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ORIGINAL ARTICLE

Thermostable allergens in canned fish: Evaluating risks for fish allergy

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Abstract

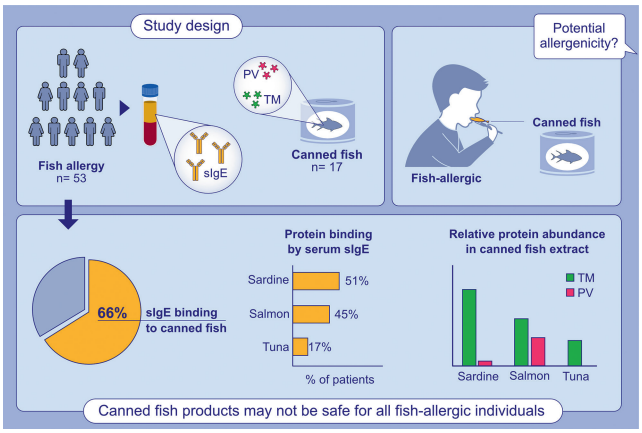
Background: Major fish allergens, including parvalbumin (PV), are heat stable and can withstand extensive cooking processes. Thus, the management of fish allergy generally relies on complete avoidance. Fish-allergic patients may be advised to consume canned fish, as some fish-allergic individuals have reported tolerance to canned fish. However, the safety of consuming canned fish has not been evaluated with comprehensive immunological and molecular analysis of canned fish products.

Methods: We characterized the in vitro immunoreactivity of serum obtained from fish-allergic subjects to canned fish. Seventeen canned fish products (salmon $n=8$; tuna $n=7$; sardine $n=2$) were assessed for the content and integrity of PV using allergen-specific antibodies. Subsequently, the sIgE binding of five selected products was evaluated for individual fish-allergic patients ($n=53$). Finally, sIgE-binding proteins were identified by mass spectrometry.

Results: The canned fish showed a markedly reduced PV content and binding to PV-specific antibodies compared with conventionally cooked fish. However, PV and other heat-stable fish allergens, including tropomyosin and collagen, still maintained their sIgE-binding capacity. Of 53 patients, 66% showed sIgE binding to canned fish proteins. The canned sardine contained proteins bound to sIgE from 51% of patients,

followed by canned salmon (43%–45%) and tuna (8%–17%). PV was the major allergen in canned salmon and sardine. Tropomyosin and/or collagen also showed sIgE binding. **Conclusion:** We showed that canned fish products may not be safe for all fish-allergic patients. Canned fish products should only be considered into the diet of individuals with fish allergy, after detailed evaluation which may include in vitro diagnostics to various heat-stable fish allergens and food challenge conducted in suitable environments.

KEYWORDS
canned fish, fish allergy, food allergy management, parvalbumin, thermostable allergen, tropomyosin



GRAPHICAL ABSTRACT

The potential risk of thermostable allergens in canned fish for fish allergy sufferers: This is the first study that comprehensively examines the in vitro immunogenicity of canned fish, employing a cohort of fish-allergic patients ($n = 53$; largest to date). Canned fish contains thermostable proteins, including major fish allergens (parvalbumin and tropomyosin), which bind to the sIgE of fish-allergic patients. 66% of patients showed sIgE binding to canned fish. Canned fish may not be safe for all fish-allergic patients.

1 | INTRODUCTION

IgE-mediated fish allergy is a worldwide concern that affects both adults and children.[1,2] Currently, there is no effective treatment; thus, the current management of fish allergy relies on complete avoidance of implicated fish in the diet of affected individuals as well as education and the provision of emergency medicine such as EpiPen®.[3] The prevalence of fish allergy can be as high as 6% amongst children in countries frequently consuming seafood, such as Finland and Vietnam.[4,5] Fish allergy, similar to nut allergy, is amongst the most common causes of anaphylaxis and death from food allergy[6–8] and persists for life in up to 90% of patients with a similar trend also observed in peanut allergy.[9–11]

Some fish-allergic individuals are reported to tolerate fish in canned form,[12] although the exact mechanisms for this tolerance remain unknown.[13] Recommendations to consume canned fish are sometimes advised by clinicians and medical practitioners to maintain a healthy dietary intake for fish-allergic patients, since the nutrient contained in fish, such as omega-3 fatty acids and minerals, is known for their beneficial properties.[14–17] The possible reasons for tolerance of canned fish in such patients include a decreased allergenicity caused by the extreme thermal treatment during the canning (retorting) process, possibly resulting in conformational changes in allergenic proteins.[18–21] A reduction in IgE binding in canned products has also been reported for tuna.[19] However, comprehensive molecular and/or immunological analysis of allergens in canned fish products have not yet been undertaken. There are very limited studies on canned fish products, and they often lacked to investigate different fish species or canned products or using an inadequate number of allergic patients/serum samples. The potential of some fish species or canned fish products to maintain their allergenicity cannot be dismissed, as allergic reactions to canned fish have been reported in the past.[13,22,23] In fact, canned tufish has been used to commit suicide by a man with known fish allergy.[24]

Given the global consumption of canned tuna, it is crucial to detect and address any potential risks to human health associated with the consumption of these products. Ensuring the safety of canned tuna is vital for maintaining the integrity of the global food security chain.[25] Furthermore, within national and international regulations that are related to the labelling of the allergen-causing compounds in food products, the importance of the detection of allergens has been well understood for public health safety over the past years.

While tuna species are generally considered to be less allergenic compared with other commonly consumed fish species like salmon and herring,[3,26] the presence of major allergen has been detected in both fresh and processed tuna.[27] Undeclared tuna allergen was also classified as a primary cause for commercial tuna recalls in the United States between 2002 and 2020.[28]

Numerous studies have reported the heat stability of major fish allergen, parvalbumin (PV; 10–14 kDa), which is present in many different fish species and how it can withstand extensive cooking processes.[29,30] Other highly allergenic fish proteins, such as tropomyosin (TM) and collagens, are also heat stable and are present in wide variety of fish species including Asian seabass (a.k.a. Barramundi), *Pangasius*/catfish, salmon and tuna,[31–33] although TM and collagens are not as well studied as fish PV. These major allergens and their reactivity must be carefully considered when characterizing the canned fish products. In the present study, we explored the potential allergenicity of a wide variety of canned products using the largest cohort of individuals with well-characterized and clinically confirmed fish allergy, with the aim of re-evaluating the recommendations on consumption of canned fish products for IgE-mediated fish-allergic patients.

2 | MATERIALS AND METHODS

2.1 | Patient recruitment

Well-characterized and clinically confirmed fish-allergic paediatric patients ($n=53$; 1–18 years old; see Table 1) were recruited at the Children's Hospital at Westmead, Australia, based on a convincing recent clinical history of IgE-mediated adverse reactions to any fish. Sensitization to fish was confirmed by skin prick testing (SPT) using commercial preparations for cod, tuna and salmon, including to culprit fresh fish (where possible) and/or the presence of specific sIgE (ImmunoCAP; Phadia-Thermo Fisher Scientific, Uppsala, Sweden), to cod (*Gadus morhua*; f3), tuna (*Thunnus albacares*; f40) and salmon (*Salmo salar*; f41). Patients underwent oral food challenges (OFC) with canned fish (tuna and/or salmon) if they had a desire to include canned fish into their diet, where parents consented to the OFC being conducted, and where a clear history of tolerance to canned fish was not obtained. The fish challenges used the index cooked fish- or canned (when it is a canned tuna or salmon challenge) and were semi-logarithmic doses up to a standard serving size which exceeds a 20 g protein per total cumulative dose. Serum from non-seafood-allergic, atopic patients ($n=4$) served as negative controls. The study was approved by the Sydney Children's Hospital Network (approval number: LNR-14/SCHN/185). Parents of all participants gave written informed consent and patient anonymity was preserved.

2.2 | Canned fish products

Canned fish products from salmon ($n=8$), tuna ($n=7$) and sardine ($n=2$), were purchased from major supermarket retailers in Australia. These 17 canned fish products were produced by nine different manufacturers and are all prepared in salt water (see Table S2 for more detail). The selection of canned fish products evaluated in this study represents a great portion of the products available at the two major supermarket retailers in Australia.

2.3 | Preparation of fish protein extracts

Protein extracts were prepared from 30 g of each canned fish product, and muscle tissues of Atlantic salmon (*Salmo salar*), yellowfin tuna (*Thunnus albacares*) and sardine (*Sardina pilchardus*) as cooked fish extracts (denoted as CE), using an established protocol[34] (Method S1). PVs from salmon and cod were purified from protein extracts.[35] Fish was purchased from specific retailers, and authenticity of specimens was confirmed using species identification guides and consulting fishery experts at the Centre for Sustainable Tropical Fisheries and Aquaculture (James Cook University, Townsville, Australia).

2.4 | Immunoblot analysis with parvalbumin-specific antibodies

For the detection of PV by immunoblotting, proteins (10 μg/lane) from all fish extracts and purified PVs from salmon and cod (0.5, 1 and 2 μg) were resolved by 16% SDS-PAGE and transferred onto an activated PVDF membrane (GE Lifesciences). PV was detected using a monoclonal anti-PV antibody,[35–39] (PARV-19; Sigma-Aldrich), and either the in-house generated polyclonal rabbit antibodies against salmon PV or Asian seabass PV.[40] The antibody bindings were visualized using a protocol described in Methods S2 and S2.1.

2.5 | Immunoblot analysis with sera from fish-allergic children

For the initial characterization of IgE-binding proteins in canned fish extract, pooled serum from five selected patients was used for immunoblotting. Patients were selected based on their clinical history and/or sIgE binding (salmon; tuna; and sardine), or positive SPT. Proteins from canned fish extracts (10 μg/lane) and CE from salmon (1 μg), tuna (10 μg) and sardine (5 μg) were resolved by 16% SDS-PAGE and transferred onto activated PVDF membranes. The sIgE bindings were visualized using a protocol described in Methods S2 and S2.2.

To investigate the individual sIgE binding of 53 patients, five canned products (see Table S2) were selected based on the results from pooled serum sIgE binding. The sIgE-binding profiles of individual patients were established against the selected five canned products and three fish CEs. The selection of five canned products was made as follows: *canned salmons* (I-2 and I-7) are both Pink salmon from different manufactures and origins, selected based on sIgE

TABLE 1 Demographic and clinical characteristics of 53 fish-allergic patients recruited for this study.

Patient			sIgE level (kU/L)			Skin prick test (mm)			Clinical history		OFC
ID	Sex	Age (years)	Cod	Tuna	Salmon	Cod	Tuna	Salmon	Implicated fish	Symptoms	Passed Canned fish
1[a]	F	7	0.05	1.27	0.58	0.0	0.0	0.0	Snapper, tuna	AE, GIS, U	Salmon
2	F	5	–	–	–	6.0	0.0	5.5	Salmon	GIS, U	
3[a]	M	4	–	–	–	5.0	3.0	4.0	Trout, tuna	AN, OAS	
4	M	10	3.79	1.17	8.09	8.5	4.5	11.0	Catfish, salmon, tuna	RD, U	
5	M	7	–	–	–	4.0	3.5	3.0	Salmon, white fish	AE, C, RD	
6	M	5	–	–	–	0.0	4.0	0.0	Bream	AE, U	
7	F	5	–	–	–	4.5	3.5	5.5	Snapper	AE, U	
8	M	15	–	–	–	7.0	0.0	3.0	White fish	AE, U	Salmon, tuna
9	F	4	–	–	–	4.0	5.0	5.0	Salmon	U	
10	F	8	–	–	–	6.5	0.0	4.0	Catfish	U, OAS	Salmon
11	M	18	–	–	–	5.5	0.0	4.5	Catfish, white fish	GIS	
12[b]	M	14	1.29	1.53	3.96	13.5	0.0	0.0	Snapper	AE, OAS	
13[a]	M	5	–	–	–	10.5	5.0	8.5	Canned salmon, canned sardine	AN, U	Salmon, tuna
14	M	9	–	–	–	0.0	0.0	0.0	Unknown	AE, U	
15	F	7	–	–	–	5.0	6.0	3.0	Catfish	GIS, U	
16[b]	M	9	–	–	–	12.5	0.0	6.5	Unknown, whitebait, white fish	AE, C, U	
17	M	10	–	–	–	4.0	4.5	5.0	Ling	AE, OAS	
18	M	1	–	–	–	7.0	3.0	6.0	Asian seabass, salmon, white fish	AE, OAS, U	
19[a]	M	5	–	–	–	6.5	0.0	5.0	Bream, cod	U	Tuna
20[a]	M	5	–	–	–	4.5	4.5	4.5	Cod, white fish	AN, GIS, RD	
21	F	13	–	–	–	3.5	4.5	0.0	Asian seabass, catfish	GIS, RD, U	Salmon, tuna
22	F	10	–	–	–	0.0	0.0	0.0	Salmon, white fish	AE, GIS, U	
23	M	11	88.50	4.83	25.30	8.5	4.0	12.0	Tuna, white fish	AE, GIS, OAS, U	
24	M	14	–	–	–	5.5	6.0	0.0	Asian seabass	AN	
25	F	5	–	–	–	6.0	0.0	5.5	Salmon	GIS, U	Salmon, tuna
26	M	9	–	–	–	5.5	2.0	7.5	Salmon, unknown	U	
27	M	10	–	–	–	4.5	3.0	4.0	White fish	AN, RD, U	Salmon, tuna

TABLE 1 (Continued)

Patient			sIgE level (kU/L)			Skin prick test (mm)			Clinical history		OFC
ID	Sex	Age (years)	Cod	Tuna	Salmon	Cod	Tuna	Salmon	Implicated fish	Symptoms	Passed Canned fish
28	F	8	–	–	–	3.5	0.0	2.5	Unknown	GIS, U	
29	M	10	<0.01	0.01	<0.01	0.0	2.5	0.0	Asian seabass	OAS, U	
30	M	7	2.29	0.10	0.64	8.0	0.0	5.0	White fish	OAS, U	
31	M	13	2.99	1.99	1.24	8.0	2.5	4.5	White fish	OAS, U	
32	M	14	0.10	0.28	0.21	2.0	2.5	0.0	Salmon	AE	
33	M	3	0.02	0.03	0.03	0.0	4.0	0.0	Catfish	AE, U	
34	F	12	2.66	1.41	3.43	13.5	4.0	11.0	White fish	OAS, U	
35	F	2	0.14	0.78	0.60	0.0	0.0	3.5	Salmon	AE, U	
36	M	1	–	–	–	2.5	0.0	4.5	Catfish	AE	
37[b]	M	12	10.00	5.91	9.01	9.5	9.0	8.0	Asian seabass, salmon	U	
38	M	9	7.25	2.15	5.33	10.0	5.5	3.0	Trevally	AN, RD, U	Salmon
39	M	11	0.11	0.14	0.11	3.5	0.0	0.0	White fish	AE, AN, RD, U	
40	F	1	5.47	5.14	5.58	6.5	0.0	3.0	Asian seabass	AE, RD, U	
41	M	11	92.10	39.50	66.10	14.0	5.0	4.5	Salmon	AE	Tuna
42	M	8	11.50	4.67	9.80	4.5	4.5	2.0	Salmon, tilapia	OAS	Salmon
43[b]	M	15	18.70	12.10	17.60	15.0	5.5	8.5	Ling	AE, OAS	
44	F	2	90.40	31.00	73.40	13.5	5.5	13.5	Cod	AE, U	
45	M	8	75.00	21.00	70.50	14.0	6.5	12.0	White fish	GIS, RD, U	Tuna
46	F	10	16.50	7.33	9.08	7.0	2.0	3.5	White fish	AE, OAS, RD	
47[b]	F	6	–	–	–	8.0	3.0	4.5	Salmon, white fish	AE	Salmon
48	M	9	0.13	0.06	0.46	3.0	2.0	6.5	Croaker	AE	
49	M	2	–	–	–	7.5	4.0	7.5	Salmon	U	
50	F	10	0.21	0.15	0.14	3.5	0.0	0.0	Catfish, white fish	GIS, RD, OAS	
51	M	5	10.20	6.78	11.50	6.5	5.0	6.0	Leather jacket	AE	
52	M	7	1.81	0.70	7.63	4.5	0.0	6.0	Trout	AE, OAS, U	
53	F	7	2.87	1.19	3.08	4.5	2.5	0.0	White fish	U	

Note: Symptoms are listed as abbreviated. A, asthma; AN, anaphylaxis; AE, angioedema (lip swelling); E, erythema; GIS, gastrointestinal syndrome (vomiting); H, hypotension (low blood pressure); OAS, oral allergy syndrome (itchy mouth or swelling throat); R, rhinitis (irritation inside nose, sneezing); RD, respiratory distress (breathing difficulty); U, urticaria (hives, rash). OFC, oral food challenge. '–', Not performed. Patient 14 = SPT Barramundi (Asian seabass; in-house SPT preparation) 3.5 mm; Patient 22 = SPT Barramundi 3.0 mm, catfish 4.0 mm.

[a]Patients selected for pooled serum analysis.

[b]Patients sIgE reactive to fish collagen.

binding to 10–15 kDa proteins; *canned tuna* (T-1 and T-3) are different species (skipjack and yellowfin tuna, respectively) and selected based on sIgE binding to only the latter species; and *canned sardine* (S-2) was selected based on higher sIgE binding. The Surf-Blot antibody screening system using a manifold (Idea Scientific) was used to investigate serum IgE binding from all patients to the same extract. Proteins (100 μg) of each extract were resolved by 16% SDS–PAGE and transferred onto a nitrocellulose membrane. IgE-binding intensities were determined in comparison with negative controls and other patients as well as signals to other proteins, and were classified into three levels (1 = weak, 1–500; 2 = moderate, 501–2000; 3 = strong, >2000; normalized within each membrane) based on the binding intensity measured by densitometric analysis.

2.6 | Identification of sIgE-binding proteins by mass spectrometry

The prominent sIgE-binding bands from five select canned fish (see Section 3.2) and whole extracts (200 μg/mL), and the respective fish CEs were subjected to mass spectrometric analysis after tryptic digestion (Method S3).[33,34,41] For canned fish products, small sections, not a band, containing sIgE-binding proteins were excised instead from the gel due to smearing (see Figure S1).

Results were analysed using MaxQuant (v. 1.6.10.43), against an NCBI database containing amino acid sequences of all proteins from the corresponding species or closest higher classification with at least three annotated genomes/transcriptomes (July 2020). The relative protein abundance is expressed in relative intensity-based absolute quantification (iBAQ%) value.[42] Fixed modification carbamidomethyl-C and variable modifications of deamidation N, deamidation Q and oxidation of M were selected. Identified protein groups with at least one unique peptide and a minimum of two razor/unique peptides were included in the analysis.

2.7 | In silico conservation analysis of fish parvalbumin and tropomyosin

PV and TM sequences, identified in canned salmon and sardine products by mass spectrometric analyses after in-solution digestion, were analysed and compared for amino acid sequence identities using Clustal Omega.[43]

3 | RESULTS

3.1 | Protein profile and detection of parvalbumin in canned fish products

The protein profile of 17 canned fish extracts differed greatly to that of the CEs of corresponding fish (Figure 1A). The proteins in canned fish products consistently extended over 10–150+ kDa as undefined bands or smears likely due to extensive aggregation and/or degradation as compared to the CEs with distinguished bands. The protein profile within each fish group was also compared. For canned salmons, no distinct differences were observed for species or manufacturers. For canned tuna, the differences were evident between species that skipjack tuna (T-1, 2, 4, 5, 7) contained more smaller proteins while yellowfin tuna (T-3, 6) contained more of larger proteins. For canned sardines, two products showed different profiles.

PV could not be detected in any of the canned fish products using PARV-19 or in-house generated anti-salmon and anti-Asian seabass PV-specific polyclonal antibodies (Figure 1B,C). PVs in salmon and sardine CEs, but not in tuna CE, were detected at 11–14 kDa in reference to the purified salmon and cod PVs (Figure 1B,C). For salmon and sardine CEs, at least two isoforms of PVs and dimers (~25 kDa) and polymers (~50 kDa and ~150 kDa) were also detected.

3.2 | Detection of sIgE-binding proteins in canned fish products

Some proteins in canned fish products and three fish CEs bound to sIgE from pooled sera (Figure 1D). Most of the sIgE binding occurred at >30 kDa for all canned fish products. Contrary to the protein profiles of canned fish differing between fish groups (Figure 1A), the sIgE binding was rather similar (Figure 1D). The sIgE binding for canned salmon products was independent of the species, origin or manufacturers. The sIgE binding changed based on the tuna species; yellowfin tuna (*Thunnus albacares*; T-3, 6) showed considerably higher sIgE binding than skipjack tuna (*Katsuwonus pelamis*; T-1, 2, 4, 5, 7). A canned sardine (*Clupea harengus*; S-2) contained more sIgE-binding proteins than the other product (*Sardinella* spp.; S-1); however, the differing factor was inconclusive due to sample size. Canned salmon (I-2 and I-7) and sardine (S-2) showed sIgE binding to proteins at 11–14 kDa while no canned tuna did.

Strong sIgE binding to proteins at 11–14 kDa was observed for salmon and sardine CEs but not for tuna CE. In addition, strong sIgE binding to proteins at 30–40 kDa was observed for tuna and sardine CEs but not for salmon CE.

3.3 | Identification of sIgE-binding proteins in canned fish products by mass spectrometry

The in-gel tryptic digestion mass spectrometry revealed that sIgE-binding proteins in the selected canned fish products as major fish allergens (Figure S1 and Tables S2–S4). Canned pink salmon (I-7) contained PV (11 kDa; iBAQ 75.2%), TM (40–42 kDa; iBAQ 47%) and type I collagen α-1 (250+ kDa; iBAQ 22%; Table S2). Both canned tuna (*Thunnus* spp.; T-3 and T-6) contained TM (35–37 kDa; iBAQ 48% and 44%; 38–46 kDa; iBAQ 33%; Table S3; T-3). Canned sardine (*C. harengus*; S-2) contained PV (12 kDa;

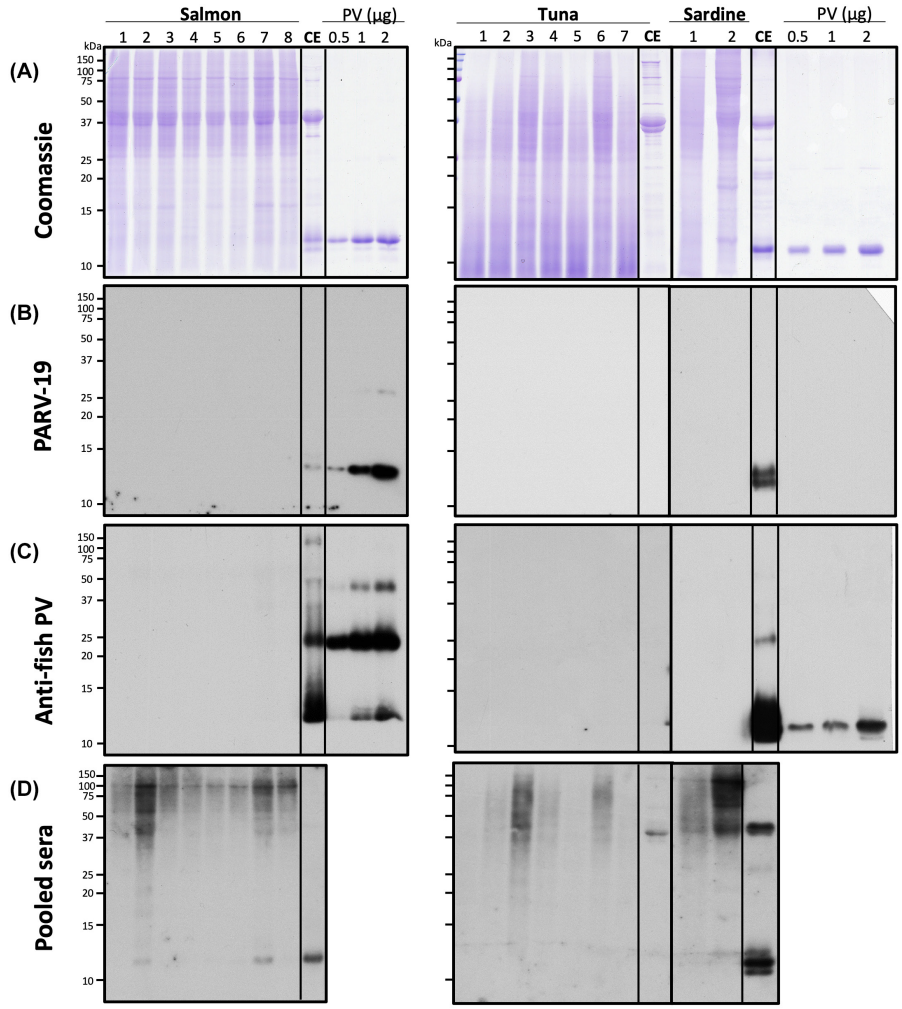


FIGURE 1 Protein profiling (A) and immunoblot analysis of 17 different canned fish products using monoclonal PARV-19 antibody (B), rabbit anti-salmon PV or anti-Asian seabass PV antibodies (C) and pooled patient sera (D). Cooked fish extracts (CE) from salmon, tuna and sardine were used as references. Purified PVs from salmon and Atlantic cod were used as quantitative controls (0.5, 1 and 2 μg).

iBAQ 85%) and TM (38–50 kDa; 74%, 78% and 30%; Table S4), which were highly abundant. Though not officially registered as a fish allergen, myosin heavy chain (MHC) and its fragments were detected throughout the 60–250+ kDa regions in all canned products.

In fish CEs, PV (10–12 kDa) and/or TM (36–40 kDa) were also found in all three species (Tables S2–S4), at the expected size. The adjacent bands to the PVs and TMs were identified as their isoforms. Besides PV and TM, MHC (tuna CE), troponin (tuna CE) and myosin light chain (sardine CE) were found as sIgE-binding proteins.

In-solution digestion mass spectrometry showed that TM was highest in abundance in four canned fish products (I-2, I-7, T-3 and S-2) and relatively high in canned Skipjack tuna (T-1), followed by MHC, myosin light chain, actin and PV (Figure 2). The canned tuna (T-1 and T-3) contained much higher levels of MHC than other fish products. PV was high in abundance for canned sardine (S-2), and low in canned salmon (I-2 and I-7). Most heat-labile proteins, including aldolase and enolase, were low in abundance in all canned products.

3.4 | sIgE-binding profiles of individual patients to canned fish proteins

The immunoblot analysis on sera from 53 fish-allergic patients (Figures 3 and 4; Figures S2–S4) revealed that 35 out of 53 patients (66%) showed sIgE binding to canned fish proteins. Canned sardine (S-2) had the highest number of patient (51% of the cohort) sIgE binding, followed by canned salmon (43% and 45%; I-2 and I-7, respectively) and canned tuna (8% and 17%; T-1 and T-3, respectively).

For canned salmon, IgE binding was mostly against small proteins (11.1 and 11.7 kDa; Figure 3). Most patients' serum binding to these small proteins (~46% of the cohort) also bound to the proteins of same size in cooked salmon (Figure 4). Some patient sera bound to proteins of 38.5 kDa in both canned salmon. No other differences in binding frequencies and proteins were observed.

For tuna, only <20% of the cohort bound to proteins in canned or cooked form (Figure 4). Of which, 8% bound to proteins at 11.0 and 11.2 kDa in both canned tuna and CE (Figures 3 and 4). About

20% of patients' serum bound to a 35.2 and/or 150 kDa proteins in CE but not in canned tuna.

For sardine, the most frequent and strongest sIgE bindings were observed across three fish groups. Proteins at 11.1 and 12.2 kDa binding to 14% and 37% of cohort (Figure 3), respectively, at high intensities. Some patients' serum bound to proteins at 35.9, 38.1 and 150 kDa in canned sardine, which was also observed for canned salmon and tuna at similar frequencies (Figures 3 and 4).

Up to 5% of cohort showed binding to larger proteins (35–100 kDa) which molecular weight could not be defined (in I-2, T-1, T-3 and S-2; Figure 4). These were the smear-like bands observed on SDS-PAGE gels (refer to Section 3.1 and Figures S2–S4). Those patients' serum with binding to this smear region showed binding to all four of these canned products as well as several large proteins in CEs of corresponding species (i.e. 150 kDa protein in salmon [19%], tuna [17%] and sardine [14%]).

Patient sera bound to proteins in CEs of salmon, tuna and sardine at frequencies of 70%, 45% and 81%, respectively (Figure 3). The sIgE binding and frequency were much higher against proteins in CEs than canned fish.

3.5 | Amino acid sequence conservation of fish allergens

Sequence identity matrix analysis performed for PVs and TMs, identified in the canned salmon, tuna and sardine by mass spectrometry, showed that TM was more conserved across three species than PV (Figure 5). TM of two sardine α-isoforms and two salmon α- and one β-isoforms were 84–90% similar to each other, while the majority of β-PV isoforms were only up to 80% similar, including within each of the analysed fish species.

4 | DISCUSSION

Here, in a comprehensive analysis of 17 canned fish, we identified heterogeneity in the protein composition between canned and cooked forms of fish, and found sIgE-binding proteins in all canned fish products using serum from 53 fish-allergic patients. The major fish allergen PV was detected in canned salmon and sardine, which bound to sIgE but not the PV-specific monoclonal and polyclonal antibodies. Along with PV, other fish allergens such as TM and collagen were also abundantly present in all canned fish products. Other proteins yet to be registered as allergens including MHC were found in all canned fish, which also bound to sIgE. Thus, the consumption of canned fish for fish-allergic patients requires a careful assessment on an individualized basis.

Parvalbumin, as it is a major fish allergen, was found to be the major IgE-binding protein in canned salmon and sardine products. Interestingly, PVs in canned fish could not be detected by neither mono- or polyclonal antibodies specific to PV. Furthermore, PVs in canned fish and cooked fish had similar sIgE-binding intensity when no bands could be detected using SDS-PAGE. In an effort to enhance the binding of anti-PV antibodies to canned fish extracts, we also carried out assays with two times (20 μg) and four times (40 μg) the loaded amount of protein extracts, as well as an extended development time of up to 24 h (data not provided). Despite these efforts, we were unable to observe any binding. The evidence of this work strongly suggests that the lack of binding was primarily influenced by the extreme processing conditions applied to the fish proteins and the consequent loss of the recognition of specific epitopes for these antibodies.[36] By contrast, there were clearly distinct sIgE bindings to PVs in the same canned fish extracts (see Figure 1D), suggesting the protein amount was not the cause of this phenomenon. This highlights that common protein-based analytical methods lack sensitivity and thus require other methods for the detection of PV in canned fish as an in vitro diagnostic tool.[44]

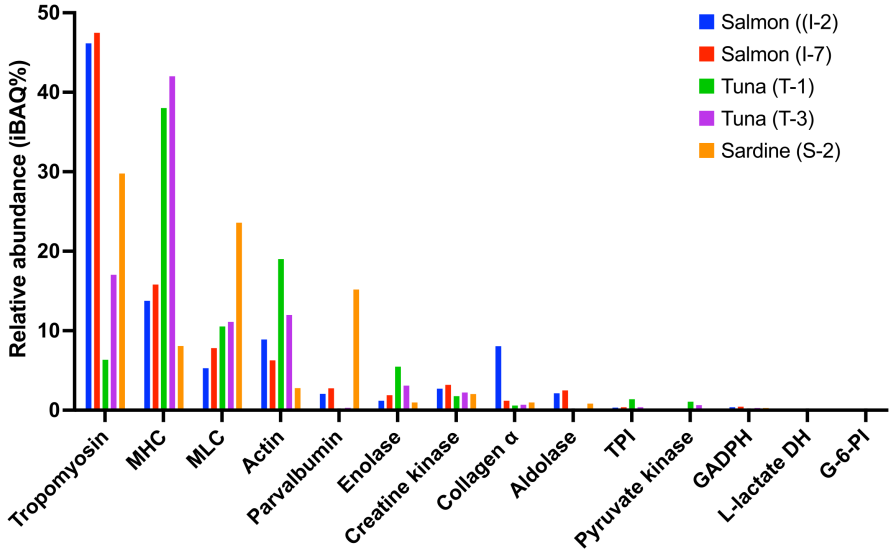


FIGURE 2 In-solution mass spectrometric analysis of relative protein abundance in selected canned fish extracts. The relative protein abundance (iBAQ%) value represents the relative abundance of each protein including isoforms. The relative abundance was determined for IgE-binding proteins. DH, dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MLC, myosin light chain; MHC, myosin heavy chain; TPI, triosephosphate isomerase.

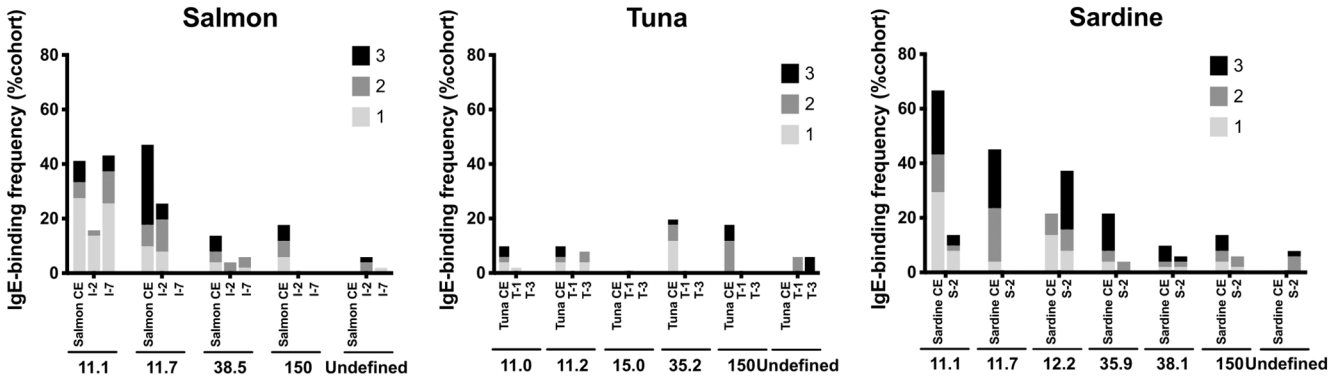


FIGURE 3 Characterization of sIgE binding of a fish-allergic cohort ($n = 53$) to canned and cooked fish proteins. IgE-binding intensities were determined in comparison with negative controls and other patients as well as signals to other proteins and were classified into three levels (1 = weak, 1–500; 2 = moderate, 501–2000; 3 = strong, >2000; normalized within each membrane) based on the binding intensity measured by densitometric analysis against bands in various sizes (in kDa; x-axis) from the Western immunoblotting. Main sIgE-binding proteins from select canned fish and cooked fish extracts (CE) were analysed.

Contrary to the common belief, we found that PV in canned fish was not *destroyed* during the retorting process despite the common belief. While the cooked extracts in this study represent the conventional heating preparation of fish (such as at home), all canned fish products investigated would have gone through an extreme heating process of 110–121°C.[45,46] The ability of these salmon and sardine PVs to maintain IgE reactivity after retorting process certainly warrants further investigation to evaluate the thermostability for each species. PVs of other fish species (i.e. Pacific mackerel) have been reported to lose IgE binding at 140°C.[29] PV from yellowfin tuna lost its binding capacity to sIgE in canned form (in comparison with cooked yellowfin tuna), while canned skipjack tuna maintained its IgE-binding capacity—this finding also supports the thermostability of PVs to be species-specific.[20]

Thermal processing can lead to alterations in protein solubility and detectability in fish, as well as fragmentation of proteins in varying molecular weights and the loss of well-defined bands on SDS-PAGE.[47] For PV, heating not only leads to structural changes but it also causes the presence of monomeric and oligomeric forms of the protein in thermally processed fish.[35] Stable allergenic polymers can exist in cod,[48] snapper[49] and several other species including pilchard.[50] Generally, about 80% of all well-characterized allergens are forming dimers or oligomers naturally[51]; however, their role in triggering allergic reactions remains unclear and needs further investigations.

In our study, we were unable to detect clear dimers using PV-specific monoclonal antibody (PARV-19) besides a faint band observed at 25 kDa for purified salmon PV. By contrast, the polyclonal anti-PV antibodies exhibited strong binding at 25 and 50 kDa for purified salmon PV, cooked extracts from salmon and sardine, indicating the presence of dimers and polymers. However, none of the canned products including salmon showed such dimers or polymers when tested with the antibodies. A comprehensive biochemical study of PV and its biological and physiochemical properties has highlighted the presence of cysteine residues in certain PVs,[52] which may assist in dimerization of this protein. Specifically, PV from salmon contains four cysteine residues which could form disulphide bonds under oxidizing conditions, which may be encountered during the intense thermal processing involved in canning fish. Our study corroborates this finding by confirming the polymerization of salmon PV, while no such polymerization was observed in tuna or sardine PVs. The formation of aggregated polymers is not solely determined by disulphide bonds but also influenced by the abundance of different PV isoforms present in various fish species, as demonstrated in the case of cod fish and mackerel. Although the allergenicity of a specific fish species is dependent on several factors, including protein (i.e. PV) sequences, concentration impacting folding, stability and interaction properties,[53] the conclusions drawn in our study supports the findings where PV polymers could be detected using the anti-fish PV polyclonal antibody in salmon and sardine samples while exhibiting no sIgE bindings.

To our surprise, TM showed significant sIgE binding, greater than PV, in canned fish, and it appeared to be a major IgE-binding protein in canned fish. We have recently identified TM as major fish allergen of unrecognized importance.[33] Importantly, TM demonstrated frequent and stronger sIgE binding in cooked tuna and canned tuna as compared to PV, highlighting its relevance for tuna-allergic individuals. TM was found in higher abundance than PV in all canned fish examined, emphasizing its significance as a major allergen in canned fish. Collagen is another allergen of interest in canned fish products. We have previously reported collagen as a key fish allergen in the same cohort of patients[32] (see Table 1). We now show serum from the same patients binding to collagen in canned fish. Collagen is another highly thermostable protein with a long, repetitive structure[31,32]; thus, its allergenicity would be maintained after the retorting process. MHC was also present in high abundance in all canned fish (in-solution analysis), however, in the form of fragments (60–250 kDa; in-gel analysis). Myosins play a crucial role in contractile movements in cells and tissues of fish. This large, hexameric protein constitutes over 50% of the total mass of skeletal muscles and is composed of two myosin heavy chains (MHC) of over 200 kDa and four myosin light chains (MLC) of 20 kDa. Due to the extreme processing conditions of canned fish and the high abundance of these protein subunits, the presence of fragments of MHC

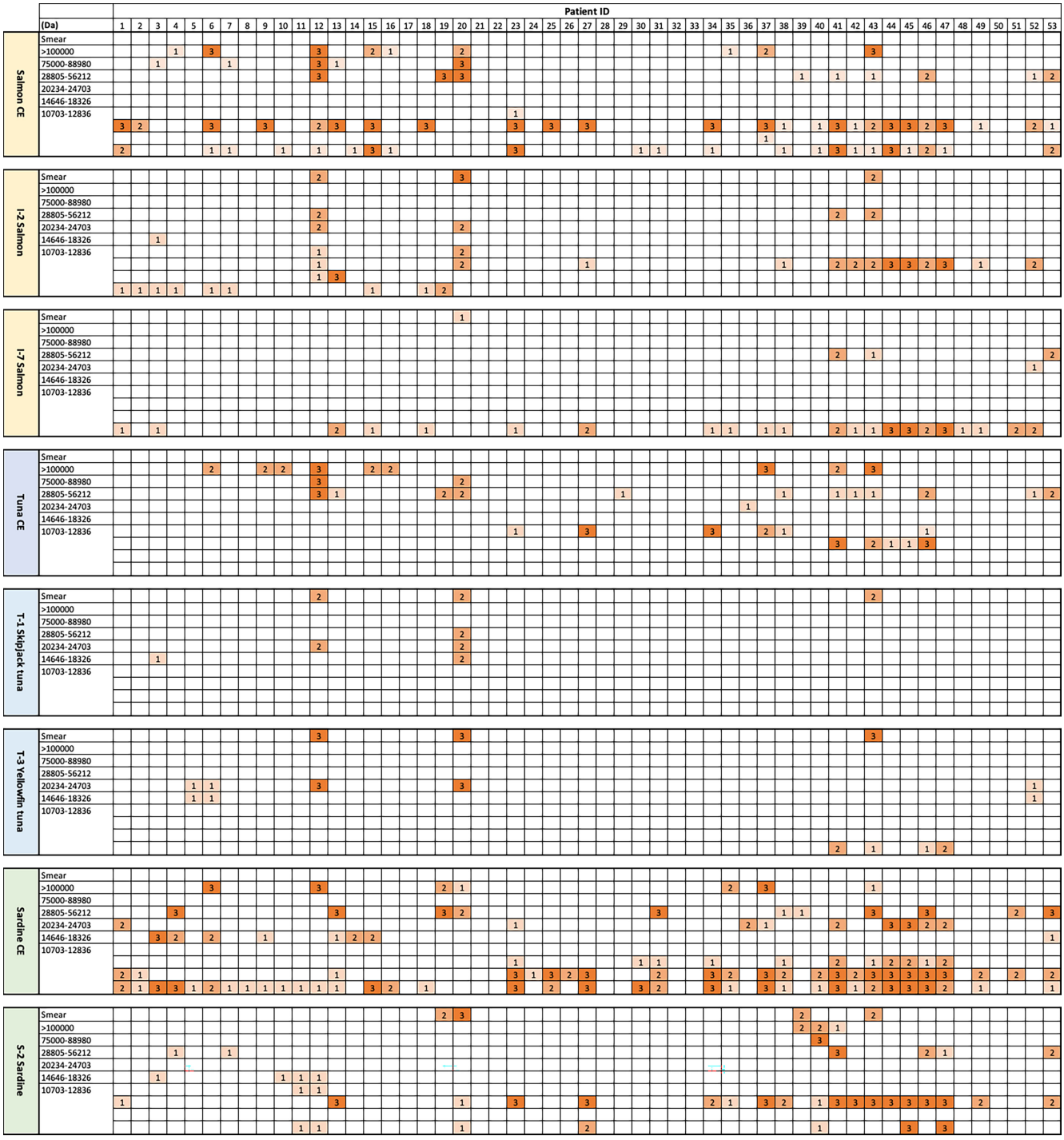


FIGURE 4 sIgE-binding profile of 53 fish-allergic subjects to canned and cooked fish proteins. Three sIgE-binding levels (1 = weak, 1–500; 2 = moderate, 501–2000; 3 = strong, >2000; normalized within each membrane) were classified using densitometric analysis of individual bands from the Western immunoblotting to each of the main sIgE-binding proteins from select canned fish and cooked fish extracts (salmon, tuna and sardine CE).

in multiple protein bands was likely. While MHC is not (*yet*) registered as an official fish allergen by the WHO/International Union of Immunological Societies (IUIS; www.allergen.org) and is susceptible to heat treatment,[54] it has been identified as a strong IgE-binding protein in patients with salmon fish allergy and atopic dermatitis.[55] Thus, its potential reactivity should not be disregarded.[55,56] Consequently, MHC could potentially be an allergen of concern in the current study.

The answer to the anticipated question on which canned fish is safe (–*r*) for consumption for fish-allergic patients must be carefully considered on an individualized basis. It can be postulated that the consumption of canned sardine may lead to severe allergic reaction compared with consuming canned salmon or tuna, hypothesized based on the stronger and/or more frequent IgE binding to canned sardine proteins overall. Furthermore, individuals who exhibit significant IgE binding to TM and/or collagen rather than PV may be

FIGURE 5 Conservation analysis of fish parvalbumin (PV) and tropomyosin (TM). Sequence identity matrix was performed for PVs and TMs identified in the canned salmon and sardine products by mass spectrometry analysis after in-solution tryptic digest.

(a) - Parvalbumin (PV)

Accession I.D.	PV	Canned	#	1	2	3	4	5	6	7	8	9	10
XP_029500831.1	β-1	Salmon	1	###									
P86431.1	β-1	Salmon	2	84	###								
XP_031435403.1	β	Sardine	3	70	57	###							
BAF98924.1	β	Tuna	4	71	61	72	###						
XP_012694365.1	β-2	Sardine	5	72	61	73	70	###					
XP_029540956.1	β-2	Salmon	6	70	62	71	75	75	###				
CBA35351.1	β-2	Tuna	7	70	60	73	70	72	75	###			
CAQ72967.1	β-1	Tuna	8	70	60	73	70	72	75	###	###		
CBA73697.1	β-2	Sardine	9	71	60	72	79	77	81	72	72	###	
XP_012694367.1	β-2	Sardine	10	72	62	71	75	74	78	79	79	80	###

(b) - Tropomyosin (TM)

Accession I.D.	TM	Canned	#	1	2	3	4	5	6	7	8	9
XP_031441857.1	α-4	Sardine	1	###								
XP_012697822.1	α-1	Sardine	2	72	###							
BAJ11924.1	α-1	Tuna	3	68	87	###						
BAD01050.1	α-1	Tuna	4	73	92	90	###					
XP_020360961.1	α-3	Salmon	5	75	87	85	91	###				
XP_012678334.1	α-3	Sardine	6	74	84	81	87	90	###			
XP_029523883.1	α-1	Salmon	7	72	92	91	95	89	84	###		
XP_021457286.1	β	Salmon	8	73	86	83	88	87	84	89	###	
YFT_comp15422	α-3	Tuna	9	78	83	80	84	84	85	83	91	###

50	60	70	80	90	###

Percentage Identity (%)

required to avoid consuming canned tuna due to the high abundance of these allergens. To gain a comprehensive understanding of the potential reactivity, or lack thereof, to canned fish, a more comprehensive and in-depth investigation is required to uncover the underlying mechanisms behind tolerance in individual patients. In our cohort of 53 patients, 13 have passed OFC with canned salmon and/or canned tuna (see Table 1). Although this was expected for the majority of the 13 patients based on our individual sIgE analysis, in particular for canned tuna with patients often not showing IgE binding to cooked tuna, the results however did not explain tolerance for three patients who passed canned salmon OFCs. Prior to our study, there have been only two clinical studies conducted on canned fish (in 1992 and 2021) that specifically investigated the tolerance to canned tuna in subjects (18 and 25 individuals, respectively) with fish allergies.[12,19] In line with our current understanding, these studies revealed no evident correlation with SPT results and/or IgE reactivity that could explain the tolerance to canned tuna. This intriguing finding suggests that there are likely additional factors at play in determining an individual's ability to tolerate canned tuna despite having a fish allergy. Elucidating the correlations between observed IgE binding, SPT results and patients detailed clinical history, including reported tolerance to specific fish species and preparations, might assist in the development of tools to predict tolerance to selected canned fish products.

The reactivity to canned fish products of different species could also be influenced by cross-reactivity of isoforms of major allergens. Salmon PV is well recognized for its mono-sensitivity, as IgE-binding regions are least similar to other fish PVs (see Figure S5). Although the epitopes of pink and red salmons (*Oncorhynchus* spp.) investigated in the present study are yet to be characterized, the epitopes of Sal s 1 from Atlantic salmon (*Salmo salar*) are highly similar; 97% and 95% sequence identities between canned salmon PV 1 and 6 (see Figure 5) with Sal 1 s β1 and β2, respectively. Our conservation analysis shows that cross-reactivity to TMs of different fish species is much more likely to occur than PVs. Therefore, the patients who were identified with strong IgE binding to TM might be recommended to avoid canned fish consumption completely.

It would be beneficial to conduct further investigations to establish IgE-reactivity profiles of individual patients to purified fish allergens (i.e. PV, TM and collagen) from each fish species through ELISA or another suitable method. However, we acknowledge the limitations associated with conducting such experiments using a cohort of paediatric patients. Our major limitations include: (i) the presence of multiple isoforms of major allergens in each fish species,[36] as this complexity significantly increases the number of test samples required. Additionally, (ii) the limited availability of serum samples, particularly from young or infant patients included in the study, poses a challenge in obtaining an adequate volume for such comprehensive analysis. Lastly, (iii) the lack of, or inaccurate, information regarding fish species on the labels of canned products,[57] especially in the case of canned salmon and sardine, poses an additional challenge. Furthermore, sensitization to various fish species can vary significantly across different geographical regions. Studies

with different cohorts will certainly demonstrate results custom to specific types of fish found in the region, with an example of canned sardine and salmon being often favoured over tuna in many Eastern regions. Considering these limitations, it is important to carefully consider and address these challenges in future studies that aim to conduct comprehensive allergen-specific analyses.

In conclusion, we found that canned fish products may not be safe for all fish-allergic patients. All three canned fish, from species commonly used for canned fish products, contained fish allergens (i.e. PV, TM and/or collagen). Thus, there is a risk of canned fish products to trigger allergic reactions in fish-allergic patients due to considerable IgE binding to these proteins. To the best of our knowledge, this is the first study to comprehensively examine in vitro immunogenicity of canned fish employing a well-characterized cohort of fish-allergic patients, which is the largest to date. Based on our findings, we recommend that immunogenicity of canned fish should be further investigated, with the potential to develop suitable diagnostic tools for selection of fish-allergic patients who may tolerate such products. Further research will help shed light on the complex nature of allergic reactions to canned fish and provide valuable insights for clinical management and patient care. In the interim, we recommend an individualized approach with the use of food challenges to determine tolerance prior incorporating canned fish into the diet of fish-allergic individuals.

AUTHOR CONTRIBUTIONS

AT, DC and AL contributed to the conceptualization. AT, TR and AL contributed to the methodology. AT and AL contribution to the validation, investigation and project administration. AT, TR and RN contributed to the formal analysis. TR, SM and DC contributed to the resources. AT, TR, RN, SK, SN and ML contributed to the data curation. AT contributed to the writing—original draft preparation. AT, TR, RN, SK, NW, SN, ML, SM, DC, AL contributed to the writing—review and editing. AT and TR contributed to the visualization. DC and AL contributed to the funding acquisition. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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