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Prevalence, molecular characterization, and clinical relevance of sensitization to *Anisakis simplex* in children with sensitization and/or allergy to *Dermatophagoides pteronyssinus*

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Summary
Prevalence of the Anisakis Simplex*'s (AS) sensitization in children sensitized to* Dermatophagoides pteronissynus *(DP) is not known, neither it is to which percentage it might be due to cross-reactivity. The primary objective of the present retrospective cross-sectional study is to evaluate the prevalence of sensitization to AS in children sensitized or allergic to DP. Secondary outcomes were the prevalence of cross-reactivity and clinical relevance of the condition. The prevalence of sensitization to AS differs significantly among patients sensitized and not to DP (13.43% vs. 3.80%; p=0.019). The higher prevalence is mainly due to cross-reactivity with Der p10 (OR=8.86; 95% CI=4.33–40.74; p=0.0001). Currently, the sensitization to AS seems to have no clinical relevance in the pediatric population.*

Introduction

The nematode *Anisakis simplex* (AS) is a common parasite of fish, marine mammals and invertebrates. The life cycle comprises the stage of egg, various stages of larva and adult nematode. The encapsulated larvae of the third stage are common in fish organs and muscles. They infest fish of various species over large geographical areas. Eating raw or undercooked fish can result in infestation with the larva and can cause severe gastrointestinal symptoms (anisakiasis). Several studies suggest that AS can be a food allergen and induce IgE-mediated reactions such as urticaria, angioedema, and anaphylaxis. AS can cause allergic reactions even when well cooked, because some allergens are thermostable. There have been reports of occupational allergy with asthma and conjunctivitis, and a causal relationship was demonstrated between AS and chronic urticaria (1). Finally, AS can also be responsible for delayed, cell-mediated allergic reactions, specifically eczema, caused by live, cooked, and frozen larvae after repeated handling of raw fish (2).

In recent years there has been an increase in cases of anisakiasis probably due to several factors: increase in the number of sea mammals, "global cuisine", faster cooking methods (microwave) also to preserve vitamins, increase in fish consumption for healthier lifestyles (3).

About 90% of the cases worldwide occurs in Japan, followed by Italy, Spain, Hawaii, and other countries where raw or undercooked fish consumption is a tradition. The prevalence of sensitization in Norway, where fish is eaten baked or fried, is around 2%, while in Spain and Japan is 12% (4,5,6). The hypersensitivity, as well as the susceptibility to the disease, may be explained by genetic predisposition (7).

Clinical manifestations of AS are essentially gastric, intestinal, ectopic and systemic disorders, probably according to the route of sensitization (8).

The prevalence of sensitization in Italy is on average 4.5%, with significant geographical differences (variation from 0.4% to 12.7%) due to local culinary traditions, being greater on the Tyrrhenian and Adriatic coasts where it is common to eat raw and marinated fish. In the mainland, the sensitization to AS is essentially related to migration: in Milan and Turin 60% of people sensitized to AS is native to Southern Italy and non-EU countries (9).

The cause of sensitization is not clear. It has been suggested that sensitization in asymptomatic individuals is due to early infestations. However, cross-reactivity may also be a possible explanation, demonstrated between AS and other nematodes, arthropods (eg. *Blatta germanica*) and some types of dust mite (*Acarus siro, Lepidoglyphus destructor, Tyrophagus putrescentiae*, DP) (10). In a recent study of adult patients in Sicily, a region in which the consumption of raw or marinated fish is very common, the co-sensitization to dust mites results in an OR of 1.98 for AS allergy (9).

The cross-reactivity is due to a thermostable protein, highly conserved, present in muscle and non-muscle cells: the tropomyosin. This protein is the major allergen of shellfish, and it is the main allergen responsible for the molecular and clinical cross-reactivity by ingestion of shellfish and by inhalation of other invertebrates, such as dust mites and insects (11). Actin and myosin together have an important role in contractile activity, i.e. motility adjustment and cell morphology (12). Der p10 (DP), Ani s3 (AS) and Pen a1 (shrimp) are among the tropomyosins currently identified (11). In a subject sensitized to multiple allergens, eg. DP and AS, the presence of IgEs against tropomyosin and not against the genuine allergens is an indication of cross-reactivity. The sensitization to tropomyosin may involve allergic reactions of highly variable severity, with very low clinical impact until anaphylaxis (11).

Data on the prevalence of sensitization to AS in the Italian pediatric population are limited. A 2001 study on patients consecutively referred to an Allergology Center, reported a prevalence of 6.1%. The sensitized patients had no allergic symptoms to AS. The sensitization was significantly associated with sensitization to DP and other allergens such as cod and soia (13).

The importance of cross-reactivity in patients sensitized to the AS, in particular in the pediatric age, is not known, both with respect to the immunological profile (prevalence of cross-reactivity or sensitization to genuine allergens) and to the clinical manifestations (allergic reactions or asymptomatic sensitization). Therefore, the primary objective of this study is to assess the prevalence of sensitization to the AS in children sensitized and/or allergic to DP.

Secondary endpoints are:

1. to evaluate the cross-reactivity between AS and DP
2. to assess the clinical relevance of sensitization to AS in children sensitized and/or allergic to DP (presence and severity of allergic reactions following ingestion / inhalation of AS).

Methods

We conducted a retrospective cross-sectional study by extracting and analyzing data from the medical records of a specific sub-population of patients referring to the Paediatric Allergy Day Hospital (DH) of the Sacro Cuore di Gesù Fatebenefratelli Hospital in Benevento (Italy), from January 1st 2013 to October 30th 2016. Every year, about 500 patients refer to the Pediatric Allergy DH, for a total of about 1800 medical records during the period of the study. Of these, 294 were selected, on the basis of inclusion and exclusion criteria.

Inclusion criteria. Pediatric patients (1 - 18 years) with suspected sensitization or allergy to DP, who underwent allergy testing for DP and AS, and refer consecutively to the Paediatric Allergy DH of the Fatebenefratelli Hospital in Benevento. *Exclusion criteria*. Patients who do not meet the inclusion criteria, with suspicion of allergy not related to the DP, or who meet the inclusion criteria but have malformations or are suffering from severe chronic illness, or assume antihistamines, corticosteroids or immunosuppressants. Data regarding medical history, Skin prick tests (SPT) and serum IgEs were extracted from the medical records of the included patients.

Medical history was collected by the referent of the Paediatric Allergy DH of the Fatebenefratelli Hospital in Benevento, with specific reference to respiratory allergic symptoms, food allergy and allergy to DP. Skin prick tests (SPT) were performed with commercial extracts for *Dermatophagoides farinae* (DF), *Dermatophagoides pteronyssinus* (DP), *Anisakis*, cockroach, shrimp, (Alk-Abello by International Pharmaceutical Immunology, SA, Madrid, Spain: protein concentration =1 mg/ml). The SPT results were considered positive (wheal diameter >3 mm) in line with the recommendations of the European Academy of Allergology and Clinical Immunology (15). Serum IgEs against DF, DP, Der p1, p2 Der, Der p10, Pen a1 (Immuno CAP Phadia) were measured. Values >0.35 KU/L were considered positive (13).

Outcomes

Primary outcome. Prevalence of STP positivity (sensitization) to AS among patients positive to the DP. *Secondary outcomes*. Cross-reactivity (associations with sensitization to cockroach, shrimp, tropomyosins Der p10 and Pen a1) and clinical relevance (suggestive clinical history of allergic reactions occurring some minutes up to 2 hours after ingestion of raw or marinated fish, confirmed by a positive SPT to AS) of sensitization to AS.

Data collection

All the data relevant for the study were collected using an ad hoc database in Excel (Microsoft software).

Statistical analysis

Sample size. For an expected prevalence of sensitization to AS=6%, based on the data already known in the literature (12), with the probability alpha=0.05 and beta=0.20, with a precision=4%, the minimum sample size calculated was equal to 140 patients.

Statistical tests. Mean±standard deviation (SD), median and range (interquartile range, minimum and maximum) were calculated for continuous variables, whereas proportions (prevalences) were calculated for categorical variables. To evaluate differences between frequencies, χ_2 test was used. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated between some variables (DP, Der p10, Shrimp, Pen a1 sensitization, DP allergy, Food allergy) and AS sensitization. The p value <0.05 was considered statistically significant. For statistical analysis STATA 13 software was used. The sample size was calculated with StatCalc of EpiInfo ver. 7.2.

Results

A total of 294 patients were selected, with a mean age of 7.7±3.5 years (median=7; range=0–16). Data on sensitization and discharge diagnoses are summarized in **Table I**.

The prevalence of SPT positivity to the tested allergens was: *Dermatophagoides farinae* 68.4%, DP 69.4%, AS 10.5%, cockroach 8.2%, shrimp 2.4%, Der p1 43.2%, Der p2 38.8%, Der p10 5.8%, Pen a1 5.1%. IgEs titer (KU/L) against DP and tropomyosins Der p10 and Pen a1 were respectively 19 (0– >100), 0.01 (0–37), and 0.02 (0–38.4)

Table I *- Age, sensitization (IgEs; SPT) and allergies (allergy to DP, food allergy).*

Variable	N°	Positives N° (%)	Mean (SD)	Median (interquartile; range min–max)
Age (years)	254		7.67 (3.54)	7 (5; 0–16)
Dermatophagoides farinae (DF) IgEs (KUA/L)	177	140 (79.10)	28.17 (34.08)	13.3 (42.67; 0– >100)
Dermatophagoides pteronyssinus (DP) IgEs (KUA/L)	183	153 (83.61)	39.05 (41.32)	19 (92.00; 0– >100)
Der p1 IgEs (KUA/L)	289	127 (43.94)	27.63 (35.33)	5.52 (70.67; 0– >100)
Der p2 IgEs (KUA/L)	232	114 (49.14)	26.12 (35.33)	2.59 (53.07; 0– >100)
Der p10 IgEs (KUA/L)	226	16 (7.08)	0.94 (4.82)	0.01 (0.06; 0–37)
Pen a1 IgEs (KUA/L)	110	15 (13.64)	1.23 (5.12)	0.02 (0.01; 0–38.4)
DF SPT	282	201 (71.28)		
DP SPT	283	204 (72.08)		
Anisakis simplex (AS) SPT	281	31 (11.03)		
Cockroach SPT	281	24 (8.54)		
Shrimp SPT	204	7 (3.43)		
Food Allergy	294	27 (9.18)		
DP Allergy	291	126 (43.30)		

Table II *- Comparison between patients sensitized and not sensitized to Anisakis Simplex.*

		Patients not sensitized to Anisakis simplex N° (%)	Patients sensitized to Anisakis simplex N° (%)	OR (95% IC)	P value[1]
Dermatophagoides pteronyssinus (DP) sensitization	yes	174 (86.57)	27 (13.43)	3.93 (1.15–20.78)	**0.019**
	no	76 (96.20)	3 (3.80)		
Der p10 sensitization	yes	8 (50.00)	8 (50.00)	8.86 (2.56–29.82)	**<0.0001**
	no	186 (93.00)	14 (7.00)		
Shrimp sensitization	yes	1 (16.67)	17 (8.63)	52.94 (5.25–2516.05)	**<0.0001**
	no	180 (91.37)	5 (83.33)		
Pen a1 sensitization	yes	8 (53.33)	7 (46.67)	11 (2.51–46.79)	**<0.0001**
	no	88 (92.63)	7 (7.37)		
DP allergy	yes	105 (84.68)	19 (15.32)	2.35 (1.01–5.70)	**0.028**
	no	143 (92.86)	11 (7.14)		
Food allergy	yes	23 (88.46)	3 (11.54)	1.06 (0.19–3.85)	**0.931**
	no	227 (89.02)	28 (10.98)		

[1]In bold statistically significant results.

With regards to the discharge diagnosis, the prevalence of DP allergy and food allergies was 43% and 9%, respectively.

No patients had allergic symptoms to AS. The prevalence of sensitization to AS, in the subgroups with DP sensitization and allergy, was (**Table II**):

- 13.4% and 3.8% in patients with and without sensitization to DP, respectively
- 50% and 7%, in patients with and without sensitization to Der p10, respectively
- 15.3% and 7.1%, in patients with and without DP allergy, respectively

Sensitization to AS was not associated with age [OR (95% CI)=1.09 (0.98–1.22); p=0.113]. Associations were found with sensitization to DP [OR (95% CI)=3.93 (1.15–13.35); p=0.028)], DP allergy [OR (95% CI)=2.35 (1.07–5.15); p=0.033)], tropomyosin Der p10 [OR (95% CI)=8.86 (3.01–26.1); p<0.0001)], shrimp [OR (95% CI)=52.94 (5.84–479.7); p<0.0001], and tropomyosin Pen a1 [OR (95% CI)=11 (3.08–38.30); p<0.0001] (**Table II**).

Discussion

Sensitization to AS is still under investigation in many clinical, epidemiological and laboratory-based studies aiming at the molecular characterization of the allergens. The latest Italian data are on 3,419 adult patients referred to the Catania University Allergy Center (9). In this sample, the prevalence of sensitization to AS was about 15%, similar to the one recorded in other coastal areas of Italy (~12%), with the only unexplained exception of Messina (0.8%) (1).

Approximately 30% of sensitized patients showed a single sensitization. The age was a risk factor for sensitization to AS, more common in older patients, along with the sensitization to dust mite and mildew.

Despite the reported association between sensitization to DP and to AS, the authors have neither quantified the cross-reactivity, nor they have been able to correlate the AS allergy (for most gastrointestinal and respiratory symptoms, but also, in approximately 7% of cases, for anaphylaxis) with cross-reactivity or sensitization.

In the first study on pediatric patients published in Italy, patients consecutively referred to the Pediatric Allergy Centre of the University of Florence. The sensitization to AS resulted associated with DP and Alternaria sensitization, atopy, allergy to soya, cod and, weakly but significantly, age. The prevalence of sensitization was 6.1%; none of the sensitized patients showed allergic reactions to AS (13).

Another recent study assessed the AS sensitivity in 443 Italian children living in an endemic area, consecutively presenting at three Pediatric Allergy Centers in Rome and one Center in Naples (14). The prevalence of sensitization was 4.5%, and the *Anisakis*-sensitized children were significantly older than control

children; none showed gastrointestinal symptoms or allergic reactions to AS or fish. The authors came to the conclusion that in endemic areas the sensitization to AS is equally frequent in children and adults. Data on the cross-reactivity were not reported. On the basis of these documented associations, with the present study we firstly wanted to quantify the prevalence of sensitization to AS in patients sensitized and not to DP, which resulted being 13.4% and 3.8% (p=0.028), respectively. The low prevalence (3.8%) in non-sensitized to DP is in line with expectations, considering that those patients generally live in the mainland and non-coastal areas, which do not have a traditional habit to consume raw or marinated fish.

Similarly, in patients with allergic symptoms to dust mite, the prevalence of sensitization to AS (15.3%) was significantly higher than in non-allergic patients (7.1%; p=0.033). The high prevalence recorded in sensitized/allergic patients to DP was strongly suggestive of the importance of cross-reactivity, confirmed by the results of the regression analysis: there is a statistically significant association with sensitization, DP allergy, and especially with positivity to tropomyosin Der p10, DP pan allergen (OR=8.86; 95% CI =3.01–5.26, p<0.0001).

Another fact in favor of cross-reactivity is the strong association with the positivity of sensitization to shrimp tropomyosin and its Pen a1 in asymptomatic patients for allergy to this crustacean.

Although the identity of amino acid sequences between Der p10 and Anis s3 is not proven, contemporary positivity of sensitization to AS and DP with Der p10 positivity is considered an expression of cross-reactivity. Recently, identity of amino acid sequences of some AS proteins and other homologous DP allergens (Der p4, Der p8, Der p14, Der p15, Der p18, Der p20) have been demonstrated, although they are not yet available for allergy diagnosis in clinical practice (3).

Based on these new acquisitions, the sensitization to AS found in 7% of Der p10 negative patients cannot definitely be attributed to genuine allergens, but it is likely that a proportion of this sensitivity is also due to cross-reactivity with antigens other than Der p10.

Despite the strength of the association, from our results it is not possible to consider the sensitization to Der p10 as certainly predictive of sensitization to AS, so that, in absence of further data, it is necessary to assess sensitization to AS with specific allergy tests, when indicated.

Contrary to previous publications and according to our results, sensitization to AS is neither associated with age, nor with concomitant food allergies, but the small number of cases does not allow us to determine if those are real data due to different epidemiological conditions, or if they are due to a statistical beta error (false negative).

In our study, as in that of Bernardini et al. (13), patients sensitized to AS did not have symptoms of allergic reactions to AS, so the clinical relevance of the sensitization and the related cross-reactivity with DP is, at least in the pediatric age, apparently inexistent.

A strength of our study is the demonstration, in patients sensitized to DP and AS, of the importance of cross-reactivity, also confirmed by the results of molecular investigations, specifically by dosages of Tropomyosins Der p10 and Pen a1.

The limits are as follows: we have no data on recombinant allergen-specific IgE Anis s3, and we do not know if this result could be confirmed in larger or different populations, or if it could be confirmed on the same patients with an adequate follow-up. The studies on adults published to date do not resolve the issue.

Conclusions

This study shows that sensitization, in patients asymptomatic for AS allergy but sensitized to DP, is essentially due to cross-reactivity and is associated with positivity of tropomyosin Der p10, DP pan allergen. Currently, the sensitization to AS seems to have no clinical relevance in the pediatric population, since it does not entail an increased risk of allergic reaction to AS, but we do not know if the risk increases with age, and if it may change in relation to genetic and/or environmental factors. Therefore, further studies are needed to confirm these results, as well as to complete the identification and characterization of AS allergens and the counterparts in nematodes, insects and crustaceans.

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