

# Mite-Induced Asthma and IgE Levels to Shrimp, Mite, Tropomyosin, Arginine Kinase, and Der p 10 Are the Most Relevant Risk Factors for Challenge-Proven Shrimp Allergy

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## Keywords

Shrimp allergy · Tropomyosin · House dust mite allergy · Asthma

## Abstract

**Background:** Shrimp sensitization is common in the general population, but the presence of symptoms is only moderately related to sensitization. A point still at issue is which in vivo and/or in vitro tests (food challenge, component-resolved diagnosis, house dust mite [HDM] sensitization) can help in distinguishing shrimp-allergic subjects from subjects that are sensitized but tolerant. **Methods:** The aim of this study was to evaluate the role of IgE to the different shrimp and mite allergens in distinguishing shrimp challenge-positive from challenge-negative patients. Subjects with suspected hypersensitivity reactions to shrimp, positive skin prick tests (SPTs), and/or anti-shrimp IgE were sub-

mitted to open and double-blind placebo-controlled food challenges (DBPCFC). Specific IgE to shrimp, mites, and the recombinants rPen a 1, rDer p 1, 2, and 10 were tested using ImmunoCAP-FEIA. IgE immunoblotting was performed to identify the patients' allergenic profiles. **Results:** In total, 13 out of 51 (25.5%) patients with reported reactions to shrimp were truly shrimp allergic (7 DBPCFC positive and 6 with documented severe reactions). These patients had significantly higher skin test wheal diameters than nonallergic patients, as well as higher levels of IgE to rPen a 1 and rDer p 10. HDM-induced asthma and the simultaneous presence of anti-nDer p 1, 2, and 10 IgE levels increased the risk of true shrimp allergy. **Conclusion:** Food challenge tests are mandatory for the diagnosis of shrimp allergy. Tropomyosin is associated with clinical reactivity. HDM-induced asthma and anti-mite IgE are risk factors for shrimp allergy.

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## Introduction

Shrimp is considered one of the “big 8” food allergens in children [1], and represents one of the most common causes of allergic reactions to foods in this age group [2]. As reported in a recent meta-analysis [3], the prevalence of self-reported shellfish allergy in Europe is 1.3%, while that of shrimp challenge positivity is about 0.1%, and is more common in older patients. On the contrary, the sensitization rate to shrimp is much higher, as shown by Burney et al. [4], who found a 10.2% sensitization prevalence rate to shrimp in Italy, where seafood consumption per capita increased from 25 kg/year in 2010 to 26 kg/year in 2015 ([www.fao.org](http://www.fao.org)). This data clearly shows a high difference among sensitization perception and real allergy to this food item when a standardized methodology is applied for the diagnosis. Accordingly, Gámez et al. [5] demonstrated that only 18 out of 36 subjects (50%) with suspected reactions to shrimp could be classified as truly shrimp allergic on the basis of the positivity of double-blind placebo-controlled food challenges (DBPCFC), skin prick tests (SPTs), and specific IgE (sIgE) to shrimp. Similarly, Pascal et al. [6] found that only 58 out of 86 (67%) patients with positive SPTs to shrimp were diagnosed as shrimp allergic on the basis of a positive shrimp challenge or documented anaphylaxis. The low concordance between sensitization and provocation tests could be explained either by cross-reactivity with house dust mite (HDM) and other arthropod allergens, or by the presence of additional factors, the so-called trigger factors, able to elicit clinical symptoms, e.g., higher allergen concentration, physical exercise, alcohol, and/or anti-inflammatory drugs intake. This makes it difficult to identify a relationship between symptoms and laboratory data [7–10]. The major shrimp allergen is tropomyosin (TM), which shares a high sequence homology with all crustaceans, molluscs, HDM, and cockroaches [11]. Other allergens have been identified, some of which are cross-reactive with HDM homologues, such as arginine kinase (AK) and hemocyanin, and others that are not cross-reactive, such as sarcoplasmic calcium-binding protein (SCBP) and myosin light chain (MLC). The clinical role of many allergens still needs to be ascertained [6]. Considering all of these aspects, we conducted a study on shrimp-sensitized patients in order to confirm the relevance of TM in distinguishing symptomatic from asymptomatic shrimp sensitization, and evaluate the role of other allergens associated with the hypersensitivity reactions. With this aim, we enrolled subjects with positive shrimp sIgE and compared different clinical and immunological

parameters between patients diagnosed by a positive challenge or by a history of documented severe reactions versus shrimp-sensitized but tolerant subjects.

## Materials and Methods

### Patients

All subjects consecutively referred to the Allergy and Immunology Unit of the Niguarda Ca' Granda Hospital (Milan, Italy) for a history of suspected hypersensitivity reactions to shrimp and those with clinically relevant HDM-induced respiratory allergy but who were shrimp tolerant, were evaluated for a shrimp IgE sensitization. Subsequently, patients with a positive SPT to commercial shrimp extract and/or anti-shrimp IgE >0.10 kUA/L were recruited for the study. Then, the study population of shrimp IgE-positive subjects was divided into 2 groups: group A (GA), subjects with suspected shrimp clinical reactions, and group B (GB), patients with a totally negative clinical history for shrimp reactions. We investigated all GA subjects for the presence of the following symptoms: oral allergy syndrome, urticaria, angioedema, pruritus, erythema, gastrointestinal symptoms, asthma, rhinitis, and anaphylaxis. The reported symptoms were classified into 3 grades of severity: grade I, mucocutaneous symptoms (oral allergy syndrome, urticaria, or angioedema) with or without rhinitis or gastrointestinal symptoms; grade II, the presence of gastrointestinal symptoms plus asthma and/or dizziness, in addition to at least 1 of the grade I symptoms, and grade III, life-threatening symptoms (anaphylaxis). Patients were also investigated for the presence of HDM allergy, with rhinitis and/or asthma history according to GINA (<http://www.ginasthma.org>) and ARIA guidelines (<http://www.progetto-aria.it/>), and the presence of HDM sIgE and its recombinant allergens, as specified in the following sections. The study protocol was approved by the Ethical Committee of Niguarda Ca' Granda Hospital (Milan, Italy) and registered at ClinicalTrials.gov, protocol ID NCT01634737. The primary endpoint was to evaluate the role of rPen a 1 sIgE in distinguishing symptomatic from asymptomatic patients. Secondary endpoints were the evaluation of the diagnostic role of other shrimp allergens identified by IgE immunoblotting N-terminal amino acid sequence and mass spectrometry (MS) analysis, and the identification of the possible confounding meaning of HDM cross-reactive allergens and sIgE.

### Skin Prick Tests

All of the patients underwent SPTs with crude and boiled shrimp (boiled at 100°C for 15 min in ion-depleted water) using the prick-prick (P+P) technique on the volar surface of the forearm. A histamine and saline prick test was used as the positive and negative control, respectively. The shrimps tested through the entire study, belonging to the Penaeidae family, were bought at a local market. Shrimp SPT were considered positive when eliciting a wheal with a mean diameter of at least 3 mm.

### Food Challenges

Patients reporting grade I and II symptoms to shrimp were submitted to open food challenge (OFC) with boiled shrimp. In the case of a positive result, patients underwent a DBPCFC with boiled shrimp. Patients with a history of severe reactions (grade III) were not challenged. Before the challenge, serum tryptase assay was per-

formed, blood pressure and FEV<sub>1</sub> were measured, and the oral cavity and skin were carefully inspected for preexisting lesions. A positive reaction for asthma was defined as a decrease in FEV<sub>1</sub> of at least 15% with respect to the baseline value. During the OFC, 7 raw shrimps (30 g) were boiled for 3 min and administered to the patients at increasing doses, starting with 5 mg. Patients were asked to chew and subsequently swallow every dose of shrimp at 20-min intervals until the final dose of 30 g [12]. In case of a negative OFC, the patients were invited to regularly eat shrimps on the following days, at increasing doses, and, if no symptoms appeared, were considered as shrimp sensitized but tolerant. Patients who developed clinical symptoms after OFC were scheduled for DBPCFC in order to confirm the diagnosis. The test meal was developed by the dieticians of our hospital. The active meal was composed of peeled boiled shrimp (30 g), canned tuna (40 g), steamed rice (20 g), water (20 mL), olive oil (1 tablespoon), salt, and saffron. The placebo meal was prepared without shrimp. Shrimp meals were administered at increasing doses as follows: 100 mg, 400 mg, 1.5 g, 3.5 g, 7.0 g, and 17.5 g. All the doses were administered at 20-min intervals unless symptoms developed. In the case of subjective symptoms, the test was stopped until the symptoms resolved.

#### Specific IgE Detection

Serum levels of anti-shrimp (*P. borealis*, *P. monodon*), anti-rPen a 1, anti-HDM (Dpt), anti-nDer p 1, anti-rDer p 2, and anti-rDer p 10 IgE antibodies were determined in all the patients by the ImmunoCAP system (Thermo Scientific, Uppsala, Sweden). A positive result was attributed to a range of 0.10–100 kUA/L. The ISAC 112 microarray platform (Thermo Scientific) was used to detect serum IgE for AK (rPen m 2), and SCBP (rPen m 4). IgE levels higher than 0.3 ISU/L were considered positive.

#### Shrimp Extraction and SDS-PAGE/Immunoblotting

Shrimp proteins were extracted by disrupting the raw shrimp in a mixer and diluting 1:20 with extraction buffer (0.1 M phosphate buffer pH 7.4). After stirring for 2 h at 4–8°C and centrifuging at 4,200 rpm for 30 min at 4–6°C, the extract was passed through a 10-μm filter, followed by a 1.2-μm PVDF membrane filter, desalted through a PD-10 column (GE Healthcare), and stored at –70°C. The protein concentration of the shrimp extract, determined by the colorimetric Lowry method [13], was 6 mg/mL. SDS-PAGE (4.2 mg/cm gel of shrimp extract) and immunoblotting were performed as previously described by Pastorello et al. [14]. The IgE-binding proteins were detected by incubation with <sup>125</sup>I-labelled anti-human-IgE antibodies (PANTEC Srl, Turin, Italy) and exposed to an autoradiographic film (Hyperfilm, Amersham) at –70°C for 4–7 days.

#### Protein Identification

Protein identification was achieved by the N-terminal amino acid sequencing technique and/or by MS analyses. For N-terminal amino acid sequencing, the selected bands indicated in Figure 2 were excised from the SDS-PAGE gel, and passively eluted and microsequenced on a Procise 492 protein sequencer (Applied Biosystems, Foster City, CA, USA) as described by Pessione et al. [15]. The amino acid sequence was searched using MS-Homology software at the Protein Prospector website (<http://prospector.ucsf.edu/prospector>) against both the UniProt KB 2015.3.15 and NCBI nr.2015.6.17 database. The parameters used were: order selected Decapoda, no limit for protein MW, from 1 to 5 possible

amino acid changes allowed. The Blast tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to characterize homologies with annotated proteins. Alternatively, MS analysis was performed: 2 × 2-mm band pieces were excised from the gel and trypsin digestion was performed using a Ultraflex III MALDI-TOF/TOF instrument (Bruker Daltonik GmbH, Germany), as already described [16]. Manual/visual evaluation of the mass spectra was performed using Flex Analysis software (Bruker Daltonik GmbH, Germany). The tandem mass spectra were acquired on the same mass spectrometer, which ran LID experiments using LIFT TOF/TOF acquisition. The MS/spectra were automatically analyzed by Flex Analysis software (Bruker Daltonik GmbH). The MS-Fit and MS-Tag software packages (<http://prospector.ucsf.edu>) were used to search against the same databases already mentioned using the peptide mass fingerprinting method. The following parameters were set for the searches: S-carbamidomethyl derivative on cysteine as the fixed modification, oxidation on methionine as the variable modification, and 3 missed cleavage sites for trypsin digestion. The peptide mass tolerance was 30 ppm.

#### Statistical Methods

The number of patients to be selected was fixed to 50 for each group and was established assuming a 70% positivity of IgE to TM in GA and 40% in GB. All data were checked for validation, and then submitted to the usual descriptive analyses. The Shapiro-Wilk test was used to evaluate the normality of the distribution of the continuous variables. The Kruskal-Wallis test, followed if significant by the post hoc test according to Conover, was used for the between-group continuous variable analysis, while the Fisher exact test was used for the categorical variables. Logistic regression (using both the standard asymptotic and the exact algorithms in the calculus procedures) was used to verify the association between continuous variables and the binary endpoint; in some cases this was followed by an ROC analysis, together with Youden J statistics, which were used for the search for an optimal cut-off value for the continuous variables. The data were analyzed with the Stata/SE 14.1 statistical package.

## Results

#### Study Population

A total of 99 patients, 55 females (55.6%) and 44 males (44.4%), with a median age of 41 years (range 18–73), presenting with positive anti-shrimp IgE and shrimp SPT, were enrolled in the study. Fifty-one (51.5%) reported a history of allergic reactions to shrimp (GA) and 48 (48.5%) did not report any reactions (GB). Ninety-one patients were sensitized to HDM, of whom 77 (84.6%) had persistent rhinitis and 14 (15.3%) also had asthma. Forty-four of the 51 patients with a suspected shrimp allergy underwent OFC, while 6 were not challenged due to a history of severe reactions. Thirty-eight of these 45 patients had a negative OFC, confirmed by the subsequent regular consumption of shrimp, while 7 (15.5%) had a positive OFC and DBPCFC to shrimp. On the basis of

**Table 1.** Demographics and test results for the 13 shrimp-allergic patients (GA2)

| Patient No. | Sex/age, years | Shrimp severity symptoms | HDM respiratory symptoms | P+P, mm      |               | OFC with shrimp |        | DBPCFC with shrimp |    |          |        |
|-------------|----------------|--------------------------|--------------------------|--------------|---------------|-----------------|--------|--------------------|----|----------|--------|
|             |                |                          |                          | crude shrimp | boiled shrimp | SYM             | CPD, g | AC                 | PL | SYM      | CPD, g |
| 1           | F/45           | III                      | A, R                     | 6.0          | 6.2           | nt              | nt     | nt                 | nt | nt       | nt     |
| 2           | M/38           | III                      | A, R                     | 12.0         | 10.0          | nt              | nt     | nt                 | nt | nt       | nt     |
| 3           | M/22           | III                      | A, R                     | 9.5          | 10.0          | nt              | nt     | nt                 | nt | nt       | nt     |
| 4           | M/22           | III                      | A, R                     | 8.0          | 8.5           | nt              | nt     | nt                 | nt | nt       | nt     |
| 5           | F/41           | III                      | R                        | 4.0          | 4.5           | nt              | nt     | nt                 | nt | nt       | nt     |
| 6           | M/26           | III                      | A, R                     | 4.2          | 4.3           | nt              | nt     | nt                 | nt | nt       | nt     |
| 7           | F/61           | II                       | R                        | 16.0         | 16.5          | E, P            | 30     | +                  | –  | E, P     | 3.5    |
| 8           | M/39           | II                       | R                        | 6.0          | 7.0           | E, P            | 30     | +                  | –  | E, P     | 3.5    |
| 9           | F/27           | II                       | R                        | 5.0          | 6.0           | U, P            | 30     | +                  | –  | AE, U, P | 17.5   |
| 10          | M/22           | II                       | R                        | 2.23         | 3.0           | U, P            | 30     | +                  | –  | U, P     | 30     |
| 11          | F/52           | II                       | R                        | 3.0          | 3.4           | E, P            | 30     | +                  | –  | E, P     | 3.5    |
| 12          | M/28           | II                       | A, R                     | 8.0          | 8.3           | U, P            | 30     | +                  | –  | U, P     | 30     |
| 13          | M/31           | II                       | NP                       | 3.0          | 3.2           | U, P            | 30     | +                  | –  | U, P     | 30     |

E, erythema; U, urticaria; AE, angioedema; P, pruritus; AC, active meal; PL, placebo meal; SYM, challenge symptoms; CPD, cumulative provocation dose; nt, not tested; R, rhinitis; A, asthma; +, positive test; –, negative test; NP, not present; OFC, open food challenge with shrimp; DBPCFC, double-blind placebo-controlled food challenge with shrimp.

**Table 2.** IgE levels to shrimp, HDM, and recombinant allergens for the 13 shrimp-allergic patients (GA2)

| Patient No. | Anti-shrimp IgE (CAP) | Anti-Dpt IgE (CAP) | rPen a 1 |      | rPen m 2 | rPen m 4 | nDer p 1 |      | rDer p 2 |      | rDer p 10 |      |
|-------------|-----------------------|--------------------|----------|------|----------|----------|----------|------|----------|------|-----------|------|
|             |                       |                    | nPen m 1 | ISAC |          |          | CAP      | ISAC | CAP      | ISAC | CAP       | ISAC |
| 1           | 100.0                 | 27.1               | 100.0    | 71   | <0.3     | <0.3     | 0.17     | <0.3 | 1.43     | 2.5  | 100.0     | 76   |
| 2           | 97.5                  | 61.4               | 70.1     | 65   | 10       | <0.3     | 0.62     | <0.3 | 1.24     | <0.3 | 67.8      | 64   |
| 3           | 66.2                  | 21.9               | 45.6     | 45   | 26       | <0.3     | 0.28     | <0.3 | 6.8      | 9.9  | 49.9      | 40   |
| 4           | 2.86                  | 6.18               | 1.87     | 6.7  | <0.3     | <0.3     | 1.92     | 2.8  | 3.51     | 9.2  | 2.15      | 8.2  |
| 5           | 7.99                  | 5.08               | 5.41     | 14   | 7.1      | <0.3     | <0.10    | <0.3 | <0.10    | <0.3 | 6.08      | 14   |
| 6           | 5.72                  | 76.3               | 0.55     | <0.3 | <0.3     | 1.2      | 33.1     | 28   | 2.77     | <0.3 | 1.13      | <0.3 |
| 7           | 2.07                  | 0.60               | 0.15     | 0.5  | <0.3     | <0.3     | <0.10    | <0.3 | <0.10    | <0.3 | 0.49      | 1.6  |
| 8           | 8.95                  | 7.95               | <0.10    | <0.3 | 8.2      | <0.3     | 0.26     | <0.3 | <0.10    | <0.3 | <0.10     | <0.3 |
| 9           | 4.42                  | 1.80               | 3.24     | 8.9  | <0.3     | <0.3     | <0.10    | <0.3 | <0.10    | <0.3 | 3.32      | 9.5  |
| 10          | 2.81                  | 16.1               | 1.11     | 3.8  | <0.3     | 2        | 0.93     | <0.3 | 16.3     | 25   | 0.25      | 0.9  |
| 11          | 5.23                  | 6.69               | <0.10    | <0.3 | <0.3     | <0.3     | <0.10    | <0.3 | <0.10    | <0.3 | <0.10     | <0.3 |
| 12          | 2.75                  | 28.5               | 1.62     | 0.6  | 4.6      | <0.3     | 11.5     | 8.9  | 19.5     | 26   | 3.11      | 5.8  |
| 13          | 8.94                  | 0.89               | <0.10    | 0.3  | <0.3     | <0.3     | <0.10    | <0.3 | <0.10    | <0.3 | <0.10     | <0.3 |

Pen a 1, IgE to tropomyosin was detected by ImmunoCAP; Pen m 1, Pen m 2, and Pen m 4 were detected by ISAC test; Der p 1, Der p 2, and Der p 10 IgE were detected by ImmunoCAP and ISAC test.

their clinical history, challenge results, and/or documented anaphylaxis, the 99 shrimp-sensitized patients were grouped as follows: group A1 (GA1), 38 subjects with a positive history but negative challenge; group A2 (GA2), 13 subjects with shrimp allergy diagnosed by positive challenge or documented anaphylaxis, and GB, 48 sub-

jects who were HDM positive and had a negative history for shrimp. Thus, the GA1 plus GB subjects were termed from this point as “shrimp tolerants.” Six of the 13 (46%) shrimp-allergic patients (GA2) had HDM-induced asthma, as compared to only 8 (9.3%) asthmatic patients out of the 86 shrimp-tolerant patients. Table 1 reports demo-

**Table 3.** Comparison of mean wheal diameters of P+P with crude and boiled shrimp and IgE levels to raw and recombinant allergens among the 3 groups

|                     | GA              |                  | GB (n = 48)       | p value | Pairwise test adjusted by Bonferroni |                |            |
|---------------------|-----------------|------------------|-------------------|---------|--------------------------------------|----------------|------------|
|                     | GA2 (n = 13)    | GA1 (n = 38)     |                   |         | GA2 vs. GA1                          | GA2 vs. GB     | GA1 vs. GB |
| P+P, mm             |                 |                  |                   |         |                                      |                |            |
| Crude shrimp        | 6 (0–16)        | 4 (0–18)         | 0 (0–5)           | 0.0001  | 0.5634                               | <0.0002        | <0.0002    |
| Boiled shrimp       | 6.2 (0–16.5)    | 4.4 (0–18.2)     | 0 (0–5.5)         | 0.0001  | 0.9738                               | <0.0002        | <0.0002    |
| Specific IgE, kUA/L |                 |                  |                   |         |                                      |                |            |
| Shrimp              | 5.72 (2.07–100) | 0.84 (0.24–5.75) | 0.68 (0.12–14.20) | 0.0001  | <0.0002                              | <0.0002        | 0.3525     |
| rPen a 1            | 1.62 (0–100)    | 0 (0–0.44)       | 0 (0–5.76)        | 0.0001  | <0.0002                              | <0.0002        | 0.7947     |
| Dpt                 | 7.95 (0.6–76.3) | 0.75 (0–49.9)    | 1.08 (0.12–100)   | 0.0008  | 0.0009                               | 0.0039         | 0.9963     |
| nDer p 1            | 0.26 (0–33.1)   | 0 (0–30.3)       | 0 (0–88.6)        | 0.1339  |                                      | not applicable |            |
| rDer p 2            | 1.24 (0–19.5)   | 0 (0–58.7)       | 0 (0–100)         | 0.1569  |                                      | not applicable |            |
| rDer p 10           | 2.15 (0–100)    | 0 (0–0.38)       | 0 (0–6.18)        | 0.0001  | <0.0002                              | <0.0002        | >0.9999    |

Kruskal-Wallis test was followed by a post hoc pairwise test. GA, patients with a shrimp-positive history; GA2, shrimp-allergic patients; GA1, shrimp-negative challenge patients; GB, patients with a shrimp-negative history.

graphics, clinical symptom severity, HDM respiratory symptoms, mean wheal diameter of the P+P tests with crude and boiled shrimp, and the results of OFC and DBPCFC with boiled shrimp with the provocative threshold dose and elicited symptoms in GA2. The results of shrimp IgE levels, rPen a 1, Dpt, nDer p 1, rDer p 2, and rDer p 10 by the ImmunoCAP system, and rPen m 2 and rPen m 4 by the ISAC method are reported in Table 2.

### *Sensitization to Shrimp and Dust Mites among the 3 Groups*

A strong statistical association was found between skin reactivity to crude and boiled shrimp (Spearman  $\rho = 0.969$ ;  $p < 0.00005$ ). The mean wheal diameters of the P+P tests with crude and boiled shrimp were significantly higher in GA2 than in GB ( $p < 0.0002$ ), and in GA1 as compared to GB ( $p < 0.0002$ ). No differences were found between GA2 and GA1 ( $p = 0.5634$  and  $p = 0.9738$ , respectively). Anti-shrimp and anti-rPen a 1 IgE levels were significantly higher in GA2 than in GA1 ( $p < 0.0002$ ), and in GA2 than GB ( $p < 0.0002$ ); no differences were found between GA1 and GB ( $p = 0.3525$  and  $p = 0.7947$ , respectively). Furthermore, anti-Dpt and anti-rDer p 10 IgE levels were significantly higher in GA2 than GA1 ( $p = 0.0009$  and  $p < 0.0002$ , respectively) and in GB ( $p = 0.0039$  and  $p < 0.0002$ , respectively); no differences were found between GA1 and GB ( $p = 0.9963$  and  $p > 0.9999$ , respectively). No significant differences were found between anti-rDer p 1 and anti-rDer p 2 IgE levels among the 3 groups (pairwise test adjusted by Bonferroni not appli-

cable). Table 3 summarizes the results of the sensitization to shrimp and to HDM between the 3 groups.

### *Cut-Off Values for Sensitization to Shrimp and HDM*

Considering GA2 patients versus GA1 plus GB patients, we analyzed the sensitivity (SE) and specificity (SP) of the SPT mean wheal diameter, and anti-shrimp and anti-rPen a 1 IgE levels in order to identify the best cut-off value (Fig. 1). P+P with crude shrimp revealed the best cut-off value with a mean wheal diameter of 6 mm (with 53.8% SE and 76.3% SP; positive predictive value [PPV] = 43.8% and negative predictive value [NPV] = 82.1%). The area under the ROC curve for P+P with boiled shrimp was too small (0.59) to allow a cut-off search. Regarding anti-rPen a 1 IgE levels, the maximum accuracy was observed at a cut-off value of 0.55 kUA/L (with 69.2% SE and 100% SP; PPV = 100% and NPV = 90.5%), while for anti-shrimp IgE levels the cut-off value was 2.07 kUA/L (with 100% SE and 81.6% SP; PPV = 65% and NPV = 100%). Regarding anti-Dpt IgE levels, the maximum accuracy was observed at a cut-off value of 5.00 kUA/L (with 76.9% SE and 81.4% SP; PPV = 38.5% and NPV = 95.9%), while for anti-rDer p 10 IgE levels the cut-off value was 3.00 kUA/L (with 46.2% SE and 98.8% SP; PPV = 85.7% and NPV = 92.4%).

### *Relationship between Shrimp Allergy and HDM-Induced Asthma*

We found that the presence of asthma was associated with a 736% increase in the odds of developing allergic

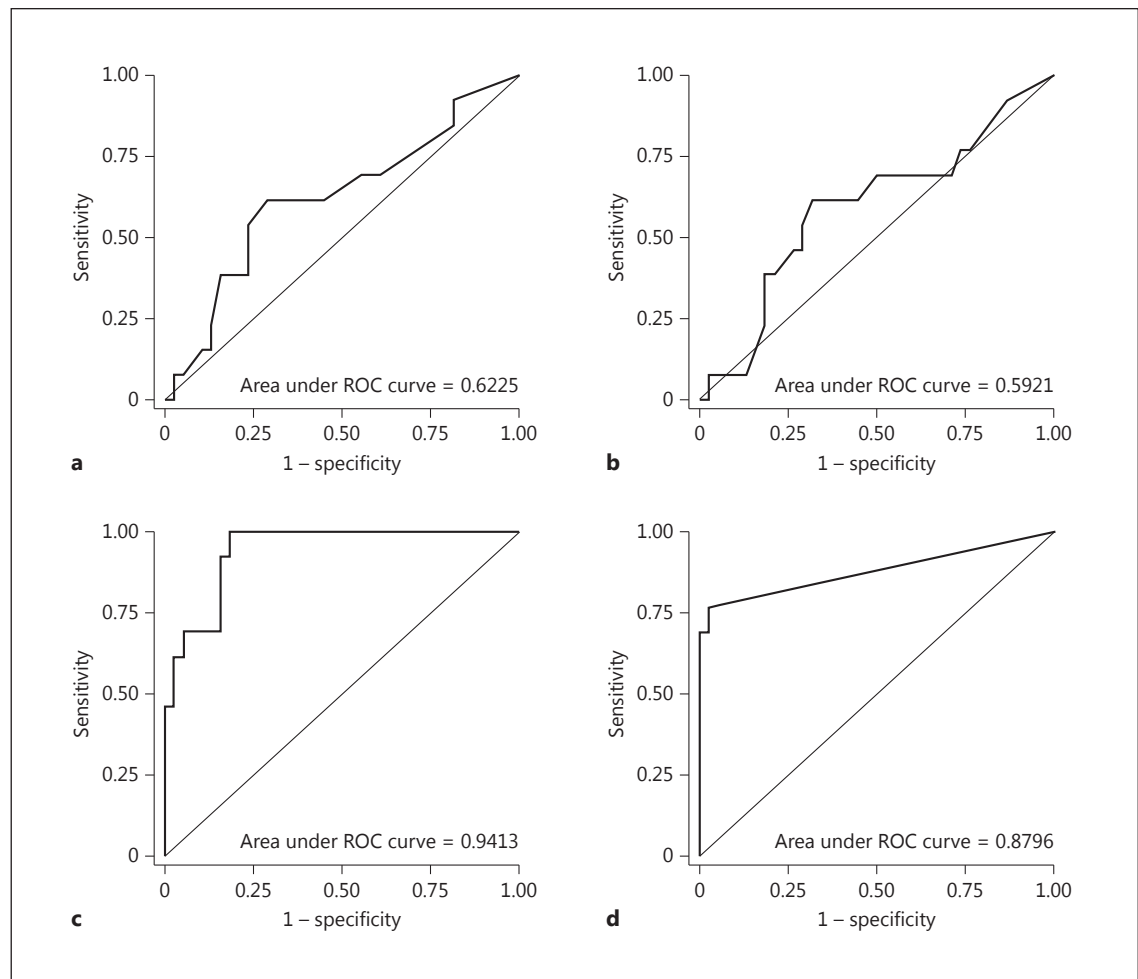


**Fig. 1.** ROC curves regarding P+P with crude (a) and boiled shrimp (b), and anti-shrimp (c) and anti-rPen a 1 (d) IgE levels.

symptoms after shrimp ingestion (Wald test:  $p = 0.002$ ). We found that any unitary increase of anti-Dpt IgE levels corresponded to a 4.4% increase in the odds of developing asthma (Wald test:  $p = 0.004$ ), and that there was a significant correlation between anti-Dpt and anti-rDer p 10 IgE values (Spearman  $\rho = 0.348$ ;  $p = 0.0001$ ), and anti-shrimp IgE values (Spearman  $\rho = 0.443$ ;  $p < 0.00005$ ), and anti-rPen a 1 IgE values (Spearman  $\rho = 0.310$ ;  $p = 0.0018$ ). In order to better explain this result, we investigated which HDM allergen best correlated with asthma. Interestingly, there was no association between positivity of anti-nDer p 1 ( $p = 0.277$ ) or anti-rDer p 2 ( $p = 0.077$ ) or anti-rDer p 10 ( $p = 0.085$ ) IgE levels and asthma. However, considering these values as a single variable, their positivity was associated with a 4,050% increase in the odds of developing asthma (Wald test:  $p <$

0.0005). Moreover, we evaluated which mite allergens were correlated with shrimp allergy, and found a significant association between the positivity of anti-rDer p 10 IgE and shrimp allergy (Fisher test:  $p < 0.0005$ ), such as between anti-rPen a 1 IgE levels and shrimp allergy (Spearman  $\rho = 0.916$ ;  $p < 0.00005$ ). In particular, any arbitrary unitary increase in anti-rDer p 10 IgE values corresponded to a 124% increase in the odds of developing shrimp allergy (Wald test:  $p = 0.0005$ ), with 80.0% PPV (95% CI 44.4–97.5) and 94.4% NPV (95% CI 87.4–98.2). Interestingly, simultaneous positivity of all HDM recombinant sIgE allergens (nDer p 1, rDer p 2 and rDer p 10) corresponded to a 4,800% increase in the odds of developing shrimp allergy (Wald test:  $p < 0.0005$ ), with 77.8% PPV (95% CI 40.0–97.2) and 93.0% NPV (95% CI 86.1–97.5).

**Table 4.** Protein bands identified from the SDS-PAGE profile of raw shrimp extract

| SDS-PAGE band No. | Protein                              | UNIProt entries | Allergen name WHO-IUIS | Species             | MW observed/ from database, kDa | Method of identification |                                                                                                                                                                                                      |                                                          |
|-------------------|--------------------------------------|-----------------|------------------------|---------------------|---------------------------------|--------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------|
|                   |                                      |                 |                        |                     |                                 | PMF by MALDI-TOF         | PSD fragmentation                                                                                                                                                                                    | N-terminal amino acid sequencing                         |
| 1                 | Hemocyanin subunit Y                 | B0L612          |                        | <i>P. japonicum</i> | 75/75.14                        | 6/41                     | 1843.93<br>( <sup>376</sup> YNLPPGVLEHFETATR <sup>391</sup> )                                                                                                                                        | No results                                               |
|                   | Hemocyanin                           | Q26180          |                        | <i>L. vannamei</i>  | 75/74.98                        | 3/41                     | 1448.73<br>( <sup>276</sup> YGGQFPARPDNVK <sup>288</sup> )                                                                                                                                           | No results                                               |
| 2                 | Phosphopyruvate hydratase            | O96656          | Pen m enolase          | <i>P. monodon</i>   | 48/47.26                        | 10/45                    | 1798.87<br>( <sup>33</sup> AAVPSGASTGVHEALEMoxR <sup>50</sup> )<br>1230.68<br>( <sup>121</sup> AGAAELGIPLYR <sup>132</sup> )                                                                         | No results                                               |
| 3                 | β-Actin                              | A0T2V0          |                        | <i>P. monodon</i>   | 42/42.00                        | 7/33                     | 1790.89<br>( <sup>87</sup> SYELPDGQVITIGNER <sup>97</sup> )                                                                                                                                          | –                                                        |
| 4                 | Fructose-bisphosphate aldolase       | A0A068FCL9      |                        | <i>F. chinensis</i> | 41/39.86                        | 16/47                    | 1269.71<br>( <sup>205</sup> VTETVLAFVYK <sup>215</sup> )<br>1893.93<br>( <sup>157</sup> NTPSYQGMLNANVLR <sup>173</sup> )<br>2142.05<br>( <sup>1</sup> (M(Met-loss) TTYFTYPEPALREELRR <sup>18</sup> ) | –                                                        |
| 5                 | AK                                   | P51545          | Pen m 2                | <i>P. japonicum</i> | 37/40.00                        | 19/60                    | 1796.76<br>( <sup>265</sup> LGFLTFCamPTNLGTTVR <sup>280</sup> )                                                                                                                                      | <sup>1</sup> ADAAKLEKLRAEK<br><sup>1</sup> ADAAVIEKLEAGF |
|                   |                                      | T2DQW9          |                        | <i>P. monodon</i>   | 37/40.00                        | –                        | 1657.62<br>( <sup>217</sup> TFLVWVNEEDHLR <sup>229</sup> )<br>1050.52<br>( <sup>194</sup> FLQAANACamR <sup>202</sup> )<br>1796.76<br>( <sup>265</sup> LGFLTFCamPTNLGTTVR <sup>280</sup> )            |                                                          |
| 6                 | Tropomyosin                          | A1KYZ2 E7CGC1   | Pen m 1                | <i>P. monodon</i>   | 32/32.85                        | 24/58                    | 1257.75<br>( <sup>91</sup> IQLLEEDLER <sup>100</sup> )                                                                                                                                               | <sup>10</sup> AMKLEKANALVR<br><sup>10</sup> AMKLEKDNAMDR |
| 7                 | Sarcoplasmic calcium-binding protein | E7CGC4          | Pen m 4                | <i>P. monodon</i>   | 22/22.08                        | –                        | 953.45<br>( <sup>1</sup> Met-loss+Acetyl AYSWDNR <sup>7</sup> )<br>1721.91<br>( <sup>111</sup> AEDVNGDGLVGLDEYR <sup>126</sup> )                                                                     | –                                                        |
|                   | MLC                                  | D4P8F7          | Pen m 3                | <i>P. japonicus</i> | 18/17.50                        | 4/36                     | –                                                                                                                                                                                                    | –                                                        |
| 8                 | Sarcoplasmic calcium-binding protein | P02636          | Pen m 4                | <i>Penaeus</i> sp.  | 19/22.08                        | –                        | 967.43                                                                                                                                                                                               | –                                                        |
|                   |                                      | H7CHW2          |                        | <i>P. monodon</i>   | 19/22.12                        | 4/36                     | ( <sup>1</sup> Acetyl-AYTWDNR <sup>7</sup> )                                                                                                                                                         |                                                          |
|                   |                                      | H7CHW3          |                        | <i>P. monodon</i>   | 19/22.12                        | 4/36                     | 1675.88<br>( <sup>39</sup> NLWNEIAELADFNK <sup>73</sup> )                                                                                                                                            |                                                          |
| 9                 | MLC                                  | D4P8F7          | Pen m 3                | <i>P. japonicus</i> | 17/17.50                        | 7/36                     | –                                                                                                                                                                                                    | –                                                        |

Bold letters indicate different amino acids to those present in the database. PMF, mass finger printing; PSD, post source decay; AK, arginine kinase; MLC, myosin light chain.

### SDS-PAGE/Immunoblotting

The SDS-PAGE profile of raw shrimp extract shows different protein bands within a molecular weight ranging from 10 to 150 kDa (Table 4). The numbers indicate the bands corresponding to the identified proteins. As first choice, we attempted to identify proteins by the N-terminal sequencing technique, but the identification was not always possible, probably due to the presence of multiple N-terminal sequences in a single band. For this reason, MALDI-TOF/TOF analyses were performed on the selected bands. Figure 2 shows shrimp IgE immunoblotting of the true allergic patients (GA2). Sera from 11 out of the 13 patients (84.6%) scored positive in the 34- to 38-

kDa band, which we identified as TM. Another component ranging from 38 to 40 kDa, corresponding to AK, was recognized by 7 out of 13 allergic subjects (53.8%), with an ISAC test positive in 5 out of 13 patients (38.5%). It is noteworthy that 12 out of 13 subjects (92.3%) reacted, with a different radiostaining, towards bands in the 45- to 50-kDa range, which corresponds to a shrimp fructose-bisphosphate aldolase (FBPA; access N.O96656). Five out of 13 patients (38.5%) reacted towards hemocyanin at 75–80 kDa. Only 1 patient (patient 6) recognized the 22- to 25-kDa SCBP. IgE reactivity to the 20-kDa MLC was detected in 5 out of 13 patients (38.5%). Interestingly, 1 patient (No. 13) with anaphylaxis after shrimp ingestion

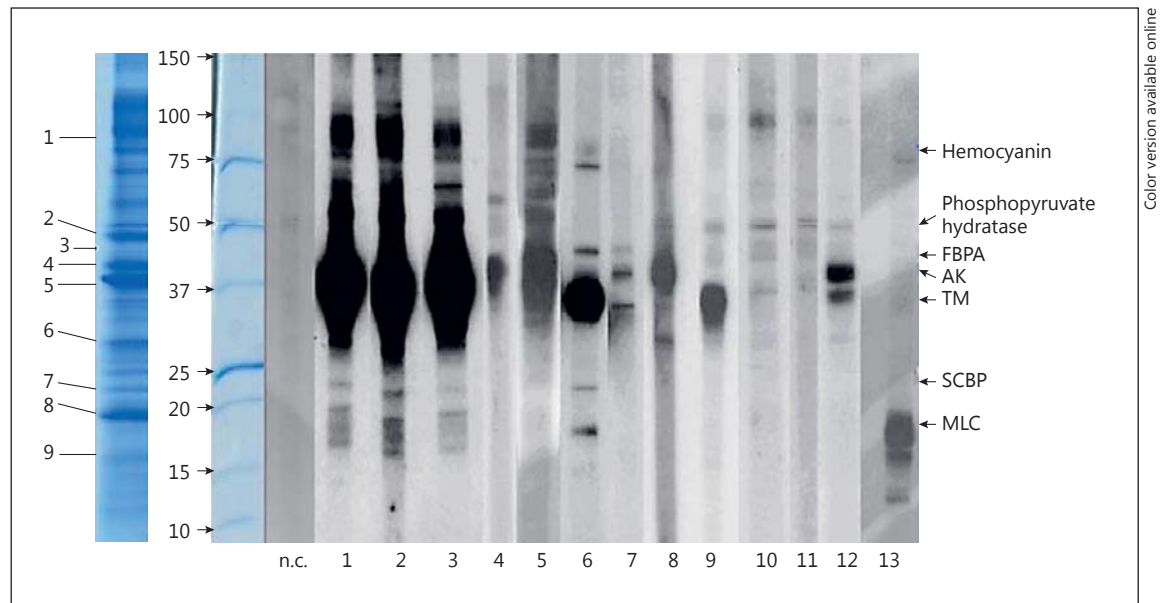


**Fig. 2.** SDS-PAGE profile of shrimp extract. Raw shrimp IgE immunoblotting of the sera of the 13 allergic patients. Patients 1–6 had severe allergic symptoms (grade III) and patients 7–13 had moderate allergic symptoms (grade II) and were positive to OFC and DBPCFC. The first line shows IgE immunoblotting of the serum-negative control (n.c.). The numbers indicate the bands corresponding to the allergens identified. FBPA, fructose-bisphosphate adolase; AK, arginine kinase; TM, tropomyosin; SCBP, sarcoplasmic calcium-binding protein; MLC, myosin light chain.

was positive for only MLC. Figure 3 shows IgE immunoblotting of a representative group of 41 nonallergic patients. Twenty-one (51.2%) recognized the 75- to 80-kDa band, corresponding to hemocyanin. By contrast, among the tolerant patients, only 6 (No. 2, 3, 15, 16, 23, and 37) recognized the protein in the 38- to 40-kDa range, corresponding to AK. Sera from 6 out of 41 patients scored positive in the 34- to 38-kDa band, identified as TM, but only 2 patients (No. 23, 37) showed IgE reactivity to rPen a 1 by ImmunoCAP and to nPen m 1 by ISAC test. Regarding IgE binding in immunoblotting, no association was observed for hemocyanin or for SCBP between allergic and nonallergic patients (Fisher exact test:  $p = 0.753$ , and  $p > 0.999$ , respectively). On the contrary, an association was found for AK, TM, and MLC between allergic and nonallergic patients (Fisher exact test:  $p = 0.001$ ,  $p < 0.0005$ , and  $p < 0.005$ , respectively).

## Discussion

The most relevant finding in our study was the high number of negative-challenge patients among shrimp IgE-positive subjects. In fact, only 13 out of 99 (13.13%)

shrimp-sensitized patients could be considered truly allergic as 7 had a positive shrimp DBPCFC and 6 had a documented severe shrimp allergic reaction. If we consider only the 51 patients out of 99 (51.51%) who also reported a suspected clinical history, we have a 25.5% positivity, which is quite similar to Gámez et al. [5], who reported only 18 out of 36 patients (50%) as positive to the challenge. Our results underline how the diagnosis of shrimp allergy should always be confirmed by food challenge. A second relevant observation was the dual role of HDM hypersensitivity in the expression of shrimp-allergic reactions. Our major finding was that, when present, HDM-induced asthma determined a significant increase in the odds of developing shrimp allergy ( $p = 0.002$ ). We also found that anti-Dpt IgE levels were significantly associated with asthma ( $p = 0.004$ ), and significantly higher in true shrimp-allergic patients than in shrimp-tolerant subjects ( $p = 0.0008$ ), thus revealing a clear importance in the conditioning of shrimp allergy. Subsequently, we searched for a possible relationship among some major mite allergens, HDM-induced asthma, and shrimp allergy. We found that no single allergen IgE positivity among Der p 1, Der p 2, and Der p 10 was related to asthma, but considering all these allergens as a single variable, its pos-

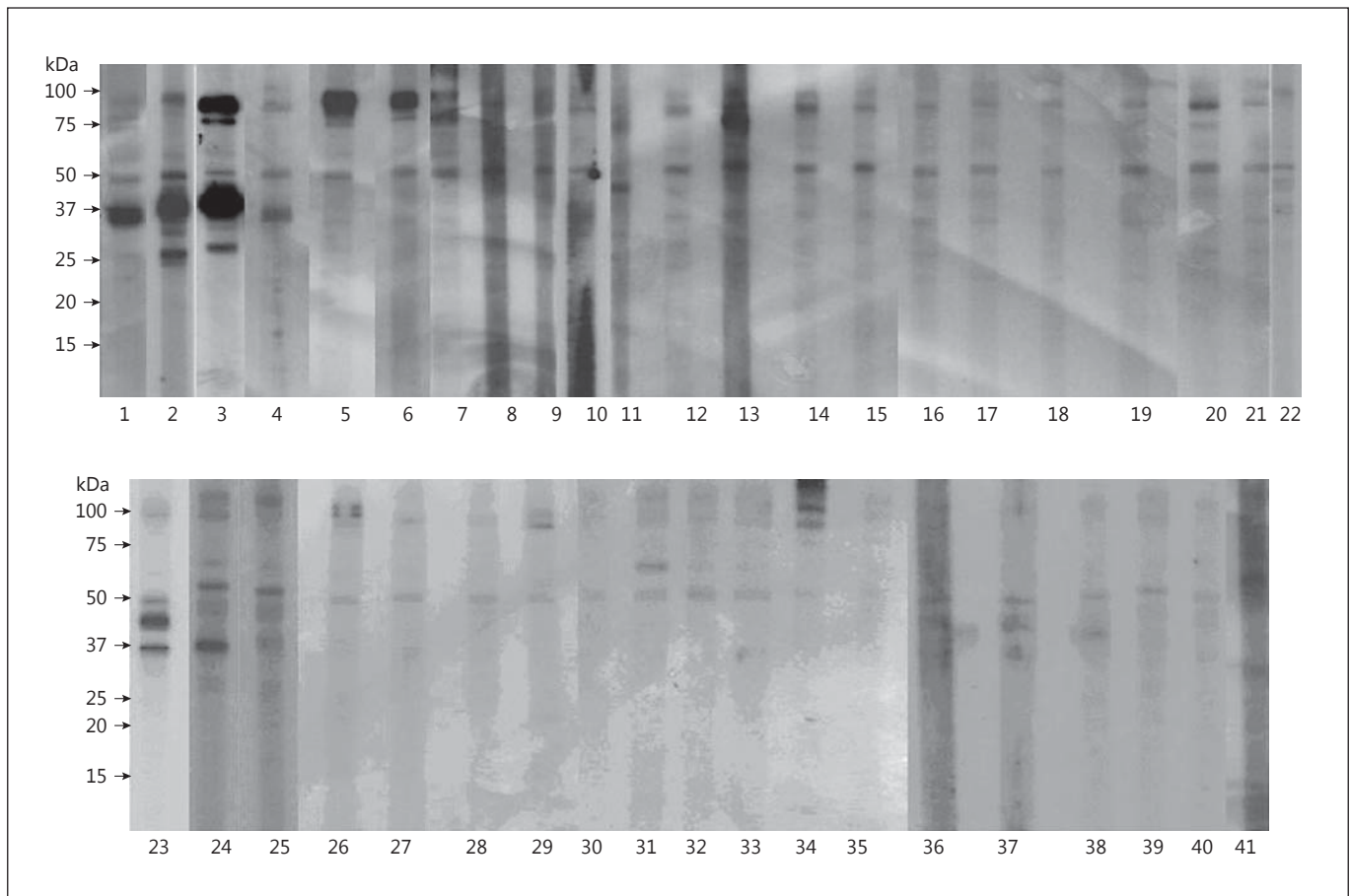


**Fig. 3.** IgE immunoblotting of a representative group of 41 shrimp-tolerant patients (GA1 and GB).

itivity was largely and significantly associated with asthma ( $p < 0.0005$ ). Interestingly, anti-Dpt IgE levels were significantly related either with anti-rDer p 10 ( $p = 0.0001$ ) or anti-rPen a 1 ( $p = 0.0018$ ) IgE levels, which were significantly interrelated ( $p < 0.0005$ ), as well as Der p 10 IgE levels, with anti-shrimp IgE levels ( $p < 0.00005$ ). These results clearly demonstrated that TM positivity is strictly determined by rDer p 10 sensitization. The role of other mite allergens is still partially unknown, and is connected to the other side of HDM sensitization in shrimp allergy, as also the majority of shrimp IgE-positive but tolerant patients were HDM-positive. IgE sensitization to shrimp may be due to cross-reacting allergens, especially TMs, between shrimp and other invertebrates, like mites and cockroaches. For example, Fernandes et al. [17] found IgE reactivity to shrimp TM in a population of Orthodox Jews who were not previously exposed to crustaceans for religious beliefs. Indeed, in HDM-sensitized patients, it is not clear what distinguishes shrimp-tolerant subjects from

those that occasionally reported reactions to shrimp (even if they were challenge negative). In our study the comparison between GA1 (challenge-negative patients) and GB (shrimp-sensitized but tolerant subjects) showed no significant differences in anti-Dpt IgE levels ( $p = 0.9963$ ), but a relevant difference in SPT reactivity. In fact, SPT diameters were greater in GA1 (positive history and negative challenge) than in GB (sensitized subjects with a negative history;  $p < 0.000001$ ). This could be explained by a different IgE affinity or by a higher releasing ability in vivo.

Besides TM, we detected other relevant allergens associated to true shrimp allergy. In particular, AK was the second most important allergen, being positive in 7 out of the 13 truly shrimp-allergic patients. Moreover, AK was more frequently recognized by shrimp-allergic patients than nonallergic patients, being the only recognized allergen in 2 allergic patients. A relevant point was that its positivity was detected in 5 out of 6 asthmatic pa-

tients. This is in line with the results of Gámez et al. [18], who recently observed how climate is important in determining different patterns of sensitization to shrimp in HDM-allergic patients, and found that 34- to 38-kDa AK was more frequently recognized by asthmatic HDM-sensitized patients from humid climate regions. Considering the high humidity in Northern Italian regions, which ranges from 58 to 80% (<http://www.isprambiente.gov.it/>), we confirmed that HDM allergy is the leading sensitizer to shrimp in patients living in humid climatic conditions, making them more prone to developing sensitization to shrimp AK than to other shrimp allergens.

We also evaluated IgE reactivity to the high molecular weight hemocyanin, recognized by only 3 patients with a true shrimp allergy but by the majority of nonallergic subjects, as expected according to the literature [6, 19, 20]. Hence, in contrast to AK, hemocyanin sensitization seems to be a direct consequence of HDM sensitization.

In our study, we also confirmed the role of other recently described shrimp allergens. The 47- to 48-kDa phosphopyruvate hydratase (or enolase) is a protein involved in the glycolytic and gluconeogenesis pathways, and has been suggested to act as an allergen in cod and cockroach. At present, it is not clear whether enolase is an allergen in shellfish or not [21]. In our study it was clearly positive on immunoblotting in 4 patients with real shrimp allergy and only slightly positive in another 4 patients. Moreover, similarly to Gámez et al. [18], we detected a 39–41 kDa FBPA, which is a glycolytic enzyme previously reported as an allergen in fish, molds, and *Anisakis*, apparently recognized by the majority of our patients with a true shrimp allergy. The clinical relevance

of these allergens could be understood when available for an IgE quantitative immune assay.

Two low molecular weight proteins, SCBP and MLC, are considered crustacean-specific minor allergens [22, 23]. Our results showed an IgE binding to SCBP and MLC only in patients with a true shrimp allergy, even if the frequency of sensitization was very low. Interestingly, 1 patient (No. 13) with anaphylaxis after shrimp ingestion presented IgE reactivity only to this protein.

Thus, in our study population, the real risk factors identified for shrimp allergy were: sensitization to HDM with high sIgE levels; the presence of HDM-induced asthma; positivity for Der p 1, Der p 2, and Der p 10, and for Pen a 1, AK, and anti-shrimp IgE levels ( $p < 0.0001$ ). On the other hand, shrimp allergy was rarely unrelated to HDM allergy and, in these cases, was associated to SCBP and MLC minor allergens.

In conclusion, in this study we found that, in the Mediterranean Italian area, shrimp allergy is strictly associated and almost always dependent upon HDM sensitization, and in particular upon HDM-induced allergic asthma. Shrimp food challenge is essential in evaluating the clinical relevance of shrimp sensitization inasmuch as it can only be an expression of previous HDM sensitization. Therefore, the dual role of HDM allergy should always be taken into account when making the final diagnosis. This study shows that in our country TM and AK are the most relevant shrimp allergens.

## Disclosure Statement

All authors declare that they have no conflicts of interest.

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