Mollusk allergy in shrimp-allergic patients: still a complex diagnosis. An Italian multicenter study

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Abstract

Introduction |Shellfish allergy is an important cause of food allergies worldwide. Both *in vivo* and *in vitro* diagnostics failure nowadays is caused by the poor quality of the extracts associated with the scarce availability of allergenic molecules in the market. It is known that not all patients with shellfish allergies experience adverse reactions to mollusks. It is still unclear how to detect and diagnose correctly these patients. **Aim** |To investigate the features of shrimp-allergic patients either reactive or tolerant to mollusks, with the currently available diagnostic methods. **Methods|** Nineteen centers, scattered throughout Italy, participated in the study, enrolling patients allergic to shrimp with or without associated reactions to mollusks. Patients underwent skin tests using commercial extracts or fresh raw and cooked foods, and IgE reactivity to currently available allergenic

extracts and molecules was measured *in vitro*. **Results|** Two hundred and forty-seven individuals with a history of adverse reaction to crustaceans participated in the study. Only 47.8% of them reacted after cephalopod or bivalve ingestion. None of the tests used, either *in vivo* or *in vitro*, was able to detect all selected patients. Accordingly, a great heterogeneity of results was observed with an agreement between *in vivo* and *in vitro* tests ranging between 52% and 62% of cases. Skin tests were able to identify the cephalopod and bivalve reactors ($p < 0.001$), also using fresh cooked or raw food ($p < 0.001$). The reactivity profile of mollusk reactors was dominated by Pen m 1, over Pen m 2 and Pen m 4 compared to the tolerant subjects, but 33% of patients allergic to shellfish were not detected by any of the available molecules. A higher frequency of shrimp hypersensitivity was recorded in northern Italy, while mollusk reactivity was more frequent in the center-south. **Conclusion |**The current diagnostic methods are inadequate to predict the cross-reactivity between crustaceans and mollusks. The detection of mollusks hypersensitivity must still rely on skin tests with fresh material. There is no need to exclude mollusks from shrimp allergic patients' diet unless clinical history, the available diagnostic instruments, and/or tolerance tests support such a decision. Primary sensitization to mollusks seems possible.

Mollusk allergy in shrimp-allergic patients: still a complex diagnosis.

An Italian multicenter study

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1 | Abstract

Introduction | Shellfish allergy is an important cause of food allergies worldwide. Both *in vivo* and *in vitro*diagnostics failure nowadays is caused by the poor quality of the extracts associated with the scarce availability of allergenic molecules in the market. It is known that not all patients with shellfish allergies experience adverse reactions to mollusks. It is still unclear how to detect and diagnose correctly these patients.

Aim | To investigate the features of shrimp-allergic patients either reactive or tolerant to mollusks, with the currently available diagnostic methods.

Methods| Nineteen centers, scattered throughout Italy, participated in the study, enrolling patients allergic to shrimp with or without associated reactions to mollusks. Patients underwent skin tests using commercial extracts or fresh raw and cooked foods, and IgE reactivity to currently available allergenic extracts and molecules was measured *in vitro* .

Results| Two hundred and forty-seven individuals with a history of adverse reaction to crustaceans participated in the study. Only 47.8% of them reacted after cephalopod or bivalve ingestion. None of the tests used, either *in vivo* or *in vitro* , was able to detect all selected patients. Accordingly, a great heterogeneity of results was observed with an agreement between *in vivo* and*in vitro* tests ranging between 52% and 62% of cases. Skin tests were able to identify the cephalopod and bivalve reactors ($p < 0.001$), also using fresh cooked or raw food ($p < 0.001$). The reactivity profile of mollusk reactors was dominated by Pen m 1, over Pen m 2 and Pen m 4 compared to the tolerant subjects, but 33% of patients allergic to shellfish were not detected by any of the available molecules. A higher frequency of shrimp hypersensitivity was recorded in northern Italy, while mollusk reactivity was more frequent in the center-south.

Conclusion | The current diagnostic methods are inadequate to predict the cross-reactivity between crustaceans and mollusks. The detection of mollusks hypersensitivity must still rely on skin tests with fresh material. There is no need to exclude mollusks from shrimp allergic patients' diet unless clinical history, the available diagnostic instruments, and/or tolerance tests support such a decision. Primary sensitization to mollusks seems possible.

Key messages

1. current diagnostic methods are inadequate to predict cross-reactivity between crustaceans and mollusks
2. the detection of mollusks hypersensitivity must still rely on skin tests with fresh material (and oral challenges where possible);
3. clinically, there is no need to exclude a priori mollusks from shrimp allergic patients' diet,
4. albeit rarely, primary sensitization to mollusks seems possible

Author contribution statement

ES, EC, and GMe carried out the experiments and data collection. ES; AA; EE; IB; FC; EC; FB; GD; AD; LF; FLR; LML; DM; GMa; GMe; MM; EN; RO; EAP; SP; AR; FR; AR; MG; LC, VP; DV and RA recruited the patients. ES performed the statistical analysis. ES and RA conceived the study and assisted in data interpretation. All authors reviewed, edited and approved the final manuscript.

KeyWords

Multiplex Analysis

IgE diagnosis

Tropomyosin

Crustaceans

Mollusks

Food Allergy

Urticaria/angioedema

Anaphylaxis

2 | INTRODUCTION

Seafood is included among the "Big Eight" food groups responsible for most cases of food allergy. It is estimated that depending on the different geographic areas, about 3% of the general population is allergic to shellfish (1,2). This generic term includes more than 300 different invertebrate species which are divided into two large groups: crustaceans (shrimps, crabs, lobsters etc.) and mollusks (*Bivalvia* , such as clam, mussels, scallops or oysters, and*Cephalopoda* , like squids, cuttlefish or octopuses etc.)(3)(4). These taxonomic classifications are essential to predict the structural, immunological and allergological similarities that underlie a possible cross-reactivity. The availability and consumption of seafood vary greatly in different parts of the world, representing the second cause of primary food allergy in Italy, after Lipid transfer proteins (5). Even eating habits, including the different methods of food processing, may exert a strong impact on the incidence and severity of allergy to crustaceans and seafood, since in some cases physical treatments can increase or reduce IgE reactivity(6), depending on the molecule involved in patient sensitization (7). So far, it has been argued that due to the cross-reactivity among allergens in invertebrates, primarily tropomyosin, the patient allergic to crustaceans should also exclude cephalopods and Bivalvia from the diet. However, recent studies seem to suggest that this is not the case; in fact, a significant proportion of crustacean allergic patients report that they usually tolerate other invertebrates (8). One of the main diagnostic problems is that all the molecules commercially available for *in vitro* tests come from crustaceans and not from mollusks. As a consequence, IgE diagnostic approach for mollusks adverse reaction is always indirectly based on a presumptive but not fully demonstrated cross-reactivity, unless tests with fresh, raw or cooked cephalopod or Bivalvia are carried out, with all the drawbacks associated with such approach.

The main aims of this study were (i) to investigate the prevalence of patients allergic to crustaceans with or without cross-reactivity to cephalopods and/or bivalve mollusks; (ii) to define the molecular profile in both cross-reacting and non-cross-reacting individuals; (iii) to highlight any differences in the severity of the allergic reactions, and (iv) to investigate the geographical differences in seafood sensitization profiles in Italy.

3 | PATIENTS AND METHODS

Nineteen Italian allergy centers took part in a multicenter cross-sectional observational clinical survey on crustacean allergy. In every participating center, doctors consecutively enrolled the largest possible number of patients with unequivocal crustacean (shrimp) and/or mollusks (*Bivalvia and/* or *Cephalopoda*) allergy, between May 15th and November 15th, 2021. The patients with a history of food allergy to shellfish confirmed by a positive skin prick test (SPT) with commercial shrimp extract and/or with prick-prick test (PPT) with fresh raw or cooked food were carefully interviewed about seafood, crustaceans and/or mollusks tolerance.

Diagnosis of tolerance was based on patient's clinical history (i.e., consumption of the culprit food without problems).

Shellfish-induced allergic reactions reported by patients included both mild (such as oral allergy syndrome or isolated gastrointestinal reactions) and moderate-severe reactions (i.e., urticaria/angioedema, anaphylaxis). Given the observational nature of the study, no randomization procedure was be implemented during enrollment. The clinical histories were confirmed by a positive skin prick test (SPT) with fresh food (either raw or cooked) and/or commercial extracts of shrimp and mollusks currently available in Italy (Alk-Abello' s.p.a., Allergy Therapeutics Italia, Anallergo, Firma Srl, Roxall Italia, Lofarma, and Stallergenes Italia Srl) and/or by the detection of IgE specific for seafood extracts by ImmunoCAP (shrimp, mussel, clam, octopus or squid), and Pen a 1 (tropomyosin) (Thermo Fisher, Uppsala, Sweden). In a subset (98 individuals) a more in-depth evaluation was carried out by the Allergy Explorer-ALEX2® (Macroarray Diagnostics, Vienna) multiplex platform evaluating a broader profile of seafood molecules (9), including Pen m 1 (tropomyosin), Pen m 2 (arginine kinase), Pen m 3 (myosin light chain), and Pen m 4 (sarcoplasmic calcium binding protein) all from Black-Tiger shrimp (*Penaeus monodon*), Cra c 6 (the troponin-c from brown shrimp, *Crangon crangon*), Der p 10 (tropomyosin), and Der p 11 (paramyosin) both from*Dermatophagoides pteronyssinus* , as well as of seafood extracts, including crab, lobster, northern shrimp, white shrimp, squid, mussel, oyster, clam, and scallop. IgE levels >0.3kUA/L were considered positive as for manufacturer's instructions.

The diagnosis of seafood allergy was not confirmed by blinded or open oral food challenges due both to the severity of several reactions and the fact that many centers were not sufficiently equipped in terms of facilities or personnel to manage possible severe allergic reactions.

3.1 | Statistics

The sampled data were recorded and analyzed by SPSS (version 27.0.1.0 SPSS, Inc, Chicago, III).

In univariate analysis, the non-parametric Mann-Whitney U-test (two groups) was first used to compare continuous IgE values in subjects with or without a given clinical involvement; then, each variable of interest was dichotomized (as negative or positive) to study the proportion of subjects with symptoms in the two groups thus obtained.

Pearson's χ^2 test or Fisher's exact test (used for two-by-two contingency tables with less than 50 cases) were used to assess if paired observations on two variables expressed in a contingency table, were independent of each other.

We performed multiple logistic regression for the clinical variables with dichotomous scores (present, absent) to see whether the association between clinical symptoms and different shellfish allergens reactivity was present after simultaneously adjusting for the other variables of interest.

The degree of relationship between the quantitative variables studied was analyzed using Spearman Correlation (rho) test, and the most commonly used bivariate correlation technique. A value of $p < 0.05$ was considered statistically significant.

3.2 | Ethical issues

The study was approved by the Ethical Committee of the coordinating centre (IDI-IRCCS CE | 667/2021). Data collection takes place anonymously, using only data obtained through routine specialist surveys. Recruited patients gave informed consent to the use of their clinical data in an anonymous form.

4 | RESULTS

4.1 | General findings

Two hundred forty-seven individuals (M 48.2%, F 51.8%; mean age 39 ± 17, range 2 - 79 years) enrolled in the 19 centers participating in the study, represented the study group.

Forty-four per cent (n= 108) of the patients came from the central-southern Italy , while the remaining 56% (n= 139) of cases had been recruited in northern Italy.

All patients selected for the study reported a clinical history suggesting an adverse reaction to crustaceans confirmed by positive in-vivo and/or in-vitro testing, but only 47.8% of them (n=118) reported adverse reactions after the ingestion of cephalopods or bivalves; of these, 38.5% (n=95) experienced urticaria/angioedema, and 9.3% (n=23) anaphylaxis.

No age and sex differences were detected concerning symptom severity.

4.2 | *In vivo* vs *in vitro* diagnostic

As previously shown(10), diagnostics with commercially available seafood extracts did not always lead to the correct identification of sensitized patients. None of the tests used, either *in vivo* or *in vitro* , was able to detect all selected patients as positive. Indeed, 60,9% per cent of the patients scored positive on SPT for shrimp; 78.3% scored on skin testing with fresh food, 65,8% with cooked food, and 72,4% were positive on the detection of specific IgE to shrimp by ImmunoCAP. Thirty-nine (16.6%) individuals were negative for skin tests with both commercial extracts and raw or cooked shrimp but scored positive on specific IgE assays.

As shown in Table 1, a concordance of commercial SPT and IgE assay for crustaceans was found only in 56% of patients; the concordance rose to 62% between skin tests with raw or cooked fresh shrimp and the *in vitro* test. Comparing the in-vivo and in-vitro evaluations with cephalopods (squid or octopus) or mollusks (mussels or clams), and even higher heterogeneity of results was observed (Table 1).

No correlation was found comparing IgE levels to seafood extracts, shrimp, cephalopods and mollusks, except for a moderate relationship between cephalopods and mussels (rho 0.634, p <0.001)

To evaluate the prevalence of IgE reactivity to cephalopods or mollusks in shrimp reactive subjects, our patients were extensively studied*in vitro* with both extracts and allergenic molecules, using singleplex and multiplex methods. As shown in Table2, IgE sensitization to shrimp was significantly associated with hypersensitivity to cephalopods (p <0.005), but not to mollusks on singleplex testing. Notably, specific IgE levels did not differ in the two patient groups.

On the other hand, when shrimp immunoreactive patients were evaluated by the multiplex system, they were also more frequently positive for crab, lobster, northern and white shrimp, squid, mussel, and clam, but not for oyster, and scallop (Table 2). Crustacean reactivity was accompanied by significantly higher levels of specific IgE towards all extracts tested, except for crab, oyster and scallop, where specific IgE levels did not differ.

From the molecular point of view, IgE reactivity to crustaceans was associated with a significantly higher frequency and higher levels of specific IgE to tropomyosins (both Pen m 1 and Der p 10) and sarcoplasmic calcium-binding protein (Pen m 4), in comparison with the patient that scored negative for specific IgE to crustaceans (Table 2).

4.3 | Sensitization profiles and clinical correlations

One hundred-eighteen (47,8%) patients enrolled because allergic to crustaceans, reported also moderate (80.5%) to severe (19.5%) reactions after ingestion of mollusks.

There was no difference in the frequency of reactive episodes related to sex or age.

Interestingly, a higher risk of developing a severe reaction after mollusks intake was associated with skin test reactivity to commercial extracts of cephalopod (OR: 4.81; CI 1.8-9.3; p <0.001) or bivalve (OR: 14.970; CI 4.2-53.3; p <0.001). Similarly, mollusk reactors showed a high frequency of reactivity to both raw (OR: 4.492; CI 1.9-10.4; p <0.001) and cooked squid (OR: 3.040; CI 1.3-7.3; p <0.001), and to both raw (OR: 20.907; CI 5.8-75.2; p <0.001) and cooked clam (OR: 17.249; CI 3.1-41.1; p <0.001).

The multiple logistic regression analysis, when simultaneously adjusted for the presence of specific IgE to all the extracts studied, age, and sex, showed a significant relationship between a history of adverse reaction to mollusks and IgE reactivity to both cephalopods (OR_{adj} 4.0, 95% CI 1.5-10.7, p <0,01) or bivalve (OR_{adj} 7.6, 95% CI 3.1-18.6, p <0.0001) extracts. Moreover, as shown in Table 3, allergy to mollusks was associated with IgE reactivity to northern shrimp (*Litopaenaeus setiferus*), squid (*Loligo* spp.), mussel (*Mytilus edulis*), oyster (*Ostrea edulis*), clam (*Ruditapes* spp.), and scallop (*Pecten* spp.) extracts. Pen m 1 (*Penaeus monodon* tropomyosin) hypersensitivity was significantly associated with an increased risk of severe reaction to mollusks.

Three patients scoring SPT positive to crustaceans showed *in vitro* sensitization to bivalves (both oyster and mussel) in two cases and squid in one case, with no sign of IgE reactivity against any the extracts and molecules of crustaceans *in vitro* , with only a low reactivity in one case to paramyosin (0.24 kU/L) and tropomyosin (0.39 kU/L) from house dust mite (Der p 11 and Der p 10 respectively).

As summarized in Figure 1, the molecular sensitization profile was dominated by Pen m 1, but more frequently in those who did not tolerate mollusks. Moreover, mollusks reactors had also two times higher frequency of sensitization to arginine kinase and sarcoplasmic Ca^{++} binding protein (Figure 1). Interestingly, the number of crustacean allergic patients who did not react to any of the molecules currently available for *in vitro* diagnostics was very high, particularly among those who tolerated cephalopods and bivalves (43%).

4.4 |Geographical differences in seafood sensitization profiles

When we analyzed the data as a function of the geographical distribution of patients, we found a significantly higher frequency of sensitization to crustaceans in northern Italy, irrespective of the diagnostic tests used (skin prick test, prick-prick test with fresh or cooked food, and specific IgE measurement for allergen extracts), whilst in centre-southern Italy, a more frequent sensitization to Bivalvia was recorded (Figure 2 A-C). This observation was in keeping with a higher frequency of reactions to bivalve in the south, whilst the sensitization levels to cephalopod were comparable among south and north (Figure 2 D).

5 | Discussion

In our multicenter study, involving 19 centers scattered throughout Italy, we evaluated the prevalence and the sensitization profile of allergic subjects to crustaceans reactive or not reactive also to mollusks

We confirmed once more (11), that the diagnostics with both the extracts and the molecules currently available in the market is inadequate to detect satisfactorily patients hypersensitive to crustaceans and mollusks. We observed an extreme heterogeneity of results from one patient to another using the common diagnostic approaches, including skin prick testing with commercial extracts, skin testing with fresh material and specific IgE measurements. We previously showed the unreliability of commercial extracts for SPT available on the market due to differences in allergenic proteins concentrations (10), thus potentially leading to confounding results.

The currently available molecular diagnostics for seafood shows two major pitfalls. First, not all allergen molecules are present on the diagnostic platforms and, second, practically all the molecules available derive from crustaceans and none from mollusks. The diagnosis of mollusks allergy is therefore always indirect, based on the presumption of cross-reactivity with crustaceans. Of the 58 shellfish molecules currently registered as allergens by the WHO/IUIS Allergen Nomenclature Sub-Committee, only 8 belong to the mollusks (see Table in the repository). If one considers, for instance, that the tropomyosins from mollusks share no more than 60% amino acid sequence identity with the other allergenic tropomyosins isolated so far from crustaceans, insects, mites, and fish (1), one can easily figure out that this might lead to a failure in the detection of allergic patients (12). A 2018 study showed that in the pacific oyster extract, along with many specific allergens of invertebrates, fish, and mites, can be isolated allergens from other different biological sources such as pollen and fungi, thus prompting interesting scenarios about possible unexpected sources of sensitization(13).

Another interesting point of our study concerns the differences in sensitization profiles observed in the different geographic areas of the country. We detected that in the north sensitization to crustaceans was

much more frequent than in the center/south, where in turn sensitization to cephalopods and bivalves was more prevalent. These differences might be the result of different culinary habits between the North (where the way of life is more similar to that in Central and Northern Europe) and the South, where habits are similar to those in other Mediterranean countries such as Greece or Spain(8). These geographical differences could also underlie different sensitization mechanisms according to different environmental exposures (11). This point needs to be addressed in future studies.

The finding of three single patients who were mono-reactors to bivalves (both oyster and mussel), suggests that primary sensitization to mollusks is possible, albeit being an extremely rare event, sometimes associated with sensitization also to house dust mites (14). A study designed to verify whether reactivity is always secondary to sensitization to mite allergens should be carried out, focusing attention on a pediatric-only population.

Objective limitations of the study are the absence of oral food challenge in patient selection, which was made only on the anamnestic data, and the use of heterogeneous commercial preparations, both for *in vivo* and *in vitro* diagnostics, where it is not always declared. by the producers, the type of crustacean, prawn or shrimp used to prepare the extract.

In conclusion, this study clearly shows the following: (i) current diagnostic methods are inadequate to predict cross-reactivity between crustaceans and mollusks due to the lack of specific mollusks allergens and because these are only partially cross-reactive to crustacean homologue proteins; (ii) the detection of mollusks hypersensitivity must still rely on skin tests with fresh material (and oral challenges where possible); (iii) clinically, there is no need to exclude a priori mollusks from shrimp allergic patients' diet, unless the available diagnostic instruments support such decision; (iv) albeit rarely, primary sensitization to mollusks seems possible although the incomplete spectrum of shrimp allergens does not allow us to exclude that shrimp acts as primary sensitizer also in those few cases.

Data Avaiability

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Conflict of interest

ES has received consultant arrangements and speakers' bureau participation from Stallergenes, Thermo Fisher Scientific, and non-financial support from Microarray Diagnostics, Vienna, all outside the submitted work. LC has received honoraria from Malesci, Menarini, Mylan and Thermo Fisher Scientific. DV and RA received honoraria from Thermo Fisher Scientific.

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Data Availability Statement information

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ETHICAL APPROVAL STATEMENT

The research was conducted ethically following the World Medical Association Declaration of Helsinki. All subjects have given their written informed consent and that the study protocol was approved by the institute's committee on human research. (CE: 667 | 2021)

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Table 1. Concordance among commercial SPT and IgE assay for Crustaceans and Mollusks

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Table 2 . Mollusks extract and molecules IgE sensitization frequency and levels as per multiplex and singleplex detection in individuals with or without IgE reactivity to crustaceans. ns = not significant

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Table 3 . Extract and molecules IgE sensitization frequency in individuals tolerating or not tolerating mollusks. ns = not significant

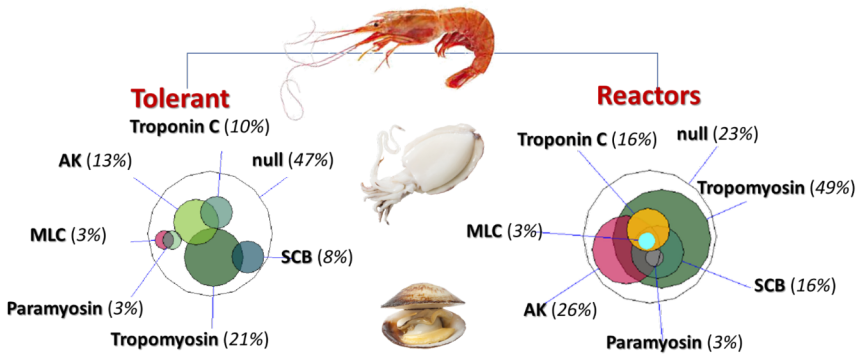

Figure 1 Molecular sensitization profile in patients allergic to crustaceans that were tolerant or reactors to cephalopods and/or mollusks. Null refers to the percentage of patients not reactive to any molecule tested.

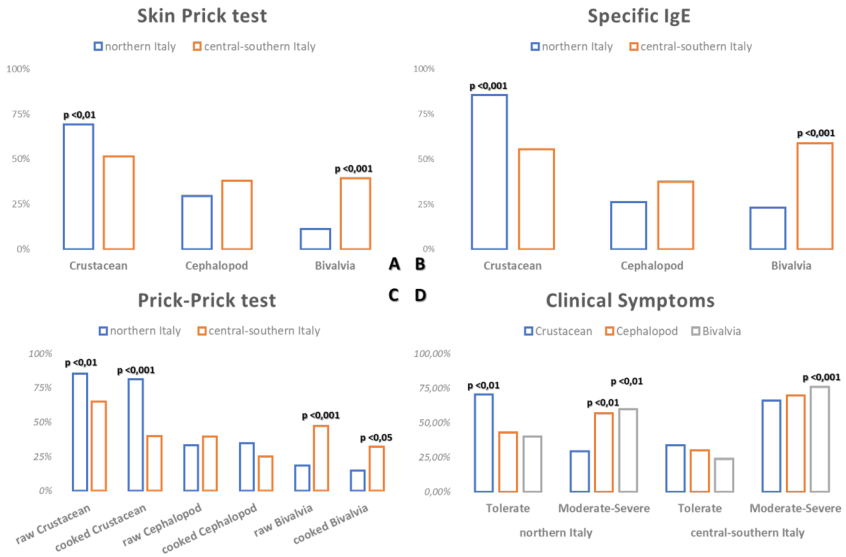

Figure 2. Skin prick test (A), prick-prick test with fresh or cooked food (C), and specific IgE measurement for allergen extracts(B) in northern and central-southern Italy. Sensitization differences are shown in D.

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