were divided into five groups according to mean *P. falciparum* density. Although no association has been observed between antibody levels and parasitemia, this analysis revealed that antibody production was significantly higher in children with a low parasite density (1001–40 000; 0·21 ± 0·034 AU) than in those with a very high parasite density (> 120 000; 0·027 ± 0·055 AU; P = 0·03). No significant difference was found between anti-PfA-M1 responses according to the parasite density in others groups (Fig. 4b).

Anti-PfA-M1 antibody response according to anemia

We then analyzed anti-PfA-M1 antibody response in children from Makokou and Franceville with UAM, MMA and SMA. In the children from Makokou, the antibody response differed significantly across the clinical groups (P = 0·002). Compared to the uninfected children (0·08 ± 0·02 AU), the antibody levels increased significantly in both children with simple malaria (0·2 ± 0·06 AU,

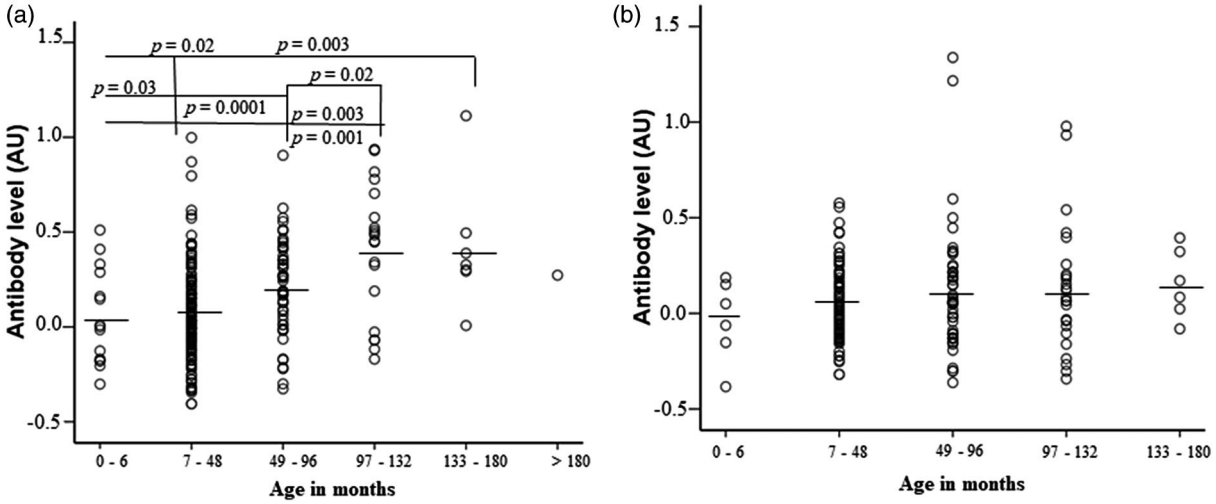

Fig. 3. Levels of malaria-specific *Plasmodium falciparum* aminopeptidase 1 (PfA-M1) antibodies in children from Makokou and Franceville according to age. Plasma concentrations of anti-PfA-M1 antibodies in children from (a) Makokou and (b) Franceville were quantified by enzyme-linked immunosorbent assay (ELISA). Children were subdivided into six groups according to their age. Group 1 consisted of children aged 0–6 months, group 2 of children aged 7–48 months, group 3 of children aged 49–96 months, group 4 of children aged 97–132 months, group 5 of children aged 133–180 months and group 6 included children more than 180 months of age. (a) The anti-PfA-M1 antibody level in plasma from children from Makokou for groups 1 (n = 15), 2 (n = 142), 3 (n = 51), 4 (n = 20), 5 (n = 7) and 6 (n = 1) were analyzed. (b) The anti-PfA-M1 antibody level in plasma from children from Franceville for groups 1 (n = 6), 2 (n = 83), 3 (n = 49), 4 (n = 26) and 5 (n = 6) were analyzed. In both parts of the figure, data are shown as mean values of duplicate measurements obtained from each individual in each age group. Each circle represents the mean level of PfA-M1 antibody concentrations from each child. The mean antibody concentrations from each age group are indicated on the figure. Kruskal–Wallis tests and pairwise comparisons using Mann–Whitney *U*-tests were used for the calculation of statistical significance.

P = 0·03; Fig. 5a) and MMA (0·2 ± 0·04 AU, P = 0·01). However, no difference in antibody production was observed between the different clinical groups with anemia. In children from Franceville, the same difference was observed (P < 0·0001). The comparison between the different groups revealed that the children with UAM (0·17 ± 0·05 AU) and MMA (0·27 ± 0·07 AU) had higher antibody levels than uninfected children (0·05 ± 0·02 AU; P = 0·03 and P = 0·0005, respectively, Fig. 5b). No significant difference in antibody levels was observed between the different clinical groups with anemia.

Discussion

This study on Gabonese children is the first, to our knowledge, to evaluate the immunogenicity of PfA-M1, whose main role in the life cycle of *P. falciparum* is to provide amino acids necessary to the survival, growth and intra-erythrocyte development of the parasite. We assessed the difference in PfA-M1 antibody levels between children infected and uninfected with *P. falciparum* living in two areas with different epidemiological contexts in Gabon. The study confirmed a low prevalence of malarial infection in the urban area (Franceville) compared to the semi-urban area (Makokou), which had a very high prevalence of malaria, as previously described [37,47,48]. We observed that the mean age of infected children was significantly higher than the mean age of uninfected children in both sites. These data are consistent with results from previous studies conducted in different areas of Gabon, which showed similar patterns in terms of malaria prevalence according to the age of infected children [37,39,49]. Indeed, breastfeeding protects young children against malaria. In Gabon, it has been demonstrated that the prevalence of *P. falciparum* malaria remains significantly higher in rural and semi-urban areas compared to urban areas [47,50]. In addition, the mean age of children developing clinical malaria has increased significantly [42,48,50]. Thus, the difference in malaria prevalence between the two areas could be due, on one hand, to better management of children aged under 5 years by the anti-malarial programs in Gabon and on the other hand to poor access to malaria control measures in semi-urban areas. This difference in malaria prevalence could also be explained by socio-economic level [50] and heterogeneity of transmission in Gabon, which have been demonstrated in other endemic areas [51,52]. Similarly, in this study anemia remained significantly higher in infected children living in the semi-urban area (Makokou). Although the etiology of anemia in tropical areas is multi-factorial, our data are consistent with several other studies showing that anemia during *P. falciparum* malaria is closely associated with

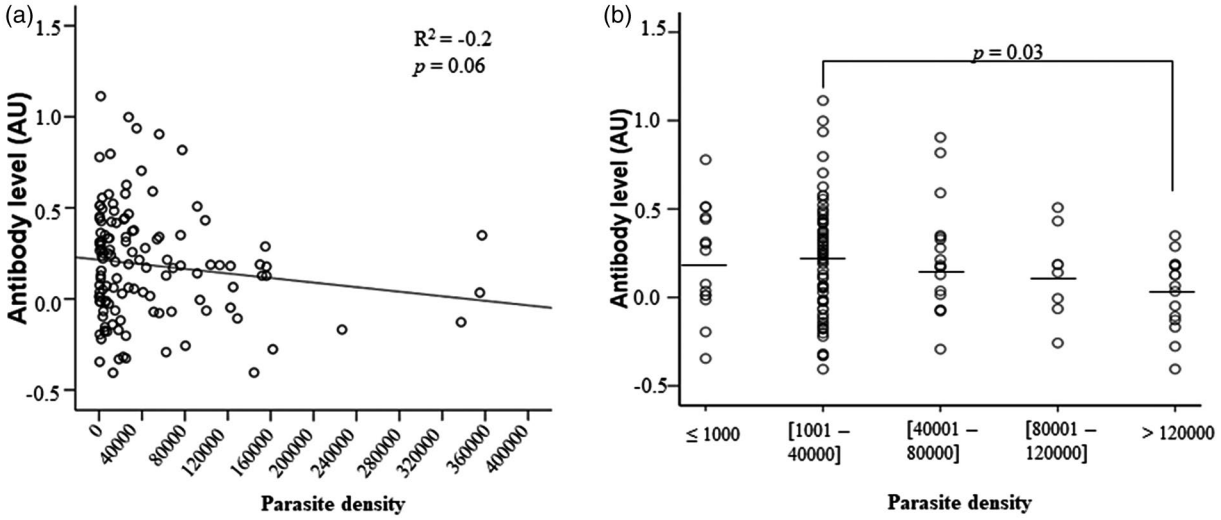

Fig. 4. Correlation between the malaria-specific anti-*Plasmodium falciparum* aminopeptidase 1 (PfA-M1) antibody levels and parasitemia in children from Makokou. Plasma concentrations of malaria-specific antibodies were quantified in *P. falciparum*-infected children from Makokou by enzyme-linked immunosorbent assay (ELISA). (a) Dot-plot represents the correlation analysis between malaria-specific antibodies production and the parasite density in Makokou infected children. Data are shown as mean values of duplicate measurements obtained from each individual. Each circle represents the mean value of antibody concentration from each child. Spearman's rank non-parametric test using for correlation analysis. (b) Anti-PfA-M1 antibody level according to the parasitemia groups in children from Makokou. The children were subdivided into five groups based on their parasite density. The 'very low density' group was characterized by a parasite density of ≤ 1000 parasites/μl of blood (n = 13). The 'low density' group was defined by a parasite density from 1001 to 40 000 parasites/μl blood (n = 71). 'Medium', 'high' and 'very high density' groups were defined by a parasite density from 40 001 to 80 000 parasites/μl (n = 18) and from 80 001 to 120 000 parasites/μl (n = 8), and > 120 000 parasites/μl of blood (n = 15), respectively. Data are shown as mean values of duplicate measurements obtained from each individual in each parasite density group. Each circle represents the mean value of antibody concentration from each child in each parasite density group. The mean antibody concentrations for each parasite density group are indicated on the figure. Kruskal–Wallis tests and pairwise comparisons using Mann–Whitney U-tests were used to calculate statistical significance.

Table 2. Correlation of malaria-specific anti-PfA-M1 antibodies levels with age and parasitemia in children from Makokou

	Parasitemia	Age	PfA-M1
Parasitemia	1	0·2 (0·1)	0·2 (0·06)
Age		1	0·4 (< 0·0001)
PfA-M1			1

Spearman's rank correlation analysis was carried between parasitemia, age and anti- *Plasmodium falciparum* aminopeptidase 1 (PfA-M1) antibody levels in Makokou children. Correlation coefficient is given. Correlation is significant at $P < 0·05$.

malaria parasitemia [40,52–55]. The high prevalence of anemia in rural areas could be due to the high circulation of intestinal parasites in these areas [56].

A significantly higher seroprevalence was found in the semi-urban area. Compared to the children living in Franceville, the data showed that children living in Makokou, where malaria prevalence was highest, had significantly higher levels of anti-PfA-M1 antibodies, suggesting that the acquisition of host immunity could vary according to the living area. This may be due to the fact that the level of malaria transmission is most intense in semi-urban areas, thus providing a better immunity boost [51]. Our findings on the PfA-M1 antigen, which show the heterogeneity of antibody levels between the two localities, is consistent with one of our previous studies, which highlighted that the anti-Pf-AMA1 and anti-PfRh5 antibody levels were significantly higher in rural areas than in urban settings [42]. These same data have also been demonstrated in Senegal, Uganda and Indonesia, where a variation of antibody levels of the vaccine candidates such as AMA1, MSP1 and circumsporozoite protein (CSP) was observed between rural, peri-urban and urban areas [52,57,58].

In both study sites, data showed that the level of anti-PfA-M1 antibodies was higher in infected than uninfected children. These findings suggest a stimulation of antibodies during acute phases of malaria. The study showed that the antibody level against PfA-M1 increased with age at both sites, especially in Makokou, with statistically significant differences between different age groups. The antibody level was higher in children with *P. falciparum* infection compared to children without *P. falciparum* infection. These findings demonstrate that IgG levels and seroprevalence of anti-PfA-M1 antibodies increase with age; this is consistent with previous immunological studies on

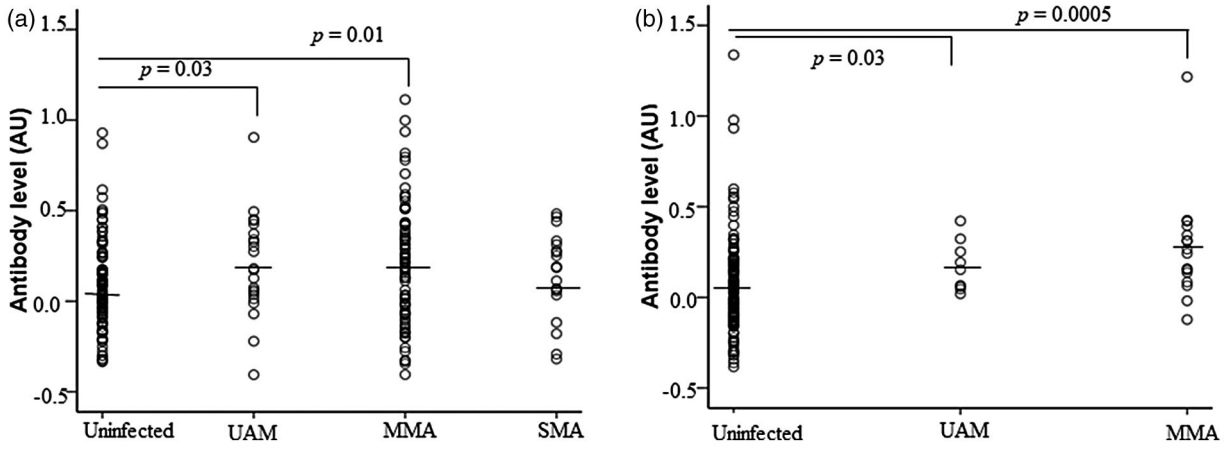

Fig. 5. Levels of malaria-specific anti-*Plasmodium falciparum* aminopeptidase 1 (PfA-M1) antibodies in children from Makokou and Franceville according to malaria anemia status. Plasma concentrations of anti-PfA-M1 antibodies in *P. falciparum*-exposed children from (a) Makokou and (b) Franceville were quantified by enzyme-linked immunosorbent assay (ELISA). Children were subdivided into the following groups: uninfected, malaria with unanemic malaria (UAM): Hb > 10 g/dl, mild malarial anemia (MMA: 5 ≤ Hb ≤ 10 g/dl) and severe malarial anemia (SMA: Hb < 5 g/dl). (a) Anti-PfA-M1 antibody levels in plasma from children from Makokou for uninfected subjects (n = 110), UAM (n = 22), MMA (n = 85) and SMA (n = 19) were analyzed. (b) Anti-PfA-M1 antibody levels in plasma from children from Franceville for uninfected subjects (n = 141), UAM (n = 9) and MMA (n = 16) were analyzed. In both parts of the figure, data are shown as mean values of duplicate measurements obtained from each individual in each clinical group. Each circle represents the mean level of PfA-M1 antibody concentration from each child. The mean antibody concentrations from each clinical group are indicated on the figure. Kruskal–Wallis tests and pairwise comparisons using Mann–Whitney *U*-tests were used for the calculation of statistical significance.

other vaccine candidates, which showed that the magnitude of antibody responses was associated with an increase in age [57]. Another study on the protection against clinical malaria in Ghana reported that the levels of IgM and IgG anti-MSP1-19, anti-MSP3, anti-MSP2, anti-GLURP and anti-AMA1 increased with age [59]. In addition, in children aged 49–132 months, anti-PfA-M1 antibodies levels increased with age and those values are significantly higher in Makokou compared to Franceville. In Makokou, the level of antibodies was higher among older children compared to younger children aged under 49 months. In both semi-urban and urban areas, the highest antibody levels were observed in children aged from 9 to 180 months. These results support the general trends associating age with increased antibody responses for many plasmodial antigens in endemic areas, which probably reflects the increased parasite exposure with age [51,60,61]. There may be an association between anti-parasite immunity, intensity of transmission and age, which has been reported in some endemic areas [57,62]. This would suggest that an acquired clinical immunity develops over several years after repeated exposure to infected mosquito bites. Therefore, age, reflecting cumulative malaria exposure, is an important determinant of protection against clinical malaria. Thus, like AMA1, MSP1 and CSP, PfA-M1 could be a marker of the intensity of malaria transmission [63,64].

Although a decreasing risk for clinical malaria [65], severe disease [66] and an increase of antibody responses [67,68] with age are commonly reported, our results in Franceville showed no significant difference between antibody response and age. This could be explained by a lower level of malaria transmission in Franceville. In fact, unlike malaria in hyperendemic rural areas, malaria transmission is less important and more focused in urban centers [19,69], leading to a delayed acquisition of immunity in these areas. It has been shown in Central Africa, and particularly in Brazzaville, that 63% of children residing since birth in urban areas still have no anti-plasmodial antibodies at the ages of 6 and 7 years. This percentage is still 16% at the age of 14. In rural areas, however, all children are seropositive by the age of 5 [69]. As a result, due to the lack of premunition in individuals living in urban areas, the epidemiological risk is potentially present and the clinical forms affecting all age classes can become severe [70].

The highest level of anti-PfA-M1 antibodies was found in children with malaria and low parasitemia. This is consistent with a study from Senegal, which showed that the levels of IgG anti-MSP3 were higher in patients, particularly in children, with low parasitemia [71]. Our study shows a trend association between the anti-PfA-M1 response and parasitemia in children living in semi-urban areas of Gabon. This suggests that anti-PfA-M1 antibodies could contribute in controlling the level of parasitemia, and confirms PfA-M1 as a potential promising vaccine candidate.

Malarial anemia appears to affect the level of the anti-PfA-M1 antibody response in children living in semi-urban

(Makokou) and urban (Franceville) areas. IgG anti-PfA-M1 response increased with MMA in both sites. The children with MMA had the highest antibody levels compared to children UMA and SMA. However, our results are inconsistent with other studies that showed an association between higher levels of vaccine candidates and decreased malaria incidence. These results suggest that anti-PfA-M1 antibodies could contribute to the pathogenesis of malarial anemia. Further studies are needed to provide a clearer picture.

Conclusion

To conclude, this study provides the first useful baseline information on the immunogenicity of PfA-M1 antigen in populations exposed to malaria transmission in Gabon. The PfA-M1 antigen showed immunological characteristics similar to those of some vaccine candidates (AMA1, MSP1, CSP) whose immunogenicity has already been proved. Antibody response against PfA-M1 increases with infection, area of residence and age. Thus, this age-dependent acquired immune response to PfA-M1 appeared to reflect malaria transmission intensity. Finally, currently considered as a promising drug target candidate, according to this study PfA-M1 also appears as a possible diagnostic biomarker for both the presence of malaria parasite and anemia level in young patients, but obviously additional experiments will be necessary to further consolidate this novel proposal based on our observations. However, although our findings show immunogenic characteristics of PfA-M1, its relevance as a potential malaria vaccine candidate remains to be proved.

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Disclosures

The authors declare no conflicts of interest.

Author contributions

S. L. O. L. coordinated the study, performed the statistical analysis, conducted data analysis and wrote the manuscript. R. K. I. L. carried out the immunoassays. L. C. K. participated in data collection. F. B. participated in data collection and carried out the immunoassays. M. S. and I. F. produced and purified recombinant PfA-M1 and participated in the writing of the manuscript. J. B. L. K. conceived, designed and coordinated the study and conducted data analysis. All authors have read and approved the final manuscript.

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