Institut Pasteur
Microbes and Infection 17 (2015) 409–416
www.elsevier.com/locate/micinf

Original article

The *Anopheles gambiae* cE5 salivary protein: a sensitive biomarker to evaluate the efficacy of insecticide-treated nets in malaria vector control

Alexandra Marie [a,*], Raffaele Ronca [b], Anne Poinsignon [a], Fabrizio Lombardo [c], Papa M. Drame [a], Sylvie Cornelie [a,d], Patrick Besnard [e], Jacques Le Mire [e], Gabriella Fiorentino [b], Filomeno Fortes [f], Pierre Carnevale [g], Franck Remoue [a], Bruno Arcà [c,*]

[a] *MIVEGEC, UMR IRD-224 CNRS-5290 UM1-UM2, 911 Av. Agropolis, 34394 Montpellier Cedex 5, France*
[b] *Department of Biology, "Federico II" University, Via Cinthia, 80126 Naples, Italy*
[c] *Department of Public Health and Infectious Diseases, Parasitology Section, Sapienza University, Piazzale Aldo Moro 5, 00185 Rome, Italy*
[d] *MIVEGEC-IRD-CREC, 01 BP44 RP Cotonou, Benin*
[e] *Malaria Control Program-SONAMET Clinic, Lobito, Angola*
[f] *Malaria Control Program, Ministry of Public Health, Luanda, Angola*
[g] *IRD, Montpellier, France*

Received 11 December 2014; accepted 22 January 2015
Available online 28 January 2015

Abstract

Evaluation of vector control is crucial for improving malaria containment and, according to World Health Organization, new complementary indicators would be very valuable. In this study the IgG response to the *Anopheles*-specific cE5 salivary protein was tested as a tool to evaluate the efficacy of insecticide-treated nets in reducing human exposure to malaria vectors. Sera collected during a longitudinal study carried out in Angola, and including entomological and parasitological data, were used to assess the IgG response to the *Anopheles gambiae* cE5 in both children and adults, before and after the application of insecticide-treated nets. Seasonal fluctuation of specific IgG antibody levels according to exposure was only found in children (up to ≈14 years old) whose anti-cE5 IgG response dropped after bed nets installation. These results were fully consistent with previous findings obtained with the same set of sera and indicating a substantial reduction of human-vector contact shortly after nets implementation. Overall, children IgG response to the cE5 protein appeared a very sensitive biomarker, which allowed for the detection of even weak exposure to *Anopheles* bites, indicating it may represent a reliable additional tool to evaluate the efficacy of vector control interventions.

Keywords: cE5 salivary protein; *Anopheles gambiae*; Malaria; Biomarker; Vector exposure; Insecticide-treated nets

1. Introduction

Despite substantial efforts and increasing international funding malaria is still a devastating human parasitic disease, with 627,000 deaths and 207 million cases estimated to have occurred in 2012. *Plasmodium falciparum*, the most dangerous *Plasmodium* species, is responsible for ≈90% of cases, with more than 85% of deaths occurring in children from sub-Saharan Africa, where the major parasite vectors are members of the *Anopheles gambiae* complex [1]. Nowadays, no vaccine is available, and malaria containment in endemic areas is largely based on control measures against the parasites (mainly artemisin-based combination therapy) and the vectors (insecticide-based control). Different vector control strategies are currently employed, for example indoor residual spraying (IRS) or insecticide-treated plastic sheeting, but the use of

* Corresponding authors.
E-mail addresses: alexandra.marie@ird.fr (A. Marie), bruno.arca@uniroma1.it (B. Arcà).

http://dx.doi.org/10.1016/j.micinf.2015.01.002
1286-4579/

insecticide-treated nets (ITNs) is so far the most efficient and used strategy to reduce malaria transmission and morbidity by decreasing human-vector contact. However, parasite resistance to antimalarial treatment [2] and mosquito vector resistance to insecticides [3,4] seriously impair the success of malaria control measures.

Currently, the WHO recommendation for phase 3 evaluation of ITNs efficacy is based on the assessment of *P. falciparum* parasite numbers in the human blood. Classical entomological methods are also used to evaluate vector control strategies, usually assessing *Anopheles* mosquito density and the Entomological Inoculation Rate (EIR), i.e. the number of infective bites per person per unit of time. However, these techniques present several limitations when it comes to large-scale field studies, particularly in areas of low exposure and transmission (dry season, high altitude, urban setting) or also in highly endemic areas after vector control, such as after implementation of ITNs. Moreover, some entomological methods (CDC traps, IRS) cannot be applied for evaluating the human-vector contact at the individual level and human landing catches on adult volunteers, which is the reference method to evaluate exposure at the individual level, may raise ethical concerns and it is not applicable to children. Considering these limits, new complementary indicators to evaluate the efficacy of vector control strategies would be very valuable.

A substantially different and innovative approach is based on the concept that human exposure to arthropod vectors may be assessed by monitoring the antibody response to their saliva [5,6]. When blood-sucking arthropods bite their hosts, they inject saliva while probing in search for blood. Saliva carries several bioactive molecules whose main physiological role is to facilitate blood feeding counteracting host hemostatic and inflammatory reactions [7–9]. Moreover, salivary proteins may also be immunogenic and induce in vertebrates an anti-saliva antibody (Ab) response that may be taken as a measure of exposure to vector bites. This strategy has been investigated for several vectors of human pathogens such as ticks [10], triatomine bugs [11–13], sand flies [14,15], tsetse flies [16,17] and mosquitoes [18–22]. More specifically, as far as malaria is concerned, it should be pointed out that IgG Ab response to *Anopheles* saliva or salivary gland extracts has been shown to represent an effective biomarker of human exposure to bites of malaria vectors [23–26]. In addition, such epidemiological indicator has also been used to evaluate the efficacy of both bed nets and other vector control strategies [27–30]. In spite of being a potentially very useful tool, the use of saliva or salivary extracts has several limitations. First, some saliva components are ubiquitous in arthropod species and can induce immune cross-reactivity. Second, saliva collection is fastidious and the composition of different batches may vary according to environmental factors or physiological status (i.e. age), inducing a lack of reproducibility [31]. For these reasons, a suitable and reliable biomarker cannot be based on whole saliva and should better rely on a single recombinant protein or synthetic peptide.

The first anopheline salivary protein identified as immunogenic to humans and specific to mosquitoes of the *Anopheles* genus is the *A. gambiae* gSG6 [32–34]. The human IgG response to this protein was validated as serological indicator of exposure to the three main Afrotropical malaria vectors: *A. gambiae*, *Anopheles arabiensis* and *Anopheles funestus* [35–38], and similar results were also obtained using the gSG6-based peptide gSG6-P1 [34,39–42]. In addition, gSG6-P1 has also been employed to evaluate different vector control strategies in Senegal [43] and in Angola [44]. In this last study, a longitudinal evaluation (including parasitological, entomological and immunological assessments) was conducted on children and adults from a malaria-endemic area before and after the introduction of ITNs. A significant decrease of anti-gSG6-P1 IgG was observed shortly after ITN implementation suggesting that this peptide may be suitable for vector control assessment. However, a very low IgG response to gSG6-P1 was found in a rather large proportion of individuals (39%) already before the installation of ITNs, suggesting that it may not be enough sensitive to detect very low levels of exposure to *Anopheles* bites.

More recently the genus-specific *A. gambiae* salivary protein cE5, a member of the anophelin family of anti-thrombin polypeptides [45], was expressed in recombinant form [46] and shown to be more immunogenic to humans than gSG6 [47], suggesting that due to its sensitivity it may be especially useful as a marker of exposure to anopheline mosquitoes in conditions of low vector density. To verify the suitability of the cE5 antigen for evaluation of vector control, and eventually improve the serological toolbox available to malaria epidemiological studies, we analyzed the IgG response to the *A. gambiae* cE5 salivary protein in a cohort from the study area in Angola mentioned above [28,44]. This longitudinal survey included sera collected before and after ITNs implementation, and was conducted in a study area (Lobito) were vector density was relatively low according to entomological and immunological data, making it especially suitable for validation of the cE5 salivary antigen. The aim of the present study was, therefore, to assess if the specific anti-cE5 IgG Ab response could be associated with the level of exposure to *Anopheles* bites in an epidemiological setting characterized by low vector density, at least in comparison to previously studied areas in Burkina Faso [47], and if it could be used as a biomarker of ITNs efficacy.

2. Materials and methods

2.1. Ethics statement

This study was conducted in accordance with the Edinburgh revision of the Helsinki Declaration, and was approved by the National Malaria Control Program of the Minister of Health of Angola (October 17th 2008), the only one Ethical authority in 2008 for approving studies on malaria research in Angola. Written informed consent (signed by the head of each household) was obtained for all individuals enrolled in the study by the SONAMET Malaria Control Program (MCP), which control malaria infection of all workers for SONAMET and their family. This consent procedure was regularly

approved by SONAMET workers, who benefited to several malaria studies/survey by MCP, and was approved by the involved Ethical authority in Angola.

2.2. Study population

This study was conducted in Lobito, a coastal city of Western Angola, from March 2005 to December 2006. The site is in the tropical Savannah with a rainy season from October to May and approximately 600–700 mm of rain per year. The duration of malaria transmission season varies between 7 and 12 months with a peak between March and May. The major malaria vectors are members of the *A. gambiae* species complex [28]. The study population was previously described [28,44]. Briefly, all workers of the Société Nationale de Métallurgie (SONAMET) Company lived in 250 households in the Bella Vista district. Residents were followed in the SONAMET in-patient clinic. In 2004, the presence of malaria parasite was diagnosed in 60 households by the SONAMET Malaria Control Program (MCP). Twenty-one of these 60 households were then randomly selected for the longitudinal follow-up. In total, 230 individuals (children and adults) were included in the study, with evaluation every 6 weeks on two periods: from March 2005 to January 2006 and from April 2006 to December 2006. The number of individuals per household varied between 2 and 23. In February 2006 long-lasting insecticide nets (LLINs) treated with deltamethrin (Permanet®) were distributed to the families (according to the number of rooms and beds per households). At each visit, thick blood smear and dried blood spot (filter paper) samples were collected from each individual for parasitological tests and immunological analysis, respectively. Parasite density (parasitemia) was calculated as the number of *P. falciparum* per microliter of blood and presented as the geometric mean of parasitemia values $(x + 1)$, as previously indicated [28]. Immunological tests reported in the present study were performed on a sub-sample (of the whole study population) represented by 71 individuals living in 14 different households and among these: 33 were young children aged from 1 to 6 years old (mean: 3.67; 95% CI: 3.14–4.20); 22 were 7–14 years old (mean: 9.68; 95% CI: 8.67–10.69); 16 individuals were more than 14 years old (mean: 26; 95% CI: 20.56–31.44). For these individuals blood spots were available for at least 10 out of 12 visits and this explains the imperfect balance in numbers of individuals among these three different age groups. Filter papers were kept at 4 °C in Silica gel before testing.

2.3. Entomological analysis and survey of LLIN use

Mosquitoes were collected every six weeks during the study at 6 reference households, representative of the studied area. The number of individuals in the 6 reference household was as follows: hld 1 (n = 6), hld 2 (n = 7), hld 3 (n = 12), hld 4 (n = 7), hld 5 (n = 17) and hld 6 (n = 13). *A. gambiae* density was evaluated using capture by CDC light trap from 7 h PM to 7 h AM for two consecutive nights. PCR was used to confirm species in order to estimate the number of *A. gambiae*/trap/night. After the introduction of LLINs, their use by individuals and their quality were inspected the night before each blood sampling by the MCP team. Information on net use was then collected for all studied individuals by questionnaires, covering: i) the number of installed LLINs, ii) the number of exchanged LLINs, and iii) the number of damaged LLINs (hole, torn, etc.), as previously described [28].

2.4. Evaluation of human IgG antibody level (ELISA)

Standardized dried blood spots (0.6 cm diameter) were eluted by incubation in 200 μL of phosphate buffer saline containing Tween 0.1% (PBST) at 4 °C for 24 h. Enzyme-Linked ImmunoSorbent Assays (ELISA) were carried out on eluates to measure the level of IgG Ab reacting to the cE5 antigen. The cE5 recombinant protein was expressed and purified as previously described [46]. Maxisorp plates (Nunc, Roskilde, Denmark) were coated with cE5 protein (5 μg/mL) in carbonate/bicarbonate buffer (0.1 M $NaHCO_3$, 0.1 M Na_2CO_3, pH9.6) at 37 °C for 2 h and 30 min. After five washings in Tween 0.1%, wells were blocked (1 h at 37 °C) in Blocking Buffer (Pierce® Protein-Free, Thermo Scientific, Rockford, USA), washed again and each eluate was incubated at 4 °C overnight at a 1/20 dilution in PBST. Eluates were analyzed in duplicate with the antigen and once without antigen. After washing, plates were incubated (1 h and 30 min at 37 °C) with a mouse biotinylated Ab against human IgG (BD Pharmingen, San Diego, CA, USA) at a 1/2000 dilution. After washing, peroxidase conjugated streptavidin (Amersham, Les Ulis, France) was added at a 1/2000 dilution for 1 h at 37 °C. After washing, colorimetric development was carried out using ABTS [2,2′-azino-bis (3-ethylbenzthiazoline 6-sulfonic acid) diammonium; Sigma, St Louis, MO, USA] in 50 mM citrate buffer (pH 4) containing 0.003% H_2O_2. Optical density (OD) was measured at 405 nm. IgG levels were expressed as final OD (ΔOD) calculated for each eluate as the mean OD value with antigen minus the OD value without antigen. Intra- and inter-assay variation of samples was below 15% as evaluated by standard positive control samples analyzed on each plate. Eluates presenting duplicates with coefficient of variation (CV) ≥20% were excluded from the analysis and re-assessed by ELISA. French individuals (n = 32) with no known exposure to *A. gambiae* mosquitoes were used as unexposed controls; anti-cE5 IgG levels measured in these subjects were used to calculate the specific immune response threshold (TR). A subject was considered as an "immune responder" if his/her ΔOD was higher than the TR = mean $\Delta OD_{unexposed} + 3SD = 0.257$.

2.5. Statistical analysis

Data were analyzed using GraphPad Prism 6 Software® (San Diego, CA, USA). After verifying the non-Gaussian distribution, the non-parametric Mann–Whitney U-test was used to compare Ab levels between two independent groups, the Wilcoxon matched pairs test was used for comparison

between two paired groups, the non-parametric Kruskall–Wallis test for comparison between more than two groups. The Chi-square test was used for the comparison of two proportions. All differences were considered significant at $p < 0.05$.

3. Results

A total number of 71 individuals (1–55 years old) living in fourteen different households located in Lobito (Angola) were analyzed in this longitudinal study. Blood spots were collected during twelve independent visits at six weeks intervals during two different periods: from March 2005 to January 2006 and from April to December 2006. Deltamethrin-treated LLINs were distributed in February 2006. Eluates from dried blood spots were used to measure IgG antibodies against the *A. gambiae* cE5 salivary protein. Entomological, parasitological and immunological measurements were also recorded as previously reported [28,44] and briefly described in the methods section.

3.1. Seasonal variation of the IgG Ab response to the cE5 protein in different age groups

Median levels of IgG antibodies against the cE5 protein in the studied individuals during the longitudinal survey are reported in Fig. 1. Significant fluctuations of anti-cE5 IgG antibody levels were found (thick solid line, $p < 0.0001$), with an evident decrease in August 2005 during the dry and low vector density season ($p = 0.01$), and a drop in April 2006 ($p < 0.0001$) shortly after the application of ITNs in February 2006. However, this variation in the response was not evenly distributed among different age groups. Indeed, when the same analysis was done after splitting the cohort in three different age groups, no significant changes were observed in the older group (>14 years old), whereas the seasonal variation of IgG levels was still highly significant in the two younger groups (1–6 years: $p < 0.0001$; 7–14 years: $p < 0.0001$). More specifically, the fall of the anti-cE5 IgG responses in August 2005 and in April 2006 mentioned above were completely absent in the >14 years old group. On the contrary, the decrease was still present and similar in both 1–6 years (August 2005 $p = 0.0431$, April 2006 $p < 0.0001$) and 7–14 years old children (August 2005 $p = 0.0230$, April 2006 $p = 0.0007$). These observations suggest that the IgG response against the cE5 protein persists for longer in adults than in children, at least in the conditions of *Anopheles* density/exposure such as those found in the Lobito area.

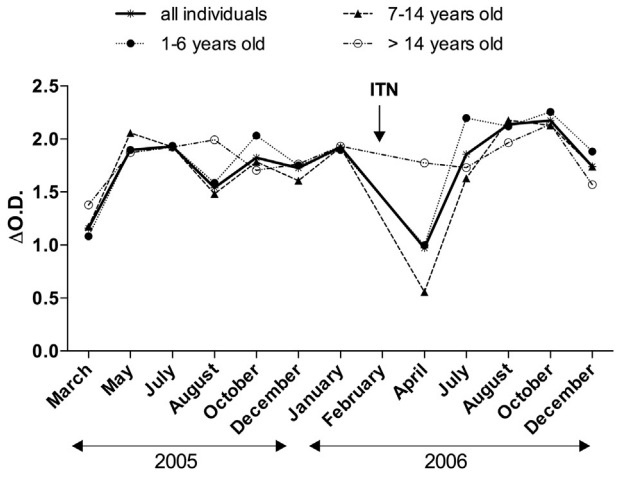

Fig. 1. Seasonal variation of the IgG Ab response to cE5. Median values of anti-cE5 IgG levels are reported for all individuals (solid tick line) and for the different age groups as indicated. The arrow points to the installation of ITNs in February 2006. Age group, number of individuals, mean age and 95% CI were as follows: 1–55 years old, n = 71, 10.6 years old, CI 8.2–12.9; 1–6 years old, n = 33, 3.7 years old, CI 3.2–4.2; 7–14 years old, n = 22, 9.7 years old, CI 8.7–10.6; >14 years old, n = 16, 26.0 years old, CI 21.0–31.0. Seasonal variation for each age group was tested using the Kruskal–Wallis test: 1–55 years old, $p < 0.0001$; 1–6 years old, $p < 0.0001$; 7–14 years old, $p < 0.0001$; >14 years old, $p = 0.0547$. Wilkoxon matched-pairs test was used to compare the IgG response between paired groups.

3.2. IgG Ab response to cE5 before and after ITNs use in children <14 years old

According to the age-dependent results mentioned above the individual IgG response against the cE5 protein was analyzed in further detail in the periods before (March 2005 to January 2006) and after (April 2006 to December 2006) installation of ITNs (February 2006) by grouping together children from 1 to 14 years old. Considerable individual variation of the anti-cE5 IgG level was observed during each visit, suggesting that the IgG response was specific to the individual exposure (Fig. 2). At population level (median values), a seasonal variation of the IgG response to the cE5 protein was also observed ($p < 0.0001$). The first peak of specific IgG response was associated with the peak of *Anopheles* density in May 2005. We observed then a decrease

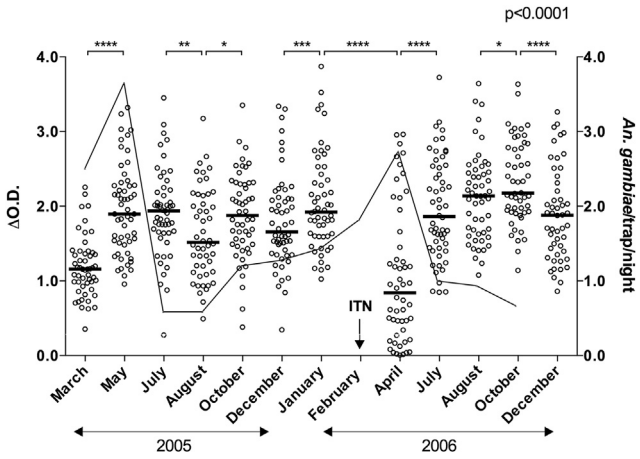

Fig. 2. Individual anti-cE5 IgG levels in children 1 to 14 years old and entomological data. Scatter plots reporting levels of IgG antibodies against the cE5 protein in children 1–14 years old (n = 55, mean age 6.1 years old, CI 5.2–7.0) in the twelve different visits. Bars represent median values. The arrow indicates the installation of ITNs in February 2006. The indicated p value was determined according to the Kruskal–Wallis test. Pairwise comparisons refer to the Wilcoxon matched pairs test (*, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; ***, $0.0001 < p < 0.001$; ****, $p < 0.0001$). The mean number of *A. gambiae*/trap/night is shown as a solid line.

from July to August 2005 and again an increase of the anti-cE5 IgG response associated with a rise of *A. gambiae* density in October 2005 ($p < 0.05$). Interestingly, after ITNs installation a significant decrease of the anti-cE5 IgG level was observed in April 2006 ($p < 0.0001$), despite the fact that the number of *A. gambiae* mosquitoes peaked this month. Moreover, in comparison to measurements made in the same period one year earlier, the specific IgG Ab response in April 2006 was not significantly different compared to March 2005, but significantly lower compared to May 2005 ($p < 0.0001$). This IgG decrease in April 2006 was also associated to a decline of *P. falciparum* parasitemia, as previously described [28,44]. The decrease of the parasitemia during a period of growth of the mosquito population highlights the clear efficacy of ITNs in decreasing the human-vector contact. Afterward, from July to October 2006, the anti-cE5 IgG level increased despite the wane of *Anopheles* density. This phenomenon was also observed in previous studies [28,44] and explained by the incorrect use and/or damage of the nets.

3.3. Individual evolution of anti-cE5 IgG level in children and short-term ITNs efficacy

The relationship between ITNs implementation and anti-cE5 IgG levels was also analyzed at individual level by comparing results of immunoassays in April 2006 (shortly after ITNs installation) to January 2006 (just before). As previously observed at population level (Fig. 1), no significant difference was found in the adult group when the anti-cE5 IgG levels were compared before and after introduction of ITNs ($p = 0.1876$, Fig. 3). On the contrary, both in 1–6 and in 7–14 years old children, the IgG response to the cE5 protein decreased in most individuals, even if some of them presented increased or unchanged IgG responses (Fig. 3). Some individuals, especially in the age groups 1–6 and 7–14 years old, exhibited high levels of anti-cE5 IgG antibodies in January, a low transmission and moderate vector density period. However, this is not surprising considering both the overall high inter-individual variability and the progressive increase of *Anopheles* mosquito density from August to January (see also Fig. 2).

A more effective graphic representation and evaluation of the trend of the anti-cE5 IgG response (positive, negative or unchanged) in relation to ITN implementation can be obtained reporting the difference between the response in April and in January ($\Delta OD_{ITNs} = \Delta OD_{April} - \Delta OD_{January}$) and introducing a threshold value of 0.257, which was obtained measuring the anti-cE5 response in unexposed individuals (Fig. 4). By applying this threshold and analyzing the ΔOD_{ITNs} it was found: (i) a decrease in the specific IgG response in 74.2% (23/31) of 1–6 years old children and in 75% (15/20) of 7–14 years old children; (ii) an increased response in 12.9% (4/31) and 20% (4/20) of 1–6 and 7–14 years old individuals, respectively; (iii) no changes in the response in 12.9% (4/31) of 1–6 years and 5% (1/20) of 7–14 years old children. No significant difference between the two age groups was found for the three different pairs of proportions (Fisher's exact test $p > 0.05$). Among individuals older than 14 years old, 46.7% (7/15) presented a decrease and 46.7% (7/15) presented an increase of their anti-cE5 IgG response whereas 1 individual (6.7%) revealed no significant change.

4. Discussion

In the present study, the IgG Ab response to the *A. gambiae* cE5 salivary protein was investigated in children and adults before and after the installation of ITNs during a two-year longitudinal survey. According to previously reported parasitological data, ITNs were effective in decreasing parasite density, which represents the current WHO criterion for evaluating vector control efficacy, whereas the *A. gambiae* mosquitoes were still present [28,44]. Therefore, this cohort appeared an adequate support to verify whether the Ab response to the cE5 salivary protein might be a reliable indicator to evaluate the real efficacy of ITNs on decreasing human-vector contact.

The IgG Ab response against cE5 was found to be different in the three age groups in which the studied population was divided: 1 to 6, 7 to 14 and >14 years old. No significant seasonal variation of specific IgG levels and no decrease after ITNs installation were noticed in individuals older than 14 years. The difference observed between children (up to 14

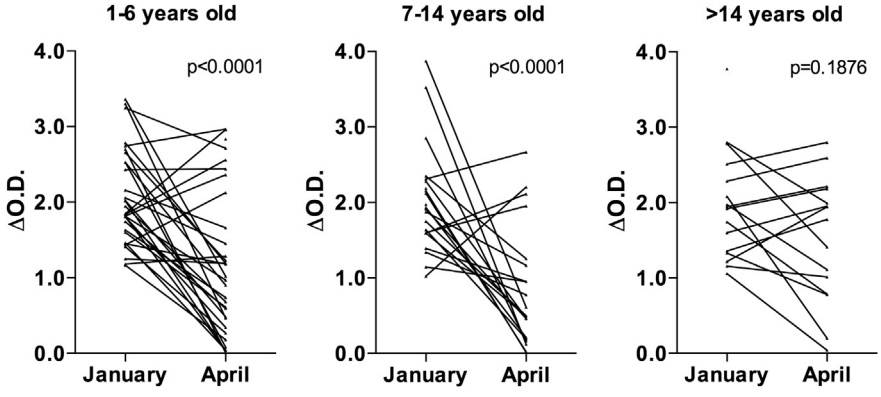

Fig. 3. Changes in anti-cE5 IgG levels before and after the introduction of ITNs. Individual changes in Ab response from January 2006 (just before ITNs) to April 2006 (just after ITNs) are presented according to the three different age groups as indicated. P values according to Wilkoxon matched-pairs test.

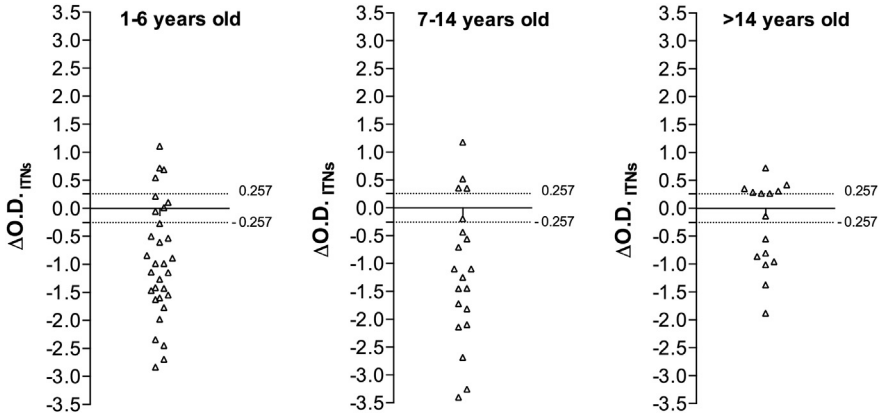

Fig. 4. IgG response to the cE5 protein as biomarker for short-term ITNs efficacy. Individual changes of IgG levels from January 2006 to April 2006 are presented by individual ΔOD_{ITNs} values ($\Delta OD_{ITNs} = \Delta OD_{April} - \Delta OD_{January}$). The threshold for specific variation in the response (TR = 0.257) is indicated by dotted lines. Significant positive ($\Delta OD_{ITNs} > 0.257$) or negative ($\Delta OD_{ITNs} < 0.257$) changes are individually presented according to age.

years old) and the older group may partly be explained by an overall more intense exposure of adults to *Anopheles* bites: for example because of their longer stay outdoor or their different sleeping schedule, which may result in a lower protection by ITNs. However, a major contribution to the absence of seasonal- or ITN-linked variation in adults is most likely related to the specific kinetic of rise and decline of the anti-cE5 IgG response, and this idea is strengthened by at least two independent observations. First, in the same individuals analyzed here the IgG response to the gSG6-P1 peptide, which is known to be short-lived, decreased after ITNs implementation indicating that the adult-vector contact was indeed disrupted [44]. Second, the anti-cE5 IgG response is relatively longer-lasting than the anti-gSG6 response, as suggested by a study in Burkina Faso where no seasonal variation and no decrease during the dry season were observed [37,47]. Therefore, the absence of any significant fluctuation may be ascribed to the fact that adults may establish a stronger and more persistent specific Ab response to the cE5 antigen, which may then need a more extended time of non-exposure (or very low exposure) to vanish.

On the other side, both a seasonal variation of the anti-cE5 IgG response and a significant decrease shortly after the introduction of ITNs were observed in children up to ≈14 years old. The seasonal variation in accordance to *Anopheles* density indicates the potential validity of the cE5 antigen as indicator of children exposure to *A. gambiae*. The rapid anti-cE5 IgG decrease observed two months after the introduction of ITNs: (i) confirms that proper net use was effective in reducing human-vector contact (even during the period corresponding to the peak of the *A. gambiae* population) and (ii) suggests that the anti-cE5 IgG response may be a suitable marker to evaluate ITNs effectiveness, provided that serological measures are performed in children. It should be emphasized that the IgG response to whole saliva and to the gSG6-P1 peptide also showed in the same cohort a similar significant decrease shortly after ITNs application [28,44]. However, the fluctuations of the anti-cE5 IgG response and its drop shortly after ITNs applications observed in children are in conflict with the results of a previous study in Burkina Faso; here, with the only exception of very young children (1–5 years old), no seasonal variation of the specific IgG response was observed [47]. We believe that this apparent discrepancy may be explained by the different vector density in the two study areas. Indeed, the IgG antibody levels against a salivary protein will be the result of both recent and past exposure to *Anopheles* bites, and the intensity and temporal continuity of exposure may deeply affect the development of the response. During the periods of maximum vector density, 3.7 *A. gambiae*/trap/night were captured in Lobito (Angola) using CDC light traps, whereas 12.1 *Anopheles*/person/night were found in Barkoumbilen (Burkina Faso) employing pyrethrum spray catches. Obviously, these entomological measures cannot be directly compared. Nevertheless, these data suggest that exposure to anophelines was much lower in Lobito, especially considering that the number of *Anopheles* is expressed per trap and that the number of individuals per household varied from 6 to 17 in the 6 reference household used for the entomological measures. It is likely that in conditions of significantly lower exposure to anophelines the anti-cE5 IgG response requires more years/seasons of exposure to become more intense and persistent and, therefore, it reaches full development and a steady state only later in life (in individuals older than 14 in Lobito).

Overall our observations indicate that the drop of the anti-cE5 IgG response was associated with the correct use of ITNs, which disrupts the human-vector contact, and not due to a wane of *Anopheles* density. Noteworthy, in July 2006 the IgG response against the cE5 protein increased and stayed high in the following months, despite the seasonal decline of mosquito density. It should be pointed out that also the IgG response to whole saliva and gSG6-P1 showed a similar increase in July and during the following months, and that also an increased parasitemia was found after the drop observed in April 2006 [28,44]. These observations are most likely linked to the incorrect use and/or damage of the nets, an hypothesis that is supported by a survey on ITNs use indicating that only 53% of ITNs were well-installed and undamaged in June 2006 in the total population [28].

The data reported here also confirm the higher immunogenicity/sensitivity of the cE5 protein in comparison to gSG6

[47]. In a previous study, including the same cohort analyzed here, Drame et al. showed that the anti-gSG6-P1 IgG response was very low in several individuals already in January 2006, before the introduction of ITNs [44]. Consequently ≈39% of the subjects showed no significant change of the IgG response to gSG6-P1 between January 2006 and April 2006. Here, in the same individuals, the Ab response against cE5 was higher in January 2006, indicating that the absence of IgG response against gSG6-P1 was due to the low sensitivity of the peptide rather than to the absence of exposure to mosquito bites.

In conclusion, the cE5 antigen does not appear to be much helpful in epidemiological settings characterized by high and continued exposure, where the gSG6 protein or gSG6-based peptides seem to perform better. On the contrary, the high immunogenicity of cE5 may be better exploited in conditions of low vector density to detect weak exposure to malaria vectors and evaluate efficacy of ITNs; however, both results reported here and previous studies [47] indicate that serological measures of the anti-cE5 IgG response should be essentially performed in young children, mainly because of the kinetic of mounting and declining of the anti-cE5 IgG response. This perspective appears very relevant considering that young children (less than 5 years old) are the first vulnerable/susceptible population to malaria and, therefore, the first target for preventive measures as ITNs. It is possible that the *A. gambiae* cE5 may also be useful to evaluate exposure to malaria vectors in individuals transiently exposed to *Anopheles* bites, such as travelers or soldiers living in malaria transmission areas just for a few weeks. Finally, it is known that *A. funestus* and *A. arabiensis* are important vectors of malaria in Tropical Africa and can play a major role in malaria transmission, especially when *A. gambiae* is not present. The IgG response to the *A. gambiae* cE5 will also reveal exposure to *A. arabiensis* (the two proteins are 100% identical), whereas other studies will be needed to evaluate cross-reactivity to the *A. funestus* proteins (55% identity, 74% similarity) and verify the possibility to use the cE5 protein to evaluate exposure to all three main Afrotropical malaria vectors. In the absence of a recombinant cE5 protein from *A. funestus*, indication of cross-reactivity could be obtained using sera from individuals living in areas where this malaria vector is largely predominant.

Conflict of interest

The authors declare they have no conflict of interest.

Acknowledgments

The authors gratefully acknowledge the population of the Bella Vista area (Lobito, Angola) for their participation in this study, the technical personal of the Malaria Control Program team, especially Maria Chilombo (SONAMET Lobito) and Dr Jean-François Foucher from Acer-Energy (ACERGY) Company. This work was supported by the Institut de Recherche pour le Développement (IRD; Département de Valorisation au Sud) and the European Community's Seventh Framework Programme (FP7) (grant agreement N° 242095, EviMalaR). Alexandra Marie was supported by an EviMalaR scholarship.

References

[1] WHO. World malaria report. 2013.
[2] O'Brien C, Henrich PP, Passi N, Fidock DA. Recent clinical and molecular insights into emerging artemisinin resistance in *Plasmodium falciparum*. Curr Opin Infect Dis 2011;24:570–7.
[3] Aikpon R, Agossa F, Osse R, Oussou O, Aizoun N, Oke-Agbo F, et al. Bendiocarb resistance in *Anopheles gambiae* s.l. populations from Atacora department in Benin, West Africa: a threat for malaria vector control. Parasit Vectors 2013;6:192.
[4] Magesa SM, Aina O, Curtis CF. Detection of pyrethroid resistance in *Anopheles* mosquitos. Bull World Health Organ 1994;72:737–40.
[5] Billingsley PF, Baird J, Mitchell JA, Drakeley C. Immune interactions between mosquitoes and their hosts. Parasite Immunol 2006;28:143–53.
[6] Remoue F, Cornelie S, Ngom A, Boulanger D, Simondon F. Immune responses to arthropod bites during vector-borne diseases. In: Garraud O, Fort PO, editors. Update in tropical immunology. Trivandrum, Keraka: Research Signpost; 2005. p. 377–400.
[7] Ribeiro JM. Blood-feeding arthropods: live syringes or invertebrate pharmacologists? Infect Agents Dis 1995;4:143–52.
[8] Ribeiro JM, Francischetti IM. Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. Annu Rev Entomol 2003;48:73–88.
[9] Ribeiro JMC, Arcà B. From sialomes to the sialoverse: an insight into salivary potion of blood-feeding Insects. Adv Insect Physiol 2009;37:59–118.
[10] Schwartz BS, Ford DP, Childs JE, Rothman N, Thomas RJ. Anti-tick saliva antibody: a biologic marker of tick exposure that is a risk factor for Lyme disease seropositivity. Am J Epidemiol 1991;134:86–95.
[11] Nascimento RJ, Santana JM, Lozzi SP, Araujo CN, Teixeira AR. Human IgG1 and IgG4: the main antibodies against *Triatoma infestans* (Hemiptera: Reduviidae) salivary gland proteins. Am J Trop Med Hyg 2001;65:219–26.
[12] Schwarz A, Helling S, Collin N, Teixeira CR, Medrano-Mercado N, Hume JC, et al. Immunogenic salivary proteins of *Triatoma infestans*: development of a recombinant antigen for the detection of low-level infestation of triatomines. PLoS Negl Trop Dis 2009;3:e532.
[13] Schwarz A, Medrano-Mercado N, Billingsley PF, Schaub GA, Sternberg JM. IgM-antibody responses of chickens to salivary antigens of *Triatoma infestans* as early biomarkers for low-level infestation of triatomines. Int J Parasitol 2010;40:1295–302.
[14] Barral A, Honda E, Caldas A, Costa J, Vinhas V, Rowton ED, et al. Human immune response to sand fly salivary gland antigens: a useful epidemiological marker? Am J Trop Med Hyg 2000;62:740–5.
[15] Souza AP, Andrade BB, Aquino D, Entringer P, Miranda JC, Alcantara R, et al. Using recombinant proteins from *Lutzomyia longipalpis* saliva to estimate human vector exposure in visceral Leishmaniasis endemic areas. PLoS Negl Trop Dis 2010;4:e649.
[16] Dama E, Cornelie S, Bienvenu Somda M, Camara M, Kambire R, Courtin F, et al. Identification of *Glossina palpalis gambiensis* specific salivary antigens: towards the development of a serologic biomarker of human exposure to tsetse flies in West Africa. Microbes Infect 2013;15:416–27.
[17] Poinsignon A, Remoue F, Rossignol M, Cornelie S, Courtin D, Grébaut P, et al. Human IgG antibody response to *Glossina* saliva: an epidemiologic marker of exposure to *Glossina* bites. Am J Trop Med Hyg 2008;78:750–3.
[18] Das MK, Mishra A, Beuria MK, Dash AP. Human natural antibodies to *Culex quinquefasciatus*: age-dependent occurrence. J Am Mosq Control Assoc 1991;7:319–21.
[19] Doucoure S, Mouchet F, Cornelie S, DeHecq JS, Rutee AH, Roca Y, et al. Evaluation of the human IgG antibody response to *Aedes albopictus* saliva as a new specific biomarker of exposure to vector bites. PLoS Negl Trop Dis 2012;6:e1487.

[20] Doucoure S, Mouchet F, Cournil A, Le Goff G, Cornelie S, Roca Y, et al. Human antibody response to *Aedes aegypti* saliva in an urban population in Bolivia: a new biomarker of exposure to dengue vector bites. Am J Trop Med Hyg 2012;87:504–10.

[21] Remoue F, Alix E, Cornelie S, Sokhna C, Cisse B, Doucoure S, et al. IgE and IgG4 antibody responses to *Aedes* saliva in African children. Acta Trop 2007;104:108–15.

[22] Trevejo RT, Reeves WC. Antibody response to *Culex tarsalis* salivary gland antigens among sentinel chickens in California. Am J Trop Med Hyg 2005;72:481–7.

[23] 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.

[24] 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;47:1156–63.

[25] Remoue F, Cisse B, Ba F, Sokhna C, Herve JP, Boulanger D, et al. Evaluation of the antibody response to *Anopheles* salivary antigens as a potential marker of risk of malaria. Trans R Soc Trop Med Hyg 2006;100:363–70.

[26] Waitayakul A, Somsri S, Sattabongkot J, Looareesuwan S, Cui L, Udomsangpetch R. Natural human humoral response to salivary gland proteins of *Anopheles* mosquitoes in Thailand. Acta Trop 2006;98:66–73.

[27] Brosseau L, Drame PM, Besnard P, Toto JC, Foumane V, Le Mire J, et al. Human antibody response to *Anopheles* saliva for comparing the efficacy of three malaria vector control methods in Balombo, Angola. PloS One 2012;7:e44189.

[28] Drame PM, Poinsignon A, Besnard P, Le Mire J, Dos-Santos MA, Sow CS, et al. Human antibody response to *Anopheles gambiae* saliva: an immuno-epidemiological biomarker to evaluate the efficacy of insecticide-treated nets in malaria vector control. Am J Trop Med Hyg 2010;83:115–21.

[29] Gidwani K, Picado A, Rijal S, Singh SP, Roy L, Volfova V, et al. Serological markers of sand fly exposure to evaluate insecticidal nets against visceral leishmaniasis in India and Nepal: a cluster-randomized trial. PLoS Negl Trop Dis 2011;5:e1296.

[30] Schwarz A, Juarez JA, Richards J, Rath B, Machaca VQ, Castro YE, et al. Anti-triatomine saliva immunoassays for the evaluation of impregnated netting trials against Chagas disease transmission. Int J Parasitol 2011;41:591–4.

[31] Choumet V, Carmi-Leroy A, Laurent C, Lenormand P, Rousselle JC, Namane A, et al. The salivary glands and saliva of *Anopheles gambiae* as an essential step in the *Plasmodium* life cycle: a global proteomic study. Proteomics 2007;7:3384–94.

[32] Lanfrancotti A, Lombardo F, Santolamazza F, Veneri M, Castrignanò T, Coluzzi M, et al. Novel cDNAs encoding salivary proteins from the malaria vector *Anopheles gambiae*. FEBS Lett 2002;517:67–71.

[33] Lombardo F, Ronca R, Rizzo C, Mestres-Simon 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;39:457–66.

[34] Poinsignon A, Samb B, Doucoure S, Drame PM, Sarr JB, Sow C, et al. First attempt to validate the gSG6-P1 salivary peptide as an immuno-epidemiological tool for evaluating human exposure to *Anopheles funestus* bites. Trop Med Int Health 2010;15:1198–203.

[35] Ali ZM, Bakli M, Fontaine A, Bakkali N, Vu Hai V, Audebert S, et al. Assessment of *Anopheles* salivary antigens as individual exposure biomarkers to species-specific malaria vector bites. Malar J 2012;11:439.

[36] Rizzo C, Ronca R, Fiorentino G, Mangano VD, Sirima SB, Nébié I, et al. Wide cross-reactivity between *Anopheles gambiae* and *Anopheles funestus* SG6 salivary proteins supports exploitation of gSG6 as a marker of human exposure to major malaria vectors in tropical Africa. Malar J 2011;10:206.

[37] Rizzo C, Ronca R, Fiorentino G, Verra F, Mangano V, Poinsignon A, et al. Humoral response to the *Anopheles gambiae* salivary protein gSG6: a serological indicator of exposure to afrotropical malaria vectors. PloS One 2011;6:e17980.

[38] Stone W, Bousema T, Jones S, Gesase S, Hashim R, Gosling R, et al. IgG responses to *Anopheles gambiae* salivary antigen gSG6 detect variation in exposure to malaria vectors and disease risk. PloS One 2012;7:e40170.

[39] Badu K, Siangla J, Larbi J, Lawson BW, Afrane Y, Ong'echa J, et al. Variation in exposure to *Anopheles gambiae* salivary gland peptide (gSG6-P1) across different malaria transmission settings in the western Kenya highlands. Malar J 2012;11:318.

[40] Drame PM, Machault V, Diallo A, Cornelie S, Poinsignon A, Lalou R, et al. IgG responses to the gSG6-P1 salivary peptide for evaluating human exposure to *Anopheles* bites in urban areas of Dakar region, Senegal. Malar J 2012;11:72.

[41] Poinsignon A, Cornelie S, Ba F, Boulanger D, Sow C, Rossignol M, et al. Human IgG response to a salivary peptide, gSG6-P1, as a new immuno-epidemiological tool for evaluating low-level exposure to *Anopheles* bites. Malar J 2009;8:198.

[42] 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.

[43] Drame PM, Diallo A, Poinsignon A, Boussari O, Dos Santos S, Machault V, et al. Evaluation of the effectiveness of malaria vector control measures in urban settings of Dakar by a specific *Anopheles* salivary biomarker. PloS One 2013;8:e66354.

[44] Drame PM, Poinsignon A, Besnard P, Cornelie S, Le Mire J, Toto JC, et al. Human antibody responses to the *Anopheles* salivary gSG6–P1 peptide: a novel tool for evaluating the efficacy of ITNs in malaria vector control. PloS One 2010;5:e15596.

[45] Valenzuela JG, Francischetti IM, Ribeiro JM. Purification, cloning, and synthesis of a novel salivary anti-thrombin from the mosquito *Anopheles albimanus*. Biochemistry 1999;38:11209–15.

[46] Ronca R, Kotsyfakis M, Lombardo F, Rizzo C, Currà C, Ponzi M, et al. The *Anopheles gambiae* cE5, a tight- and fast-binding thrombin inhibitor with post-transcriptionally regulated salivary-restricted expression. Insect Biochem Mol Biol 2012;42:610–20.

[47] Rizzo C, Lombardo F, Ronca R, Mangano V, Sirima S, Nébié I, et al. Differential antibody response to the *Anopheles gambiae* gSG6 and cE5 salivary proteins in individuals naturally exposed to bites of malaria vectors. Parasit Vectors 2014;7:549.