IgG antibody response against Anopheles salivary gland proteins in asymptomatic infections in Narino – Colombia.

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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 risk of malaria infection by measuring antibody responses against salivary glands extracts from An. (Nys.) albimanus and An. (Nys.) darlingi and also against the gSG6-P1 peptide of An. gambiae in people residing in a malaria endemic area in the Colombian Pacific coast. Methods: We eluted dried blood spots samples to measure the IgG antibodies against salivary gland extracts of An. (Nys.) albimanus strains STECLA (STE) and Cartagena (CTG) and An. (Nys.) 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. (Nys.) 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. (Nys.) albimanus salivary glands extracts are a potential source of new Anopheles salivary biomarkers to identify exposure to the main malaria vector and to calculate risk of disease 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.

Background
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 primary malaria vectors in Colombia belong to the subgenus Nyssorhynchus, with An. (Nys.) nuneztovari, An. (Nys.) albimanus and An. (Nys.) darlingi as the most important malaria vectors in areas of high malaria transmission (5). On the South Pacific coast, several species has been associated with malaria transmission with An. (Nys.) albimanus is the main vector (6, 7). Previous studies reported that the An. (Nys.) albimanus lineage circulating the Southern region may be different from the one found the in the Northern part of the country suggesting that two different lineages are circulating in the country (8-10). Interestingly, malaria prevalence in these sites is significantly different. and further studies evaluating vector competence and susceptibility to both, P. vivax and P. falciparum (10) as well as to measure potential changes in salivary content that could impact pathogen transmission (11) are necessary.

Extensive entomological research has been done in the Nariño Department (10, 12, 13). This research suggests that mosquitoes from the subgenus Anopheles, An. (An.) calderoni and An. (An.) punctimacula are also important malaria vectors in the area. However, these two species are often misclassified due to their high morphological similarities (12). However, An. (An) calderoni was found infected with both P. vivax and P. falciparum with an annual entomological inoculation rate (EIR) of 2.84 bites/human/year in Nariño between 2012 and 2013 (12). Also, a previous study reported EIR for An. (An.) calderoni between 1.7 and 14.7 from 2009 to 2010, while EIR reported for An. (Nys.) albimanus during the same period was found between 0.1 and 2.6 (13). Suggesting that An. calderoni is a primary vector of malaria in Nariño. Furthermore, in the Tumaco city, located in the Narino Department), Ahumada et. al., reported different malaria incidence in places where An. (Nys.) albimanus and An. (An.) calderoni were found in the 2011 – 2012 study. Specifically, they reported a high Annual Parasite Index (API) (73 cases/1000 inhabitant) in places where An (An.) calderoi is the
predominant species compared to lower (27 cases/1000) where An. (Nys.) albimanus was predominant (10).

To design a proper vector control method, it is necessary to accurately determine human-vector interaction and the proportion of those vectors that are infected. Vectorial capacity (VC) and EIR are quantitative entomological indicators used to determine epidemiology of vector-borne diseases such as malaria. The VC is used as the measure of a mosquito population’s proficiency to transmit an infectious agent to a susceptible population (14), while EIRs are useful to establish a direct estimation of transmission risk (15, 16). In the case of malaria, the EIR is the gold standard for measuring transmission intensity. EIRs are based on the number of mosquitoes captured and the proportion of mosquitoes infected with *Plasmodium* (17). However, estimation of EIR is expensive and may be insufficient in areas of low or seasonal transmission (18, 19). Human Landing Collection (HLC) is currently the only mosquito catching method that can directly measure the biting rates of human-seeking mosquitoes. Unfortunately, it is only applicable to mosquitoes seeking human adults and results are difficult to extrapolate to children or to pregnant women that are the most vulnerable to malaria (20). Furthermore, during HLC, the human bait is exposed to the diseases transmitted by the landing mosquitoes posing ethical concerns on implementation of this technique (21). As an alternative, catching traps such as the CDC (Center for Disease Control) light trap and the bed net traps have been developed and the data collected is useful in estimating vector populations when the studies are properly controlled. However, these trapping methods often differ in the number of host-seeking mosquito population sampled (22). Still, in spite the high number of mosquitoes captured on these studies (up to 12,000 specimens) a few mosquitoes (up to 4 specimens) were found positive for *Plasmodium* parasites even in their high abundance months (12, 13). So, the question remains on how much is people being exposed to mosquito bites and acquiring the parasite. Thus, it is important to design alternative methods able to reflect the vector-human contact and complement the data collected by mosquito trapping methods.

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 (23). 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. (24-28).

Thus, the use of salivary gland and saliva antigens has been previously validated as an indirect proxy to determine mosquito bite exposure. Significant higher IgG antibody levels against *An. (Nys.) albimanus* and *An. (Nys.) 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 (24, 29). A similar pattern has been observed in areas where *An. (Cel.) gambiae* and *An. (Cel.) 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* (30). 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 (25, 28, 31). Although there are no known species of the subgenus *Cellia* in South America, the responses observed against the gSG6-P1 peptide could be hypothesized to result from the presence of mosquitoes belonging to the subgenus *Anopheles* such as *An. (An.) pseudopunctipennis* and *An. (An.) punctimacula* and *An. (An.) calderoni* (32).

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. Since the use of salivary gland extract as antigen to indirectly measure exposure to mosquito species circulating in a region has been validated by several groups the main objective of this work was to measure IgG antibodies in humans living in an area where low-density *P. falciparum* infections are frequent. Thus, we explored if human IgG responses to *Anopheles* salivary gland extracts (SGE) are associated with low-density infections by *P. falciparum* and risk of disease we aimed to evaluate whether gSG6-P1 peptide
continues as a useful marker to detect exposure in areas where mosquitoes from the sub-genus *Anopheles* are important vectors of malaria 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 (1850’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. During our study, *P. falciparum* was reported as the predominant species (96%) in Tumaco with an API of 13.5 cases/1000 inhabitants in 2017 and 10.4 cases/1000 inhabitants in 2018. No entomological data was collected during the time of our study (33).

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 Loop-mediated isothermal amplification -LAMP or nested polymerase chain reaction- nPCR 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. (Nys.) darlingi* were maintained under insectary conditions until salivary gland dissection. Based on our recent studies suggesting that time of colonization has an influence on arthropod salivary gland content (34), and that two different *An. (Nys.) albimanus* lineages are circulating in two geographically distant regions of Colombia, we wanted to evaluate potential differences in antibody responses against salivary content of two different strains of *An. (Nys.)*
albimanus, one from a long-established colony strain STECLA (STE) versus a recently colonized strain Cartagena (CTG). Briefly, An. (Nys.) albimanus strains originated from El Salvador (STE) and Colombia (CTG), respectively, and were maintained in the insectary at the CDC (Atlanta, GA, USA). The An. (Nys.) darlingi laboratory strain originated from Iquitos, Peru (35), 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 (24). Mosquitoes were blood fed 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 50μL 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 (Plasmodium falciparum Merozoite Surface Protein) peptide (Fitzgerald, USA) was used to evaluate exposure to malaria parasites.

Indirect ELISA (Enzyme Linked Immunosorbent Assay)

ELISA conditions were standardized as described elsewhere (24, 25). Also, DBS samples were prepared as by eluting half of a card circle into 300μL 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. (Nys.) darlingi and An. (Nys.) 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 tetramethyl-benzidine (Abcam) as a substrate. In parallel, each assessed microplate contained in
duplicate: a positive control, a negative control, and a blank; wells containing no sample. The positive control was a pool of DBS of people with positive malaria diagnosis. The negative control was a sample of people from US (n=36) with no exposure to malaria parasites. The blank was composed by wells containing no sample. The reaction was stopped with 0.25 N sulfuric acid, and the optical density (OD) was measured at 450 nm.

**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 (25). Briefly, antibody levels were expressed as the ΔOD value: ΔOD = ODx − ODb, where ODx represents the mean of individual OD in both antigen wells and ODb the mean of the blank wells. For each tested peptide, positive controls of each plate were averaged and divided by the average of the ODx 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. Assay variation of samples (inter and intra assay) tested in the study was below 20% and we only included in the analysis serum samples with a coefficient of variation ≤20% duplicates between duplicate (36). The mean ΔOD of negative US controls plus 3 standard deviations (SD) was used to determine cut-off value for responsiveness to antigens. The ΔOD cut off value to determine exposure to malaria antigens as 0.263. 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).

Odd ratios (OR) were calculated to evaluate risk of malaria. For this, the median was used to classify IgG antibody levels as high (ΔOD higher than the median) and low (ΔOD equal or lower than the median) and the samples were classified as cases (Asymptomatic and submicroscopic infections) and controls (uninfected). In addition, 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. (Nys.) 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 demographics, sociocultural variables and antibody responses to mosquito antigens**

We studied exposure to mosquito bites in the area of Tumaco in Nariño (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. (Nys.) albimanus* (STE and CTG), *An. (Nys.) 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. **Detection of IgG antibody against Anopheles SGE and gSG6-P1 peptide by infection status**

Our study shows that the level of antibodies against *An. (Nys.) 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. (Nys.) darlingi* (Mann-Whitney
test p value= 0.2746). This is consistent with information provided by previous studies showing *An. (Nys.) 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. We calculated OR to measure risk of suffering a malaria infection based in presenting low or high antibody levels. Our analysis revealed a significant higher risk of suffering malaria if the patient present higher levels of antibodies against CTG (OR=3.4, 95%CI 1.468 – 8.131, Fisher’s Exact test *p*=0.0023), STE (OR=2.68, 95%CI 1.166 – 6.234, Fisher’s Exact test *p*=0.138) and gSG6=P1 (OR=2.30, 95%CI 1.009 – 5.309, Fisher’s Exact test *p*=0.0374) but not for *An. darlingi* SGE (OR=1.4, 95%CI 0.656 – 3.349, Fisher’s Exact test *p*=0.3454).

3. Detection of IgG antibody levels by *P. falciparum* detection threshold (microscopic vs. sub-microscopic)

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 gSGS-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. (Nys.) 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. (Nys.) darlingi and STE SGE.

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

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 EIR, which is defined by the number of infected bites received per human per unit of time; nevertheless, this strategy has shown limitations in low endemic settings for malaria (26, 37). 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 as tool to detect IgG antibodies in humans that could be used as indirect estimation of exposure to *Anopheles* bites in a malaria-endemic area in Colombia where there is an important proportion of asymptomatic infections. Based in previous reports suggesting at least two *An. (Nys.) albimanus* lineages in Colombia (9-11), we used
SGE from two *An. (Nys.) albimanus* strains to try to capture potential differences in immunogenicity of salivary proteins from colony mosquitoes isolated from different geographical regions and with differences in the colonization time. Specifically, we compared immunogenicity of the CTG strains, a recently colonized strains of *An. (Nys.) albimanus* collected in Colombia that could potentially resemble more closely responses to “wild mosquito antigens” than those observed against the STE strains, isolated in Central America in 1974.

*An. (Nys.) albimanus* has been reported as one of the main malaria vectors in Nariño displaying EIR up to 2.6 in recent studies. Consistent with previous studies, we observed that the *An. (Nys.) albimanus* SGEs (STE and CTG) were associated with the infectious status, where people with active *Plasmodium* infection presented significantly higher IgG antibody levels against the salivary proteins. We also found that people with higher antibody levels against STE, CTG and gSG6-p1 have between 2 and 4 times more probability of suffering a malaria infection. These results agree with previous findings in Haiti were the IgG antibody levels against *An. (Nys.) albimanus* SGE were higher in patients with clinical malaria than those in uninfected people living in the same region (24). These studies suggest that the IgG antibody response against *An. (Nys.) albimanus* SGE is associated with *Plasmodium* exposure and highlights the relevance of using whole salivary content in the form of SGE as potentially useful antigen to measure risk of infection in areas of low and seasonal transmission. Interestingly, the relationship between parasitemia and IgG antibodies against *Anopheles* antigens was significant when using the antigen from the CTG strain and not the STE, suggesting that the antigens contained on the SGE from the CTG may be more closely related to the one the study subjects are exposed in the field. However, we did not find an association between antibodies against *An. (Nys.) darlingi* SGE and malaria infection. This could be explained due to the low abundance (or probable absence) of *An. (Nys.) darlingi* mosquito previously reported in areas where samples were collected (6, 7). Still, the observed antibody response against the *An. (Nys.) darlingi* SGE may be explained by a potential cross reactivity between salivary proteins present in mosquitoes from the subgenus *Nyssorhynchus*, which *An. (Nys.) darlingi* belongs to.

Previous studies suggest that *An. (An.) calderoni* is a primary malaria vector in Narino (12). This may
explain our current findings showing 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 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 (25). 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 (38, 39). Three of these species (An. (An.) calderoni, An. (An.) pseudopunctipennis and An. (An.) punctimacula) are present along the Pacific coast, 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 previously published about the usefulness of the gSG6-P1 peptide in Colombia (25) should be interpreted with caution (40), previous work also showed that a deduced gSG6 from the New World species An. (An.) freeborni and An. (An.) quadririmaculatus (from the subgenus Anopheles) had between 67 and 71% of degree of identity with the gSG6 from Old World Anopheles species (41). 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 hypothetically represent exposure to minor vectors in the country (42). Thus, our current results suggest that the gSG6-P1 peptide could be a useful marker for malaria risk in areas of Colombia where mosquitoes belonging to subgenus other than Nyssorhynchus are present.

When comparing IgG levels against An. albimanus among villages, we observed that SGE from both STE and CTG, were higher in infected than uninfected people in all villages except California. 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. To evaluate further, 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. (Nys.) albimanus but also against the gSG6-P1. Suggesting the importance of using a panel of exposure biomarkers (mosquito antigens)
and concurrent entomological data to accurately evaluate risk especially in areas where several *Anopheles* species are implicated in malaria transmission. Also, our antibody model revealed a negative association between age and IgG antibodies against all *Anopheles* antigens. Similar trend has been observed in other studies measuring antibody responses against mosquito salivary antigens and has been associated with the development of tolerance against certain mosquito allergens (36, 43, 44).

Our recent studies revealed important differences in salivary content in arthropods collected in the field when compared to the same species maintained in a colony (34). Also, a previous study suggests the possibility of two *An. (Nys.) albimanus* lineages circulating two geographically distant regions of Colombia. Thus, we aimed to determine if risk of infection can be affected by the salivary content of mosquitoes from the same species but from different origin. So, we used a recently colonized strain (CTG) and a long term established laboratory colony (STE) each isolated from a distinct geographical region (Colombia and El Salvador) to account for potential changes in IgG responses based on salivary content. As our results indicate, the SGE from the CTG strain showed significant association with the Pf-MSP1 and not with the SGE from STE suggesting potential differences. Determination and confirmation of these differences are subject of further studies aimed to characterize salivary gland content of the two *An. (Nys.) albimanus* lineages circulating in Colombia and comparing those to *An. (Nys.) albimanus* isolates from other countries. This is important since the use of salivary antigens as vaccines for malaria are undergoing (45) and characterization of the main immunogenic salivary proteins of the main vectors circulating in endemic areas are important for the success of such vaccine.

This study has several 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. Also, the lack of concurrent entomological data in our study is a significant limitation. Since this study did not
included mosquito collection or other concurrent entomological surveillance, our current results should be interpreted as an indirect measurement of disease risk (currently calculated by OR) until further determination of the specific mosquitoes circulating in an area where these antibodies are measured. We plan to complete our serological data with entomological data in future anopheline collection to further validate the findings of this study. Despite these limitations, these results are useful to identify new potential biomarkers for malaria risk in Colombia.

Conclusion
This study demonstrates that SGE from An. (Nys.) albimanus strains CTG and STE could be a potential source of new Anopheles salivary biomarkers to determine risk of malaria in Colombia, supports previous findings 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.

List Of Abbreviations

**IgG**: Immunoglobulin G  
**STE**: STECLA  
**CTG**: Cartagena  
**EIR**: annual entomological inoculation rate  
**API**: Annual Parasite Index  
**VC**: Vectorial capacity  
**HLC**: Human Landing Collection  
**CDC**: Center for Disease Control  
**SGE**: salivary gland extracts  
**DBS**: Dried blood spots  
**LAMP**: Loop-mediated isothermal amplification  
**nPCR**: nested polymerase chain reaction  
**LM**: light microscopy
**Pf-MSP:** *Plasmodium falciparum* Merozoite Surface Protein

**ELISA:** EnzymeLinkedImmunosorbentAssay

**IQR:** interquartile range

**OD:** optical density

**SD:** standard deviations

**IQR:** interquartile range

**OR:** Odds Ratio

**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

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its supplementary information files. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**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.

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Tables

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

Legend: *Normalized Optical density, **interquartile range

| Characteristic     | Uninfected  | Asymptomatic malaria | Total |
|--------------------|-------------|-----------------------|-------|
|                    | n=50        | n=63                  | n=113 |
| Age                |             |                       |       |
| <5                 | 4           | 3                     | 7     | 6. |
| 5 -15              | 16          | 17                    | 33    | 29 |
| > 15               | 30          | 43                    | 73    | 64 |
| Site               |             |                       |       |
| California         | 15          | 24                    | 39    | 34 |
| Tangareal          | 19          | 31                    | 50    | 44 |
| Robles             | 10          | 5                     | 15    | 13 |
| Candelillas        | 6           | 3                     | 9     | 8. |
| Gender             |             |                       |       |
| Male | 20 | 40.0 | 28 | 44.4 | 48 | 42 |
| Female | 30 | 60.0 | 35 | 55.6 | 65 | 57 |
| Episodes of malaria | | | | | | |
| 0 | 25 | 50.0 | 21 | 33.3 | 46 | 40 |
| 1 | 11 | 22.0 | 16 | 25.4 | 27 | 23 |
| >1 | 14 | 28.0 | 26 | 41.3 | 40 | 35 |
| Malaria last year | | | | | | |
| No | 38 | 76.0 | 42 | 66.7 | 80 | 70 |
| Yes | 12 | 24.0 | 21 | 33.3 | 33 | 29 |
| Education level | | | | | | |
| High school or lower | 35 | 70.0 | 49 | 77.8 | 84 | 74 |
| Undergraduate or Graduate | 15 | 30.0 | 14 | 22.2 | 29 | 25 |
| Occupation | | | | | | |
| Housewife | 14 | 28.0 | 19 | 30.2 | 33 | 29 |
| Farmer | 4 | 8.0 | 9 | 14.3 | 13 | 11 |
| Student | 19 | 38.0 | 24 | 38.1 | 43 | 38 |
| Others | 13 | 26.0 | 11 | 17.5 | 24 | 21 |
| IgG Levels (DOD*) | Median (IQR**) | Median (IQR) | Median (IQR) |
| An. (Nys.) darlingi | 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 (0.126-0.246) |
| STE | 0.194 (0.148-0.303) | 0.248 (0.185-0.386) | 0.219 (0.168-0.312) |
| gSG6-P1 | 0.170 (0.072-0.244) | 0.224 (0.169-0.291) | 0.203 (0.141-0.252) |

Table 2. Linear Mixed Effects models to explain exposure to *Anopheles* in a malaria-endemic area in Colombia.
| Fixed effects | Anti-An. darlingi IgG | Anti-CTG IgG | Anti-STE IgG | Anti-gSG6-P1 IgG |
|---------------|-----------------------|--------------|--------------|------------------|
| Intercept     | 0.4 0.0               | 0.1 0.0      | 0.0 0.0      | 0.128 0.024      |
|              | 28  46                 | 91  36       | 3  0         | 1  4             |
| &nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;Intercept |                      |              |              |                  |
| Uninfected    | 1                      | 1            | 1            | 1                |
|              | -0.0 -0.0 -0.0 -0.0    | 0.0 0.0 -0.0 -0.0 | -0.0 0.0 -0.0 | 0.021 0.020      |
|              | 02 37 09               | 51 07 29 05  | 64 0.0 0.0 0.0 |                  |
| Infected      | 2 6                    | 1            | 0 3 8 6      |                  |
|              | -0.0 -0.0 -0.0 -0.0    | -0.0 0.0 -0.0 -0.0 | -0.0 0.0 -0.0 | 0.001 0.000      |
|              | 00 01 00               | 00 01 00     | 00 0.0 0.0 0.0 |                  |
| Age           | 3 4 1 2 3 1            | 1            | 0 0 0 0      |                  |
| Residency time| 1                      | 1            | 1            | 1                |
| <5 years      | -0.0 -0.0 -0.0 -0.0    | -0.0 0.0 -0.0 -0.0 | -0.0 0.0 -0.0 | -0.035 0.016     |
|              | 03 30 09 29 00 24 05  | 40 0.0 0.0 0.0 | 3 3 9 2      |                  |
| &nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;Residency time |          |              |              |                  |
| ≥ 5 years     | 1 0                    | 7 4          | 2 1 3 9      |                  |
|              | 0.0 0.0 -0.0 0.1      | 0.1 0.0 0.0 0.1 | 0.0 0.0 -0.0 | 0.070 0.029      |
|              | 66 54 04 72 06 43 22  | 89 0 0 0 2   | 9 5 1 0      |                  |
| Pf-MSP        | 1                      |              | 2 5 7 0      |                  |
| Random effect | 6.5 1.5                | 4.3 9.7      | 7.0 1.0      | 2.46E- 1.96E-    |
|               | 1E- 6E- 22 20          | 1E- 1E- 27 26 | 1E- 1E- 3 8 |                  |
| Village level | 7 5                    |              | 2 2          |                  |

**Figures**
Figure 1

Study sites in Tumaco, Nariño, Colombia
IgG responses to Anopheles per status of infection. Figure 2a shows the individual anti CTG IgG levels, 2b STE, 2c An. (Nys.) darlingi SGE and 2d gSG6-P1 peptide. Legend: 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.
IgG responses to Anopheles per status of infection and per site. Legend: Figure 3a shows the individual anti CTG IgG levels, 3b STE, 3c An. (Nys.) 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.
IgG responses to Anopheles per infection group: uninfected, submicroscopic (positive PCR and negative LM) and microscopic (positive by both PCR and LM). Legend: Figure 4a shows the individual anti CTG IgG levels, 4b STE, 4c An. (Nys.) 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.
Correlation between anti- Anopheles IgG levels and anti-Plasmodium IgG levels in the whole population Legend: Anti CTG and anti-Pf-MSP (3a), STE and anti-Pf-MSP (3b), An. (Nys.) darlingi and anti-Pf-MSP (3c), and gSG6-P1 anti-Pf-MSP (3d). The red solid line indicates the correlation curve.