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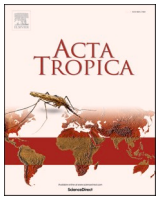

Biomarker of *Anopheles* exposure in Ghanaian children with hemoglobin S and C

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ABSTRACT

Heterozygous carriers of hemoglobin S and C (HbAS and HbAC) have a reduced risk of severe malaria but are not protected from *Plasmodium falciparum* infection, suggesting that the protection involves acquired immunity. During a blood meal, female *Anopheles* mosquitoes inject saliva that can elicit a host antibody response, which can serve as a proxy for exposure to *Plasmodium* infection. Previous studies have shown that the peptide gSG6-P1 of *An. gambiae* saliva is antigenic and highly *Anopheles* specific. Here, we used plasma samples from 201 Ghanaian children with wild-type hemoglobin (HbAA), HbAS, and HbAC to evaluate antibody levels against gSG6-P1 as a serological biomarker of *Anopheles* exposure and, therefore of *P. falciparum* infection risk. Malaria antigen (PfCSP, GLURP, Pfs230, and HB3VAR06)-specific IgG levels, demographic data, and data regarding *P. falciparum* infection and malaria control practices were also analyzed. Children with active *P. falciparum* infection had higher antibody levels against all antigens, and those with HbAS and HbAC had significantly higher antibody levels against Pfs230. Pfs230-specific IgG correlated negatively with gSG6-P1-specific IgG in children with HbAC. Our results highlight the importance of studying the role of hemoglobinopathies in malaria transmission to improve control interventions.

1. Introduction

Plasmodium falciparum is a mosquito-borne protozoan that causes most malaria cases in Sub-Saharan Africa and almost all the associated mortality and severe morbidity (WHO, 2020). Despite significant progress towards malaria elimination in several countries worldwide, 247 million malaria cases and more than 600,000 malaria deaths were estimated in 2021, most in Africa (~95 %) (WHO, 2020). In Ghana, malaria prevalence varies widely, with the Northern region having as high as 51 % prevalence compared with 16–28 % in the Southern part of the country (Amoah et al., 2019; Diallo et al., 2017; Dieng et al., 2019; Seidu et al., 2023a). A recent study also confirmed *Anopheles gambiae* as the primary vector and showed variable entomological inoculation rates within Northern Ghana, ranging from 26 to 109 infective bites/person/year (VectorLink, 2020).

During their blood feeding, mosquitoes deposit salivary proteins in the skin and blood vessels of the host to help in the feeding process. We have previously shown that several proteins in the saliva of important malaria vectors are immunogenic and that levels of IgG specific for these antigens correlate directly with the risk of infection (Londono-Renteria et al., 2020a, 2020b, 2010). Among several factors associated with the risk of malaria, hemoglobinopathies are important, with the structural hemoglobin variants S (β6Glu → Val) and C (β6Glu → Lys) as frequent polymorphisms of adult hemoglobin (HbA). HbAS and HbAC have reached carrier frequencies of up to 30 % in malaria-endemic areas, particularly in sub-Saharan Africa, the Middle East, India and among people of African descent living in Europe and North, Central and South America (Lamptey et al., 2023; Piel et al., 2013, 2010;

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Vargas-Hernandez et al., 2023). Individuals carrying on those traits do not display symptoms of sickle cell disease. Although they are at markedly reduced risk of severe malaria, they are not protected from *P. falciparum* infection per se (Taylor et al., 2012), implying that the protection is complex and acquired immunity to several malaria antigens seems to play an important role (Oleinikov et al., 2023; Tan et al., 2011; Verra et al., 2007). Previous studies have suggested that these hemoglobinopathies are associated with higher transmissibility to the mosquito vector since gametocytes, the sexual stages infective to *Anopheles* mosquitoes, are carried more often by HbAC (Goncalves et al., 2017) and HbAS (Andolina et al., 2023) individuals than by their HbAA counterparts. They are also more infectious and tend to have infections that last longer than in HbAA individuals (Goncalves et al., 2017), which could increase attractiveness to mosquitoes (Lacroix et al., 2005; Robinson et al., 2018). Together, these findings suggest that carriers of hemoglobinopathies are an important parasite transmission reservoir (Ngou et al., 2023).

We explored the potential relationship between HbAS, HbAC and *P. falciparum* infection on the one hand and exposure to mosquito bites on the other by measuring IgG levels against the peptide gSG6-P1 of *An. gambiae* saliva, which has been validated as a marker of exposure to *Anopheles* bites (Londono-Renteria et al., 2015; Poinsignon et al., 2009). Our results suggest an association between antibody responses against mosquito antigens and the type of hemoglobin in *P. falciparum* carriers. To the best of our knowledge, this is the first study evaluating the presence of anti-saliva antibodies in people infected with *P. falciparum* and different hemoglobinopathies.

2. Material and methods

2.1. Study site and participants

We assessed IgG responses to *P. falciparum* and *An. gambiae* saliva antigens in 201 plasma samples collected from resident children during a cross-sectional study conducted between August and September 2020 in rural communities of Kumbugu, Nanton, and Tolon in Northern Ghana. The study area is relatively dry, with one rainy season from April to September or October. A detailed description of the study area has been given elsewhere (Seidu et al., 2023a). Plasma samples from HbAA, HbAS, and HbAC children were matched 1:1:1 by age. Hb phenotypes and circulating *P. falciparum* parasites were determined by isoelectric focusing electrophoresis and PCR, respectively (Lamptey et al., 2023; Seidu et al., 2023a). PfHRP2 in plasma was determined with the Quantimal™ CELISA kit (Cellabs, Australia) following the manufacturer's instructions and used to calculate the total parasite biomass as described before (Hendriksen et al., 2012). Additionally, we used information regarding malaria control practices collected in a cross-sectional household survey conducted between June and July 2020 in the same communities (Seidu et al., 2023a).

2.2. Antigens

The gSG6-P1 and circumsporozoite protein (PfCSP) peptides were synthesized by Genscript. The domain R0 of glutamate-rich protein (GLURP) (Theisen et al., 1995) was produced in *Escherichia coli*, whereas the 230-kDa sexual stage protein (Pfs230) (Acquah et al., 2017) was produced in *Lactococcus lactis*. The entire ectodomain of HB3VAR06 was produced in baculovirus-transfected Sf9 insect cells (Stevenson et al., 2015).

2.3. Specific IgG response measured by ELISA

IgG reactivity against the recombinant proteins and synthetic peptides was measured by ELISA as described elsewhere (Londono-Renteria et al., 2010; Lopez-Perez et al., 2021). Briefly, 96-well flat-bottom microtiter plates (Nunc MaxiSorp; Thermo Fisher Scientific) were coated with recombinant protein in phosphate-buffered saline (PBS) and incubated overnight at 4 °C. After blocking, plasma samples were added in duplicate, followed by horseradish peroxidase-conjugated rabbit anti-human IgG (1:3000; Dako). Bound antibodies were detected by adding TMB PLUS2 (Eco-Tek) and the reaction stopped with 0.2 M H_2SO_4. The optical density was read at 450 nm (HiPo MPP-96 microplate reader; Molecular Devices). To normalize data between plates, the specific antibody levels were calculated in arbitrary units (AU) as described elsewhere (Lopez-Perez et al., 2021). Plasma samples from Danish adults without malaria exposure and a pool of Ghanaian adults with previous *P. falciparum* infection were included as negative and positive controls, respectively.

2.4. Statistical analysis

Data were analyzed and plotted using GraphPad Prism version 9.5 (GraphPad Software, San Diego, California, USA). The Mann-Whitney U or Kruskal-Wallis test followed by Dunn's multiple-comparison test were used to compare two or more than two groups, respectively. Spearman's rank correlation (r_s) was used to assess the correlation between numeric variables. Multiple regression models were used to evaluate the effect of potential confounders on the association between antibody levels and relevant independent factors. A chi-square test was used to compare differences in proportions. P-values < 0.05 were considered statistically significant. The antibody levels were normalized using z-score to generate the breadth scores, and then each individual's *z*-score to *P. falciparum* antigens were summed up. Antibody levels to gSG6-P1 were then categorized into three groups (low, medium, and high) based on tertiles with an analysis comparing the low and high groups.

3. Results

3.1. Study population

We analyzed a subset of 201 plasma samples from children living in Northern Ghana (Table 1). The age of the study children ranged from 2 to 15 years, with no differences among municipalities ($p = 0.30$). Likewise, no differences in the Hb levels were observed among municipalities ($p = 0.87$) or Hb phenotypes ($p = 0.57$). About 21 % of the children had a PCR-positive *P. falciparum* infection, with no significant differences in age ($p = 0.48$) or Hb levels ($p = 0.22$) between infected and uninfected participants. A similar frequency of circulating *P. falciparum* parasites ($p = 0.68$), but lower total parasite biomass ($p = 0.03$) was observed in children with HbAS. The frequency of PCR-positive infection ($p = 0.02$) and the total parasite biomass ($p = 0.02$) were significantly higher in Tolon than in Kumbugu and Nanton (Fig. 1).

Table 1
Demographic characteristics of the studied population.

Parameter	Category	Total[a] n = 201
Age	Years	7 [4 - 10]
Hb	g/dL	11 [10.2 – 11.6]
PfHRP2[b]	pg/mL	103.3 [12.2 – 561.5]
Sex	Female	80 (40 %)
PCR	*P. f* positive	43 (21 %)
Municipality	Kumbugu	75 (37 %)
	Tolon	95 (47 %)
	Nanton	31 (15 %)
Hb phenotype	HbAA	67 (33 %)
	HbAS	67 (33 %)
	HbAC	67 (33 %)

[a] Interquartile ranges are presented in brackets
[b] data available for 32 of the 43 children with *P. falciparum* infection.

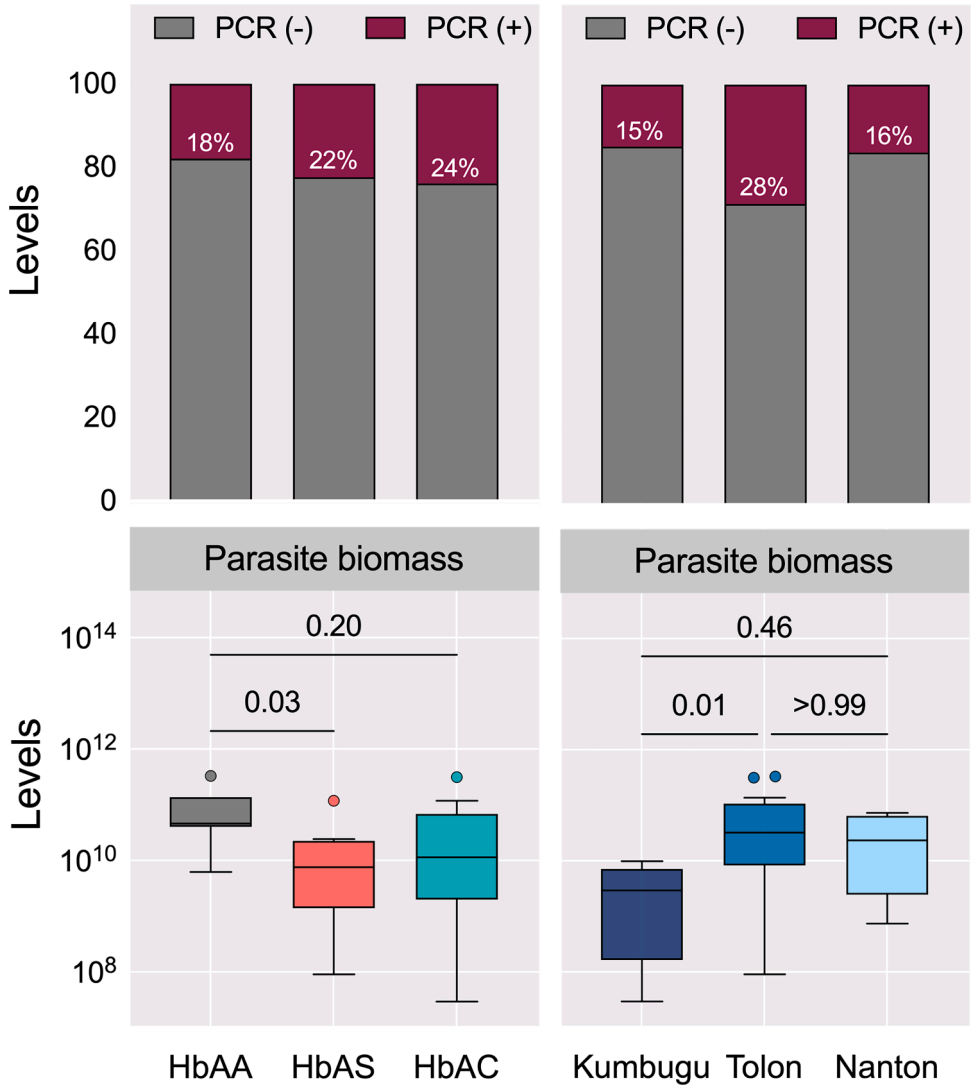

Fig. 1. *P. falciparum infections and plasma levels of PfHRP2.* Frequency of *P. falciparum* infections by PCR (percentage; n = 43) and total parasite biomass (Log scale; n = 30) according to the Hb phenotype (left) and municipally (right). Median, interquartile range (IQR), and whiskers (1.5 times the IQR) are shown. P values using the Kruskal-Wallis test followed by Dunn's multiple comparisons test, are also indicated.

3.2. IgG response to Anopheles and malaria antigens

To further evaluate the differences in the frequency of *P. falciparum* infection and total parasite biomass among Hb phenotypes and municipalities, we next explore the specific IgG response to *Anopheles* and malaria antigens. For this, we included a mosquito salivary peptide (gSG6-P1) and antigens from *P. falciparum* sporozoites (PfCSP), gametocytes (Pfs230), merozoites (GLURP), and mature asexual blood stages (HB3VAR06).

Plasma levels of Pfs230-specific IgG were significantly higher in children with HbAS and HbAC than in HbAA children, whereas levels of IgG specific for PfCSP and GLURP were similar among the groups. In contrast, IgG to HB3VAR06 was slightly lower in HbAS ($p = 0.07$) (Fig. 2). Although, IgG levels to gSG6-P1 were not significantly different among Hh phenotypes, children highly exposed to gSG6-P1 were more frequent in HbAS and HbAC groups than in their HbAA counterpart (76% vs 58 %, $p = 0.01$, OR = 2.3, 1.2-4.4). Overall, the breadth score for *P. falciparum* antigens was significantly higher in children highly exposed to gSG6-P1 (−1.02 vs −0.55; $p = 0.049$), but no differences were observed among Hb phenotypes or infection status. Children living in Kumbugu, where the lowest frequency of *P. falciparum* infection was observed, had the highest levels of IgG to gSG6-P1 but lowest to PfCSP, Pfs230, GLURP and HB3VAR06 (Fig. 3).

Children with PCR-positive *P. falciparum* infections had significantly higher IgG levels against gSG6-P1, Pfs230, GLURP, and HB3VAR06 (Fig. 4). Levels of hemoglobin and IgG to all the tested *P. falciparum* antigens correlated positively among uninfected HbAA children, indicative of acquired protective immunity, whereas their hemoglobin levels correlated negatively with the levels of gSG6-P1-specific IgG. Moreover, in infected children carrying HbAC, hemoglobin and HB3VAR06-specific IgG levels were positively correlated and the association remains after adjusting by age, sex, and total parasite biomass ($\beta = 2.33$; $p = 0.003$) (Table 2). Among children with PCR-detectable *P. falciparum* infection, we found a significant negative correlation between IgG antibody levels to gSG6-P1 and Pfs230 only in individuals with HbAC, and this continues being significant after adjusting by age ($\beta = -0.13$; $p = 0.03$) (Table 3).

3.3. Malaria control and prevention practices

We used information about malaria control and prevention practices collected from 394 households in a cross-sectional survey conducted in June-July 2020 in the same communities (Seidu et al., 2023a). Overall, 59 % of the households used indoor insecticide sprays/coils, and 47 %

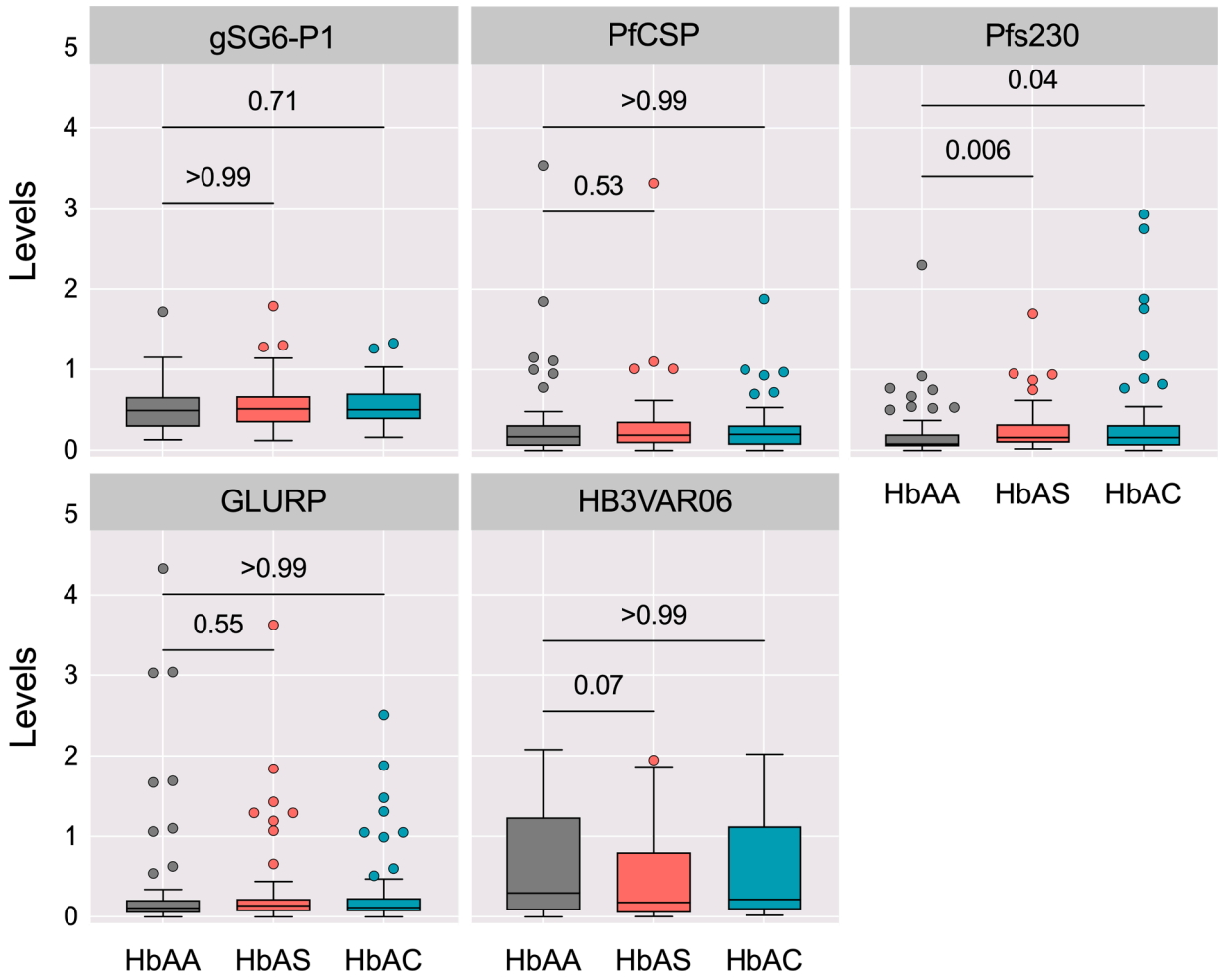

Fig. 2. *IgG response to An. gambiae and P. falciparum antigens.* Specific IgG levels against *An. gambiae* salivary antigen (gSG6-P1) and recombinant antigens from several parasite stages (PfCSP, Pfs230, GLURP, and HB3VAR06) were determined by ELISA in plasma samples from HbAA, HbAS, and HbAC children (n = 64-67, each group). Median, interquartile range (IQR), and whiskers (1.5 times the IQR) are shown. P values using the Kruskal-Wallis test followed by Dunn's multiple comparisons test are also indicated. All panels express the values in Log arbitrary units (AU).

used bed nets. In contrast, only 13 % mentioned clearing bush/weeds around homes, and 6 % avoid sitting outside at night as malaria control and preventive measures. Some differences were observed among municipalities (Table 4).

4. Discussion

P. falciparum malaria continues as the main vector-borne parasitic infection in Africa, and hemoglobinopathies drastically reduce the risk of developing severe malaria (Taylor et al., 2012). Here, we did not observe significant differences in the Hb levels associated with the municipality, the Hb phenotype, or the infection status. However, lower total parasite biomass (reflecting both sequestrated and peripheral parasites) was observed in children with HbAS in all municipalities. We observed the lowest frequency of *P. falciparum* infection in participants living in Kumbugu, yet, here we also observed the highest levels of IgG antibodies against gSG6-P1 but the lowest to PfCSP, Pfs230, GLURP and HB3VAR06 suggesting the possibility that these children were mainly exposed to uninfected bites. Although we did not collect mosquito specimens to prove this hypothesis, the use of IgG antibodies against gSG6-P1 peptide has been extensively tested worldwide and validated as a biomarker of mosquito exposure (Badu et al., 2012; Cheteug et al., 2020; Drame et al., 2012; Poinsignon et al., 2008; Sagna et al., 2013). A recent report on entomological monitoring reported a slightly higher mean of human bite rates in Kumbugu than in Tolon (8.8 vs 6.5 bites per person per night), with a similar preference for feeding indoors or outdoors in both places. However, the entomological inoculation rates (EIR) were lower in Kumbugu than in Tolon (17 vs 87 infective bites/-person/year, respectively) (VectorLink, 2020). The same study also found a higher outdoor EIR in Tolon, suggesting that prevention practices among households are not in agreement with the malaria transmission dynamics found in the study sites.

As shown previously (Montiel et al., 2020), we found that children with PCR-detectable *P. falciparum* infection had higher levels of *P. falciparum*-specific IgG, perhaps due to a boosting effect. Then, we used the IgG anti-gSG6-P1 antibody levels to evaluate exposure to mosquito bites and found that those highly exposed (reflected by higher anti-gSG6-P1 antibodies), also presented higher levels of *P. falciparum*-specific IgG. Antibody levels against the sexual stage-related parasite antigen Pfs230, which has been strongly associated with transmission-reducing activity (Bousema et al., 2010; Jones et al., 2015), were higher in children with HbAS and HbAC. As shown in previous studies (Bousema et al., 2010), increased IgG levels to Pfs230 in these individuals are consistent with the longer duration of infection and higher probability of having gametocytes described in HbAS (Gong et al., 2012). Furthermore, studies have shown an increased attractiveness to mosquitoes in the presence of sexual stages of *P. falciparum* (Debebe et al., 2020; Robinson et al., 2018) leading to suggestions of HbAS and HbAC carriers as a parasite important reservoir involved in maintaining *P. falciparum* transmission (Goncalves et al., 2017;

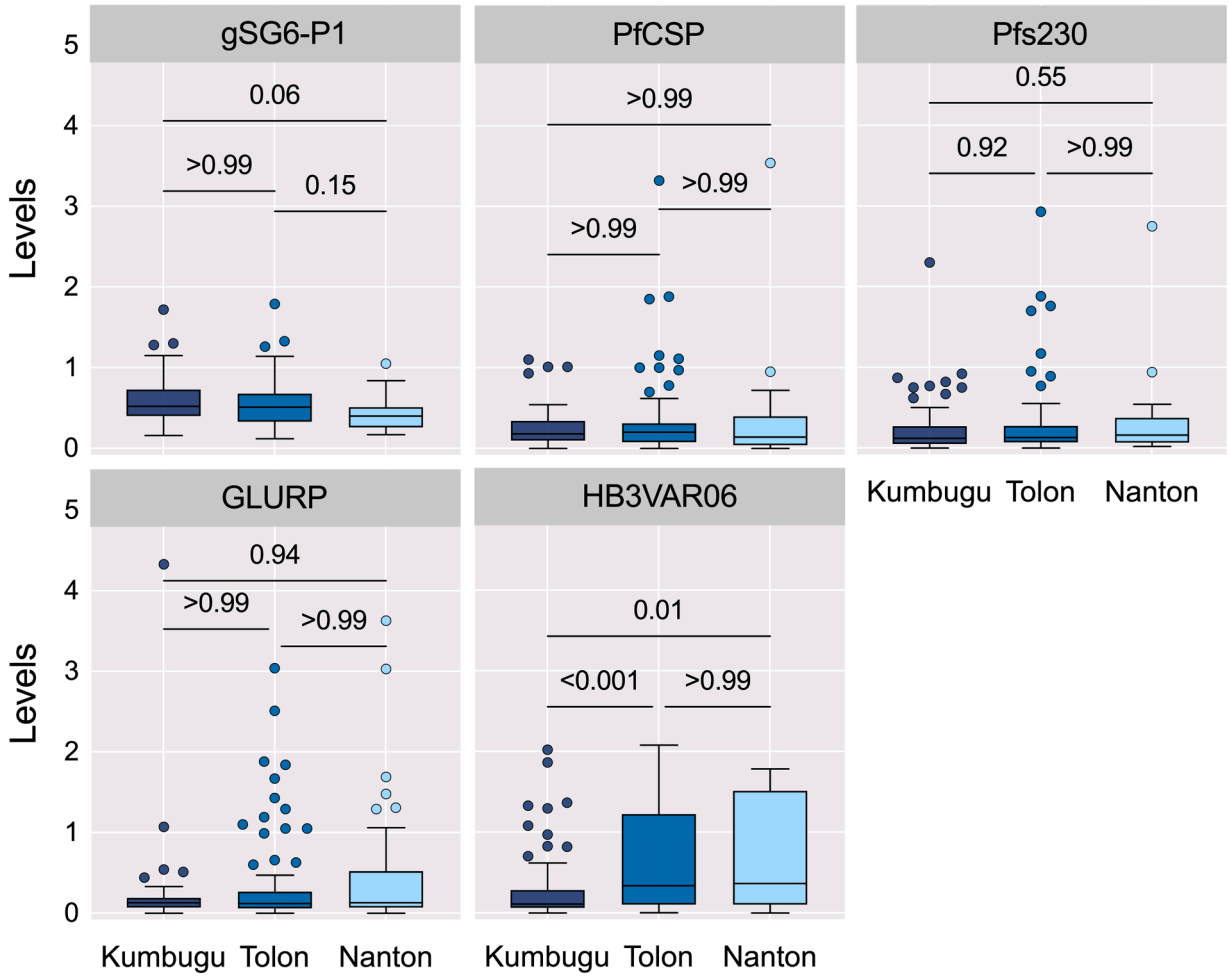

Fig. 3. *Specific-IgG response in the municipalities.* Specific IgG levels against *An. gambiae* salivary antigen (gSG6-P1) and recombinant antigens from several parasite stages (PfCSP, Pfs230, GLURP, and HB3VAR06) determined in plasma samples from children living in Kumbugu (n = 75), Tolon (n = 95), and Nanton (n = 31). Median, interquartile range (IQR), and whiskers (1.5 times the IQR) are shown. P values using the Kruskal-Wallis test followed by Dunn's multiple comparisons test are also indicated. All panels express the values in Log arbitrary units (AU).

Gouagna et al., 2010). Here, we observed that high exposure to gSG6-P1 was more frequent among children with HbAS and HbAC. Moreover, in parasitaemic HbAC children, a negative correlation between IgG levels to gSG6-P1 and Pfs230 was observed, suggesting that those children can be less effective in reducing transmission. Alternatively, those children may have short-duration infections that are not able to produce gametocytes and boost gSG6-P1-specific IgG, but did not analyze this. Recently, it was reported that a high proportion of infections in HbAA and HbAS individuals seems to be too short in duration and low density to contribute to the transmission (Andolina et al., 2023). Regrettably, we lack the information about mosquito biting rates and gametocytaemia in our cohort to explore the association of IgG levels and contribution to parasite transmission.

In addition, lower IgG levels to HB3VAR06 observed in children with HbAS are consistent with the reduced expression of PfEMP1 on the HbAS IE surface (Cholera et al., 2008; Sanchez et al., 2019; Seidu et al., 2023b). High exposure to *Anopheles* mosquitoes was associated with a lower antibody level to PfMSP1 in Côte d'Ivoire (Aka et al., 2020), suggesting the immunomodulatory activity of mosquito saliva in response against the parasite and consistent with many studies demonstrating the importance of salivary proteins in vector-borne pathogen transmission and pathogenesis, and new vaccine candidates aim to block their enhancing effect (Olajiga et al., 2021). For instance, Imutex's AGS-v and AGS-v PLUS vaccines, which have been advocated as potential universal vaccines against vector-borne pathogens, are based on salivary proteins of multiple mosquito vectors and have shown good immunogenicity and safety in their initial clinical trials (Friedman--Klabanoff et al., 2022; Manning et al., 2020).

To the best of our knowledge, this is the first study evaluating the presence of antibodies to mosquito salivary antigens in individuals infected with *P. falciparum* and their association with hemoglobinopathies. However, we acknowledge that our study has some limitations that should be addressed in future studies, in particular, the lack of entomological data collected at the time of the study. Likewise, gametocyte carriage and infection duration could help measure the impact of hemoglobinopathies on transmission. Taking into account the relatively high parasite carriage rates reported here, it appears that more efficient interventions are needed in agreement with the transmission dynamics reported previously (VectorLink, 2020).

In conclusion, our study shows that antibodies against the salivary peptide gSG6-P1 increase with exposure to *P. falciparum,* and the type of host hemoglobin influences it. Malaria is still one of the most important vector-borne parasitic diseases in the world and HbAS/HbAC are frequent hemoglobinopathies in sub-Saharan African countries, with carriers being potential infectious reservoirs. Accurate data on malaria burden, mosquito abundance, and mosquito-human contact intensity are important factors in estimating disease risk and should be used to guide control measures in higher transmission areas.

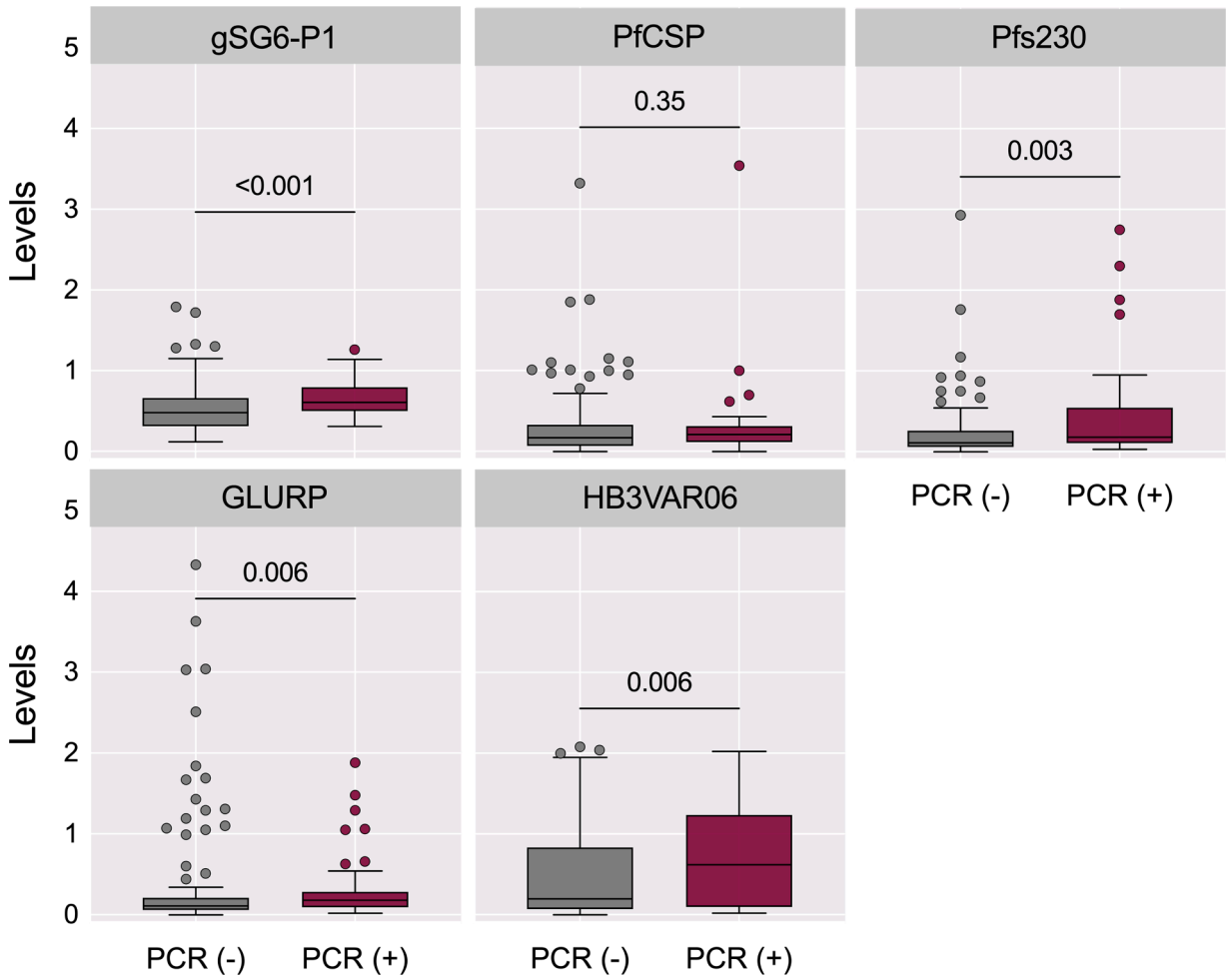

Fig. 4. *IgG response according to P. falciparum infection.* Specific IgG levels in plasma samples from children with (n = 43) and without (n = 158) *P. falciparum* infection determined by PCR. Median, interquartile range (IQR), and whiskers (1.5 times the IQR) are shown. P values using the Mann-Whitney test are also indicated. All panels express the values in Log arbitrary units (AU).

Table 2
Correlation between Hb concentration and antigens according to the Hb phenotype.[a]

Uninfected chidlren Hemoglobin concentration	gsg6-p1	pfcsp	pfs230	glurp	hb3var06
HbAA	**−0.280***	**0.278***	**0.403****	**0.416****	0.257
HbAS	**0.360****	−0.004	0.140	0.070	−0.067
HbAC	−0.145	−0.082	−0.035	−0.034	0.184
Infected chidlren					
hemoglobin **concentration**	**gsg6-p1**	**pfcsp**	**pfs230**	**glurp**	**hb3var06**
HbAA	−0.032	−0.506	−0.228	0.132	−0.196
HbAS	−0.421	−0.144	−0.186	0.471	0.046
HbAC	0.044	0.149	0.283	0.055	**0.670****

[a] Spearman's rank correlation (r_s) values and significant p values (* $p < 0.05$, ** $p < 0.001$).

Table 3
Correlation between IgG levels to gSG6-P1 peptide and *P. falciparum* antigens according to the Hb phenotype.[a]

Uninfected children gSG6-P1	PfCSP	Pfs230	GLURP	HB3VAR06
HbAA	0.136	0.102	0.085	0.075
HbAS	0.079	0.018	−0.014	0.145
HbAC	0.161	0.078	0.215	0.029
Infected children				
gSG6-P1	**PfCSP**	**Pfs230**	**GLURP**	**HB3VAR06**
HbAA	0.164	−0.355	0.018	−0.218
HbAS	0.353	0.285	−0.145	0.082
HbAC	0.124	**−0.597***	−0.170	−0.029

[a] Spearman's rank correlation (r_s) values and significant p values (* $p < 0.05$).

Ethics approval and consent to participate

The original study, from which the samples used here were obtained, was approved by the Ethics Review Committee of the Ghana Health Service (GHS; GHS-ERC 008/07/19) and by the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (Federal-wide Assurance FWA 00001824, NMIMR-IRB CPN 006/19). A declaration of free willingness to participate in the study and written informed consent were obtained from all participants or guardians before enrolment.

Consent for publication

Not applicable.

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Table 4
Malaria control and prevention practices among households.

Parameter	Kumbugu (n = 137)	Tolon (n = 127)	Nanton (n = 130)	p-value[a]
Have a bed net	101 (74 %)	108 (85 %)	**113 (87 %)**	**0.01**
Use a bed net	63 (46 %)	57 (45 %)	66 (51 %)	0.60
Use insecticide sprays/coils	89 (65 %)	68 (54 %)	75 (58 %)	0.16
Bush clearing around homes	**27 (20 %)**	17 (13 %)	9 (6.9 %)	**0.009**
Avoid sitting outside	4 (2.9 %)	**13 (10 %)**	6 (4.6 %)	**0.031**

[a] p-value using Chi-square test.

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CRediT authorship contribution statement

Berlin Londono-Renteria: Conceptualization, Formal analysis, Methodology, Resources, Visualization, Writing – original draft, Writing – review & editing. **Zakaria Seidu:** Investigation, Methodology, Resources, Writing – review & editing. **Helena Lamptey:** Resources, Writing – review & editing. **Michael F. Ofori:** Funding acquisition, Resources, Supervision, Writing – review & editing. **Lars Hviid:** Funding acquisition, Supervision, Writing – review & editing. **Mary Lopez-Perez:** Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors declare that all the data related to this study are cited in the text.

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