

Humoral immune response against *Anopheles* bites as a measure of exposure to *Plasmodium falciparum* in asymptomatic infections in a malaria endemic area of Colombia

Jehidys Montiel

Universidad de Antioquia

Luisa Fernanda Carbal

Universidad de Antioquia

Alberto Tobón-Castaño

Universidad de Antioquia

Gissella M. Vásquez

Naval Medical Research Unit No 6

Michael L. Fisher

Naval Medical Research Unit No 6

Berlin L. Londono (✉ blondono@ksu.edu)

Kansas State University <https://orcid.org/0000-0001-5956-5274>

Research

Keywords: Asymptomatic malaria, *An. albimanus*, *An. darlingi*, antibodies, bite exposure

Posted Date: December 3rd, 2019

DOI: <https://doi.org/10.21203/rs.2.18019/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Version of Record: A version of this preprint was published at Malaria Journal on January 23rd, 2020. See the published version at <https://doi.org/10.1186/s12936-020-3128-9>.

Abstract

Background The humoral immune response against *Anopheles* salivary glands proteins in the vertebrate host can reflect the intensity of exposure to *Anopheles* bites and the risk of *Plasmodium* infection. In Colombia, the identification of exposure biomarkers is necessary due to the several *Anopheles* species circulating. The purpose of this study was to evaluate human exposure to *Anopheles* bite by measuring antibody responses against salivary glands extracts from *An. albimanus* and *An. darlingi* and also against the gSG6-P1 peptide of *An. gambiae* in asymptomatic *P. falciparum* infections in the Colombian Pacific coast.

Methods We eluted dried blood spots samples to measure the IgG antibodies against salivary gland extracts of *An. albimanus* and *An. darlingi* and the gSG6-P1 peptide by ELISA in uninfected people and microscopic and submicroscopic *Plasmodium* carriers from the Colombia Pacific Coast. A multiple linear mixed regression model, Spearman correlation, and Mann-Whitney U-test were used to analyze IgG data.

Results Significant differences in specific IgG levels were detected between infected and uninfected groups for salivary glands extracts from *An. albimanus* and for gSG6-P1, also IgG response to CTG and gSG6-P1 peptide were positively associated with the IgG response to *P. falciparum* in the mixed model.

Conclusion The CTG and STE *An. albimanus* salivary glands extracts are a potential source of new *Anopheles* salivary biomarkers to identify exposure to the main malaria vector in the Colombian Pacific coast. Also, the gSG6-P1 peptide has the potential to quantify human exposure to the subgenus *Anopheles* vectors in the same area.

Introduction

Malaria is caused by the protozoan parasite *Plasmodium* and is transmitted by female *Anopheles* mosquitoes. Although significant advances have been made towards its elimination in several previously endemic countries, malaria remains a significant public health concern (1). The World Malaria Report in 2018 estimated that the global burden of malaria comprised around 219 million reported cases and 435,000 deaths worldwide (2). Specifically, in Colombia, there was a decrease in the estimated number of malaria cases by more than 20% between 2016 and 2017 (2). Despite this, malaria remains one of the foremost public health concerns in some states in Colombia such as Nariño, which is located along the Pacific coast of the country. In 2017, 26% of malaria cases in Colombia came from Nariño where, unlike other regions, *P. falciparum* is the most common species (96.3%) (3).

More than 47 *Anopheles* species in five subgenera have been reported in Colombia (4). The majority of malaria vectors in Colombia belong to the subgenus *Nyssorhynchus*, with *An. nuneztovari* and *An. darlingi* as the most important malaria vectors in areas of high malaria transmission such as Urabá, Bajo Cauca and Alto Sinú regions (5). On the South Pacific coast, where comparatively little is known about the current spatial distribution of malaria vector species (5), it has been reported that *An. albimanus* is the main vector (6, 7). However, the subgenus *Anopheles*, is the second most important group of mosquito vectors in the region, with *An. pseudopunctipennis* and *An. punctimacula* as the most representative (8).

Malaria is acquired when *Plasmodium* spp. sporozoites are injected into human skin through the bite of a female *Anopheles* mosquito along with the mosquito salivary proteins (9). Previous studies have shown that a significant number of mosquito salivary proteins are immunogenic and able to induce antibody responses, mainly IgG isotype. These antibodies can reflect the intensity of human exposure to mosquito bites and represent good indicators of the risk of infection with *Plasmodium* spp. (10–14). Significant higher IgG antibody levels against *An. albimanus* and *An. darlingi* salivary proteins have been observed in people with active malaria infection in Central and South America when compared to uninfected people living in the same region (10, 15). A similar pattern has been observed in areas where *An. gambiae* and *An. stephensi* are among the most important vectors. A significant number of these studies were performed evaluating IgG responses against the *An. gambiae* salivary protein gSG6, a highly conserved protein among *Anopheles* species from the Subgenus *Cellia* and *Anopheles* (16). The peptide, gSG6-P1, was designed from the original *An. gambiae* gSG6 sequence. IgG responses specific to this salivary peptide has been validated as a biomarker of human exposure not only in Africa but also in Asia and South America (11, 14, 17). Although there are no known species of the subgenus *Cellia* in South America, the responses observed against the gSG6-P1 peptide can be attributed to the presence of *An. pseudopunctipennis* and *An. punctimacula* members of the subgenus *Anopheles* as described above (18).

Consequently, it is necessary to characterize a broader panel of biomarkers able to identify the risk of disease more closely in areas with a great diversity of *Anopheles* mosquitoes. Our research group plans to identify exposure markers that include not only the primary malaria vectors but also markers for the majority of the circulating species playing an important role in malaria transmission in Latin America, even when these vectors species are in a smaller proportion. Thus, the main objective of this work was to characterize *Anopheles* bite exposure in an area where low-density *P. falciparum* infections are frequent and where *An. albimanus* and *An. darlingi* (lacking the gSG6 protein) are present. Thus, we explored if human IgG responses to salivary gland extracts (SGE) from these two species are associated with low-density infections by *P. falciparum*. We also aimed to evaluate whether gSG6-P1 peptide continues as a useful marker to detect exposure to minor malaria vectors in Colombia.

Methods

Samples selection

The samples used in this study were collected as part of a longitudinal study in which the purpose was to evaluate the dynamic of submicroscopic *Plasmodium* infections in Colombia.

Dried blood spots (DBS) in Whatman® 903 protein saver card (GE Healthcare, US) were collected by passive case detection in the transversal phase of the study, conducted between August 2017 to March 2018 in four villages (California, Tangareal, Robles, and Candelillas) in Tumaco city located in the south of

Colombia (1 850'N, 78845'W) (Figure 1).. The first village represents a typical suburban zone. The following two sites are characterized as rural areas, and the last one is classified as a peri-urban zone. In 2017, *P. falciparum* was reported as the predominant species (96%) in Tumaco with an annual parasite index (parasite incidence per 1,000 population) of 13.5 (19). The main malaria vector in the area is *An. albimanus* with a HBR of 2.6 in 2006 (20)

To compare the vector exposure between infected and uninfected individuals, all positive *P. falciparum* samples were selected (n = 63) from the 958 people that were enrolled in the main study. All of these infections were afebrile (axillary temperature <37.5°C), and 48 (76.2%) were submicroscopic (detected by LAMP or PCR but not by light microscopy (LM)). Furthermore, 50 uninfected samples were randomly selected by age (5 years) and sex from the total of non-infected individuals by using an Excel random list.

ELISA antigens and SGE preparations

Anopheles albimanus and *An. darlingi* were maintained under insectary conditions until salivary gland dissection. The STECLA (STE) and Cartagena (CTG) *An. albimanus* strains originated from El Salvador and Colombia, respectively, and were maintained in the insectary at the CDC (Atlanta, GA, USA). The *An. darlingi* laboratory strain originated from Iquitos, Peru (21), and was maintained in the NAMRU-6 insectary (Iquitos, Loreto, Peru). Salivary glands from 8 to 10 days old female mosquitoes were extracted by dissection and pooled into 1X PBS (10). Mosquitoes were blood feed at day 3 or 4 after emergence. A pool of 100 salivary gland pairs from each strain was then frozen and thawed three times to prepare the SGE. The concentration of the SGE was determined using a NanoDrop™ (Thermo Scientific, Wilmington, DE, USA) and 50uL aliquots were stored at -80°C until use. The *An. gambiae* gSG6-P1 peptide was synthesized by Genscript (Piscataway, NJ, USA) and the *P. falciparum* Pf-MSP peptide (Fitzgerald, USA) was used to evaluate exposure to malaria parasites.

Indirect ELISA

ELISA conditions were standardized as described elsewhere (10, 11). Also, DBS samples were prepared as by eluting half of a card circle into 300uL of elution buffer (PBS 1X, Tween 20 0.05%) and incubated overnight at 4°C. Testing of serial dilutions (1:50, 1:100 and 1:200) showed better performance of the ELISA using a 1:50 dilution. Briefly, Nunc-Maxisorp 96-well plates (Nalgene Nunc International, Rochester, NY) were coated with 50 µL/well of gSG6-P1 peptide (2µg/mL), *An. darlingi* and *An. albimanus* SGE (1µg/mL) or Pf-MSP (1µg/mL) diluted 1X PBS. Plates were incubated overnight at 4°C and blocked with 200 µL of 5% skim milk solution in PBS-tween 20 (0.05%) (Blocking buffer) for 1.5 hours at 37°C. The DBS eluted was used to prepare a 1:50 sample dilution in blocking buffer, this optimal dilution had been determined by preliminary experiments and 50 µL of diluted samples were added to each well (individual samples were tested in duplicate). Plates were incubated at 37°C for 1.5 hours, washed three times, then incubated 1h at 37°C with 50 µL/well of a 1/1,000 dilution of goat monoclonal anti-human IgG conjugated with horseradish peroxidase (AbCam, Cambridge, MA). After three final washes, colorimetric development was carried out using tetra-methyl-benzidine (Abcam) as a substrate. The reaction was stopped with 0.25 N sulfuric acid, and the optical density (OD) was measured at 450 nm. In parallel, each assessed microplate contained in duplicate: a positive control, a negative control, and a blank; wells containing no sample.

Statistical analysis

All data from questionnaires and forms were entered into a Microsoft Access database, and statistical analyses were conducted in STATA 14 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP) and GraphPad Software V5. OD normalization and plate to plate variation was performed as described elsewhere (11). Briefly, antibody levels were expressed as the ΔOD value: $\Delta OD = OD_x - OD_b$, where OD_x represents the mean of individual OD in both antigen wells and OD_b the mean of the blank wells. For each tested peptide, positive controls of each plate were averaged and divided by the average of the OD_x of the positive control for each plate to obtain a normalization factor for each plate as previously described. Each plate normalization factor was multiplied by plate sample ΔOD to obtain normalized ΔOD that were used in statistical analyses.

We estimated the median of antibody level for each antigen in uninfected people (negative PCR and negative LM) in submicroscopic (positive PCR and negative LM) and microscopic (positive PCR and positive LM) carriers. The medians are shown with their respective interquartile range (IQR). Spearman correlation coefficients were calculated to measure the strength of association between each *Anopheles* antigen with Pf-MSP IgG levels. Finally, a Mann-Whitney U-test was used to estimate differences between medians of each *Anopheles* antigen by the status of infection in the whole sample and by sites and a Kruskal–Wallis test to estimate differences between groups of infection. A multiple linear mixed regression model was constructed to determine the correlation between anti-*Anopheles* IgG levels (anti-gSG6-P1, CTG, STE, and *An. darlingi*) with anti Pf-MSP IgG levels. A random intercept at the village level was introduced in the model to correct the inter-village variations. The model was adjusted by *Plasmodium* infection, age and time of residence in a malarial endemic area; these factors showed significant p values in simple models.

Results

1. Study sample characteristics and exposure to mosquito bites

We studied exposure to mosquito bites in the area of Tumaco in Narino (Colombia) (Figure 1).. Table 1 shows the characteristics of participants according to the status of infections. The gender and age groups distribution seem to be equally represented between infected and uninfected individuals. The majority of infected people came from California and Tangareal (78.7%). There was a higher proportion of people with malaria history on infected people (42/63, 66.6%) compared uninfected people group (25/50, 50%), and 33.0% of them, got at least one episode of malaria in the previous year.

Pairwise comparison of the level of IgG antibodies against *An. albimanus* (STE and CTG), *An. darlingi* or gSG6-P1 by gender, education level and occupation did not show significant differences (Mann-Whitney test $p > 0.05$) data no showed.

2. Exposure levels and IgG response against *Anopheles* SGE and gSG6-P1 peptide

Our study shows that the level of antibodies against *An. albimanus* salivary proteins from both strains (STE and CTG) and against the gSG6-P1 peptide was significantly higher in volunteers with *Plasmodium* infection (CTG, Mann-Whitney test $p = 0.0004$; STE, Mann-Whitney test $p = 0.033$; and gSG6-P1, Mann-Whitney test $p = 0.0016$) antibody levels (Figure 2). However, this difference was not observed when testing IgG antibodies against the whole SGE from *An. darlingi* (Mann-Whitney test p value = 0.2746). This is consistent with information provided by previous studies showing *An. albimanus* as one of the important vectors in the region.

We also tested whether the difference observed in antibody level between infected and uninfected will be influenced by the village where samples were collected. Figure 3 shows the median of anti-*Anopheles* IgG levels between infected and uninfected samples by the site. Except for the California neighborhood, the IgG levels in infected samples were higher than uninfected. Nevertheless, there were only significant associations for CTG and STE in Tangareal village.

3. Human IgG antibody levels and *P. falciparum* parasitemia:

All of our *Plasmodium* infected patients were afebrile and considered as asymptomatic carriers. However, we grouped them according to the diagnostic test results into microscopic (if parasites were detected by LM and PCR) or submicroscopic if parasites were only detected by PCR (Figure 4). Accordingly, our results showed that IgG levels might change according to parasitemia. Specifically, we observed a tendency of increased antibody levels in samples where parasitaemia was detected by light microscopy compared to infections only detected by molecular tests and also in uninfected specimens. There were significant differences in the median IgG antibody levels against CTG (Kruskal-Wallis test $p = 0.0016$) and gSG6-P1 (Kruskal-Wallis test p value = 0.0067) between the three groups of infections. Although the tendency was also observed when using STE and *An. darlingi* as antigen, the differences were not significant.

4. Association between exposure to *Anopheles* antigens and antibodies against *Plasmodium* pf-MSP1 protein

We evaluated whether there was any correlation between the level of IgG antibodies against the Pf-MSP1 protein and exposure to mosquito bite reflected by the levels of IgG antibodies against the salivary antigens. We observed a positive association between Pf-MSP IgG levels with anti CTG (Spearman $r = 0.2722$, $p = 0.0035$) and gSG6-P1 peptide (Spearman $r = 0.3872$; $p < 0.000$) (Figure 5) but not for *An. darlingi* and STESGE.

5. Antibody-based model to evaluate factors of variation in exposure to *Anopheles* and *Plasmodium*.

Independent of location (random intercept at village levels), IgG response to CTG and gSG6-P1 peptide were positively associated with the IgG response to *P. falciparum* (regression coefficient (RE) = 0.105; 95% CI 0.0223- 0.189 and RE = 0.070; 95% CI 0.013-0.126 respectively). In contrast with the IgG Pf-MSP, a negative association for all IgG responses to *Anopheles* was found with age showing there is a decreasing of IgG immune response with increased age (Table 2). A similar situation occurred with the time of residence in an endemic area for malaria; IgG responses to gSG6-P1 peptide was 3.4% lower in samples from people who had lived in a malarial area for more than five years (RE = -0.035; 95% CI -0.070 to -0.003). Finally, no significant variation of specific anti-*Anopheles* IgG was observed according to the status of infection (Table 2).

Discussion

The intensity of malaria transmission has been traditionally evaluated using the entomological inoculation rate, which is defined by the number of infected bites received per human per unit of time (22); nevertheless, this strategy has shown limitations in low endemic settings for malaria (12, 23), such as Colombia. As a result, alternative methods to estimate human exposure to *Anopheles* bites have been proposed, including the detection of IgG responses to *Anopheles* SGE and salivary peptides. The purpose of the present study was to explore the possibility of using whole SGE from different *Anopheles* species to estimate exposure to *Anopheles* bites in a malaria-endemic area in Colombia where there is an important proportion of asymptomatic infections. We used SGE from two *An. albimanus* strains to try to capture potential differences in immunogenicity of salivary proteins from mosquitoes maintained for long periods in colony. Specifically, we compared immunogenicity of the CTG strains, a recently colonized strains of *An. albimanus* isolated in Colombia that could potentially resemble more closely exposure to "wild mosquito populations) against the STECLA strains, isolated in Central America in 1974, that has been previously used in our studies. We also wanted to evaluate the use of the gSG6-P1 peptide to evaluate exposure to minor malaria vectors in the area belonging to the subgenus *Anopheles*.

Consistent with our previous studies, we observed that the *An. albimanus* SGEs (STE and CTG) were associated with the infectious status, where people with infection presented significantly higher antibody levels against the salivary proteins. These results agree with previous findings in Haiti where the IgG antibody levels against *An. albimanus* SGE salivary proteins were higher in patients with clinical malaria than those in uninfected people living in the same region (10).

However, the fact that previous studies suggest that the antibody response against *An. albimanus* SGE is associated with *Plasmodium* exposure, highlights the relevance of using whole salivary content in the form of SGE as potentially useful antigen to measure mosquito bite exposure and risk of infection in areas of low and seasonal transmission where *An. albimanus* is one of the main vectors. Interestingly, the relationship between parasitemia and exposure was significant when using the antigen from the CTG strain and not the STE. This suggests that the antigens contained on the SGE from the CTG may be more closely related to the one the study subjects are exposed to in the field. Still, more studies are needed to evaluate the effect of mosquito colonization on immunogenicity changes of salivary proteins of vectors.

However, we did not find an association between antibodies against *An. darlingi* SGE and malaria infection. This could be explained due to the low or probably absence of *An. darlingi* mosquito in areas where samples were collected (6, 7). Still, the observed antibody response against the *An. darlingi* SGE may be explained by a potential cross reactivity between salivary proteins present in mosquitoes from the subgenus *Nyssorhynchus*, which *An. darlingi* belongs to. We are currently working on the design and validation of specific peptides designed from immunogenic proteins of *An. albimanus* SGE to assess exposure to the most abundant malaria vectors in Central and South America.

Our current study shows a high IgG response against gSG6-P1 peptide in samples from infected compared to uninfected people. These findings agree with our previous study in Colombian and Chilean volunteers, where we found that the concentration of gSG6-P1 antibodies was significantly correlated with malaria infection status and that people with clinical malaria presented significantly higher levels of IgG anti-gSG6-P1 antibodies than healthy controls (11). Although, *Anopheles* species from the subgenus *Nyssorhynchus* are the main vectors of malaria in Colombia, at least six species from the sub-genus *Anopheles* have been described as potential malaria vectors in the region (8, 24). Two of these species (*An. pseudopunctipennis* and *An. punctimacula*) are present along the Pacific coast (4), the main area where *P. falciparum* is transmitted in Colombia (3). Although Arcà *et al.* reported that gSG6 had no degree of identity with orthologous proteins from vectors in Central and South America, and therefore serological data published about the usefulness of this peptide in Colombia previously (11) should be interpreted with caution (25), previous work also showed that a deduced gSG6 from the New World species *An. freeborni* and *An. quadrimaculatus* (which belongs to the subgenus *Anopheles*) had between 67 and 71% of degree of identity with the gSG6 from Old World *Anopheles* species (26). In the same way, Pollard *et al.* (2019) suggested that the antibodies to the gSG6-P1 peptide in the Colombian population may represent exposure to *An. punctimacula*, which is a member of the *Anopheles* subgenus or could represent exposure to minor vectors in the country (27).

When comparing IgG levels against *An. albimanus* among villages, we observed that SGE was higher in infected than uninfected people in all villages except California. However, antibodies against the gSG6-P1 were equally high in infected and uninfected people from California residents; this is interesting because California is an area with urban characteristics, unlike Tangareal which is a sub-urban area and Robles and Candelillas which are rural areas. The evaluation of both SGE and peptides could help to refine diagnostic tools regarding malaria risk (28), especially in endemic areas with a high proportion of low density infections.

As discussed previously, Colombia has a high *Anopheles* species biodiversity (4), but only a few of them have been recognized as primary malaria vectors or secondary or local vectors, (i.e. *An. albimanus* and *An. darlingi*). Nevertheless some studies have shown additional *Anopheles* species naturally infected with *Plasmodium* spp. (i.e. *An. neivai*, *An. nuneztovari* and *An. punctimacula*), but the implication of these species as malaria vectors is not yet well understood in Colombia, and more evidence is needed to determine their role in malaria transmission (29). The above becomes particularly relevant if we consider the low natural infection *Plasmodium* rate reported in main malaria vectors in Colombia (0.13% in *An. darlingi* and 1.3% in *An. nuneztovari*) (29) suggesting that other *Anopheles* species could also be implicated in malaria transmission (29, 30). More importantly, our multilevel analysis demonstrated that independent of site, both age and anti-Pf-MSP IgG levels were associated not only with IgG antibody levels against the CTG strains of *An. albimanus* but also gSG6-P1. The fact that the population has shown exposure not only to the main malaria vectors (*An. albimanus*) but also to other possible related species to the subgenus *Anopheles*, highlight the need to have a panel of exposure biomarkers to have a whole view about the implicated vectors in malaria transmission.

This study has some limitations. First, because this study was cross-sectional, association with the anti-*Anopheles* IgG levels should be interpreted with caution as they do not imply causality. Second, due to the lack of a symptomatic group, we could not analyze risk factors for this kind of infection, and we could not explore the differences in the anti-*Anopheles* IgG levels between uninfected, asymptomatic (both, submicroscopic and microscopic infections) and symptomatic groups. Despite these limitations, these results are useful to identify new potential biomarkers of exposure to *Anopheles* in Colombia.

Conclusion

This study demonstrates that SGE from *An. albimanus* strains CTG and STE could be a potential source of new *Anopheles* salivary biomarkers for the primary vectors on the Colombian Pacific Coast, and that gSG6-P1 peptide has the potential to quantify human exposure to some malaria secondary vectors. All of them could be useful to estimate the risk of malaria transmission and could provide relevant tools to better understand malaria transmission dynamics and orient control strategies according to the specific characteristics in low-endemic settings.

Declarations

Acknowledgements

The authors thank the Department of Entomology at Kansas State University, the Vector Control Unit at the Nariño Department of Health Institute and the Epidemiology Group in Public Health Faculty at Universidad de Antioquia for their support in this research. We also thank Dr. Audrey Lenhart and Dr. Franck Remoue for their valuable comments to improve our manuscript. A special thanks to the people from California, Tangareal, Robles, and Candelillas in Tumaco for allowing and participating in this study, and to Flor Portocarrero, Zully Toloza, Yuri Pino and Leidy González for their hard work and commitment in the fieldwork

Declarations

Ethics approval and consent to participate

The methods and protocols were reviewed and approved by the Ethics Committee at the Medicine faculty, Universidad de Antioquia in Medellín, Colombia (Record 14 dated 9 August 2017) and by the Kansas State University Institutional Review Board in compliance with all applicable federal regulations governing the protection of human subjects (Proposal number 8952).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by COLCIENCIAS (RC-766-2016, Code 111574455032) Colombia and the CDC- USAID grant # 200-2018-M-00025

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Authors' contributions

JM, GMV, MLF, ATC and BLR designed the study and provide funding. BLR, dissected mosquitoes and prepare SGE. JM performed the testing to measure antibodies. JM, LFC and AT design and execute human sample collection protocols. JM and BLR performed the data analysis. All authors participated in manuscript writing and approved the final manuscript.

Disclaimer

The views expressed in this article reflect the results of research conducted by the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. Government.

Copyright statement

Some authors of this manuscript are military service members and employees of the United States Government. This work was prepared as part of their official duties. Title 17 U.S. C. §105 provides that "copyright protection under this title is not available for any work of the United States Government". Title 17 U.S. C. §101 defines a U.S. Government work as a work prepared by a military service member or employee of the U.S. Government as part of that person's official duties.

References

- 1.Recht J, Siqueira AM, Monteiro WM, Herrera SM, Herrera S, Lacerda MVG. Malaria in Brazil, Colombia, Peru and Venezuela: current challenges in malaria control and elimination. *Malar J.* 2017;16(1):273.
- 2.World Health Organization. (2018). World malaria report 2018. World Health Organization. <http://www.who.int/iris/handle/10665/275867>. Licencia: CC BY-NC-SA 3.0 IGO.
- 3.Instituto Nacional de Salud. (2017). Informe de Evento Malaria, Colombia, 2017. Instituto Nacional de Salud. <https://www.ins.gov.co/buscador-eventos/Informesdeevento/Malaria%202017.pdf>. FOR-R02.4000-001.
- 4.Gonzalez, R., Carrejo, N., 2009. Introducción al estudio taxonómico de Anopheles de Colombia: Claves y notas de distribución, 2nd edition. Universidad del Valle, Cali, Colombia.
- 5.Naranjo-Diaz N, Rosero DA, Rua-Urbe G, Luckhart S, Correa MM. Abundance, behavior and entomological inoculation rates of anthropophilic anophelines from a primary Colombian malaria endemic area. *Parasit Vectors.* 2013;6:61.

6. Ahumada ML, Orjuela LI, Pareja PX, Conde M, Cabarcas DM, Cubillos EF, et al. Spatial distributions of Anopheles species in relation to malaria incidence at 70 localities in the highly endemic Northwest and South Pacific coast regions of Colombia. *Malar J.* 2016;15(1):407.
7. Gutierrez LA, Naranjo N, Jaramillo LM, Muskus C, Luckhart S, Conn JE, et al. Natural infectivity of Anopheles species from the Pacific and Atlantic Regions of Colombia. *Acta Trop.* 2008;107(2):99–105.
8. Montoya-Lerma J, Solarte YA, Giraldo-Calderón GI, Quiñones ML, Ruiz-López F, Wilkerson RC, et al. Malaria vector species in Colombia: a review. *Mem Inst Oswaldo Cruz.* 2011;106 Suppl 1:223–38.
9. Ménard R, Tavares J, Cockburn I, Markus M, Zavala F, Amino R. Looking under the skin: the first steps in malarial infection and immunity. *Nat Rev Microbiol.* 2013;11(10):701–12.
10. Londono-Renteria BL, Eisele TP, Keating J, James MA, Wesson DM. Antibody response against Anopheles albimanus (Diptera: Culicidae) salivary protein as a measure of mosquito bite exposure in Haiti. *J Med Entomol.* 2010;47(6):1156–63.
11. Londono-Renteria B, Drame PM, Weitzel T, Rosas R, Gripping C, Cardenas JC, et al. An. gambiae gSG6-P1 evaluation as a proxy for human-vector contact in the Americas: a pilot study. *Parasit Vectors.* 2015;8:533.
12. Ya-Umphap P, Cerqueira D, Parker DM, Cottrell G, Poinsignon A, Remoue F, et al. Use of an Anopheles Salivary Biomarker to Assess Malaria Transmission Risk Along the Thailand-Myanmar Border. *J Infect Dis.* 2017;215(3):396–404.
13. Noukpo MH, Damien GB, Elanga-N'Dille E, Sagna AB, Drame PM, Chaffa E, et al. Operational Assessment of Long-Lasting Insecticidal Nets by Using an Anopheles Salivary Biomarker of Human-Vector Contact. *Am J Trop Med Hyg.* 2016;95(6):1376–82.
14. Drame PM, Poinsignon A, Dechavanne C, Cottrell G, Farce M, Ladekpo R, et al. Specific antibodies to Anopheles gSG6-P1 salivary peptide to assess early childhood exposure to malaria vector bites. *Malar J.* 2015;14:285.
15. Andrade BB, Rocha BC, Reis-Filho A, Camargo LM, Tadei WP, Moreira LA, et al. Anti-Anopheles darlingi saliva antibodies as marker of Plasmodium vivax infection and clinical immunity in the Brazilian Amazon. *Malar J.* 2009;8:121.
16. Arca B, Lombardo F, Struchiner CJ, Ribeiro JM. Anopheline salivary protein genes and gene families: an evolutionary overview after the whole genome sequence of sixteen Anopheles species. *BMC Genomics.* 2017;18(1):153.
17. Poinsignon A, Cornelie S, Mestres-Simon M, Lanfrancotti A, Rossignol M, Boulanger D, et al. Novel peptide marker corresponding to salivary protein gSG6 potentially identifies exposure to Anopheles bites. *PLoS One.* 2008;3(6):e2472.
18. Pollard EJM, Patterson C, Russell TL, Apairamo A, Oscar J, Arca B, et al. Human exposure to Anopheles farauti bites in the Solomon Islands is not associated with IgG antibody response to the gSG6 salivary protein of Anopheles gambiae. *Malar J.* 2019;18(1):334.
19. Instituto Departamental de Salud de Nariño. Informe ENOS por Municipio y Departamento, años 2008 a 2017 [Available from: <http://www.idsn.gov.co/index.php/subdireccion-de-salud-publica/epidemiologia/429-eventos-de-notificacion-enos>].
20. Naranjo-Diaz N, Altamiranda M, Luckhart S, Conn JE, Correa MM. Malaria vectors in ecologically heterogeneous localities of the Colombian Pacific region. *PLoS One.* 2014;9(8):e103769.
21. Villarreal-Treviño C, Vásquez GM, López-Sifuentes VM, Escobedo-Vargas K, Huayanay-Repetto A, Linton YM, et al. Establishment of a free-mating, long-standing and highly productive laboratory colony of Anopheles darlingi from the Peruvian Amazon. *Malar J.* 2015;14:227.
22. Birley MH, Charlewood JD. Sporozoite rate and malaria prevalence. *Parasitol Today.* 1987;3(8):231–2.
23. Sagna AB, Sarr JB, Gaayeb L, Drame PM, Ndiath MO, Senghor S, et al. gSG6-P1 salivary biomarker discriminates micro-geographical heterogeneity of human exposure to Anopheles bites in low and seasonal malaria areas. *Parasit Vectors.* 2013;6:68.
24. Freitas LA, Russo CA, Voloch CM, Mutaquiha OC, Marques LP, Schrago CG. Diversification of the Genus Anopheles and a Neotropical Clade from the Late Cretaceous. *PLoS One.* 2015;10(8):e0134462.
25. Arcà B, Lombardo F, Struchiner CJ, Ribeiro JM. Anopheline salivary protein genes and gene families: an evolutionary overview after the whole genome sequence of sixteen Anopheles species. *BMC Genomics.* 2017;18(1):153.
26. Lombardo F, Ronca R, Rizzo C, Mestres-Simòn M, Lanfrancotti A, Currà C, et al. The Anopheles gambiae salivary protein gSG6: an anopheline-specific protein with a blood-feeding role. *Insect Biochem Mol Biol.* 2009;39(7):457–66.
27. Pollard EJM, Patterson C, Russell TL, Apairamo A, Oscar J, Arcà B, et al. Human exposure to Anopheles farauti bites in the Solomon Islands is not associated with IgG antibody response to the gSG6 salivary protein of Anopheles gambiae. *Malar J.* 2019;18(1):334.
28. Doucoure S, Drame PM. Salivary Biomarkers in the Control of Mosquito-Borne Diseases. *Insects.* 2015;6(4):961–76.

29. Montoya C, Bascuñán P, Rodríguez-Zabala J, Correa MM. [Abundance, composition and natural infection of Anopheles mosquitoes from two malaria-endemic regions of Colombia]. *Biomedica*. 2017;37(0):98–105.

30. Rosero DA, Naranjo-Díaz N, Alvarez N, Cienfuegos AV, Torres C, Luckhart S, et al. Colombian *Anopheles triannulatus* (Diptera: Culicidae) Naturally Infected with *Plasmodium* spp. *ISRN Parasitol*. 2013;2013:927453.

Tables

Table 1. Socio-demographic characteristics, malaria history and anti-Anopheles IgG levels in the study population

Characteristic	Uninfected n=50		Infected Asymptomatic n=63		Total n=113	
	n	%	n	%	n	%
Age						
<5	4	8.0	3	4.8	7	6.2
5 -15	16	32.0	17	27.0	33	29.2
> 15	30	60.0	43	68.3	73	64.6
Site						
California	15	30.0	24	38.1	39	34.5
Tangareal	19	38.0	31	49.2	50	44.2
Robles	10	20.0	5	7.9	15	13.3
Candelillas	6	12.0	3	4.8	9	8.0
Gender						
Male	20	40.0	28	44.4	48	42.5
Female	30	60.0	35	55.6	65	57.5
Episodes of malaria						
0	25	50.0	21	33.3	46	40.7
1	11	22.0	16	25.4	27	23.9
>1	14	28.0	26	41.3	40	35.4
Malaria last year						
No	38	76.0	42	66.7	80	70.8
Yes	12	24.0	21	33.3	33	29.2
Education level						
Primary education or lower	35	70.0	49	77.8	84	74.3
Secondary education or upper	15	30.0	14	22.2	29	25.7
Occupation						
Housewife	14	28.0	19	30.2	33	29.2
Farmer	4	8.0	9	14.3	13	11.5
Student	19	38.0	24	38.1	43	38.1
Others	13	26.0	11	17.5	24	21.2
IgG Levels (DOD*)	Median (IQR**)		Median (IQR)		Median (IQR)	
<i>An. darlingi</i>	0.332 (0.234-0.415)		0.355 (0.287-0.430)		0.352 (0.258-0.430)	
CTG	0.139 (0.101-0.202)		0.207 (0.143-0.285)		0.172 (0.126-0.275)	
STE	0.194 (0.148-0.303)		0.248 (0.185-0.386)		0.219 (0.168-0.324)	
gSG6-P1	0.170 (0.072-0.244)		0.224 (0.169-0.291)		0.203 (0.141-0.267)	

*Normalized Optical density, **interquartile range

Table 2. Linear Mixed Effects models to explain exposure to Anopheles in a malaria-endemic area in Colombia

	Anti- <i>An. darlingi</i> IgG			Anti-CTG IgG			Anti-STE IgG			A
	Estimated coefficient	SE	95% CI	Estimated coefficient	SE	95% CI	Estimated coefficient	SE	95% CI	
Fixed effects										
Intercept	0.428	0.046		0.191	0.036		0.313	0.047		0.128
Infectious status										
Uninfected	1			1			1			1
Infected	-0.022	0.037	-0.096 - 0.051	0.007	0.029	-0.051 - 0.064	-0.005	0.038	-0.080 - 0.069	0.021
Age	-0.003	0.001	-0.004 - -0.001	-0.002	0.001	-0.003 - -0.001	-0.003	0.001	-0.005 - -0.001	0.001
Residence time										
<5 years	1			1			1			1
≥ 5 years	-0.031	0.030	-0.090 - 0.029	-0.007	0.024	-0.054 - 0.040	-0.032	0.031	-0.093 - 0.029	-0.035
Pf-MSP	0.066	0.054	-0.041 - 0.172	0.106	0.043	0.022 - 0.189	0.092	0.055	-0.017 - 0.200	0.070
Random effect										
Village level	6.51E-22	1.56E-20		4.31E-27	9.71E-26		7.13E-27	1.48E-25		2.46E-05

Figures

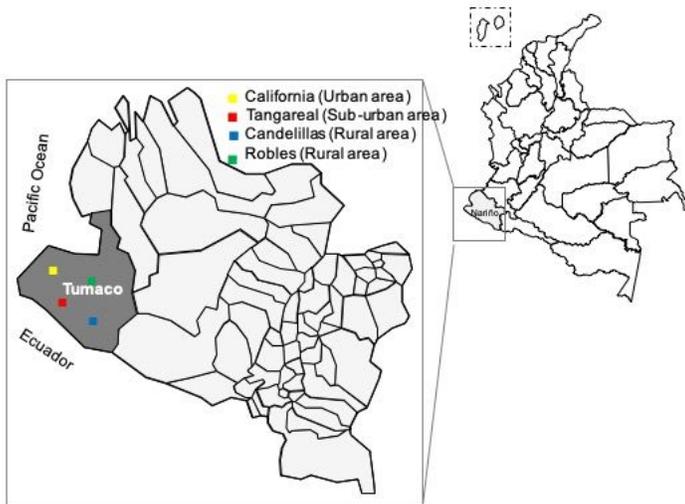


Figure 1

Study sites in Tumaco, Nariño, Colombia Modified from: http://moe.org.co/home/doc/comunicados/mapa_colombia_MOE_vectores.pptx Mapa político en vectores de Colombia – Departamentos y Municipios MOE”

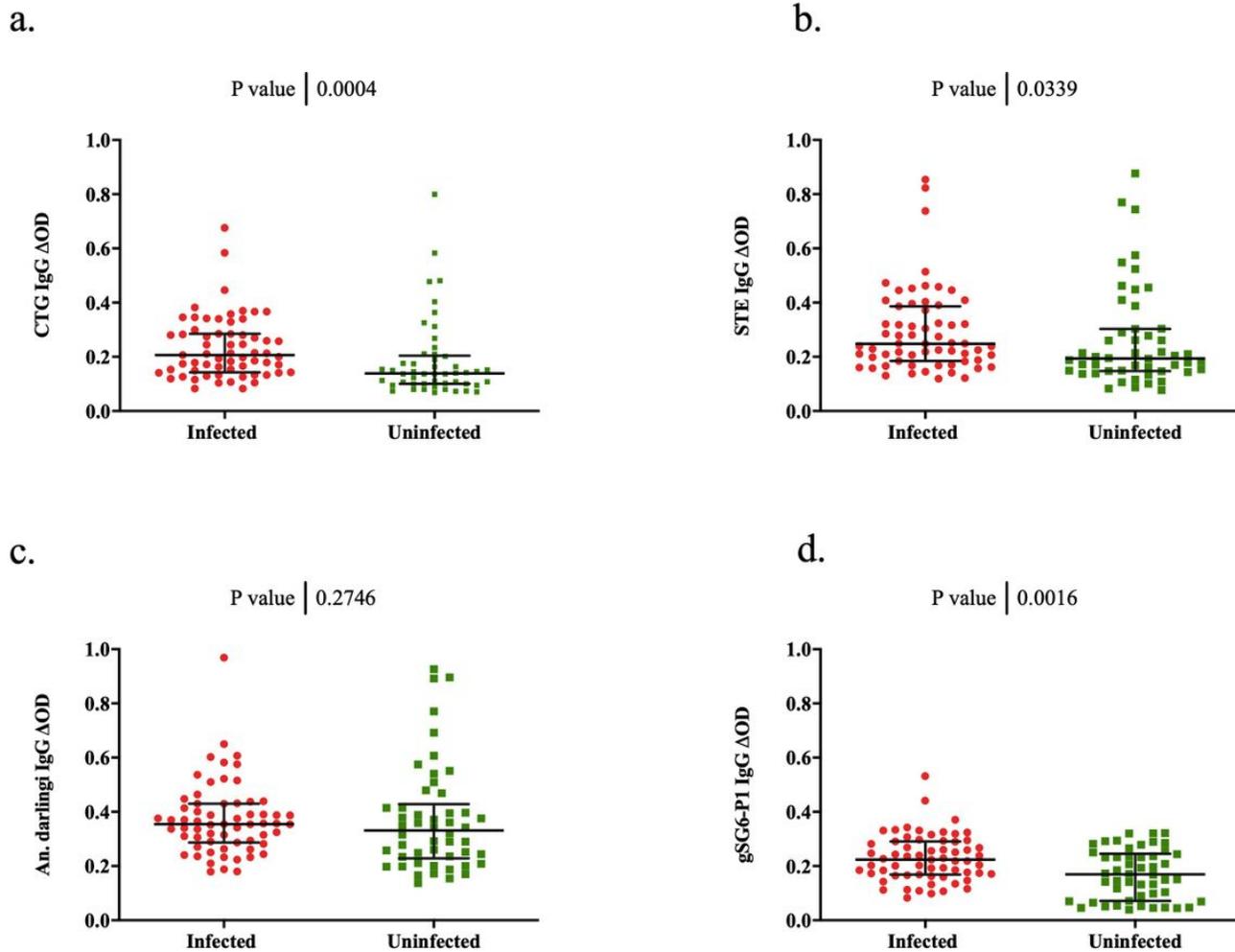


Figure 2

IgG responses to Anopheles per status of infection. Figure 2a shows the individual anti CTG IgG levels, 2b STE, 2c An. darlingi SGE and 2d gSG6-P1 peptide. Horizontal lines in the boxes indicate median values; lengths of boxes correspond to the inter-quartile ranges. Pairwise significance was tested with Mann-Whitney test

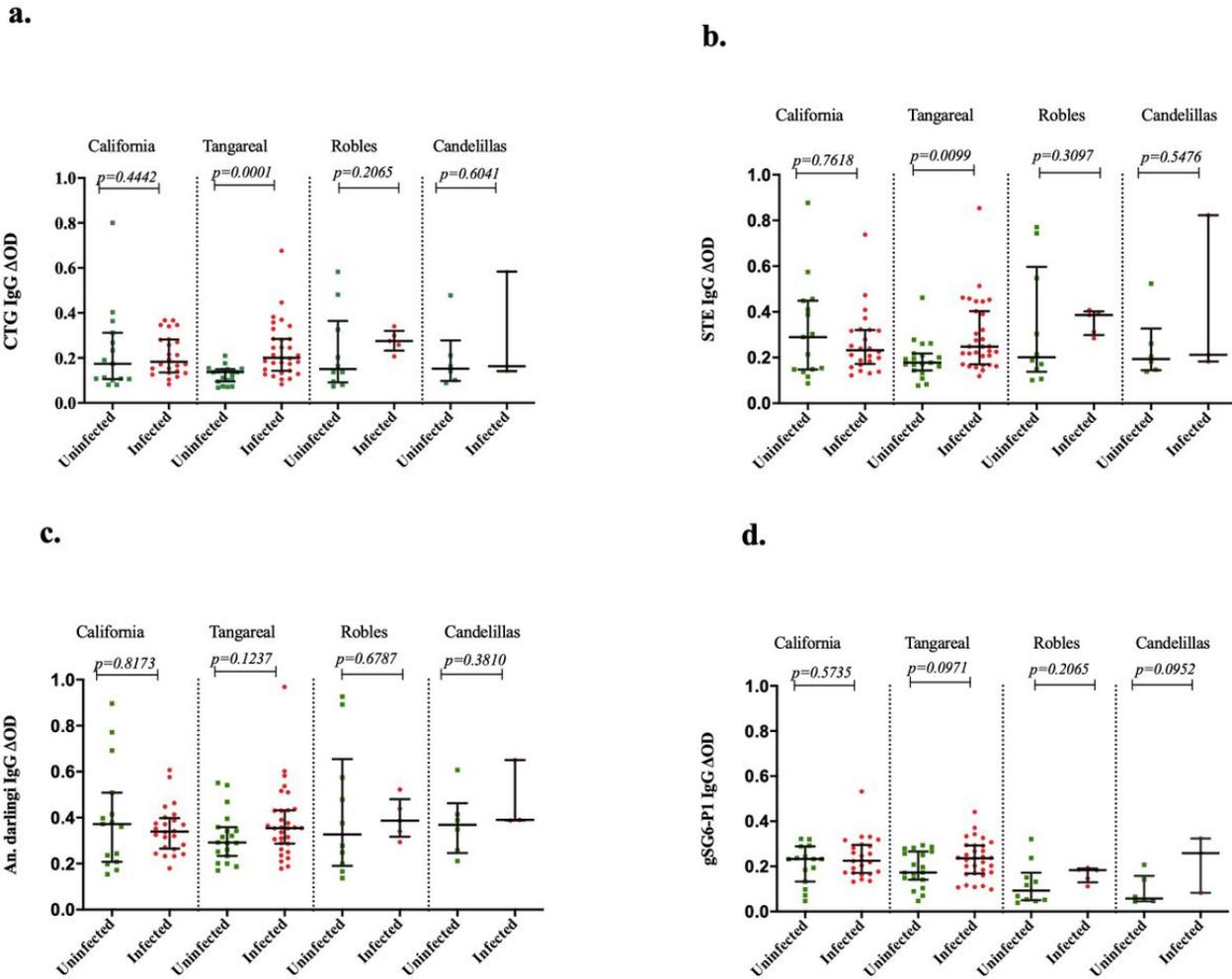


Figure 3

IgG responses to *Anopheles* per status of infection and per site. Figure 3a shows the individual anti CTG Ig levels, 3b STE, 3c *An. darlingi* and 3d gSG6-P1 peptide. Horizontal lines in the boxes indicate median values; lengths of boxes correspond to the inter-quartile ranges. Pairwise significance was tested with Mann-Whitney test

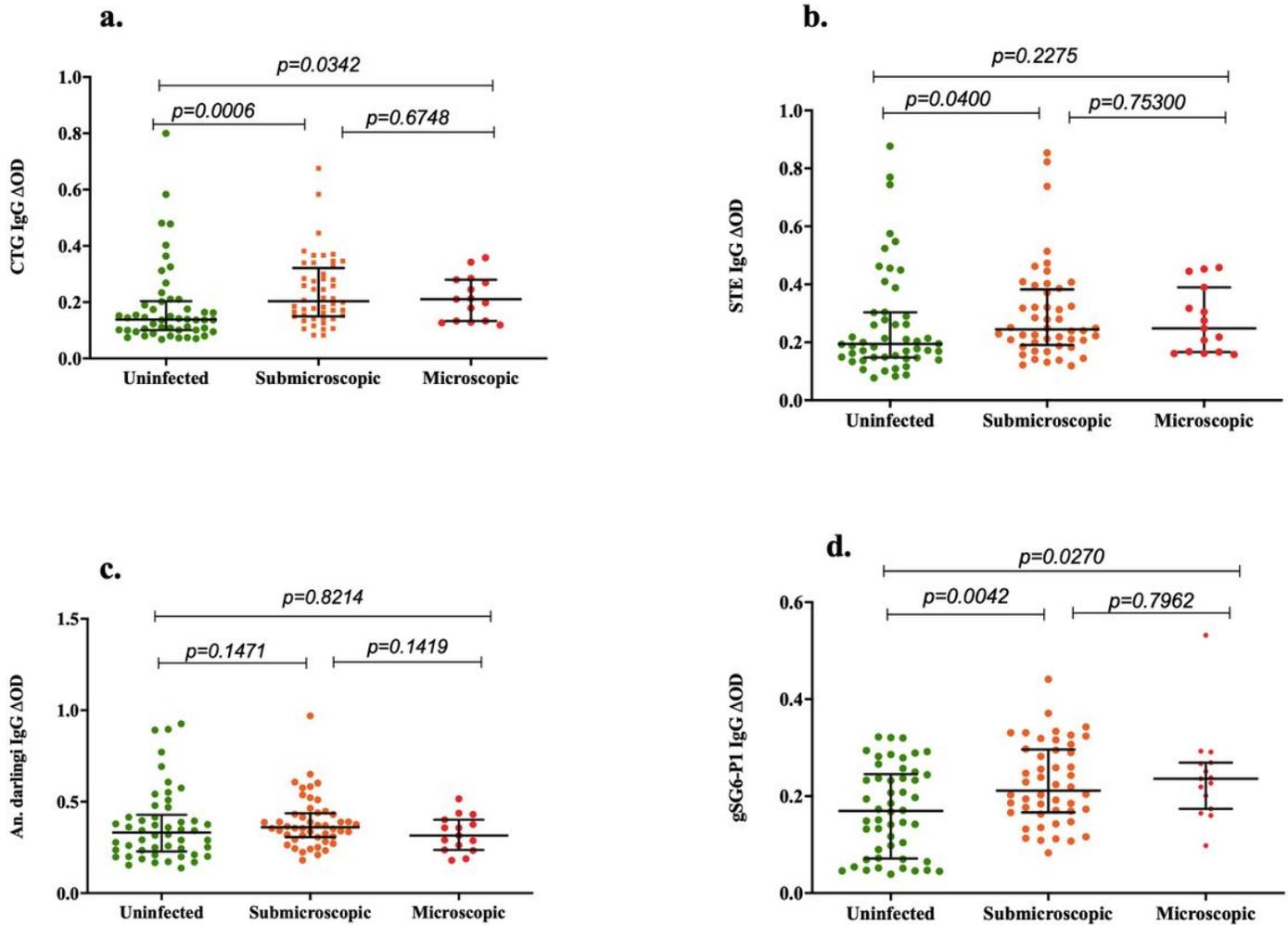


Figure 4

IgG responses to Anopheles per infection group: uninfected, submicroscopic (positive PCR and negative LM) and microscopic (positive by both PCR and LM). Figure 4a shows the individual anti CTG IgG levels, 4b STE, 4c An. darlingi and 4d gSG6-P1 peptide. Horizontal lines in the boxes indicate median values; lengths of boxes correspond to the inter-quartile ranges. Pairwise significance was tested with Mann-Whitney test.

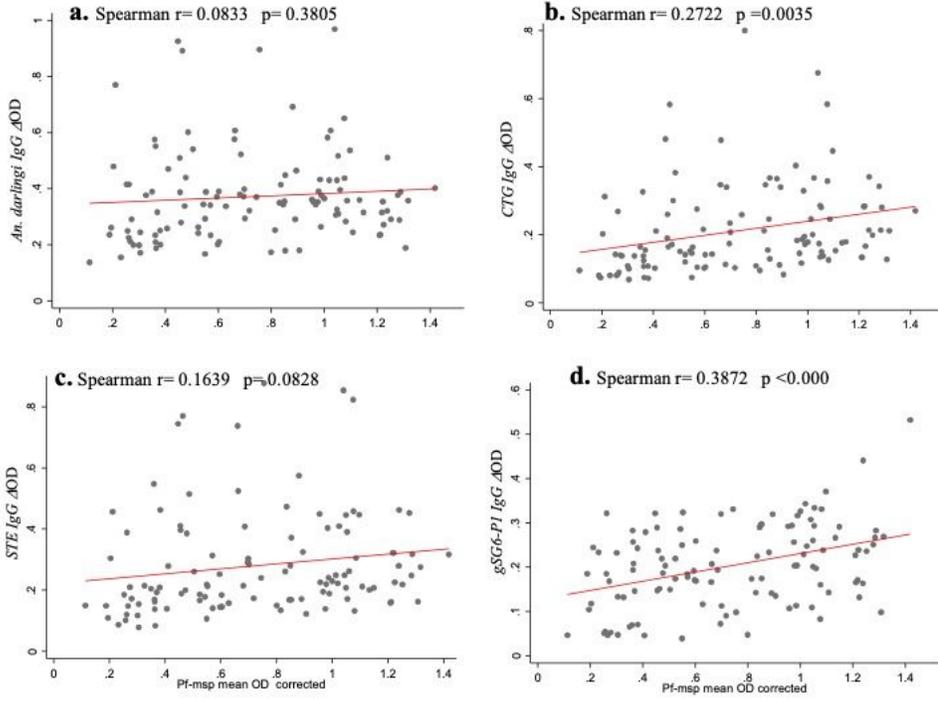


Figure 5

Correlation between anti- Anopheles IgG levels and anti-Plasmodium IgG levels in the whole population: Anti CTG and anti-Pf-MSP (3a), STE and anti-Pf-MSP (3b), An. darlingi and anti-Pf-MSP (3c), and gSG6-P1 anti-Pf-MSP (3d). The red solid line indicates the correlation curve.