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An. gambiae gSG6-P1 evaluation as a proxy for human-vector contact in the Americas: a pilot study

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Abstract

Background: During blood meal, the female mosquito injects saliva able to elicit an immune response in the vertebrate. This immune response has been proven to reflect the intensity of exposure to mosquito bites and risk of infection for vector transmitted pathogens such as malaria. The peptide gSG6-P1 of *An. gambiae* saliva has been demonstrated to be antigenic and highly specific to *Anopheles* as a genus. However, the applicability of gSG6-P1 to measure exposure to different *Anopheles* species endemic in the Americas has yet to be evaluated. The purpose of this pilot study was to test whether human participants living in American countries present antibodies able to recognize the gSG6-P1, and whether these antibodies are useful as a proxy for mosquito bite exposure and malaria risk.

Methods: We tested human serum samples from Colombia, Chile, and the United States for the presence of IgG antibodies against gSG6-P1 by ELISA. Antibody concentrations were expressed as delta optical density (ΔOD) of each sera tested in duplicates. The difference in the antibody concentrations between groups was tested using the nonparametric Mann Whitney test (independent groups) and the nonparametric Wilcoxon matched-pairs signed rank test (dependent groups). All differences were considered significant with a $P < 0.05$.

Results: We found that the concentration of gSG6-P1 antibodies was significantly correlated with malaria infection status and mosquito bite exposure history. People with clinical malaria presented significantly higher concentrations of IgG anti-gSG6-P1 antibodies than healthy controls. Additionally, a significant raise in antibody concentrations was observed in subjects returning from malaria endemic areas.

Conclusion: Our data shows that gSG6-P1 is a suitable candidate for the evaluation of exposure to *Anopheles* mosquito bites, risk of malaria transmission, and effectiveness of protection measures against mosquito bites in the Americas.

Background

Globally, an estimated 3.3 billion people are at risk of being infected with malaria [1, 2]. Although the majority of cases and deaths occur in Africa, approximately 170 million people in the Americas live at risk of malaria

infection, with 74 % of cases caused by *Plasmodium vivax* and 25 % by *P. falciparum* [3, 4]. Malaria is transmitted to humans when the infective sporozoites, located in salivary glands, are injected into the human skin through the bite of a female *Anopheles* mosquito. These sporozoites are submerged in mosquito saliva [5] that is used by the mosquito to facilitate blood uptake. Mosquito saliva contains physiologically active components able to counteract blood coagulation, active complement, and the response to hinder the bite injury. Interestingly, some of these salivary components can also elicit and modify immune

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responses in the vertebrate host and can therefore modify the outcome of vector-transmitted diseases [6, 7].

The gold standard to measure the intensity of malaria transmission is the entomological inoculation rate (EIR), which is defined as the number of infectious *Anopheles* bites per person in a given time [8]. EIR measurement is highly dependent on the density of human-biting *Anopheles* [9]. The latter is estimated by trapping methods such as human-landing catches (HLCs) of adult mosquitoes. However, the technique of HLC poses ethical concerns as the human “bait” could be exposed to transmission of malaria and other mosquito-borne diseases. In addition, this trapping technique is only applicable to human adults. It is difficult to extrapolate HLC results to children or to pregnant woman that are the most vulnerable to malaria [10]. As an alternative, catching traps such as the CDC light trap, CDC light trap associated with CO₂ or the Mbitrap have been developed [11]. However, studies have shown that these alternative methods have several limitations, like preferentially capturing mosquitoes with higher sporozoite rate and consequently overestimating EIR [12] and can not replace HLC [13]. Therefore, new tools able to evaluate vector–host contact as well as the changes of this contact over time are needed to monitor both population and individual exposure and disease risk.

Previous works have shown that the immune response against mosquito salivary proteins, specially IgG antibodies, can reflect the intensity of exposure to mosquito bites as well as the risk of infection for vector-borne pathogens [14–16]. The serological evaluation of this immune response and its association with the exposure to malaria vectors is receiving increasing attention, especially regarding the major malaria vectors in Africa and India, *An. gambiae sensu lato* and *An. stephensi*, respectively. Transcriptome analysis of the salivary glands of *An. gambiae*, identified a protein named *An. gambiae* salivary gland protein 6 (gSG6), a member of the SG protein family known to be exclusively expressed in the adult *Anopheles* female [17]. Most of the SG proteins seem to be very well conserved among Old World species, *An. gambiae*, *An. arabiensis*, and *An. funestus*. However, clustal alignment of gSG6 showed similarities with the New World *Anopheles* species, *An. quadrimaculatus* and *An. freeborni* [18, 19]. The peptide gSG6 is restricted to the *Anopheles* genus based on substantial previous research, which has also demonstrated that specific IgG response to gSG6-P1 could serve as a biomarker for exposure to malaria vectors [2, 17, 19–25]. Moreover, the use of the gSG6-P1 salivary peptide has enabled the collection of relevant data on the efficacy of long lasting insecticide treated nets (LLIN) over time [26] and to compare the effectiveness of individual protection tools such as spray bombs and mosquito coils to LLINs [27].

The development of accurate and sensitive tools to identify variations in vector exposure and malaria risk is important to assess the effectiveness of control efforts. So far, the relevance of antibody response against gSG6-P1 has only been evaluated in subjects exposed to bites of *Anopheles* species in the Old World. The purpose of this study was to evaluate whether gSG6-P1 is a suitable biomarker for exposure to bites of American species of *Anopheles*, serving as an indicator of the efficacy of individual/collective malaria vector control tools and a proxy for *Plasmodium* transmission risk in the New World.

Methods

Ethics statement

Universidad de Pamplona, Los Patios Hospital in Norte de Santander-Colombia, approved this study. Additionally, an IRB approval from University of North Carolina at Chapel Hill was granted for the previously described double-blind RCT examining effects of permethrin-treated uniforms on the incidence of tick bites in North Carolina outdoor workers. The Military Hospital and the Chilean Army Health Command in Santiago, Chile, granted the approval for the inclusion of Chilean military personnel. Written consent was obtained from each volunteer.

Study participants

Chile

A sample of Chilean soldiers participating for a period of 6 months in the United Nations Stabilization Mission in Haiti (MINUSTAH) in 2011 were included in the present study (*n* = 45) (Table 1). They were part of a surveillance project of the Department of Preventive Medicine of the Chilean Military to evaluate the risk of vector-borne disease for peace troops working in Haiti. Samples were obtained from troops that were stationed

Table 1 Sample description

Country	Sample description	Sample groups	<i>n</i>	Species potential exposure
Chile	Chilean soldiers serving in Haiti	Before Deployment	45	<i>An. albimanus</i>
		Return	45	
Colombia	Healthy subjects	Before traveling	26	<i>An. albimanus</i> <i>An. darlingi</i> <i>An. punctimacula</i>
		After traveling	26	
	Febrile patients	Malaria (+)	7	
		Malaria (–)	35	
USA	Park Rangers	Before protective clothing	42	<i>An. punctipennis</i> <i>An. quadrimaculatus</i>
		After protective clothing	42	

in Port-au-Prince and responsible for road and bridge building. Daily activities included exposure to swamps, rivers, and flooded roads, as well as diurnal and nocturnal patrolling and convoy security duties in different rural sites. All soldiers received training to avoid mosquito exposure before departure. In Haiti, measures against vector-borne infections included personal use of skin repellent containing 34 % DEET (Ultrathon™, 3M, St. Paul, MN, USA), impregnation of occupational clothes with a permethrin-based repellent, weekly oral dose of 300 mg of chloroquine (base), sleeping in container-type facilities with air-conditioning, and indoor residual spraying and space spraying (adulticiding) with permethrin derivate every 15 days. During off-duty hours troops were authorized to wear sport and casual clothing. Almost all soldiers spent their 15-days UN leave at beach resorts in the Dominican Republic. The main malaria vector in both Haiti and the Dominican Republic is *An. albimanus* [28, 29]. Serum samples were collected before and after returning from Haiti and kept at -20°C until processing.

Colombia

In Colombia, the highest reported altitude for *Anopheles* species is 2000 m above the sea level (m.a.s.l.) [30]. For this study, we included 26 participants (Colombia population 1), aged 18 to 21 years, living in a malaria free area located at 2300 m.a.s.l. in Pamplona City, but traveling for at least 30 days to endemic areas in the Caribbean Coast of Colombia (States of Guajira, Cordoba, Sucre, Magdalena and Santander), where the main malaria vectors are *An. albimanus*, *An. darlingi*, and *An. punctimacula* [31]. These participants were included in a follow-up study to test reactivity to vector salivary proteins before and after traveling to endemic areas. Participants were bled before and within a week after returning to Pamplona.

A second group from Colombia (Colombia population 2) was formed by febrile participants ($n = 42$) seeking malaria diagnosis and medical services at the Local Hospital of Los Patios and Ocana in the Norte de Santander department, which is a known low malaria endemic area, and at the San Francisco de Asis Hospital in Quibdo in the Choco department, considered the second highest endemic area for malaria in the country. Serum samples were collected and kept at -20°C until processing.

United States

We selected 42 North Carolina Parks and Forestry rangers participating in a randomized controlled trial to test the efficacy of long-lasting permethrin-impregnated (LLPI) clothing. Serum samples were collected on time 1 (before implementation of protective clothing) and time 2 (after implementation of protective clothing) from participants

of the control group ($n = 24$) and the test group ($n = 18$). Aliquots of the samples were kept at -20°C until testing.

Malaria testing by PCR

Total DNA was isolated from human samples using the gDNA Blood Kit (Life technologies, Carlsbad, CA, USA) according to manufacturer's instructions. Plasmodium-specific PCR was performed using a multiplex PCR for the small sub-unit (SSU) rRNA gene described elsewhere [32]. DNA from in vitro culture of the Haiti I/CDC strain of *P. falciparum* and a blood sample from a thick-smear *P. vivax* positive patient were used as positive controls.

Antibody detection by ELISA

The ELISA conditions followed in this study were published elsewhere [26]. Briefly, 96-well ELISA plates were coated with 100 μL /well of 0.5 $\mu\text{g}/\text{ml}$ of gSG6-P1 in coating solution (Kirkegaard & Perry Laboratories, Inc. Gaithersburg, MD) serum samples were incubated at 37°C for 2 h in a 1/100 dilution. After washing, plates were incubated with 100 μL /well of horseradish peroxidase (HRP)-conjugated goat anti-human IgG (1:1000) antibodies at 37°C for 1.5 h. Later, each well received 100 μL /well of tetra-methyl-benzidine (TMB) (GenScript, Piscataway, NJ) and 100 μL /well solution of 1 M phosphoric acid to stop the reaction after 5 min incubation with the TMB. Absorbance was measured at 450 nm.

Statistical analysis

Antibody concentrations were expressed as delta optical density (ΔOD) calculated for each sample subtracting the mean OD value of the negative control and blank wells from the mean OD value of the duplicates for each sample. Positive control ΔOD values from all plates were recorded and averaged. This average was then divided by individual plate positive control ΔOD values to obtain a "calculation factor" for each plate. To correct for plate-to-plate variations, we then multiplied each sample ΔOD value by their respective plate calculation factor to obtain normalized ΔOD 's. The difference in the antibody concentrations between two independent groups was tested using the Mann Whitney *U* test while the difference between two dependent groups was calculated using Wilcoxon matched-pairs rank test. To determine risk of disease by Odd Ratios (OR), we divided the study sample into two groups with high and low IgG anti-gSG6-P1 antibody levels. As a threshold the median ΔOD of 1.3075 was used. Significant differences were established at p values higher lower 0.05. All statistical testing was performed with Prism version 6.0 (Graph Pad Software Inc.) and STATA™ version 10.1 (Stata Corporation).

Results and discussion

Anti-gSG6-P1 IgG as biomarker of exposure to “New World” *Anopheles* mosquito bites

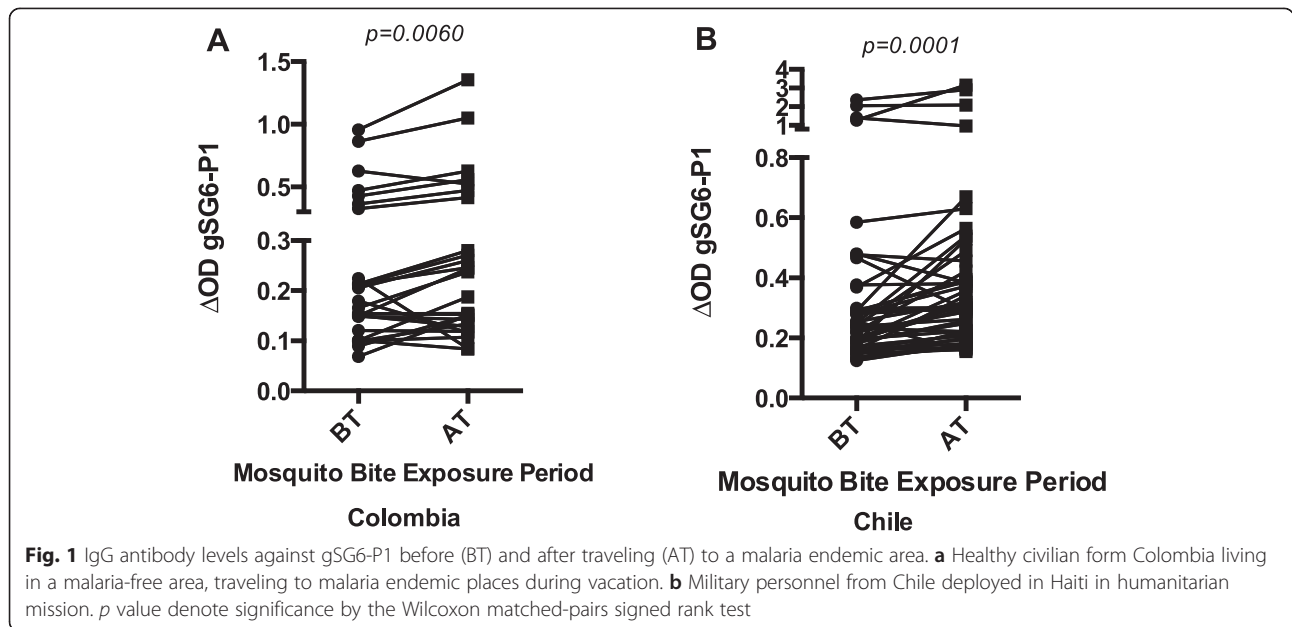
Estimating mosquito-human contact rate in endemic areas for vector transmitted diseases like malaria, is an important tool to measure the risk of transmission of such diseases. Since saliva from arthropods is injected at the time of transmission, efforts have been directed to discover the salivary proteins able to elicit an immune response that correspond to the level of bites a person has received in a determined period of time. Recently, the protein gSG6 from *An. gambiae*, has been reported as a useful marker for the degree of exposure to mosquito bites in malaria endemic areas of Africa. Research shows that the gSG6 protein presents little sequence variation within species of the *An. gambiae* complex (i.e. *An. melas*, *An. quadriannulatus*, *An. arabiensis*). However, sequence comparison of the gSG6 with more distant related species like *An. stephensi* (Asia) and *An. freeborni* (America) shows a 67 to 71 % sequence identity [17]. There is not available information on the sequence similarity between gSG6 and the South or Central American *Anopheles* species (*An. albimanus*, *An. darlingi* or *An. nuneztovari*). Nevertheless, salivary transcriptome analysis of the Nyssorhynchus subgenus did not reveal SG6 presence in the salivary content of these species [33, 34]. These reports suggested that a possible reason for this divergence is an independent evolutionary event of the gSG6 protein among anopheline subgenera although they also state that more data was necessary to confirm that hypothesis. In spite of the few available information on the American species homology with gSG6 protein [17, 35], several reports showed the gSG6 protein to be specific to the genus *Anopheles*. Consequently, we wanted to evaluate whether participants exposed to bites of American endemic *Anopheles* species develop antibodies specific to gSG6-P1, a salivary antigen validated as a biomarker of exposure to *An. gambiae s. l.* and *An. funestus* (main malaria vectors in Africa) bites. To do this, we used the gSG6-P1 peptide to assess exposure to *Anopheles* bites in healthy participants from Colombia living in a malaria-free area before and after travel to endemic malaria regions (Fig. 1a). In this group, we noticed a significant increase of individual levels of anti-gSG6-P1 IgG after traveling (AT) to malaria areas in comparison to those before traveling (BT) ($P = 0.006$). A similar increase in specific gSG6-P1 IgG levels was observed in Chilean soldiers after a 6-months stay in malaria endemic areas in Haiti and the Dominican Republic ($P < 0.0001$; Fig. 1b). These results suggest that IgG response to gSG6-P1 is closely associated with the degree of exposure to the bites of *An. albimanus*, *An. darlingi* or *An. punctimacula*, the malaria vectors described in these endemic areas in Latin America.

These results implicate that gSG6-P1 peptide, although based on the Old World *An. gambiae s. s.* mosquito, can be used to evaluate the level of exposure to bites of New World (Americas) species of *Anopheles* malaria vectors. Previous studies conducted in Senegal, Angola, and Benin have described a correlation between the level of gSG6-P1 IgG antibodies and the intensity of human exposure to *Anopheles* bites [21, 22, 26]. Cross-response to gSG6-P1 peptide bases on the fact that gSG6 is a well conserved polypeptide presenting homologues in both the Old and New World *Anopheles* species [36]. In conclusion, gSG6 polypeptide as well as its gSG6-P1 peptide is suitable as a “bite” biomarker of Old World as well as New World species of *Anopheles* malaria vectors.

IgG specific to gSG6-P1 as a proxy of malaria transmission risk

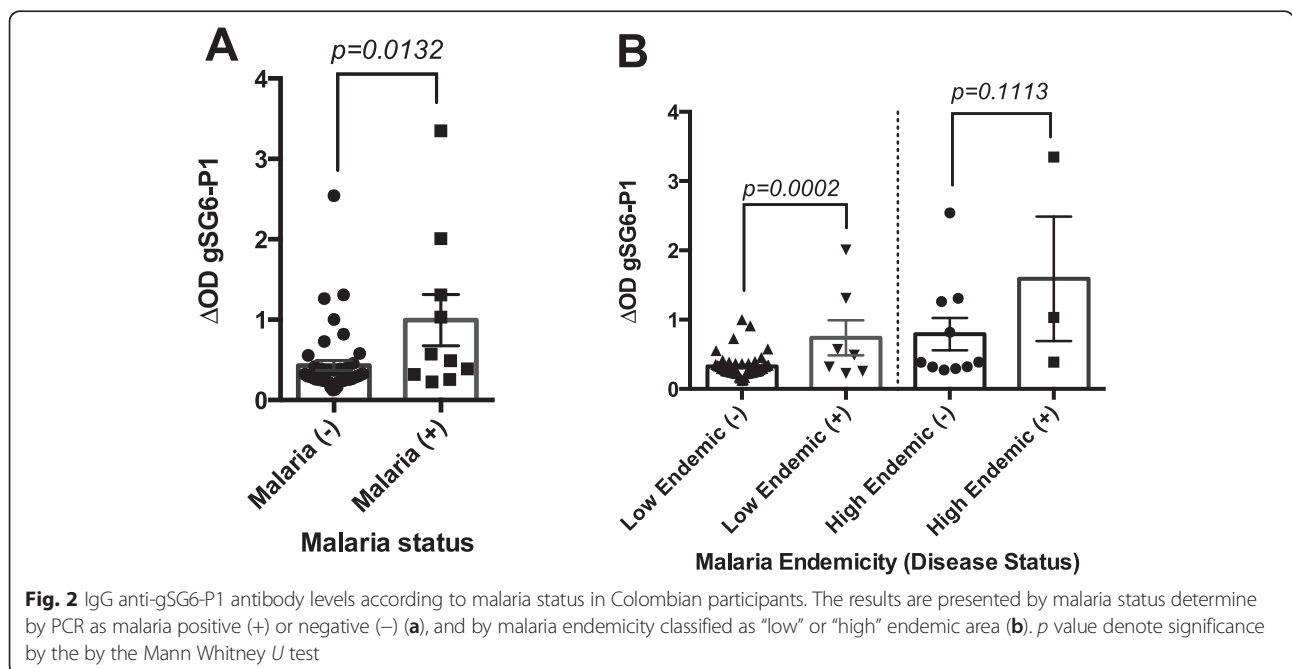
Anti-gSG6-P1 IgG levels were assessed in malaria negative (–) and malaria positive (+) groups of participants residing in malaria endemic areas of Colombia where *An. albimanus*, *An. darlingi*, and *An. punctimacula* are the main vectors. Our results demonstrated that the median level of specific IgG antibodies against gSG6-P1 was significantly higher in the malaria infected group compared to the uninfected group ($P = 0.0132$) (Fig. 2a). Serum samples in Colombia were collected in two different states: 1) Choco with an annual parasitic incidence (API) of 49 cases/1000 habitants, which in 2010 was classified as a high malaria transmission region [37] and 2) Norte de Santander, which is classified as low transmission area (API: 1.3 cases/1000). Interestingly, the difference in median anti-gSG6-P1 IgG levels between malaria infected and uninfected participants was only significant in participants living in areas of low malaria endemicity ($P = 0.0002$). In the high transmission area, no significant difference in anti-gSG6-P1 levels was observed ($P = 0.1113$) (Fig. 2b).

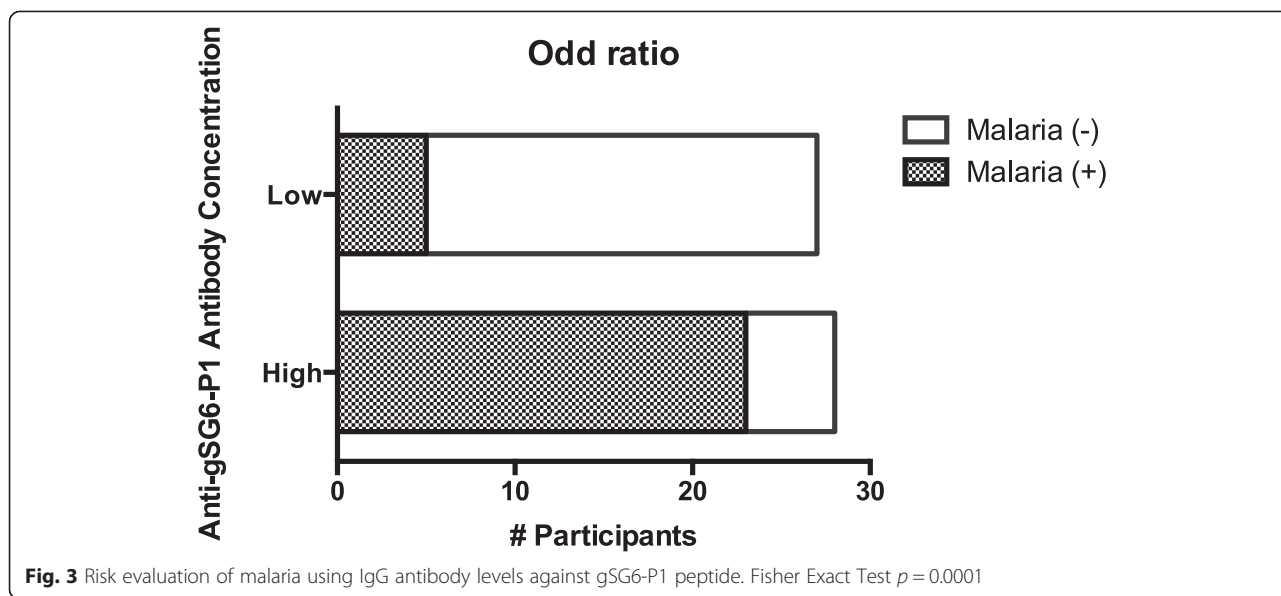
We also tested the hypothesis that IgG-gSG6-P1 antibody concentration serves as a proxy for the risk of acquiring malaria. For that, we divided our study subjects into two categories: “low antibody levels” (with a ΔOD value < 0.774) and “high antibody levels” (with a ΔOD value ≥ 0.775). Participants with “high” levels of IgG anti-gSG6-P1 antibodies had a 20.24 times higher risk for acquiring malaria ($P = 0.0001$) than people with lower levels of those antibodies (Fig. 3) suggesting that more mosquito bites elicit higher anti-gSG6-P1 antibodies and that higher antibody levels are also associated with a higher possibility of being exposed to a bite from an *Plasmodium*-infected mosquito. This suggests that the level of gSG6-P1 specific IgG could be used as biomarker for the risk of malaria infection. Our data confirm previous studies conducted in different parts of the Old World, in particular a recent study in a low to



moderate malaria transmission area of Senegal, which reported an association between IgG response to gSG6-P1 and the status *P. falciparum* infection of children, even during the dry season [22]. Furthermore, this study showed different levels of anti-gSG6- IgG P1 antibodies in asymptomatic *Plasmodium* carriers compared to clinical malaria cases. In Kenya, an association between the prevalence of *P. falciparum* and the levels of anti-gSG6-P1 IgG was also described [38]. Another study in Burkina-Faso revealed a positive correlation between malaria incidence and IgG response against the gSG6

recombinant protein [39]. All together, the anti-salivary gSG6-P1 peptide IgG response, previously demonstrated to be a biomarker of exposure to *Anopheles* bites in numerous contexts and in this study, could also be a useful indicator of malaria exposure and infection. This could also be useful to estimate the malaria risk for non-indigenous populations taking chemoprophylaxis such as the Chilean soldiers of our study, which helps to adapt protection strategies for these populations. In a high transmission situation in Colombia, we found no significant differences in anti-gSG6-P1 levels of malaria (+)





and malaria (-) groups suggesting that the biomarker might have limitation in this setting. Although the sample size of this study group was small, it suggested that in hyperendemic malaria areas, residents might be highly exposed to infectious and non-infectious bites. They thus present high levels of anti-gSG6-P1 antibodies, which do not necessarily reflect the risk of malaria transmission. Therefore, for such situations there is a need for more specific biomarkers.

It is important to clarify that these findings are applicable for malaria transmission settings in the absence of antimalarial drugs as prophylactic treatments. In such cases, individuals will be still exposed to vector bites with a reduced risk of presenting symptoms and evaluation of the gSG6 antibody levels will be suitable marker for human-vector contact.

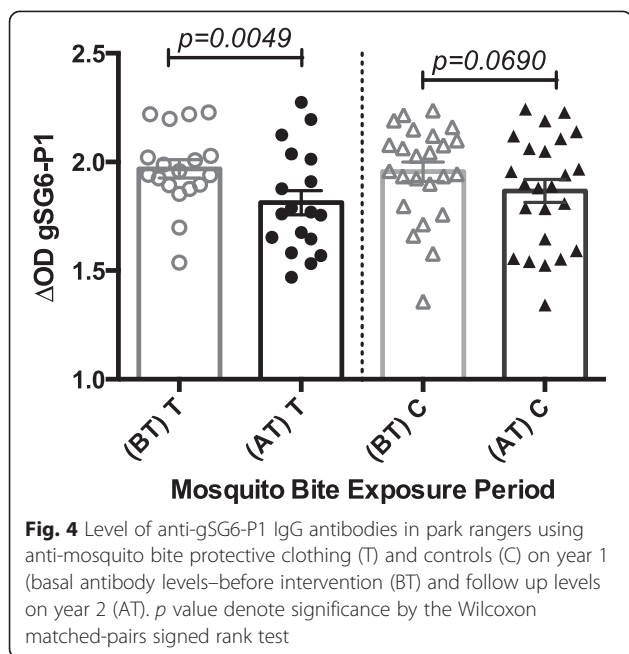
In the African settings where previous studies with gSG6-P1 took place, entomological records were made to evaluate the abundance of *Anopheles* species present in these areas. So, it was known that the majority of the *Anopheles* species present in these areas were potential vectors of malaria. In Colombia, between 70 and 90 % of the mosquitoes captured during entomological surveillance belong to genus *An. albimanus*, *An. darlingi* or *An. nuneztovari* considered the main vectors of malaria in the country [40, 41] while *An. albimanus* is the most abundant *Anopheline* species in the Hispaniola Island [42, 43]. However, if same studies will be undertaken in area where only half of the *Anopheles* are malaria vectors, we might observe the same non significance between infected and non-infected individuals, as in hyperendemic transmission area, probably because people would receive as many uninfected that infected bites and no association between the level of

IgG to gSG6-P1 peptide and the risk of transmission would be observed.

Human IgG response to gSG6-P1 for evaluating effectiveness of individual protection against mosquito bites

A major application for an immunologically based vector-human contact biomarker is the assessment of the efficacy of vector control methods. For instance, Drame et al. proved that the use of insecticide-treated bednets and spray bombs drastically reduced the IgG levels specific to gSG6-P1 peptide, independently of age or transmission season [22, 27]. Other studies also suggest that the IgG response against gSG6-P1 is a reliable alternative to accurately assess the effectiveness of malaria control methods and surveillance programs in areas of low malaria risk [44, 45]. In this study, we evaluated and compared the IgG-gSG6-P1 levels of US park rangers using LLPI clothing during working hours and control participants wearing untreated clothes before and after the implementation of the strategy. At baseline, both groups presented similar levels of IgG specific to gSG6-P1 peptide. One year after the implementation of LLPI clothing, we observed a significant decrease in the concentration of IgG-gSG6-P1 antibodies in the treatment group ($P = 0.0049$), whereas no changes were observed in the controls (Fig. 4).

Anopheles punctipennis is the most abundant *Anopheles* species in North Carolina (US), mostly biting outdoors after dusk. However, it will attack humans also during daytime in dense woodlands or close to their daylight resting places, where the park rangers might be performing their work activities. Monitoring the efficacy of individual use of anti-malaria vector tools is very challenging. Previous data suggested that the gSG6-P1 salivary biomarker can



be used to assess and monitor the effectiveness of individual or collective protection tools/strategies against *Anopheles* mosquito bites [22, 26, 27]. Our study confirmed this by showing that the level of *Anopheles* bites was significantly reduced in those rangers wearing LLPI clothes. We therefore believe that gSG6-P1 salivary biomarker is a useful marker to assess and monitor the effectiveness of protection measures also against New World *Anopheles* bites. This raises the importance of personal protection to vector bites in the control of malaria. This is in accordance with previous data showing significant reduction of specific IgG levels, human-*Anopheles* contact and bites in association with the use of spray bombs [26]. However all personal protection tools do not seem to reduce the level of mosquito bites as pointed by the same authors who described a none-significant effect of the use of mosquito coils or electric fans/air conditioning. In addition, by comparing the efficacy of individual protection tools such as spray bombs and mosquito coils to bed-nets, the same authors indicate that the decreases in *Anopheles* aggressiveness has essentially been due to the use of bed-nets, meaning that these individual protection tools can be complementary but cannot replace bed-nets in the fight against malaria.

In the case of the Chilean troops in Haiti, air conditioning facilities and bednets were used for all the troops at base. Additionally, they use of military uniforms, left neck and wrists exposed so although most of the body is protected these areas can still be reached by mosquitoes during the night work. These observations suggest that the use of these uniforms, reinforced by chloroquine treatment, reduced the risk of presenting malaria in the

presence of higher mosquito bite exposure (There has been only one case of malaria in 10 years of mission in Haiti), and that gSG6-P1 is a useful marker to show that in the event of increased exposure to mosquito bites subjects were protected against infection.

Conclusion

The synthetic peptide gSG6-P1 is useful as a proxy indicator of human-vector contact with American *Anopheles* species. Further investigation is needed to compare the immune response against the specific mosquito species vectors of malaria in America with the response elicited against gSG6-P1 in areas with different malaria endemicity. It is also a useful tool for the evaluation vector control interventions and devices designed to decrease mosquito bite and disease transmission in human populations.

Competing interests

None of the authors have a relationship or support that can be perceived as constituting competing interests.

Authors' contributions

BLR participated in the study design, acquisition of data and data analysis. PMD, FR and AP helped in the production of recombinant gSG6-P1 as well as the in the study design and data analysis. TW and RR participated in the sample collection of Chilean samples, data analysis and manuscript drafting. CG helped in the acquisition of data. JCC and MA carried out the sample collection in Colombia and helped in the data analysis. DMW and TMC participate in the design of the study and the manuscript preparation. All authors read and approved the final version of the manuscript.

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