IgE response to Aed al 13 and Aed al 14 recombinant allergens from Aedes albopictus saliva in humans

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ABSTRACT

Background: Mosquito bite is normally associated with mild allergic responses, but severe localized or systemic reactions are also possible. Reliable tools for the diagnosis of mosquito allergy are still unavailable. Here, we investigated the IgE response to 3 potential salivary allergens identified in the saliva of the tiger mosquito Aedes albopictus.

Methods: Serum from 55 adult individuals (28 controls and 27 allergic people), were analysed using an in-house Enzyme Linked ImmunoSorbent Assay (ELISA) against the Salivary Gland Extract (SGE) and the recombinant proteins albD7l2 (Aed al 2), albAntigen5-3 (Aed al 13) and albLIPS-2 (Aed al 14).

Results: Fifteen of the 27 (56%) individuals having hypersensitive reactions to mosquito bites had IgE serum levels recognizing SGE. Negative sera did not show detectable levels of IgE targeting the SGE from the most common sympatric mosquito Culex pipiens. Among the positive individuals, 2 subjects displayed IgE targeting Aed al 2 (13%), while IgE recognizing Aed al 13 and Aed al 14 were detected in ten (67%) and seven (47%) individuals, respectively. Two sera from non-hypersensitive subjects had detectable levels of IgE targeting Aed al 13, suggesting possible cross-reaction with the homologue salivary proteins of multiple mosquito species or, more generally, of hematophagous insects.

Conclusions: Our results indicate that Aed al 13 and Aed al 14 hold the potential to be developed as tools for the diagnosis of allergy to Ae. albopictus bites. Such tools would facilitate epidemiological studies on tiger mosquito allergy in humans and might foster the development of further protein-based assays to investigate cross-species allergies.

Keywords: Venom hypersensitivity, Insect proteins, Saliva, Culicidae, Allergens
INTRODUCTION

Mosquito bites are the most common cause of acute itch in humans.\textsuperscript{1,2} The itching is caused by a local immune reaction to mosquito salivary allergens, which are injected by female mosquitoes in the skin to favour the blood meal needed to complete egg development. Non-salivating mosquitoes do not elicit any response in human skin,\textsuperscript{3} while intradermal injections with salivary gland extracts (SGEs) induce both an immediate and a delayed response, as typically described after a mosquito bite.\textsuperscript{4} The small local reaction exhibits immediate wheal and flare about 230 min after the bite, followed by pruritic indurations 24–36 h later, or delayed reaction.\textsuperscript{4,5} At an individual level, the history of the response can be subdivided into 5 stages.\textsuperscript{6,7} First exposures (Stage I) typically do not elicit any reaction. When repeatedly bitten, the individuals firstly develop delayed reactions only (Stage II), then immediate reactions also take place (Stage III). During the desensitisation phase, which naturally occurs in a timeframe of 2–20 years,\textsuperscript{8} the delayed reaction progressively disappears (Stage IV), followed by the immediate reaction (Stage V).

The reactions are usually self-limited, as they vanish within a few days, but scratching can lead to other consequences, such as bacterial superinfections, hyperpigmentation, and scarring. Less frequently, subjects can develop more severe response, known as "Skeeter syndrome", displaying large localized reactions with papules >30 mm and mild fever, extended or atypical local reactions, or systemic responses such as anaphylaxis, angioedema, generalised urticaria, wheezing, referred to as mosquito allergy or hypersensitivity to mosquito bites (HMB).\textsuperscript{9–11} Additionally, hypersensitivity can be associated with Epstein-Barr virus infections or with hematologic malignancies.\textsuperscript{12,13}

There is only limited information about the prevalence of these disorders in humans, but a study in the Centre of Allergy and Clinical Immunology of Monterrey in Mexico reported that 2.5% of the patients displayed large local reactions and one patient (0.2%) had a history of systemic reaction to mosquito bites.\textsuperscript{14}

Human response to mosquito bites is both antibody- (IgG and IgE) and immune cell-mediated (lymphocytes, eosinophils, and neutrophils).\textsuperscript{15,16} In particular, IgE and IgG are involved in the immediate response, while lymphocyte activation is associated with the delayed reaction.\textsuperscript{15}

The first exposure to mosquito saliva causes the activation of T lymphocytes and B lymphocytes, while further exposures cause proliferation of B cells and production of IgG antibodies against mosquito salivary proteins, while T lymphocytes differentiate to memory cells. In this respect, IgG levels can be used as a marker of mosquito bites exposure.\textsuperscript{17–19} Additionally, IgE antibodies are specifically produced against salivary allergens and IgE saliva-specific antibodies are thought to be the main cause of allergic reactions.\textsuperscript{10,16,20}

Dosage of serum IgE levels against mosquito extracts, determined using Enzyme Linked ImmunoSorbent Assay (ELISA), is useful to diagnose mosquito allergy, in association with clinical diagnosis, based on the observation of atypical or exaggerated reaction to mosquito bite, and skin prick test (SPT).\textsuperscript{21} The use of the whole mosquito body extract as source of allergens displays several limits in terms of sensitivity and specificity,\textsuperscript{21} while saliva or salivary gland extract (SGE) preparation results in a more accurate diagnosis of mosquito allergy.\textsuperscript{22} However, dissecting the salivary glands and collecting saliva from female mosquitoes is a time-consuming process and can cause cross-reactivity due to the presence of highly conserved proteins throughout different mosquito species.\textsuperscript{22,23} Therefore, the use of recombinant mosquito salivary allergens in diagnostic tests could be a powerful alternative to these preparations.

In this regard, the present study investigates the IgE reactivity to the SGE of the Asian tiger mosquito, \textit{Aedes albopictus}, and to 3 recombinant proteins (\textit{albD712} or Aed al 2, \textit{albAgS5-3} or Aed al 13, and \textit{albLIPS-2} or Aed al 14), selected from its salivary proteome. Additionally, an evaluation of the IgE response to the SGE of the mosquito species \textit{Culex pipiens}, sympatric to \textit{Aedes albopictus} in the research area, was carried out. The study was conducted in Italian subjects affected by mosquito allergy, according to their clinical history. We emphasize that \textit{Ae. albopictus} are the mosquitoes...
most frequently associated with systemic allergic reactions to mosquito bites; the species is widespread in Italy, where it was first described in 1990. This species displays an aggressive diurnal behaviour and it has rapidly spread worldwide, exposing a high number of people to its bites.

The availability of recombinant allergens from this mosquito species is, therefore, a prerequisite to study the prevalence of *Ae. albopictus* allergy in human populations and to design strategies to alleviate its nuisance.

**METHODS**

**Collection of sera from allergic and control patients**

Fifty-five serum samples were obtained from the “Area Allergologica presso l’Unità di Immunologia, Reumatologia, Allergologia e Malattie Rare” at the IRCCS Ospedale San Raffaele (Milan, Italy) through 2018–2020. The protocol was approved by the institutional review board (IRB) of the hospital, the independent ethics committee (IEC). Of the 55 samples, 27 were from people displaying severe hypersensitivity reactions to mosquito bites (HMB) and 28 from control subjects (CTR), all recruited after informed consent. Mosquito allergy was diagnosed according to the clinical history of allergy of the subjects (Table 1).

**Mosquito rearing and preparation of the salivary gland extract (SGE)**

*Aedes albopictus* and *Culex pipiens* mosquitoes were reared in the insectarium in standard conditions (28°C, 65% humidity, 12:12 h light:dark and 25°C, 35% humidity, 12:12 h light:dark, respectively). *Ae. albopictus* are from the Rimini strain, established decades ago from mosquitoes coming from Italy. The *Culex pipiens* colony has been established using mosquitoes collected in 2020 in the Bergamo district. For each species separately, 3- to 10-day old female mosquitoes were ice-anaesthetised and salivary glands (SGs) were dissected with a pair of forceps. After removal, SGs (about n = 60) were collected in PBS and homogenised with a pestle. Following high-speed centrifugation (4°C, 10’000 g, 30 min), the supernatant was collected, filtered and protein concentration was assessed (DC protein assay, Biorad). Salivary gland extract (SGE) was stored at −20°C until use.

**Cloning of the allergens and expression in *Escherichia coli***

DNA fragments coding for three putative *Ae. albopictus* salivary protein allergens have been cloned in a modified version of pETSUMO vectors (Invitrogen), such that the recombinant proteins carry an 8xHis-tag followed by the SUMO protein fused at the N-terminus of the target recombinant protein.

*aBlIPS-2* (MW: 34 kDa) was cloned, expressed and purified as previously described. Briefly, its coding sequence (mRNA AY826118, protein AAV90690, uniprot Q5MIU2) deprived of the region encoding for the signal peptide (residues 1–25, as predicted using SignalP software) was amplified from salivary glands cDNA using the primers LIPS-2fw AGTCGGATCCAACCCAACCAACAAGTCG and LIPS-2rv CGTAGCGGCCGCTATTACAATGTACCTTAAGCCC.

The *aBlAg5-3* (MW: 27 kDa; mRNA AY826105, protein AAV90677, UniProt: Q5MIV5), and the previously identified allergen *aBlD7l2* (*Aed al 2*, MW: 35 kDa, mRNA GAPW01002627, protein JAC10971.1, UniProt: A0A023EQS5), deprived of the signal peptides, were codon-optimised, synthesised, and cloned by Genewiz into the modified pETSUMO vector.

Recombinant proteins were produced in *E. coli* Shuffling K12 strain (New England Biolabs) using ZYP-5052 autoinducing medium at 30°C at 180 rpm for 4.5 h, then temperature was lowered at 20°C at 180 rpm ON.

Ag5-3 and LIPS-2 have now been registered to the WHO/IUIS Allergen Nomenclature Database as *Aed al 13* and *Aed al 14* respectively.

**Recombinant allergens purification**

Bacterial cells were harvested by centrifugation after overnight incubation (5’000 g, 15 min, 10°C), resuspended in Buffer A (50 mM HEPES/NaOH, 500 mM NaCl, pH 8.0), then lysed by sonication. Cell debris were removed by centrifugation (50’000 g, 45 min, 4°C) and recombinant proteins were purified at room temperature using liquid chromatography (Åkta purifier, GE Healthcare)
Table 1. Description of Hypersensitivity Reactions (HRs) to mosquito bites. HR to mosquito bite and the respective grading are reported for every allergic subject of the present study. CTCAE, Common Terminology Criteria for Adverse Events (National Institutes of Health, 2017); ELR, Extended local reaction.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Hypersensitivity Reaction</th>
<th>grading CTCAE</th>
</tr>
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<tbody>
<tr>
<td>A01</td>
<td>ELR (urticaria)</td>
<td>1</td>
</tr>
<tr>
<td>A02</td>
<td>ELR (edema, erythema)</td>
<td>1</td>
</tr>
<tr>
<td>A03</td>
<td>ELR (edema, erythema)</td>
<td>1</td>
</tr>
<tr>
<td>A04</td>
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<td>1</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td>A07</td>
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<td>ELR (edema, erythema)</td>
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<td>ELR (edema, erythema), diffuse itching</td>
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</tr>
<tr>
<td>A14</td>
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</tr>
<tr>
<td>A15</td>
<td>ELR (urticaria)</td>
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<td>ELR (edema, erythema)</td>
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<td>A22</td>
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</tr>
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<td>local reaction, extended rash, facial edema</td>
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</tr>
<tr>
<td>A27</td>
<td>ELR (edema, erythema)</td>
<td>2</td>
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through immobilised metal ion affinity chromatography (IMAC; 5 mL HisTrap excel column). The fractions containing the proteins of interest eluted after the application of 250 mM imidazole to Buffer A. The samples were then incubated with His-tagged SUMO-protease in a 1:300 M ratio during dialysis at 4 °C in Buffer A. A second IMAC step was used to remove the cleaved His-tag; fractions containing the proteins of interest underwent concentration using Vivaspin Turbo 15 filters MWCO 10 kDa (Sartorius) and subsequent injection into a Superdex 75 10/300 GL column (GE Healthcare) equilibrated with 25 mM HEPES/NaOH, 100 mM NaCl, pH 8.0.

Protein quality was assessed throughout the purification using reducing and non-reducing SDS-PAGE analysis (Supplementary Fig. S1). Protein concentrations were measured at 280 nm using a NP80 spectrophotometer (IMPLEN), using 0.82 as extinction coefficient for albLIPS-2, 2.00 for albAg5-3 and 1.03 for albD712, calculated using the Expasy ProtParam tool prediction. Purified proteins were stored at −80 °C until use.

In-house Enzyme Linked ImmunoSorbent Assay (ELISA)

The IgE ELISA protocol was initially optimised by testing different protein, serum, and anti-IgM immunoglobulin (Ig) E concentrations. A checkerboard titration was performed to assess sera and SGE concentrations. Particularly, we tested SGE at 25, 10, 5, 2.5 and 1 μg/mL, sera diluted 1:2, 1:5, 1:10 and 1:20 in blocking buffer (BB), and anti-IgE diluted 1:500, 1:1000 and 1:2000 in BB. The final selected conditions were: 10 μg/mL SGE or recombinant allergen concentration for coating, sera diluted 1:5 and anti-IgE diluted 1:500 in BB.

SGE, albLIPS-2, albAg5-3, albD712, and the mixture of albLIPS-2 and albAg5-3 were diluted at 10 μg/mL in carbonate coating buffer (Sigma Aldrich) to coat 96-well ELISA plates (ThermoFisher Scientific). After 2 h incubation at 37 °C, plates were washed with PBS-Tween 0.05% (washing buffer, WB) using an automated system (Immunowash Microplate Washer, Bio-Rad) and incubated with 5% Non-Fat Dry Milk diluted in WB (blocking buffer, BB) for 1 h at RT. After washing, plates were incubated overnight at RT with sera diluted 1:5 in BB. After an additional washing, each well was incubated with horseradish peroxidase conjugated anti-human IgE antibody (Goat anti-Human IgE Secondary Antibody, HRP, Invitrogen) diluted 1:500 in BB for 1 h at RT. Finally, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added after washing. The colorimetric reaction was stopped with H2SO4 2 N and the absorbance at 450 nm was recorded (EnSight Multimode Plate Reader). Each sample was analysed in duplicate. Wells incubated overnight with blocking buffer instead of sera were used as an internal control for the in-house ELISA test.

ELISA for IgG detection was performed as described above for IgE but using Horseradish peroxidase conjugated anti-human IgG antibody (Anti-Human IgG-Fc Fragment, HRP conjugated, Bethyl Labs) diluted 1:100’000 in BB.

For each sample, the OD450 was also evaluated after performing the ELISA for IgE and IgG as described above, except for coating with 5% milk in coating buffer (no allergens) at the beginning of the test. For each serum, this OD450 was subtracted to that of the test performed by coating with the allergen, ie, the extract or the recombinant proteins (ΔOD450).

Statistical analysis

The response variable of the tests of the present work is the Optical Density (OD) at 450 nm obtained by coating with SGE e/o recombinant allergens (OD450) subtracted with the OD at 450 nm recorded by coating with 5% milk in coating buffer (ΔOD450). The individuals were subdivided into 2 groups, ie, subjects displaying or not severe hypersensitivity reactions to mosquito bites (HMB and CTR, respectively), as determined by the anamnestic information. Each dataset was tested for normal distribution and Mann-Whitney and Unpaired t-test were used respectively for non-normally distributed and normally distributed datasets. Correlations between IgE response to AeSGE and Culex pipiens SGE, to AeSGE and recombinant proteins, or between IgE and IgG responses targeting AeSGE were assessed by Spearman test using ΔOD450 as parameter. The statistical analysis was performed using Prism software (GraphPad). P-values were adjusted applying the Bonferroni correction in R studio.
Immunoglobulins (Ig) targeting salivary gland extract (SGE) and recombinant salivary proteins. IgG (A) and IgE (B) targeting *Aedes albopictus* SGE (AeSGE) were detected by performing an in-house ELISA on sera from subjects showing severe hypersensitivity reactions to mosquito bites (HMB) and from control individuals (CTR). In (B), light blue dots represent subjects showing a positive IgE response targeting AeSGE, while dark blue dots represent individuals having a negative IgE response to the extract (cut-off value is represented by a red dotted line). IgE targeting Aed al 2 (C), Aed al 13 (D) and Aed al 14 (E) recombinant salivary proteins were detected by performing an in-house ELISA on sera from HMB individuals showing a level of IgE higher than CTR subjects (HMB \textit{AeSGE-IgE+}) and on control subjects (CTR). In (F), Aed al 13 and Aed al 14 were combined in a unique assay. In all graphs, the IgE level is reported on the y axis as the difference between the absorbance at 450 nm detected coating with recombinant proteins or with SGE and that recorded coating with 5 % milk (\(\Delta OD_{450}\)). In (C)-(F), cut-off values for IgE positivity for each analysis were defined as described in the main text and are represented by a red dotted line (sera positive to the recombinant proteins are reported as red dots, sera positive to AeSGE but negative to recombinant proteins as light blue dots, and sera negative to AeSGE and recombinant proteins as dark blue dots). Sensitivity and specificity of each marker are reported. In (G), two sera (red arrows) from HMB \textit{AeSGE-IgE+} group showing a positive level of IgE targeting Aed al 14 display an
To strictly select allergic subjects displaying IgE-mediated hypersensitivity reactions against the bite of the tiger mosquito, the cut-off value for positivity to IgE targeting AeSGE corresponded to the maximum value of ΔOD₄₅₀ retrieved in the CTR group.

To evaluate the maximum potential effectiveness of the recombinant proteins in the in-house ELISA, Receiving Operator Characteristic (ROC) curves were generated in RStudio. The cut-off value (c) selected for each biomarker maximized sensitivity (Se) and specificity (Sp), based on the calculation of Youden index (J), which can be formally defined as $J = \max_c \{Se(c) + Sp(c) - 1\}$. The Area Under the Curve (AUC) was determined for each ROC curve to assess the performance of each marker in predicting positivity to IgE against the whole AeSGE.

RESULTS

Allergic individuals show increased serum levels of IgE targeting Aedes albopictus salivary gland extract (AeSGE)

Sera collected from individuals showing severe hypersensitivity reactions to mosquito bites (HMB) and from control individuals (CTR) were firstly screened for the presence of IgG targeting SGE of Ae. albopictus female mosquitoes (AeSGE) by performing an in-house ELISA to assess exposure of subjects to the bites of the tiger mosquito. We detected IgG in all samples (except for 1 subject in the CTR group), and a modest increase of this immunoglobulin in the HMB group, which was not statistically significant (Fig. 1A, Unpaired t-test: $t_{53} = 1.923, P = 0.0599$).

As expected, samples from HMB group showed a higher median absorbance of IgE targeting AeSGE than controls (Fig. 1B, Mann-Whitney: $U = 125; P < 0.0001$). Nevertheless, considering as threshold value the maximum ΔOD₄₅₀ of the CTR group, only 15/27 HMB subjects showed a positive level of IgE targeting AeSGE.

No correlation between IgG and IgE levels targeting AeSGE was observed, suggesting that the strength of the IgE response to AeSGE is independent from the level of exposure to mosquito bites (Spearman: $rs = 0.18, P = 0.1824$).

The individuals belonging to the HMB group and displaying a level of IgE targeting AeSGE higher than control individuals (HMB AeSGE-IgE+) were chosen to carry out the following analyses, oriented to the selection of recombinant salivary allergens to be used as an alternative to SGE in the in-house ELISA.

Recombinant salivary allergens Aed al 13 and Aed al 14 are powerful markers of mosquito allergy

Three salivary proteins from Aedes albopictus, namely albD712 (Aed al 2), Antigen 5-3 (albAg5-3) (Aed al 13) and Labrum-Interacting Protein of the Saliva (albLIPS)-2 (Aed al 14) were selected for their suspected allergenicity based on previous literature, as described in the discussion section. The proteins were produced in recombinant form (Supplementary Fig. S1).

Presence of IgE recognizing the three recombinant proteins (10 μg/mL) tested alone was evaluated by in-house ELISA on HMB AeSGE-IgE+ and CTR sera. IgE targeting Aed al 2 measured in the sera of HMB AeSGE-IgE+ subjects were comparable with those observed in controls (Fig. 1C, Mann-Whitney: $U = 183, adjp = 0.4652$).

On the contrary, an increase in IgE was observed in the sera of HMB AeSGE-IgE+ subjects recognizing Aed al 13 (Fig. 1D, Mann-Whitney: $U = 81.50, adjp = 0.0004$) and Aed al 14 (Fig. 1E, Mann-Whitney: $U = 112, adjp = 0.0008$).

Using ROC analysis to define the threshold of IgE positivity, 10/15 (67 %) subjects of the HMB AeSGE-IgE+ group were positive to Aed al 13, 7/15 (47 %) to Aed al 14, and 2/15 (13 %) to Aed al 2.

Additionally, 2/28 (7%) CTR individuals were positive to Aed al 13, which thus displayed lower specificity if compared to the other two
recombinant proteins. Despite this loss in specificity and due to its high sensitivity, the in-house ELISA based on Aed al 13 displayed the highest AUC within the tests carried on using single recombinant proteins (Supplementary Table S1, Supplementary Fig. S2, AUC Aed al 13 = 0.806, AUC Aed al 14 = 0.733, AUC Aed al 12 = 0.5667).

Considering only individuals belonging to HMB AeSGE-IgE+ no correlation between IgE levels targeting recombinant salivary proteins and the extract was observed (Supplementary Fig. S3, Spearman correlation: S3A: Aed al 2, rs = −0.1207, adjp = 1; S3B: Aed al 13, rs = +0.5928, adjp = 0.0904; S3C: Aed al 14, rs = +0.2014, adjp = 1).

When combining Aed al 13 and Aed al 14 in a unique in-house ELISA, we observed an increase in the level of IgE in the sera of HMB AeSGE-IgE- subjects (Fig. 1F, Mann-Whitney: U = 37, adjp = 0.0004), while no correlation with IgE targeting AeSGE was highlighted (Supplementary Fig. S3D, Spearman correlation: rs = +0.4629, adjp = 0.3356). When using ROC analysis to define the threshold for IgE positivity, 13/15 (87 %) subjects of HMB AeSGE-IgE- group were positive to the combined recombinant proteins, indicating an increase in the sensitivity of the test. No correlation was detected between the IgE responses of HMB AeSGE-IgE- subjects to the two recombinant proteins tested alone (Fig. 1G, Spearman correlation: rs = +0.1420, p = 0.6094), but two subjects displayed a high level of IgE targeting Aed al 14, while showing a null level of IgE targeting Aed al 13 in single-protein ELISA (Fig. 1G). The combination of the two recombinant proteins in a unique in-house ELISA allowed to obtain the best AUC (0.91, Supplementary Table S1), thus indicating the most powerful test in individuating HMB subjects having a level of IgE targeting AeSGE higher than CTR subjects.

Sera of HMB subjects showing undetectable IgE levels to Aedes albopictus SGE show undetectable IgE response against Culex pipiens SGE

Given the sympatry of Culex pipiens mosquitoes with Aedes albopictus in the vast majority of Italy and considering that cross-reactivity between SGE of different mosquito species was previously observed,34 IgE recognizing Cx. pipiens mosquito SGE (CxSGE) were evaluated in the sera of HMB subjects. A positive correlation was detected between the two datasets (Fig. 1H, Spearman correlation: rs = +0.4170, p = 0.0305). Fig. 1H clearly highlights that only 4 sera were positive to both SGEs and that the sera of the HMB subjects showing a nearly null level of IgE targeting AeSGE (or rather comparable to that found in CTR individuals) also have undetectable/low levels of IgE targeting CxSGE. This observation suggests that, based on these assays, the hypersensitivity reactions described in the anamnesis of these individuals are not related to the occurrence of a relevant IgE response to salivary proteins of neither mosquito species.

DISCUSSION

Mosquito allergy is difficult to diagnose, partially due to the exposure of individuals to the bite of multiple blood-feeding arthropods in the same area, and partially to the lack of dedicated diagnostic tools. Whole body extracts are commercially available for Ae. communis mosquitoes (CAP-System, Thermo Fisher Scientific, Waltham, MA, USA) and Cx. pipiens (ALK-Abelló, Hørsholm, Denmark), but they contain a low amount of salivary allergens and they lack sensitivity.2,34-36

This difficulty is reflected in our data, as only 56% of people with hypersensitivity to mosquito bites showed a level of IgE recognizing the AeSGE higher than control subjects. The lack of IgE might be due to multiple factors, such as a lack of exposition to the bites of one mosquito species and the concomitant exposure to a different mosquito species. We exclude this possibility by analysing the IgG levels against the Ae. albopictus SGE, as a proxy of exposure, and evaluating the presence of IgE against the SGE of Cx. pipiens, which is highly diffused in Italy, but less relevant as human-biting species, and elicit a minor IgE response compared to Aedes mosquitoes.37

This observation underlies the importance of establishing clear clinical criteria and diagnostic tools for the diagnosis of mosquito allergy, suggesting the possibility that only a portion of the patients showing strong topical reactions to bites would be considered as allergic to Ae. albopictus and/or Cx. pipiens bites.
The identification of antigens responsible for sensitisation is a fundamental step toward the development of standardised species-specific tools to improve mosquito-allergy diagnosis, such as a component-resolved diagnostic (CRD), in which individual allergens are used to detect IgE-mediated sensitisation to complex mixtures.

Although recombinant salivary allergens have been already individuated and/or tested for allergy mediated by *Ae. aegypti* similar tools are still underdeveloped for *Ae. albopictus*. Indeed, potential candidates had previously been selected by Western blot analysis of allergic patients sera, but none of these was expressed and purified to assess effectiveness in diagnosing mosquito allergy.

In our study, 3 recombinant salivary antigens, namely albD712 (Aed al 2), albAg5-3 (Aed al 13), and albLIPS-2 (Aed al 14), were produced to test the IgE response of individuals affected by mosquito allergy. Aed al 2 is a member of the D7 family, belonging to the salivary odorant-binding protein superfamily, which was recently hypothesised to elicit an IgE response in individuals affected by mosquito allergy. Aed al 13 is part of the CAP (cysteine-rich secretory proteins, antigen 5 and pathogenesis-related 1 proteins) superfamily, particularly of the antigen 5 family, comprising salivary proteins from blood-feeding insects and wasp venom proteins eliciting strong allergic responses. Aed al 14 belongs to a culicine-specific salivary protein family involved in controlling mosquito feeding, which was previously reported to be a valuable marker of exposure to *Aedes* mosquito bites by eliciting an IgG response. Aed al 13 was the best antigenic marker, followed by Aed al 14, while IgE targeting Aed al 2 were retrieved at low level only in 2 HMB subjects.

This is the first time an antigen-5 protein is characterised as an allergen in mosquitoes. The role as major allergens of antigen-5 proteins is well characterised in the venom of almost all allergy-relevant Vespioidea species and of *Apis mellifera*. Notably, antigen 5 proteins from *Vespuca vulgaris* (Ves v 5) and *Polistes dominula* (Pol d 5), are currently the sole commercially available recombinant antigens for singleplex or multiplex testing of wasp allergy. However, the Aed al 13 antigen showed lower specificity than Aed al 14, possibly to cross-species reactivity with other mosquito species.

Based on our analysis, the Aed al 2 recombinant protein is probably a minor allergen in *Ae. albopictus* saliva, even if it elicited a positive IgE response in a subject who displayed a null response towards Aed al 13 and Aed al 14 tested in single-protein ELISAs. We have used this protein because long forms of D7 proteins were recently identified as putative allergens of *Ae. albopictus* and *Cx. quinquefasciatus* mosquitoes using Western blot and mass-spectrometry analyses. Furthermore, the D712 protein from *Ae. aegypti* saliva was observed to elicit an IgE response and was previously used to assess immediate and delayed responses in epicutaneous tests in allergic subjects.

Studying the yellow jacket venom, a better diagnostic sensitivity was achieved by combining 2 major allergens, ie, Antigen 5 and phospholipase proteins. In the present study, the combination of Aed al 13 and Aed al 14 in a unique in-house ELISA increased the performance of the test in individuating the HMB subjects positive to IgE targeting AeSGE, and thus showing a level of IgE higher than that of controls.

**CONCLUSIONS**

During the last decades, multiple mosquito species have been introduced in novel areas and spread. Therefore, the need for new diagnostic, preventive and therapeutic tools to monitor exposure to mosquito bites and their allergic potential has grown. In this study, we expressed and purified three recombinant proteins secreted in *Ae. albopictus* saliva, i.e., Aed al 2, Aed al 13, and Aed al 14, and we evaluated the IgE response targeting these allergens in allergic and control subjects. IgE response against Aed al 14 and particularly Aed al 13 was detected in 47% and 67% of allergic individuals showing a high level of IgE targeting SGE of *Aedes albopictus*. When combining the 2 recombinant proteins in a unique assay, 87% of these individuals displayed a positive IgE response targeting the allergens. In this regard, the potential use of Aed al 13 and Aed al 14 recombinant proteins in the diagnosis of
mosquito allergy has to be taken into account in future studies and in the development of new diagnostic tools.

**Abbreviations**

SGE, Salivary Gland Extract; ELISA, Enzyme Linked Immunosorbuttent Assay; SPT, Skin Prick Test; HMB, subjects displaying severe hypersensitivity reactions to mosquito bites; CTR, subjects not displaying severe hypersensitivity reactions to mosquito bites.

**Availability of data and materials**

All data supporting the findings of this study are available within the paper and its Supplementary Information.

**Author contributions**

IA and MV prepared the SGE, designed and performed the in-house ELISA tests; GM produced recombinant allergens; IVB helped in designing and performing the ELISA; YMR, AC, MA, CS and LD collected the human sera; SE and CB helped in analysing the data and revising the manuscript; FF supervised the production of the recombinant proteins; PG conceived the study and supervised it.

**Ethics statement**

The study was approved by the institutional review board (IRB) of the IRCCS Ospedale San Raffaele hospital (Milan, Italy), the independent ethics committee (IEC).

**Authors’ consent for publication**

All the authors have read the manuscript and agreed to send it to the journal for publication.

**Submission declaration**

The manuscript is original, has not been published before, is not currently being considered for publication elsewhere, and has not been posted to a preprint server.

**Declaration of competing interest**

The authors report no competing interests.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.waojou.2023.100836.

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