



ORIGINAL ARTICLE

Molecular diagnosis of allergy to *Anisakis simplex* and *Gymnorhynchus gigas* fish parasites



A. Armentia^{a,*}, J. Santos^b, Z. Serrano^a, B. Martín^a, S. Martín^c, J. Barrio^b,
S. Fernández^c, M. González-Sagrado^d, F. Pineda^e, R. Palacios^e

^a Servicio de Alergia, Hospital Universitario Río Hortega, Spain

^b Servicio de Gastroenterología, Hospital Universitario Río Hortega, Spain

^c Servicio de Pediatría, Hospital Universitario Río Hortega, Spain

^d Unidad de apoyo a la Investigación, Hospital Universitario Río Hortega, Spain

^e Laboratorios Diater, Madrid, Spain

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Abstract

Background: There has been an increase in the prevalence of hypersensitivity to *Anisakis simplex*. There are fish parasites other than *Anisakis simplex* whose allergenicity has not yet been studied.

Objective: To assess IgE hypersensitivity caused by fish parasite allergens in patients with gastro-allergic symptoms after consumption of fish, shellfish or cephalopods, compared with healthy subjects, pollen allergic individuals and children with digestive symptoms after eating marine food.

Methods: We carried out in vivo tests (skin prick) and in vitro tests (specific IgE determination, Western blot) and component resolved diagnostics (CRD) using microarray analysis in all patients.

Results: CRD better detected sensitisation to allergens from marine parasites than skin prick tests and determination of specific IgE by CAP. Sensitisation to *Gymnorhynchus gigas* was detected in 26% of patients measured by skin prick tests and 36% measured by IgE.

Abbreviations: CRD, component resolved diagnostics; rAni s3, *Anisakis* tropomyosin; rAni s1, *Anisakis* serinprotease inhibitor; rCyp c1, carp parvalbumin; rGad s1, cod parvalbumin; nPen i 1, prawn tropomyosin; rDer p 10., *Pteronyssinus* tropomyosin; nBla g7, cockroach tropomyosin; Groups 1, 4, 5 and 6 of pollens, (Pol. Group 1, 4, 5, 6).; nDer p1 and Der p2, Cysteine-proteases of *Dermatophagoides pteronyssinus* and *farinae*, respectively.; RAlt a 1, acidic glycoprotein of *Alternaria alternata*; nApi m1, phospholipase A2 of apis venom; Ves v5, phospholipase A2 of yellow jacket venom; rAni s1, serin-protease inhibitor of *Anisakis simplex*; nPrup3, peach lipid transfer protein; rCor a 8, hazelnut lipid transfer protein; nArt v3, mugwort lipid transfer protein; Prof, pollen profilin; Prol, pollen polcalcin; CCD, cross-reactivity carbohydrate determinants.

* Corresponding author.

E-mail address: aliciaarmentia@gmail.com (A. Armentia).

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Conclusions: The prevalence of hypersensitivity to marine parasite allergens other than *Anisakis simplex* should be studied, and the most appropriate technique for this is CRD.
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Introduction

There are many unknown allergen sources, including various fish parasites other than *Anisakis simplex*, whose allergenicity has not yet been studied and which should be investigated to maintain legislation on food information up to date.¹

Fish and fish products occupy a prominent place in the human diet, and provide proteins, minerals and vitamins. However, due to globalisation and immigration, new exotic fruits, vegetables and fish are now included in our diets and their allergenicity should be investigated and updated.

Most fish and shellfish for consumption are often infested by certain species of parasites that could produce parasitosis and/or allergic reactions. The main groups of parasites affecting fish are protozoa, helminths (trematodes, tapeworms, acanthocephalan, nematodes) and arthropods.^{1–8}

The best known marine parasite so far is *A. simplex*, a nematode that is distributed worldwide and may infect consumers of raw, undercooked, salted, smoked and marinated fish, resulting in anisakiasis, a disease caused by an inflammatory reaction of the mucosa of the gastric wall when penetrated by *A. simplex* larvae. The most common symptoms are abdominal pain, nausea, vomiting and intestinal rhythm disorders.⁹ Fortunately, this can be avoided with correct freezing and cooking of fish before consumption.⁸

However, hypersensitivity reactions to *A. simplex* after a first contact with the parasite and subsequent sensitisation (synthesis of specific IgE to heat-resistant antigens of *A. simplex* larvae) may occur. These reactions are immediate and may present with pruritus, urticaria, angio-oedema and even anaphylactic shock.^{1–8} Cases of occupational disease (asthma and conjunctivitis) in fishmongers, *A. simplex* allergy after eating chicken fattened with feed from fish viscera, contact dermatitis and joint pain after exposure to *A. simplex* have also been reported.^{10–13}

In addition, there have been reports of mixed symptoms, characterised by allergic (urticaria and/or angio-oedema) and gastrointestinal symptoms, known as gastroallergic anisakiasis.³

Recently, an increasing prevalence of hypersensitivity to *A. simplex* has been observed despite preventive measures such as freezing fish.^{1,3,6,14,15} There are other common fish parasites, such as *Gymonorhynchus gigas* and *Kudoa*⁷ whose allergenicity has not yet been studied and which could cause allergic symptoms (Fig. 1).

A. simplex is an elongated, cylindrical nematode with pointed ends, whose larvae are whitish and 7–30 mm in length and are usually found coiled up in the musculature of most fish (cod, whiting, haddock, hake, mackerel, tuna, mackerel, halibut, salmon, sardines, herring, megrim, anglerfish, conger eels, etc.). In contrast, *G. gigas* is a dark white, almost transparent cestode which, with age, becomes bigger, more yellowish and more opaque. It is found primarily in the musculature of pomfret and hake.⁷

Most patients with gastrointestinal and/or allergic symptoms after eating fish present specific IgE skin tests to *A. simplex*. However, although these tests using full *Anisakis* extracts have a high sensitivity, their specificity is low as they do not identify the causal protein or whether it is heat sensitive.¹⁶ Molecular analysis (microarrays or component resolved diagnosis [CRD]) that detects specific allergens of *A. simplex* increases diagnostic specificity.¹⁷ The main recombinant allergens detected using these techniques are: r Ani s1 (serine protease inhibitor, the salivary enzyme of *Anisakis*) which is sensitive to freezing and r Ani s3 (tropomyosin, the muscular enzyme of *Anisakis*) which is heat stable.

In the case of sensitisation to Ani s1 (whose allergenicity partly disappears after correct freezing), freezing fish at –20 °C for 72 h is recommended. However, if sensitisation is to somatic antigens such as Ani s3, it would be prudent to recommend the exclusion of fish, seafood and cephalopods from the diet as this is a heat-stable, gastro-resistant protein which resists freezing and cooking and the gastric juices. Therefore, recommendations to patients allergic to *A. simplex* should be made according to the protein to which they are sensitised.

Since provocation techniques in foods infested by marine parasites are risky, and therapeutic measures used until now do not resolve the symptoms of gastroallergic anisakiasis (which causes severe deterioration in the quality of life), we sought to carry out an allergic analysis and a molecular diagnosis of allergens to the most common fish parasites.

The aim of this study was to assess IgE hypersensitivity caused by allergens of fish parasites in patients with gastroallergic symptoms after consumption of fish, shellfish or cephalopods, compared with healthy subjects, pollen allergic individuals and children with digestive symptoms after eating marine food.

Material and methods

Patients

We carried out a 12-month transversal observational study in 203 patients.

- Group 1: Patients with major digestive symptoms and allergic symptoms (rhinitis, urticaria, asthma or anaphylaxis) triggered after ingestion of a food of marine origin. In one year we attended 78 patients with these symptoms of whom 50 were randomly selected.
- Group 2: Healthy blood donors (non-smokers, not exposed to tobacco). We randomly selected 50 subjects from the Blood Donation Unit, SACYL, which rejects patients with any clinical allergy.



Figure 1 *Gymnorhynchus gigas*.

- Group 3: Patients with the most common allergic picture in our area (hypersensitivity to grass pollens) to determine whether existing atopy could influence the responsiveness to marine nematodes. Sensitisation to pollens was defined as the presence of: (a) ≥ 1 positive skin tests to pollens, (b) a CAP (IgE) positive > 0.35 IU/mL to these allergens or (c) positive specific challenge. Fifty patients were randomly selected.
- Group 4: Children not yet tested for allergies studied by our paediatric service with clinical symptoms of possible hypersensitivity after the ingestion of fish. We randomly selected 53 children. The reason was to include children who usually eat fish of species other than those consumed by adults (filleted, spineless, surimi paste derivatives) in order to investigate the presence of parasites.
- All patients were considered to have been exposed to similar levels of pollen, pollution and other environmental factors, as confirmed by weekly analysis of environmental quality standards by the Environmental Health Control Section of the Ministry of Health of our region (SACYL). All patients followed a standard Mediterranean diet, verified by nutritional surveys.

To calculate the sample size, an alpha risk of 0.05 and a beta risk of 0.2 in a bilateral contrast was accepted. Forty-eight subjects were required in each group to detect a minimum difference of eight between the two groups, assuming that there were four groups, and a standard deviation of 10. The estimated loss to follow up was 20%.

The study was approved by the Clinical Research Ethics Committee of the HURH and all patients who participated provided signed informed consent.

Methods

History and examination: In addition to signs and symptoms, we investigated the possible causal foods and any other factor (medicines, exercise, the manner of cooking of fish, condiments used) that could have influenced the clinical presentation.

Skin prick tests were performed with commercial allergens and with purified proteins of native (n) and recombinant (r) allergens. These tests were performed according to the standards of the European Academy of Allergy and

Clinical Immunology: After placing a drop of each allergen in the anterior forearm, a small puncture that did not reach the dermis was made through the drop with a lancet. The result was read after 15 min. A wheal with a diameter ≥ 3 mm was considered positive. Each allergen was tested twice.

A standard battery of aeroallergens and foods including pollen (trees, grasses, weeds and flowers), mites (*Dermatophagoides* and storage mites), fungi, antigens to animals and common foods (ALK-Abelló, Madrid, Spain), and some main drug classes (antibiotics, non-steroidal analgesics, omeprazole). Extracts of *A. simplex* and *G. gigas* were added to this battery.

The extract of *G. gigas* was prepared from parasites of pomfret (*Brama Brama*) purchased at a local fishmonger. After zoological identification of the parasite, proteins were extracted using the following method: water soluble extraction was made in PBS (phosphate buffered saline) in a cold chamber. The mixture was centrifuged at 5000 rpm for 30 min at 4°C and the supernatant was dialysed by soft magnetic stirring overnight, keeping the pH at 7.5–8.5. The supernatant was filtered through a 0.45 μ m filter and dialysed in a 3500 dalton cassette overnight. Finally, the material was lyophilised and the extraction was completed using the intermediate product obtained from the lyophiliser.

Specific IgE: specific IgE was determined for all allergens by radioimmunoassay using the CAP system (Thermo Fisher Scientific®, Uppsala, Sweden). Results > 0.7 kU/l were considered positive.

CRD was performed on serum using the ISAC 112 panel (Thermo Fisher Scientific®, Uppsala, Sweden) according to the manufacturer's instructions. The response to 112 proteins of different origins was measured and the possible response due to cross-reactivity with other allergens (see abbreviations) was assessed. This technique includes the recombinants r Ani s1 (*Anisakis* serinprotease inhibitor) and r Ani s3 (tropomyosin of *Anisakis*), and other tropomyosins of prawns, cockroaches, and parvalbumins from cod and carp.

Immunoblotting, Western blot: Protein allergen extracts of *G. gigas* and *A. simplex* were transferred to a PVDF membrane for possible IgE-mediated recognition after being incubated with serum from patients with suspected allergy to *G. gigas* and serum of patients diagnosed with allergy to *A. simplex* (positive controls). Sera from patients not sensitised to either source were used as negative controls.

Statistical analysis: The statistical analysis was performed using the SPSS version 15.0 statistical package. The chi-square test was used to analyse qualitative data. The Student *t* test for non-paired data and ANOVA test were used to compare mean values of parametric data. Fisher's exact test was used when the number of cells with expected values ≤ 5 was greater than 20%. Non-parametric data were analysed using the Mann-Whitney test (for two groups) or the Kruskal-Wallis *H* test (for more than two groups).

Clinical evolution after the diagnosis: Patients were advised to avoid the causative allergen identified. In the case of heat-stable marine allergens, total exclusion of any food from the sea was recommended. Conventional depot immunotherapy (ALK-Abelló, Denmark) was initiated in patients in whom CRD showed hypersensitivity to allergens for which there is effective immunotherapy. The treatment was maintained during one year and its

Table 1 Clinical data and source of allergens.

(a) Demographic and clinical data according to study group						
		Group 1	Group 2	Group 3	Group 4	p-Value
Sex	Female	18 (36%)	15 (30%)	23 (46%)	12 (22.6%)	0.081
	Male	32 (64%)	35 (70%)	27 (54%)	41 (77.4%)	
Age (DS)		31.62 ± 11.482	25.82 ± 10.261	5.92 ± 4.415	25.94 ± 17.937	0.000
Rhinitis	n/%	26 (32%)	0 (0%)	30 (60%)	35 (66%)	0.000
Asthma	n/%	5 (10%)	0 (0%)	50 (100%)	53 (100%)	0.000
Urticaria	n/%	32(64%)	0 (0%)	1 (2%)	35 (66%)	0.000
Digestive symptoms	n/%	50 (100%)	0 (0%)	0 (0%)	2 (3.8%)	0.000
Pollen allergy	n/%	8 (16%)	0 (0%)	50 (100%)	2 (3.8%)	0.000
Anaphylaxis	n/%	18 (36%)	0 (0%)	1 (2%)	0 (0%)	0.000
Healthy	n/%	0 (0%)	50 (100%)	0 (0%)	0 (0%)	0.000
(b) Fish and cofactors related to positive symptoms						
	Group 1	Group 2	Group 3	Group 4	p-Value	
Hake	5(10%)	1 (2%)	1 (2%)	0 (0%)	0.032	
Blue whiting	8 (16%)	1 (2%)	0 (0%)	0 (0%)	0.000	
Sole	2 (4%)	1 (2%)	0 (0%)	5 (9.4%)	0.053	
Panga	8 (16%)	1 (2%)	0 (0%)	3 (5.7%)	0.002	
Sardines	10 (20%)	2 (4%)	0 (0%)	0 (0%)	0.000	
Tuna	7 (14%)	0 (0%)	0 (0%)	0 (0%)	0.000	
Trout	1 (2%)	0 (0%)	0 (0%)	0 (0%)	0.421	
Salmon	1 (2%)	0 (0%)	0 (0%)	0 (0%)	0.421	
Pomfret	8 (16%)	0 (0%)	0 (0%)	0 (0%)	0.000	
Prawn	2 (4%)	0 (0%)	0 (0%)	0 (0%)	0.203	
Squid	1 (2%)	0 (0%)	0 (0%)	0 (0%)	0.515	
Cuttlefish	2 (4%)	0 (0%)	0 (0%)	0 (0%)	0.203	
Surimi	5(10%)	0 (0%)	0 (0%)	3 (5.7%)	0.002	
NSAIDs	6 (12%)	0 (0%)	2 (4%)	1 (5.8%)	0.019	

NSAIDs: non-steroidal anti-inflammatory drugs.

effectiveness was evaluated in Spring–Summer of the following year. Patients were considered to have a favourable evolution if clinical signs and symptoms improved or disappeared.

Results

Descriptive study

The overall mean age of our sample was 25.94 ± 17.94 years and 5.92 ± 4.42 years in children. The digestive symptoms mainly affected males (adults 64% and children 77%).

Patients in group 1 had a significant risk ($p < 0.0001$) of anaphylaxis. In group 1, 18 patients (36%) were attended by the outpatient clinic due to digestive and allergic symptoms (rhinitis and/or asthma) or digestive symptoms and urticaria. The remaining 32 (64%) patients went to the emergency room within the first year with anaphylaxis after ingesting seafood (fish 82%). In group 4, 51 (96.2%) children had digestive symptoms, 35 (66%) rhinitis and urticaria, and all had episodes of childhood asthma, but not anaphylaxis.

Table 1 shows the signs and symptoms and the possible cause. The most common causes in adults were

sardines and/or anchovies (ten patients, 20%), blue whiting (eight patients, 16%), panga (*Pangasianodon hypophthalmus*) (eight patients, 16%) and pomfret (eight patients 16%), while 82% of patients reported having eaten frozen fish. Twelve per cent of patients with digestive symptoms and two patients with pollen asthma were taking NSAIDs.

In group 4, symptoms were associated primarily with panga (three patients, 5.7%) and sole (five children, 9.4%). In all cases the fish was frozen.

Molecular microarray analysis

In group 1, the most frequent allergens identified were Ani s1 (34, 68%), group 1 grass pollens (16, 32%), and Ani s3 (14, 28%). No sensitivity to fish parvalbumins was observed, but 10 (20%) patients were positive to the allergen rPol d 5 (antigen 5 of the European paper wasp (*Polistes dominula*)) and this was significant compared with the other groups. Although this is the predominant wasp in our area, no patients reported previous stings.

Two patients in group 2 were sensitive to Ani s1.

In group 3 the most frequent allergens identified were grass pollens group 1 (72%), group 2 (68%), and groups 4 and

Table 2 Positivity to specific allergens in component-resolved diagnosis.

	Group 1	Group 2	Group 3	Group 4	p-Value
Pol1	16 (32%)	1 (2%)	36 (72%)	8 (15.1%)	0.000
Pol2	4 (8%)	0 (0%)	34 (68%)	0 (0%)	0.000
Pol4	1 (2%)	0 (0%)	21 (42%)	0 (0%)	0.000
Pol5	3 (6%)	0 (0%)	6 (12%)	4 (7.5%)	0.031
Pol6	3 (6%)	0 (0%)	14 (28%)	0 (0%)	0.000
Cynd1	11 (22%)	0 (0%)	17 (34%)	1 (1.9%)	0.000
Dp1	7 (14%)	0 (0%)	1 (2%)	3 (5.7%)	0.007
Dp2	7 (14%)	1 (2%)	1 (2%)	3 (5.7%)	0.046
Alt1	8 (16%)	0 (0%)	2 (4%)	5 (9.4%)	0.004
Apim1	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.000
FAv5	10 (20%)	0 (0%)	0 (0%)	0 (0%)	0.002
Dog	3 (6%)	0 (0%)	0 (0%)	2 (3.8%)	0.066
Cat	9 (18%)	0 (0%)	1 (2%)	4 (7.5%)	0.001
Anis1	34 (68%)	2 (4%)	3 (6%)	0 (0%)	0.000
Anis3	14 (28%)	0 (0%)	1 (2%)	6 (11.3%)	0.000
nPen i1	3 (6%)	0 (0%)	0 (0%)	2 (3.8%)	0.066
rDer p10	7 (14%)	1 (2%)	1 (2%)	3 (5.7%)	0.007
rCyp c1	0 (0%)	0 (0%)	0 (0%)	1 (2%)	0.000
rGad s1	0 (0%)	0 (0%)	0 (0%)	1 (2%)	0.000
nBla g7	3 (6%)	0 (0%)	0 (0%)	2 (3.8%)	0.066
Art3	6 (12%)	0 (0%)	2 (4%)	7 (13.2%)	0.007
ProfT	3 (6%)	0 (0%)	2 (4%)	2 (3.8%)	0.224
ProfL	7 (14%)	0 (0%)	2 (4%)	2 (3.8%)	0.011
ProfG	1 (2%)	0 (0%)	7 (14%)	1 (1.9%)	0.004
PolT	0 (0%)	0 (0%)	1 (2%)	1 (1.9%)	0.434
PolG	0 (0%)	0 (0%)	3 (6%)	1 (1.9%)	0.081
CCD	2 (4%)	0 (0%)	0 (0%)	2 (3.8%)	0.138
PR_hazel	5 (10%)	0 (0%)	3 (6%)	0 (0%)	0.007
PR_apple	0 (0%)	0 (0%)	3 (6%)	0 (0%)	0.000
PR_pea	0 (0%)	0 (0%)	0 (0%)	1 (1.9%)	0.440
Soy	0 (0%)	0 (0%)	0 (0%)	1 (1.9%)	0.440
PR_peanut	0 (0%)	0 (0%)	1 (2%)	1 (1.9%)	0.434
Kiwi	2 (4%)	1 (2%)	1 (2%)	5 (9.4%)	0.245
Walnut	7 (14%)	0 (0%)	3 (6%)	3 (5.7%)	0.016
Egg	2 (4%)	0 (0%)	0 (0%)	0 (0%)	0.129
Milk	3 (6%)	0 (0%)	1 (2%)	0 (0%)	0.077

6, and profilin (14%). No greater prevalence of sensitisation to marine allergens (Ani s1 6% and Ani s3 2%) was observed.

In group 4, the most frequent allergens identified were grass pollens group 1 (15%), lipid transporter proteins (13%), Ani s3 (11.3%), kiwi (9.4%) and hazelnut (5.7%). (Table 2).

Skin prick tests

The most frequent positive tests in group 1 were grass pollens (14 patients, 28%), *Anisakis* (14 patients, 28%), *G. gigas* (13 patients, 26%), and latex (seven patients, 14%).

In group 3 the most frequent positive tests were *Lolium* pollens (44 patients, 88%), *cynodon* (20 patients, 40%) and only two patients had positive IgE to *Anisakis*.

In group 4, there were positive tests to wheat, peach, fish and tomato in one patient each (Table 3).

Allergen specific IgE

IgE testing significantly ($p < 0.0001$) detected *Anisakis* (24 patients, 48%) and *G. gigas* (18 patients, 36%) in group 1 and in seven children (13.2%). There was no sensitisation to these parasites in groups 2 and 3 (Table 4).

Determination by Western blot of the proteins present in allergenic extracts in sera from patients diagnosed with allergy to *G. gigas* identified proteins 18, 27, 37, 40 and 50 kDa of *G. gigas* but no protein of *A. simplex*. In sera from patients with allergy to *A. simplex*, Western blot identified various proteins of *A. simplex* of different molecular weights but no proteins of *G. gigas* (Fig. 2).

Comparing the three diagnostic techniques employed (prick, IgE and CRD), CRD showed greater sensitivity and specificity than prick tests and specific IgE for the diagnosis of hypersensitivity to *Anisakis* (Table 5).

Table 3 Positive skin prick tests.

	Group 1	Group 2	Group 3	Group 4	p-Value
Prick <i>lolium</i>	14 (28%)	2 (4%)	44 (88%)	3 (5.7%)	0.000
Prick <i>cynodon</i>	9 (18%)	1 (2%)	20 (40%)	3 (5.7%)	0.000
Prick <i>olea</i>	5 (10%)	0 (0%)	14 (28%)	0 (0%)	0.000
Prick <i>platanus</i>	3 (6%)	0 (0%)	12 (24%)	0 (0%)	0.000
Prick <i>arizonica</i>	2 (4%)	0 (0%)	4 (8%)	0 (0%)	0.024
Prick <i>phleum</i>	1 (2%)	0 (0%)	16 (32%)	0 (0%)	0.000
Prick <i>artemisia</i>	1 (2%)	0 (0%)	7 (14%)	0 (0%)	0.001
Prick <i>chenopodium</i>	2 (4%)	0 (0%)	9 (18%)	0 (0%)	0.000
Prick dog	1 (2%)	1 (2%)	0 (0%)	1 (1.9%)	0.634
Prick cat	3 (6%)	0 (0%)	2 (4%)	1 (1.9%)	0.198
Prick rabbit	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0.421
Prick horse	1 (2%)	1 (2%)	0 (0%)	1 (1.9%)	0.634
Prick <i>d.pterony</i>	5 (10%)	0 (0%)	1 (2%)	0 (0%)	0.008
Prick <i>d.farinae</i>	4 (8%)	1 (2%)	1 (2%)	0 (0%)	0.086
Prick <i>lepidoglyph</i>	7 (14%)	0 (0%)	1 (2%)	0 (0%)	0.001
Prick <i>alternaria</i>	2 (4%)	0 (0%)	2 (4%)	0 (0%)	0.125
Prick <i>cladospo</i>	0 (0%)	0 (0%)	2 (4%)	0 (0%)	0.129
Prick aspergill	0 (0%)	0 (0%)	2 (4%)	0 (0%)	0.129
Prick wheat	2 (4%)	0 (0%)	2 (4%)	1 (1.9%)	0.334
Prick barley	2 (4%)	0 (0%)	2 (4%)	0 (0%)	0.125
Prick rye	2 (4%)	0 (0%)	2 (4%)	0 (0%)	0.125
Prick white egg	0 (0%)	0 (0%)	2 (4%)	0 (0%)	0.129
Prick yolk	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Prick milk	2 (4%)	0 (0%)	1 (2%)	0 (0%)	0.200
Prick pea	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0.421
Prick peanut	0 (0%)	0 (0%)	2 (4%)	0 (0%)	0.129
Prick hazelnut	5 (10%)	0 (0%)	3 (6%)	0 (0%)	0.007
Prick chestnut	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0.421
Prick pine nut	1 (2%)	0 (0%)	2 (4%)	0 (0%)	0.200
Prick legume	1 (2%)	0 (0%)	2 (4%)	0 (0%)	0.200
Prick mustard	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0.421
Prick peach	10 (20%)	0 (0%)	3 (6%)	1 (1.9%)	0.000
Prick fish	1 (2%)	0 (0%)	0 (0%)	1 (1.9%)	0.434
Prick <i>anisakis</i>	14 (28%)	3 (6%)	2 (4%)	0 (0%)	0.000
Prick <i>Gymnor</i>	13 (26%)	0 (0%)	0 (0%)	0 (0%)	0.000
Prick shell	3 (6%)	0 (0%)	0 (0%)	0 (0%)	0.036
Prick tomato	0 (0%)	0 (0%)	2 (4%)	1 (1.9%)	0.210
Prick melon	1 (2%)	0 (0%)	1 (2%)	0 (0%)	0.415
Prick banana	1 (2%)	0 (0%)	0 (0%)	0 (0%)	0.421
Prick almond	0 (0%)	0 (0%)	2 (4%)	0 (0%)	0.129
Prick apple	0 (0%)	0 (0%)	2 (4%)	0 (0%)	0.129
Prick chicken	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Prick parsley	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0.421
Prick pistachio	2 (4%)	0 (0%)	0 (0%)	0 (0%)	0.129
Prick cashew	1 (2%)	0 (0%)	1 (2%)	0 (0%)	0.415
Prick latex	7 (14%)	0 (0%)	0 (0%)	0 (0%)	0.000

Evolution after aetiological treatment

Treatment consisted of avoiding the food allergens detected (fish, shellfish and cephalopods in the case of fish parasites) and immunotherapy directed at positive allergens. 74% of group 1 patients, 72% of group 3 patients and 52.8% of group 4 patients evolved favourably. The improvement was based on a score that included a quality of life questionnaire, the absence of symptoms and no requirement for medication.¹⁸

Discussion

There has been an increase in the prevalence of hypersensitivity to *A. simplex*, together with that of still-unknown marine parasites causing allergy.¹⁻⁶ The allergenicity of most of these parasites has not been studied in humans, even though the consumption of fish is very high worldwide. This is the case of *G. gigas*, a very common marine parasite that has been studied in animals but not in humans.⁷

Table 4 Positive allergen-specific IgE.

	Group 1	Group 2	Group 3	Group 4	p-Value
IgE lolium	4 (8%)	0 (0%)	25 (50%)	4 (7.5%)	0.000
IgE cynodon	5 (10%)	0 (0%)	12 (24%)	5 (9.4%)	0.002
IgE Eolea	1 (2%)	0 (0%)	6 (12%)	1 (1.9%)	0.012
IgE platanus	0 (0%)	0 (0%)	6 (12%)	0 (0%)	0.001
IgE arizonica	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0.421
IgE phleum	1 (2%)	0 (0%)	1 (2%)	1 (1.9%)	0.000
IgE artemisia	0 (0%)	0 (0%)	4 (8%)	0 (0%)	0.009
IgE chenopodium	0 (0%)	0 (0%)	4 (8%)	0 (0%)	0.009
IgE dog	0 (0%)	0 (0%)	0 (0%)	1 (1.9%)	0.440
IgE cat	0 (0%)	0 (0%)	1 (2%)	2 (3.8%)	0.221
IgE rabbit	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0.421
IgE horse	0 (0%)	0 (0%)	0 (0%)	1 (1.9%)	0.440
IgE pterony	1 (2%)	0 (0%)	1 (2%)	0 (0%)	0.415
IgE farinae	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0.421
IgE pidoglyph	2 (4%)	0 (0%)	1 (2%)	0 (0%)	0.200
IgE alternaria	2 (4%)	0 (0%)	2 (4%)	1 (1.9%)	0.334
IgE cladosporium	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
IgE asperg	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
IgE wheat	0 (0%)	0 (0%)	1 (2%)	1 (1.9%)	0.434
IgE barley	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
IgE Erye	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
IgE white	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
IgE yolk	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
IgE milk	2 (4%)	0 (0%)	1 (2%)	0 (0%)	0.200
IgE pea	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
IgE peanut	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0.421
IgE hazelnut	1 (2%)	0 (0%)	4 (8%)	0 (0%)	0.026
IgE chestnut	1 (2%)	0 (0%)	1 (2%)	0 (0%)	0.415
IgE pinenut	0 (0%)	0 (0%)	2 (4%)	0 (0%)	0.129
IgE legume	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0.421
IgE mustard	0 (0%)	0 (0%)	1 (2%)	1 (1.9%)	0.434
IgE peach	2 (4%)	0 (0%)	7 (14%)	1 (1.9%)	0.006
IgE fish	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
IgE anisakis	24 (48%)	2 (4%)	2 (4%)	0 (0%)	0.000
IgE Gymnor	18 (36%)	0 (0%)	0 (0%)	7 (13.2%)	0.000
IgE shellfish	2 (4%)	0 (0%)	0 (0%)	0 (0%)	0.129
IgE lettuce	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0.421
IgE tomato	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0.421
IgE melon	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0.421
IgE banana	1 (2%)	0 (0%)	0 (0%)	0 (0%)	0.421
IgE almond	0 (0%)	0 (0%)	2 (4%)	0 (0%)	0.129
IgE apple	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0.421
IgE chicken	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
IgE parsley	0 (0%)	0 (0%)	2 (4%)	0 (0%)	0.129
IgE pistachio	1 (2%)	0 (0%)	0 (0%)	0 (0%)	0.421
IgE Latex	7 (14%)	0 (0%)	0 (0%)	0 (0%)	0.036

Since provocation techniques in foods infested by marine parasites are risky, and therapeutic measures used until now do not resolve the symptoms of gastroallergic anisakiasis (which causes severe deterioration in the quality of life), we sought to make an allergic analysis and a molecular diagnosis of allergens to the most common fish parasites.

A 2001 study by Vazquez-Lopez in experimental animals found that the pleocercoides larvae of *G. gigas* had a major allergen component.⁷ Twenty days after mice were orally inoculated with a crude extract there was a significant

increase in serum IgE together with a significant accumulation of eosinophils 14–48 h after intraperitoneal inoculation. This suggests that ingestion of the parasite could cause of some of the allergic reactions which occur after eating certain fish and/or seafood.

The main objective of our study was to assess allergic IgE-mediated hypersensitivity to allergens of marine parasites including *A. simplex* and *G. gigas* by determining specific IgE and molecular analysis in patients with gastroallergic symptoms following consumption of fish, shellfish or cephalopods

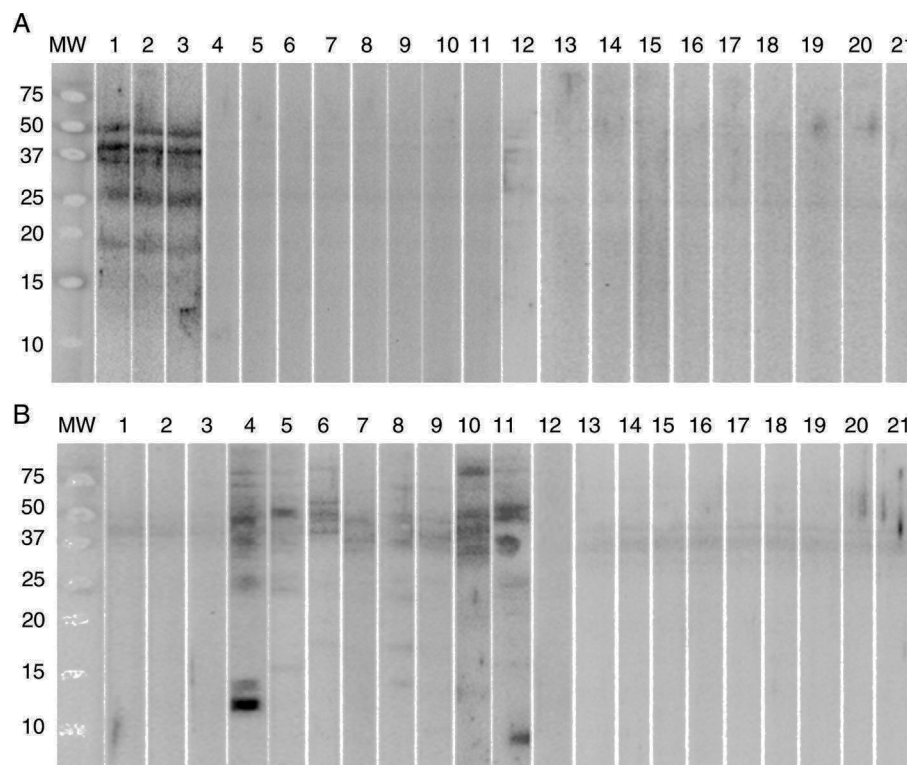


Figure 2 Western blot. (A) Extract of *G. gigas* transferred to membrane. (B) Extract of *A. simplex* transferred to membrane. MW, Marker of molecular weight. Lanes 1–3: patients positive to *G. gigas*. Lanes 4–12: patients positive to *A. simplex*. Lanes 13–21: Negative controls.

Table 5 Sensitivity and specificity of diagnostic methods.

Diagnostic method	Group 1 <i>n</i> = 50	Group 4 <i>n</i> = 53	Sensitivity*	Specificity*
Prick positivity <i>Anisakis simplex</i>	14	0	28% (14.55–41.45)	96% (89.57–100)
IgE positivity <i>Anisakis simplex</i>	24	0	48% (33.15–62.85)	96% (89.57–100)
Prick positivity <i>G. gigas</i>	13	0	26% (15.08–40.61)	96% (91.58–99.83)
IgE positivity <i>G. gigas</i>	18	7	36% (23.28–90.86)	86.79 (74.05–99.09)
Ani s1 positive	34	0	68% (66.92–69.08)	96% (94.97–97.03)
Ani s3 positive	14	6	48% (26.92–29.08)	100% (99–100)

* 95% confidence intervals.

compared with healthy subjects, patients with pollen allergies and allergic children with digestive symptoms.

Our results, in agreement with other reports,^{16,17} show that molecular microarray analysis is better detects sensitisation to allergens of marine parasites, detecting 20 more patients than prick tests and 10 more than measurement of CAP by specific IgE.

Provocation tests of foods infested by marine nematodes are not risk free. Likewise, the therapeutic measures used until have not cured the symptoms of gastroallergic anisakiasis.¹⁴ An aetiological diagnosis made by CRD, avoidance measures and targeted immunotherapy resulted in

improvements in at least half of our patients within a few months.

A significant percentage of patients who suffer clinical symptoms of allergy (urticaria, asthma) after eating fish have hypersensitivity to marine parasites, which is due not only to heat-sensitive allergens such as serine protease Ani s1 but also to heat-stable allergens such as Ani s3, which are not altered by freezing, meaning that freezing fish is not sufficient.

In children, sensitivity was especially to Ani s3 and was related to the ingestion of panga and sole, boneless white fish that are suitable for children's diets. In adults,

the most common source was anchovy, blue whiting and hake.

In patients with polypharmacy, allergic symptoms tend to be attributed to the effect of drugs rather than to a hypersensitivity reaction caused by marine allergens, whose consumption may increase as fish is assumed to be more digestible in the sick. Anaphylactic reactions followed ingestion of NSAIDs in 12% of patients, suggesting this may act as a cofactor. These reactions always occurred in patients who had previously suffered digestive symptoms.

Sensitisation to *G. gigas* affected 26% of patients measured by skin prick and 36% measured by IgE, although this does not rule out possible cross-sensitisation by common amino acid sequences. *G. gigas* was most often found in pomfret, while *Anisakis* was found most often in anchovies and blue whiting. Therefore, a useful preventive measure in patients with these symptoms would be to avoid these fish.

In conclusion, our study suggests that the prevalence of hypersensitivity to allergens to marine parasites should be studied and that molecular diagnostic microarrays are the most suitable diagnostic technique.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Author contribution

I declare that all the authors have participated in the conception, design of the study, analysis and interpretation of the data. All authors should have made substantial contributions to all of the following: in the conception and design of the study (Armentia and Santos), acquisition of data (Martín, Fernandez), analysis and interpretation of data (Pineda, Martín, Palacios, González), drafting the article or revising it critically for important intellectual content (Barrio, Armentia) and final approval of the version to be submitted (all authors).

All authors have participated in the preparation and critical revision of the letter and all authors have seen and approved the final version of the manuscript. I also declare that any authors have no conflict of interest in connection with this paper, other than any noted in the covering letter to the editor.

Ethical disclosures

Confidentiality of data. The authors declare that they have followed the protocols of their work centre on the publication of patient data and that all the patients included in the study have received sufficient information and have given their informed consent in writing to participate in that study.

Right to privacy and informed consent. The authors have obtained the informed consent of the patients and/or

subjects mentioned in the article. The author for correspondence is in possession of this document.

Protection of human subjects and animals in research.

The authors declare that the procedures followed were in accordance with the regulations of the responsible Clinical Research Ethics Committee and in accordance with those of the World Medical Association and the Helsinki Declaration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aller.2016.12.008>.

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