



ORIGINAL ARTICLE

Inflammatory cytokine responses in children with asymptomatic malaria infection living in rural, semi-urban and urban areas in south-eastern Gabon

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Abstract

Cytokines are soluble mediators of the immune response, and their evolution influences the disease outcome. Gaining knowledge on cytokines has become important, as they can constitute biomarkers allowing the diagnosis of malaria and preventing severe forms of the disease. Here, we investigated 10 cytokines and their circulating levels in asymptomatic Gabonese children with *Plasmodium falciparum* infection living in urban, semi-urban and rural areas. Blood samples were collected from 273 school-children (153 uninfected and 120 infected) aged 6 to 192 months. Hematological parameters were determined and *P. falciparum* diagnosis was performed using a rapid diagnosis test, microscopy and nested polymerase chain reaction (PCR). Plasma pro-[interferon (IFN)- γ , tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-12p70, IL-17A and IL-22] and anti-inflammatory [IL-10, IL-4, IL-13 and transforming growth factor (TGF)- β] cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA) and compared between asymptomatic-infected and uninfected children. Results revealed that without distinction of area, IL-10 and IL-6 levels were higher in infected compared to uninfected children; however, the pro- and anti-inflammatory ratios (IL-6/IL-10 and TNF- α /IL-10) were similar. Furthermore, with area distinction significantly elevated levels of IL-10 in these asymptomatic children were always accompanied by either significantly low or high levels of a proinflammatory cytokine. Also, comparison between asymptomatic-infected children from the three areas showed significantly lower IL-17A, IL-22 and TGF- β levels in urban area compared to semi-urban and rural areas. These results suggest that asymptomatic malaria infections induce significantly high inflammatory cytokine levels without modifying the balanced between pro- and anti-inflammatory cytokines and underline the higher exposure to infections of children in rural areas.

KEYWORDS

asymptomatic *Plasmodium falciparum* infection, children, cytokines, rural area, semi-urban area, urban area

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INTRODUCTION

Malaria is a major health problem, with an incidence of 228 million cases and 405 000 deaths reported worldwide in 2018 [1]. On the African continent, among the five species responsible for human infections, *Plasmodium falciparum* is responsible for one of five deaths in children [2]. According to the World Health Organization (WHO), in 2018 approximately 24 million children were estimated to be infected with *P. falciparum* in sub-Saharan Africa and, among these, 1.8 million were likely to have severe anemia [1]. *P. falciparum* malaria infection causes a wide range of presentations of the clinical disease – asymptomatic infections that are poorly understood and slightly complicated and mild or severe forms. While efforts are increasing to achieve the millennium goals for malaria elimination, asymptomatic plasmodial infection remains widespread in endemic malaria regions and has become a major cause for concern.

Indeed, due to the absence of symptoms, asymptomatic plasmodial infections generally remain untreated and constitute a silent natural reservoir which supports the transmission of malaria and complicates malaria elimination in these regions [3]. Asymptomatic *P. falciparum* infection remains high, and represents approximately 96% of malaria infections in rural areas of Gabon [4,5], meaning that almost all its population can then serve as a reservoir for malaria transmission. In Gabon, the urban area of Franceville, semi-urban area of Makokou and rural area of the Mulundu district are characterized by their high level of malaria transmission throughout the year, as in other regions of Gabon. However, although Gabon is a hyperendemic country for malaria, endemicity levels may vary from one region to another, with significant disparities observed in the distribution of malaria in urban, semi-urban and rural areas [6–9]. According to a recent study, asymptomatic malaria is more frequent in rural (25.5%) and semi-urban (20.5%) areas than in urban areas (4.8%) [10]. In addition to the heterogeneity of the malaria burden which has been reported in Gabon [8], the prevalence of co-infections with *P. falciparum* in asymptomatic parasite carriers is 6.8%, with a predominance in helminthes infections in the rural area compared to semi-urban and urban areas [10].

People living in malaria-endemic areas become clinically immune after multiple reinfections over time and remain infected without apparent symptoms. Age and exposure to malaria infection are critical to the development of natural immunity to *P. falciparum* malaria. Therefore, the increasing numbers of multi-resistance to anti-malarial drugs [11] associated with vector resistance to available insecticides have increased the need to understand the immunological basis of protective immunity against malaria.

The modulation of pro- and anti-inflammatory cytokines has been associated with different clinical malaria manifestations [5,11,12]. The evolution of cytokine

balance is decisive in the outcome of the disease. The balance between pro- and anti-inflammatory cytokines appears to be key in the determination of the clinical outcome of *P. falciparum* infection [13]. Indeed, a balance in favor of anti-inflammatory cytokines such as interleukin (IL)-10 suppresses the inflammatory immune response as well as parasite clearance [13], and therefore promotes the development of asymptomatic infections [14]. Finding cytokines which can constitute biomarkers would be an asset to prevent severe forms of the disease by allowing the diagnosis and indirectly the control of asymptomatic *P. falciparum* infections [15]. A recent study reported that in asymptomatic malaria there is a balance between pro- and anti-inflammatory cytokines [16]. Also, significantly high levels of IL-10 and IL-4 were observed in asymptomatic cases compared to healthy individuals, whereas levels of T helper type 1 (Th1) cytokines such as IFN- γ and tumor necrosis factor (TNF)- α and Th17 (IL-17, IL-23) were higher in symptomatic patients compared to healthy controls and asymptomatic individuals [17,18]. During asymptomatic *P. vivax* infection lower levels of the regulatory T cell (T_{reg}) cytokine IL-10 have been observed compared to controls [19], whereas in another study no significant difference in IL-10 levels was found between asymptomatic cases and uninfected controls [16]. Conversely, an overall reduction in proinflammatory cytokines (TNF, IFN- γ , IL-6) and increased levels of regulatory cytokines (IL-10 and TGF- β) were observed when compared with those of the symptomatic groups [20]. It has also been shown that increased levels of IL-10 were linked to asymptomatic malaria in pregnant women [21].

Although the relative balance between anti- and proinflammatory cytokines is thought to be a crucial factor in the outcome of malaria infection, studies on asymptomatic children living in malaria-endemic areas with different epidemiological facies, particularly in Gabon, are relatively rare. The aim of our present study was to investigate and compare pro- and anti-inflammatory cytokine responses in asymptomatic *P. falciparum* infections in children from urban, semi-urban and rural areas in Gabon. We explore the hypothesis that the immune response to asymptomatic *P. falciparum* is mediated by anti-inflammatory cytokines, and this response can vary according to the level of exposure to infections.

METHODS

Study sites

The study was conducted between June and July 2017 and 2019 during a voluntary screening campaign organized by the Centre Interdisciplinaire de Recherches Médicale de Franceville (CIRMF). The study took place in the primary

schools of Ombellé in Franceville and Notre Dame de Victoire in Makokou and in three villages of the Mulundu district (Matsatsa, Mana-Mana and Malende), near the city of Lastourville (Figure 1). Franceville is an urban area of south-eastern Gabon ($0^{\circ}41'60''S$ and $13^{\circ}34'54''E$). Demographically, it is Gabon's third largest city after Libreville and Port-Gentil. The city of Makokou is a semi-urban area of north-eastern Gabon ($0^{\circ}29'10''N$ and $12^{\circ}52'4''E$). Mulundu is a rural area of eastern-central Gabon ($0^{\circ}49'S$ and $12^{\circ}42'E$). Although Gabon is a malaria-endemic country, these three areas are characterized by different prevalence rates of plasmodial infection in children: 79.5% at Lastourville, 70.2% at Makokou and 21.2% at Franceville [22,23].

Study participants and design

Two hundred and seventy-three schoolchildren aged from 6 to 192 months were included in this study (Supporting

information, Figure S1). Children were classified according to their *P. falciparum* infection status in two groups: asymptomatic-infected (asymptomatic *P. falciparum* carriers) and uninfected (healthy controls); they were then distributed according to their living area (urban, semi-urban and rural area) into six groups. Negative status was defined by the negativity of either the rapid diagnostic test or thick blood drop and positive status was defined by a positive rapid diagnostic test and/or thick blood drop; nested polymerase chain reaction (PCR) was then performed for species determination. Children with a febrile syndrome or a history of fever during the 2 weeks before sampling (temperature $\geq 37.5^{\circ}C$) and children infected with *Plasmodium* species other than *P. falciparum* were excluded. Among the uninfected children, those with high white blood cell levels above normal ($>10.00 \times 10^3$ cells/mm³) were also excluded. All asymptomatic *Plasmodium*-infected children were treated with artemether/lumefantrine.

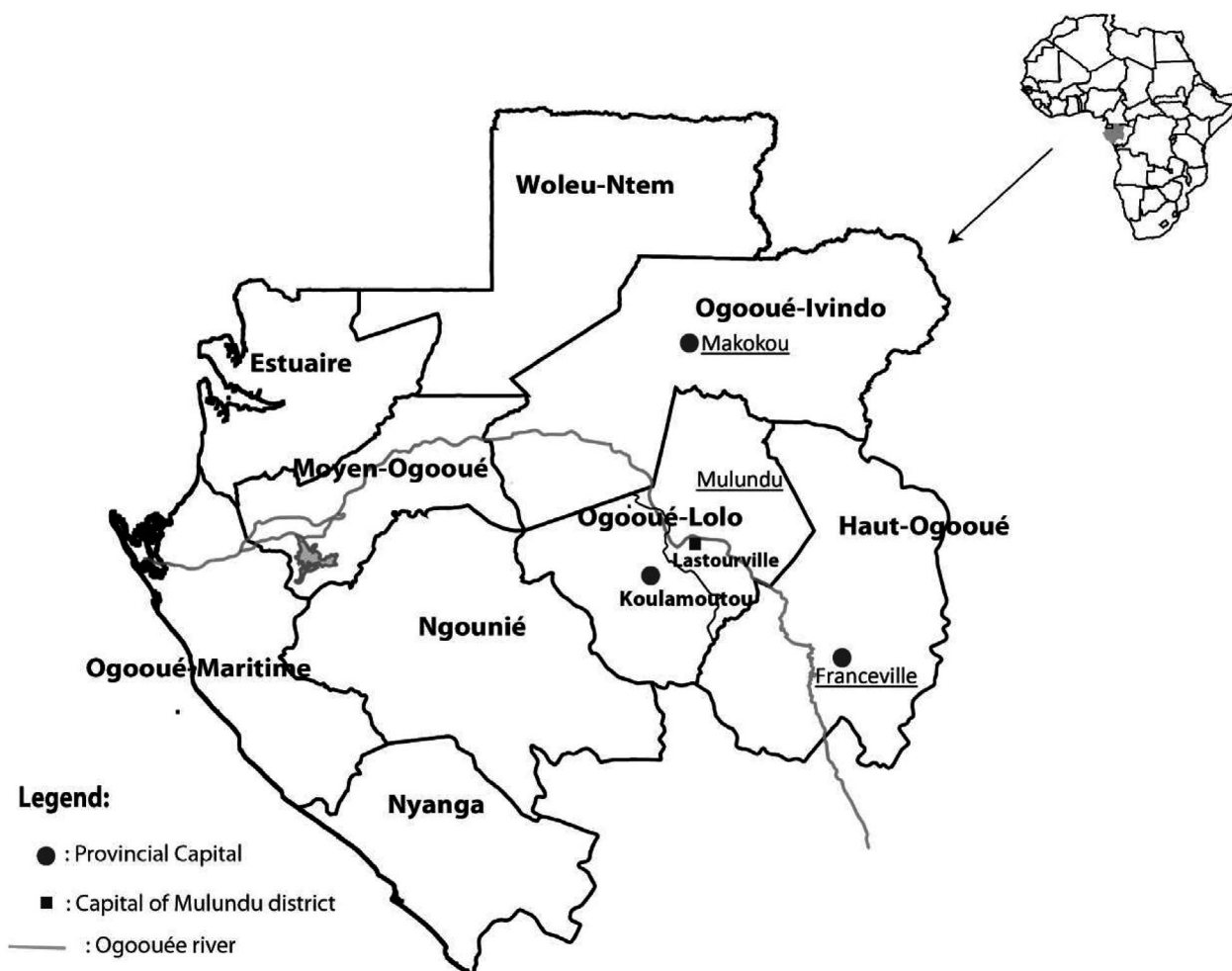


FIGURE 1 Map of Gabon. The study sites are underlined: urban (Franceville), semi-urban (Makokou) and rural (Mulundu district) areas. The rural study site is made up of three villages (Mana-Mana, Malende and Matsatsa) on the outskirts of Lastourville located at the south of the Mulundu district in the Ogooué-Lolo province

Sample collection, hematological analyses and malaria diagnosis

Venous blood was collected in ethylenediamine tetraacetic acid (EDTA) tubes (2–5 ml). The hemogram (also named 'blood count'), was performed using COBAS ABX (Roche Diagnostics, Indianapolis, Indiana, USA) counters.

For malaria diagnosis, rapid diagnostic testing (RDT) [OptiMAL-IT[®] test (HRP2)] and thick drops were used for the parasite load count using the Lambarene method [24]. Thick blood smears from samples were stained with 10% Giemsa, and the number of *P. falciparum* parasites was determined by microscopy. The thick droplet slides were read after washing and drying by two qualified microscopists from the CIRMF, Gabon. The remainder of the whole blood was then immediately centrifuged and, after separation, the plasma was placed at -80°C and the pellet was used for the extraction of *P. falciparum* DNA.

Extraction of the DNA from blood was conducted using the Qiagen kit (QIAamp DNA Mini kit; Qiagen, Hilden, Germany). Then, 5 μl of extracted DNA were amplified with 1 \times Taq polymerase buffer (Invitrogen, San Diego, California, USA), 0.8 μM for each of the primers rPLU5 and rPLU6 for the first PCR reaction and rFAL1, rFAL2, rMAL1, rMAL2, rVIV1, rVIV2, rOVA1 and rOVA2 for the nested PCR (Eurogentec, Seraing, Belgium) [25], 0.2 mM dNTP, 2 mM MgCl_2 and 0.024 U of Taq DNA polymerase (Invitrogen) using the cycling program used by Maghendji-Nzondo *et al.* [22]. Primer sequences were 5'-CCT GTT GTT GCC TTA AAC TTC-3' (rPLU5) and 5'-TTAAAATTGTTGCAGTTAAAACG-3' (rPLU6) for the *Plasmodium* genus with 1100 base pairs, and for each species were rFAL1 (5'-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3') and rFAL2 (5'-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3') for *P. falciparum* with 206 base pairs, rMAL1 (5'-ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC-3') and rMAL2 (5'-AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA-3') for *P. malariae* with 145 base pairs, rVIV1 (5'-CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC-3') and rVIV2 (5'-ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA-3') for *P. vivax* with 121 base pairs and rOVA1 (5'-ATC TCT TTT GCT ATT TTT TAG TAT TGG AGA-3') and rOVA2 (5'-ATC TAA GAA TTT CAC CTC TGA CAT CTG-3') for *P. ovale* with 226 base pairs. The products of nested PCR were analyzed by electrophoresis on 2% agarose gel.

Cytokine measurements

Detection of human IL-12 p70, TNF- α , TGF- β , IL-4, IL-6, IL-13, IL-22, IFN- γ , IL-17 and IL-10 were determined by enzyme-linked immunosorbent assay (ELISA) in the

plasmas according to the manufacturer's instructions (Bender MedSystems, Vienna, Austria). Optical densities (OD) were measured at 450 nm with a reference at 620 nm using an ELISA plate reader (Stat Fax 3200[®]; Bioblock/Fisher Scientific, Vienna, Austria). The detection limits were as follows: 2 pg/ml for IL-6, IL-10 and IL-4; 4 pg/ml for IFN- γ , TNF- α , IL-13, IL-12p70 and IL-17A; and 8 pg/ml for IL-22 and TGF- β . All samples were tested in duplicate, and the mean of the two OD values was used for analyses.

Statistical analysis

Variables were compared between the two subgroups (uninfected and infected) of the study participants and of each study site (urban, semi-urban and rural areas). In addition, comparison was performed between all infected groups (infected cases of urban, semi-urban and rural areas). Differences between the two subgroups were analyzed for statistical significance using the Mann–Whitney *U* non-parametric test and between three groups using the Kruskal–Wallis non-parametric test. Correlations between cytokines and between circulating levels of cytokines and parasite density or age were analyzed using Spearman's rho test. The multiple *t*-tests were used for ratio determination. We also performed a multivariate analysis using a generalized linear model of the Gaussian family using the stats package, and then the best model was determined using the Dredge function of the MumIn package. All statistical analyses were performed using Graph Pad Prism software version 8.4 and the R statistical software version 3.6.2. *p* values < 0.05 were considered statistically significant.

RESULTS

Socio-demographic and clinical characteristics of the study participants

Among the 273 children included in this study, 120 had asymptomatic *P. falciparum* infection (asymptomatic-infected group) and 153 were uninfected (uninfected group). The demographic and clinical features for children according to the study areas are presented in Table 1. The sex ratio (men/women) was slightly in favor of males in infected children from urban and semi-urban areas (sex ratio = 1.13 and 1.38) and in uninfected children from the rural area (sex ratio = 1.05).

The median age in asymptomatic-infected children was significantly higher than in uninfected children from the urban area [108 (84–124) months versus 84

TABLE 1 Demographic and clinical parameters of the participants in the study (median, interquartile range)

	Urban area (Franceville)		Semi-urban area (Makokou)		Rural area (Mulundu)		<i>p</i>
	Uninfected	Asymptomatic-infected	Uninfected	Asymptomatic-infected	Uninfected	Asymptomatic-infected	
	<i>n</i> = 33	<i>n</i> = 17	<i>n</i> = 32	<i>n</i> = 19	<i>n</i> = 88	<i>n</i> = 84	
Sex ratio	0.83	1.13	0.68	1.38	1.05	0.87	
Age (IQR) months	84 (72–102)	108 (84–124)	96 (84–132)	108 (72–132)	92 (52–133)	90 (60–118)	0.921 ^a
Temperature (IQR) °C	36.5 (36.3–36.8)	36.6 (36.6–37.2)	37.0 (36.4–37.2)	37.0 (36.6–37.1)	36.6 (36.2–36.9)	36.7 (36.1–37.1)	0.260 ^a
White blood cells (IQR) 10 ³ cells/mm ³	5.90 (5.50–7.25)	6.20 (5.80–6.95)	7.15 (6.23–7.98)	7.50 (5.20–9.70)	ND	ND	
Red blood cells (IQR) 10 ³ cells/mm ³	4.57 (4.35–4.81)	4.27 (3.95–4.49)	4.29 (4.03–4.57)	4.42 (3.63–4.75)	ND	ND	
Hemoglobin (IQR) g/dl	10.5 (9.70–11.2)	10.3 (8.85–10.7)	10.6 (10.0–11.0)	9.70 (9.0–11.0)	ND	ND	
Platelets (IQR) 10 ³ cells/mm ³	253 (206–298)	241 (203–271)	354 (276–433)	306 (219–364)	ND	ND	
Parasitemia (IQR) parasites/μl		1295 (560–3815)		4590 (1820–9785)		3500 (1260–6580)	0.028^{ab}

Sex ratio, age, temperature, leukocyte counts, hemoglobin concentrations and parasite densities in asymptomatic uninfected and infected children according to the study site (urban, semi-urban and rural areas). The uninfected children were defined as *Plasmodium falciparum*-exposed children, negative for parasites in thick blood smears and/or in rapid detection test kits.

Abbreviations: IQR, interquartile range; ND, not determined.

Significant levels were calculated using ^a Mann–Whitney rank sum analysis and ^b Kruskal–Wallis analysis.

The bold-type figures are to highlight the significant *p*-values.

* *p* < 0.05.

(72–102) months; $p = 0.02$]. In the semi-urban and rural areas, the mean age was similar between asymptomatic-infected and uninfected children. However, comparisons between the ages of asymptomatic-infected children in the three areas showed that even though rural children tended to have a younger age than urban and semi-urban children, there was no statistical difference ($p = 0.095$, data not shown). In addition, significantly higher parasitemia loads were found in the semi-urban and rural areas than in the urban area [4590 (1820–9785) parasites/ μl , 3500 (1260–6580) parasites/ μl and 1295 (560–3815) parasites/ μl , respectively; $p = 0.028$].

Regarding red blood cells, the concentration was significantly lower in asymptomatic-infected children compared to uninfected children in the urban area [$4.27 (3.95\text{--}4.49) \times 10^3 \text{cells}/\text{mm}^3$ versus $4.57 (4.35\text{--}4.81) \times 10^3 \text{cells}/\text{mm}^3$; $p = 0.004$]. However, no significant difference was noted between the two subgroups in the semi-urban area [$4.42 (3.63\text{--}4.75) \times 10^3 \text{cells}/\text{mm}^3$ versus $4.29 (4.03\text{--}4.57) \times 10^3 \text{cells}/\text{mm}^3$; $p = 0.95$]. Moreover, although it was not significantly different, a decrease in the hemoglobin parameter was observed in asymptomatic-infected children compared to the uninfected children from the urban and semi-urban areas [10.3 (8.85–10.7) g/dl versus 10.5 (9.70–11.2) g/dl; $p = 0.167$ and 9.70 (9.0–11.0) g/dl versus 10.6 (10.0–11.0) g/dl; $p = 0.091$ in the urban and semi-urban areas, respectively].

Although not significant, temperature tended to increase in asymptomatic-infected children compared to uninfected children in the urban area [$36.6 (36.6\text{--}37.2)^\circ\text{C}$ versus $36.5 (36.3\text{--}36.8)^\circ\text{C}$; $p = 0.066$], but was similar between the two groups of children in the semi-urban area [$37.0 (36.6\text{--}37.1)^\circ\text{C}$ versus $37.0 (36.4\text{--}37.2)^\circ\text{C}$; $p = 0.533$] and in the rural area [$36.7 (36.1\text{--}37.1)^\circ\text{C}$ versus $36.6 (36.2\text{--}36.9)^\circ\text{C}$; $p = 0.26$].

Higher IL-6 and IL-10 levels in children with asymptomatic *P. falciparum* infection compared to other cytokines

Pro- and anti-inflammatory cytokines levels were assessed in the plasma samples collected in children from the Mulundu district, Makokou and Franceville. Pro- and anti-inflammatory cytokines IL-6 and IL-10 were significantly higher in children with asymptomatic malaria than in uninfected children (Figure 2). Indeed, the IL-6 concentration was significantly higher in asymptomatic-infected children compared to uninfected children [0.60 (0–1.41) pg/ml versus 0.29 (0–0.88) pg/ml; $p = 0.018$; Figure 2a]. IL-10 concentration was significantly higher in asymptomatic-infected children than in uninfected children [13.14 (0–29.90) pg/ml versus 1.60 (0–7.79) pg/ml; $p < 0.0001$; Figure 2b]. The concentrations of TNF- α , IL-12p70 and IFN- γ did not differ between asymptomatic-infected and uninfected children [0 (0–1.88) pg/ml versus 0.39 (0–4.82) pg/ml; $p = 0.106$ for TNF- α and 0 (0–0.09) pg/ml versus 0 (0–0.13) pg/ml; $p = 0.713$ for IL-12p70 and 2.89 (1.28–6.03) pg/ml versus 1.84 (0.26–6.61) pg/ml; $p = 0.089$ for IFN- γ ; Supporting information, Figure S2]. Also, IL-17A and IL-22 levels in the asymptomatic-infected children in comparison to uninfected children were similar [0 (0–1.85) pg/ml versus 0.23 (0–2.93) pg/ml; $p = 0.329$ for IL-17A and 67.3 (52.3–81.3) pg/ml versus 66.1 (52–85.3) pg/ml; $p = 0.859$ for IL-22, respectively; Supporting information, Figure S2]. Similarly, no difference in TGF- β , IL-4 and IL-13 levels was observed in asymptomatic-infected children compared to uninfected children [0 (0–2.92) pg/ml versus 0 (0–4.62) pg/ml; $p = 0.568$ for TGF- β ; 0 (0–0.76) pg/ml versus 0 (0–0.70) pg/ml; $p = 0.868$ for IL-4; 1.5 (0–7.0) pg/ml versus 1.5 (0–8.5) pg/ml; $p = 0.957$ for IL-13, respectively; Supporting information, Figure S2].

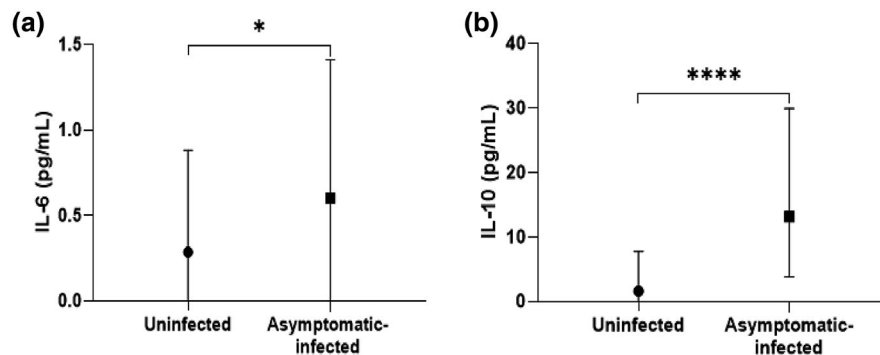


FIGURE 2 Levels of pro- and anti-inflammatory cytokines in infected and uninfected children from the three study sites combined. Plasma concentration of interleukin (IL)-6 (a) and IL-10 (b) were quantified from asymptomatic *Plasmodium falciparum*-infected and -uninfected children by enzyme-linked immunosorbent assay (ELISA). Data are represented with the median of cytokine concentrations in pg/ml with interquartile range. Significant differences between infected and uninfected children were tested using Mann–Whitney’s method and are indicated. * $p < 0.05$; **** $p < 0.0001$

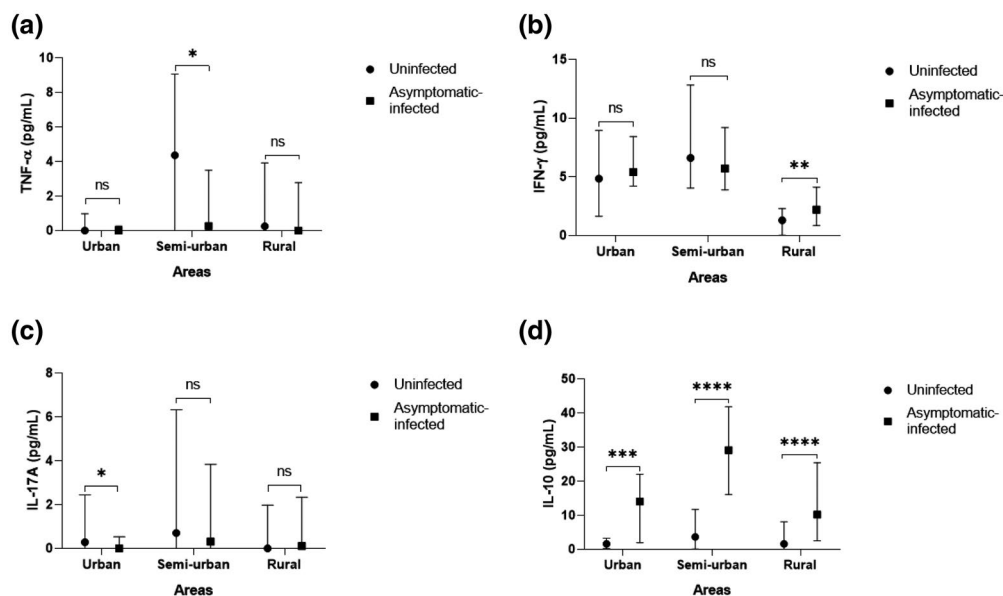


FIGURE 3 Levels of tumor necrosis factor (TNF)- α , interferon (IFN)- γ , interleukin (IL)-17A and IL-10 in plasma from infected and uninfected children according to site. Plasma concentration of TNF- α (a), IFN- γ (b), IL-17A (c) and IL-10 (d) in asymptomatic *Plasmodium falciparum*-infected and -uninfected children according to the living area (rural, semi-urban and urban areas) were quantified using enzyme-linked immunosorbent assay (ELISA). Data are represented with cytokine concentration medians in pg/ml with interquartile range. Statistically significant differences between the two groups were tested using the Mann-Whitney method and are represented as * $p < 0.05$; ** $p < 0.001$; *** $p = 0.0001$; **** $p < 0.0001$; NS = non-significant difference

Pro-/anti-inflammatory ratio in asymptomatic malaria children

The ratios of IL-6/IL-10 and TNF- α /IL-10 were determined for evaluating whether proinflammatory cytokine or anti-inflammatory cytokine predominates in asymptomatic *P. falciparum* infection. Although no significant difference was found between the pro-/anti-inflammatory (IL-6 and TNF- α to IL-10) ratio of asymptomatic-infected and uninfected children, data revealed that IL-10 was nearly five- to four-fold higher than TNF and IL-6, respectively (4.793 for TNF- α /IL-10 ratio and 3.909 for IL-6/IL-10 ratio; Table 2).

Proinflammatory cytokine levels (including IL-17A, IFN- γ and TNF- α) with higher IL-10 levels in asymptomatic malaria children for each area

With the aim of characterizing the response to asymptomatic *P. falciparum* infection according to the level of development in the regions, the asymptomatic-infected and uninfected groups were subdivided into three groups according to the participants' location (urban, semi-urban and rural area). The cytokine levels in plasma from infected and uninfected children with asymptomatic *P. falciparum* infection was determined and compared for

TABLE 2 Pro-/anti-inflammatory ratio

Ratio	Uninfected	Infected	p -value
IL-6/IL-10	1,367	3,909	0,333
TNF- α /IL-10	0,244	3,858	

Multiple t -test ratio analysis was carried out between proinflammatory [interleukin (IL)-6, tumor necrosis factor (TNF)- α] and anti-inflammatory (IL-10) cytokines in asymptomatic *Plasmodium falciparum*-infected and uninfected children. The means, standard error of the means (SEM) and n were 6.122 pg/ml, 2.541 and 117 versus 3.127 pg/ml, 1.579 and 142 for IL-6; 6.064 pg/ml, 2.773 gp/ml and 117 versus 5.136 pg/m, 1.022 mg/ml and 149 for TNF- α and 30.32 pg/m, 5.606 mg/ml and 119 versus 5.438 pg/m, 0.711 pg/m and 153 for IL-10 in infected asymptomatic compared to uninfected children. The Mann-Whitney U -test was used to compare the balance between pro- and anti-inflammatory cytokines. The difference was statistically significant for $p < 0.05$.

each area. Interestingly, the results showed that, in each site, the levels of the anti-inflammatory cytokine IL-10 were statistically elevated in the asymptomatic-infected compared to the uninfected children, making it a good marker of asymptomatic *P. falciparum* infection. At the same time, at least one proinflammatory cytokine (IFN- γ , TNF- α or IL-17A) was significantly different between the two groups of children (Figure 3 and Supporting information, Figure S2). In the urban area, results showed that significantly lower IL-17A levels with higher IL-10 levels were found in the asymptomatic-infected group compared

to the uninfected group [0 (0–0.53) pg/ml versus 0.28 (0–2.44) pg/ml; $p = 0.034$ for IL-17A; Figure 3c and 14.0 (1.98–22.0) pg/ml versus 1.60 (0.20–3.27) pg/ml; $p < 0.001$ for IL-10; Figure 3d, respectively]. There was no difference in TNF- α and IFN- γ levels in urban areas [0 (0–0.26) pg/ml versus 0 (0–0.98) pg/ml; $p = 0.299$ and 5.40 (4.21–8.43) pg/ml versus 4.84 (1.64–8.95) pg/ml; $p = 0.379$, respectively].

In the semi-urban area there were significant lower TNF- α levels with higher IL-10 levels in the asymptomatic-infected group when compared to the uninfected group [0.26 (0–3.49) pg/ml versus 4.36 (0–9.05) pg/ml; $p = 0.046$ for TNF- α ; Figure 3a and 29.6 (16.1–41.8) pg/ml versus 3.64 (0.05–11.7) pg/ml; $p < 0.0001$ for IL-10; Figure 3d, respectively]. However, there was no difference in IL-17A and IFN- γ levels [0.31 (0–3.83) pg/ml versus 0.71 (0–6.32) pg/ml; $p = 0.226$ and 5.70 (3.88–9.20) pg/ml versus 6.61 (4.03–12.8) pg/ml; $p = 0.531$, respectively].

Concerning the rural area, significant higher IFN- γ levels with higher IL-10 levels were also observed in the asymptomatic-infected group in comparison to the uninfected group [2.19 (0.86–4.12) pg/ml versus 1.29 (0.02–2.30) pg/ml; $p = 0.001$ for IFN- γ ; Figure 3b and 10.2 (2.52–25.4) pg/ml versus 1.60 (0–8.07) pg/ml; $p < 0.0001$ for IL-10; Figure 3d]. However, there was no significant difference in IL-17A and TNF- α levels in this area [0.11 (0–2.34) pg/ml versus 0 (0–1.97) pg/ml; $p = 0.713$ and 0 (0–2.78) pg/ml versus 0.25 (0–3.91) pg/ml; $p = 0.536$, respectively].

Th1-, Th17- and Th2-circulating cytokines in asymptomatic *P. falciparum*-infected children according to study area

When considering only the infected children with asymptomatic *P. falciparum* infection in the three areas, an overall comparison showed a significant difference between the urban, semi-urban and rural areas in the majority of cases: all Th1 apart from TNF- α ($p < 0.0001$, $p = 0.009$, $p < 0.001$ and $p = 0.192$ for IFN- γ , IL-6, IL-12p70 and TNF- α , respectively), Th2 ($p = 0.012$, $p < 0.001$, $p = 0.007$ and $p = 0.006$ for IL-4, IL-13, TGF- β and IL-10, respectively) and Th17 cytokines ($p < 0.0001$ for IL-22 and $p = 0.101$ for IL-17A).

However, when comparing two areas at a time results showed that between asymptomatic-infected children of urban and semi-urban areas, there were significant differences in the Th1 cytokine response for IL-12p70 and IL-6 levels but not for TNF- α and IFN- γ levels. Indeed, higher IL-12p70 and lower IL-6 levels were found in the urban area than in the semi-urban areas [0 (0–0) pg/ml versus 0.09 (0.07–0.12) pg/ml; $p < 0.0001$ and 0.33 (0.20–0.86) pg/ml versus 0 (0–0.67) pg/ml; $p < 0.010$ (Figure 4a,c), respectively]. However, TNF- α and IFN- γ levels were similar

[0 (0–0.26) pg/ml versus 0.26 (0–3.49) pg/ml; $p = 0.104$ and 5.40 (4.21–8.43) pg/ml versus 5.70 (3.88–9.20) pg/ml; $P > 0.999$] (Figure 4b,d), respectively].

Also, there were significant differences in the Th2 cytokine response between asymptomatic-infected children of urban and semi-urban areas for IL-10 and TGF- β levels but not for IL-4 and IL-13 levels. IL-10 and TGF- β levels were lower in asymptomatic-infected children of urban than those of semi-urban areas [14.0 (1.98–22.0) pg/ml versus 29.9 (16.1–41.8) pg/ml; $p = 0.009$ for IL-10 and 0 (0–0) pg/ml versus 0 (0–0.70) pg/ml respectively; $p = 0.025$ for TGF- β (Figure 4j,i), respectively]. In contrast, levels of IL-4 and IL-13 were similar between the urban and the semi-urban areas [0 (0–0) pg/ml versus 0 (0–0.06) pg/ml; $p = 0.962$ and 0 (0–0.40) pg/ml versus 0 (0–0) pg/ml; $p = 0.339$ (Figure 4g,h) respectively].

There was a significant difference in the Th17 cytokine responses (IL-17A and IL-22). The levels of IL-17A and IL-22 were lower in asymptomatic-infected children of urban than those of semi-urban areas [0 (0–0.53) pg/ml versus 0.31 (0–3.83) pg/ml; $p = 0.042$ and 47.8 (43.4–59.4) pg/ml versus 66.0 (49.5–75.0) pg/ml; $p = 0.034$ (Figure 4e,f) respectively].

Between asymptomatic-infected children of urban and rural areas there was no significant difference in the Th1 cytokine responses for IL-12p70, TNF- α and IL-6 levels except for IFN- γ . IFN- γ levels were significantly higher in the urban than in the rural area [5.40 (4.21–8.43) pg/ml versus 2.19 (0.86–4.12) pg/ml; $p < 0.001$; Figure 4d], but the levels were similar [0 (0–0) pg/ml versus 0 (0–0.11) pg/ml; $p = 0.432$ for IL-12p70; 0 (0–0.26) pg/ml versus 0 (0–2.78) pg/ml; $p = 0.087$ for TNF- α and 0.33 (0.20–0.86) pg/ml versus 0 (0–1.55) pg/ml; $p = 0.186$ for IL-6, respectively; Figure 4a–c].

In the Th2 cytokine responses there was a significant difference for IL-4, IL-13 and TGF- β , apart from IL-10. Lower levels were found in the urban area than in the rural area [0 (0–0) pg/ml versus 0.23 (0–1.18) pg/ml; $p = 0.015$ for IL-4, 0 (0–0.40) pg/ml versus 3.10 (0.80–8.50) pg/ml; $p = 0.002$] for IL-13 and [0 (0–0) pg/ml versus 0 (0–7.17) pg/ml; $p = 0.003$ for TGF- β ; Figure 4g–i, respectively]. IL-10 levels were similar between asymptomatic-infected children from urban and rural areas [14.0 (1.98–22.0) pg/ml versus 10.2 (2.52–25.4) pg/ml; $p = 0.728$; Figure 4j].

There was a significant difference in Th17 cytokine responses (IL-17A and IL-22). Lower levels were found in the urban area compared to the rural area [0 (0–0.53) pg/ml versus 0.11 (0–2.34) pg/ml; $p = 0.045$ for IL-17A and 47.8 (43.4–59.4) pg/ml versus 72.0 (60.8–93.0) pg/ml; $p < 0.0001$ for IL-22; Figure 4e,f, respectively].

Between asymptomatic-infected children of semi-urban and rural areas in the Th1 cytokine responses there was a significant difference for IL-12p70, IL-6 and IFN- γ levels, but no difference was found for TNF- α levels. IL-12p70

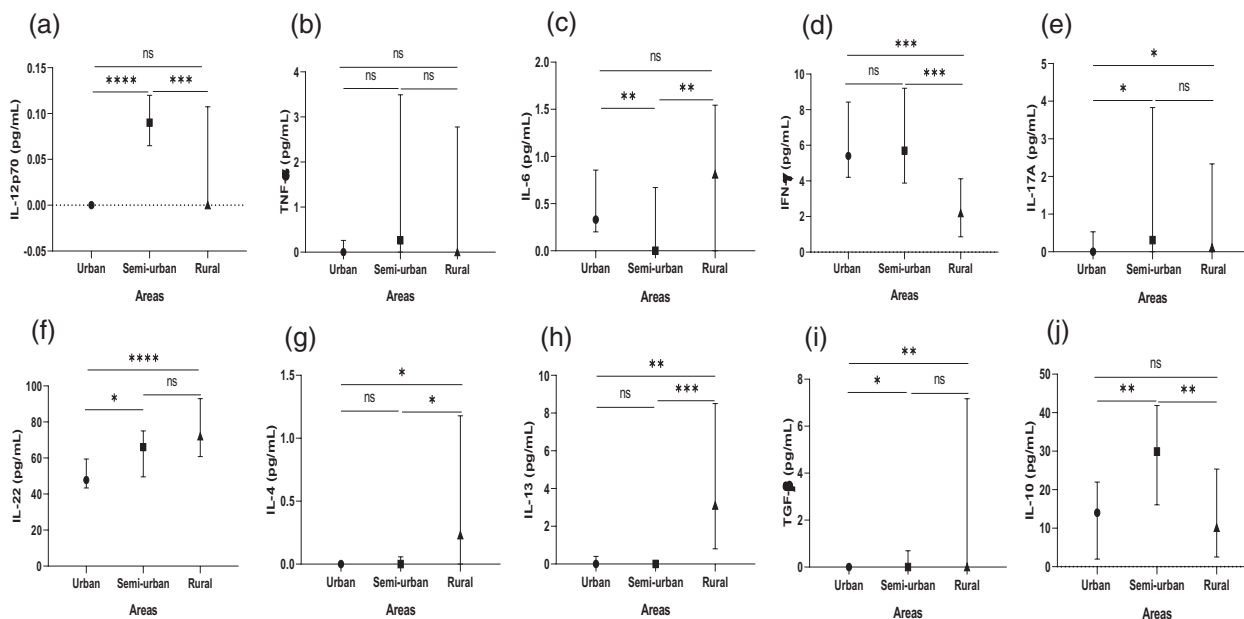


FIGURE 4 T helper type 1 (Th1), Th17 and Th2 cytokine levels (median with interquartile range) in plasma from infected children by site. Circulating levels of interleukin (IL)-12p70 (a), tumor necrosis factor (TNF)- α (b), interleukin (IL)-6 (c), interferon (IFN)- γ (d), IL-17A (e), IL-22 (f), IL-4 (g), IL-13 (h), transforming growth factor (TGF)- β (i) and IL-10 (j) in the plasma of asymptomatic *Plasmodium falciparum*-infected children were compared in pairs according to location (urban versus rural, urban versus semi-urban and semi-urban versus rural). The levels of these cytokines were measured using enzyme-linked immunosorbent assay (ELISA). Data are expressed as medians in pg/ml with the interquartile range. The Mann-Whitney method was used for comparisons and significant differences are indicated: * $p < 0.05$; ** $p = 0.001$; *** $p = 0.0001$; **** $p < 0.0001$; NS = non-significant difference

and IFN- γ levels were higher; however, IL-6 was lower in the semi-urban area compared to the rural area [0.09 (0.07–0.12) pg/ml versus 0 (0–0.11) pg/ml; $p < 0.001$ for IL-12p70, 5.70 (3.88–9.20) pg/ml versus 2.19 (0.86–4.12) pg/ml; $p < 0.001$ for IFN- γ and 0 (0–0.67) pg/ml versus 0.81 (0–1.55) pg/ml; $p = 0.005$ for IL-6; Figure 4a,c,d, respectively]. TNF- α levels were similar between asymptomatic-infected children of these two areas [0.26 (0–3.49) pg/ml versus 0 (0–2.78) pg/ml; $p = 0.806$; Figure 4b].

Furthermore, in Th2 cytokine responses there were significant differences for IL-10, IL-4 and IL-13 levels but not for TGF- β levels. IL-4 and IL-13 levels were significantly lower in the semi-urban area compared to the rural area [0 (0–0.06) pg/ml versus 0.23 (0–1.18) pg/ml; $p = 0.043$ for IL-4; 0 (0–0) pg/ml versus 3.10 (0.80–8.50) pg/ml; $p < 0.001$ for IL-13; Figure 4g,h]. However, IL-10 was significantly higher in the semi-urban than in the rural area [29.9 (16.1–41.8) pg/ml versus 10.2 (2.52–25.4) pg/ml; $p = 0.00213$; Figure 4j], while TGF- β levels were similar [0 (0–0.70) pg/ml versus 0 (0–7.17) pg/ml; $p = 0.246$; Figure 4i].

Also, no significant difference was found in the levels of IL-17A and IL-22, the Th17 cytokines, between asymptomatic-infected children of the semi-urban and rural areas [0.31 (0–3.83) pg/ml versus 0.11 (0–2.34) pg/ml; $p = 0.805$ and 66.0 (49.5–75.0) pg/ml versus 72.0 (60.8–93.0) pg/ml; $p = 0.054$; Figure 4e,f, respectively].

TABLE 3 Correlations between plasma levels of IL-6, IL-10, IFN- γ , IL-12p70 and parasitemia and between IL-10 and IL-6

Parameters	Spearman's r (or rho)	p
Parasitemia versus IL-10	0.53	<0.0001 ****
Parasitemia versus IL-6	0.20	0.046*
Parasitemia versus IFN- γ	-0.22	0.027*
Parasitemia versus IL-12 p70	0.24	0.031*
IL-10 versus IL-6	0.21	0.026*

Spearman's rank correlation analysis was carried out between cytokine and parasitemia in asymptomatic *Plasmodium falciparum*-infected children. Correlation coefficient (rho) is given. The correlation is strong for rho = 0.7 to 1, moderate for rho = 0.5 to 0.7 and weak for rho = 0.2 to 0.5 and for a value close to 0 there is no correlation. The correlation can be positive or negative. The statistically significant difference was designated from 1. Abbreviations: IFN, interferon; IL, interleukin.

* $p < 0.05$; **** $p < 0.0001$.

Association between cytokine levels and parasitemia, age, gender or residence area in asymptomatic *P. falciparum* infection

Associations between parasitemia and cytokines and between cytokines themselves are shown in Table 3. IL-10 showed a moderate positive association with parasite density ($r = 0.53$, $p < 0.0001$). Statistically significant weak

associations between cytokine levels and parasite density were also observed for IL-6 ($r = 0.20$, $p = 0.046$) and IL-12p70 ($r = 0.24$, $p = 0.031$). The correlation analysis of cytokines with each other showed that only IL-10 and IL-6 presented a statistically significant association trend ($r = 0.21$, $p = 0.026$). Conversely, a weak trend of negative association was observed between parasite load and IFN- γ ($r = -0.22$, $p = 0.027$).

Moreover, the effect of age, gender, parasitemia load and urbanization on cytokine levels was evaluated using a generalized linear model. None of the cytokine levels were associated with gender. Among the 10 cytokines only TGF- β varied with age. Otherwise, data revealed that parasitemia was a good predictor of IL-10 and the levels of three cytokines (IFN- γ , TGF- β and IL-10) were influenced by urbanization or residence area (Table 4 and Supporting information, Table S1).

DISCUSSION

This study analyzed and compared the levels of inflammatory cytokines of children with *P. falciparum* asymptomatic infection and uninfected children living in three different hyperendemic regions of Gabon.

In the urban areas, asymptomatic-infected children were significantly older than uninfected children. Previous studies have found that malaria-infected children were older than uninfected children in urban, but also in the semi-urban as well as rural areas [22], and an increase in the age of the population at risk of malaria in different regions of Gabon [8]. Furthermore, an occurrence of subclinical infections in all age groups has been reported that the more age increases, the more the risk of infection and of developing the disease significantly decrease [26]. Conversely, the significant higher parasitemia in semi-urban and rural areas in comparison with the urban area suggests that children living in these two areas are more exposed to malaria infection [10] and therefore are always asymptomatic with higher parasite loads than those living in urban areas.

There were no clinical symptoms of malaria and anemia in the children; however, the levels of red blood cells were significantly lower in infected compared to uninfected children in the urban area. Indeed, by multiplying, *P. falciparum* is responsible for a breakdown of red blood cells which leads to anemia in some patients [27]. During malaria, the immune system suppresses the infected erythrocytic cells and an over-stimulation leads to excessive production of proinflammatory cytokines; this activation of immune cells can cause severe malaria symptoms such as anemia [28].

In this study we found a significantly higher level of cytokines such as IL-6 and IL-10 in the plasma of infected children with asymptomatic *P. falciparum* parasitemia compared to uninfected children, while IFN- γ , TNF- α , IL-12 p70, IL-17A, IL-22, IL-4, IL-13 and TGF- β were similar.

IL-6 is a pleiotropic cytokine with pro- and anti-inflammatory properties [29,30]. It is produced by various innate and adaptive cells including macrophages, dendritic cells, T and B cells. This cytokine is involved in the production of antibody differentiation of monocytes to macrophage and can exert regulatory role upon CD4⁺ T cells to become Th1 or Th2 effector cells [31]. It has been suggested that the cytokine network as a whole (not just one cytokine) may contribute to serious illness [32]. Moreover, a recent study has showed that asymptomatic malaria among children is maintained by a homeostasis between pro- and anti-inflammatory cytokine responses [16]. This can explain the minimally but significantly increased levels of IL-6 and IL-10 observed in our asymptomatic *P. falciparum*-infected children. This result suggests that IL-6 plays a role in parasite control. A similar study in Gabonese children by Guiyedi *et al.* has reported a high plasma level of IFN- γ and IL-10 associated with high antigen-specific antibody responses in asymptomatic *P. falciparum* infection [33]. These authors suggested that the antibody response may have a protective immune effect. A study in Indonesia also found that levels of TNF- α increased in adult subjects with asymptomatic malaria [34].

Elevated levels of IL-10 in asymptomatic *P. falciparum* infection in pregnant women, adult subjects and children

TABLE 4 Association between plasma pro- and anti-inflammatory cytokine levels and age, urbanization, parasitemia or gender

	Intercept	Urbanization	Parasitemia	Age	Gender	Degree of free	Δ AIC	Weight
IL-10	0.33	+	0.22			6	0	0.74
IFN- γ	-0.2	+				4	0	0.96
TGF- β	0.54	+		0.003		5	0	0.62

Generalized linear model of the Gaussian family was carried out between cytokine levels, age, urbanization, parasitemia and gender in children. The Δ AIC = 0 designates the best model. The association can be positive or negative depending on the sign of intercept.

Abbreviations: AIC, Akaike information criterion; IFN, interferon; IL, interleukin; TGF, transforming growth factor.

have been reported in different studies from Uganda, Ghana, Indonesia, Mali and Gabon [17,21,33–36]. IL-10 is an anti-inflammatory cytokine that exerts an immunoregulatory role. Its presence in these asymptomatic-infected children suggests that IL-10 suppresses parasite clearance, thus preventing the development of anti-parasitic immunity and the progression to clinical forms of malaria [15]. Thus, IL-10 promotes the preservation of asymptomatic infections [17]. Many studies have reported that repeated exposures to infection lead to the development of anti-inflammatory immunity (by producing IL-10) [17,37–39]. During a *Plasmodium* infection, IL-10 is produced by a wide variety of innate and adaptive immune cells, including macrophages, dendritic cells (DCs), Th1 cells, T_{reg} cells, IL-17-producing CD4⁺ T (Th17) cells, Tfh cells, CD8⁺ T cells, regulatory B cells, natural killer (NK) cells and $\gamma\delta$ T cells [15]. Through its action on different cells, IL-10 promotes the tolerance of the pathogen and inhibits the production of IL-4, IL-5 and TNF- α in addition to IL-2 and IFN- γ , thus promoting the development of parasites [40–43].

Moreover, in endemic areas, repeated infections due to *Plasmodium* in children lead to the development of asymptomatic forms to the detriment of serious or severe forms. Monunill *et al.* have found that the number of *P. falciparum* malaria infections does not affect the levels of IL-5, IL-6 and IL-10, unlike other cytokines [37]. Depending on the number of malaria episodes, no difference has been found between three groups of patients in both their IL-6 and IL-10 levels [44]. All this is consistent with our current results.

In addition, the positive association between cytokines IL-10, IL-6, IL-12p70 and parasitemia in asymptomatic *P. falciparum* infection suggests that these three cytokines are produced concurrently. IL-6 and IL-12p70 display a proinflammatory cytokine role promoting parasite suppression, whereas IL-10 plays an anti-inflammatory role. However, the absence of significant differences in IL-12p70 between asymptomatic *P. falciparum*-infected children revealed that this cytokine is minimally produced during asymptomatic malaria. The negative association of IFN- γ with parasitemia shows that this cytokine is not produced during asymptomatic parasitemia and highlights the regulatory role of IL-10 in the inhibition of the effects of IFN- γ , as already described above.

Frimpong *et al.* have found significant increased levels of TNF- α , IL-6, IFN- γ , IL-17 and IL-4 in asymptomatic malaria infection, although a lack of difference was observed in IL-10 levels and the ratios of IFN- γ /IL-10, TNF- α /IL-10 and IL-6/IL-10 when compared to controls, suggesting the maintenance of the balance between pro- and anti-inflammatory cytokines [16]. Indeed, the latter is crucial in the outcome of the disease. Here, data demonstrated

that even though asymptomatic *P. falciparum* infection seems to be up-regulated by anti-inflammatory cytokine there is a balance between pro- and anti-inflammatory cytokines. In fact, a balance in favor of anti-inflammatory cytokines such as IL-10 or maintenance of the balance between pro- and anti-inflammatory responses suppresses parasite clearance and contributes to parasite growth in increasing parasitemia [13,16], and therefore promotes the development of asymptomatic infection. Due to the absence of clinical signs, as asymptomatic carriers remain untreated they constitute a silent natural reservoir which supports the transmission of malaria. All this has led to postulating that people with asymptomatic malaria infection can be either in acute, mild or late stages of infection, as in the case of symptomatic infection. Indeed, it has been observed that in symptomatic malaria infection the acute phase may reflect an early and effective immune response regulated by proinflammatory cytokines for controlling the parasite load, whereas the mid-stage might be down-regulated by anti-inflammatory cytokines that is put in place for suppressing the proinflammatory effect and a very unbalanced response leads to severe disease [45,46].

The significant lower levels of IL-17A and TNF- α found, respectively, in the infected group from the urban and semi-urban areas with higher IL-10 levels suggest that asymptomatic *P. falciparum* infection does not induce these proinflammatory cytokines. These results were close to previous reports. In Uganda, Jagannathan *et al.* found a low level of IFN- γ and TNF- α in asymptomatic children with malaria and concluded that repeated exposure to malaria infection leads to a decrease in the frequency of TNF-producing CD4⁺ T cells and autoregulating Th1 CD4⁺ cells that co-produce IFN- γ together with IL-10 [35]. Production of Th1 and Th17 cytokines (such as TNF- α and IL-17A, respectively) was shown to decrease in asymptomatic *P. falciparum* infection, while IL-10 levels were high [36]. This is consistent with the suppressive activity that IL-10 would play upon production of proinflammatory cytokines in our asymptomatic children.

However, there are conflicting reports on IFN- γ and TNF- α levels resulting from asymptomatic *P. falciparum* infection in Gabonese children and Indonesian adults. In these studies, the authors report that high IFN- γ and TNF- α levels were associated with asymptomatic forms of the disease [33,34]. Interestingly enough, these results corroborate with the significantly elevated levels of IFN- γ and IL-6 with higher IL-10 levels found in our asymptomatic children from both the rural area and the general study population, and re-emphasize the possible existence of a balance between pro- and anti-inflammatory cytokines in asymptomatic *P. falciparum* infection. Given that the urbanization was predictive of IFN- γ and IL-10 levels, especially in the urban and semi-urban areas and in the

semi-urban area, all the above suggests that IL-10 in asymptomatic *P. falciparum* carriers could counter-regulate the IFN- γ , TNF- α and IL-17A effects to maintain tissue integrity.

The following comparisons relate only to the asymptomatic *P. falciparum*-infected children between areas. Significantly elevated differences in cytokine levels found in the rural area when compared to the semi-urban and/or urban area but also observed in the semi-urban area when compared to the urban area may highlight the effect of immune activation due to repeated exposures to infections observed in high endemicity regions. Indeed, it has been shown that infection frequencies are higher in rural than in urban and semi-urban areas [10]. Another study stipulated that chronic exposure to infection and immune activation may affect the development and preservation of natural acquired immunity [47]. An association between repeated malaria infection in children and the loss or decrease of cytokine production has been observed; this dysfunction was correlated with clinical tolerance to malaria [48–50]. After repeated malaria infections children lost their ability to produce strong levels of cytokines [38]; this is reflected by the elevated cytokine levels observed in our asymptomatic rural children and accordingly accounts for their exposure to infections compared to the urban area.

In particular, IL-17 and IL-22 are known as Th17 cytokines [39,51], and their significant difference between infected children from urban and semi-urban areas and between children from urban and rural areas to the disadvantage of the urban area may imply that the Th17 response is also modified by repeated exposition to infections. The Th17 pathway appears to be playing an extremely important role in immunity against *P. falciparum* [52]. It has been stipulated that IL-17 could play a dual role in malaria in the early phase of infection through the expression of a high production of IL-17, which can prevent severe malaria, and during the acute phase by overproduction of IL-17, which induces severe malaria anemia [12].

In addition, similar to that of IL-17A and IL-22, the profile observed by TGF- β would bring to light the role played by this regulatory cytokine upon Th17 response. Development of Th17 cells in humans depends upon cytokine microenvironments rich in IL-1 β , IL-6 and TGF- β that initiate the differentiation process of the Th17 cells [52]. A study has shown that after many exposures to infection the standard baseline level of cytokines of people living in hyperendemic regions remained more elevated compared to people living in hypo-endemic areas [53]. This underlines the higher level of exposure to infections of children in rural than in semi-urban and urban areas.

Moreover, that IL-4 and IL-13 levels were significantly lower in the urban and semi-urban compared to the rural

area suggests that Th2 immune response is modulated differently in these two areas. It has been established that these Th2 cytokines are involved in anti-helminth responses [54]. A study conducted in Gabon found 6.8% polyparasitism among asymptomatic parasite carriers living in urban, semi-urban and rural areas, and that the prevalence of helminthic infections in rural areas was higher than in semi-urban and urban areas [10].

The significantly higher IFN- γ levels in the urban and semi-urban areas compared to the rural area suggest a probable inhibition of IFN- γ production in the rural area, the most hyperendemic region. In a hyperendemic context, the higher regulatory cytokine (TGF- β and IL-10) levels can inhibit IFN- γ production and thus suppress proinflammatory responses and promote the preservation of parasites through asymptomatic forms of infection.

In contrast, the significant difference between urban and semi-urban values of IL-12p70 and IL-10 levels in favor of the semi-urban area supports the idea that the semi-urban area is more exposed than the urban area.

The significantly elevated levels of IL-10 in the semi-urban area and IL-6 in the urban area compared, respectively, to the rural and urban areas suggest a probable inhibition of their secretion. However, the similarity between the urban and rural areas for the IL-12p70, IL-6 and IL-10 levels could be due to the difference in sample size observed.

This study has some limitations. First, some participants in the uninfected group might have been wrongly classified due, on one hand, to the existence of submicroscopic parasitemia load that only a PCR-based method can detect, and on the other hand to the fact that the uninfected group was classified based on the presence of infection as determined by either a rapid diagnostic test or a thick blood smear. Secondly, the participants were not followed to determine if any of them progressed to symptomatic infection and therefore note the cytokine variations in the onset of these clinical signs. A final limitation could be the lack of detection of other infections such as intestinal parasites, which are common in children. Nevertheless, this study was able to show that asymptomatic *P. falciparum* infection results in increased pro- and anti-inflammatory cytokines levels without modifying the balance between pro- and anti-inflammatory cytokines.

In conclusion, this study demonstrates that higher levels of IL-6 and IL-10 among the 10 cytokines evaluated in the Gabonese children in this study are associated with asymptomatic *P. falciparum* infection. IL-10 may be a clearer indicator of the asymptomatic malaria, as significantly higher levels were associated with the asymptomatic form in all three distinct areas and with parasitemia load, in contrast to IL-6. Our studies reinforce recent studies reporting that the asymptomatic malaria infection results

in maintenance of the balance between pro- and anti-inflammatory cytokines [16], and underline the higher exposure to infections of asymptomatic-infected children in rural areas than in the two other areas.

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CONFLICTS OF INTEREST

The authors have no financial conflicts of interest.

AUTHOR CONTRIBUTIONS

C.N.M.M.N. co-ordinated the study, performed the cytokine measurements and all analyses and wrote the manuscript. S.L.O.L. co-ordinated the study and contributed to the cytokine measurements and drafting of the manuscript. C.L.K. and R.K.I.L. contributed to the collection of samples and data and performed the *P. falciparum* diagnosis. APO contributed to the sample and data collections and performed *P. falciparum* diagnosis and with S.S.O. contributed the cytokine measurements. N.P.T.T. contributed to the sample collection and prescribed the treatment to the asymptomatic malaria participants. J.B.L.D. conceived and designed the study, contributed to the drafting of the manuscript and will be responsible for communication with the journal during the manuscript submission, peer review, publication process and after publication. All authors critically reviewed and approved the final version of the manuscript.


ETHICAL STATEMENT

This study was approved by the Gabonese National Research Ethics Committee (no. 0023/2013/SG/CNE). Written informed consent was obtained from the parents or guardians before each child's participation in the study.

DATA AVAILABILITY STATEMENT

Due to the confidentiality of participants' data, the data associated with this study are not directly accessible via a link, but readers or researchers can access it by contacting the corresponding author.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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