Original Article

Collagen—An Important Fish Allergen for Improved Diagnosis

Tanja Kalic, PhD[a], Sandip D. Kamath, PhD[b,c], Thimo Ruethers, MSc[b,c], Aya C. Taki, PhD[b,c,d], Roni Nugraha, PhD[b,e], Thu T.K. Le, PhD[b], Piotr Humeniuk, PhD[a], Nicholas A. Williamson, PhD[f], Diamond Hira, MD[g], Jennifer M. Rolland, PhD[h,i], Robyn E. O'Hehir, MD, PhD[h,i], Danyi Dai, MA[j], Dianne E. Campbell, MD, PhD[c,j], Heimo Breiteneder, PhD[a,*], and Andreas L. Lopata, PhD[b,c,*] *Vienna, Austria; Townsville, QLD, Australia; Melbourne, VIC, Australia; Jawa Barat, Indonesia; and Sydney, NSW, Australia*

What is already known about this topic? Clinical relevance of fish collagen for fish-allergic patients is poorly understood, likely due to its low abundance in commercial diagnostic tests. Patients may be exposed to such collagens via pharmaceutical products, food, beverages, and cosmetics.

What does this article add to our knowledge? We demonstrated the potential clinical relevance of sensitization to fish collagen in fish-allergic patients, some of whom were not sensitized to the major fish allergen parvalbumin.

How does this study impact current management guidelines? Current diagnostic tests for fish allergy contain low quantities of collagen due to its insolubility in aqueous solutions. Inclusion of collagen in diagnostic tests is indicated to improve patients' safety.

BACKGROUND: Fish collagen is widely used in medicine, cosmetics, and the food industry. However, its clinical relevance as an allergen is not fully appreciated. This is likely due to collagen insolubility in neutral aqueous solutions, leading to low abundance in commercially available *in vitro* and skin prick tests for fish allergy.
OBJECTIVE: To investigate the relevance of fish collagen as an allergen in a large patient population (n = 101).
METHODS: Acid-soluble collagen type I was extracted from muscle and skin of Atlantic salmon, barramundi, and yellowfin tuna. IgE binding to collagen was analyzed by ELISA for 101 fish-allergic patients. Collagen-sensitized patients' sera were tested for IgE binding to parvalbumin from the same fish species. IgE cross-linking was analyzed by rat basophil leukemia assay and basophil activation test. Protein identities were confirmed by mass spectrometry.
RESULTS: Purified fish collagen contained type I α1 and α2 chains and their multimers. Twenty-one of 101 patients (21%) were sensitized to collagen. Eight collagen-sensitized patients demonstrated absence of parvalbumin-specific IgE to some fish species. Collagen induced functional IgE cross-linking, as shown by rat basophil leukemia assay performed using 6 patients' sera, and basophil activation test using fresh blood from 1 patient. Collagen type I α chains from barramundi and Atlantic salmon were registered at www.allergen.org as Lat c 6 and Sal s 6, respectively.
CONCLUSIONS: IgE sensitization and IgE cross-linking capacity of fish collagen were demonstrated in fish-allergic

[a]Institute of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria
[b]College of Public Health, Medical and Veterinary Sciences, Australian Institute of Tropical Health and Medicine, Molecular Allergy Research Laboratory, James Cook University, Townsville, QLD, Australia
[c]Centre for Food and Allergy Research, Murdoch Children's Research Institute, Melbourne, VIC, Australia
[d]Veterinary Biosciences, Melbourne Veterinary School, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Melbourne, VIC, Australia
[e]Department of Aquatic Product Technology, Bogor Agricultural University, Bogor, Jawa Barat, Indonesia
[f]Bio21 Mass Spectrometry and Proteomics Facility, The Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Melbourne, VIC, Australia
[g]Allergy Centre, Townsville, QLD, Australia
[h]Department of Immunology and Pathology, Monash University, Melbourne, VIC, Australia
[i]Department of Allergy, Immunology and Respiratory Medicine, Central Clinical School, Monash University and The Alfred Hospital, Melbourne, VIC, Australia
[j]Department of Allergy and Immunology, Children's Hospital at Westmead, Sydney, NSW, Australia

Funding for this research was provided by the Centre for Food and Allergy Research, Murdoch Childrens Research Institute; the National Health and Medical Research Council (project grants nos. GNT1086656 and GNT1124143); the Austrian Science Fund (project grant W1248-B30); and the Medical University of Vienna.
Conflicts of interest: The authors declare that they have no relevant conflicts of interest.
Received for publication February 22, 2020; revised April 9, 2020; accepted for publication April 23, 2020.
Available online May 7, 2020.
Corresponding author: Andreas L. Lopata, PhD, College of Public Health, Medical and Veterinary Sciences, Australian Institute of Tropical Health and Medicine, Molecular Allergy Research Laboratory, James Cook University, Townsville, QLD, Australia. E-mail: andreas.lopata@jcu.edu.au.
* These authors contributed equally to this work.
2213-2198
© 2020 The Authors. Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
https://doi.org/10.1016/j.jaip.2020.04.063

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

Abbreviations used
BAT- basophil activation test
CBB- Coomassie Brilliant Blue
RBL- rat basophilic leukemia
SPT- skin prick test
WHO/IUIS- World Health Organization and International Union of Immunological Societies

patients. Inclusion of relevant collagen allergens in routine diagnosis is indicated to improve the capacity to accurately diagnose fish allergy. **© 2020 The Authors. Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).** **(J Allergy Clin Immunol Pract 2020;8:3084-92)**

Key words: *Fish allergy; Collagen; IgE; Allergy diagnosis; IgE cross-linking*

INTRODUCTION

Fish collagen type I, a major structural protein abundant in the skin, bones, and muscle of fish, is widely used in cosmetics, pharmaceutical products, and the food industry.[1,2] Although several studies reported anaphylactic reactions upon exposure to products containing fish collagen and gelatin (denatured form of collagen),[3,4] this protein has not received broad attention and its allergenic properties have not been well characterized. To date, only a few studies have investigated IgE-binding properties of fish collagen and its potency to induce allergic reactions. The outcomes of these studies were sometimes conflicting and opinions about the importance of fish collagen as an allergen remain controversial. A study by Andre et al[5] demonstrated IgE binding to collagen from tuna skin for only 3% of patients with fish allergy.[5] In contrast, in a Japanese patient cohort, specific IgE to fish collagen was detected in as many as 50% of the fish-allergic patients and demonstrated to be cross-reactive between different fish species.[6] Moreover, IgE cross-linking—induced luciferase expression upon exposure to fish collagen in rat basophilic leukemia (RBL) cells sensitized by patients' sera was demonstrated for 4 patients.[6]

Because of the incomplete understanding of its allergenic potential, fish collagen is not included in diagnostic tests for fish allergy. In addition, it was not registered as an allergen by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-Committee (www.allergen.org). A possible reason for the poor understanding of IgE-binding properties of collagen is its insolubility in neutral aqueous solutions. Common extraction buffers, such as phosphate-buffered saline, extract only low quantities of collagen.[7] Subsequently, IgE binding to collagen may be missed when using commercially available *in vitro* and skin prick tests (SPTs) for fish allergy.[8] To successfully extract collagen from tissues, acid-solubilization or enzyme-assisted extraction can be used.[9] Being an acid-soluble molecule, significant amounts of collagen may be released in the stomach following fish consumption. Understanding the allergenicity of collagen is therefore crucial for the safety and optimal management of fish-allergic patients.

Collagen type I has a unique structure of α chains that wind around each other to form triple helices. Triple helices further form elongated fibrils, which are the main components of the extracellular matrix of connective tissues where they play a structural role.[10] When heated, α chains of collagen separate and upon subsequent cooling form gelatin, a denatured form of collagen.[11] It was demonstrated that upon thermal denaturation, collagen retained its IgE-binding ability.[12]

Detailed understanding of the allergenic potential of fish collagen is of immense importance for the safety of fish-allergic patients, because unexpected exposure to fish collagen may induce serious allergic reactions. In the present study, we extracted collagen from skin and muscle tissues of frequently consumed fish species and investigated IgE binding to fish collagen in a large cohort of Australian fish-allergic patients (n = 101). In addition to IgE binding, we investigated the ability of fish collagen to induce IgE cross-linking, hence rendering it a clinically relevant allergen.

Our study demonstrated IgE sensitization to fish collagen in 21% of patients with fish allergy. Moreover, collagen induced functional IgE cross-linking, which emphasizes the importance of its wider recognition as an allergen. Based on these results, collagen α chains from Atlantic salmon and barramundi were submitted to the WHO/IUIS Allergen Nomenclature Sub-Committee and following positive evaluation designated as Sal s 6 and Lat c 6, respectively.

METHODS

Collagen extraction from skin and muscle tissue of fish

Acid-soluble collagen was extracted from skin and muscle of fish species commonly consumed in Australia and worldwide (yellowfin tuna [*Thunnus albacares*], barramundi [Asian sea bass, *Lates calcarifer*], and Atlantic salmon [*Salmo salar*]) according to the protocols described in this article's Online Repository at www.jaci-inpractice.org. After successful extraction, protein identity was confirmed by immunoblotting using a commercial anticollagen antibody (ab23730, Abcam, Cambridge, UK), as detailed in this article's Online Repository at www.jaci-inpractice.org. Collagen extract purity was confirmed by Coomassie Brilliant Blue (CBB) staining of the Any kD Mini-PROTEAN TGX Precast Protein gel (Bio-Rad, Irvine, Calif) and imaging by the Odyssey CLx imaging system (LI-COR Biosciences, Lincoln, Neb).

Fish extracts and parvalbumin purification

Protein extracts and purified parvalbumins were generated from muscle tissues of yellowfin tuna, barramundi, and Atlantic salmon, according to the methods described in this article's Online Repository, at www.jaci-inpractice.org.

Patient characteristics

Seventy-five children (under 18 years old) and 26 adults (≥18 years old) with a convincing clinical history of fish allergy were recruited at the Children's Hospital at Westmead (Sydney, NSW, Australia), The Alfred Hospital (Melbourne, VIC, Australia), and the Translational Research Facility at the Australian Institute of Tropical Health and Medicine of James Cook University (Townsville, QLD, Australia). Sensitization to fish was confirmed by ImmunoCAP (Thermo Fisher Scientific, Waltham, Mass) and/or SPT for 92 patients. ImmunoCAP was considered positive if a minimum of 0.1 kUA/L of specific IgE was detected. SPT results were considered positive if the average wheal diameter was equal to

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

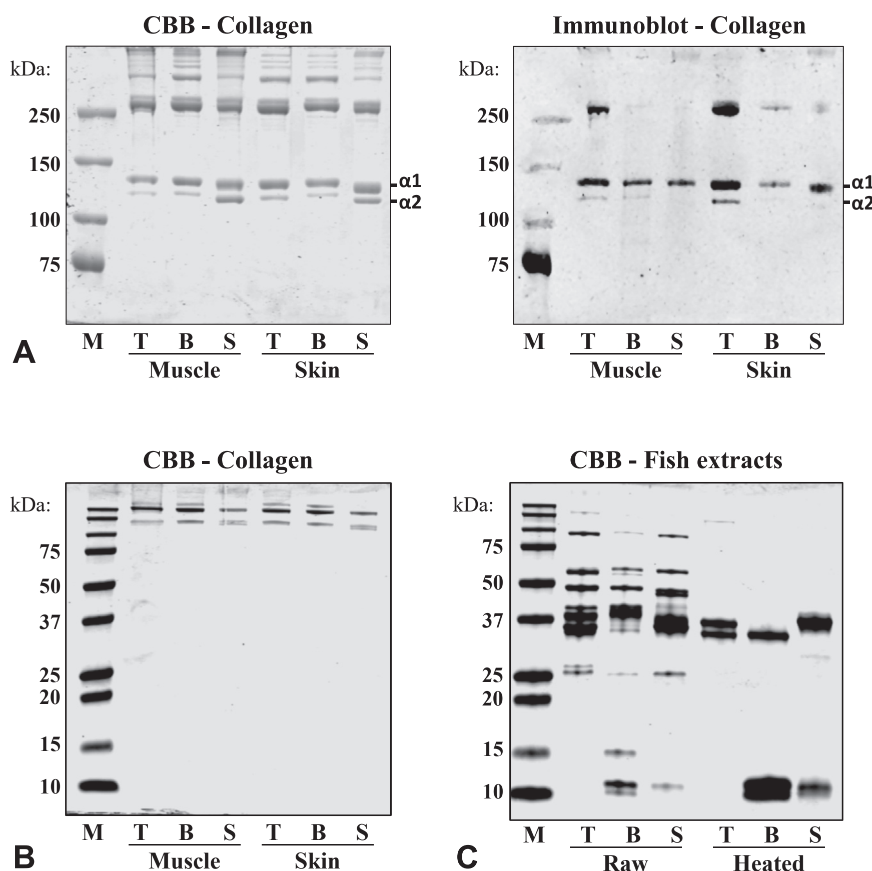

FIGURE 1. (**A**) Collagen extracted from fish muscle and skin, visualized by CBB staining of 8% gels and immunoblotting with a collagen-specific antibody. (**B**) CBB staining of purified collagen demonstrating absence of low-molecular-weight proteins (gradient gels). (**C**) Low abundance of collagen in fish extracts prepared in PBS. *B*, Barramundi; *M*, protein marker; *PBS*, phosphate-buffered saline; *S*, salmon; *T*, tuna.

or greater than 3 mm compared with that with negative control. For tuna and salmon SPT, commercial SPT preparations were used. The barramundi preparation for SPT was generated by homogenizing raw minced muscle tissue with 1 part (wt/vol) Hanks' Balanced Salt Solution (Gibco, Thermo Fisher Scientific), and aliquots were stored at −80°C until single use. Demographic and clinical characteristics of all patients are presented in Table E1 in this article's Online Repository at www.jaci-inpractice.org. Information about implicated fish species is based on patients' history of allergic reactions upon eating specific fish.

As negative controls, 2 nonatopic individuals without history of type I allergy and 3 atopic individuals with allergies other than to fish were recruited (see Table E2 in this article's Online Repository at www.jaci-inpractice.org).

Written informed consent was obtained from all participants or their legal representatives, and patient anonymity was preserved. Ethics approvals were obtained from the Sydney Children's Hospitals Network (LNR-14/SCHN/185), and the ethics committees of the Alfred Hospital (project no. 192/07), Monash University (MUHREC CF08/0225), and the James Cook University (H4313 and H6829).

IgE ELISA

All fish-allergic patients were analyzed for IgE binding to fish collagen using ELISA. Collagen-positive patients were further tested for IgE binding to parvalbumins from the same 3 fish species. Sera of 5 negative controls (Ctr 1 to Ctr 5) were used for determination of the threshold for a positive signal, calculated as the average signal from the negative controls plus 3 standard deviations. Detailed methods used for the ELISA are specified in this article's Online Repository at www.jaci-inpractice.org.

IgE immunoblotting

To analyze serum IgE binding of fish-allergic patients to the collagen α chains derived from muscle and skin of the 3 fish species, and to proteins present in whole-fish extracts, immunoblotting was performed as described in this article's Online Repository at www.jaci-inpractice.org. Densitometric analysis of IgE-binding intensity to each collagen α chain obtained by IgE immunoblotting was performed using the Image Studio Lite software (version 5.2; LI-COR Biosciences).

RBL assay

To demonstrate the ability of fish collagen to induce IgE cross-linking and subsequent allergic reaction, we used the RS-ATL8 cell line. This cell line comprises RBL cells stably transfected with the α chain of human FcεRI, with the nuclear factor of activated T-cell—responsive luciferase reporter gene. Allergen-induced IgE cross-linking is detected by the expression of luciferase.[13] RBL assays were performed according to the protocol established and validated by Ali et al,[14] with slight modifications described in this article's Online Repository at www.jaci-inpractice.org.

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

Basophil activation test

The ability of fish collagen to activate human basophils was shown by direct basophil activation test (BAT) with fresh blood from 1 patient (A7) and 1 atopic control (Ctr 6). BAT was performed using the Flow-CAST kit (Bühlmann Laboratories AG, Schönenbuch, Switzerland) according to the manufacturer's protocol. Detailed methods used are specified in this article's Online Repository at www.jaci-inpractice.org.

Mass spectrometric identification of collagen α chains using LC-MS/MS

To explore the isoform composition of the collagen α chains, purified collagens from barramundi and Atlantic salmon were separated by 8% SDS-PAGE and stained by CBB. Bands corresponding to the molecular weight of collagen α chains were excised and subjected to mass spectrometric analysis after tryptic digestion as explained in this article's Online Repository at www.jaci-inpractice.org.

Statistical analysis

Significant differences in RBL cell activation upon exposure to collagens or parvalbumins in comparison with unstimulated cells were determined by 1-way ANOVA followed by Dunett test for multiple comparisons. *P* values below .05 were regarded as significant ($*P < .05$; $**P < .01$; $***P < .001$). Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, Calif).

RESULTS

Efficient extraction of collagen from fish tissues

Acid-soluble collagen was successfully extracted from muscle and skin tissues of yellowfin tuna, barramundi, and Atlantic salmon. The generated collagen extracts contained collagen type I α1 and α2 chains, as well as β chains, γ chains, and their multimers, as demonstrated by CBB staining of 8% gels (Figure 1, *A*, left). The identity of collagen α chains was confirmed by immunoblotting using an anticollagen antibody (Figure 1, *A*, right). The purity of the collagen preparations was demonstrated by CBB staining of gradient gels where no proteins with a molecular weight below that of collagen α chains could be observed (Figure 1, *B*). In addition, we demonstrated that fish extracts prepared in phosphate-buffered saline, the most frequent approach for preparing commercial extracts for allergy diagnosis, contain very low quantities of collagen. This was shown by negligible CBB staining of proteins of molecular weights corresponding to collagen in whole-fish extracts (Figure 1, *C*).

IgE binding to fish collagen by ELISA

Sera of 101 patients were tested for IgE reactivity to a pool of purified collagens from muscle and skin tissues of yellowfin tuna, barramundi, and Atlantic salmon using ELISA. Collagen-specific IgE was demonstrated in 21% of the patients (Figure 2, *A*). A higher proportion of adults (28%) than children (18%) demonstrated sensitization to fish collagen (Figure 2, *B*). Moreover, *in vitro* IgE-binding intensity to collagen was stronger for sensitized adults than for children (Figure 2, *C*). All identified collagen-sensitized patients are labeled with an asterisk (*) in Table E1.

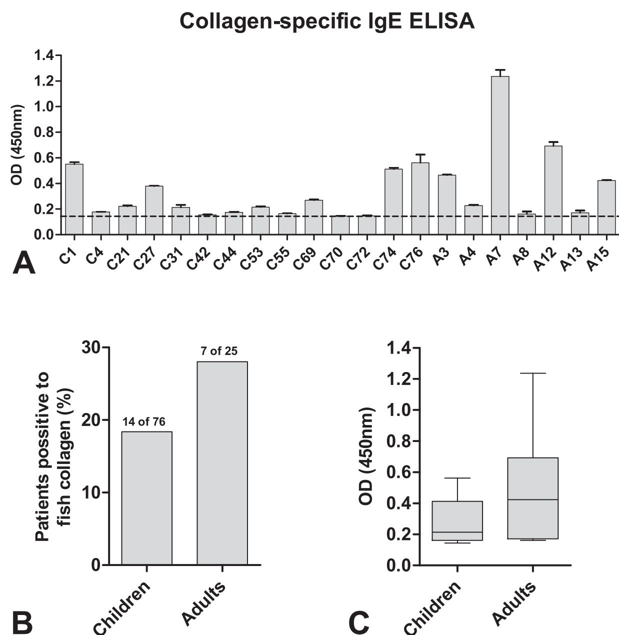

FIGURE 2. (**A**) IgE binding to a pool of purified collagens from muscle and skin of yellowfin tuna, barramundi, and Atlantic salmon. Results for collagen-positive (21 of 101) patients are shown. The threshold for positivity (dashed line) was calculated using the average signal from 5 negative controls + 3 SD. (**B**) Higher frequency of IgE binding to fish collagen in adults than in children demonstrated by ELISA. (**C**) Box plots showing stronger IgE binding to collagen for adults than for children. Black lines indicate median. *A*, Adults; *C*, children.

Patient-specific IgE binding to different collagen α chains

To investigate IgE binding to species- and tissue-specific collagen α chains, 13 collagen-positive patients from the prescreen (performed by ELISA with the pool of collagens) were analyzed by IgE immunoblotting using single collagen preparations. Patient-specific IgE-binding intensities to collagen α1 and α2 chains from muscle and skin of fish were observed (Figure 3; see Figure E1 in this article's Online Repository at www.jaci-inpractice.org). For example, stronger IgE binding to tuna skin than to tuna muscle α1 and α2 collagen chains was observed for most of the patients. In the case of salmon, IgE binding to muscle collagen was stronger than to skin collagen for some patients, for example, C1, C21, C27, and C76. In addition, serum of patient A12 showed stronger IgE binding to α1 than to α2 chains from both fish tissues, demonstrating the absence of a tissue-specific response. IgE binding to collagen by sera of patients A8 and A15 was much weaker in comparison to other patients (Figures 3 and E1).

IgE to fish parvalbumins in collagen-sensitized patients

Subsequently, all 21 collagen-sensitized patients from our cohort were tested for IgE binding to the major allergen parvalbumin from the same fish species. Most of the patients demonstrated IgE to parvalbumins (Figure 4). However, 8

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

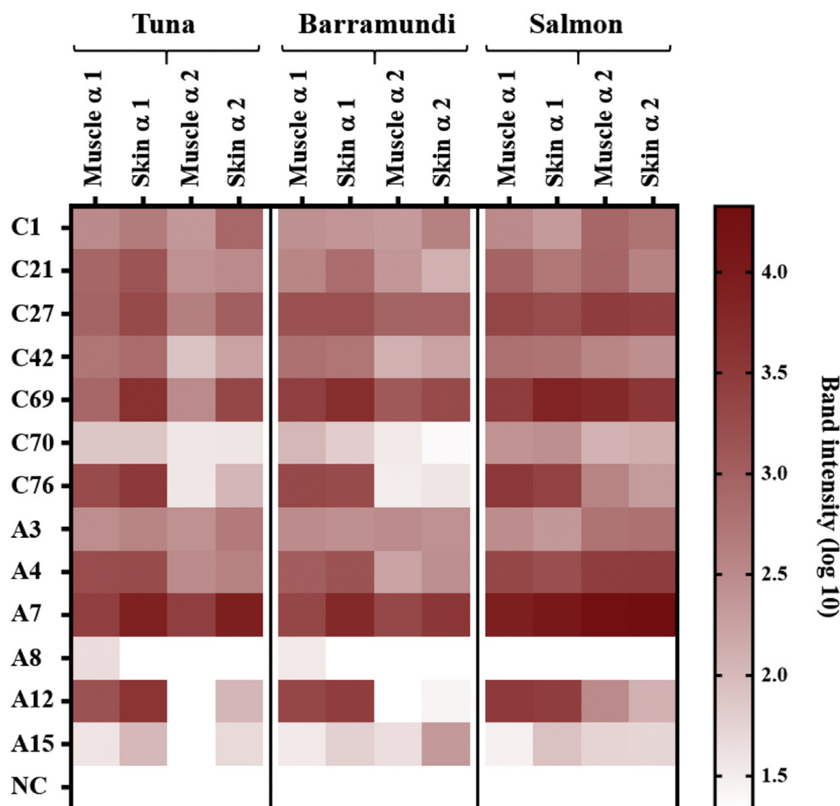

FIGURE 3. Densitometric analysis of IgE-binding intensity to collagen α1 and α2 chains obtained by IgE immunoblotting using sera of collagen-sensitized patients. *A*, Adults; *C*, children; *NC*, immunoblot using pooled sera from 5 negative controls.

patients showed absence of parvalbumin-specific IgE while demonstrating moderate to strong IgE binding to collagen from specific fish species. All patients with sensitization to collagen but absence of IgE to parvalbumin from the corresponding species are depicted with gray circles below the patient code. For example, IgE of patient A7, with clinically confirmed fish allergy and anaphylactic symptoms, failed to bind barramundi parvalbumin and bound only weakly to parvalbumins from tuna and salmon in ELISA. In contrast, IgE of this patient demonstrated strong binding to collagen from all 3 fish species as previously shown (Figure 3). Similarly, IgE of C69 and C76 demonstrated moderate to strong binding to tuna collagen (Figure 3), whereas IgE to tuna parvalbumin was absent (Figure 4).

To elucidate whether some of the patients with limited IgE binding to parvalbumin have IgE specific to other fish allergens except for collagen, sera of 2 collagen-sensitized patients (A7 and C76) were further tested by IgE immunoblots using whole-fish extracts. As a control, a collagen-negative patient (C54) was used. IgE binding to proteins corresponding to molecular weights of aldolase A, β-enolase, or tropomyosin was shown for both collagen-sensitized patients when raw fish extracts were used (see Figure E2 in this article's Online Repository at www.jaci-inpractice.org). However, when heated extracts were used, IgE binding to these proteins was diminished, especially for tuna and barramundi. In contrast to patient C54, no IgE binding to parvalbumin was detectable for A7 and C76 by immunoblots.

Collagen-induced IgE cross-linking

Sera of 6 fish-allergic patients and 3 negative controls were used for sensitization of RS-ATL8 cells. Of 6 tested patients, 5 (C27, C69, C76, A7, and A12) demonstrated a significant response to fish collagen (Figure 5). Signal intensity was patient-dependent and the strongest signal upon exposure of the cells to collagen was observed for A7 and A12, which were also strongly positive to collagen in ELISA (Figure 2, *A*). RBL cells sensitized by serum of patient C76 demonstrated a positive signal upon exposure to collagens but not to parvalbumins from all 3 fish species (Figure 5). Cells sensitized by sera of negative control individuals were not stimulated by any of the collagens nor parvalbumins, confirming specificity of the assay.

In addition to the RBL assay, the ability of fish collagen to induce functional IgE cross-linking was confirmed by BAT using fresh blood of patient A7 (see Figure E3 in this article's Online Repository at www.jaci-inpractice.org). Salmon collagen showed the highest potency to activate basophils, followed by tuna collagen, reflecting the immunoblot data for this patient (Figure E1). Barramundi collagen demonstrated a positive BAT result only at a very high concentration of 100 μg/mL (data not shown). BAT result with parvalbumins from all 3 species was negative for A7 (Figure E3).

Identification of collagen α chains by LC-MS/MS and allergen registration

Collagen identities of IgE-binding bands observed in immunoblots were confirmed by tryptic digestion and subsequent LC-

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

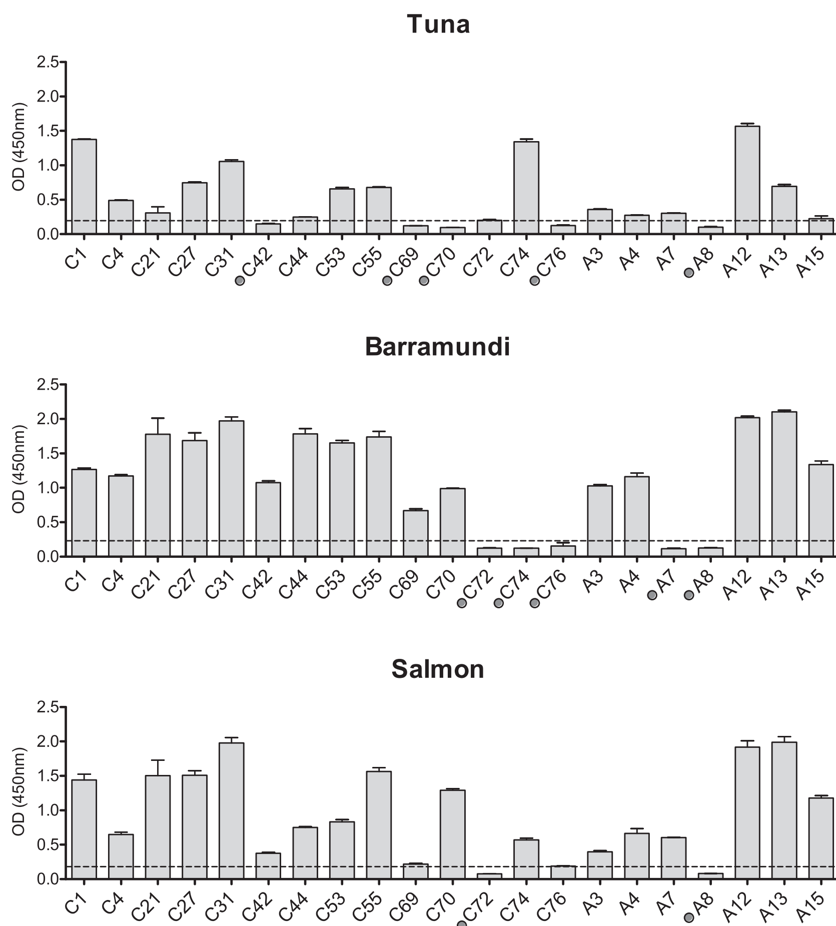

FIGURE 4. Binding to fish parvalbumins by IgE from collagen-sensitized patients' sera. Patients with sensitization to collagen but absence of IgE to parvalbumin from the corresponding species are depicted with gray circles. The threshold for positivity (dashed line) was calculated using the average signal from 5 negative controls + 3 SD. *A*, Adults; *C*, children.

MS/MS analysis of the resulting peptides for barramundi and salmon (see Table E3 in this article's Online Repository at www.jaci-inpractice.org). Collagen α chains from barramundi and salmon were submitted to the WHO/IUIS Allergen Nomenclature Sub-Committee and were designated as Lat c 6 and Sal s 6, respectively.

DISCUSSION

Fish collagen is widely used in the pharmaceutical industry (tissue engineering, dental applications, wound dressings), food industry (gummy candies, jellies, collagen supplements, beer clarification), and recently cosmetics.[15,16] In recent years its use has increased, in part to replace bovine- or porcine-derived collagen due to dietary and religious food restrictions.[15,17] Because anaphylactic reactions have been reported following consumption of products containing collagen and gelatin from various sources, it is clear that fish-allergic patients are at risk of exposure to this protein not only by whole-fish consumption but also accidentally, by using the above-mentioned sources.[3,18] Comprehensive understanding of collagen's allergenic properties is therefore of immense importance for patient safety.

Studies in Japan have reported allergenic properties of fish collagen and demonstrated frequent IgE sensitization to collagen in fish-allergic patients. In addition, thermal stability of IgE-binding epitopes and cross-reactivity between different fish species was shown.[12,19] However, the clinical relevance of this protein elsewhere in the world has not been widely investigated. Two European studies based on food challenges showed a low relevance of collagen for fish-allergic patients.[5,20] In contrast, other studies demonstrated anaphylactic reactions upon exposure to this protein, for example, while consuming marshmallows.[3] Importantly, although fish collagen is declared on food products in the United States, it is exempted from mandatory labeling in Europe.[21]

The present study aimed to improve the understanding of the allergenic properties of fish collagen derived from frequently consumed species—Atlantic salmon, yellowfin tuna, and barramundi. We extracted acid-soluble collagen from muscle and skin tissues of fish using 0.5 mol acetic acid. Harsh acidic or enzymatic treatment of tissues is generally required to successfully extract collagen, due to its insolubility in aqueous solutions.[22] This may also be the reason why commercial fish extracts used in fish allergy diagnostics contain very low quantities of collagen, leading to a misjudgment of the

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

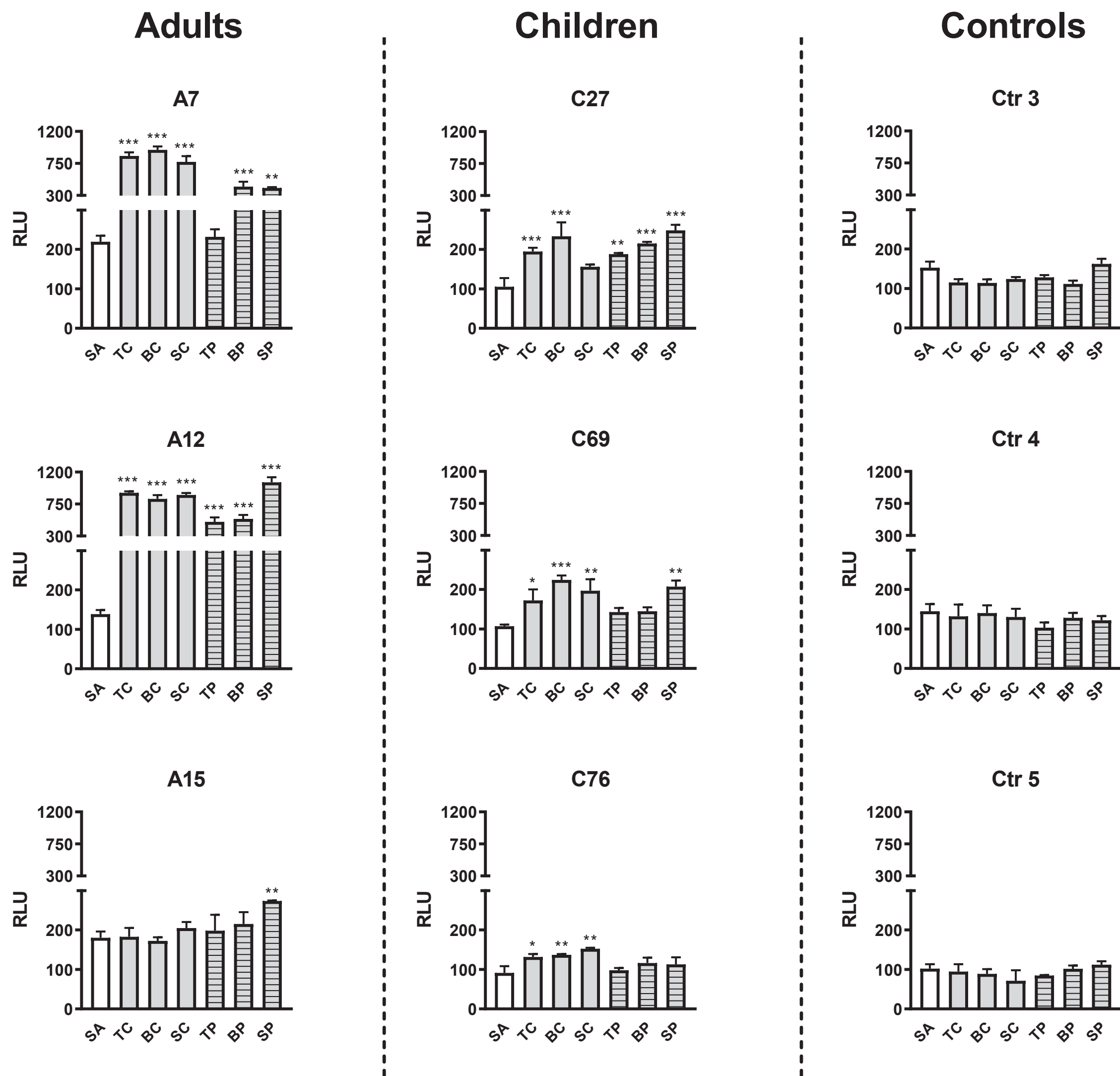

FIGURE 5. Activation of RS-ATL8 cells sensitized by sera of 3 collagen-sensitized children, 3 adults, and 3 negative controls, upon exposure to collagens and parvalbumins from tuna, barramundi, and salmon. *A*, Adult; *BC*, barramundi collagen; *BP*, barramundi parvalbumin; *C*, children; *Ctrl*, control; *RLU*, relative luminescence units; *SA*, spontaneous activation; *SC*, salmon collagen; *SP*, salmon parvalbumin; *TC*, tuna collagen; *TP*, tuna parvalbumin. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$ (significantly different from spontaneous activation of cells).

frequency of IgE sensitization to this protein and discordance with clinical suspicion of fish allergy in some individuals.[8] As recently reported by Ruethers et al,[8] collagen was underrepresented in commercially available fish SPT extracts from several manufacturers. Another study demonstrated false-negative results in prick-to-prick tests with raw fish due to insolubility of collagen.[23] In addition, it was reported that other relevant allergens might be low-abundant or missing from some of the commercial tests, further decreasing the accuracy of fish allergy diagnosis.[8]

Our collagen samples consisted of type I collagen α1 chains, α2 chains, as well as their dimers and multimers, as expected on the basis of previously published literature.[24,25] We first analyzed sera of 101 fish-allergic patients for IgE binding to a pool of collagens extracted from all 3 fish species. Twenty-one percent of all patients demonstrated IgE sensitization to fish collagen. Collagen specificity of our IgE ELISA was confirmed by demonstrating absence of contamination of collagen samples with other proteins, such as low-molecular-weight parvalbumins, by CBB staining of collagen extracts, and sensitive imaging.

Previous studies indicated strong cross-reactivity of collagen between different fish species.[6,19] However, differences in IgE binding to collagen derived from muscle or skin, as well as to specific α chains, have not been investigated. We hence next tested collagen-sensitized patients' sera for IgE to specific

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

collagen α chains from skin or muscle tissue of the 3 fish species. Most patients reacted to all collagen α chains, but IgE-binding intensities differed. Reactivity to all α chains by most patients indicated possible presence of cross-reactive IgE epitopes. Different, patient-dependent binding intensities to different α chains may in contrast indicate possible presence of distinct IgE epitopes on the 2 collagen α chains. A study by Shiomi et al[26] identified an immunodominant IgE epitope from rainbow trout collagen α2 chain (MKGLRGHGGLQGMPGPNGPS). The identical amino acid sequence is found in collagen α2 chain of salmon collagen (XP_013998297.1), and a homologous region with 90% sequence identity is present in barramundi collagen α2 chain (XP_018522130.1). Based on high sequence identities in this region, patient IgE cross-reactivity to different fish species may therefore be expected for patients with IgE specific for this epitope.

Most fish-allergic patients are sensitized to the major allergen parvalbumin, which is highly cross-reactive.[27] However, monosensitization to parvalbumin from certain species has been reported.[28] Patients can also be sensitized to other fish allergens.[28] Therefore, the absence of parvalbumin-specific IgE for a particular fish species does not exclude the possibility of sensitization to other fish allergens such as collagen. We thus analyzed the collagen-sensitized patients from our cohort for presence of IgE to parvalbumins from the same fish species. Most of the collagen-sensitized patients' sera contained IgE to parvalbumins. However, 8 of the collagen-sensitized fish-allergic patients (40%) had no parvalbumin-specific IgE while demonstrating IgE binding to collagen from specific fish species. This finding emphasizes the importance of recognizing collagen as a relevant allergen and its inclusion in diagnostics, because these patients could be in danger of misdiagnosis as not allergic to fish using standard extracts or only those containing parvalbumin.

In addition to IgE-binding properties, we characterized the ability of fish collagen to induce functional IgE cross-linking by RBL assay and BAT. In RBL assays, sera from 6 collagen-sensitized individuals were analyzed, with 5 demonstrating a positive result upon exposure of cells to collagen from all 3 fish species (Figure 5). One of the patients, C76, was positive to collagen in RBL but negative to parvalbumin, reflecting the previously observed direct IgE-binding pattern for this patient. The patient A15, who tested negative to collagen by RBL assay, demonstrated only weak IgE binding to collagen α chains by immunoblot. In this study, we also demonstrate for the first time IgE cross-linking and basophil activation by fish collagen using a fish-allergic patient's fresh blood. However, because BAT was performed only for 1 fish-allergic patient, further investigation of the potency of collagen to activate basophils, especially in patients not sensitized to parvalbumin, is required in a larger cohort.

On the basis of our IgE reactivity findings, and the identification of collagen α chains for these assays by LC-MS/MS, we submitted collagen α chains from barramundi and Atlantic salmon to the WHO/IUIS Allergen Nomenclature Subcommittee and they were assigned the allergen names Lat c 6 and Sal s 6, respectively. Identifying sequences of IgE-reactive collagen α chains from tuna and subsequent allergen registration will be part of further studies.

CONCLUSIONS

We demonstrate the importance of collagen as a fish allergen in a large patient cohort. Collagen from skin and muscle tissues of fish bound IgE of 21% of fish-allergic individuals and was able to induce basophil activation. Current diagnosis of fish allergy relies on parvalbumins from several species and whole-fish extracts.[27] Extracts however may contain very low quantities of clinically relevant collagen. Recognizing collagen, from both skin and muscle tissue, as an important allergen and its inclusion in fish allergy diagnostics is therefore required to help prevent adverse reactions to certain fish species and to other products containing fish collagen.

REFERENCES

1. Felician FF, Xia C, Qi W, Xu H. Collagen from marine biological sources and medical applications. Chem Biodivers 2018;15:e1700557.
2. Avila Rodriguez MI, Rodriguez Barroso LG, Sanchez ML. Collagen: a review on its sources and potential cosmetic applications. J Cosmet Dermatol 2018;17:20-6.
3. Kuehn A, Hilger C, Hentges F. Anaphylaxis provoked by ingestion of marshmallows containing fish gelatin. J Allergy Clin Immunol 2009;123:708-9.
4. Fujimoto W, Fukuda M, Yokooji T, Yamamoto T, Tanaka A, Matsuo H. Anaphylaxis provoked by ingestion of hydrolyzed fish collagen probably induced by epicutaneous sensitization. Allergol Int 2016;65:474-6.
5. Andre F, Cavagna S, Andre C. Gelatin prepared from tuna skin: a risk factor for fish allergy or sensitization? Int Arch Allergy Immunol 2003;130:17-24.
6. Kobayashi Y, Akiyama H, Huge J, Kubota H, Chikazawa S, Satoh T, et al. Fish collagen is an important panallergen in the Japanese population. Allergy 2016;71:720-3.
7. Telis V, Wolf K, Sobral P. Characterizations of collagen fibers for biodegradable films production. Presented at: 13th World Congress of Food Science & Technology; September 17-21; 2006:929. Nantes, France.
8. Ruethers T, Taki AC, Nugraha R, Cao TT, Koeberl M, Kamath SD, et al. Variability of allergens in commercial fish extracts for skin prick testing. Allergy 2019;74:1352-63.
9. Anand S, Kamath S, Chuang L, Kasapis S, Lopata AL. Biochemical and thermo-mechanical analysis of collagen from the skin of Asian Sea bass (*Lates calcarifer*) and Australasian Snapper (*Pagrus auratus*), an alternative for mammalian collagen. Eur Food Res Technol 2013;236:873-82.
10. Fidler AL, Boudko SP, Rokas A, Hudson BG. The triple helix of collagens—an ancient protein structure that enabled animal multicellularity and tissue evolution. J Cell Sci 2018;131:jcs203950.
11. Bozec L, Odlyha M. Thermal denaturation studies of collagen by microthermal analysis and atomic force microscopy. Biophys J 2011;101:228-36.
12. Kobayashi Y, Kuriyama T, Nakagawara R, Aihara M, Hamada-Sato N. Allergy to fish collagen: thermostability of collagen and IgE reactivity of patients' sera with extracts of 11 species of bony and cartilaginous fish. Allergol Int 2016;65:450-8.
13. Nakamura R, Uchida Y, Higuchi M, Nakamura R, Tsuge I, Urisu A, et al. A convenient and sensitive allergy test: IgE crosslinking-induced luciferase expression in cultured mast cells. Allergy 2010;65:1266-73.
14. Ali EA, Nakamura R, Falcone FH. Use of humanized RS-ATL8 reporter system for detection of allergen-specific IgE sensitization in human food allergy. Methods Mol Biol 2017;1592:147-61.
15. Silva TH, Moreira-Silva J, Marques AL, Domingues A, Bayon Y, Reis RL. Marine origin collagens and its potential applications. Mar Drugs 2014;12:5881-901.
16. Venkatesan J, Anil S, Kim SK, Shim MS. Marine fish proteins and peptides for cosmeceuticals: a review. Mar Drugs 2017;15:E143.

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

17. Raman M, Gopakumar K. Fish collagen and its applications in food and pharmaceutical industry: a review. EC Nutr 2018;13:752-67.
18. Land MH, Piehl MD, Burks AW. Near fatal anaphylaxis from orally administered gelatin capsule. J Allergy Clin Immunol Pract 2013;1: 99-100.
19. Kobayashi Y, Huge J, Imamura S, Hamada-Sato N. Study of the cross-reactivity of fish allergens based on a questionnaire and blood testing. Allergol Int 2016; 65:272-9.
20. Hansen TK, Poulsen LK, Stahl Skov P, Hefle SL, Hlywka JJ, Taylor SL, et al. A randomized, double-blinded, placebo-controlled oral challenge study to evaluate the allergenicity of commercial, food-grade fish gelatin. Food Chem Toxicol 2004;42:2037-44.
21. Kourani E, Corazza F, Michel O, Doyen V. What do we know about fish allergy at the end of the decade? J Investig Allergol Clin Immunol 2019; 29:414-21.
22. Wang J, Pei X, Liu H, Zhou D. Extraction and characterization of acid-soluble and pepsin-soluble collagen from skin of loach (*Misgurnus anguillicaudatus*). Int J Biol Macromol 2018;106:544-50.
23. Chikazawa S, Hashimoto T, Kobayashi Y, Satoh T. Fish-collagen allergy: a pitfall of the prick-to-prick test with raw fish. Br J Dermatol 2015;173:1330-1.
24. Muralidharan N, Jeya Shakila R, Sukumar D, Jeyasekaran G. Skin, bone and muscle collagen extraction from the trash fish, leather jacket (*Odonus niger*) and their characterization. J Food Sci Technol 2013;50: 1106-13.
25. Gistelinck C, Gioia R, Gagliardi A, Tonelli F, Marchese L, Bianchi L, et al. Zebrafish collagen type I: molecular and biochemical characterization of the major structural protein in bone and skin. Sci Rep 2016;6:21540.
26. Shiomi K, Yoshida S, Sawaguchi T, Ishizaki S. A major IgE epitope of rainbow trout collagen alpha2 chain. Shokuhin Eiseigaku Zasshi 2010;51:153-9.
27. Ruethers T, Taki AC, Johnston EB, Nugraha R, Le TTK, Kalic T, et al. Seafood allergy: a comprehensive review of fish and shellfish allergens. Mol Immunol 2018;100:28-57.
28. Kuehn A, Swoboda I, Arumugam K, Hilger C, Hentges F. Fish allergens at a glance: variable allergenicity of parvalbumins, the major fish allergens. Front Immunol 2014;5:179.

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

ONLINE REPOSITORY

METHODS

Extraction of collagen from fish tissues

Fillets with skin from yellowfin tuna (*Thunnus albacares*), barramundi (Asian sea bass, *Lates calcarifer*), and Atlantic salmon (*Salmo salar*) were purchased at the local seafood store (Townsville, Australia) and used for extraction of collagen. The fillets were washed with ultrapure water and skin separated from muscle. Skin and muscle tissues were cut into small pieces (∼0.5 cm^3), washed with 10 volumes of ultrapure water, and centrifuged (3000*g*, 5 minutes, 4°C). Supernatants were discarded, tissue samples resuspended in 10 volumes of cold 0.1 mol NaOH, and incubated for 24 hours at 4°C with gentle agitation. After centrifugation, supernatants were discarded, and samples washed with cold ultrapure water until neutral pH was obtained. For defatting, 10 volumes of 10% butyl alcohol were added and samples incubated at 4°C for 24 hours with gentle agitation. Samples were centrifuged, washed with 10 volumes of ultrapure water, and resuspended in 0.5 mol acetic acid. Collagen was extracted by stirring the samples in acetic acid for 24 hours at 4°C. Samples were centrifuged (20,000*g*, 1 hour, 4°C) and supernatant containing the extracted collagen collected and sterile filtered. For successful collagen extraction from salmon and barramundi skin and muscle tissue, 10 volumes of acetic acid were used. For collagen extraction from skin and muscle of tuna, 2 extraction steps were required: first using 15 volumes and second using 10 volumes of acetic acid. All obtained collagen extracts were concentrated using Amicon Ultra-15 Centrifugal Filter Units with 50-kDa cutoff (Merck Darmstadt, Germany).

Fish extracts and parvalbumin purification

Frozen muscle tissues of yellowfin tuna, barramundi, and Atlantic salmon were used for the purification of parvalbumins and for preparation of total protein extracts. Tissues were homogenized using a rotor-stator homogenizer in PBS (10 mmol/L phosphate; pH 7.2; 2 mL/g tissue). After gentle agitation overnight at 4°C, subsequent centrifugation (20,000*g*), and filtration (0.2-μm filters) of the supernatants, extracts were stored at −20°C until further use and referred to as raw protein extracts. For preparation of heated extracts, tissue was heated in PBS (95°C-100°C) for 20 minutes before homogenization. Parvalbumins were purified from heated extracts by ammonium sulfate precipitation with subsequent dialyses against 100 mmol/L ammonium bicarbonate buffer as described previously.[E1]

Western blot

To confirm the collagen identity of the collagen preparations, extracted collagen samples (5 μg of proteins) were separated by SDS-PAGE (8% polyacrylamide gels) and transferred to a nitrocellulose membrane. After blocking (1 hour at room temperature) using casein blocking buffer (B6429, MilliporeSigma, St. Louis, Mo) diluted in PBS, the membrane was incubated (1 hour at room temperature) with a rabbit polyclonal antibody to collagen type I (ab23730, Abcam) diluted 1:5000 in 10% blocking buffer in PBS with 0.05% tween-20 (PBST). After washing, IRDye 800CW antirabbit IgG secondary antibody (926-32213, LI-COR) was added to the membrane diluted 1:10,000 in 50% blocking buffer in PBST and incubated for 45 minutes at room temperature. The membrane was washed and subsequently scanned using the Odyssey CLx imaging system (LI-COR Biosciences, Lincoln, Neb). Acquired images were analyzed using Image Studio Lite software (LI-COR, Lincoln, Neb).

To analyze binding of serum IgE of fish-allergic patients to the collagen α chains, extracted collagen samples were applied to SDS-PAGE (8% gels), transferred to a nitrocellulose membrane, and blocked using casein blocking buffer as described above. Blocked membranes were incubated with patients' sera derived from 7 children and 6 adults, as well as a pool of sera from 5 negative controls (Ctr 1-Ctr 5). All sera were diluted 1:15 in 20% blocking buffer in PBST. After overnight incubation, membranes were washed and incubated (1 hour at room temperature) with polyclonal rabbit antihuman IgE antibody (A0094, DAKO, Agilent, Santa Clara, Calif) diluted 1:8000 in 20% blocking buffer in PBST. Bound IgE was detected by antirabbit IgG IRDye 800CW.

Binding of serum IgE of fish-allergic patients to whole-fish extracts (raw and heated) was analyzed using 12% gels, according to previously published protocols.[E2]

RBL assay

RS-ATL8 cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2, in minimum essential media (Thermo Fisher Scientific, Waltham, Mass, 11095-080) supplemented with 10% FBS (Gibco, Thermo Fisher Scientific, 10270-106), 100 U/mL penicillin-streptomycin (Gibco, 15140122), 0.5 mg/mL geneticin (Thermo Fisher Scientific, 10131035), and 0.2 mg/mL Hygromycin B (Thermo Fisher Scientific, 10687010). Passive sensitization of the cells was performed using sera of collagen-sensitized patients (C27, C69, C76, A7, A12, or A15) or control individuals (Ctr 3, Ctr 4, or Ctr 5). All sera were used in final dilution of 1:30 in cell culture medium and sensitization was performed for 18 hours. After washing with PBS, cells were stimulated with collagens or parvalbumins from muscle tissues of tuna, barramundi, and salmon for 4 hours. All allergens were applied at a concentration of 10 μg/mL, which was determined to be optimal for both collagen and parvalbumin in a separate experiment (data not shown). After 4 hours of stimulation with allergens, ONE-Glo Luciferase Assay System (Promega, Madison, Wis) was added and luminescence measured using the Spark multimode microplate reader (Tecan, Switzerland).

BASOPHIL ACTIVATION TEST

As stimulants, purified collagens from muscle tissues of tuna, barramundi, and salmon, as well as parvalbumins from the same species, were used. Collagens were used in 10-fold serial dilutions between 0.01 μg/mL and 100 μg/mL. Parvalbumins were used in 10-fold serial dilution ranges between 0.01 μg/mL and 10 μg/mL, which was demonstrated to be optimal on the basis of previous research.[E3] For the atopic control (Ctr 6), 0.01, 1, and 10 μg/mL collagen extracts, as well as 0.1 and 10 μg/mL parvalbumins, were used for the basophil stimulation. In addition, as the atopic control had a confirmed sensitization to house-dust mite, we used the house-dust mite extract (Stallergenes Greer Laboratories, Sydney, Australia) as an additional positive control for activation of basophils, in concentrations of 0.001, 0.1, and 10 μg/mL. House-dust mite extract (0.1 μg/mL) induced activation of 17% of basophils (data not shown). Activation of more than 10% of the basophils was considered a positive result.

Stimulation buffer was used as a negative control. Anti-FcεRI antibody and formyl-methionyl-leucyl-phenylalanine were used as positive controls. For both tested individuals, the positive controls

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

activated more than 10% of basophils (data not shown). The percentage of activated basophils in each sample was determined by expression of the activation marker CD63 measured by flow cytometry. Basophils were gated on the basis of high CCR3 expression and low side scatter ($CCR3^{high}$, SSC^{low}). Data acquisition was performed using a BD FACS Canto II Flow Cytometer. Data were analyzed using FlowJo software (FloJo LLC, Ashland, Ore).

Mass spectrometric identification of allergens using LC-MS/MS

The mass spectrometric analysis of peptides obtained after tryptic digestion of collagen bands from barramundi and salmon was performed as published previously.[E4] Briefly, the extracted peptides were analyzed using an LTQ Orbitrap Elite (Thermo Scientific) coupled to an Ultimate 3000 RSLC nanosystem (Dionex, Thermo Fischer Scientific). The nanoLC system was equipped with an Acclaim Pepmap nano-trap column and an Acclaim Pepmap analytical column. The peptide mix was loaded onto the trap column before the enrichment column was switched in-line with the analytical column. The LTQ Orbitrap Elite mass spectrometer was operated in the data-dependent mode, spectra acquired first in positive mode at 240 k resolution followed by collision-induced dissociation fragmentation. Twenty of the most intense peptide ions with charge states greater than or equal to 2 were isolated and fragmented using normalized collision energy of 35 and activation Q of 0.25 (collision-induced dissociation). All results were analyzed with Mascot search engine and cross-referenced against NCBI protein databases containing sequences of all barramundi or Atlantic salmon proteins (March 2019). Variable modifications of carbamidomethyl-C and N-terminus, deamidation N, deamidation Q, and oxidation of M were selected. Mass spectrometric analyses were performed at the Melbourne Mass Spectrometry and Proteomics Facility of The Bio21 Molecular Science and Biotechnology Institute at The University of Melbourne, Melbourne, Australia.

LC-MS/MS analysis of collagen extracts from yellowfin tuna was not performed because of absence of genome annotation for this species.

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

TABLE E1. Demographic and clinical characteristics of fish-allergic patients

Patients	Age (y)	Sex	Clinical symptoms	SPT Tuna (mm)	SPT Barramundi (mm)	SPT Salmon (mm)	ImmunoCAP Tuna (kUA/L)	ImmunoCAP Salmon (kUA/L)	Implicated fish
Pediatric patients									
C1*	14	M	AE, OAS	0.0	13.0	0.0	1.5	4.0	ND
C2	5	M	AN, U	5.0	15.0	10.0	ND	ND	Salmon, sardine
C3	7	F	GIS, U	6.0	ND	3.0	ND	ND	Catfish
C4*	9	M	AE, C, U	10.0	15.0	15.0	ND	ND	White bait
C5	10	M	ND	5.5	8.0	4.5	ND	ND	Ling
C6	1	M	AE, OAS, U	4.5	24.5	2.5	ND	ND	Sea bass, salmon, barramundi
C7	5	M	U	2.0	0.0	8.0	ND	ND	Cod
C8	13	F	GIS, RD, U	3.0	12.0	0.0	ND	ND	Barramundi, catfish
C9	11	M	AE, GIS, OAS, U	4.0	11.5	12.0	4.8	25.3	Tuna
C10	14	M	AN	3.0	9.5	0.0	ND	ND	Barramundi
C11	5	F	GIS, U	0.0	7.0	5.5	ND	ND	Salmon
C12	10	M	U	2.0	5.0	7.5	ND	ND	Tuna, salmon, yellowtail
C13	7	F	AE, OAS, U	5.0	16.0	3.0	ND	ND	Salmon
C14	10	M	AN, RD, U	3.0	8.0	4.0	ND	ND	White bait
C15	7	M	OAS, U	0.0	18.5	5.0	0.1	0.6	White fish
C16	13	M	OAS, U	2.5	21.0	4.5	2.0	1.2	White fish
C17	14	M	AE	2.5	10.5	0.0	0.3	0.2	Salmon
C18	3	M	AE, U	4.0	5.0	0.0	0.0	0.0	Basa
C19	12	F	OAS, U	4.0	19.0	11.0	1.4	3.4	White fish
C20	2	F	AE, U	0.0	0.0	3.5	0.8	0.6	Salmon
C21*	12	M	U	9.0	16.5	8.0	5.9	9.0	Barramundi
C22	9	M	AN, RD, U	5.5	15.5	3.0	2.2	5.3	Trevally
C23	11	M	AE, AN, RD, U	0.0	3.5	0.0	0.1	0.1	White fish
C24	1	F	AE, RD, U	0.0	15.5	3.0	5.1	5.6	Barramundi
C25	11	M	AE	5.0	21.0	4.5	39.5	66.1	Salmon
C26	8	M	OAS	4.5	7.0	2.0	4.7	9.8	Salmon
C27*	15	M	AE, OAS	5.5	20.0	8.5	12.1	17.6	Ling
C28	2	F	AE, U	5.5	18.5	13.5	31.0	73.4	Cod
C29	8	M	GIS, RD, U	6.5	19.0	12.0	21.0	70.5	White fish
C30	10	F	AE, OAS, RD	2.0	9.0	3.5	7.3	9.1	White fish
C31*	6	F	AE	3.0	19.0	4.5	ND	ND	White fish
C32	9	M	AE	2.0	6.5	6.5	0.1	0.5	Croaker
C33	2	M	U	4.0	4.5	7.5	ND	ND	Salmon
C34	10	F	GIS, RD, OAS	0.0	17.0	0.0	0.2	0.1	Basa
C35	5	M	AE	5.0	12.5	6.0	6.8	11.5	Leather jacket
C36	7	M	AE, OAS, U	0.0	7.5	6.0	0.7	7.6	Trout
C37	7	F	U	2.5	16.0	0.0	1.2	3.1	White fish

(continued)

TABLE E1. *(Continued)*

Patients	Age (y)	Sex	Clinical symptoms	SPT Tuna (mm)	SPT Barramundi (mm)	SPT Salmon (mm)	ImmunoCAP Tuna (kUA/L)	ImmunoCAP Salmon (kUA/L)	Implicated fish
C38	4	M	AP, U	0.0	10.0	7.0	17.3	21.0	White fish
C39	14	M	OAS, U	1.0	9.5	10.5	0.7	1.6	Salmon
C40	7	M	C, GIS	2.0	17.5	2.0	1.0	0.5	Tilapia
C41	4	F	AE, E, U	0.0	17.0	0.0	0.7	0.5	Basa
C42*	15	F	AE, U	2.5	55.0	5.0	0.9	1.4	Barramundi
C43	12	M	RD, U	3.0	14.0	3.0	0.2	0.2	ND
C44*	9	M	AE	4.0	14.0	6.5	2.4	3.7	Silverperch
C45	2	F	U	4.5	17.0	7.0	ND	ND	Salmon, yellowtail
C46	12	F	AE	3.0	14.0	4.5	14.9	38.9	Flathead
C47	7	M	AN, RD, U	3.5	3.5	3.5	ND	ND	ND
C48	15	F	AE, U	4.5	16.0	4.0	ND	ND	ND
C49	12	M	E, U	0.0	0.0	7.0	3.9	31.8	Salmon, tuna
C50	9	M	AE, E, U	0.0	10.0	4.5	ND	ND	ND
C51	11	F	AN, AE, OAS, RD	5.5	4.0	0.0	<0.1	<0.1	Silver bream
C52	17	F	GIS	4.0	7.0	0.0	ND	ND	Basa
C53*	8	M	AN, R, RD, U	4.5	10.0	4.5	ND	ND	Australian cod
C54	7	M	AE, U	4.0	13.5	7.5	ND	ND	Ling
C55*	13	F	U	5.0	15.5	9.5	9.4	20.6	Salmon
C56	1	M	AE, U	1.0	12.0	6.0	ND	ND	Yellowtail
C57	5	M	AE, OAS, U	0.0	13.5	3.0	ND	ND	White fish
C58	15	M	U	4.0	19.0	12.0	ND	ND	Milkfish
C59	10	M	U	0.0	14.0	4.5	ND	ND	Perch
C60	8	M	AN, AE, RD	0.0	20.5	6.5	ND	ND	Bream
C61	17	F	AE, AN, GIS, RD	3.5	0.0	0.0	ND	ND	White fish
C62	16	M	U	5.5	17.0	4.5	ND	ND	Snapper
C63	10	M	AE	5.5	17.5	10.5	13.5	54.2	Barramundi
C64	11	M	OAS, AP	0.0	9.5	9.0	ND	ND	White fish
C65	15	F	E, U	5.5	16.5	5.0	ND	ND	Salmon
C66	11	M	AE, C, RD	3.5	3.5	3.0	ND	ND	Salmon
C67	15	M	AP, GIS, RD	2.0	8.5	2.5	ND	ND	ND
C68	13	M	C, RD, U	0.0	16.0	4.5	ND	ND	Milk fish, tilapia
C69*	17	F	OAS, R	1.0	11.5	2.5	3.2	2.9	White fish
C70*	14	M	RD, U	4.5	6.5	11.0	1.2	8.1	ND
C71	13	F	OAS, U	0.0	5.0	0.0	ND	ND	White fish
C72*	13	F	RD	ND	ND	ND	0.3	0.1	ND
C73	10	F	AE	ND	ND	ND	ND	ND	ND
C74*	5	M	OAS, U	ND	ND	ND	3.4	0.9	ND
C75	13	F	ND	7 mm	ND	ND	ND	3.3	ND
C76*	17	M	AE, U	ND	ND	ND	7.8	7.1	ND

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

Adult patients									
A1	18	M	RD, U	4.0	13.0	6.5	1.1	3.2	Tuna
A2	20	M	AE, U	0.0	14.5	3.0	ND	ND	White fish
A3*	21	F	AE, GIS, U	0.0	0.0	0.0	1.3	0.6	Snapper, tuna
A4*	18	M	AE, AN, C, RD	3.0	9.5	6.0	ND	ND	Salmon, silver perch, flake
A5	18	M	GIS	0.0	5.0	4.5	ND	ND	Basa
A6	48	F	AN	ND	ND	ND	0.3	0.6	Tout, herring, red grouper
A7*	24	F	AN	ND	ND	5.0	ND	21.4	ND
A8*	18	M	AN	ND	3.0	ND	<0.1	0.1	ND
A9	72	M	OAS, U	ND	ND	ND	<0.1	<0.1	ND
A10	36	F	GIS	0.0	0.0	0.0	ND	ND	ND
A11	48	F	NA	3.0	ND	ND	ND	ND	ND
A12*	21	F	AE, U	ND	ND	ND	53.0	>100	ND
A13*	18	F	AE	ND	ND	ND	29.9	53.3	ND
A14	19	F	OAS	ND	ND	ND	11.9	ND	ND
A15*	29	F	AE, U	ND	ND	ND	5.7	13.6	ND
A16	33	F	AE, E	ND	ND	ND	0.1	<0.01	Mackerel
A17	32	F	RD	ND	ND	ND	ND	ND	Salmon, mackerel, whiting, tuna
A18	31	F	E	ND	ND	ND	0.1	0.9	Salmon, tuna
A19	51	M	AE, OAS	ND	ND	ND	0.3	0.4	Salmon
A20	49	F	GIS	ND	ND	ND	<0.1	<0.1	Salmon
A21	43	M	AE, E	ND	ND	ND	<0.1	<0.1	ND
A22	49	F	AP, GIS	ND	ND	ND	<0.1	<0.1	Salmon
A23	78	M	AE, OAS, RD	ND	ND	ND	<0.1	<0.1	Barramundi
A24	57	F	AE, GIS, OAS	ND	ND	ND	0.7	1.1	Tuna, mullet, billfish
A25	29	F	GIS, OAS, E	ND	ND	ND	<0.1	<0.1	ND

AE, Angioedema; *AN*, anaphylaxis; *AP*, abdominal pain; *C*, conjunctivitis; *E*, eczema; *F*, female; *GIS*, gastrointestinal symptoms; *M*, male; *NA*, not available/applicable; *ND*, not determined; *OAS*, oral allergy syndrome; *R*, rhinitis; *RD*, respiratory distress; *U*, urticaria.

*Patients identified as collagen-sensitized in our study.

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

TABLE E2. Demographic and clinical characteristics of control individuals

Participant no.	Age (y)	Sex	Allergies
Ctr 1	23	F	Shrimp
Ctr 2	26	M	Shrimp
Ctr 3	33	F	None
Ctr 4	29	F	None
Ctr 5	37	F	None
Ctr 6	32	M	House-dust mite

F, Female; *M*, male.

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

TABLE E3. Collagen sequences identified by LC-MS/MS

Sample	Molecular weight of a band (according to SDS-PAGE)	Hit	Protein ID (NCBI)	Protein name	Mass (Da)	Mascot score	emPAI value
Barramundi muscle	130 kDa	1	*XP_018522130.1	Collagen alpha-2(I) chain isoform X1	126,912	1,025	0.89
		2	XP_018521723.1	Collagen alpha-1(I) chain-like isoform X1	136,649	422	0.39
		3	XP_018553992.1	Collagen alpha-1(I) chain-like isoform X1	136,922	408	0.33
	140 kDa	1	*XP_018553992.1	Collagen alpha-1(I) chain-like isoform X1	136,922	1,037	0.72
		2	XP_018521723.1	Collagen alpha-1(I) chain-like isoform X1	136,649	950	0.76
		3	XP_018522130.1	Collagen alpha-2(I) chain isoform X1	126,912	445	0.32
Barramundi skin	130 kDa	1	*XP_018522130.1	Collagen alpha-2(I) chain isoform X1	126,912	1,447	1.63
		2	XP_018521723.1	Collagen alpha-1(I) chain-like isoform X1	136,649	783	0.72
		3	XP_018553992.1	Collagen alpha-1(I) chain-like isoform X1	136,922	550	0.42
	140 kDa	1	*XP_018521723.1	Collagen alpha-1(I) chain-like isoform X1	136,649	1,618	1.57
		2	XP_018553992.1	Collagen alpha-1(I) chain-like isoform X1	136,922	914	0.72
		3	XP_018522130.1	Collagen alpha-2(I) chain isoform X1	126,912	551	0.46
Salmon muscle	130 kDa	1	*XP_013998297.1	Collagen alpha-2(I) chain isoform X1	126,924	2,317	2.31
		2	*XP_014033985.1	Collagen alpha-2(I) chain isoform X1	126,930	2,046	2.3
		3	XP_014048044.1	Collagen alpha-1(I) chain-like	136,832	944	0.53
	140 kDa	1	*XP_014059932.1	Collagen alpha-1(I) chain	136,851	2,107	1.13
		2	*XP_014048044.1	Collagen alpha-1(I) chain-like	136,832	2,039	0.98
		3	XP_013998297.1	Collagen alpha-2(I) chain isoform X1	126,924	1,089	1.26
Salmon skin	130 kDa	1	*XP_013998297.1	Collagen alpha-2(I) chain isoform X1	126,924	2,695	3.26
		2	*XP_014033985.1	Collagen alpha-2(I) chain isoform X1	126,930	2,406	2.95
		3	XP_014059932.1	Collagen alpha-1(I) chain	136,851	903	0.49
	140 kDa	1	*XP_014059932.1	Collagen alpha-1(I) chain	136,851	2,609	1.76
		2	*XP_014048044.1	Collagen alpha-1(I) chain-like	136,832	2,426	1.39
		3	XP_014035319.1	Collagen alpha-1(I) chain-like	137,390	1,667	1.88

emPAI, Exponentially modified protein abundance index.
*Sequences registered in the database of the WHO/IUIS Allergen Nomenclature Sub-Committee.

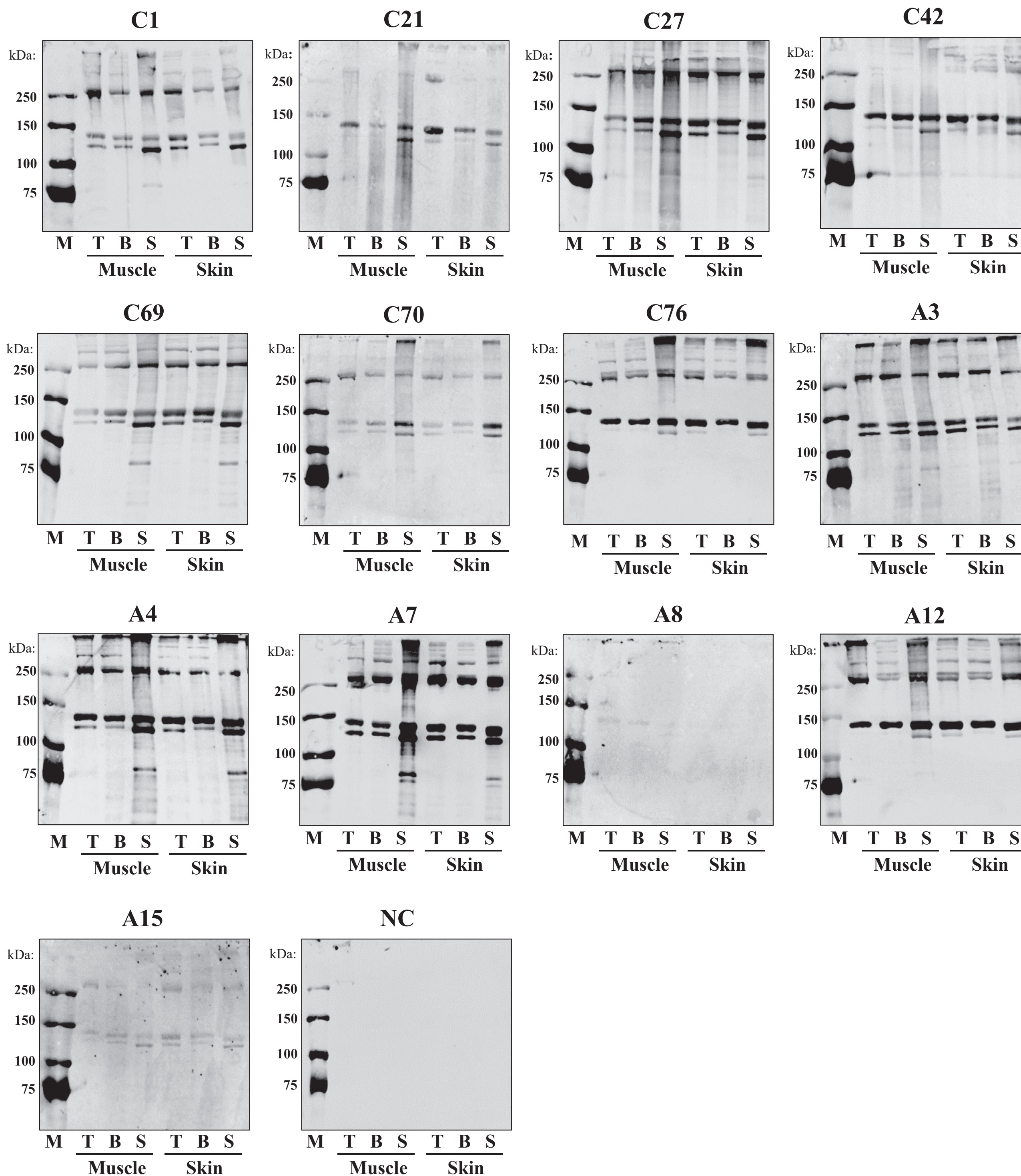

FIGURE E1. IgE sensitization to fish collagens demonstrated by immunoblots using purified collagens from muscle and skin tissues of yellowfin tuna, barramundi, and Atlantic salmon. *A*, Adults; *B*, barramundi; *C*, children; *M*, protein marker; *NC*, negative control; *S*, salmon; *T*, tuna.

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

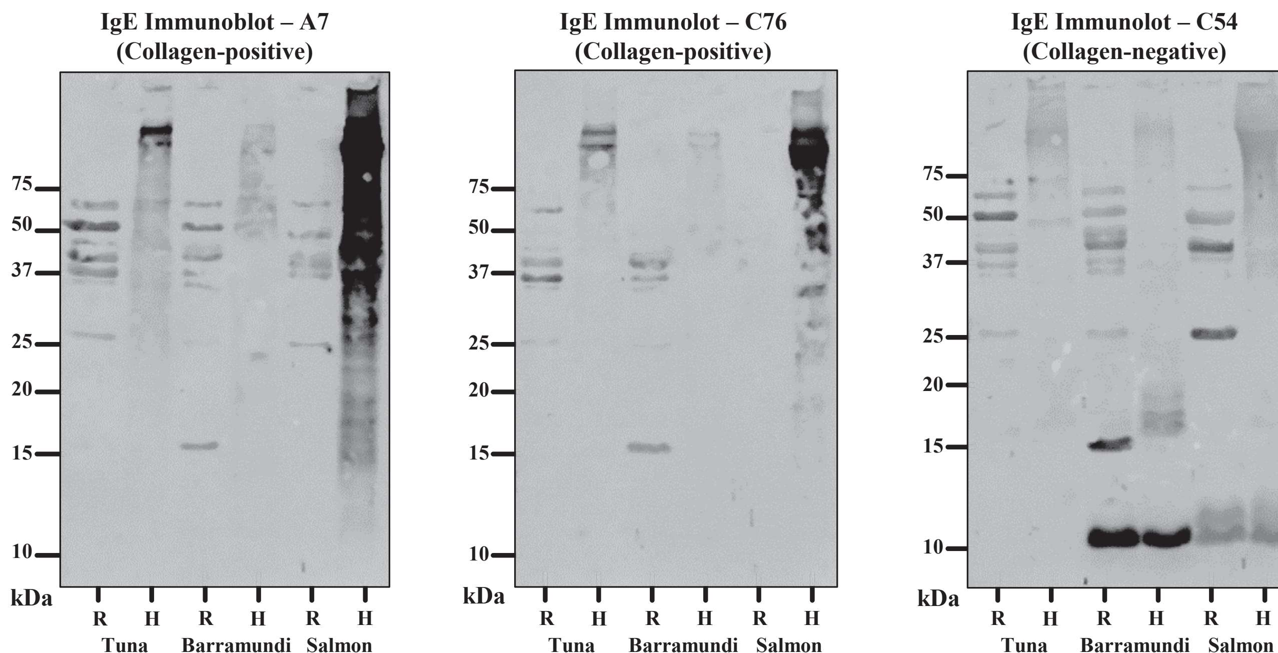

FIGURE E2. IgE binding to proteins from whole-fish extracts detected by immunoblots. *A*, Adults; *C*, children; *H*, heated; *R*, raw.

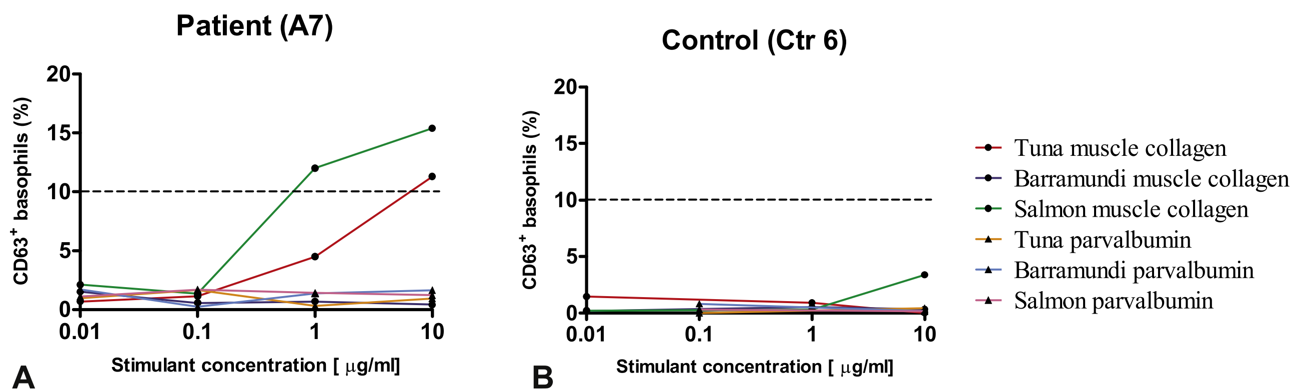

FIGURE E3. BAT using fresh blood of a fish-allergic patient (A7) and an atopic control (Ctr 6). Data indicate percentage of $CD63^+$ basophils in response to stimulation with collagens and parvalbumins from yellowfin tuna, barramundi, and Atlantic salmon. Dashed line indicates threshold for positive signal. *A*, Adult.

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.

REFERENCES

E1. Ruethers T, Raith M, Sharp MF, Koeberl M, Stephen JN, Nugraha R, et al. Characterization of Ras k 1 a novel major allergen in Indian mackerel and identification of parvalbumin as the major fish allergen in 33 Asia-Pacific fish species. Clin Exp Allergy 2018;48:452-63.

E2. Ruethers T, Taki AC, Nugraha R, Cao TT, Koeberl M, Kamath SD, et al. Variability of allergens in commercial fish extracts for skin prick testing. Allergy 2019;74:1352-63.

E3. Kalic T, Morel-Codreanu F, Radauer C, Ruethers T, Taki AC, Swoboda I, et al. Patients allergic to fish tolerate ray based on the low allergenicity of its parvalbumin. J Allergy Clin Immunol Pract 2019;7:500-8.

E4. Nugraha R, Kamath SD, Johnston E, Zenger KR, Rolland JM, O'Hehir RE, et al. Rapid and comprehensive discovery of unreported shellfish allergens using large-scale transcriptomic and proteomic resources. J Allergy Clin Immunol 2018;141:1501-1504.e8.

Downloaded for Anonymous User (n/a) at James Cook University from ClinicalKey.com.au by Elsevier on January 21, 2021.
For personal use only. No other uses without permission. Copyright ©2021. Elsevier Inc. All rights reserved.