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Possible Allergenic Role of Tropomyosin in Patients with Adverse Reactions after Fish Intake

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ABSTRACT

In a recent case report, patient's anti-fish tropomyosin IgE was associated with gastrointestinal symptoms. We aimed to demonstrate on a wider scale that the panallergen tropomyosin should not be limited to invertebrate species and that clinically relevant reactions could be elicited by vertebrate tropomyosin. On the whole, 19 patients with adverse reactions after fish intake and showing negative skin tests with commercial fish extracts were included. Fish tropomyosin was recognized by 10/19 patients' IgE by immunoblotting. All patients with gastrointestinal complaints after fish intake (6/6) showed an IgE band matching with tropomyosin. Cod, albacore, and swordfish tropomyosins were recognized by most patients although 3/10 patients did not claim adverse reactions to these fish species. Immunoblotting with a battery of antigens from different fish species have a high yield of positivity at a band matching with tropomyosin molecular weight, even if they have not been claimed to be causative agents of symptoms. Tropomyosin is therefore a good candidate to be investigated as a clinically relevant fish allergen in patients who report adverse reactions after fish intake.

KEYWORDS

Albacore; allergen; cod; fish; swordfish; tropomyosin; vertebrates

Introduction

Tropomyosins are a family of alpha-helix folded proteins with numerous isoforms in muscle and non-muscle cells of animals and fungi, not being documented in prokaryotes, protists, and plants (Geeves et al., 2015). Tropomyosin is considered an invertebrate panallergen because it can be recognized by human IgE whenever it comes from any species of invertebrate (Reese et al., 1999). Vertebrate tropomyosins have classically been considered as non-allergenic. Tilapia tropomyosin is the only one characterized as an allergen (Liu et al., 2013) and included in the IUIS/WHO Allergen Nomenclature Database (www.allergen.org) so far.

When working up with suspected adverse reactions associated with fish consumption, allergic and non-allergic mechanisms have to be addressed. Food allergy is found in the

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latest reviews as an emerging disease of industrialized countries, as is the special case of fish (Kuehn et al., 2014; Mourad and Bahna, 2015) with a variation in prevalence ranging from 0% to 2% considering the cases of self-reported fish allergy or less than 0.5% considering only symptomatic and sensitized subjects (Rona et al., 2007).

Other possible causes of adverse symptoms after fish intake are: intolerance reactions, diminished available diamine-oxidase production (Daschner et al., 2015), or underlying digestive abnormalities, such as irritable bowel syndrome or inflammatory bowel disease (IBD) (Prester, 2015). In fact, 6 out of 10 patients studied to characterize tilapia tropomyosin as an allergen were diagnosed with IBD (Liu et al., 2013). In our region, *Anisakis* sensitization is frequent and fish-intake-associated symptoms have to rule out parasitism by this nematode.

In food allergy, double-blind, placebo-controlled food challenge is the gold standard to evidence the patient's allergic reaction after the ingestion of the suspected food. However, in the normal clinical practice, *in vitro* tests such as specific IgE measurement and/or *in vivo* tests such as skin prick tests are evaluated together with a detailed anamnesis to demonstrate sensitization (Asero et al., 2009). These routine tests in fish allergy are not always straightforward and negative results do not rule out allergic mechanisms (Barbarroja-Escudero et al., 2016). Gastrointestinal symptoms may not be accompanied by the presence of positive skin prick tests or specific serum IgE (Chikazawa et al., 2015). A recent study showed reactivity against fish tropomyosin in a patient with only gastrointestinal symptoms. In this report, clinically relevant fish tropomyosin sensitization with gastrointestinal symptoms could be demonstrated in spite of negative skin prick tests (González-Fernández et al., 2016).

With the exception of this report, bibliographic work-up show that skin prick tests with fish extracts have not yielded suspicion of tropomyosin being an allergen in fish.

The purpose of the present work was to test the hypothesis that the panallergen tropomyosin is not limited to invertebrate species and that clinically relevant reactions could be elicited by vertebrate tropomyosin. We therefore sought to perform an exploratory investigation, where we included patients with allergic or gastrointestinal symptoms (see Material and Methods section for specifications) after fish intake in order to search for tropomyosin being a potential allergen in different clinical settings. Our hypothesis was first that at least some patients with shellfish allergy, where tropomyosin is a known major allergen (Khora, 2016), could evolve to react also against tropomyosin in fish, such as in a previous report (González-Fernández et al., 2016). Further, we explored also the possibility that some patients with repeated episodes of gastrointestinal symptoms after fish intake could be sensitized to tropomyosin.

For this purpose, we determined IgE immunorecognition patterns against a battery of fish species commonly consumed in our area and compared the molecular weights of the patients' IgE bands with the tropomyosin ones using a commercial monoclonal anti-tropomyosin antibody.

Material and methods

Patients and serum samples

A total of 19 adult (22–73 years old) patients were consecutively included in this study during three years when they had a history of at least two adverse reactions after fish

intake. These consisted of repeated allergic hypersensitivity symptoms after consumption of different fish and who already had allergic-type symptoms after shellfish consumption were included. We also included patients who complained of gastrointestinal symptoms repeatedly after consumption of at least two different fish species (Table 1).

Allergic hypersensitivity symptoms were defined as urticaria, pruritus, oral allergy syndrome, or bronchial asthma and gastrointestinal symptoms as abdominal pain, diarrhea, nausea, or vomiting.

As explained above, we included only patients who were negative for commercial fish extracts against the offending fish species in every case. If patients were sensitized against *Anisakis*, acute parasitism (gastric or gastro-allergic anisakiasis) accounting for the described adverse effects of fish was excluded by a cautious history as well as serial measurements of IgE, as previously proposed (Daschner et al., 1999). Further, even if tropomyosin has been shown to be a potential allergen of *Anisakis*, it is only a minor allergen accounting for only 0–13% of recognition rate in sensitized patients (Asturias et al., 2000). Thus, sensitization against *Anisakis* due to a previous gastro-allergic anisakiasis episode was not an exclusion criterion, if the new claimed health effects by fish intake were not due to new parasitic episodes. Further, *Anisakis*-sensitization-associated chronic urticaria is also endemic and patients were included with the described selection criteria, if ongoing urticaria was clinically exacerbated by fish consumption, but not by *Anisakis* parasitism, as they consumed only frozen or well-cooked fish. Written consent was obtained from all patients studied. The project was approved by the Ethics Committee of the University Hospital La Princesa, Madrid, Spain.

Table 1. Epidemiological and clinical characteristics of included patients.

Subject	Gender	Age	Shellfish symptoms	Shrimp IgE >0.35 kU/l	Fish symptoms	CU	<i>Anisakis</i>	
							Sensitisation	GAA
1	Female	40	URT		URT		X	X
2	Male	67	PRU		PRU		X	
3	Female	44	URT		URT		X	X
4	Male	23	URT		URT		X	
5	Female	33	URT+GI	X	URT+GI+OAS			
6	Female	30	URT+BA	X	URT		X	
7	Female	60	URT		URT		X	
8	Female	46	URT	X	URT	X _c	X	
9	Female	47	URT		URT	X	X	
10	Female	49	URT	X	URT	X	X	X
11	Female	73	URT	X	URT		X	
12	Female	22	URT		URT	X _c	X	X
13	Female	45	URT		PRU	X		
14	Female	62	URT		GI			
15	Male	48			GI		X	
16	Female	58			PRU+GI	X		
17	Female	46		X	URT+GI			
18	Female	36			GI			
19	Female	65			GI	X	X	

CU: chronic urticaria; GAA: gastro-allergic anisakiasis; URT: urticaria; PRU: pruritus; GI: gastrointestinal complaints; OAS: oral allergy syndrome; BA: bronchial asthma; subscript "c" indicates cold urticaria.

Shellfish allergy confirmed either by Skin Prick Test, Prick by Prick, or specific serum IgE against one of the following species: shrimp, crayfish, or clam.

Crude extract selection and preparation

The following fish species were selected because individual patients referred adverse reactions after eating at least two of them. We then decided to test these fish species as a whole also to the other included patients even if they did not had contact with them or did not have adverse reaction to them: raw muscle of *Merluccius merluccius* (European hake), *Dicentrarchus labrax* (European seabass), *Lophius piscatorius* (monkfish), *Gadus morhua* (Atlantic cod), *Lepidorhombus whiffiagonis* (megrim), *Xiphias gladius* (swordfish), *Thunnus alalunga* (albacore), *Engraulis encrasicolus* (European anchovy), and *Anisakis simplex sensu lato* third stage larvae. They were homogenized in Phosphate-Buffered Saline (PBS) [$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 2.2 mM; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 7.3 mM; NaCl, 146 mM] and sonicated six times for 10 s to break the cell membranes releasing the proteins. The homogenate was extracted in PBS at 4°C overnight and subsequently delipidized with n-hexane. After centrifugation at 6700 g at 4°C, the supernatant was dialyzed overnight in PBS at 4°C (Medicell Intl. Ltd., London, UK). The protein content was quantified by the Bradford protein assay (BioRad, München, Germany).

IgE, IgG4, IgA immunoblotting and tropomyosin identification

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as was previously described (Hames, 1986; Laemmli, 1970) using a Mini Protean® III cell (BioRad). The gels consisted of a 4% stacking gel and 12% separating gel. Crude extracts were dissolved in a sample buffer (50 mM Tris-HCl buffer, pH 8.6, containing 2% SDS, 20% glycerol, and 0.02% bromophenol blue) diluted 1:1 in electrode buffer (25 mM Tris, 192 mM glycine, pH 8.3). Samples were loaded (400 µg) and electrophoresis was performed for 2 h at a constant 100 V in Tris-glycine electrode buffer. Broad range molecular mass markers (10–250 kDa Kaleidoscope®, BioRad) were incorporated into each electrophoresis run. Following the SDS-PAGE of antigens, the protein bands were transferred onto a 0.22 µm pore size nitrocellulose membrane (Hybond® ECL, GE Healthcare, Amersham, UK) in a Mini Trans-blot® Electrophoretic Transfer Cell (BioRad) with 25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3. The transblot was carried out at a constant 100 V for 1 h. The membrane was blocked overnight at 4°C with PBS containing 5% non-fat dry milk, prior to immunorecognition by overnight incubation with the anti-tropomyosin monoclonal antibody (mAb) TM311 (mouse IgG1) (Sigma, St Louis, MO) at 1/500 in PBS plus 0.05% Tween 20 (PBS-Tween 20) containing 1% non-fat dry milk (this diluent was used for all antibody and sera dilutions). TM311 recognizes the epitope from the position 14 to 32 among tropomyosins from mammals, birds, fish, and some invertebrates (González-Fernández et al., 2016; Nicholson-Flynn et al., 1996). Nitrocellulose membranes were cut into strips. Each membrane strip was then washed with PBS-Tween 20 (3 × 5 min) and incubated for 2 h with goat anti-mouse IgG1 (γ1), horseradish peroxidase conjugate (HRP) (Molecular Probes Inc., Eugene, OR) at 1/500. To visualize bands, the nitrocellulose was washed with PBS-Tween 20 (3 × 5 min) and the strips were treated with a solution of 0.5 mg/ml of 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) with hydrogen peroxide 0.05% in PBS.

For IgE determination, after blocking step, strips were incubated for 3 h with sera diluted at 1/10. After that, nitrocellulose strips were washed with PBS-Tween 20

(3 × 5 min) and they were incubated with a murine mAb against human IgE (IgG1k, E21A11; INGENASA, Madrid, Spain) at 1/500 followed by the goat anti-mouse IgG1 HRP conjugate (see above) at 1/500.

For specific IgG4 and IgA determination, the serum was diluted at 1/25. An antihuman IgG4-HRP mAb (clone HP6025) (SouthernBiotech) at 1/500 or an antihuman IgA-HRP (Biosource, Camarillo, CA) at 1/1500 was used. Finally, all the strips were equally treated with DAB solution (see above).

Negative controls served five adult subjects without any history of food allergy/intolerance or respiratory allergy recruited from the research department. In addition, blocked nitrocellulose strips of the different electrophoresed extracts were incubated with diluent (PBS-Tween 20 containing 1% non-fat dry milk) without serum, following the above-mentioned determinations for IgE, IgG4, or IgA.

Results

Clinical data

Table 1 summarizes data about the epidemiological and clinical characteristics of the included patients. During three years, we were able to prospectively include 19 patients with the noted criteria. On the whole, 16 were female, 3 were male. Mean age was 47 ± 14.5 years old.

A total of 14 patients belonged to the group of previous or concomitant allergic symptoms (urticaria, pruritus, gastrointestinal complaints, or bronchial asthma) after shellfish intake (patients 1–14); of these, five had specific serum IgE against shellfish.

Five other patients without previous history of shellfish allergy (patients 15–19) claimed of recurring gastrointestinal symptoms after fish intake. One patient claimed shellfish allergy was (subclinically) positive against shrimp.

Overall, 10 patients reported urticaria after fish intake, 2 patients claimed urticaria and gastrointestinal symptoms (one of them also oral allergy syndrome), 3 patients claimed of pruritus, and 4 patients had only gastrointestinal symptoms.

Seven patients had active chronic urticaria (two of them had cold urticaria) and reported exacerbation after fish intake, one of them claimed of gastrointestinal symptoms after fish intake.

A total of 13 patients were sensitized against *Anisakis*, whereas only 4 of them had a clear past history of gastro-allergic anisakiasis not related to the new complaints after fish intake and which could not be assigned to a new parasitic episode.

IgE determinations

Table 2 summarizes the specific symptoms to each fish and the presence of detected bands in IgE immunoblotting analysis, as well as possible anti-tropomyosin IgE for all the studied patients and fish species.

A total of 17 patients displayed at least one visible band and in 10 patients at least one band was detected between 25 and 37 kDa with identical molecular weight to the monoclonal anti-tropomyosin antibody. Otherwise, in only seven of them, these IgE bands were detected in fish species with claimed symptoms. This means that the possible

Table 2. Specified symptoms and detected bands in IgE immunoblotting analysis for all the studied patients and fish species.

Subject	Hake	Seabass	Monkfish	Cod	Megrim	Swordfish	Albacore	Anchovy
1			ORB				URT	
2	PRU			PRU TM+ORB	PRU	PRU		ND
3			ORB	TM+ORB		URT		
4	URT ORB			URT ORB	URT	URT		ND
5								URT+OAS*
6	URT	ORB					C	ND
7				URT TM+ORB				URT
8				URT ORB			ORB	ND
9				ORB			URT ORB	ND
10								URT
11		ORB	ORB	ORB				URT
12			ORB	ORB		ORB	TM+ORB	URT
13	PRU	PRU	PRU ORB	PRU	PRU	PRU		ND
14	GI	ORB	GI				GI TM+ORB	ND
15			GI	TM		GI	GI	GI
16		PRU+GI	PRU+GI	PRU+GI TM			PRU+GI TM+ORB	ND
17			ORB	GI+URT TM		GI+URT		URT
18	GI		ORB	GI ORB		TM	GI TM+ORB	ND
19					TM	GI TM	GI	GI

Hake, *Merluccius merluccius*; seabass, *Dicentrarchus labrax*; monkfish, *Lophius piscatorius*; cod, *Gadus morhua*; megrim, *Lepidorhombus whiffiagonis*; swordfish, *Xiphias gladius*; albacore, *Thunnus alalunga*; anchovy, *Engraulis encrasicolus*.

URT: urticaria; PRU: pruritus; OAS: oral allergy syndrome; GI: gastrointestinal complaints; C: conjunctivitis. TM: tropomyosin recognition; ORB: other recognised bands; Empty: negative; ND: not determined.*Asterisk indicates GI symptoms observed after eating sardines. Anti-tropomyosin monoclonal antibody TM311 was used as control (González-Fernández et al., 2016).

yield of IgE positivity against claimed fish species is low, but can importantly be elevated by performing the diagnostic procedures including other fish species.

When analyzing the 10 patients with this possible anti-fish tropomyosin IgE by subgroups, the following positivity rates could be ascertained: 5/14 patients with concomitant claimed shellfish symptoms, but 0/5 patients with confirmed specific IgE against shellfish; all (6/6) patients included with gastrointestinal complaints after fish intake; 6/13 patients sensitized against *Anisakis*; 3/7 patients with chronic urticaria.

A total of 4 out of 19 patients showed IgE against different proteins in the immunoblot of *Anisakis* extract, 3 of them (3/4) belonged to the group of previous gastro-allergic anisakiasis (Figure 1).

Concerning the analysis of patients positive to this possible anti-fish tropomyosin IgE (Figures 2–5 and Table 2), IgE band matching with tropomyosin molecular weight was frequently detected against cod and albacore, only twice against swordfish and once against megrim. No positivity could be observed against hake, seabass, and monkfish (immunoblotting not shown). No sufficient data could be collected for anchovy. It is noteworthy that there is a common shadow in some more patients that match with the

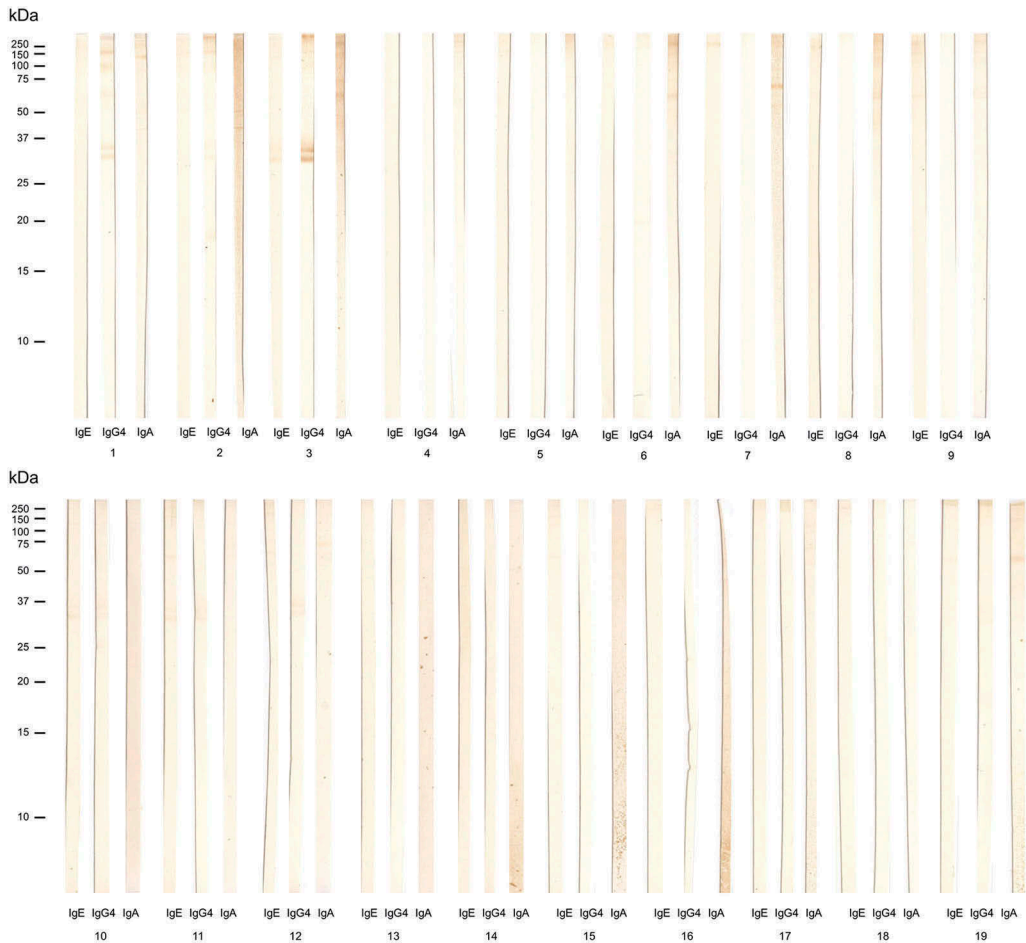


Figure 1. Immunoblotting analysis of IgE, IgG4, and IgA binding to *Anisakis simplex sensu lato* (third stage larvae) crude extract with the patients' sera.

tropomyosin in the extracts from cod, swordfish, and albacore (Figures 2, 4, and 5). Only the clear observed bands have been considered positive. Patients frequently claimed of symptoms with three or four species and were only positive by immunoblotting in one or two species. In three (3/10) cases, positivity was ascertained with fish-species to which patients did not clinically react. This means that the yield of positivity with respect to this possible anti-fish tropomyosin IgE can further be elevated searching with a predetermined battery of fish species in selected patients.

IgG4 and IgA determinations

Possible anti-fish tropomyosin IgG4 was observed only for swordfish in all (6/6) studied sera and in 1/4 studied sera for albacore (Table S3). Possible anti-fish tropomyosin IgA was positive in 3/6 sera for swordfish, in 1/3 for monkfish, 1/3 for cod, and 1/4 for albacore (Table S4). No pattern of reactivity could be detected when analyzing for symptoms or IgE-reactivity.

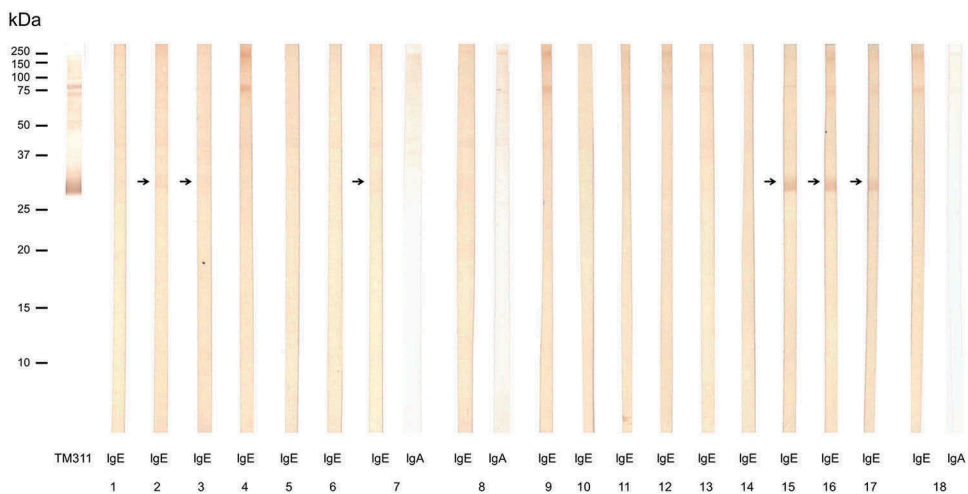


Figure 2. Immunoblotting analysis of IgE, IgG4, and IgA binding to *Gadus morhua* (Atlantic cod) crude extract with the patients' sera which showed detectable reactions. Arrows indicate putative tropomyosin bands.

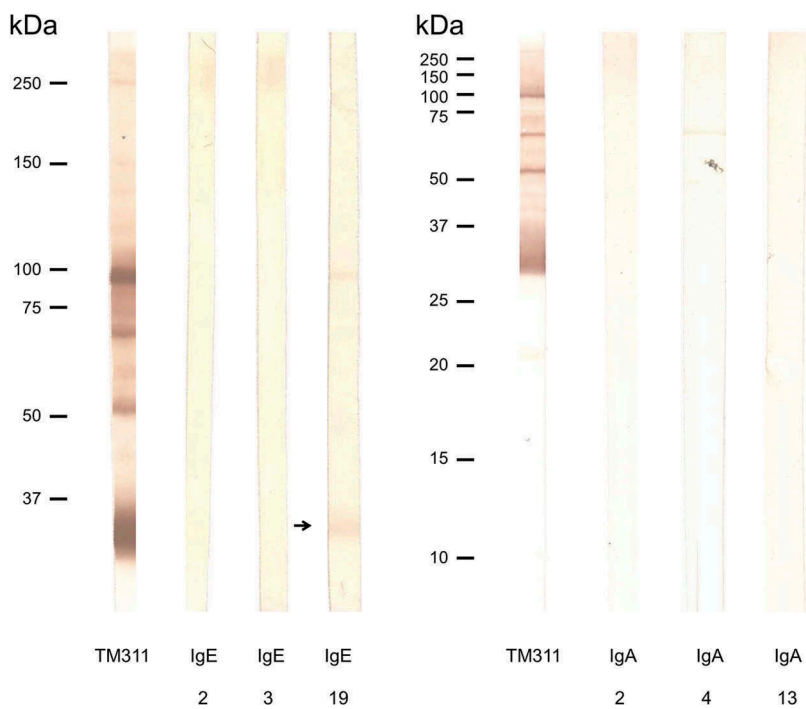


Figure 3. Immunoblotting analysis of IgE, IgG4, and IgA binding to *Lepidorhombus whiffiagonis* (megrim) crude extract with the patients' sera which showed detectable reactions. Arrows indicate putative tropomyosin bands.

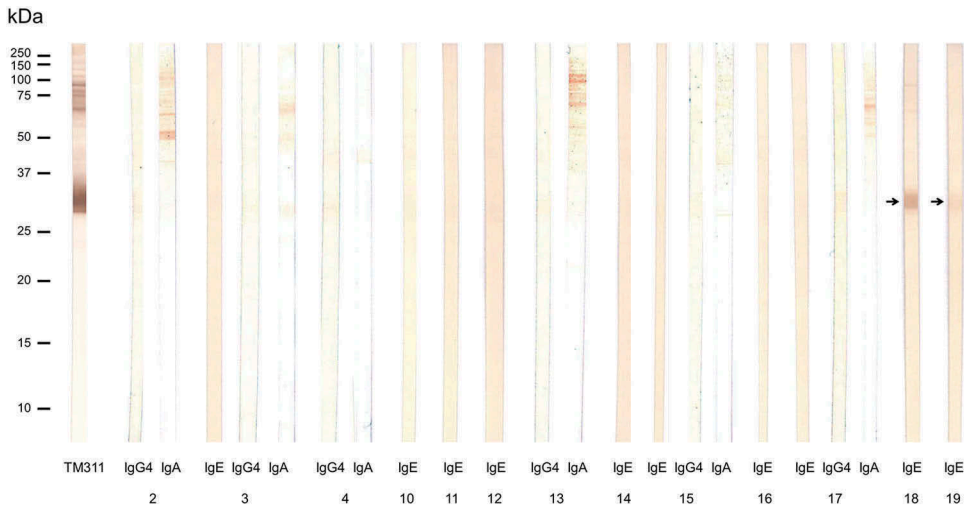


Figure 4. Immunoblotting analysis of IgE, IgG4, and IgA binding to *Xiphias gladius* (swordfish) crude extract with the patients' sera which showed detectable reactions. Arrows indicate putative tropomyosin bands.

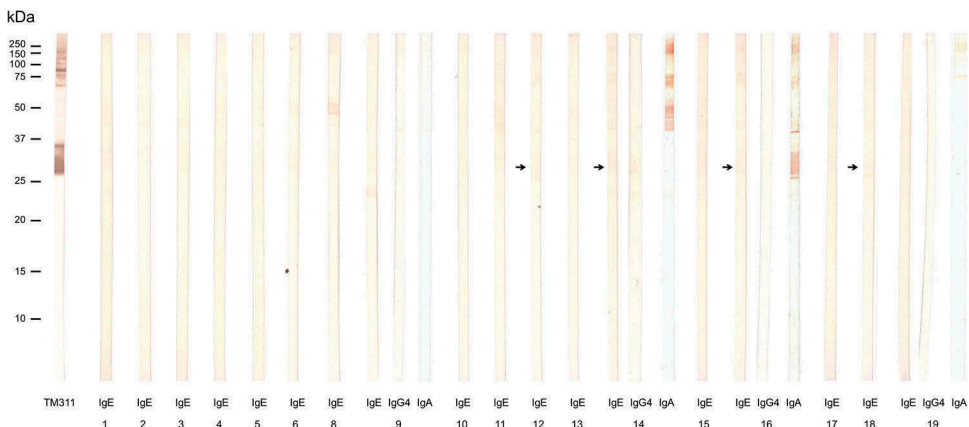


Figure 5. Immunoblotting analysis of IgE, IgG4, and IgA binding to *Thunnus alalunga* (albacore) crude extract with the patients' sera which showed detectable reactions. Arrows indicate putative tropomyosin bands.

Discussion

In this work, a relevant proportion of patients, who repeatedly reported adverse reactions after fish intake, seem to recognize tropomyosin by IgE.

Females made up 84% of our patients. This strong gender bias is also observed in most autoimmune diseases, in which three out of four patients are women (Rubtsova et al., 2014) and could be attributed to the differences observed in the female immune response (Oertelt-Prigione, 2012). On the other side, allergy to fish parvalbumins has also been reported to have a significant female bias (van Do et al., 2005). Autoantibodies directed to tropomyosin have been documented in diseases such as ulcerative colitis, Alzheimer's

disease, autoimmune hepatitis, and Behçet's disease (Mahesh et al., 2005). It has been discussed that allergenic tilapia tropomyosin could be acting as a cross-reacting molecule with human tropomyosin isoform 5, which is a known auto-antigen that binds specific IgG auto-antibodies in ulcerative colitis patients (Liu et al., 2013). In the referred study, IgG isotype was not detected but it should not be forgotten that IgE is also associated with autoimmune disorders such as systemic lupus erythematosus, bullous pemphigoid, chronic spontaneous urticarial, uveitis, rheumatoid arthritis, sclerosis, Hashimoto's thyroiditis, and Graves' disease amongst others (Sanjuan et al., 2016). Fish tropomyosins recognition might be due to an autoimmune reaction by the fact of being vertebrate tropomyosins with a very high identity (at least 90% of identical amino acids) with a human tropomyosin isoform (Jenkins et al., 2007).

In spite of the idea of autoimmunity mentioned above because of the high sequence conservation of the tropomyosin genes, it is evident that each tropomyosin from each fish species has particularities that make it immunologically singular. This idea of "uniqueness" of an antigen was also suggested in a study in which allergens without homologues or with low levels of sequence conservation in microbial/helminth or human proteins were the most allergenic (Santiago et al., 2012).

Although invertebrate tropomyosin is a panallergen, the anti-tropomyosin IgE specificity has been demonstrated between invertebrates, for example with mollusks and crustaceans. There are patients who are sensitized only to molluscan tropomyosin and not to tropomyosin in crustacean species (Taylor, 2008). *In vitro* tests also give us the idea of specificity in the group of tropomyosins. For example, the mAb TM311 (IgG1) against chicken gizzard tropomyosin recognizes common epitopes in vertebrate tropomyosin (mammals, birds, and fish) and some invertebrates such as snail and bivalves but neither crustaceans nor cephalopods (González-Fernández et al., 2016; Sereda et al., 2010).

Our first hypothesis was based on a previous report of a patient with shellfish allergy who extended his symptoms and showed tropomyosin positive to some fish tropomyosins (González-Fernández et al., 2016). Therefore, we suspected that some of the patients included in the current study with confirmed specific IgE against shellfish should also be reacting against fish tropomyosin in the same way as the patient from the mentioned report. Surprisingly, our results showed that the five patients with shrimp-specific IgE did not cross-react with fish tropomyosin. Still, five out of the remaining nine patients with fish-associated symptoms and concomitant-shellfish-induced symptoms displayed the IgE band matching with tropomyosin molecular weight (Tables 1 and 2). On the one hand, we can think that fish tropomyosins should have this particular IgE specificity because, for example, in the previous report the patient did not recognize all tested fish tropomyosins. He only recognized *in vitro* tropomyosin from monkfish, hake, albacore, and swordfish and he presented symptoms in addition to megrim and blonde ray without showing IgE bands (González-Fernández et al., 2016). On the other hand, it is possible that this specific IgE against shellfish detected in our five shellfish positive patients is directed to other shellfish allergens different to tropomyosin. In spite of the general assumption, a recent study showed that tropomyosin is probably not a major shellfish allergen in Spain. Only 21 out of 60 patients with shellfish allergy were positive to purified shrimp tropomyosin (Pen a 1) by skin prick test (López-Matas et al., 2016). This previous study showed that there was no *in vivo* cross-reactivity between purified Pen a 1 and *Anisakis* crude extract *in vivo* but there was an association between symptoms with shellfish and with *Anisakis*. This

clinical association matches perfectly with our 14 shellfish allergic patients, of whom 11 were sensitized against *Anisakis* (Table 1). These authors also state that tropomyosin does not seem to be the responsible for this association due to the low recognition of tropomyosin by *Anisakis* patients (Asturias et al., 2000).

Our second hypothesis, which states that some patients with repeated episodes of gastrointestinal symptoms after fish intake could be sensitized to tropomyosin, was confirmed because all of six patients (patients 14 to 19) showed intense bands matching with tropomyosin molecular weight (between 25 and 37 kDa) detected by the anti-tropomyosin monoclonal antibody TM311. As it was mentioned in the introduction, IBD was common in the patients of the tilapia study after the intake of the fish (Liu et al., 2013). It has been claimed that food allergy is related to an excess of permeability of the gastrointestinal epithelial junctions (Vickery et al., 2011). Indeed, one proposed hypothesis for the allergenicity of tropomyosin as an invertebrate panallergen is the fact that vertebrate tropomyosins are fragmented during the digestion in shorter peptides than invertebrates ones. Its small size is responsible for the absence of the complete three-dimensional IgE epitopes in vertebrate tropomyosins (Mikita and Padlan, 2007). Gastrointestinal permeability problems could be the reason for the presence of anti-fish tropomyosin IgE and could also account for the local gastrointestinal symptoms associated with fish. It, therefore, could be interesting to perform in future fish allergy evaluations, studies for detecting IBD or ulcerative colitis to confirm the hypothetical connection between fish allergy and IBD or ulcerative colitis.

Monosensitization against tropomyosin and in general against panallergens is rare and usually a long exposure to the allergen source is necessary (McKenna et al., 2016). Concerning our patients with *Anisakis* sensitization and its relationship with fish tropomyosin positivity, it is unlikely that their primary *Anisakis* sensitization is due to *Anisakis* tropomyosin (Asturias et al., 2000) and the proteins that they recognize on the immunoblotting around 37 kDa match exactly with the molecular weight of the major allergen Ani s 13 (hemoglobin) (González-Fernández et al., 2015). In addition, *Anisakis* tropomyosin is recognized as a dimer around 75 kDa by the mAb TM311 (González-Fernández et al., 2016).

IgG4 and IgA isotypes are considered protective antibodies because they are able to compete, inhibiting IgE binding to the allergen (Flicker and Valenta, 2003). IgA is secreted into the gut lumen and it has functions of protection against viral and bacterial pathogens and maintaining the homeostasis of the gut flora (Fagarasan and Honjo, 2003). Furthermore, it has been reported that intestinal secretory IgA production is affected by stress in mice (Peña-Juárez et al., 2016). IgG4, in addition to its ability to reduce allergic inflammation, inhibits IgE-facilitated allergen presentation to T cells decreasing the allergen-induced increase of memory IgE production (Ozdemir et al., 2011). Interpretation of our results is limited due to the lack of data. However, it is noteworthy that IgG4 was not detectable in cod being one of the most IgE-positive extracts but was observed in all six studied sera against swordfish. IgG4 was not present in the fish species which were IgE positive to tropomyosin in the abovementioned case of a tropomyosin allergic patient (González-Fernández et al., 2016). Anti-tropomyosin IgA-matching band was present in more species but without any clinical observable pattern of protection. There are two possible cases: the first is the absence of IgA with symptoms, in which there is no protection as for example Patient 16 to seabass. The second case is subdivided in two: the presence of IgA with symptoms (IgA insufficient to block all IgE epitopes, for example

Patient 7 with cod or Patient 16 with albacore) or without symptoms (IgA is enough to prevent IgE binding insufficient data). In the previous study (González-Fernández et al., 2016), IgA was also induced against swordfish, albacore, and tuna, and its absence was hypothesized to be responsible for digestive symptoms.

Strengths of the study are the high number of patients in the specific series and the design, which allowed to test not only the fish species against which the patients reported symptoms in the clinical history, but also to include a battery of fish species. Moreover, we highlight the higher frequency of possible anti-tropomyosin IgE band in cod, albacore, and swordfish even being positive in patients that did not refer symptoms with them. Finally, the results lead us to propose that they should be included in a battery of fish for *in vitro* fish allergy diagnostic tests.

On the other hand, because we do not have recombinant or purified tropomyosins from each fish in this study, immunoblotting was the only molecular technique used. Although conformational epitopes are likely to be better recognized, the very simple folding of tropomyosin presents similar epitopes even in the denatured form. This initial study should now be completed with proteomics and other immunological techniques to definitely assign a new role to tropomyosin as a fish panallergen. Another limitation of the study is the lack of resources to complete the anchovy and all the IgG4 and IgA.

Tropomyosin is a stable protein and we do not know why commercial extracts are not able to demonstrate sensitization even if immunoblotting is able to demonstrate IgE against it. A very low quantity in the extracts, inactivating protein–protein interactions within the extract; or inhibition phenomena by other immunoglobulin isotypes, are some possibilities.

As we mentioned in the introduction, negative results of routine skin prick tests do not rule out allergic mechanisms (Barbarroja-Escudero et al., 2016; Chikazawa et al., 2015; and González-Fernández et al., 2016). Some fish allergens have been underestimated as it was the case of fish collagen because of solubility problems that significantly affected the results of immunological tests (Kobayashi et al., 2016).

Conclusions

We have been able to demonstrate that immunoblotting with a battery of antigens from different fish species, even if they have not been claimed to be causative agents of symptoms, have a high yield of positivity at a band matching with tropomyosin molecular weight. Fish tropomyosin was apparently recognized by 10/19 patients' IgE that referred adverse reactions after fish intake by immunoblotting and is therefore a good candidate to be investigated as a relevant fish allergen in selected patient groups. These preliminary results are important for the diagnosis of fish allergy and the safety for human consumption. Actually, from our seven fish species tested, two of them (cod and albacore) were especially reactive, even if patients did not refer symptoms against them. In the clinical practice, cases of fish allergy should be studied considering not only all species which the patients refer, but also a battery, which could include cod, albacore, and swordfish. In addition, diseases such as IBD or ulcerative colitis should be investigated for affecting gastrointestinal permeability and their relationship with fish allergy.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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