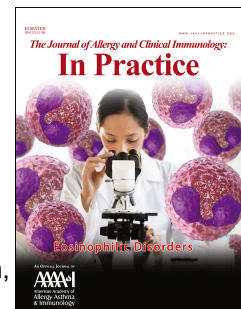


Journal Pre-proof

Collagen - an important fish allergen for improved diagnosis

Tanja Kalic, PhD, Sandip D. Kamath, PhD, Thimo Ruethers, MSc, Aya C. Taki, PhD, Roni Nugraha, PhD, Thu T.K. Le, PhD, Piotr Humeniuk, PhD, Nicholas A. Williamson, PhD, Diamond Hira, MD, Jennifer M. Rolland, PhD, Robyn E. O'Hehir, MD, PhD, Danyi Dai, MA, Dianne E. Campbell, MD, PhD, Heimo Breiteneder, PhD, Andreas L. Lopata, PhD



PII: S2213-2198(20)30423-2

DOI: <https://doi.org/10.1016/j.jaip.2020.04.063>

Reference: JAIP 2854

To appear in: *The Journal of Allergy and Clinical Immunology: In Practice*

Received Date: 22 February 2020

Revised Date: 9 April 2020

Accepted Date: 23 April 2020

Please cite this article as: Kalic T, Kamath SD, Ruethers T, Taki AC, Nugraha R, Le TTK, Humeniuk P, Williamson NA, Hira D, Rolland JM, O'Hehir RE, Dai D, Campbell DE, Breiteneder H, Lopata AL, Collagen - an important fish allergen for improved diagnosis, *The Journal of Allergy and Clinical Immunology: In Practice* (2020), doi: <https://doi.org/10.1016/j.jaip.2020.04.063>.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology

Submission to: The Journal of Allergy and Clinical Immunology: In Practice

Manuscript type: Original article

Title: Collagen - an important fish allergen for improved diagnosis

Authors

Tanja Kalic, PhD^a, Sandip D. Kamath, PhD^b, Thimo Ruethers, MSc^b, Aya C. Taki, PhD^{b,c}, Roni Nugraha, PhD^{b,d}, Thu T. K. Le, PhD^b, Piotr Humeniuk, PhD^a, Nicholas A. Williamson, PhD^e, Diamond Hira, MD^f, Jennifer M. Rolland, PhD^{g,h}, Robyn E. O’Hehir, MD, PhD^{g,h}, Danyi Dai, MAⁱ, Dianne E. Campbell, MD, PhDⁱ, Heimo Breiteneder, PhD^a, Andreas L. Lopata, PhD^{b*}

*Corresponding author

Institutions

^aInstitute of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

^bCollege of Public Health, Medical and Veterinary Sciences, Australian Institute of Tropical Health and Medicine, Molecular Allergy Research Laboratory, James Cook University, Townsville, Queensland, Australia

^cVeterinary Biosciences, Melbourne Veterinary School, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Victoria, Australia

^dDepartment of Aquatic Product Technology, Bogor Agricultural University, Bogor, Jawa Barat, Indonesia

^eBio21 Mass Spectrometry and Proteomics Facility, The Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Melbourne, Victoria, Australia

^fAllergy Centre, Townsville, Queensland, Australia

^gDepartment of Immunology and Pathology, Monash University, Melbourne, Victoria, Australia

^hDepartment of Allergy, Immunology and Respiratory Medicine, Central Clinical School, Monash University and The Alfred Hospital, Melbourne, Victoria, Australia

ⁱDepartment of Allergy and Immunology, Children's Hospital at Westmead, Sydney, New South Wales, Australia

Corresponding author

Professor Andreas L. Lopata

College of Public Health, Medical and Veterinary Sciences

Australian Institute of Tropical Health and Medicine

Molecular Allergy Research Laboratory

James Cook University, Townsville, Queensland, Australia

Telephone: +61 7 4781 4563

E-mail: andreas.lopat@jcu.edu.au

Funding: Funding for this research was provided by the Austrian Science Fund (FWF) grant W1248-B30, the Medical University of Vienna, National Health and the Medical Research Council Australia (project grants GNT1086656 and GNT1124143) and the Centre for Food and Allergy Research, Australia.

Word counts:

Abstract: 250

Text: 3491

Conflicts of interest: All authors declare that they have no conflicts of interest regarding the work presented in this manuscript.

Abstract:

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 solution, leading to low abundance in commercially available *in vitro* and skin prick tests for fish allergy.

Objective: We investigated 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 RBL (rat basophil leukemia) assay and basophil activation test (BAT). Protein identities were confirmed by mass spectrometry.

Results: Purified fish collagen contained type I $\alpha 1$ and $\alpha 2$ 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 RBL assay using six patients' sera, and BAT using fresh blood from one 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.

Conclusion: IgE sensitization and IgE cross-linking capacity of fish collagen were demonstrated in fish-allergic patients. Inclusion of relevant collagen allergens in routine diagnosis is indicated to improve the capacity to accurately diagnose fish allergy.

Highlights box:**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.

Keywords: Fish allergy, collagen, IgE, allergy diagnosis, IgE cross-linking

Abbreviations

CBB – Coomassie Brilliant Blue

PBS – Phosphate-buffered saline

SPT – Skin prick test

BAT – Basophil activation test

RBL – Rat Basophilic Leukemia

RLU – Relative luminescence units

1. 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. 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 four patients.⁶

Due to 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 (PBS), 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 (SPT) for fish allergy.⁸ To successfully extract collagen from tissues, acid-solubilization or enzyme-assisted extraction can be utilized.^{9, 10} 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 safety and optimal management of fish-allergic patients.

Collagen type I has a unique structure of α chains which 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.¹¹ When heated, α chains of collagen separate and upon subsequent cooling form gelatin, a denatured form of collagen.¹² It was demonstrated that upon thermal denaturation, collagen retained IgE-binding ability.¹³

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 Subcommittee and following positive evaluation designated as Sal s 6 and Lat c 6, respectively.

2. Methods

2.1 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 the Online Repository. After successful extraction, protein identity was confirmed by immunoblotting using a commercial anti-collagen antibody (ab23730, Abcam, USA), as detailed in the Online Repository. Collagen extract purity was confirmed by Coomassie Brilliant Blue (CBB) staining of the Any kD™ Mini-PROTEAN® TGX™ Precast Protein gel (Bio-Rad, CA, USA) and imaging by the Odyssey CLx imaging system (LI-COR, Nebraska, USA).

2.2 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.

2.3 Patient characteristics

Seventy five children (<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, New South Wales, Australia), The Alfred Hospital, (Melbourne, Victoria, Australia) and the Translational Research Facility at the Australian Institute of Tropical Health and Medicine of James Cook University (Townsville, Queensland, Australia). Sensitization to fish was confirmed by ImmunoCAP (Thermo Scientific, MA, USA) and/or skin prick test (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 or greater than 3 mm compared to 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 one part (w/v) Hanks' Balanced Salt Solution (Gibco®, ThermoFisher Scientific), and aliquots were stored at -80°C until single use.

Demographic and clinical characteristics of all patients are shown in Table EI. Information about implicated fish species is based on patients' history of allergic reactions upon eating specific fish.

As negative controls, three non-atopic individuals without history of type I allergy and three atopic individuals with allergies other than to fish were recruited (Table EII).

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 number 192/07), Monash University (MUHREC CF08/0225), and the James Cook University (H4313 and H6829).

2.4 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 three fish species. Sera of five negative controls (Ctr 1 – 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 (SD). Detailed methods used for the ELISA are specified in the Online Repository.

2.5 IgE immunoblotting

To analyze serum IgE binding of fish-allergic patients to the collagen α chains derived from muscle and skin of the three fish species, and to proteins present in whole fish extracts, immunoblotting was performed as described in this article's Online Repository. 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 (Nebraska, USA).

2.6 Rat basophil leukemia assay

To demonstrate the ability of fish collagen to induce IgE cross-linking and subsequent allergic reaction, we utilized the RS-ATL8 cell line. This cell line comprises rat basophilic leukemia (RBL) cells stably transfected with the α chain of human Fc ϵ RI, with the nuclear factor of activated T-cells (NFAT)-responsive luciferase reporter gene. Allergen-induced IgE cross-linking is detected by the expression of luciferase.¹⁴ RBL assays were performed according to the protocol established and validated by Ali et al.¹⁵ with slight modifications described in the Online Repository.

2.7 Basophil activation test

The ability of fish collagen to activate human basophils was shown by direct basophil activation test (BAT) with fresh blood from one patient (A7) and one 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.

2.8 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 the Online Repository.

2.9 Statistical analysis

Significant differences in RBL cell activation upon exposure to collagens or parvalbumins in comparison with unstimulated cells were determined by one-way ANOVA followed by Dunett's test for multiple comparisons. P values below 0.05 were regarded as significant (*p

< 0.05; **p < 0.01; ***p < 0.001). Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1 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 $\alpha 1$ and $\alpha 2$ chains, as well as β chains, γ chains and their multimers, as demonstrated by CBB staining of 8% gels (**Figure 1A, left**). The identity of collagen α chains was confirmed by immunoblotting using an anti-collagen antibody (**Figure 1A, 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 1B**). In addition, we demonstrated that fish extracts prepared in PBS, 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 1C**).

3.2 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 2A**). A higher proportion of adults (28%) than children (18%) demonstrated sensitization to fish collagen (**Figure 2B**). Moreover, *in vitro* IgE-binding intensity to collagen was stronger for sensitized adults than children (**Figure 2C**). All identified collagen-sensitized patients are labelled with an asterisk (*) in the Table EI.

3.3 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 α 1 and α 2 chains from muscle and skin of fish were observed (**Figure 3 and Figure E1**). For example, stronger IgE binding to tuna skin than to tuna muscle α 1 and α 2 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, e.g. C1, C21, C27 and C76. In addition, serum of patient A12 showed stronger IgE binding to α 1 than to α 2 chains from both fish tissues, demonstrating 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 (**Figure 3 and Figure E1**).

3.4 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, eight 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 grey 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 three fish species as previously shown (**Figure 3**). Similarly, IgE of C69 and C76 demonstrated moderate-to-strong binding to tuna collagen (**Figure 3**), while 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 two 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 (**Figure E2**). 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.

3.5 Collagen-induced IgE cross-linking

Sera of six fish-allergic patients and three negative controls were used for sensitization of RS-ATL8 cells. Out of six tested patients, five (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 2A**). RBL cells sensitized by serum of patient C76 demonstrated a positive signal upon exposure to collagens but not to parvalbumins from all three 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 (**Figure E3**). 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 μ g/ml (data not shown). BAT with parvalbumins from all three species was negative for A7 (**Figure E3**).

3.6 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-MS/MS analysis of the resulting peptides for barramundi and salmon (**Table EIII**). 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.

4. 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.^{16, 17} In recent years its use has increased, in part to replace bovine- or porcine-derived collagen due to dietary and religious food restrictions.^{16, 18} As 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, 19} 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.^{13, 20} 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, 21} In contrast, other studies demonstrated anaphylactic reactions upon exposure to this protein, for example while consuming marshmallows.³

Importantly, while fish collagen is declared on food products in the USA, it is however exempted from mandatory labelling in Europe.²²

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 M acetic acid. Harsh acidic or enzymatic treatment of tissues are generally required to successfully extract collagen, due to its insolubility in aqueous solutions.²³ This may also be the reason why commercial fish extracts used in fish allergy diagnostics contain very low quantities of collagen, leading to a misjudgement of the 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, collagen was underrepresented in commercially available fish SPT extracts from several manufacturers.⁸ Another study demonstrated false negative results in prick-to-prick tests with raw fish due to insolubility of collagen.²⁴ 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 $\alpha 1$ chains, $\alpha 2$ chains, as well as their dimers and multimers, as expected based on previously published literature.^{25, 26} We first analyzed sera of 101 fish-allergic patients for IgE binding to a pool of collagens extracted from all three 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,}

²⁰ However, differences in IgE binding to collagen derived from muscle or skin, as well as to specific α chains have not been investigated. So we next tested collagen-sensitized patients'

285 sera for IgE to specific collagen α chains from skin or muscle tissue of the three fish species.
286 Most patients reacted to all collagen α chains, but IgE-binding intensities differed. Reactivity
287 to all α chains by most patients indicated possible presence of cross-reactive IgE epitopes.
288 Different, patient-dependent binding intensities to different α chains may in contrast indicate
289 possible presence of distinct IgE epitopes on the two collagen α chains. A study by Shiomi et
290 al. identified an immunodominant IgE epitope from rainbow trout collagen α 2 chain
291 (MKGLRGHGGLQGMPGPNGPS).²⁷ The identical amino acid sequence is found in
292 collagen α 2 chain of salmon collagen (XP_013998297.1), and a homologous region with
293 90% sequence identity is present in barramundi collagen α 2 chain (XP_018522130.1). Based
294 on high sequence identities in this region, patient IgE cross-reactivity to different fish species
295 may therefore be expected for patients with IgE specific for this epitope.

296 Most fish-allergic patients are sensitized to the major allergen parvalbumin which is highly
297 cross-reactive.²⁸ However, mono-sensitization to parvalbumin from certain species has been
298 reported.²⁹ Patients can also be sensitized to other fish allergens.²⁹ Therefore, the absence of
299 parvalbumin-specific IgE for a particular fish species does not exclude the possibility of
300 sensitization to other fish allergens such as collagen. We thus analyzed the collagen-
301 sensitized patients from our cohort for presence of IgE to parvalbumins from the same fish
302 species. Most of the collagen-sensitized patients' sera contained IgE to parvalbumins.
303 However, eight of the collagen sensitized fish-allergic patients (40%) had no parvalbumin-
304 specific IgE while demonstrating IgE binding to collagen from specific fish species. This
305 finding emphasizes the importance of recognizing collagen as a relevant allergen and its
306 inclusion in diagnostics, as these patients could be in danger of misdiagnosis as non-fish
307 allergic 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 six collagen-sensitized individuals were analyzed, with five demonstrating a positive result upon exposure of cells to collagen from all three 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, as BAT was performed only for one 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.

Based on 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 Sub-committee 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.

In summary, 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.²⁸ Extracts however may contain very low quantities of clinically relevant collagen. Recognizing collagen, both from 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.

Journal Pre-proof

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, Hige 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. 13th World Congress of Food Science & Technology 2006, 2006:929-.
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. *European Food Research and Technology* 2013; 236:873-82.

- 359 10. Anand S, Kamath S, Chuang L, Kasapis S, Lopata A. Biochemical and thermo-
360 mechanical analysis of collagen from the skin of Asian Sea bass (*Lates calcarifer*) and
361 Australasian Snapper (*Pagrus auratus*), an alternative for mammalian collagen.
362 European Food Research and Technology 2013;1-10.
- 363 11. Fidler AL, Boudko SP, Rokas A, Hudson BG. The triple helix of collagens - an
364 ancient protein structure that enabled animal multicellularity and tissue evolution. J
365 Cell Sci 2018; 131.
- 366 12. Bozec L, Odlyha M. Thermal denaturation studies of collagen by microthermal
367 analysis and atomic force microscopy. Biophys J 2011; 101:228-36.
- 368 13. Kobayashi Y, Kuriyama T, Nakagawara R, Aihara M, Hamada-Sato N. Allergy to
369 fish collagen: Thermostability of collagen and IgE reactivity of patients' sera with
370 extracts of 11 species of bony and cartilaginous fish. Allergol Int 2016; 65:450-8.
- 371 14. Nakamura R, Uchida Y, Higuchi M, Nakamura R, Tsuge I, Urisu A, et al. A
372 convenient and sensitive allergy test: IgE crosslinking-induced luciferase expression
373 in cultured mast cells. Allergy 2010; 65:1266-73.
- 374 15. Ali EA, Nakamura R, Falcone FH. Use of Humanized RS-ATL8 Reporter System for
375 Detection of Allergen-Specific IgE Sensitization in Human Food Allergy. Methods
376 Mol Biol 2017; 1592:147-61.
- 377 16. Silva TH, Moreira-Silva J, Marques AL, Domingues A, Bayon Y, Reis RL. Marine
378 origin collagens and its potential applications. Mar Drugs 2014; 12:5881-901.
- 379 17. Venkatesan J, Anil S, Kim SK, Shim MS. Marine Fish Proteins and Peptides for
380 Cosmeceuticals: A Review. Mar Drugs 2017; 15.
- 381 18. Raman M, Gopakumar K. Fish Collagen and its Applications in Food and
382 Pharmaceutical Industry: A Review. EC Nutrition 2018; 13.12:752-67.

- 383 19. Land MH, Piehl MD, Burks AW. Near fatal anaphylaxis from orally administered
384 gelatin capsule. *J Allergy Clin Immunol Pract* 2013; 1:99-100.
- 385 20. Kobayashi Y, Hige J, Imamura S, Hamada-Sato N. Study of the cross-reactivity of
386 fish allergens based on a questionnaire and blood testing. *Allergol Int* 2016; 65:272-9.
- 387 21. Hansen TK, Poulsen LK, Stahl Skov P, Hefle SL, Hlywka JJ, Taylor SL, et al. A
388 randomized, double-blinded, placebo-controlled oral challenge study to evaluate the
389 allergenicity of commercial, food-grade fish gelatin. *Food Chem Toxicol* 2004;
390 42:2037-44.
- 391 22. Kourani E, Corazza F, Michel O, Doyen V. What Do We Know About Fish Allergy
392 at the End of the Decade? *J Investig Allergol Clin Immunol* 2019; 29:414-21.
- 393 23. Wang J, Pei X, Liu H, Zhou D. Extraction and characterization of acid-soluble and
394 pepsin-soluble collagen from skin of loach (*Misgurnus anguillicaudatus*). *Int J Biol*
395 *Macromol* 2018; 106:544-50.
- 396 24. Chikazawa S, Hashimoto T, Kobayashi Y, Satoh T. Fish-collagen allergy: a pitfall of
397 the prick-to-prick test with raw fish. *Br J Dermatol* 2015; 173:1330-1.
- 398 25. Muralidharan N, Jeya Shakila R, Sukumar D, Jeyasekaran G. Skin, bone and muscle
399 collagen extraction from the trash fish, leather jacket (*Odonus niger*) and their
400 characterization. *J Food Sci Technol* 2013; 50:1106-13.
- 401 26. Gistelink C, Gioia R, Gagliardi A, Tonelli F, Marchese L, Bianchi L, et al. Zebrafish
402 Collagen Type I: Molecular and Biochemical Characterization of the Major Structural
403 Protein in Bone and Skin. *Sci Rep* 2016; 6:21540.
- 404 27. Shiomi K, Yoshida S, Sawaguchi T, Ishizaki S. A major IgE epitope of rainbow trout
405 collagen alpha2 chain. *Shokuhin Eiseigaku Zasshi* 2010; 51:153-9.

- 406 28. Ruethers T, Taki AC, Johnston EB, Nugraha R, Le TTK, Kalic T, et al. Seafood
407 allergy: A comprehensive review of fish and shellfish allergens. Mol Immunol 2018;
408 100:28-57.
- 409 29. Kuehn A, Swoboda I, Arumugam K, Hilger C, Hentges F. Fish allergens at a glance:
410 variable allergenicity of parvalbumins, the major fish allergens. Front Immunol 2014;
411 5:179.
- 412

Figure legends

Figure 1. A: Collagen extracted from fish muscle and skin, visualized by Coomassie Brilliant Blue (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. M, protein marker; T, tuna; B, barramundi; S, salmon.

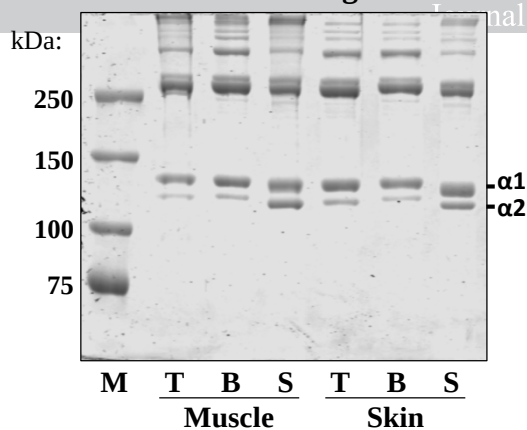
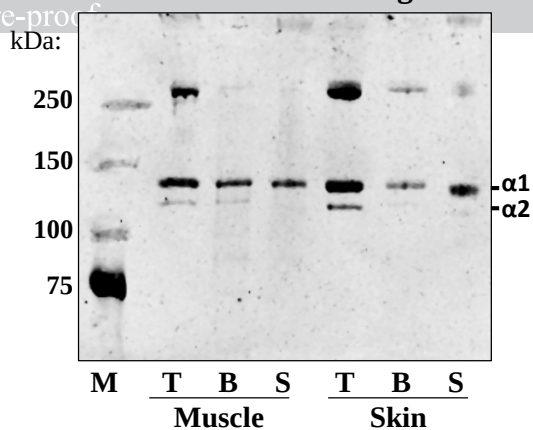
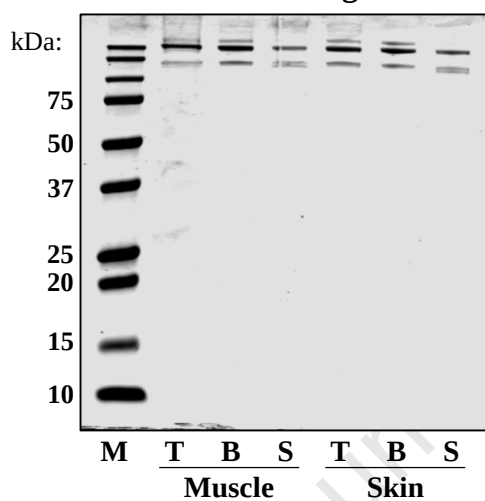
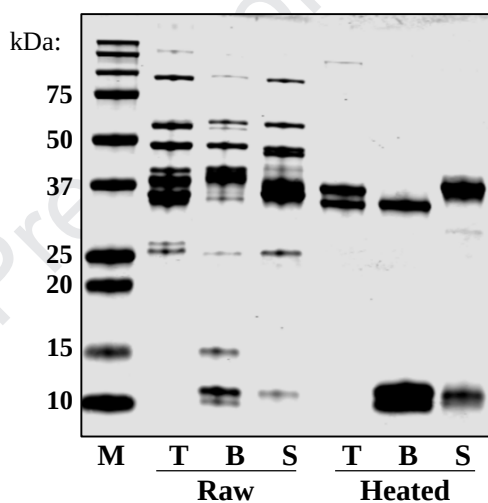
Figure 2. A: IgE binding to a pool of purified collagens from muscle and skin of yellowfin tuna, barramundi and Atlantic salmon. Results for collagen-positive (21 of 101) patients are shown (C, children; A, adults). The threshold for positivity (dashed line) was calculated using the average signal from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

Figure 3. Densitometric analysis of IgE-binding intensity to collagen $\alpha 1$ and $\alpha 2$ chains obtained by IgE immunoblotting using sera of collagen-sensitized patients. NC: immunoblot using pooled sera from five negative controls.

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 grey circles. The threshold for positivity (dashed line) was calculated using the average signal from five negative controls + 3 SD.

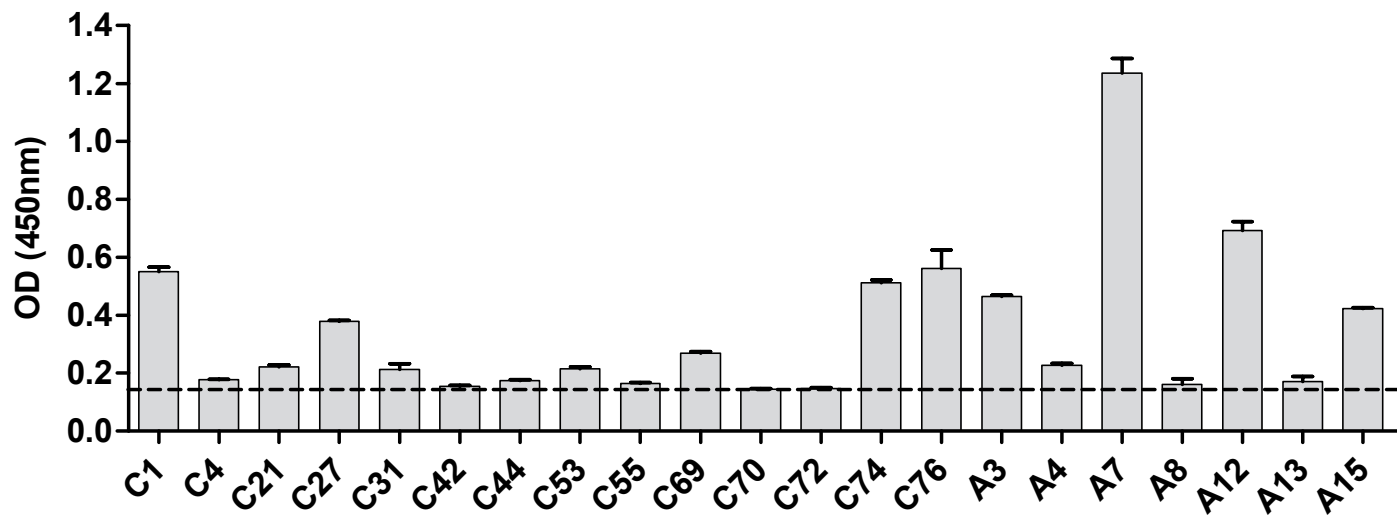
Figure 5. Activation of RS-ATL8 cells sensitized by sera of 3 collagen-sensitized children, 3 adults and 3 negative controls, upon exposure to collagens and parvalbumins from tuna, barramundi and salmon. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (significantly different from spontaneous activation of cells). SA, spontaneous activation; TC, tuna collagen; BC,

436 barramundi collagen; SC, salmon collagen; TP, tuna parvalbumin, BP, barramundi
437 parvalbumin; SP, salmon parvalbumin.

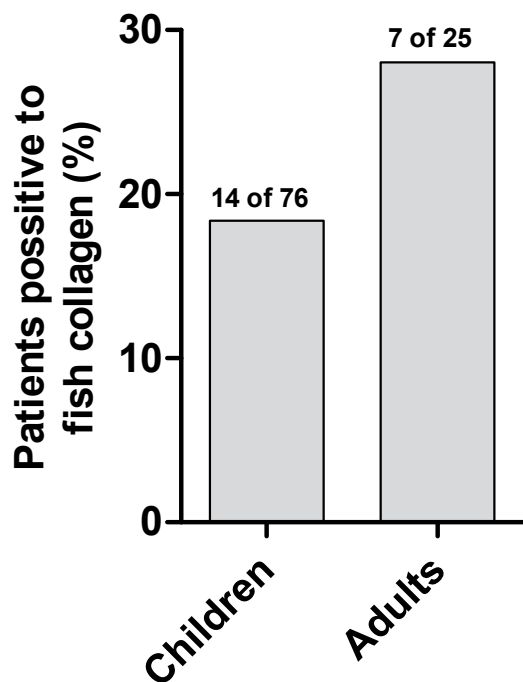
A**CBB - Collagen****Immunoblot - Collagen****B****CBB - Collagen****C****CBB - Fish extracts**

A

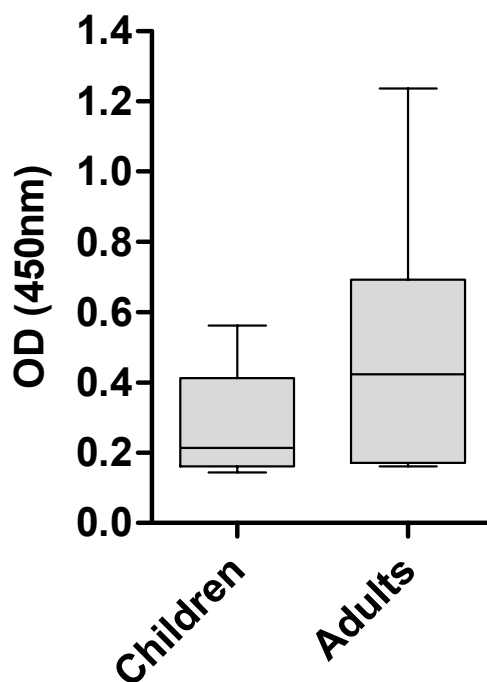
Collagen-specific IgE ELISA

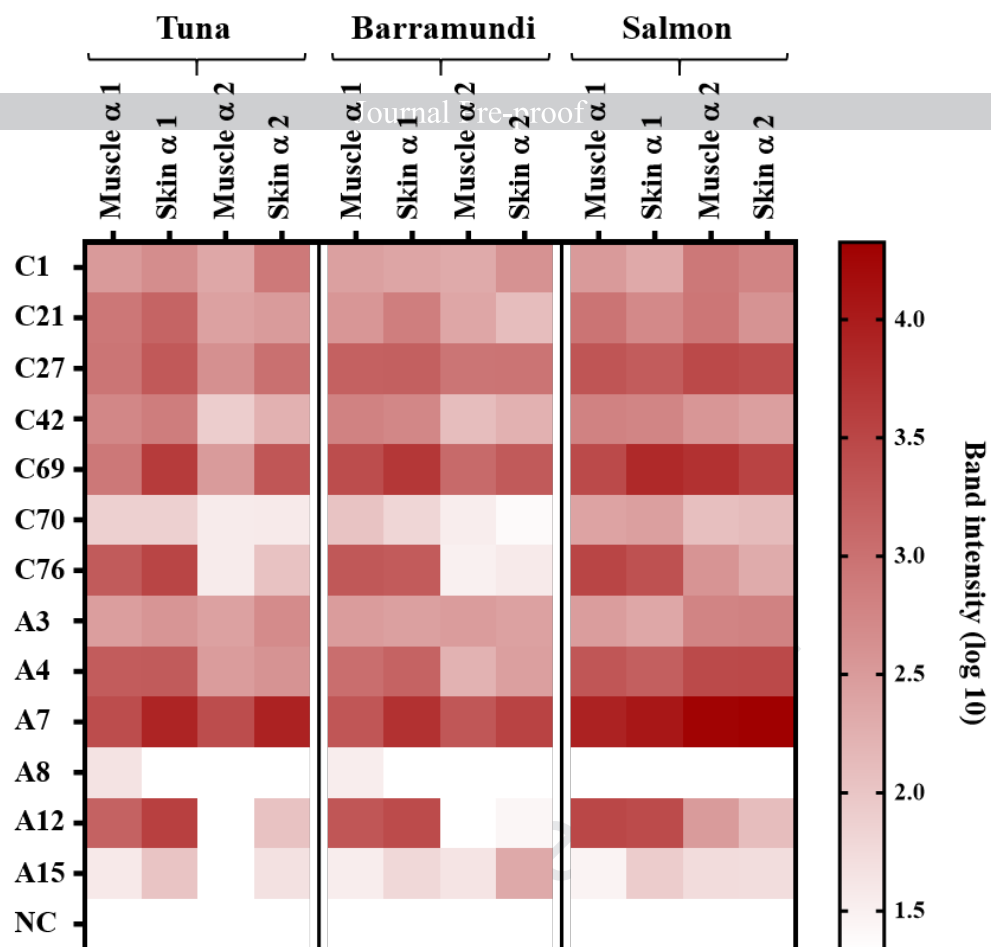


B

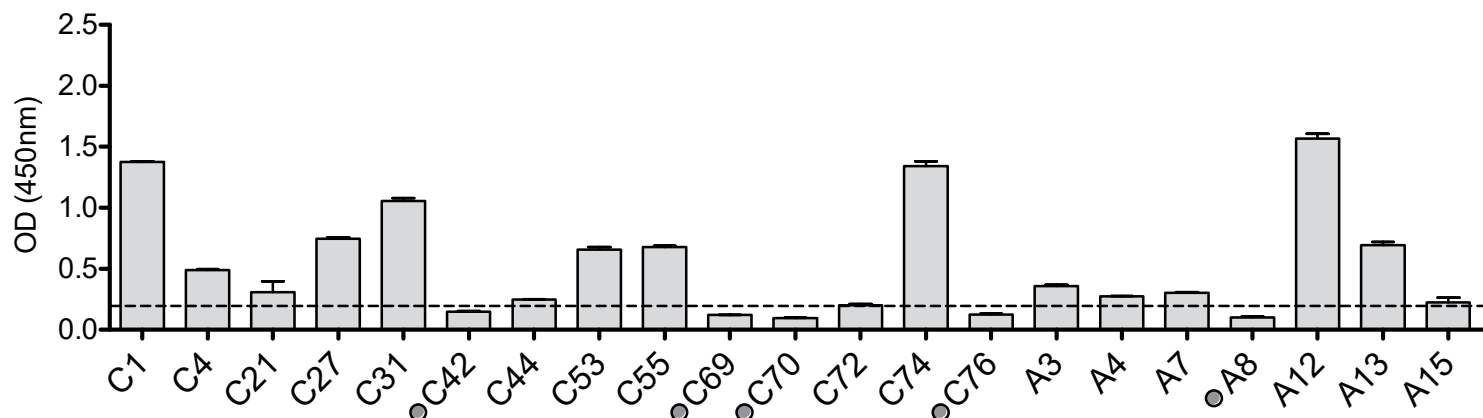


C

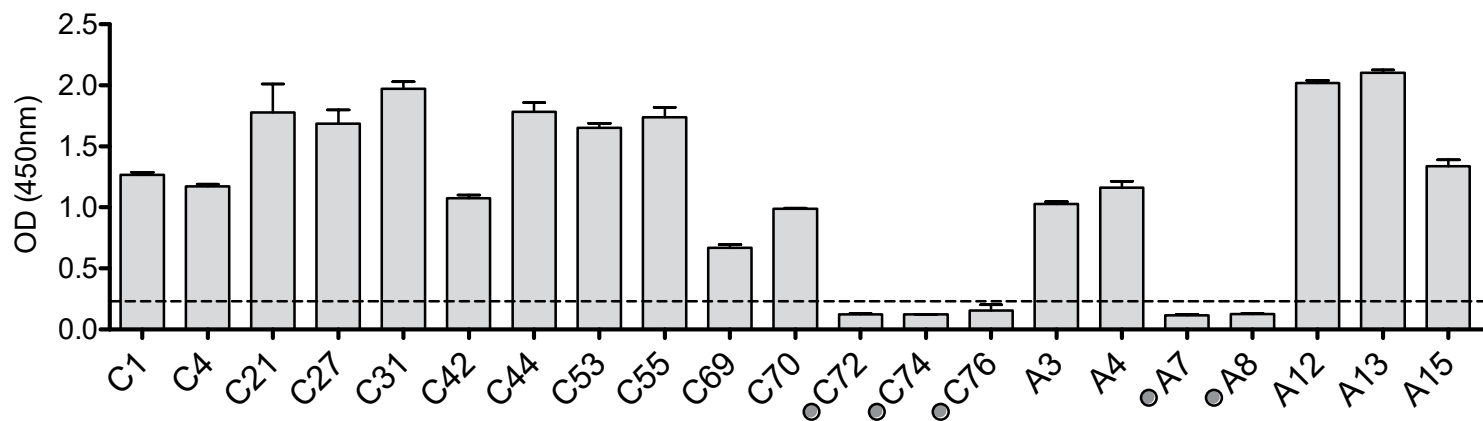




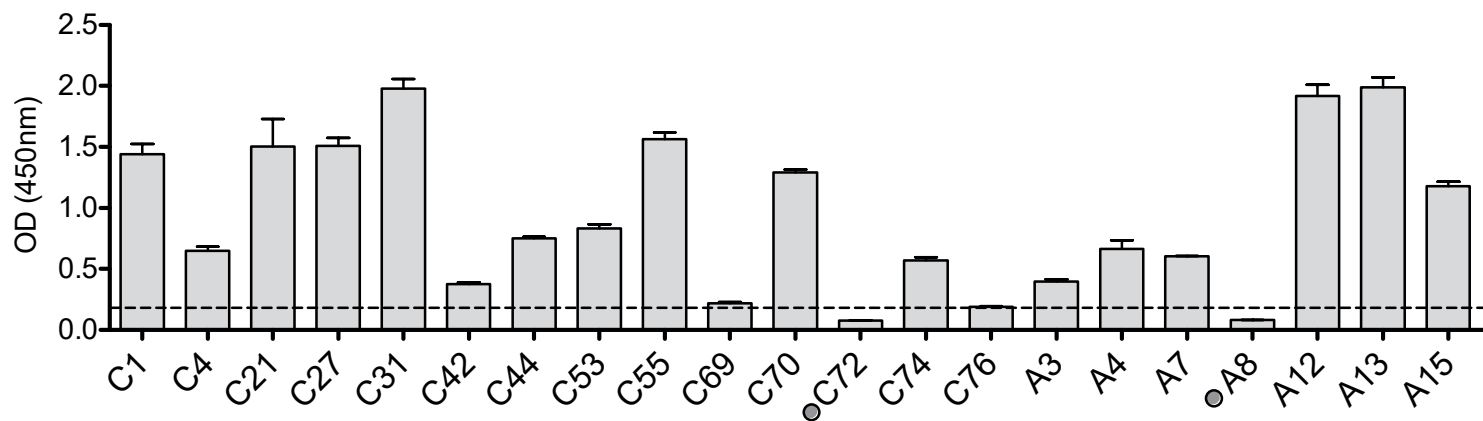
Tuna



Barramundi

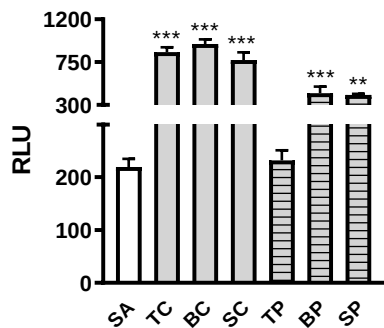


Salmon

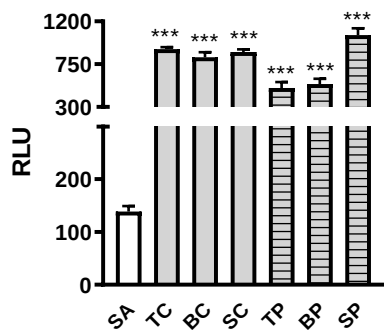


Adults

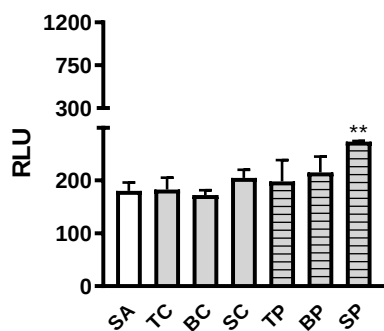
A7



A12

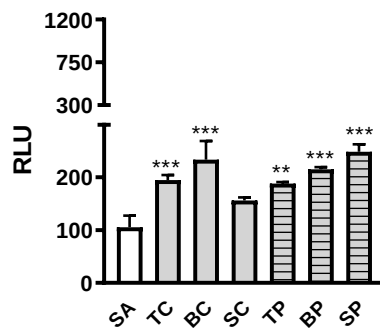


A15

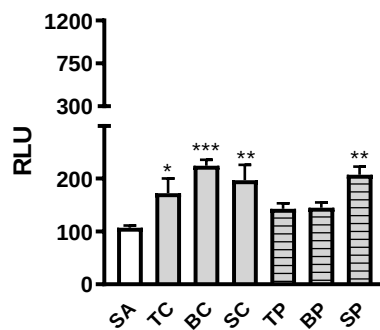


Children

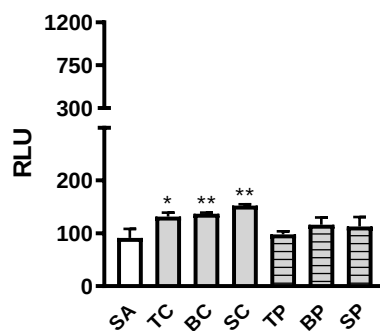
C27



C69

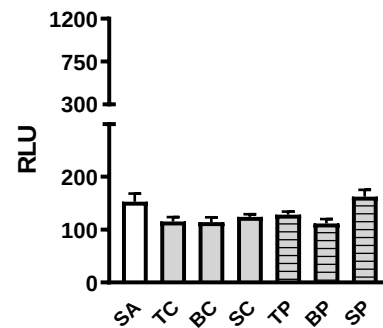


C76

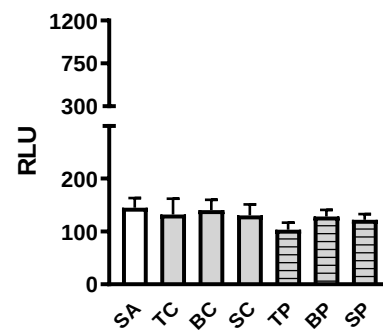


Controls

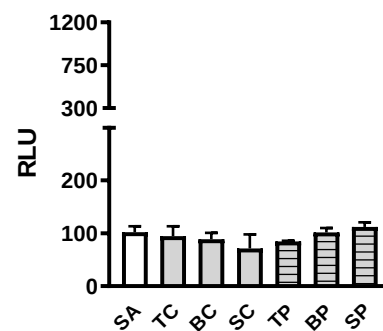
Ctr 3



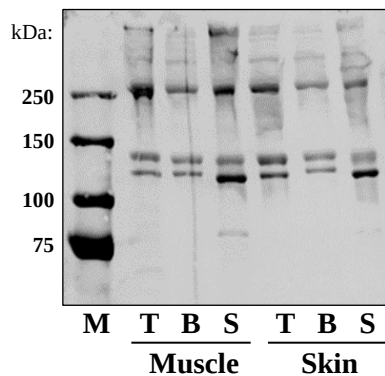
Ctr 4



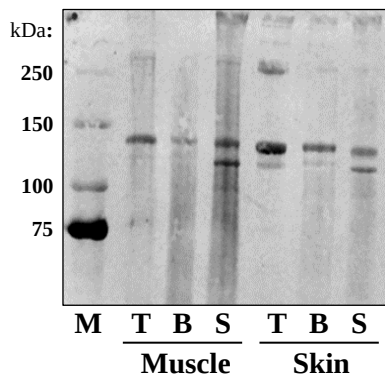
Ctr 5



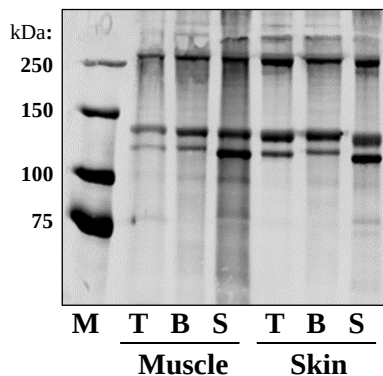
C1



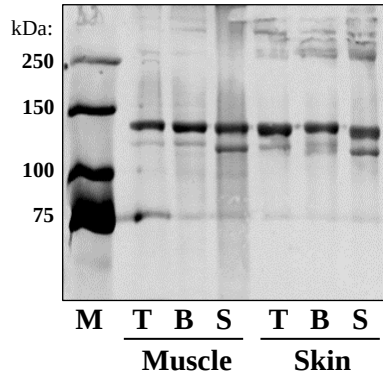
C21



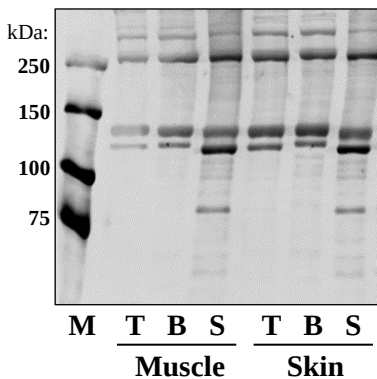
C27



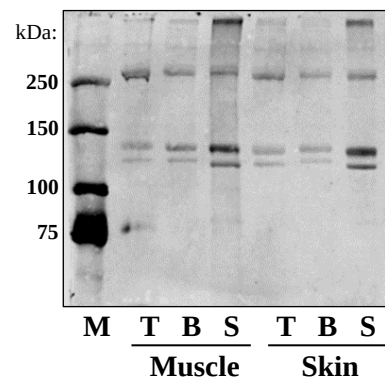
C42



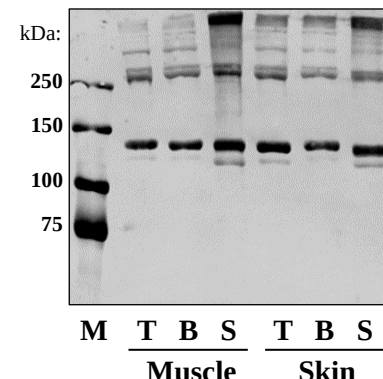
C69



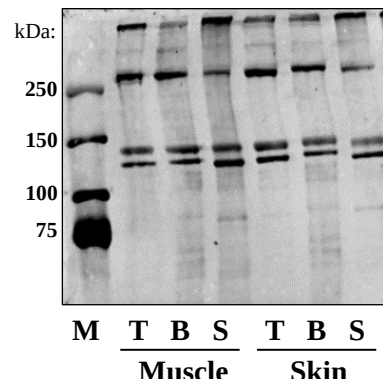
C70



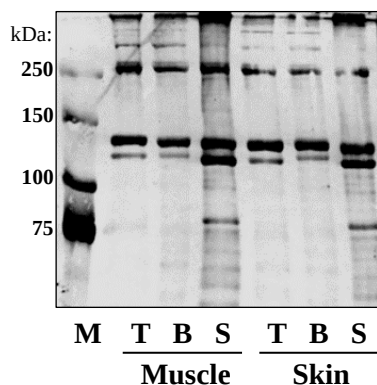
C76



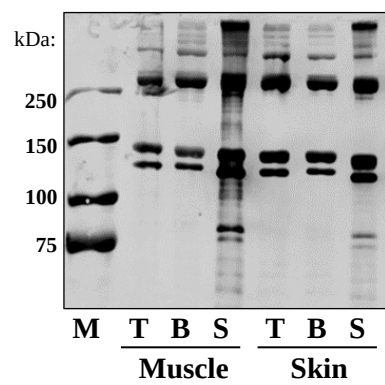
A3



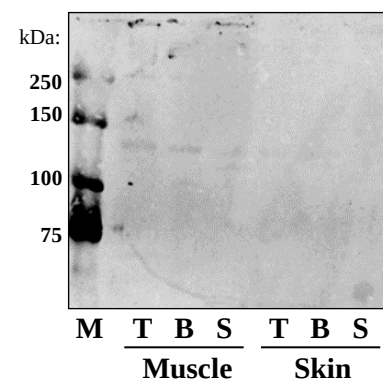
A4



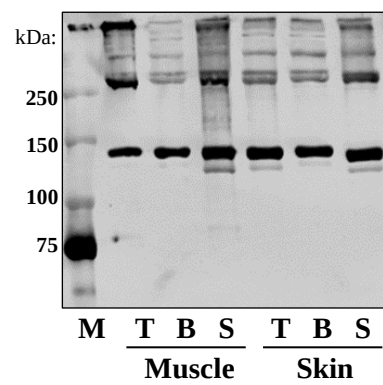
A7



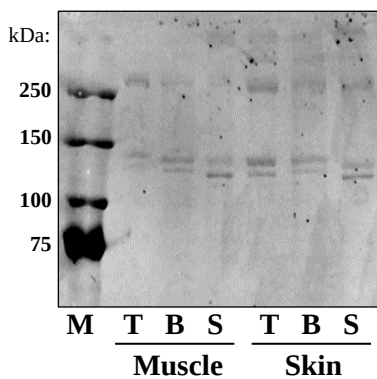
A8



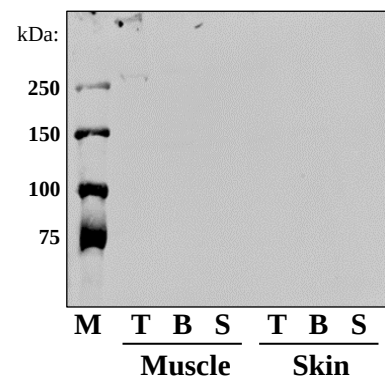
A12



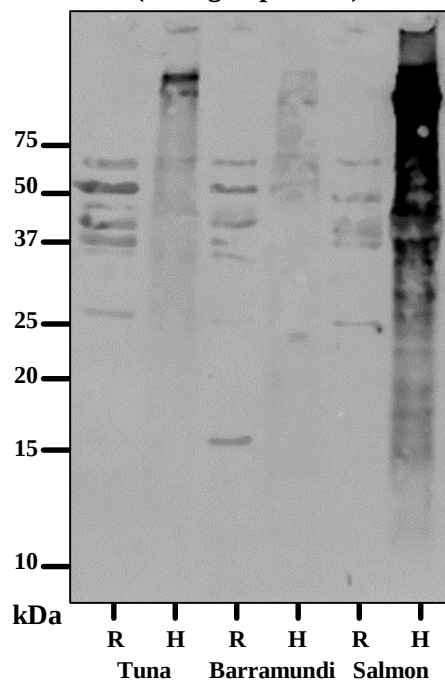
A15



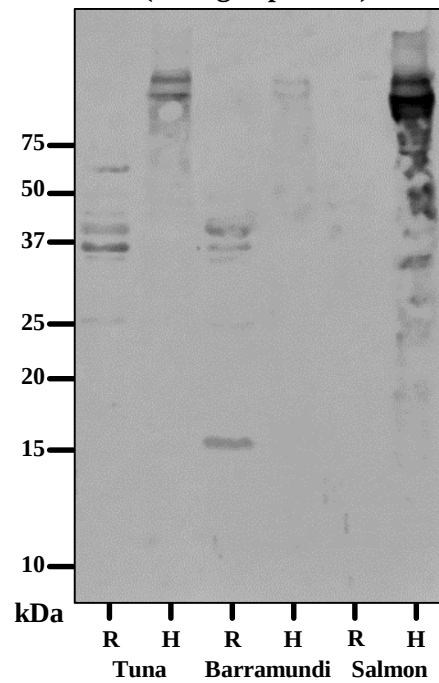
NC



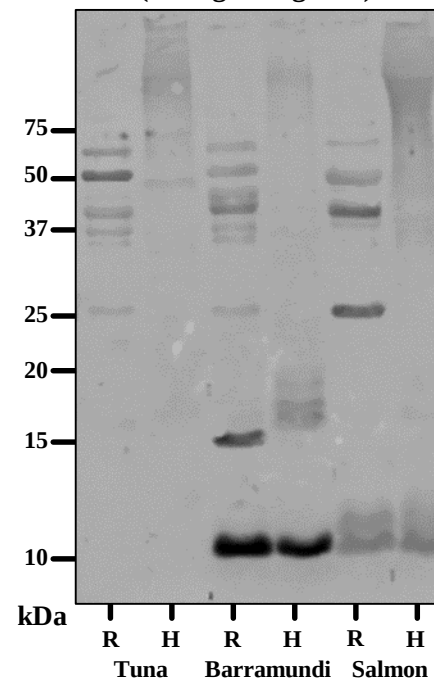
**IgE Immunoblot – A7
(Collagen-positive)**

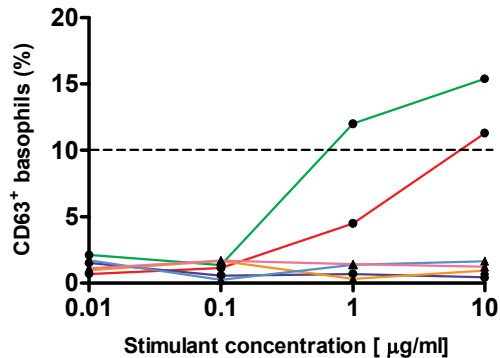
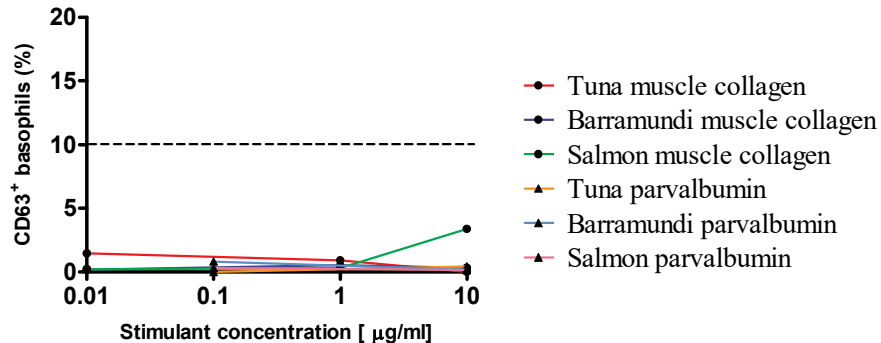


**IgE Immunoblot – C76
(Collagen-positive)**



**IgE Immunoblot – C54
(Collagen-negative)**



A**Patient (A7)****B****Control (Ctr 6)**

Online repository

Article title: Collagen - an important fish allergen for improved diagnosis

Abbreviations

PBS – phosphate-buffered saline

PBST – phosphate-buffered saline with tween-20

CID – collision-induced dissociation

Repository methods

Extraction of collagen from fish tissues

Filets with skin on 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 (approximately 0.5 cm³), washed with 10 volumes of ultrapure water and centrifuged (3000 x g, 5 min, 4°C). Supernatants were discarded, tissue samples resuspended in 10 volumes of cold 0.1 M NaOH and incubated for 24 h 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 h with gentle agitation. Samples were centrifuged, washed with 10 volumes of ultrapure water and resuspended in 0.5 M acetic acid. Collagen was extracted by stirring the samples in acetic acid for 24 h at 4°C. Samples were centrifuged (20000 x g, 1 h, 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, two extraction steps were required: firstly using 15 volumes and secondly using 10 volumes of acetic acid. All obtained collagen extracts were concentrated using Amicon Ultra-15 Centrifugal Filter Units with 50 kDa cutoff (Merck Millipore).

Fish extracts and parvalbumin purification

Frozen muscle tissues of yellowfin tuna, barramundi and Atlantic salmon were utilized for the purification of parvalbumins and for preparation of total protein extracts. Tissues were homogenized using a rotor-stator homogenizer in phosphate-buffered saline (PBS, 10 mmol/L phosphate; pH 7.2; 2 ml/g tissue). After gentle agitation overnight at 4°C, subsequent centrifugation (20000 x 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 (95-100°C) for 20 min before homogenization. Parvalbumins were purified from heated extracts by ammonium sulphate 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 h at room temperature) using casein blocking buffer (B6429, Sigma) diluted in PBS, the membrane was incubated (1 h 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 anti-rabbit IgG secondary antibody (926-32213, LI-COR) was added to the membrane diluted 1:10000 in 50% blocking buffer in PBST and incubated for 45 min at room temperature. The membrane was washed and subsequently scanned using the Odyssey

CLx imaging system (LI-COR, Nebraska, USA). Acquired images were analyzed using Image Studio Lite software (LI-COR, Nebraska, USA).

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 five 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 h at room temperature) with polyclonal rabbit anti-human IgE antibody (A0094, DAKO, USA) diluted 1:8000 in 20% blocking buffer in PBST. Bound IgE was detected by anti-rabbit 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% CO₂, in minimum essential media (Thermo Fisher Scientific, 11095-080) supplemented with 10% fetal bovine serum (Gibco, 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 h. After washing with PBS, cells were stimulated with collagens or parvalbumins from muscle tissues of tuna, barramundi and salmon for 4 h. All allergens were applied at a concentration of 10 μ g/ml, which was

determined to be optimal for both collagen and parvalbumin in a separate experiment (data not shown). After 4 h of stimulation with allergens, ONE-Glo™ Luciferase Assay System (Promega, Madison, Wisconsin, USA) 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 based on previous research.^{E3} For the atopic control (Ctr 6), 0.01 µg/ml, 1 µg/ml and 10 µg/ml collagen extracts, as well as 0.1 µg/ml and 10 µg/ml parvalbumins were used for the basophil stimulation. Additionally, as the atopic control had a confirmed sensitization to house dust mite, we used the house dust mite extract (Greer Laboratories, USA) as an additional positive control for activation of basophils, in concentrations of 0.001 µg/ml, 0.1 µg/ml and 10 µg/ml. 0.1 µg/ml of house dust mite extract 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 (fMLP) were used as positive controls. For both tested individuals, the positive controls 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 based on 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 (LLC, Ashland, USA).

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). The nanoLC system was equipped with an Acclaim Pepmap nano μ 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 (CID) fragmentation. Twenty of the most intense peptide ions with charge states ≥ 2 were isolated and fragmented using normalized collision energy of 35 and activation Q of 0.25 (CID). 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, Australia.

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

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 (2), 500–508.
- 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; 141:1501-4.e8.

Repository tables

Table EI. Demographic and clinical characteristics of fish-allergic patients.

Patients	Age	Gender	Clinical symptoms	SPT Tuna (mm)	SPT Barramundi (mm)	SPT Salmon (mm)	ImmunoCAP Tuna (kUA/L)	ImmunoCAP Salmon (kUA/L)	Implicated fish
Paediatric 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
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

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

133

134 AE, angioedema; AN, anaphylaxis; AP, abdominal pain; C, conjunctivitis; E, eczema; GIS, gastrointestinal

135 symptoms; OAS, oral allergy syndrome; R, rhinitis; RD, respiratory distress; U, urticaria; nd: not determined,.

136 *Patients identified as collagen-sensitized in our study.

137 **Table EII.** Demographic and clinical characteristics of control individuals.

Participant number	Age (years)	Gender	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

138 M, male; F, female

139

Table EIII. 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	126912	1025	0.89
		2	XP_018521723.1	Collagen alpha-1(I) chain-like isoform X1	136649	422	0.39
		3	XP_018553992.1	Collagen alpha-1(I) chain-like isoform X1	136922	408	0.33
	140 kDa	1	*XP_018553992.1	Collagen alpha-1(I) chain-like isoform X1	136922	1037	0.72
		2	XP_018521723.1	Collagen alpha-1(I) chain-like isoform X1	136649	950	0.76
		3	XP_018522130.1	Collagen alpha-2(I) chain isoform X1	126912	445	0.32
Barramundi skin	130 kDa	1	*XP_018522130.1	Collagen alpha-2(I) chain isoform X1	126912	1447	1.63
		2	XP_018521723.1	Collagen alpha-1(I) chain-like isoform X1	136649	783	0.72
		3	XP_018553992.1	Collagen alpha-1(I) chain-like isoform X1	136922	550	0.42
	140 kDa	1	*XP_018521723.1	Collagen alpha-1(I) chain-like isoform X1	136649	1618	1.57
		2	XP_018553992.1	Collagen alpha-1(I) chain-like isoform X1	136922	914	0.72
		3	XP_018522130.1	Collagen alpha-2(I) chain isoform X1	126912	551	0.46
Salmon muscle	130 kDa	1	*XP_013998297.1	Collagen alpha-2(I) chain isoform X1	126924	2317	2.31
		2	*XP_014033985.1	Collagen alpha-2(I) chain isoform X1	126930	2046	2.3
		3	XP_014048044.1	Collagen alpha-1(I) chain-like	136832	944	0.53
	140 kDa	1	*XP_014059932.1	Collagen alpha-1(I) chain	136851	2107	1.13
		2	*XP_014048044.1	Collagen alpha-1(I) chain-like	136832	2039	0.98
		3	XP_013998297.1	Collagen alpha-2(I) chain isoform X1	126924	1089	1.26
Salmon skin	130 kDa	1	*XP_013998297.1	Collagen alpha-2(I) chain isoform X1	126924	2695	3.26
		2	*XP_014033985.1	Collagen alpha-2(I) chain isoform X1	126930	2406	2.95
		3	XP_014059932.1	Collagen alpha-1(I) chain	136851	903	0.49
	140 kDa	1	*XP_014059932.1	Collagen alpha-1(I) chain	136851	2609	1.76
		2	*XP_014048044.1	Collagen alpha-1(I) chain-like	136832	2426	1.39
		3	XP_014035319.1	Collagen alpha-1(I) chain-like	137390	1667	1.88

*Sequences registered in the database of the WHO/IUIS Allergen Nomenclature Sub-Committee.

144 **Repository figure legends:**

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

149 **Figure E2:** IgE-binding to proteins from whole fish extracts (R, raw; H, heated) detected by
150 immunoblots.

151 **Figure E3:** Basophil activation test using fresh blood of a fish-allergic patient (A7) and an
152 atopic control (Ctr 6). Data indicate percentage of CD63⁺ basophils in response to stimulation
153 with collagens and parvalbumins from Yellowfin tuna, barramundi and Atlantic salmon.
154 Dashed line indicates threshold for positive signal.