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Tackling Fish Allergy

Bio-molecular studies on allergenic proteins causing fish allergy in Australian children for improved diagnosis

Thimo Ruethers

– Master of Science –

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

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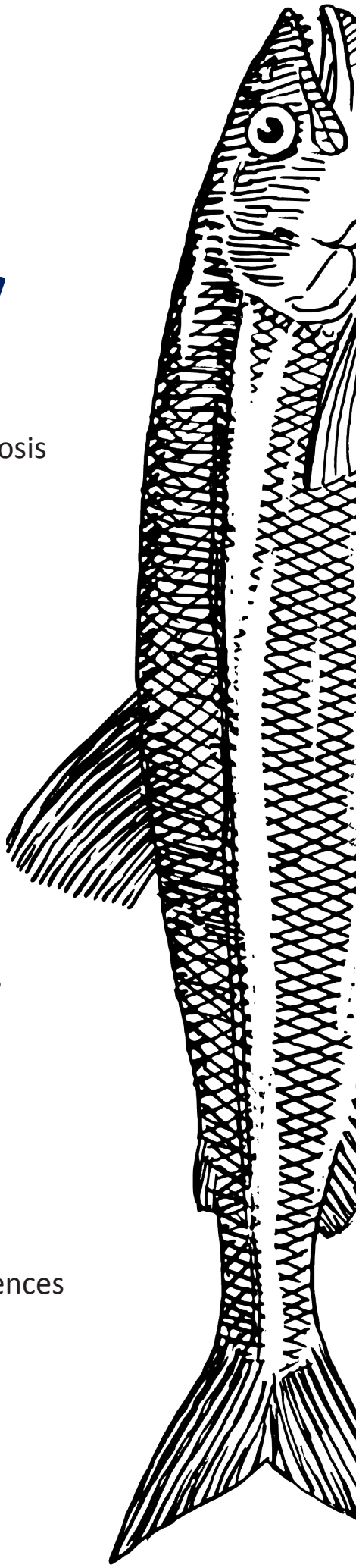

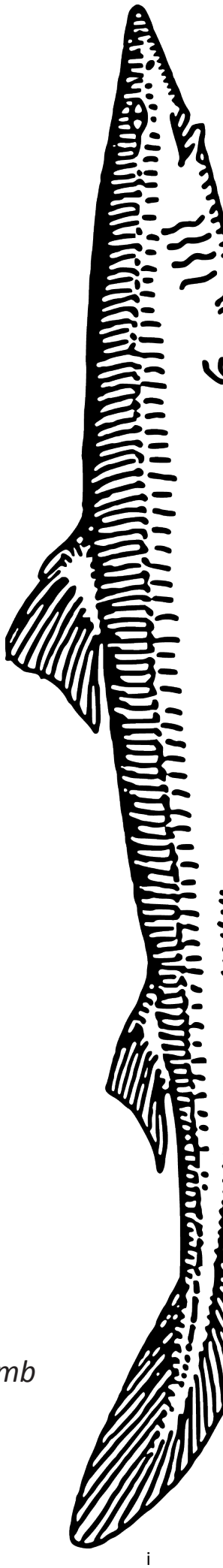

To my family in Germany and Vietnam

Allzeit bereit!
Ăn cá thôi!

Everybody is a genius. But if you judge a fish by its ability to climb a tree, it will live its whole life believing that it is stupid.

– Albert Einstein –

With sincere gratitude to
my advisory panel:

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  (James Cook University)
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Tackling Fish Allergy

Bio-molecular studies on allergenic proteins causing fish allergy in Australian children for improved diagnosis

Background:

Fish plays a critical role in sustainable food security but is also a major trigger of frequent life-threatening allergic reactions. Fish allergy is a serious chronic immune disease and more complex than most other food allergies, affecting up to 3% of the general population. Food allergies collectively cost the Australian economy over $7 billion per year and are a major and increasing global health burden. Fish as a food commodity comprises of over 1,000 edible species, of which most are under-investigated. The current diagnostic and management tools for fish allergy do not reflect this diversity and are often unreliable. Patients can be allergic to a narrow range of fish species. Consequently, the utility of fish as a food source is hampered and the lives of affected individuals are at risk. Knowledge gaps in the literature are manifold and cross-reactivities between different bony and cartilaginous fish as well as non-fish vertebrate species urgently require further investigations (**General introduction** and **Chapter I**).

Overarching objectives:

The aim of this thesis is to evaluate current diagnostic and management tools for fish allergy and subsequently provide the foundation for their improvement by expanding the allergen repertoire of fish and identifying safe food alternatives to bony fish.

Methodologies:

A total of over 100 patients with clinically confirmed fish allergy were recruited and their *in vivo* and *in vitro* sensitisation patterns determined. Specimens and derived products from over 100 different fish and non-fish vertebrate species were collected, with a focus on edible meat, and proteins solubilised following different extraction procedures. The detailed allergen repertoire was investigated utilising a variety of biochemical and immunological methods in conjunction with advanced mass spectrometry, employing available protein databases, and genomic/transcriptomic resources. Novel fish allergens were isolated and characterised, and their utility for improved diagnostics evaluated.

Outcomes and implications:

A high heterogeneity was discovered among commercial fish preparations (*n*=28) utilised for most common *in vivo* testing (skin prick test). Most preparations contained insufficient amounts of important allergens, resulting in low Immunoglobulin E (IgE) antibody-binding capacity and subsequently posing the risk of false-negative test results (**Chapter II**).

Commercial food allergen test kits (*n*=3), widely employed for the detection of fish in food products, identified only 26-61% of 57 bony fish species and no cartilaginous fish. Consequently, there is a high rate of false-negative test results, which implies that food products can be incorrectly labelled concerning fish content (**Chapter III A**).

Most investigated canned fish products (*n*=17) exhibited IgE-binding capacity and may induce allergic reactions upon consumption. Diagnostic tools should be developed to predict tolerance prior to recommending the implementation of selected canned fish products in the diet of fish-allergic individuals (**Chapter III B**).

The major fish allergen parvalbumin was identified and characterised in different Asia-Pacific fish species (*n*=33). A method for the purification of vertebrate parvalbumin was established, which built the foundation for obtaining purified allergens for downstream analysis in this research (**Chapter IV**).

Previously identified and novel fish allergens were quantified and characterised in up to 66 fish species. Tropomyosin was discovered as a novel major fish allergen of unrecognised importance and the number of fish allergens, registered with the World Health Organisation (WHO), was doubled (**Chapter V A**).

Collagen was revealed as an important allergen in fish and its under-representation in commercial diagnostic tests was demonstrated. This protein was registered as a fish allergen with the WHO for the first time (**Chapter V B**).

Cartilaginous fish species, e.g., sharks and rays, were identified as a safe food alternative for some fish-allergic individuals. Tools to predict patient's individual tolerance to this fish group are under development (**Chapter VI A**).

In contrast, it was demonstrated that reptiles and amphibians are not safe food alternatives. Parvalbumin isoforms from the Australian saltwater crocodile (salty) were registered as first reptile allergens with the WHO (**Chapter VI B**).

Conclusions:

The allergenicity of fish and other vertebrate species appears to be patient-, species- and preparation-specific, which must be reflected in diagnostic and detection tools for fish allergy sufferers to allow the consumption of selected vertebrate species and derived products. Generally, cartilaginous fish, birds and mammals appear safe for consumption while the majority of bony fish, reptiles and amphibians are not. The knowledge on the allergen repertoire of different fish species was expanded. Multiple allergens were detected, characterised and quantified in over 100 vertebrate species. Parvalbumin, tropomyosin and collagen isoforms are candidates for component-resolved diagnostics and were identified, characterised and purified for further investigations.

In summary, this thesis provides the foundation for the development of urgently needed improvement of the diagnosis and management of fish allergy. Aligning with the United Nations' sustainable development goals, this will contribute to a nutritious, safe and sustainable fish-rich diet for everyone.

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PEER-REVIEWED PUBLICATIONS

1) **T Ruethers**, AC Taki, J Khangurha; J Roberts; S Buddhadasa; D Clarke; CE Hedges, DE Campbell, SD Kamath, AL Lopata, M Koeberl; **2020**; Commercial fish ELISA kits have a limited capacity to detect different fish species and their products; ***Journal of the Science of Food and Agriculture***, in press (IF 2.6).-See **Chapter III A**.

→ *Three commercial kits for the detection of fish were evaluated together with the National Measurement Institute. The kits detected only 26-61% of 57 investigated bony fish species, highlighting a high risk of false-negative test results, putting lives at risk. Media underlined the need for improved test kits.*

2) T Kalic, SD Kamath, **T Ruethers**, AC Taki, R Nugraha, TT Le, P Humeniuk, NA Williamson, D Hira, JM Rolland, RE O'Hehir, D Dai, DE Campbell, H Breiteneder, AL Lopata; **2020**; Collagen - an important fish allergen for improved diagnosis; ***The Journal of Allergy and Clinical Immunology: In Practice***, in press (IF 7.6). See **Chapter V B**.

→ *Fish collagens, frequently used in food, cosmetics, and medicine were for the first time registered as novel fish allergens with the WHO. Over 20% of 101 fish-allergic patients reacted to fish collagen. Collagen is underrepresented in preparations used for allergy-diagnostics, leading to false-negative test results.*

3) MJ Sheridan, M Koeberl, CE Hedges, E Biros, **T Ruethers**, Dean Clarke, S Buddhadasa, S Kamath, AL Lopata; **2020**; Undeclared allergens in imported packaged food for retail in Australia; ***Food Additives and Contaminants: Part A***, 32 (2), 183-192 (IF 2.3). See **Appendix D 2**.

→ *Nearly half of 50 analysed products, imported from Asia and sold in Australia, contained undeclared allergens, posing a serious risk to allergic consumers. Findings and the risk for consumers were highlighted by the media and transferred to the responsible government authorities, requesting action.*

4) **T Ruethers**, AC Taki, R Nugraha, TT Cao, M Koeberl, SD Kamath, NA Williamson, S O'Callaghan, S Nie, SS Mehr, DE Campbell, AL Lopata; **2019**; Skin Prick Test preparations for fish allergy – A comparative biochemical and immunological analysis; ***Allergy***, 74 (7), 1352-1363 (IF 8.7).–See **Chapter II**.

→ *Twenty-eight commercial preparations used for fish allergy diagnostics were evaluated. Great variability in protein and allergen repertoire was discovered. Most extracts contained insufficient amounts of important allergens, posing the risk of false-negative test results. Current diagnostic tests are unreliable and the urgent need for improved tools to diagnose fish allergy was promoted through media.*

5) T Kalic, F Morel-Codreanu, C Radauer, **T Ruethers**, AC Taki, I Swoboda, C Hilger , K Hoffmann-Sommergruber, M Ollert, C Hafner, AL Lopata, M Morisset, H Breiteneder, A Kuehn; **2019**; Patients Allergic to Fish Tolerate Ray Based on the Low Allergenicity of Its Parvalbumin; ***The Journal of Allergy and Clinical Immunology: In Practice***, 7 (2), 500-508 (IF 7.6).-See **Chapter VI A**.

→ *Cartilaginous fish (sharks & ray) were identified as a potentially safe alternative for fish-allergic patients. Clinical trials with gummy shark (flake) and other sustainably-caught cartilaginous fish are underway in Melbourne. Media highlighted this publication as a 'ray of hope' for fish allergy sufferers worldwide.*

PEER-REVIEWED PUBLICATIONS continued

6) R Nugraha, SD Kamath, EB Johnston, S Karnaneedi, **T Ruethers**, AL Lopata; **2019**; Conservation analysis of B-cell allergen epitopes to predict clinical cross-reactivity between shellfish and inhalant invertebrate allergens; ***Frontiers in Immunology***, 10, 2676 (IF 5.1).-See **Appendix D 5**.

→ *Shellfish, including crustaceans and molluscs, mites, and cockroaches are all invertebrates with similar allergens and some reported cross-reactivity. We presented an approach for the prediction of individual's clinical cross-reactivity and provided a valuable tool for the development of immunotherapy's.*

7) TTK Le, DH Nguyen, ATL Vu, **T Ruethers**, AC Taki, AL Lopata; **2019**; A cross-sectional, population-based study on the prevalence of food allergies among children in two different socio-economic regions of Vietnam; ***Pediatric Allergy and Immunology***, 30 (3), 348-355 (IF 4.7).-See **Appendix D 1**.

→ *This was the first study investigating the prevalence of food allergy in Vietnam. A higher prevalence than in most South-East Asian countries was observed. Crustaceans were the major trigger of food-related allergic reactions followed by fish, mollusc, beef, milk, and egg.*

8) T Kalic, I Ellinger, SD Kamath, C Pallandino, V Mayr, A Tscheppe, **T Ruethers**, EE Waltl, V Niederberge, N Lengger, C Radauer, C Hafner, AL Lopata, M Bublin, H Breiteneder; **2019**; Fish-derived low molecular weight components modify bronchial epithelial barrier properties and release of pro-inflammatory cytokines; ***Molecular Immunology***, 112, 140-150 (IF 3.6).-See **Appendix D 8**.

→ *The sensitisation phase in the development of fish allergy is poorly understood. We investigated the cellular uptake of the major fish allergen parvalbumin and low molecular components. Our findings can explain an observed higher sensitisation among fish-processing workers and help protecting those at risk.*

9) **T Ruethers**, AC Taki, EB Johnston, R Nugraha, TT Le, T Kalic, TR McLean, SD Kamath, AL Lopata; **2018**; Seafood allergy: A comprehensive review of fish and shellfish allergens; Special Issue Molecular Allergology (by invitation) in ***Molecular Immunology***, 100, 28-57 (IF 3.6).-See **Chapter I**.

→ *To date, this is the most comprehensive review on seafood allergens. It is the tenth most-cited article in Molecular Immunology since 2017.*

10) **T Ruethers**, M Raith, MF Sharp, M Koeberl, JN Stephen, R Nugraha, TT Le, S Quirce, HX Nguyen, SD Kamath, SS Mehr, DE Campbell, CR Bridges, AC Taki, I Swoboda, AL Lopata; **2018**; Characterisation 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; ***Clinical and Experimental Allergy***, 48 (4), 452-463 (IF 5.1).-See **Chapter IV**.

→ *This is the first study evaluating the allergenicity of fish in Vietnam. The majority of investigated species is consumed throughout the Asia-Pacific, and most species are exported worldwide.*

11) JN Stephen, MF Sharp, **T Ruethers**, AC Taki, DE Campbell, AL Lopata; **2017**; Allergenicity of bony and cartilaginous fish – molecular and immunological properties; ***Clinical and Experimental Allergy***, 47 (3), 300-312 (IF 5.1).-See **Appendix D 3**.

→ *This is the first review highlighting the complexity of fish allergy with a focus on the two major fish classes bony and cartilaginous fish. Reports of cross-reactivity between those two classes appeared rare, initiating a detailed and ongoing evaluation of cartilaginous fish as a safe alternative for fish allergy sufferers.*

Contributions of others

Intellectual and inspirational contributions and/or support

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Grants and prizes during Candidature are listed in **Appendix A**.

Other financial contributions are disclosed where appropriate.

Declarations

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given. The contributions by others have been stated clearly and fully in the thesis. As the copyright owner of this thesis, I request the thesis to be embargoed until at least 18th August 2022 and grant James Cook University a permanent non-exclusive license to store, display or copy any or all of the thesis, in all forms of media, for use within the University after this date, and to make the thesis freely available online to other persons or organisations. The research presented and reported in this thesis was conducted with the approval of responsible Ethics Committees as stated where appropriate.

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Townsville, Australia, 20th July 2020

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Abbreviations

AK – arginine kinase

BAT – basophil activation test

CBB – Coomassie Brilliant Blue

CD – circular dichroism

CE – cooked protein extract

CFAR – Centre for Food and Allergy Research (www.foodallergyresearch.org.au)

CID – collision-induced dissociation

CRD – component-resolved diagnostics

DBPCFC – double-blind placebo-controlled food challenge

DH – dehydrogenase

DSP – diarrhetic shellfish poisoning

ELISA – enzyme-linked immunosorbent assays

FAO – Food and Agriculture Organization of the United Nations (www.fao.org)

FAOSTAT – Food and Agriculture Organization Corporate Statistical Database (http://www.fao.org/faostat)

FPIES – food protein-induced enterocolitis syndrome

GADPH – glyceraldehyde-3-phosphate dehydrogenase

GF – gel filtration

HE – heated protein extract

IEF – isoelectric focusing

IgE – Immunoglobulin E

IgG – immunoglobulin G

IQR – interquartile range

IUIS – International Union of Immunological Societies (www.iuis.org)

LC/MS – liquid chromatography coupled with mass spectrometry

LFD – lateral flow device

LOD – limit of detection

LOQ – limit of quantification

MHC – myosin heavy chain

MLC – myosin light chain

MS – mass spectrometry

MSG – monosodium glutamate

MW – molecular weight

NCBI – National Center for Biotechnology Information (www.ncbi.nlm.nih.gov)

NHMRC – National Health and Medical Research Council (www.nhmrc.gov.au)

NMI – National Measurement Institute (www.measurement.gov.au)

NMR – nuclear magnetic resonance

OD – optical density

OFC – oral food challenge

PBS – phosphate-buffered saline

PBS-T – phosphate-buffered saline with Tween-20

PCR – polymerase chain reaction

PDB – Protein Data Bank (www.rcsb.org)

pI – isoelectric point

PPT – prick-to-prick testing

PSP – paralytic shellfish poisoning

PV – parvalbumin

RBL – rat basophilic leukemia

RE – raw protein extract

SCBP – sarcoplasmic calcium-binding protein

SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEC-SAXS – small-angle X-ray scattering

SPT – skin prick test(ing)

SRSV – small round-structured viruses

TIM – triosephosphate isomerase

TM – tropomyosin

TPI – triosephosphate isomerase

UN – United Nations (www.un.org)

WHO – World Health Organization (www.who.int)

WHO/IUIS - World Health Organization and International Union of Immunological Societies Allergen Nomenclature (www.allergen.org)

General introduction: **Allergies to fish and other foods**

1. The nutritious value of fish and its role in human food security

Food security is an immediate concern in the face of population growth, climate change and increasing affluence. Planetary resources are limited and the sustainability of food systems is under growing scrutiny. Sustainably caught and produced seafood, including fish, is a nutritious food source.[1,2]

The demand for fish is on a continuous rise for over 60 years, which is increasingly met through aquaculture.[3,4] The sustainability of fisheries and aquaculture is critical to meet the United Nations' Sustainable Development Goals; foremost goal 14: Conserve and sustainably use resources and life below water.[5] The global per capita consumption of fish is thought to be about 20.5 kg as per data from 2018.[6] Fish is a staple in many cultures and its consumption is associated with numerous health benefits.[7–12] Fish is rich in proteins, essential fatty acids and micronutrients, and a fish-rich diet has been linked to the prevention of allergic diseases, including asthma and food allergies.[13–20] In Australia, fish plays an imported role for a balanced diet and its production is a major primary industry.[21–23]

However, fish is also a common trigger of allergic reactions. Food allergies and particularly fish allergies are under-investigated and thus cause avoidable allergic reactions, fatalities and dietary restrictions.[24]

2. The burden of food allergies

We are in the midst of an explosion of food allergies, which is a major and increasing health burden.[25,26] Food allergies are adverse inappropriate immune responses to food and often called as 'food hypersensitivities'.[27,28] They can be life-threatening and negatively impact on a person's quality of life.[29,30] Food allergy rates have tripled in the past decade and now over 500 million people are directly affected worldwide.[31,32] The reported prevalence usually ranges between one and ten per cent, while studies based on self-reports significantly overestimate the prevalence up to 10-fold.[25,33] In 2009/10 it was estimated that 5-8% of young children and 2-4% of adults suffer from food allergy.[34–36] More recent data, published in 2019, suggest a prevalence of 10.8% for adults in the US.[37]

Australia is considered the world's allergy capital where over four million people live with allergies.[38] One in ten infants and one in twenty children aged 10-14 years are suffering from food allergy.[39] One in five Australian adults reported avoiding specific food(s) due to an allergy or intolerance.[40] Consequently, food allergy-induced hospitalisation and fatality rates are continuously rising in Australia.[41–43]

Allergies and in particular food allergies have a significant direct and indirect cost for an affected individual (about US$5,000) and the economy.[44] All allergic diseases are estimated to cost the Australian economy over AU$30 billion collectively.[45] Food allergies account for about a third of this, with the greatest contributor being lost productivity (71% of total cost).[45]

3. The mechanism and triggers of food allergies

Allergic reactions occur upon exposure to harmless food components (proteins). Specific human antibodies detect the culprit proteins (allergens), which triggers a cascade of responses that frequently results in life-threatening systemic reactions (e.g., anaphylaxis). However, not all adverse reactions to food are food allergies. A food allergy is a chronic immune disease and needs to be strictly distinguished from other adverse reactions to food.[27,46] Adverse reactions to food can be caused for example by contamination with bacteria and toxins.[47] Food sensitivities can be secondary or primary. Primary food sensitivities are either non-immunological (e.g., anaphylactoid reactions such as strawberry reaction, metabolic reactions such as lactose intolerance or idiosyncratic reactions such as sulphite-induced asthma) or immunological.[48] Immunological reactions are either Immunoglobulin E (IgE)-mediated food allergies (immediate hypersensitivity reactions by allergen-specific IgE, e.g., fish allergy) or non-IgE cell-mediated (delayed hypersensitivity reactions, e.g., food protein-induced enterocolitis syndrome (FPIES)).[48–50] **Figure I 1** below shows the mechanism of an IgE-mediated allergic reaction with a seafood allergen as an example. Upon initial exposure and passing the cellular barrier, the allergen is processed and allergen-specific IgE antibodies are produced with the involvement of T- and B-lymphocytes (IgE memory cells) as well as IgE-secreting plasma cells. IgE antibodies are presented on mast cells and basophils. Hence, the immune system is hypersensitive to the food allergen.[51] Upon subsequent exposure, the allergen is bound via IgE-crosslinking. Pharmacologically active mediators (e.g., histamine) are then released into the bloodstream and tissues due to degranulation, triggering a cascade of cellular responses, which can result in multiple clinical symptoms.[48,52] Allergens, which were discovered in a wide variety of food commodities, are listed by the World Health Organization and International Union of Immunological Societies

(WHO/IUIS) Allergen Nomenclature Sub-committee in a peer-reviewed database (www.allergen.org).[53] Other databases include AllergenOnline by the University of Nebraska (www.allergenonline.org) and Allergome (The Platform for Allergen Knowledge, www.allergome.org). Most food allergens belong to eight protein superfamilies: prolamin, cupin, EF-hand, tropomyosin-like, profilin-like, Bet v 1-like, casein and calycin-like.[54,55] Animal-derived allergens are often stable and their ability to induce sensitisation and allergic reactions appears to correlate with evolutionary and molecular closeness to human homologies.[56,57]

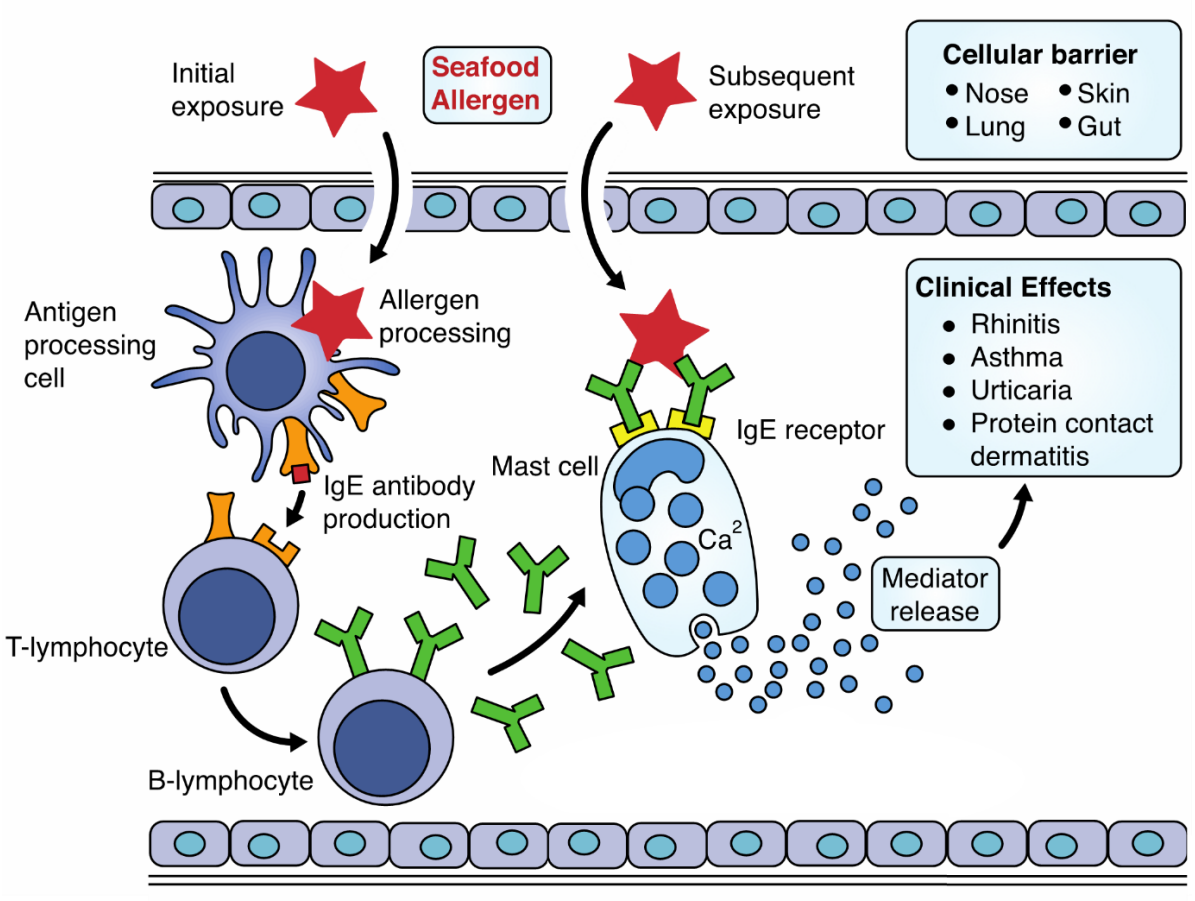

Figure I 1: Mechanism of an IgE-mediated allergic reaction. The figure (adapted from [58]) illustrates the underlying immunology of an allergic reaction with a seafood allergen (red star) as an example. After initial exposure to the cell and passing the cellular barrier, the allergen is processed. IgE antibodies (green Y), specific to the allergen, are produced by B-lymphocytes, which is triggered by T-lymphocytes. The IgE antibodies are presented on mast cells. On subsequent exposure, after passing the cellular barrier, the allergen is recognised. Upon cross-linking of IgE antibody receptors, mediators (blue), including histamine, are released, which leads to clinically relevant symptoms.

Theoretically, all foods can trigger allergic reactions, ranging from mild reactions including skin rash to life-threatening anaphylaxis, upon consumption, inhalation or skin contact.[59,60] However, over 90% of allergic reactions to food are triggered by the big eight: Peanut, tree nuts, cow's milk, soy, wheat, hen's egg, crustaceans and fish.[61] The latter two are often combined together with molluscs as seafood. The prevalence and common triggers of food allergies differ between age groups, geographical regions, tested allergens, diagnostics criteria, dietary habits and cultures.[62–64] Most common allergens for children are cow's milk, hen's egg, soy, wheat and sesame, whereas for adults it is seafood, fruits, vegetables, peanut and tree nuts in most Westernized countries.[25] Unique food allergens such as buckwheat, chestnut, bird's nest and royal jelly are found, for example, in Asia.[65] The most common food allergen and cause of life-threatening anaphylaxis in Asia is seafood, partly due to frequent consumption.[66,67] In Vietnam, for example, crustaceans are the major trigger of food allergies, followed by fish, molluscs, beef, milk and hen's egg.[68](see **Appendix D 1)** In Australia and New York City (US), seafood is the most common cause of food allergy-related fatalities.[43,69]

4. Food allergy diagnostics and management

There is no known cure for food allergies and management is generally limited to strict avoidance of allergy-triggering foods, which depends on accurate diagnostics.

Food allergies are recommended to be diagnosed by a combinatory approach of clinical history and allergen-specific sensitisation, followed by an oral food challenge (OFC) or elimination diet.[59] The most common *in vivo* test is the skin prick test (SPT) using commercial allergen preparations, which requires training and consistency.[56] The most common *in vitro* test is based on detecting food- or allergen-specific IgE antibodies and there is a wide variety

of different testing platforms and test components.[70–72] However, test results often do not correlate with clinical reactivity and current diagnostics can demonstrate a high rate of false-negative results, putting lives at risk, and limited capacity, resulting in unnecessary dietary restrictions.[73–77] One of many reasons is that the high affinity IgE antibody receptors (FcεRI) do not necessarily cross-link upon antigen-/allergen-binding, which itself not always translates to mast cell granulation and cellular reactivity.[78,79] Diagnostics are further complicated by cross-reactivity between different foods and between foods and other allergen sources.[80–83]

Once correctly diagnosed, food-allergic patients with risk of anaphylaxis are prescribed self-injectable epinephrine, often in the form of an autoinjector (e.g., EpiPen®).[84–86] However, the overall utility and cost-effectiveness of epinephrine autoinjectors depends on adequate awareness, training and prescription along with appropriate guidelines and their implementation.[87–89] Strict avoidance of allergy-triggering foods is key to complete resolution of any allergy symptoms, which is often a major challenge.[59] Many food products are inadequately labelled because of missing or unclear regulations, as well as a lack in their enforcement.[90–92](see **Appendix D 2** for 92) Many established methods for detecting allergy-triggering food components are unreliable, non-specific and not standardised between providers.[93–98] In addition, current food labelling (e.g. precautionary allergen labelling) is often unregulated and can be confusing, which poses a risk for food-allergic individuals.[99,100]

The development of food allergies is not well understood, but thought to be partially linked to environmental factors and modern lifestyle (e.g., improved hygiene, less exposure to microbial organisms, changes in diet and internal microbial milieus and reduced exposure to vitamin D).[28,101] In addition, it appears that some people are genetically predisposed to food allergies.[102,103] One strategy to avoid the development of food allergies is the early

introduction of food commodities as recently indicated for peanut and hen's egg.[104,105] For peanut, the US Food and Drug Administration approved the first food allergy immunotherapy in January 2020, allowing the treatment of a narrow range of paediatric patients.[106] Nevertheless, the microbiome plays a critical role in food allergy and its alterations might be key to the successful cure of all food allergies.[107–111]

5. Fish allergy, a brief overview

Fish allergy is an often life-long disease, which affects up to 3% of the general population and 8% of fish-processing workers.[112–118] Allergic reactions frequently include anaphylaxis and occur upon ingestion, inhalation and skin contact.[84,119,120] The deadly potential of an allergic reaction upon consumption of fish has sadly also been utilised for suicide.[121]

Cross-reactivity between fish and shellfish has been rarely reported and is not well-understood.[24,116,117,120,122–125] Therefore, fish and shellfish are generally two different allergy research fields. See **Figure 1** of **Chapter I** for a taxonomic overview of different seafood and related species. In addition, fish allergy must be strictly separated from other adverse reactions to fish as illustrated in **Figure 2** of **Chapter I**. For example, an adverse reaction to the parasite *Anisakis simplex*, frequently found in cod, black plaice, hake and herring is an allergic reaction to the parasite itself with its major allergen tropomyosin.[126–128] Other adverse reactions include responses to histamine and toxins produced by bacteria. Scombroid (histamine) poising is known for species such as mackerel, salmon and tuna, whereas ciguatera toxin is mainly found in reef fish such as snapper.[129] Nevertheless, all adverse reactions except fish allergy can usually be prevented by a mindful choice of the fish source and food preparation.

Diagnosis and management of fish allergies are however challenging due to limited knowledge, diagnostic and detection capacity. Globally over 34,000 fish species are described (www.fishbase.org) and at least 1,000 of them are consumed. In Australia, there are about 500 different fish species traded and consumed. See **Figure I 2** below for an overview of the various orders of fish relevant for consumption in the Asia-Pacific region, including Australia. Every fish is different and so is its recognition by a fish-sensitised individual.[116] It is generally accepted that fish allergy sufferers have only about a 50% chance of reacting to a wide variety

of fish.[36,130] Many patients seem to demonstrate clinical mono-sensitivity to specific fish species including salmon, tilapia, swordfish and *Pangasius*/catfish, which is sold as basa in Australia.[129,131–136] The loose term 'fish' is divided into two distinct groups: Most edible fish belong to the class of bony fish (Osteichthyes), whereas occasionally consumed sharks and rays belong to the class of cartilaginous fish (Chondrichthyes).[137,138] Flake, for example, is a trading name for gummy shark under which various sharks are sold.[139] There are indicators that most patients are allergic to bony but not cartilaginous fish (sharks and rays).[120,140–143](see **Appendix D 3** for 140) Generally, the allergenicity of a fish appears to be patient- and species-specific.[144–149]

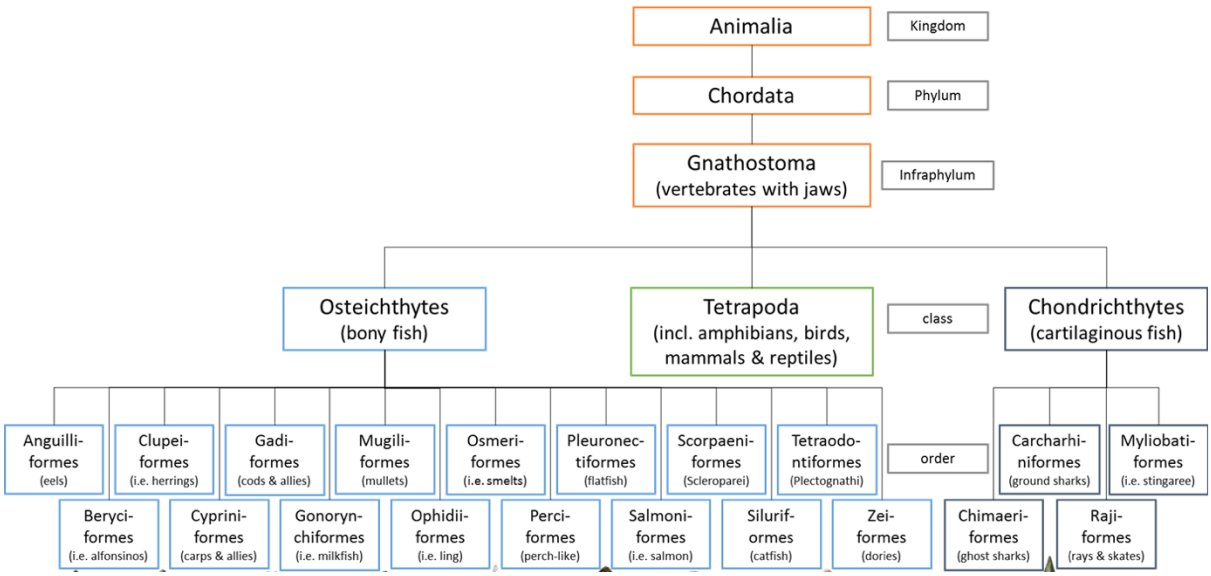

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Figure I 2: Taxonomic classification of orders of fish, which are highly relevant for consumption in the Asia-Pacific region, including Australia. The presented classification is based on The Catalogue of Life (www.catalogueoflife.org).

6. Fish allergens

The abundance of fish proteins and their capacity to bind IgE antibodies and trigger an allergic reaction largely vary between fish species and their preparation.[24,116,122,124] Most fish allergy research is focusing on muscle tissue as this is the most commonly consumed part of fish. Heat-stable calcium-binding parvalbumin is the major fish allergen, causing up to 95% of allergic reactions to fish and their products.[73,150–155] Minor allergens are aldolase A and β-enolase, which are heat-sensitive enzymes and involved in the generation of energy in eukaryotes.[156,157] Further identified allergens include actin-binding tropomyosin and vitellogenin, whereas the identification of tropomyosin (a pan-allergen for shellfish) as a fish allergen is questionable and vitellogenin is a unique allergen in caviar roe.[158–162]. In 2018, those five proteins were the only discovered fish allergens registered with the WHO/IUIS, which are listed in **Table 2** of **Chapter I** (parvalbumin from 13 species including Indian mackerel (**Chapter IV**), aldolase A and β-enolase from three species and tropomyosin and vitellogenin from one species each). Since then and apart from allergens discovered and described in this thesis, two further parvalbumins (from Atlantic mackerel and grass carp) were registered as allergens with the WHO/IUIS.

In addition, IgE-binding has been reported to numerous other fish proteins, including protamine in fish milt (seminal fluid) and collagen in fish skin and muscle.[142,163,164] **Table 1** in **Chapter I** lists most further reported IgE-binding fish proteins (aldehyde phosphate dehydrogenase, creatine kinase, glyceraldehyde-3-phosphate dehydrogenase and triosephosphate isomerase). Proteins not included in this list are actin, myosin heavy chain as well as several proteins of unknown identity.[131,132,134,165–167]

Among all these (putative) fish allergens, only parvalbumins were comprehensively analysed and evaluated for their cellular reactivity and allergenicity using recombinants (**Table 5** in

Chapter I). Amino acid sequence comparisons (**Figure 3** in **Chapter I**) demonstrate a high variety among parvalbumins, which explains (limited) immunological cross-reactivity to some extent. Interestingly, two parvalbumin isoforms from barramundi (Asian seabass) and Atlantic salmon exhibit less than 67 and 64% amino acid identity, respectively.[151,168] Very different IgE-binding epitopes of parvalbumin from five fish species (Atlantic cod, Atlantic salmon, Baltic cod, common carp and chub mackerel) have been characterised and can further explain some species-specific reactivity.[116,140,169–173](see **Appendix D 3** for 140) Nevertheless, those linear-type epitopes are not sufficient for explaining all different IgE-binding profiles as demonstrated for various mackerel and tuna species.[171] More detailed sequence and structure comparisons may allow a better understanding as demonstrated recently.[174,175] Parvalbumins in a wide variety of fish species were detected as monomers, dimers, trimers and other oligomers utilising the commercial anti-frog parvalbumin monoclonal antibody PARV-19 or in-house produced single chain, mono- or polyclonal antibodies.[154,176–187] Importantly, the parvalbumin content was found to be species- and tissue-specific.[155,188–192] The structure is resolved for parvalbumins from six fish species (**Table 11** in **Chapter I**). The structure for other fish allergens is predicted based on resolved structures of this protein in other animals (**Figure 5** in **Chapter I**).

7. Fish allergy diagnostics and management

The majority of fish species consumed worldwide has never been evaluated for their allergenicity. Consequently, a large variety of under-investigated fish species is consumed, posing a serious risk to fish-allergic individuals. While the allergenicity is patient- and species-specific, diagnostics of fish allergy are currently limited to a very few fish species.

For *in vivo* SPT, preparations for about 30 fish species are commercially available worldwide (see **Figure I 3**). Among those, only four species are commonly consumed in Australia. Prick-to-prick testing with fresh fish allows testing for a larger variety of fish but can induce anaphylaxis.[193,194] In addition, false-positive results are common due to contaminations and selected allergens can be under-represented.[195]

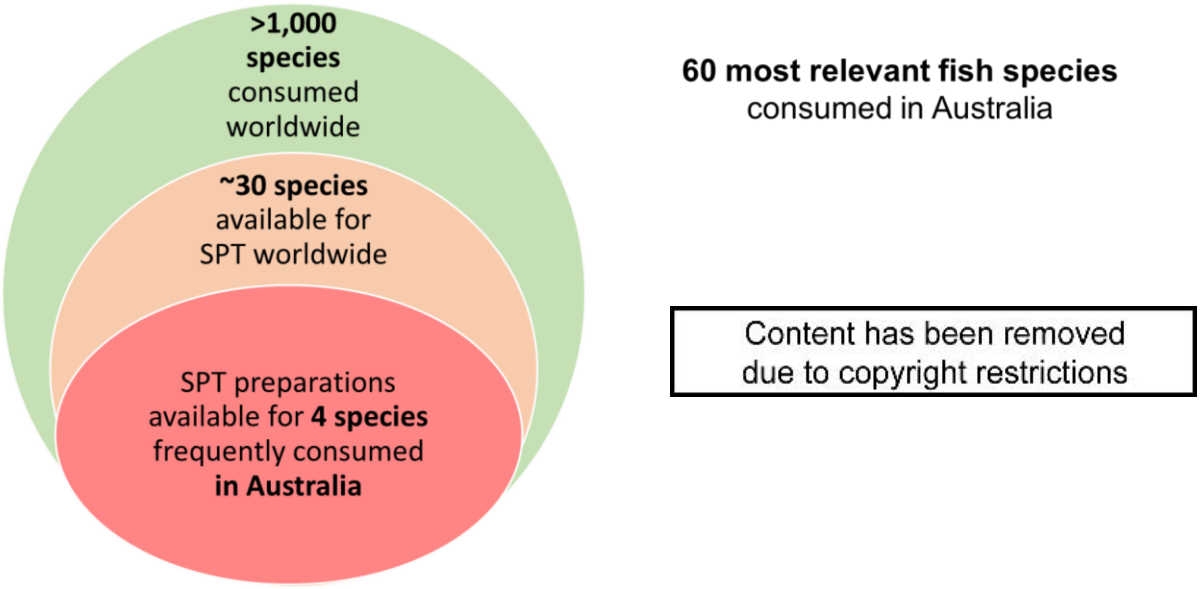

Figure I 3: Availability of commercial fish preparations for *in vivo* Skin Prick Testing (SPT). For four out of sixty most relevant fish species consumed in Australia commercial preparations are available. These are mackerel, tuna, rainbow trout and Atlantic salmon (circled in red).

Most fish allergy research has previously been conducted in Europe and North America with a focus on common native fish species. Subsequently, available *in vitro* diagnostics focus on these species, not reflecting the species diversity consumed in and exported from Australia and the Asia-Pacific. **Table 13** in **Chapter I** summarises the number of fish species and fish allergens commercially available on the three most common platforms for *in vitro* diagnostics in March 2018, presenting 28 different species. Component-resolved diagnostics were limited

to up to two recombinant parvalbumins from Atlantic cod and common carp, which are both native to Europe. Since 2019, Macro Array Diagnostics (www.macroarraydx.com) offers a platform to determine IgE levels to six fish species, including a ray, and eight parvalbumins along with a mix of aldolase A and β-enolase; however, all represented species are native to Europe. More advanced *in vitro* testing includes basophil activation testing, which is currently not (cost-)effective and not established for routine testing.[196–198]

Results from *in viv*o SPT and *in vitro* tests often do not correlate with clinical reactivity as determined by double-blind placebo-controlled food challenge (DBPCFC).[124,145,148,199,200] Therefore, the performance of an OFC, ideally DBPCFC, currently remains the gold standard for diagnosis of fish allergy, which is however challenging considering the large variety of different fish species and requirement for highly experienced and trained health professionals.

In summary, fish allergy and the allergenicity of most fish is under-investigated. The diagnostic capacity is limited and there is a high risk of false-negative test results. Cross-reactivity is often unpredictable and thus avoidance of all fish and its products is commonly recommended for individuals diagnosed with an anticipated allergy to at least one species.[24] This results in dietary restrictions, which could be avoided by more specific diagnostics.

Complete avoidance of all fish and fish-containing products requires correct food labelling. About one-third of allergic reactions, reported by fish allergic-individuals, is caused by undeclared or hidden fish allergens.[201] Furthermore, even traces of fish allergens can trigger allergic reactions and are frequently detected in food.[93,181,202,203] Fish needs to be declared on packaged foods in all countries with mandatory food labelling, including Australia, but excluding Japan.[204] The reliability and enforcement of appropriate food labelling depends on the reliable detection of fish, for which commercial test kits are available.[205–210] However,

there is no comprehensive study known, evaluating their capability to detect a wide variety of fish species (after processing). More recently non-antibody based methods including mass spectrometric approaches are emerging for the detection of fish.[211–214]

Immunotherapies for fish allergy are currently investigated but are not established. Recently, tolerance to a variety of fish species was induced through desensitisation by oral immunotherapy using gradually increasing amount of lyophilised hake.[215] The most developed approach is based on a recombinant hypoallergenic mutant of parvalbumin from common carp (mCyp c 1), which demonstrates a significant lower IgE-binding capacity and *in vivo* reactivity while maintaining the capability to induce tolerance in murine models.[216–219] Its utility was subsequently demonstrated *in vivo* and *in vitro* using fish-allergic patients' cells as well as subcutaneous immunotherapy trials.[220–223] However, its application for patients primarily sensitised to proteins other than carp parvalbumin remains unclear.

8. Summary and aims of this thesis

Fish allergy is a serious chronic immune disease with high relevance for Australians. Its diagnosis and management is hampered by limited knowledge and utility of currently available tests for allergy diagnostics and detection of fish. The focus of this thesis is to evaluate current diagnostics and management of fish allergy and subsequently provide the foundation for their improvement. See the **Graphical overview** for the structure of this PhD thesis (page 34).

- In **Chapter I**, the current literature on fish and shellfish allergy is reviewed to identify further knowledge gaps.

Utilising a range of biochemical and immunological approaches and investigating the sensitisation pattern of fish-allergic patients I aim to:

- Evaluate the utility of 28 commercial fish preparations for SPT, the most common method for *in vivo* diagnostics (**Chapter II**);
- Evaluate management tools for fish allergy (**Chapter III**) by
  - Investigating the capacity of three commercially available and widely employed enzyme-linked immunosorbent assay (ELISA) test kits to detect fish in foods (**A**),
  - Assessing the IgE-binding capacity of canned fish, which is often considered a safe food alternative for fish allergy sufferers (**B**);
- Detect, quantify and characterise the major fish allergen parvalbumin in 33 Asia-Pacific fish for improved diagnosis (**Chapter IV**);
- Discover the comprehensive repertoire of IgE-binding proteins in different fish species and evaluate their suitability for component-resolved diagnostics (**Chapter V**)
  - In most commonly investigated phosphate-buffered saline (PBS-)derived protein extracts, with focus on Atlantic salmon and *Pangasius*/catfish (basa) (**A**),
  - In acid-derived protein extracts, with focus on collagen (**B**);
- Identify safe food alternatives for bony fish-allergic patients (**Chapter VI**),
  - such as cartilaginous fish, with focus on thornback ray and gummy shark (**A**),
  - but not amphibians and reptiles, including saltwater crocodile (**B**),

  to improve management.

9. The overarching objective of tackling fish allergy

This thesis shall contribute to the implementation of a safe and sustainable fish-rich diet for all Australians and everyone worldwide, leading to improved food security, by providing a knowledgebase and tools for advancing fish allergy diagnostics and detection capacity whilst concurrently improving the management of food allergies. Everyone should be enabled to make informed and safe dietary decisions. Aligned with Sustainable Development Goals 3 (ensure healthy lives and promote well-being) and 12 (ensure sustainable consumption and production patterns),[5] allergic individuals should be empowered to consume selected nutritional and sustainable fish and their products safely.

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Chapter I – Literature review

Seafood allergy: A comprehensive review of fish and shellfish allergens

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Seafood allergy: A comprehensive review of fish and shellfish allergens

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ABSTRACT

Seafood refers to several distinct groups of edible aquatic animals including fish, crustacean, and mollusc. The two invertebrate groups of crustacean and mollusc are, for culinary reasons, often combined as shellfish but belong to two very different phyla. The evolutionary and taxonomic diversity of the various consumed seafood species poses a challenge in the identification and characterisation of the major and minor allergens critical for reliable diagnostics and therapeutic treatments. Many allergenic proteins are very different between these groups; however, some pan-allergens, including parvalbumin, tropomyosin and arginine kinase, seem to induce immunological and clinical cross-reactivity.

This extensive review details the advances in the bio-molecular characterisation of 20 allergenic proteins within the three distinct seafood groups; fish, crustacean and molluscs. Furthermore, the structural and biochemical properties of the major allergens are described to highlight the immunological and subsequent clinical cross-reactivities. A comprehensive list of purified and recombinant allergens is provided, and the applications of component-resolved diagnostics and current therapeutic developments are discussed.

1. Classification of seafood

Seafood is comprised of the distinct taxa of fish (bony and cartilaginous fish), in the phylum of Chordata, and shellfish (crustacean and mollusc), in the phyla of Arthropoda and Mollusca, in the kingdom Animalia (Fig. 1). Aquatic animals of other phyla have not been investigated comprehensively regarding allergy due to limited consumption and hence are not discussed in this review. Other seafood, such as jellyfish (phylum Cnidaria), can trigger anaphylaxis upon ingestion (Imamura et al., 2013), but was reported to be safe for patients with allergy to the seafood groups mentioned above (Amaral et al., 2018).

1.1. Types of fish

Fish are divided into the superclasses of bony fish (Osteichthyes) and cartilaginous fish (Chondrichthyes). The third superclass of Vertebrata, the tetrapods (Tetrapoda), contains all the four-limbed vertebrates including amphibians, birds, mammals, and reptiles. The bony fish are the largest group of all vertebrates consisting of 45 orders and over 435 families. They can be further divided into the two subclasses ray-finned fish (Actinopterygii) and lobe-finned fish (Sarcopterygii). Actinopterygii is the largest subclass and includes all edible bony fish. Most Sarcopterygii species are extinct, with the coelacanths being the oldest discovered order - having a continuous presence for 100 million years. Bony fish have an endoskeleton made of stable cranial bones, reproduce mostly externally, and are found in fresh and marine water whereas, cartilaginous fish have an endoskeleton made of cartilage, reproduce internally, and are found primarily in marine water. They can be further divided into the subclasses; Elasmobranchii and Holocephali. Elasmobranchii consist of sharks (Selachii), rays, skates (Batoidea), and sawfish. In contrast, the subclass Holocephali (ghost sharks) has only one surviving order (Chimaeriformes), which consists of rat fish, rabbit fish, and elephant fish. The most

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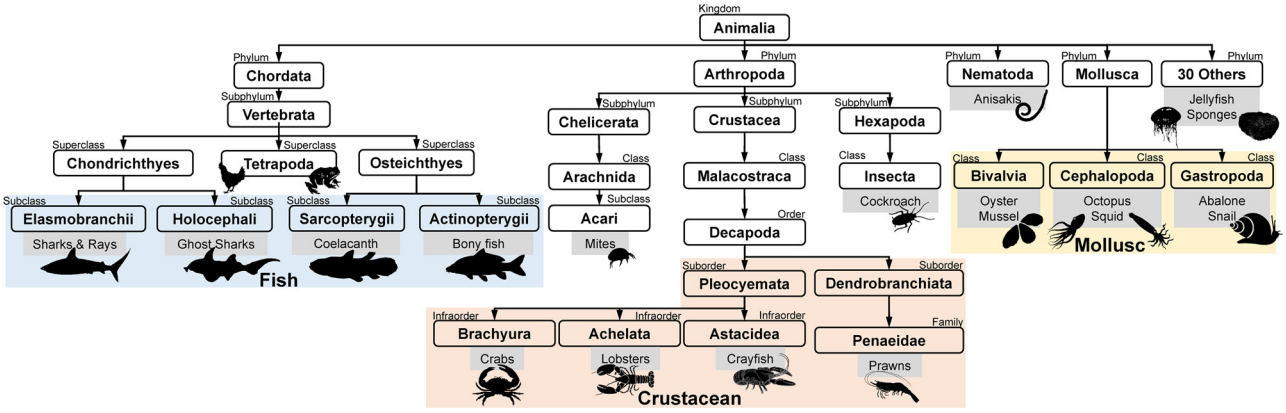

Fig. 1. Taxonomic tree of edible seafood species and related allergen sources. Classification and taxonomy of species are presented according to the NCBI (www.ncbi.nlm.nih.gov/taxonomy) and the Catalogue of Life (www.catalogueoflife.org/col/). Species in branches highlighted in blue, yellow, and red are considered as fish, mollusc, and crustacean, respectively. Allergic reactions to these and related species are discussed in this review (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

investigated species of ghost shark is the chimeric elephant fish, also known as the Australian Ghost Shark (*Callorhinchus milli*), as it is closely related to sharks, but also has distinct features like bony fish, such as gill covers. For consumption, and therefore allergy research, bony fish are the most relevant. Only in a few studies has the allergenicity of cartilaginous fish been investigated, and these suggest lower allergenicity compared to bony fish (Stephen et al., 2017).

1.2. Types of crustacean

Edible arthropods belong to the subphylum Crustacea and more specifically to the order Decapoda. This group includes prawns, shrimps, crabs, and lobsters (Fig. 1). Most crustaceans have five pairs of legs on the main thoracic body and five pairs of swimmerets on the abdomen or tail (Lee et al., 2012a). Crustaceans are closely related to arachnids (dust mites, spiders *etc.*) and insects such as cockroaches. Among the edible crustaceans, prawns constitute a major proportion of the consumed and farmed species. Decapods from the Penaeidae family are known as prawns, and those from the Caridae family, shrimps. Prawns and shrimps belong to two different taxonomical classifications with the main anatomical differences being the different overlapping pattern of the segments in the carapace and their brooding methods (Poore, 2004). Penaeids have a larger size compared to Caidae and are commercially important. Most of the species characterised for the existence and prevalence of allergens belong to this family. Shrimps are smaller in size and used mainly as additives or flavouring agents. Common examples of the caridean shrimps are the freshwater shrimp (*Macrobrachium rosenbergii*), and northern shrimp (*Crangon crangon*). However, the term “prawns” and “shrimps” are often used interchangeably in the commercial sector as well as in many research publications. The term prawn is used in the UK, Australia and other Commonwealth countries whereas the term shrimp is most commonly used in the USA and Europe. In addition, krill (*Euphausia superba*) are marine crustaceans that belong to the Euphausiidae family and are widely used in processed food products and the production of krill oil.

1.3. Types of molluscs

The mollusca are the second largest phylum of the kingdom Animalia with 104 edible species recorded by the Food and Agriculture Organization of the United Nations (FAO). This phylum consists of 9–10 taxonomic classes, with only three, Cephalopoda, Bivalvia, and Gastropoda, being commonly consumed by humans. In 2011 molluscs contributed around 12% of global fishery production; nearly 75% were farmed (Campbell and Pauly, 2013). Bivalvia is the most important class commercially, with oysters, mussels, scallops, clams, and cockles constituting a significant part of total production. Bivalves have a shell consisting of two hinged parts, while cephalopod either have an internal calcareous cuttlebone (cuttlefish), an internal chitinous pen (squid), or no shell at all (octopus). About 80% of all mollusc species belong to the class of Gastropoda including abalone, snails, and slugs with very diverse anatomic features. Molluscs mainly inhabit aquatic environments. The only edible terrestrial molluscs are the land snails of the genus *Helix*.

2. Adverse reactions to seafood including IgE-mediated allergy

The clinical presentation of seafood allergy is similar to other food allergies. Single or multiple symptoms usually appear immediately or within two hours following exposure; however, late phase reactions of up to eight hours after ingestion have been reported (Lopata et al., 1997). Respiratory reactions along with oral allergy syndrome are reported more frequently than in other food allergies, and seafoods are one of the most common triggers of life-threatening anaphylactic reactions (Matricardi et al., 2016). Sensitisation and subsequent reactions occur most frequently upon ingestion; however, they can also occur due to skin contact or inhalation of aerosolised proteins generated during cooking or processing. Unlike most other food allergies, seafood allergy persists for life in up to 90% of patients, a trend also observed with peanut allergy.

Adverse reactions to seafood can be classified into three categories, based on underlying mechanisms: 1) Immunological reactions, including allergic reaction and Food Protein-Induced Enterocolitis Syndrome (FPIES), 2) Toxic reactions, including marine biotoxins and 3) Food intolerance. Adverse reactions due to toxins and/or food intolerance often resemble clinical symptoms of seafood allergy. A good patient workup and sensitive diagnostic analysis of IgE antibody reactivity can distinguish between a true seafood allergy and other adverse reactions.

This review focuses on IgE-mediated allergic reactions to seafood and the allergenic proteins involved. In contrast, adverse clinical reactions to seafood often involve non-immunological toxic reactions as detailed below (Fig. 2).

2.1. Adverse reactions to ingested seafood

In the USA, seafood is responsible for at least 1 in 6 food poisoning outbreaks and the proportion is even higher in Japan (Trevino, 1998). Diagnosis and treatment are generally similar to those for any other food poisoning. Seafood toxins are very stable, and their toxicity is not reduced by preparation procedures (*e.g.* cooking, pickling).

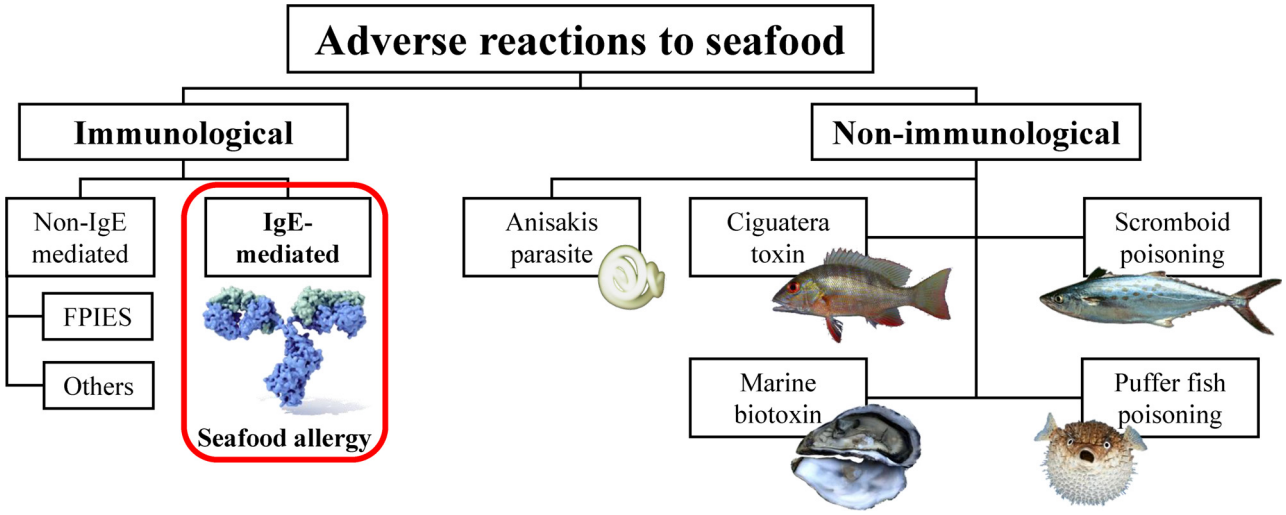

Fig. 2. Overview of adverse reactions to seafood upon ingestion. Some adverse reactions to seafood are non-immunological and/or triggered by substances other than seafood proteins. Immunological reactions are either non-IgE mediated or IgE mediated.

2.1.1. Adverse clinical reactions resembling seafood allergy

2.1.1.1. Scombroid poisoning. Scombroid fish poisoning (also termed histamine poisoning) is an allergy-like reaction that occurs after eating fish that have been improperly refrigerated after capture. Bacteria in the tissue and on the fish skin convert the amino acid histidine into histamine, one of the major mediators of allergic reactions. Enterobacteriaceae, such as *Proteus morganii* and *Klebsiella pneumonia*, are often implicated. Fish with a high content of red meat, which turns brown upon cooking is commonly involved including mackerel, tuna, herring, sardines, marlin, anchovies and bluefish. Affected fish often have a metallic or peppery taste. Symptoms usually commence within 30 min of eating, and include flushing, hives (urticaria) and nausea and severe episodes may result in anaphylactic reactions.

2.1.1.2. Marine algae toxins. A major cause of adverse seafood reactions is the presence of numerous toxins in fish, crustaceans and molluscs (Visciano et al., 2016; Friedman et al., 2017). The phenomenon of "red tides" is a significant contributor where large blooms of minute toxic dinoflagellates change the colour of the seawater to red, brown, or even green. The toxic algae are taken up by filter feeders such as mussels and oysters, accumulated in the tissue of these invertebrates, and in turn consumed by fish which absorb some of these toxins (ciguatera). Several of these phycotoxins have been identified and characterised; however, the detection in contaminated seafood is often difficult.

2.1.1.3. PSP toxins. PSP (paralytic shellfish poisoning) has been recorded frequently and has accounted for the majority of human fatalities. Minute dinoflagellates (often from the genus *Alexandrium*) produce over 20 related chemical compounds, including the well-characterized neurotoxin saxitoxin, which blocks the neuronal and muscular sodium channels. Symptoms include gastrointestinal signs (nausea, vomiting, diarrhea), but may include a prickly sensation in the fingertips and toes and even muscular paralysis.

2.1.1.4. DSP toxins. Diarrhetic shellfish poisoning (DSP) toxins, which are produced by algae of the Dinophysis or Prorocentrum genera, contain several toxic components including okadaic acid, a potent inhibitor of protein phosphatase enzymes. Chronic exposure to DSP toxins may also promote tumour formation in the digestive tract. Symptoms are mainly gastrointestinal problems such as diarrhea, nausea, vomiting, and abdominal pain.

2.1.1.5. Ciguatera. Ciguatera poisoning is caused by eating fish that has been contaminated by algae-derived toxins. Ciguatoxin, produced by single-celled dinoflagellates, is a lipid-soluble, heat-stable, and acid-resistant neurotoxin, which concentrates via the food chain in more than 400 different reef fish including Spanish mackerel (family of *Scombroidae*) and Red Emperor. Symptoms manifest mainly by gastrointestinal (diarrhea, vomiting), neurologic (temperature reversal, blurred vision) and cardiovascular (drop in blood pressure, heart block) signs. Most affected consumers recover within a few days or weeks with supportive treatment. This fish poisoning causes more human illnesses than any other seafood toxicity and affects up to 50,000 individuals annually.

2.1.1.6. Bacterial and viral contamination. Seafood poisoning outbreaks have frequently been associated with bacterial contamination of shellfish harvested in waters containing inadequately treated sewage. Especially different vibrio strains such as *V. cholerae* or *V. vulnificus* have been implicated as well as *Listeria* and *Salmonella* species. Consumption of raw shellfish, including oysters, often involves small round-structured viruses (SRSVs) and Norwalk virus. Symptoms can occur several hours after consumption and include gastrointestinal symptoms.

2.1.1.7. Anisakis parasite allergy. Allergic reactions after fish consumption can also be triggered by an intestinal fish parasite. The major nematode parasites involved are species of the genus Anisakis. This parasite infects many marine fish species as well as seals, whales and dolphins. Cooking of fish at temperatures above 60 °C or storage in industrial freezers for two days is required to kill the parasite. Anisakis can cause two major problems in humans: 1) Anisakis infection (anisakiasis) can result from eating raw, pickled or undercooked seafood, such as sushi. Infection may cause nausea, vomiting, stomach pain, and sometimes appendicitis, bowel blockage or bleeding. Diagnosis usually requires an endoscopy. However, our immune system destroys the parasite after about three weeks (Baird et al., 2014), 2) Anisakis allergy in contrast resembles a typical food allergy. In this case, the allergic reaction is directed towards allergenic proteins of this fish parasite, not to the fish itself. The allergens of *Anisakis simplex* appear to be not destroyed by heat or cooking and so allergic reactions may be triggered by dead parasites, even if the fish has been long frozen or well cooked. The presence of Anisakis specific IgE antibody can confirm allergic sensitisation (Nieuwenhuizen and Lopata, 2013, 2014).

2.1.1.8. Seafood intolerance. Adverse reactions to seafood may be caused by pharmacological properties of the ingested seafood,

resulting in intolerance reactions, reflected often with severe headaches. The substances involved include vasoactive amines such as histamine and tyramine, often found in large quantities in canned and pickled fish, and fish autolysates (Biji et al., 2016). However, vasoactive amines can also originate from fresh fish such as herring, tuna, and mackerel, while shellfish contain histamine liberators. In addition, food dyes (*e.g.* tartrazine) and food additives (*e.g.* monosodium glutamate, MSG) have also been implicated in adverse reactions.

Other adverse reactions unrelated to seafood are frequently reported including iodine reactivity, glucosamine reactivity and reactions to metabisulfite, but are not discussed in this review.

2.2. Adverse reactions through inhaling or handling seafood

While exposure via ingestion is the major route and trigger for allergic reactions, some sensitive individuals may react to the fumes when seafood is being cooked or processed, and occasionally from touch, such as when cleaning and handling fish (Jeebhay and Lopata, 2012). Symptoms include rhinitis, asthma and skin reaction and can be a problem where exposure is frequent, such as in seafood processing factories, or in some food handlers, such as chefs. These occupational allergies can affect large groups in the workforce, with up to 8% of fish processors (Jeebhay and Lopata, 2012) and even 36% of crustacean processing workers (Thomassen et al., 2016).

The major fish allergen involved seems to be parvalbumin, while tropomyosin seems to be the major crustacean allergen (Lopata and Jeebhay, 2013; Kamath et al., 2014b). Other IgE-binding proteins have also been identified in molluscs and other seafood-associated agents (*e.g.* Anisakis; proteolytic enzyme), although their exact biological and molecular nature has not been characterised (Nieuwenhuizen et al., 2006; Abdel Rahman et al., 2011). Aerosolised allergens can be identified and quantified using immunological and chemical approaches, detecting levels as low as 10 ng/m^3 (Lopata et al., 2005; Kamath et al., 2014b; Thomassen et al., 2016).

2.3. Prevalence and epidemiology of seafood allergy

It is estimated that up to 2.5% of the general population have experienced adverse reactions to seafood. Seafood allergy is more common in adults than in children (Ben-Shoshan et al., 2010; Kamdar et al., 2015) compared to the opposite distribution observed in other food allergies such as egg and milk. The prevalence of seafood allergy varies substantially across regions; however, it appears to dominate in regions where seafood is a staple food.

The highest rate of fish allergy reported for children is 5% of young Finnish (1–4 years old) (Pyrhonen et al., 2009) followed by 2.29% (95% CI, 2.02–2.56) of young Filipino (14-16 years old) populations (Connett et al., 2012) and 2.04% of American adults (95% CI, 1.7–2.38), being the highest reported rate amongst adult populations (McGowan and Keet, 2013). The prevalence of clinically confirmed fish allergy is much lower (Moonesinghe et al., 2016); however, it still affects as high as 0.7% (95% CI, 0.5–1.2) of Swedish (4 years old) (Ostblom et al., 2008) and 0.6% (95% CI, 0.3–1.3) of German (2-6 years old) (Schnabel et al., 2010) children. It is slightly higher for adults with 0.8% (95% CI, 0.2–2.5) of the European population (Burney et al., 2010) confirmed to be sensitised to fish.

The prevalence of shellfish allergy is often higher than that of fish allergy in the general population. Prawn and crab are the major causes of anaphylaxis in both children and adults. Combined crustacean and mollusc allergy reportedly affects as many as 5.5% (95% CI, 4.3–7.1) of French children (5–17 years old) and 9.0% (95% CI, 6.7–11.9) of American adults (Moonesinghe et al., 2016). The prevalence of crustacean allergy alone is considerably higher, with the highest reported prevalence of 10.3% (95% CI, 7.0–14.9) determined among Italian adults who were confirmed positive by Skin Prick Test (SPT) (Burney et al., 2010). Mollusc allergy was reported in several studies across Europe and Asia (Rance et al., 2005; Osterballe et al., 2009; Lao-araya and Trakultivakorn, 2012; Wu et al., 2012), but its prevalence seems to be less when confirmed using available *in vitro* and *in vivo* tests.

3. Immunoreactivity, biology and biochemistry of fish, crustacean and mollusc allergens

Several allergenic proteins and related isoforms have been identified in commonly consumed seafood species. Seafood allergens are generally stable, water-soluble proteins found in the edible meat. This section provides an overview of the biochemical properties, protein structure and stability and patient serum specific-IgE antibody reactivity of currently identified fish, crustacean and mollusc allergens.

3.1. Fish allergens

Fish allergens have been identified in various parts of the fish, including fish muscle (Arif, 2009), skin, bones (Hamada et al., 2001; Kobayashi et al., 2016b), roe (Perez-Gordo et al., 2008; Shimizu et al., 2009), milt (seminal fluid) (Liu et al., 2016) and blood (Nakamura et al., 2009). The most commonly consumed portion of the fish is muscle tissue, which is frequently heat-treated. Subsequently, heat-stable fish allergens found in muscle are primary focuses of research in the field of fish allergy. However, depending on region and species, other parts of fish are consumed with or without heat treatment and people may be exposed to them during food processing. Therefore, understanding the biochemistry and immunoreactivity of heat-unstable and minor fish allergens is of crucial interest for adequate diagnosis and management of patients with fish allergy.

Members of several protein families have been designated as major or minor allergens in fish, based on their IgE reactivity, and are summarised in Table 1. Parvalbumin, aldolase A, β-enolase, tropomyosin and vitellogenin have been registered as official fish allergens and are recognised by the World Health Organization (WHO)/International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee (www.allergen.org).

3.1.1. Parvalbumin

The major fish allergen is the small muscle protein parvalbumin (10–13 kDa, pI 4.1–5.2) with a conserved protein structure. The most commonly characterised allergenic fish parvalbumins from cod, salmon and carp were demonstrated to be responsible for 70–100% of allergic reactions to fish and fish products (Sharp et al., 2015; Matricardi et al., 2016). Sensitisation rates to purified and recombinant parvalbumins from different fish species have mainly been elucidated using parvalbumin-specific IgE ELISA or immunoblotting (Bugajska-Schretter et al., 1998; Van Do et al., 2005; Ma et al., 2008; Sharp et al., 2014). Functional reactivity of parvalbumins has been confirmed in some studies by basophil histamine release assays (Bugajska-Schretter et al., 2000). Furthermore, a study by Van Do et al demonstrated positive skin reaction to natural purified parvalbumins from cod, salmon and tuna in 9/10 tested fish-allergic patients (Van Do et al., 2005).

Parvalbumin belongs to one of the largest protein families of food allergens: the calcium-binding proteins (Radauer et al., 2008). It plays a role in the relaxation of muscles after contraction and is a key-protein in fast-contracting vertebrate muscle fibres and other cells in various tissues of all Chordata (Gerday, 1976; Heizmann, 1984; Gillis, 1985; Arif, 2009). Similarly to many other food allergens, parvalbumin is known for extreme heat stability (Lopata and Jeebhay, 2013; Saptarshi et al., 2014), food processing, and enzymatic digestion (Griesmeier et al., 2010a). However, it is suggested that the IgE reactivity of parvalbumin decreases when the tissue is heated to 140 °C (Kubota et al., 2016).

The two distinct phylogenetic lineages of parvalbumin, α and β, differ by isoelectric point; β-parvalbumins contain a greater proportion of acidic amino acid residues and have an isoelectric point (pI) below

Table 1
Seafood proteins with demonstrated IgE reactivity. The presented information has been retrieved from www.allergenonline.org, www.allergome.org as well as the literature. This table lists all currently known seafood proteins, which have been reported as allergens (March 2018). Proteins are listed in alphabetical order. Note: 'X' indicates in which seafood groups the protein was described as an allergen and '– ' = no details available.

Protein	Fish	Crustacean	Mollusc	Biological function	Molecular weight	Remark	References
Aldehyde phosphate dehydrogenase	X	–	–	Catalyses oxidation of aldehydes	41 kDa	–	(Das Dores et al., 2002)
Aldolase A	X	X	–	Enzyme in glycolysis	~40 kDa	–	(Liu et al., 2011b; Kuehn et al., 2013; Kamath et al., 2014a)
Arginine kinase	–	X	X	Energy metabolism	38-45 kDa	Invertebrate equivalent to creatine kinase	(Yu et al., 2003; Garcia-Orozco et al., 2007; Shen et al., 2011, 2012; Chen et al., 2013b)
Collagen	X	–	–	Structural protein	> 100 kDa	–	(Sakaguchi et al., 2000; Hamada et al., 2001; Kobayashi et al., 2016b)
Creatine kinase	X	–	–	Energy metabolism	~40 kDa	Veequivalent to arginine kinase	(Kondo et al., 2006)
β-Enolase	X	X	–	Enzyme in glycolysis	~50 kDa	–	(Liu et al., 2011b; Kuehn et al., 2013; Khanaruksombat et al., 2014)
Glycealdehyde-3-phosphate dehydrogenase	X	–	–	Enzyme in glycolysis	~37 kDa	–	(van der Ventel et al., 2011; Gonzalez-Fernandez et al., 2017)
Hemocyanin	–	X	–	Oxygen transport	77 kDa	Metalloprotein, uses copper	(Giuffrida et al., 2014; Khanaruksombat et al., 2014; Zhang et al., 2016)
Myosin light chain 1	–	X	–	Structural protein	17-23 kDa	Small subunit of myosin motor	(Bauermeister et al., 2011)
Myosin light chain 2	–	X	–	Structural protein	17-23 kDa	Small subunit of myosin motor	(Ayuso et al., 2008; Kamath et al., 2014a; Zhang et al., 2015)
Ovary development-related protein	–	X	–	Unknown ovary protein	28 kDa	Found in roe	(Zhu et al., 2016)
Paramyosin	–	–	X	Structural protein	100 kDa	Similar structure as tropomyosin	(Suzuki et al., 2011)
α-parvalbumin	X	–	–	Calcium-binding protein	10-13 kDa	Mainly cartilaginous fish	(Stephen et al., 2017)
β-parvalbumin	X	–	–	Calcium-binding protein	10-13 kDa	Mainly bony fish	(Stephen et al., 2017)
Sarcoplasmic calcium-binding protein	–	X	–	Calcium ion buffering	20-24 kDa	Dimeric; Two main isoforms in crustaceans	(Ayuso et al., 2009; Bauermeister et al., 2011; Chen et al., 2013a)
Triosephosphate isomerase	X	X	X	Glycolytic enzyme	28 kDa	Dimeric enzyme	(Bauermeister et al., 2011; Wang et al., 2011a; Yang et al., 2017)
Tropomyosin	X	X	X	Structural protein	33-39 kDa	–	(Shanti et al., 1993; Daul et al., 1994; Leung et al., 1994; Leung et al., 1998a; Leung et al., 1998b; Reese et al., 2005; Motoyama et al., 2007; Liang et al., 2008; Motoyama et al., 2008; Nakano et al., 2008; Bauermeister et al., 2011; Yadzir et al., 2012; Abramovitch et al., 2013; Liu et al., 2013; Myrset et al., 2013; Koeberl et al., 2014; Gonzalez-Fernandez et al., 2016)
Troponin C	–	X	–	Structural protein	~20 kDa	Subunit of troponin trimer	(Bauermeister et al., 2011; Kalyanasundaram and Santiago, 2015)
Troponin I	–	X	–	Structural protein	~30 kDa	Subunit of troponin trimer	WHO/IUIS
Vitellogenin	X	X	–	Yolk protein	18 kDa	Found in roe	(Mäkinen-Kiljunen et al., 2003; Perez-Gordo et al., 2008; Khanaruksombat et al., 2014; Zhu et al., 2016)

4.8 (Pechere et al., 1971; Arif, 2009). Furthermore, α and β lineages of parvalbumins have characteristic amino acids at specific positions (A-14, L-16, C-19, F-67, Q-69, F-79 for β-parvalbumins) (Beale et al., 2009). β-parvalbumins are predominantly expressed in bony fish and are known to cause IgE-mediated allergic reactions in humans (Jenkins et al., 2007). In cartilaginous fish, on the other hand, only α-parvalbumins have been found, for which a lower IgE-reactivity is proposed. Koyama et al demonstrated reduced IgE binding to extracts from several shark species compared to IgE binding to bony fish extracts in 38 Japanese fish-allergic subjects. However, the role of α-parvalbumin in lower allergenicity of cartilaginous fish has not been demonstrated (Koyama et al., 2006). To our knowledge, there has only been one report showing IgE binding to purified α-parvalbumin, with 15 fish-allergic patients sera reacting to red stingray parvalbumin (Cai et al., 2010).

3.1.2. Aldolase A and β-enolase

Aldolase A (40 kDa) and β-enolase (47–50 kDa), are essential glycolytic enzymes and have been identified as important allergens in muscle tissue of bream (Liu et al., 2011b), cod, salmon and tuna (Kuehn et al., 2013). Aldolase was first identified as a fish allergen in Pacific salmon in 2009 (Nakamura et al., 2009). Enolase was detected as a fish allergen in bream in 2011 by Liu et al., where IgE binding to this protein was demonstrated in 10 individuals with confirmed bream allergy by immunoblotting (Liu et al., 2011b). In a study by Kuehn et al. from 2013, aldolase A and β-enolase were further characterised as fish allergens using specific IgE ELISA and, in selected cases, basophil degranulation assays. This study included a total of 62 individuals with fish allergy from Luxembourg and France. Demonstrated sensitisation rates were 13–37% to aldolase A and 14-56% to β-enolase from different fish species (Kuehn et al., 2013).

Aldolase and enolase were found to be sensitive to heat treatment (Kuehn et al., 2013) and seem to be less resistant to food processing than parvalbumin. However, this has not been investigated comprehensively.

3.1.3. Tropomyosin

The structural protein tropomyosin (33–39 kDa), a pan-allergen for shellfish, has been registered as an allergen for only one fish species by WHO/IUIS, Mozambique tilapia (*Oreochromis mossambicus,* WHO/IUIS Ore m 4). Tropomyosin from fish has been reported as an allergen in only one study (Liu et al., 2013) since 2012. It was identified by immunoblotting and specific IgE ELISA using sera from 10 individuals with confirmed allergy to tilapia. However, a more recent study from 2018 has further highlighted the importance of recognising fish tropomyosin as an allergen by demonstrating IgE-reactivity to cod and albacore tropomyosin in 10/19 patients (Gonzalez-Fernandez et al., 2018).

3.1.4. Other fish allergens

Other proteins that are potential allergens have been identified in various fish species. Collagen was first described as a fish allergen in a study by Hamada et al in 2001, where it was purified from muscle tissue of tuna and demonstrated IgE binding in 5/8 patients´ sera. In another study by Kobayashi et al, IgE reactivity to collagen from Pacific mackerel skin was shown in 18/36 patients (50%) with confirmed allergy to fish by specific IgE ELISA (Hamada et al., 2001; Kobayashi et al., 2016b). Allergens identified in parts of fish other than muscle and skin include vitellogenin as the major allergen in fish roe (caviar) (Perez-Gordo et al., 2008; Shimizu et al., 2009), protamine as the major allergen in milt (seminal fluid) (Liu et al., 2016), and serum albumin as a potential allergen in blood (Nakamura et al., 2009). Some of these proteins are also expressed in muscles or present in muscle tissue due to contamination.

Other potential fish allergens include aldehyde phosphate dehydrogenase (Das Dores et al., 2002), triosephosphate isomerase (Wang et al., 2011a), glyceraldehyde-3-phosphate dehydrogenase (van der Ventel et al., 2011; Gonzalez-Fernandez et al., 2017), and creatine kinase (Liu et al., 2011b; Larco-Rojas et al., 2017) as well as several unidentified proteins of different molecular weight (4, 7, 18, 25, 40, 42, 45, 46, and 51 kDa) (Kelso et al., 1996; Asero et al., 1999; Rosmilah et al., 2005; Ebo et al., 2010). However, the allergenic relevance of these molecules is still to be determined, and they will not be discussed in this review.

3.2. Crustacean and mollusc allergens

Allergens in various crustacean and mollusc species are mainly found in the edible portions of meat. Several crustacean allergens have been identified over the past two decades, due to the advances in protein identification and allergen characterisation techniques. Crustacean allergens are proteins found mostly in the muscle tissue, cephalothorax or in roe. Their physiological functions are involved in muscle movement and energy metabolism. Prawns are a commonly consumed crustacean species and more than six prawn allergens have been identified (Table 1). Several allergens have also been identified from edible parts of mollusc species. Three allergenic proteins: tropomyosin, arginine kinase and triosephosphate isomerase, have also been identified in other shellfish groups.

3.2.1. Tropomyosin

Tropomyosin is considered the major invertebrate pan-allergen found in all edible crustacean species. Tropomyosin-specific IgE quantification in shrimp-allergic patients has demonstrated that 72–98% of patients showed positive IgE binding to purified tropomyosin (Ayuso et al., 2010; Bauermeister et al., 2011; Gamez et al., 2011). Epitope mapping studies have shown the presence of eight IgE binding epitopes (Reese et al., 2005) that are capable of binding and cross-linking IgE on basophils derived from shrimp-allergic patients (Abramovitch et al., 2013, 2017).

Tropomyosin belongs to a family of highly conserved actin-binding proteins involved in muscle contraction in vertebrates and invertebrates (Reese et al., 1999). Tropomyosin exists as a complex with troponin and is involved in muscle contractile function by interacting with actin and myosin (Nevzorov and Levitsky, 2011; Oguchi et al., 2011; Behrmann et al., 2012). It is present in muscle (skeletal, cardiac and smooth), brain, platelets, fibroblasts, and many other non-muscle cells. The physiological state of tropomyosin is a highly stable, α-helical coiled-coil homo-dimeric protein. The stability of the coiled-coil structure of tropomyosin is a function of the interaction of repeated sequence patterns of seven amino acids over the entire length of the protein, with a hydrophobic amino acid in every first and fourth position (Lees-Miller and Helfman, 1991). This form of secondary and tertiary structure imparts structural stability to tropomyosin and it is therefore able to withstand heat and high-pressure processing and retain its allergenicity (Kamath et al., 2013; Kamath et al., 2014a). Several studies have investigated the effects of thermal and enzymatic digestion on tropomyosin. Although tropomyosin is highly heat-stable, studies have demonstrated an increase in antibody binding to tropomyosin after heat treatment (Kamath et al., 2013; Kamath et al., 2014a). Simulated digestion studies have shown that tropomyosins are susceptible to gastric pepsin and trypsin degradation (Liu et al., 2011a; Gamez et al., 2015).

Tropomyosin is the major crustacean allergen, identified and registered by WHO/IUIS as an allergen in 13 different species. In crustaceans, the fast twitch and the slow twitch isoforms were identified in the tail muscles and the pincer muscles, respectively (Motoyama et al., 2007). Tod p 1 was the first major mollusc allergen submitted to WHO/IUIS and was isolated from squid (*Todarodes pacificus)*. The allergen is a 38 kDa, heat-stable protein and was later identified as tropomyosin after amino acid sequencing and alignment with tropomyosin from other organisms (Miyazawa et al., 1996). Shortly after, other tropomyosins were identified in other groups of mollusc species such as oyster (Ishikawa et al., 1997), and snail (Ishikawa et al., 1998a).

3.2.2. Arginine kinase

Arginine kinase (AK) was characterised in shrimps as a clinically relevant allergen after tropomyosin. Subsequently, AK has been identified and characterised in crabs, lobster, prawns, and octopus. Similar to tropomyosin, the diagnostic utility of AK has been investigated for shrimp allergy, with 10–51% of shrimp-allergic patients eliciting positive IgE binding (Ayuso et al., 2010; Bauermeister et al., 2011; Giuffrida et al., 2014; Pascal et al., 2015).

Arginine kinase is involved in the regulation of cellular ATP levels and is an essential enzyme in the energy metabolism of invertebrates. It catalyses the transfer of a phosphate group from ATP to L-arginine. Phosphoarginine is the most important phosphagen in invertebrates, synonymous in function to phosphocreatine in vertebrates. Sequence analysis of AK and creatine kinase concluded that they are derived from a common origin (Uda et al., 2006), with the homologs displaying a sequence identity of < 39%. AK is widely distributed throughout invertebrates and generally found as a monomer, although some dimeric AK have been observed (Uda et al., 2006). It is highly concentrated in muscle tissue (Abdel Rahman et al., 2011) and binding to the muscle protein actin has previously been demonstrated (Reddy et al., 1992).

Epitope mapping studies have shown the presence of seven IgE binding epitopes and confirmed the role of structural stability for IgE binding (Ayuso et al., 2010; Yang et al., 2015). Accordingly, loss of IgE reactivity upon heat-treatment was demonstrated (Chen et al., 2013b). AK is a heat labile protein, with previous studies showing reduced allergenicity after thermal and pH treatment (Kamath et al., 2014a). It has a molecular weight of 38–45 kDa and a pI of approximately 6 (Jarilla et al., 2014). AK the second crustacean allergen discovered and has been described in black tiger prawn (*Penaeus monodon*), white-leg shrimp (*Litopenaeus vannamei*), red swamp crayfish (*Procambarus clarkii*) (Chen et al., 2013b), and North sea shrimp (*Crangon crangon*) (Bauermeister et al., 2011). AK has also been described as a mollusc allergen, first discovered in octopus (*Octopus fangsiao*) (Shen et al., 2012). The amino acid sequence shares 54% identity between crustaceans and molluscs. It is also well-known for its role in house dust mite allergy (Abdel Rahman et al., 2011), as well as insect allergy.

3.2.3. Myosin light chain

Myosin light chain (MLC) was identified as an allergen in white-leg shrimp (Lit v 3) (Ayuso et al., 2008). It is considered a minor allergen, with the frequency of allergic sensitisation ranging from 19 to 55% (Bauermeister et al., 2011; Pascal et al., 2015). IgE binding studies indicate that recognition of MLC is higher in adults compared to children, using heated shrimp extract.

Myosin light chains form part of the large myosin macromolecular complex that make up the functional muscle protein structure (actin-binding molecular motor). The multimetric protein consists of two heavy chains (~200 kDa) and four light chains (~18-20 kDa). The light chains belong to the calmodulin-like protein family (along with parvalbumin) that bind Ca^{2+} through their EF-hand domain. There are two classes of myosin light chain, which are referred to as the essential light chain (MLC1) and the regulatory light chain (MLC2) (also referred to as α and β chains). Like many food allergens, crustacean MLCs are heat and pH stable (Zhang et al., 2015). Studies have shown that IgE recognises primarily conformational rather than linear epitopes of MLC1 (Zhang et al., 2015).

MLC1 (allergen number 5, WHO/IUIS) has been characterised as an allergen in North Sea shrimp (Bauermeister et al., 2011) and red swamp crayfish (Zhang et al., 2015). MLC2 (allergen number 3, WHO/IUIS) has been characterised as an allergen in American lobster (*Homarus americanus*), black tiger prawn (Kamath et al., 2014a), and white-leg shrimp (Ayuso et al., 2008).

3.2.4. Sarcoplasmic calcium-binding protein

Sarcoplasmic calcium-binding protein (SCBP), was first identified as a shellfish allergen in Black tiger prawn (Pen m 4) and Pacific white-leg shrimp (Lit v 4) (Shiomi et al., 2008; Ayuso et al., 2009). Although considered a minor allergen, SCBP elicits 29–50% positive IgE binding among shrimp-allergic patients. Compared to other shellfish allergens, SCBP contains only three IgE binding epitopes (Ayuso et al., 2010). However, it was demonstrated that SCBP contains conformational type IgE epitopes that may partially be dependent on calcium binding (Morii et al., 2013). Interestingly, IgE recognition was found to be higher in children than in adults, and with a greater relevance in predicting clinical reactivity to shrimps in combination with tropomyosin-specific IgE (Ayuso et al., 2009; Pascal et al., 2015).

Sarcoplasmic calcium-binding proteins belong to the EF-hand calcium-binding protein family that contain helix-loop-helix motifs to bind calcium ions (Ca^{2+}). They are found exclusively in invertebrates and are thought to act as a functional analogue to vertebrate parvalbumin. Like parvalbumin they have a role in the buffering of cytosolic calcium in muscles; however, SCBPs are larger proteins (20–24 kDa compared to 12 kDa) that form dimers, so may have other functions which are not yet known (White et al., 2011). The sequence identity varies greatly between species and multiple isoforms have been characterised. Crustacean SCBPs have two peptides (Wnuk and Jauregui-Adell, 1983; Takagi et al., 1984), subunits α and β, which can combine as either a homodimer or heterodimer isotype (α_2, αβ, β_2; SCBP-I, -II, and -III, respectively). Furthermore, multiple variants have also been reported (White et al., 2011). SCBPs contain four EF-hand domains, of which two or three retain the ability to bind either Ca^{2+} only, or mixed Ca^{2+}/Mg^{2+}, depending on the domain. Binding of Ca^{2+} occurs cooperatively (Wnuk and Jauregui-Adell, 1983), with marked structural changes occurring after binding (Wnuk et al., 1981; Christova et al., 2000). SCBPs are heat resistant and stable under acid/alkali treatment (Chen et al., 2013a).

SCBP has been described as an allergen in a number of crustacean species including black tiger prawn (Shiomi et al., 2008), white-leg shrimp (Ayuso et al., 2009), North Sea shrimp (Bauermeister et al., 2011), red swamp crayfish (*P. clarkii*), and narrow-clawed crayfish (*Pontastacus leptodactylus*) (Chen et al., 2013a).

3.2.5. Troponin

Troponin has been identified as a minor crustacean allergen with IgE sensitisation rates ranging from 12 to 29% (Bauermeister et al., 2011; Pascal et al., 2015). Troponins bind with tropomyosin to the thin filament to regulate the contraction of striated muscle. The troponin complex is comprised of three subunits, Troponin C, I and T, of which Troponin C and I have been shown to be IgE-reactive. Troponin C binds Ca^{2+} to regulate activation in response to Ca^{2+} efflux. It belongs to the large family of calcium-binding proteins along with myosin light chain, parvalbumin and calmodulin. It contains four EF-hand motifs, of which two contain functional Ca^{2+}-binding loops in crustaceans (Tanaka et al., 2013). There are three main Troponin C isoforms in crustaceans (Garone et al., 1991), with an additional eight isoforms detected using genetic techniques (Chao et al., 2010). Troponin C has been described as an allergen in North sea shrimp (Bauermeister et al., 2011), black tiger prawn (Kalyanasundaram and Santiago, 2015), and American lobster (Chao et al., 2010). The Troponin I subunit inhibits muscle contraction by binding to actin and preventing the actin-myosin interaction. Troponin I has been identified as a crustacean allergen in narrow-clawed crayfish (WHO/IUIS: Pon l 7).

3.2.6. Triose phosphate isomerase

Triose phosphate isomerase (TIM) has been characterised as a shrimp and octopus allergen (Bauermeister et al., 2011; Kamath et al., 2014a; Yang et al., 2017). It is considered a minor allergen with the frequency of IgE sensitisation rainging from 15 to 23%. It is a 26–29 kDa enzymatic protein that exists as a dimer, and its primary function is in glucose energy metabolism. Apart from crustacean and mollusc species, TIM has been identified as an allergen in cockroach and plants (Hoppe et al., 2006; Chuang et al., 2010). The cross-reactivity among these species due to TIM has not been investigated in detail.

3.2.7. Paramyosin

Paramyosin, a major structural component of thick filaments in invertebrate muscle, was identified as another allergen in abalone (*Haliotis discus discus*) (Suzuki et al., 2011, 2014) and Pacific oyster (*Crassostrea gigas*) (Nugraha et al., 2018). The discovery of allergenic paramyosin in mollusc species was not surprising since this protein has been confirmed as a major allergen in other invertebrates such as house dust mite (Lee et al., 2004) and Anisakis (Perez-Perez et al., 2000). Paramyosin is a significant component of the bivalve myofibril, making up 38–48% of the white adductor muscle and 15–30% of the red adductor muscle and 9–14% on the myofibril of the mantle muscle of squid and the foot muscle of turban shell (Kantha et al., 1990). The structure of paramyosin is similar to that of tropomyosin, and it is sometimes referred to as "water-insoluble tropomyosin" or "tropomyosin-A". The molecule is comprised of two α-helical coiled-coil proteins with short non-helical extensions at both the N- and C-termini (Kantha et al., 1990). Although the cross-reactivity of paramyosin and tropomyosin has been demonstrated, the IgE of allergic patients did not react with paramyosin from heated abalone extract, suggesting that the observed cross-reactivity may not be clinically significant, as abalone is generally cooked before consumption (Suzuki et al., 2011). Paramyosin is a heat and pH labile protein and aggregates during extraction in a buffer containing a low salt concentration.

3.2.8. Other crustacean and mollusc allergens

Several other proteins found in various crustacean species have been identified as potential allergens, based on IgE recognition from shellfish-allergic patient sera. Hemocyanin has been identified as an allergen in shrimps (Giuffrida et al., 2014; Khanaruksombat et al., 2014) and crabs (Zhang et al., 2016). It is a high molecular weight multi-subunit protein that is mostly found in the hemolymph or hepatopancreatic regions. It consists of an oxygen transport protein complex that interacts with copper ions, giving it a characteristic blue-green colour. A 2014 case study reported anaphylaxis to shrimp caused by hemocyanin and the utility of hemocyanin for shrimp allergy diagnostics was investigated along with other major shrimp allergens. Notably, hemocyanin has been implicated in shellfish-mite or shellfish-insect cross-reactivity (Giuffrida et al., 2014; Srinroch et al., 2015; Kamath et al., 2017). The clinical implications of cross-reactivity due to hemocyanin has yet to be investigated in detail. Other potential crustacean allergens are fructose bis-phosphate aldolase and titin, which require further investigation to confirm IgE reactivity (Kamath et al., 2014a).

While only tropomyosin, paramyosin and arginine kinase have been confirmed as allergens in molluscs, additional proteins may cause an allergic reaction. Indeed, many unknown proteins have been reported to elicit hypersensitivity. The common whelk (*Buccinumun datum)* was found to have several IgE binding proteins with molecular weights of 40, 71, and 82 kDa. These proteins were heat-stable but demonstrated rapid degradation in simulated intestinal fluid and moderate degradation in simulated gastric fluid. Similarly, various proteins from the brown garden snail (*Helix aspersa*) were found to bind IgE from allergic patient sera (Asturias et al., 2002). Recently 23 unreported allergens were identified in the Pacific oyster using biochemical and computational tools in addition to IgE antibody reactivity (Nugraha et al., 2018).

4. Allergen sequence similarity - molecular and clinical cross-reactivity

4.1. *Fish allergen* sequences and cross-reactivity

Most fish express several different isoforms of the major fish allergen parvalbumin (up to 5), primarily of the β-lineage, with a large molecular diversity most likely being responsible for differential clinical reactivity resulting in mono- or multiple-sensitivity (Kuehn et al., 2014). It is generally accepted that fish allergy sufferers have an approximately 50% chance of being cross-reactive to another fish species (Sicherer and Sampson, 2010; Kobayashi et al., 2016c) and mono-sensitivity to only one type of species such as tilapia and salmon has been reported (Ebo et al., 2010; Kuehn et al., 2011). Different linear IgE-binding epitopes of parvalbumin from five fish species (Atlantic cod, Atlantic salmon, Baltic cod, common carp, and chub mackerel) have been characterised and probably contribute to species-specific reactivity (Elsayed and Apold, 1983; Untersmayr et al., 2006; Yoshida et al., 2008; Perez-Gordo et al., 2012, 2013; Sharp and Lopata, 2014). However, the variation of linear IgE-binding epitopes is not sufficient to explain all the different IgE-binding profiles demonstrated for species of mackerel and tuna (Yoshida et al., 2008). Multiple parvalbumin proteins and aggregates have been identified in fish as monomers, dimers and oligomers (Saptarshi et al., 2014). Most fish parvalbumins have been identified by immunoblotting, utilising the commercial anti-frog parvalbumin monoclonal antibody PARV-19 (Chen et al., 2006; Lim et al., 2008; Beale et al., 2009; Gajewski and Hsieh, 2009; Weber et al., 2009). Polyclonal antibodies against parvalbumin from a range of different fish species, such as barramundi, basa/catfish, carp, cod, pilchard, and salmon, have recently been used successfully in the identification of parvalbumin, including in some Asian fish species (Swoboda et al., 2002b; Lee et al., 2011; Sharp et al., 2015; Ruethers et al., 2018).

Parvalbumin was first described as an allergen in the early 1970s in Baltic cod. This allergen was subsequently named Gad c 1 and as of March 2018, β-parvalbumins from 13 fish species were officially registered by WHO/IUIS and named accordingly (Table 2). The database, maintained by the United States National Library of Medicine (www.pubmed.org) listed 156 publications about the 'fish allergen parvalbumin' as of March 2018. Non-fish parvalbumins are WHO/IUIS-recognised allergens from frog (α- and β-isoform) and chicken (α-isoform) with reported cross-reactivity between chicken and fish parvalbumins (Kuehn et al., 2016).

Parvalbumins and their allergenicity have been well investigated for many bony fish species consumed in West Europe and North America. Those studies show large amino acid sequence diversity between species such as cod, carp, and salmon (Kuehn et al., 2014; Sharp and Lopata, 2014). The diversity of WHO/IUIS-recognised parvalbumin isoforms is demonstrated in Fig. 3. Notably, parvalbumin isoforms from the same species often share less than 68% amino acid sequence identity as shown for barramundi and rainbow trout, adding complexity to the detection of allergenic parvalbumins. Among all β-parvalbumins, the sequence identities range from 46 to 99%, with the lowest identity between the respective isoforms from Baltic cod and rainbow trout, suggesting the possibility of limited cross-reactivity. Parvalbumins of the α-lineage share only 43–60% amino acid sequence identity with β-parvalbumins. Human α-parvalbumin (UniProt: P20472) has a sequence identity with all WHO/IUIS-registered α- and β-parvalbumins of 44-58% and 68-79%, respectively. α-parvalbumins have been reported as allergens in cartilaginous fish (Stephen et al., 2017); however, clinical relevance is yet to be determined. In addition, the vast majority of the more than 32,300 fish species have never been investigated for their allergens (Sharp and Lopata, 2014).

The allergenicity of any given fish species is dependent on two factors; the allergenicity of the proteins (in this case parvalbumin), and on the amount of the allergens present in the tissue (Griesmeier et al., 2010b; Kuehn et al., 2010; Kobayashi et al., 2016a). A study by Kuehn et al. (Kuehn et al., 2014) demonstrated that the parvalbumin content may differ up to 48 fold between fish species (0.2–11.2 mg of parvalbumin per gram of tissue), dependant on the size of the specimen, the species and also the type of fish (sedentary or migratory type). Furthermore, the parvalbumin content within one species can differ up to 7.4-fold between different muscle parts, as demonstrated for the Mediterranean flying fish (Kobayashi et al., 2016d). Generally, higher parvalbumin content is observed in dorsal and rostral compared to ventral and caudal parts of fish muscle, respectively. Furthermore, parvalbumin

Table 2
WHO/IUIS registered fish allergens. Fish allergens registered with the WHO/IUIS (www.allergen.org) are listed in alphabetical order of given allergen names. Note: '*' reference is not directly linked to the WHO/IUIS allergen entry. MW = molecular weight in kDa and '-'= no details available.

Fish species		Allergen	Protein type	MW	Genbank protein accession number of isoforms			References
					.0101 .0102	.0201 .0202	.0301	
Atlantic herring	*Clupea harengus*	**Clu h 1**	β-parvalbumin	12	CAQ72970	CAQ72971	CAQ72972	(Swoboda et al., 2013)
Common carp	*Cyprinus carpio*	**Cyp c 1**	β -parvalbumin	12	CAC83658	CAC83659	–	(Bugajska-Schretter et al., 2000)
Baltic cod	*Gadus callarias*	**Gad c 1**	β-parvalbumin	12	P02622	–	–	(Bugajska-Schretter et al., 1998)
Atlantic cod	*Gadus morhua*	**Gad m 1**	β-parvalbumin	12	AAK63086 CAM56785	AAK63087 CAM56786	–	(Van Do et al., 2003)
		Gad m 2	β-enolase	47	B3A0L6	–	–	(Kuehn et al., 2013)*
		Gad m 3	Aldolase A	40	P86980	–	–	(Kuehn et al., 2013)*
Barramundi	*Lates calcarifer*	**Lat c 1**	β-parvalbumin	12	AAV97933/AHW83198	AAT45383	–	(Sharp et al., 2014)
Whiff	*Lepidorhombus whiffiagonis*	**Lep w 1**	β-parvalbumin	12	CAP17694	–	–	(Griesmeier et al., 2010a)*
Chum salmon (roe)	*Oncorhynchus keta*	**Onc k 5**	β-prime-component of vitellogenin	18	BAJ07603	–	–	(Shimizu et al., 2014)*
Rainbow trout	*Oncorhynchus mykiss*	**Onc m 1**	β-parvalbumin	12	P86431	P86432	–	(Kuehn et al., 2010)*
Mozambique tilapia	*Oreochromis mossambicus*	**Ore m 4**	Tropomyosin	33	AFV53352	–	–	(Liu et al., 2013)*
Indian Mackerel	*Rastrelliger kanagurta*	**Ras k 1**	β-parvalbumin	11	ANW10058.1	–	–	(Ruethers et al., 2018)*
Atlantic salmon	*Salmo salar*	**Sal s 1**	β-parvalbumin	11	CAA66403	–	–	(Lindstrom et al., 1996)*
		Sal s 2	β-enolase	50	ACH70931 ACH70932	–	–	(Kuehn et al., 2013)*
		Sal s 3	Aldolase A	40	ACH70901	–	–	(Kuehn et al., 2013)*
Pacific pilchard	*Sardinops sagax*	**Sar sa 1**	Parvalbumin	12	CAQ68366	–	–	(Beale et al., 2009)*
Ocean perch	*Sebastes marinus*	**Seb m 1**	Parvalbumin	11	CAQ72968	CAQ72969	–	(Kuehn et al., 2010)*
Yellowfin tuna	*Thunnus albacares*	**Thu a 1**	β-parvalbumin	11	CAQ72967	–	–	(Kuehn et al., 2010)*
		Thu a 2	β-enolase	50	P86978	–	–	(Kuehn et al., 2013)*
		Thu a 3	Aldolase A	40	P86979	–	–	(Kuehn et al., 2013)*
Swordfish	*Xiphias gladius*	**Xip g 1**	β-parvalbumin	12	CAR48256	–	–	(Griesmeier et al., 2010b)*

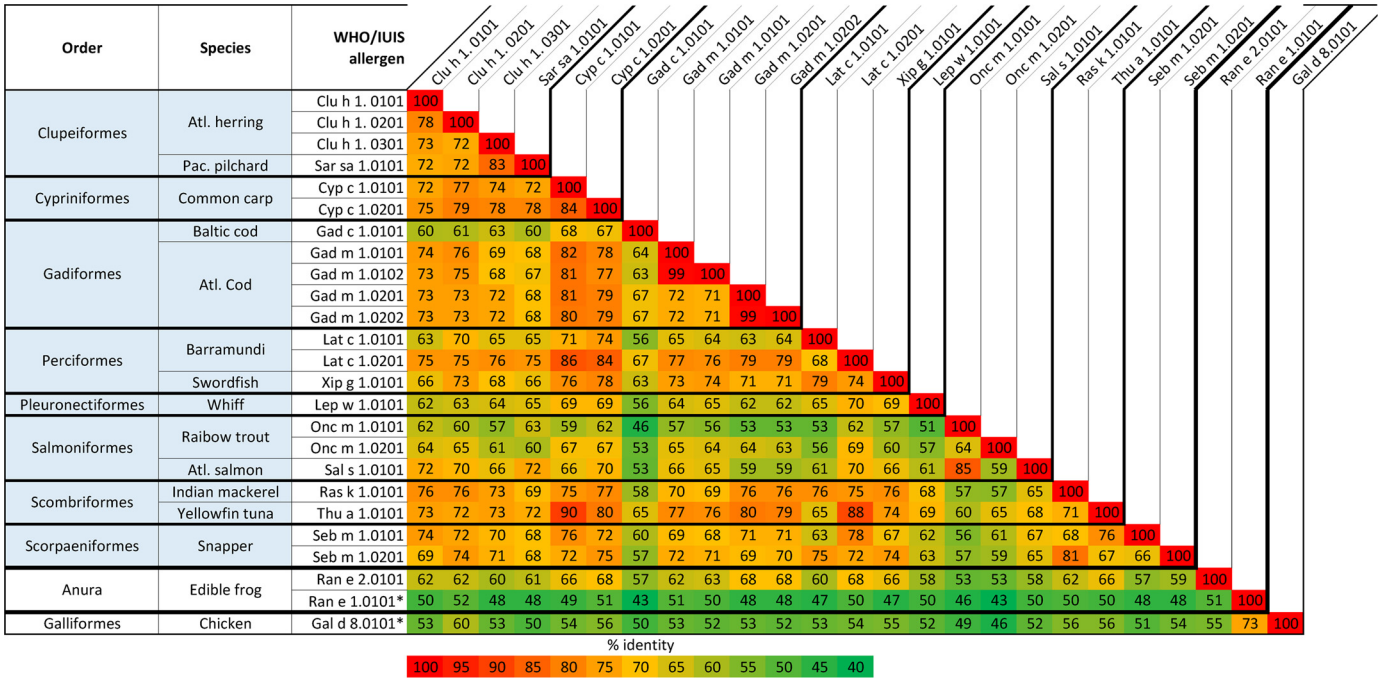

Fig. 3. Comparison of amino acid sequence identities (%) of all WHO/IUIS-registered parvalbumins. All allergens are β-parvalbumins, except the last two listed (highlighted with *), which are α-parvalbumins. The sequence identities were calculated using Multiple Sequence Alignment in Clustal Omega (EMBL-EBI).

is more abundant in white compared to dark tissue (Kobayashi et al., 2006; Lee et al., 2012b), a factor possibly leading to contradictions in the reported allergenicity of tuna (Lim et al., 2005).

Collagen has been comprehensively investigated as an allergen in the Japanese population and was identified as a highly cross-reactive pan-allergen along with parvalbumin (Kobayashi et al., 2016b). All other fish allergens have only been investigated in a limited number of species and their cross-reactivity is poorly understood. Vitellogenin and tropomyosin are the only WHO/IUIS registered allergens in chum salmon and Mozambique tilapia, respectively. For vitellogenin, an IgE cross-reactivity was demonstrated, but the clinical relevance remains unknown (Shimizu et al., 2009). Aldolase A and β-enolase from Atlantic cod and Atlantic salmon have been well characterised, and may be useful for the identification of patients with limited cross-reactivity (Kuehn et al., 2013; Sorensen et al., 2017).

4.2. *Crustacean allergen* sequences and cross-reactivity

Crustacean allergens exhibit a high degree of conservation and amino acid sequence identity among their member species as well as between molluscs, insects, mites and other invertebrates. This is the primary reason for clinical and immunological cross-reactivity among these species due to the presence of shared IgE epitopes. Moreover, the high frequency of cross-reactivity presents a challenge in identifying the primary sensitising source during routine allergy diagnosis and differentiation from other cross-reacting allergens. Tropomyosin is the major allergen implicated in clinical and IgE antibody cross-reactivity, and several studies have investigated the role of crustacean tropomyosin in cross-reactivity to other invertebrate sources (Table 3). Cross-reactivity among crustaceans and molluscs is frequently observed due to tropomyosin's potential to sensitise via occupational exposure in addition to exposure by ingestion (Goetz and Whisman, 2000; Zhang et al., 2006). Cross-reactivities between crustaceans and house dust mites, as well as insects, have been extensively investigated due to their high clinical impact on sensitised individuals upon accidental exposure and subsequent allergic reactions to the seafood allergens (Ayuso et al., 2002; Arlian et al., 2009; Boquete et al., 2011; Wang et al., 2011b, Lopez-Matas et al., 2016; Kamath et al., 2017). Tropomyosin has a highly conserved amino acid sequence among crustaceans ranging from 88 to 100% identity as shown in Fig. 4. Since tropomyosin is the major sensitising source in more than 80% of shellfish-allergic patients, the high sequence identity may explain the frequency of clinical cross-reactivity among crustacean species. Due to evolutionary lineages, crustacean tropomyosins share a high sequence identity with cockroaches and dust mites, 80–97 and 78–98%, respectively as shown in Fig. 4. The clinical implications of this similarity were evident in a 2003 study where IgE reactivity to shrimp allergens was demonstrated in a shrimp-unexposed population that was allergic to house dust mites or cockroaches. Interestingly, crustacean tropomyosin shares a lower sequence identity with mollusc tropomyosin (55–65%) as well as with vertebrate tropomyosin (54–60%).

Besides tropomyosin, arginine kinase has been implicated in cross-reactivity to moths, spiders, and silkworms (Binder et al., 2001; Liu et al., 2009; Bobolea et al., 2011). In addition, hemocyanin may be a potential cross-reactive allergen identified so far as an allergen in crustaceans, dust mites, cockroaches and molluscs (Giuffrida et al., 2014; Khanaruksombat et al., 2014; Khurana et al., 2014; Kamath et al., 2017).

4.3. *Mollusc allergen* sequences and cross-reactivity

The molecular heterogeneity of allergenic mollusc tropomyosins (70–97% sequence identity, see Fig. 4) poses a challenge for clinicians to identify the species responsible for allergic reactions. There are many cases of patients with IgE reactivity to only a very narrow range of mollusc species (Carrillo et al., 1992; Jimenez et al., 2005; Martin-Garcia et al., 2007; Rodriguez-Del Rio et al., 2009). Another study demonstrated the utility of this diversity for phylogenetic analysis of molluscs (Wang et al., 2011c). There are 56 tropomyosins from 41 different mollusc species listed by UniProt (www.uniprot.org), while only five are registered as allergens with the WHO/IUIS (Table 4). Few studies have comprehensively investigated cross-reactivities among mollusc species. The largest studies demonstrated serum IgE reactivity to ten cephalopods, seven bivalves, and four gastropods for ten shellfish-allergic patients (Motoyama et al., 2006; Emoto et al., 2009). In a clinical study with 17 patients, only ten were co-sensitised to abalone

Table 3
WHO/IUIS registered crustacean allergens. Crustacean allergens registered with the WHO/IUIS (www.allergen.org) are listed in alphabetical order of given allergen names. Note: '*' reference is not directly linked to the WHO/IUIS allergen entry. MW = molecular weight in kDa and '-' = no details available.

Crustacean species		Allergen	Protein type	MW	Genbank protein accession number of isoform		References
					.0101	.0102	
Common name unavailable	*Archaeopotamobius sibiriensis*	**Arc s 8**	Triosephosphate isomerase	28	CAD29196	–	–
Flowery crab	*Charybdis feriatus*	**Cha f 1**	Tropomyosin	34	AAF35431	–	(Leung et al., 1998b)*
North Sea shrimp	*Crangon crangon*	**Cra c 1**	Tropomyosin	38	ACR43473	–	(Bauermeister et al., 2011)*
		Cra c 2	Arginine kinase	45	ACR43474	–	(Bauermeister et al., 2011)*
		Cra c 4	Sarcoplasmic calcium-binding protein	25	ACR43475	–	(Bauermeister et al., 2011)*
		Cra c 5	Myosin, light chain 1	18	ACR43477	–	(Bauermeister et al., 2011)*
		Cra c 6	Troponin C	21	ACR43478	–	(Bauermeister et al., 2011)*
		Cra c 8	Triosephosphate isomerase	28	ACR43476	–	(Bauermeister et al., 2011)*
Chinese mitten crab	*Eriocheir sinensis*	**Eri s 2**	Ovary development-related protein	28	AAO73305	–	(Zhu et al., 2016)
American lobster	*Homarus americanus*	**Hom a 1**	Tropomyosin	–	AAC48287	AAC48288	(Leung et al., 1998b)
		Hom a 3	Myosin light chain 2	~23	EH115965	–	–
		Hom a 6	Troponin C	~20	P29291	–	–
White-leg shrimp	*Litopenaeus vannamei*	**Lit v 1**	Tropomyosin	36	ACB38288	–	–
		Lit v 2	Arginine kinase	–	ABI98020	–	(Garcia-Orozco et al., 2007)*
		Lit v 3	Myosin, light chain 2	20	ACC76803	–	(Ayuso et al., 2008)*
		Lit v 4	Sarcoplasmic calcium-binding protein	20	ACM89179	–	(Ayuso et al., 2009)*
Giant freshwater prawn	*Macrobrachium rosenbergii*	**Mac r 1**	Tropomyosin	37	ADC55380	–	(Yadzir et al., 2012)*
King prawn	*Melicertus latisulcatus*	**Mel l 1**	Tropomyosin	38	AGF86397	–	(Koeberl et al., 2014)*
Greasyback shrimp	*Metapenaeus ensis*	**Met e 1**	Tropomyosin	34	AAA60330	–	(Leung et al., 1994)
Northern shrimp	*Pandalus borealis*	**Pan b 1**	Tropomyosin	37	CBY17558	–	(Myrset et al., 2013)*
Spiny lobster	*Panulirus stimpsoni*	**Pan s 1**	Tropomyosin	34	AAC38996	–	(Leung et al., 1998b)
Brown shrimp	*Penaeus aztecus*	**Pen a 1**	Tropomyosin	36	AAZ76743	–	(Daul et al., 1994)
Indian prawn	*Penaeus indicus*	**Pen i 1**	Tropomyosin	34	–	–	(Shanti et al., 1993)

(*continued on next page*)

Table 3 (*continued*)

Crustacean species	Allergen	Protein type	MW	Genbank protein accession number of isoform		References
				.0101	.0102	
Black tiger prawn	**Pen m 1**	Tropomyosin	38	AAX37288.BAF47262, ADM34184	–	(Motoyama et al., 2007; Kamath et al., 2014a)*
	Pen m 2	Arginine kinase	40	AAO15713	–	(Yu et al., 2003)
	Pen m 3	Myosin light chain 2	–	ADV17342 ADM34185	–	(Kamath et al., 2014a)*
	Pen m 4	Sarcoplasmic calcium binding protein	–	ADV17343	–	–
	Pen m 6	Troponin C	–	ADV17344	–	(Kalyanasundaram and Santiago, 2015)*
Narrowclawed crayfish	**Pon l 4**	Sarcoplasmic calcium-binding protein	~24	P05946	–	–
	Pon l 7	Troponin I	~30	P05547	–	–
Blue swimmer crab	**Por p 1**	Tropomyosin	39	AGE44125	–	(Abramovitch et al., 2013)*

and snail as shown by RAST (Lopata et al., 1997).

Tropomyosins from molluscs share only 55–65% amino acid sequence identity with allergenic tropomyosins from crustacean, insects, mites, and fish (Fig. 4). However, it was demonstrated that tropomyosin is responsible for clinical cross-reactivity between molluscs, crustaceans, cockroach, and house dust mite (Leung et al., 1996; Bessot et al., 2010).

The other reported mollusc allergens have only been identified and characterised in abalone (paramyosin) (Carrillo et al., 1992; Jimenez et al., 2005; Martin-Garcia et al., 2007; Rodriguez-Del Rio et al., 2009) and octopus (arginine kinase and triosephosphate isomerase) (Shen et al., 2012; Yang et al., 2017) and the cross-reactivity of these allergens has only been investigated in crustaceans. However, a recent study demonstrated the presence of multiple allergens in Pacific oyster that are common in various allergen sources including fish, mites, pollen, plant, and fungi (Nugraha et al., 2018). This suggests the possibility of cross-reactivity between molluscs and other allergen sources.

4.4. Cross-reactivity between fish, crustacean, and mollusc

Recent studies on the identification and characterisation of novel allergens have identified a wide range of cross-reactive allergens. Interestingly, several allergens found independently in fish, crustacean or mollusc species originate from the same protein family or share a similar structure and function. Although there is lack of evidence proving their role in clinical cross-reactivity among fish, crustacean and mollusc species, this may be a potential cause for cross-reactivity between vertebrate and invertebrate species particularly with the highly conserved structures demonstrated for tropomyosins. Allergenicity and IgE recognition of tropomyosin has been well documented in crustacean, mollusc and fish (tilapia). A 2018 study reported the potential cross-reactivity of tropomyosin between shellfish and fish (Gonzalez-Fernandez et al., 2018). Serum from patients who have a history of adverse reactions to either shellfish or fish showed IgE-reactivity to cod and albacore tropomyosin. This highlights the need for further investigations to determine the potential of cross-reactivity across a wide range of vertebrate and invertebrate species. Triose-phosphate isomerase has been independently identified in all three groups. Aldolase A, β-enolase as well as vitellogenin have been identified in both fish and crustacean species, but further investigation is required to confirm potential IgE cross-reactivity.

5. Recombinant allergens

Fish parvalbumins and shellfish tropomyosins are the most studied seafood allergens and thus have been expressed as recombinant proteins in numerous studies. The plasmid construct is typically designed based on a published nucleotide sequence for an open reading frame, a cDNA sequence obtained from extracted total RNA or an amino acid sequence of the allergen retrieved from databases such as the NCBI GenBank and UniProt. The gene sequence is subsequently codon-optimised according to the expression host. The resulting gene sequence is usually flanked by two endonuclease (restriction enzyme) sites to facilitate cloning. An additional sequence is inserted, or often already available on the expression vector, for a hexa-histidine (His-)tag or glutathione S-transferase (GST-)tag on either the N- or C-terminus of the protein for purification by affinity chromatography (Sharp et al., 2014; Kumeta et al., 2017; Vologzhannikova et al., 2017). The pET vector system (Novagen) with a T7 or T7 *lac* promotor and His-tag was most commonly used for the expression of recombinant seafood allergens. The use of pET vectors is described in studies from 1999 through to 2017, suggesting that it is one of the most popular and possibly successful expression vectors. Another commonly used expression vector is the pGEX system (GE Healthcare), which incorporates a GST fusion tag. The *Escherichia coli* BL21 expression system is the dominant system to produce recombinant seafood allergens with the most

Classification	Species	Accesion number	#	Crustacean											Insect		Mite		Mollusc										Fish	Human
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Crustacean	Giant tiger prawn	A1KYZ2	1	100																										
	Brown shrimp	Q3Y8M6	2	100	100																									
	Whiteleg shrimp	B4YAH6	3	100	100	100																								
	Giant fresh water prawn	D3XNR9	4	98	98	98	100																							
	Blue swimmer crab	M1H607	5	98	98	98	97	100																						
	Mud crab	A7L5V2	6	93	93	93	92	92	100																					
	Snow crab	A2V735	7	89	89	89	89	88	95	100																				
	Common yabby crayfish	W5RFX0	8	98	98	98	98	99	93	89	100																			
	South African spiny lobster	K9UTQ8	9	94	94	94	94	94	96	92	94	100																		
	Red rock lobster	W5RFX7	10	95	95	95	95	95	92	88	95	93	100																	
	Flathead lobster	W5RFZ5	11	98	98	98	97	100	92	88	99	94	95	100																
Insect	American cockroach	Q9UB83	12	82	82	82	82	81	83	80	82	83	81	82	100															
	German cockroach	Q9NG56	13	82	82	82	82	82	83	80	83	84	82	82	97	100														
Mite	European house dust mite	O18416	14	80	80	80	80	80	82	78	80	82	79	80	80	80	100													
	American house dust mite	Q23939	15	82	82	82	82	81	83	80	82	84	81	81	81	81	98	100												
Mollusc	Bay scallop	A1KYZ4	16	56	56	56	57	56	57	56	57	58	58	56	56	55	58	60	100											
	Japanese scallop	Q9GUW9	17	60	60	60	61	60	61	60	60	62	61	60	59	58	60	62	88	100										
	Blue mussel	Q25457	18	57	57	57	57	56	57	56	57	58	58	57	57	57	55	57	71	71	100									
	Pacific oyster	B7XC66	19	62	62	62	62	61	62	60	62	63	62	62	61	60	60	62	74	75	79	100								
	Common octopus	Q2V0V0	20	64	64	64	64	63	64	63	64	65	64	64	62	62	63	65	71	74	71	76	100							
	Japanese flying squid	Q2V0V2	21	63	63	63	63	63	63	61	63	63	64	63	61	60	63	64	70	71	70	75	91	100						
	Golden cuttlefish	Q2V0V4	22	63	63	63	64	63	64	63	64	64	64	64	62	61	64	65	71	73	71	76	92	98	100					
	Brown garden snail	O97192	23	64	64	64	64	64	63	62	64	64	64	64	63	62	64	65	73	76	70	76	83	82	84	100				
	Horned turban	B7XC63	24	62	62	62	63	62	62	60	62	63	63	62	61	60	62	64	71	76	74	78	81	80	81	87	100			
	Disc abalone	B7XC62	25	63	63	63	64	63	63	61	63	64	64	63	62	62	63	64	71	77	75	79	83	81	81	87	97	100		
Fish	Mozambique tilapia	K4PEK4	26	57	57	57	58	57	58	58	57	60	58	57	54	54	57	58	52	54	52	55	56	55	55	55	55	55	100	
Human	Human	P06753	27	57	57	57	58	57	58	58	57	59	57	57	54	55	58	58	51	53	52	55	55	54	54	56	55	55	94	100

% identity

100 95 90 85 80 75 70 65 60 55 50

Fig. 4. Comparison of amino acid sequence identities (%) of tropomyosins. The sequence identities were calculated using Multiple Sequence Alignment in Clustal Omega (EMBL-EBI).

common subset strain being BL21(DE3). However, the users must be reminded that the proteins expressed in prokaryotic hosts do not undergo many of the post-translational modifications required for proteins originally from eukaryotic organisms to obtain their correct physiological structure and properties. Common modifications such as acetylation and phosphorylation of expressed proteins are achievable in some of the prokaryotic expression hosts (Cain et al., 2014). In the case of parvalbumin, there are no additional modifications besides the N-terminal acetylation (see Section 7.1), which negates the need for a more complex eukaryotic expression system.

5.1. Recombinant fish allergens

Successful expression and purification of recombinant parvalbumins has been demonstrated for several fish species including Atlantic cod (Van Do et al., 2003; Ma et al., 2008; Sanchez et al., 2016), Atlantic salmon (Van Do et al., 1999), barramundi (Sharp et al., 2014), carp (Swoboda et al., 2002b; Ma et al., 2008; Zuidmeer-Jongejan et al., 2015), Pacific mackerel (Hamada et al., 2004; Kumeta et al., 2017), and wolf-herring fish (Mohammadi et al., 2017), as summarised in Table 5. Recombinant fish parvalbumins have been expressed to explore potential use in immunotherapy (Swoboda et al., 2007) and treatment (Freidl et al., 2017) of fish allergy. In addition, the recombinant parvalbumins were used in studies investigating IgE-binding and differences in secondary structures between the natural and recombinant forms of the protein (Ma et al., 2008), characterisation of isovariants of parvalbumin (Sharp et al., 2014), and determination of tertiary structure by X-ray diffraction crystallography (Cates et al., 1999) and nuclear magnetic resonance (NMR) (Kumeta et al., 2017). The introduction of mutations in the nucleotide sequence can result in the production of hypoallergenic or mutated forms of parvalbumin, which are explored in the aforementioned studies. Parvalbumin requires the presence of Ca^{2+} to obtain the correct physiological protein conformation (refer to Section 8.1 for details). The recombinant parvalbumin must be introduced to Ca^{2+}-supplemented buffer post-purification to avoid a conformation similar to that of Ca^{2+}-depleted parvalbumin. The natural parvalbumin purified from muscle tissue can however retain the bound Ca^{2+} despite undergoing the purification process (Ma et al., 2008). The IgE binding of recombinant parvalbumins was often found to be comparable to that of the natural form (Ma et al., 2008; Sharp et al., 2014).

Recombinant tropomyosin has been expressed for Atlantic salmon (Jackman et al., 1996), however, this was used in a study unrelated to allergy. Other fish allergens such as β-enolase, aldolase A, collagen, and vitellogenin are yet to be produced as recombinant proteins (March 2018).

5.2. Recombinant crustacean allergens

Crustacean allergens have been recombinantly expressed and purified for several research purposes. The focus of recombinant crustacean allergen studies have been the discovery and characterisation of novel major allergens from previous uncharacterised species (Reese et al., 2005; Motoyama et al., 2007; Liang et al., 2008; Nakano et al., 2008; Albrecht et al., 2009; Kunimoto et al., 2009; Bauermeister et al., 2011; Abramovitch et al., 2013; Myrset et al., 2013). In addition, recombinant crustacean allergens have also been produced for IgE epitope mapping studies, which are primarily focused on the immunological characterisation of major allergens. These recombinant allergens have also been investigated for the development of novel allergy-diagnostic platforms for accurate quantification of allergen-specific IgE, as well as improving *in-vitro* diagnostics to better predict the severity of allergy to crustaceans, and to avoid risky food challenges (Albrecht et al., 2009; Johnston et al., 2014; Koeberl et al., 2014; Pascal et al., 2015). Studies investigating the effect of heat processing on major crustacean allergens, allergen detection (Kamath et al., 2014b), therapeutic research (Wai et al., 2014, 2016; Leung et al., 2017), and immune mechanisms of crustacean allergy (Leung et al., 2008; Lam et al., 2015; Mahajan et al., 2017) have also made use of recombinant allergens. Tropomyosin is the major crustacean allergen that has been widely expressed as a recombinant protein. Recombinant tropomyosins from species such as black tiger prawn (*Penaeus monodon*) (Pen m 1), Brown shrimp (*Penaeus aztecus*) (Pen a 1) and white-leg shrimp (*Litopenaeus vannamei*) (Lit v 1) have been commonly used for the above-mentioned research. However, arginine kinase, myosin light chain, sarcoplasmic calcium-binding protein, troponin C and triose-phosphate isomerase have also been produced as recombinant proteins primarily for novel allergen characterisation purposes (Ayuso et al., 2008, 2009; Bauermeister et al.,

Table 4
WHO/IUIS registered mollusc allergens. Mollusc allergens registered with the WHO/IUIS (www.allergen.org) are listed in alphabetical order of given allergen names. Note: '*' reference is not directly linked to the WHO/IUIS allergen entry. MW = molecular weight in kDa and '-' = no details available.

Mollusc species		Allergen	Protein type	MW	Genbank protein accession number of isoform		References
					1.0101	1.0102	
Pacific Oyster	*Crassostrea gigas*	**Cra g 1**	Tropomyosin	38	KY549366.1	AB444943.1	(Nugraha et al., 2018)*
Jade tiger abalone	*Haliotis laevigata x H. rubra*	**Hal l 1**	Tropomyosin	33	KX961689	–	–
Abalone	*Haliotis midae*	**Hal m 1**	Unknown	49	–	–	(Lopata et al., 1997)
Brown garden snail	*Helix aspersa*	**Hel as 1**	Tropomyosin	36	Y14855	–	(Asturias et al., 2002)
Sydney Rock Oyster	*Saccostrea glomerata*	**Sac g 1**	Tropomyosin	38	MF996471	–	–
Japanese flying squid	*Todarodes pacificus*	**Tod p 1**	Tropomyosin	38	–	–	(Miyazawa et al., 1996)

2011; Shen et al., 2011; Pascal et al., 2015) (Table 6). Recombinant crustacean allergens have been most commonly expressed in an *E. coli* expression system, while other expression systems such as yeast (*Pichia pastoris*) have also been used (Kumjim et al., 2016).

5.3. Recombinant mollusc allergens

Recombinant tropomyosins from different mollusc species have been expressed in *E. coli* (Table 7). Tropomyosins from abalone (*Haliotis diversicolor*), scallop (*Chlamys nobilis*), and mussel (*Perna viridsi*) were the first recombinantly expressed mollusc allergens reported (Chu et al., 2000). Subsequently, allergenic tropomyosins from three additional mollusc species were produced over the last 17 years. Besides tropomyosin, only allergenic paramyosin and triosephosphate isomerase from abalone (Suzuki et al., 2014) and octopus (Yang et al., 2017), respectively, were recombinantly expressed. Notably, a high salt concentration of 500 mM was needed to purify recombinant paramyosin from the crude extract. All recombinant mollusc allergens have solely been utilised for investigating their IgE reactivity.

6. Purified natural allergens

Purifying naturally occurring allergens in their native form is essential for the study of food allergy. The most commonly used method to extract soluble seafood allergens from the muscle tissue is by mechanical homogenisation in isotonic buffer (*i.e.* PBS and Tris-HCl). The homogenate can be left in the isotonic buffer from several hours to overnight at 4°C with gentle shaking to allow leaching of soluble proteins, which are subsequently separated from the insoluble proteins by a centrifugation step typically ranging from 5000 to 20,000 × *g* (Sharp et al., 2015; Kobayashi et al., 2016d; Ruethers et al., 2018). The use of protease inhibitors is often not necessary as most food allergens are resistant to proteolytic digestion (Moreno, 2007); however, it can be supplemented in the extraction buffer for a comprehensive protein characterisation (Vologzhannikova et al., 2017) and the identification of isovariants of the allergen (Brownridge et al., 2009). The muscle tissue, homogenate or the crude extract of soluble proteins can be heated or boiled for several minutes before the purification of heat-stable allergens, such as parvalbumin (Sharp et al., 2015; Kobayashi et al., 2016d) and tropomyosin (Kamath et al., 2014a). Due to their thermostability, the boiling step allows the preparation of a fraction highly enriched with these heat-stable proteins (Saptarshi et al., 2014; Vologzhannikova et al., 2017; Ruethers et al., 2018). Natural allergens are often successfully purified by chromatography. Several methods have been reported for seafood allergen purification, and the most commonly applied techniques are a combination of gel filtration (GF) (Kuehn et al., 2010; Kobayashi et al., 2016b; Freidl et al., 2017) and ion-exchange chromatography (IE) (Kuehn et al., 2010; Perez-Gordo et al., 2011). Purified allergens can be further refined by methods such as reversed-phase HPLC (Hamada et al., 2004; Guo et al., 2012). The addition of ammonium sulfate to saturate a clarified extract or fraction also allows the purification of allergen by precipitation of contaminating proteins (Bugajska-Schretter et al., 2000; Beale et al., 2009; Abdel Rahman et al., 2011; Ruethers et al., 2018).

6.1. Purified natural ***fish allergens***

The most commonly reported fish allergen purified from muscle tissues is parvalbumin. Parvalbumin has been purified from different fish species by ammonium sulfate precipitation and/or sequential IE and GF chromatography. Some of the extraction sources include: Atlantic cod (de Jongh et al., 2011; Freidl et al., 2017), Atlantic mackerel (Kuehn et al., 2010), Atlantic salmon (Kuehn et al., 2010; Freidl et al., 2017), carp (Kuehn et al., 2010; Freidl et al., 2017), European anchovy (Beale et al., 2009), grass carp (Li et al., 2014), hake (Beale et al., 2009), herrings (Kuehn et al., 2010), Indian mackerel

(Ruethers et al., 2018), Japanese eel (Hamada et al., 2004), Pacific mackerel (Hamada et al., 2004; Guo et al., 2012; Freidl et al., 2017), trout (Kuehn et al., 2010), yellowfin tuna (Ebo et al., 2010; Kuehn et al., 2010), and other species extensively listed in Table 8. Purification of parvalbumin from carp semen has been reported (Dietrich et al., 2014); however not yet described for other fish species. Less frequently reported purified fish allergens are: tropomyosin from Mozambique tilapia (Liu et al., 2012), Atlantic salmon and several other species (Huang and Ochiai, 2005), aldolase A and β-enolase from Atlantic cod, Atlantic salmon and yellowfin tuna (Kuehn et al., 2013), and collagen from several fish species (Hamada et al., 2001, 2003). Purification of fish tropomyosin included IE and reversed-phase HPLC, while for purification of aldolase A and β-enolase a combination of IE and GF was used. Collagen or gelatin (hydrolysed collagen) can also be purified from fish skin, bone and scale (Sakaguchi et al., 2000; Koppelman et al., 2012; Pan et al., 2012; Gistelinck et al., 2016; Kobayashi et al., 2016b) and a more vigorous extraction method must be applied. It requires delipidation by alkali-treatment (*i.e.* NaOH), and de-calcification (*i.e.* EDTA) steps, and finally purification of the acid-soluble collagen in acetic acid (Gistelinck et al., 2016). Depending on the level of purity to be achieved, lipids are removed by butyl alcohol and pigments bleached by hydrogen peroxide (Anand et al., 2013).

6.2. Purified natural crustacean allergens

Many of the known crustacean allergens have been purified from natural sources, as listed in Table 9 (note: this list is not exhaustive). The most commonly purified allergens are tropomyosin and arginine kinase. Tropomyosin has been isolated from many shrimp and prawn species (Shiomi et al., 2008; Liu et al., 2011a; Wang et al., 2012; Gamez et al., 2015; Lasekan et al., 2017) as well as American lobster (Motoyama et al., 2007), while arginine kinase has been isolated from a few shrimp species (Yu et al., 2003; Garcia-Orozco et al., 2007; Chen et al., 2013b), mud crab (Shen et al., 2011) and horseshoe crab. MLC, SCBP and troponin have been purified from a limited number of crustacean species (Ojima et al., 1995; Chen et al., 2013a; Mita et al., 2013; Morii et al., 2013; Rohrback et al., 2015; Zhang et al., 2015; Hu et al., 2017). These crustacean allergens have been purified not only for studies in allergy but also for studies of the molecular function of muscle (tropomyosin, MLC and troponin) and mechanisms of energy transfer by phosphagen kinases. Purification methods for crustacean allergens have made use of standard procedures such as salting out, isoelectric precipitation and column chromatography. Additionally, as tropomyosin, MLC and SCBP are heat stable, purification often involves a heating step.

6.3. Purified natural **mollusc allergens**

Tropomyosin from seven mollusc species, arginine kinase and triosephosphate isomerase from octopus and paramyosin have been purified for allergy studies using a variety of multiple step purification systems (Table 10). After ammonium sulfate precipitation, the purity was improved using chromatography methods. Only one of the respective studies mentions a yield: 33 mg of tropomyosin was purified from 44 g of muscle tissue from the brown garden snail (Asturias et al., 2002).

7. Post-translational modifications

Post-translational modifications of known seafood allergens are not very common. The few known modifications are described below.

7.1. Post-translational modifications of **fish allergens**

Parvalbumin, tropomyosin, β-enolase and aldolase A have been described as official WHO/IUIS-registered fish allergens. The homologous forms of all four proteins have been extensively studied as they are commonly and ubiquitously expressed within other species including human. Despite this, parvalbumin is the only fish allergen elucidated for post-translational modifications. The only described modification for fish parvalbumin is the alpha-N-terminal acetylation (Lee et al., 2009; Dietrich et al., 2014), and occurs on both the α- and β-parvalbumins (Permyakov et al., 2012). Mass spectrometry (MS) is often utilised in analysing the protein moieties. The addition of an acetyl group on the N-terminal region on the majority of the purified natural Gad m 1 was observed by ESI-QTOF intact MS analysis (Ma et al., 2008). Furthermore, the N-terminal alanine was found to undergo the modification, confirmed by LC—MS/MS using tryptic-digested peptides. The α-parvalbumin from human and β-parvalbumin from rat were subjected to ESI-MS and determined to have no additional modification (Fohr et al., 1993).

The presence of post-translational modifications on several fish tropomyosins was suggested by MALDI-TOF MS analysis based on the observed mass differences to the theoretical mass (Huang and Ochiai, 2005). There is no information on the exact modifications that cause these mass differences of fish tropomyosins; however, it is speculated that they undergo modifications other than N-terminal acetylation commonly found on tropomyosin from other species.

α- and β-enolases and aldolase A from non-fish species undergo acetylation, phosphorylation and methylation (Freemont et al., 1988; Merkulova et al., 1997). The only study describing cod, salmon and

Table 5
Recombinant allergenic parvalbumins in the literature. A summary of recombinant fish allergens and expression methods are listed. The WHO/IUIS name and accession number to either the nucleotide or amino acid sequence are provided where available. Note: '-' = no details available.

Fish species	Allergen	Accession number	Expression vector	Expression system	References
Atlantic cod	**Gad m 1.01, Gad m 1.02**	A51874 AM497927 AM497928	pET-15b; pET-17b; pET-19b	*E. coli* BL21(DE3); BL21(DE3)-RIPL; BL21Star(DE3)pLysS	(Van Do et al., 2003; Ma et al., 2008; Sanchez et al., 2016)
Atlantic salmon	**Sal s 1**	X97825	pET-19b	*E. coli* BL21(DE3)pLysS	(Van Do et al., 1999)
Barramundi	**Lat c 1.01, Lat c 1.01**	KF021278.1 KF021279.1	pPROEX HTb	*E. coli* NM522	(Sharp et al., 2014)
Common carp	**Cyp c 1.01, Cyp c 1.02**	AJ292211 AJ2922212	pET-28b+; pET-17b	*E. coli* BL21(DE3); BL21(DE3)-RIPL	(Swoboda et al., 2002a; Ma et al., 2008; Zuidmeer-Jongejan et al., 2015)
Pacific mackerel	–	P59747	pGEX-6p-1, pGEX-6p-3	*E. coli* BL21(DE3), *E. coli* JM 109	(Hamada et al., 2004; Kumeta et al., 2017)
Wolf-herring fish	–	Primers designed based on JN190495	pET-28a	*E. coli* BL21	(Mohammadi et al., 2017)

Table 6
Recombinant WHO/IUIS-registered and reported crustacean allergens in the literature. A summary of recombinant crustacean allergens and expression methods are listed. The WHO/IUIS name and accession number to either the nucleotide or amino acid sequence are provided where available. Note: '-' = no details available.

Protein	Crustacean species	Allergen	Accession number	Expression vector	Expression system	References
Tropomyosin	**Kuruma prawn**	–	BAF47263	pGEM-T	*E. coli* BL21	(Kunimoto et al., 2009)
	Northern shrimp	**Pan b 1**	FR728681	pET19b	*E. coli* Rosetta2 (DE3)	(Myrset et al., 2013)
	Krill	–	AB289604	pCold IV	*E. coli*	(Nakano et al., 2008)
	Chinese mitten crab	–	EF471314	pGEX-4	*E. coli* JM109	(Liang et al., 2008)
	Brown shrimp	**Pen a 1**	DQ151457	pET101	*E. coli* BL21 (DE3)	(Reese et al., 2005)
	North sea shrimp	**Cra c 1**	FJ457621	pET23b	*E. coli* BL21	(Bauermeister et al., 2011)
	Blue swimmer crab	**Por p 1**	JX874982	pProEXHT-B	*E. coli* NM522	(Abramovitch et al., 2013)
	King prawn	**Mel l 1**	JX171685	pProEXHT-B	*E. coli* BL21 (DE3) RIPL	(Koeberl et al., 2014)
	Black tiger prawn	**Pen m 1**	HM486525	pRSET-A	*E. coli* BL21 (DE3)	(Kamath et al., 2014a)
	Giant freshwater shrimp	**Mac r 1**	GU369816	pPICZαA	*Yeast Pichia strain KM71*	(Kumjim et al., 2016)
	Flowery crab	**Cha f 1**	AF061783	pGEX 1	*E. coli*	(Leung et al., 1998b)
Arginine kinase	**North sea shrimp**	**Cra c 2**	FJ457622	pET23b	*E. coli* BL21	(Bauermeister et al., 2011)
	Mud crab	–	GQ851626	pGEX-4T-3	*E. coli* BL21	(Shen et al., 2011)
	White-leg shrimp	**Lit v 2**	ABY57915	pGEX-4T-2	*E. coli* BL21(DE3) codon plus	(Yao et al., 2009)
	Greasyback shrimp	–	EU497674	pET28a	*E. coli* Rosetta	(Wang et al., 2009)
Myosin light chain	**White-leg shrimp**	**Lit v 3**	EU449515	pET24b	*E. coli* BL21	(Ayuso et al., 2008)
	North sea shrimp	**Cra c 5**	FJ462739	pET23b	*E. coli* BL21	(Bauermeister et al., 2011)
Sarcoplasmic calcium-binding protein	**White-leg shrimp**	**Lit v 4**	FJ184279	pET24b	*E. coli* BL21	(Ayuso et al., 2009)
	Black tiger prawn	**Pen m 4**	ADV17343	pFN6A (HQ) Flexi	*E. coli* KRX	(Mita et al., 2013)
	North sea shrimp	**Cra c 4**	FJ462737	pET23b	*E. coli* BL21	(Bauermeister et al., 2011)
	Red swamp crayfish	–	AEG79568.1, AEG79569.1, AEG79567.1	pET21b	*E. coli* BL21(DE3)	(Rohrback et al., 2015)
	Mud crab	–	AFJ80778	pET22b	*E. coli* Rosetta	(Hu et al., 2017)
Troponin C	**Black tiger prawn**	**Pen m 6**	ADV17344	pET32a	*E. coli* BL21(DE3)	(Kalyanasundaram and Santiago, 2015)
	North Sea shrimp	**Cra c 6**	FJ462740	pET23b	*E. coli* BL21	(Bauermeister et al., 2011)
Triosephosphate isomerase	**North Sea shrimp**	**Cra c 8**	FJ462738	pET23b	*E. coli* BL21	(Bauermeister et al., 2011)
	White-leg shrimp	–	AFT92034.1	pJetExpress404	*E. coli* BL21(DE3)	(Lopez-Zavala et al., 2016)
Hemocyanin	**Chinese mitten crab**	–	AEG64817	pET28a	*E. coli* Rosetta (DE3)	(Zhang et al., 2016)

tuna β-enolases and aldolase A as fish allergens analysed these protein's moieties and reported no modifications; however, supplementary data was not provided (Kuehn et al., 2013).

7.2. *Post-translational modifications of* ***crustacean and mollusc allergens***

Little is known about the post-translational modification of most crustacean and mollusc allergens. The most extensively studied allergen in this area is hemocyanin. Hemocyanin from white-leg shrimp was shown to be heavily glycosylated and this may be variant specific. It is thought this glycosylation has a role in the immune response for the agglutination and clearance of bacteria (Zhang et al., 2017). Furthermore, cleavage of the C-terminal end of hemocyanin releases a fragment that has antimicrobial activity (Wen et al., 2016). A *Vibrio parahaemolyticus* resistant white-leg shrimp was found to have a 18.4 kDa truncated hemocyanin in its serum. A small antibacterial peptide of hemocyanin from freshwater crayfish (*Pacifastacus leniusculus*), hemocyanin, has also been discovered (Lee et al., 2003). In mollusc hemocyanin (*e.g.* some snails) a variety of N-glycans have been detected as well as the methylation of hexoses, which is a novel modification

Table 7
Recombinant WHO/IUIS-registered and reported mollusc allergens in the literature. A summary of recombinant crustacean allergens and expression methods are listed. The WHO/IUIS name and accession number to either the nucleotide or amino acid sequence are provided where available. Note: '-' = no details available.

Protein	Mollusc species	Allergen	Accession number	Expression vector	Expression system	References
Tropomyosin	**Abalone**	–	X75218.1	pGEX	*E. coli* DH5α	(Chu et al., 2000)
	Scallop	–	AB021681.1	proEX HT	*E. coli* XL1-Blue	
	Mussel	–	AB000907.1			
	Pacific oyster	**Cra g 1**	KY549366.1, AB444943.1	pGEX4T 1-3	*E. coli* XL1-Blue	(Leung and Chu, 2001)
	Snail	**Hel as 1**	Y14855	pKN172	*E. coli* JM109 (DE3)	(Asturias et al., 2002)
	Chinese razor clam	–	EU082209	pET28a	*E. coli* BL21(DE3)	(Song et al., 2009)
Paramyosin	**Abalone**	–	AB571843	pFN6A (HQ) Flexi	*E. coli*	(Suzuki et al., 2014)
Triosephosphate isomerase	**Octopus**	–	KT007222	pET-28a	*E. coli* BL21(DE3)	(Yang et al., 2017)

(Staudacher et al., 2009).

Myosin lights chains are modified by phosphorylation and glycosylation. As part of the physiological function of myosins, the regulatory light chains need to be phosphorylated by myosin light-chain kinase. This is part of the muscle contractile action. MLC1 from red swamp crayfish has been shown to be a glycoprotein that contains 4.3% carbohydrates (Zhang et al., 2015). SCBP from red swamp crayfish was also identified as a glycoprotein, containing 4.7% carbohydrates (Chen et al., 2013b). No other modifications of MLC and SCBP have been reported.

Crustacean and mollusc tropomyosin have not been explored in detail for post-translational modifications; however, modifications in tropomyosins of other species (*e.g.* mammals and yeast) are described such as acetylation, phosphorylation, S-nitrosylation and disulfide bonding (Schevzov et al., 2011). One crustacean example is tropomyosin from American lobster. This species contains fast and slow tropomyosin isoforms and it is suggested that they are both generated as splicing variants of the same gene. Furthermore, N-terminal acetylation and another post-translational modifications are indicated due to differences in mass obtained by mass spectrometry (Mykles et al., 1998).

8. Available structures of seafood allergens

The determination of the tertiary structure of allergens leads to an understanding of a protein's behaviour but its foremost benefit is in the elucidation of potential IgE-binding regions. Several structures of seafood allergens have been characterised and are available through the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB) (www.rcsb.org). The following sections will introduce and discuss in detail, the features of published seafood allergen structures.

8.1. Available structures of *fish allergens*

Of the fish allergens, parvalbumin is the only protein studied for its tertiary structure. Parvalbumin consists of six α-helices (A–F) and short segments of antiparallel β-sheet, organised by the N-terminal AB domain and the C-terminal CD-EF domain. The metal ion-binding sites reside within the CD and EF loops, which are flanked by the corresponding helical elements. There are a total of 20 parvalbumin structures from six bony-fish species deposited in PDB (March 2018), which are summarised in Table 11. Most parvalbumin structures were solved by the X-ray power diffraction of crystalline parvalbumin (crystallography) and their data were often published in the 1990s, with the highest atomic resolution of 0.91 Å (Declercq et al., 1999). More recently published structures were determined by NMR and in particular, solution NMR could be used to determine epitopes important for IgE-binding (Kumeta et al., 2017). Although their allergenicity is yet to be determined, the only structure available for cartilaginous fish parvalbumin is α-parvalbumin (PDB: 5 PA L) from Leopard shark (*Triakis semifasciata*), which became available in 1993. In comparison to bony fish β-parvalbumins, α-parvalbumin differed in the C-terminal organisation due to an elongation induced by one additional amino acid residue despite the main structural features being conserved (Roquet et al., 1992).

Many of the purified natural and recombinant parvalbumins were found to form dimers and oligomers (van der Ventel et al., 2011; Sharp et al., 2014, 2015; Zuidmeer-Jongejan et al., 2015; Vologzhannikova et al., 2017). Four structures of common carp parvalbumin were resolved as homodimers (Table 11); however, none of the original studies further investigated the occurrence of dimerisation of parvalbumin. The transient dimers of allergens are speculated to have significantly higher allergenicity compared to the monomeric form (Rouvinen et al., 2010) due to feasible cross-linking between multiple IgEs bound on the surface of mast cells via Fc-epsilon receptors (FcεR), activating mast cell degranulation (Shakib et al., 2008). This has been observed for cow's milk allergen, Bos d 5 (Rouvinen et al., 2010), and more recently for Indian mackerel parvalbumin, Ras k 1. (Ruethers et al., 2018).

It is important to note that many of these parvalbumin structures were determined while bound to metal ions, particularly calcium or magnesium, regardless of the phylogenetic lineages. Parvalbumin is a calcium-binding protein and it cannot achieve its physiological conformation without the presence of divalent ions bound on the EF-hand domains (CD and EF sites) of the protein (Fig. 5A) (Declercq et al., 1999). It also affects the protein stability by significantly reducing the melting temperature and the reversibility of folding. Ca^{2+}-depleted parvalbumin prepared by EGTA-treatment is more prone to denaturation at a temperature as low as 30°C and completely loses the ability to refold after heat treatment (Bugajska-Schretter et al., 2000; Ma et al., 2008). Several studies using rat and chicken α- and β-parvalbumins elucidated the conformational change influenced by the removal of Ca^{2+}. The most prominent conformational alteration was observed on the CD-loop binding site of Ca^{2+}-depleted β-parvalbumin (Henzl and Tanner, 2007; Henzl et al., 2011), while a less extensive change was induced in β-parvalbumin.

The additional feature to be noted on parvalbumin structures is the presence of the acetyl group. One of the post-translational modifications known for parvalbumin is alpha-N-acetylation (N-terminal acetylation). The absence of N-terminal acetylation can drastically change the protein folding, degradability and increases the propensity of oligomerisation (Permyakov et al., 2012), and therefore almost all parvalbumin structures determined were acetylated.

Table 8
Natural WHO/IUIS-registered and reported fish allergens in the literature. The purified natural fish allergens from respective sources are summarised i with purification methods used. The WHO/IUIS name and accession number to either the nucleotide or amino acid sequence are provided where available. Note: ASP = ammonium sulfate precipitation, GF = gel filtration chromatography, HIC = hydrophobic interaction chromatography, IE = ion-exchange chromatography, P = precipitation, RP-HPLC = reversed-phase high-performance liquid chromatography and '-' = no details available. * gelatin is hydrolysed collagen.

Protein	Fish species	Allergen	Purification method	References
Parvalbumin	**Alaska pollack**	–	IE, GF, RP-HPLC	(de Jongh et al., 2011; Perez-Gordo et al., 2011; Freidl et al., 2017)
	Atlantic cod	**Gad m 1**	IE, GF; ASP, IE; IE	(Kuehn et al., 2010)
	Atlantic salmon	**Sal s 1**	IE, GF; IE	(Kuehn et al., 2010; Perez-Gordo et al., 2011; Kuehn et al., 2013)
	Bigeye tuna	–	GF, RP-HPLC	(Hamada et al., 2001, 2004)
	Common carp	**Cyp c 1**	ASP, IE	(Bugajska-Schretter et al., 2000; Kuehn et al., 2010; Freidl et al., 2017)
	Crimson sea bream	–	GF, RP-HPLC	(Guo et al., 2012)
	European anchovy	–	ASP, IE	(Beale et al., 2009)
	Grass carp	–	GF, HPLC	(Li et al., 2014)
	Hake	–	ASP, IE	(Beale et al., 2009)
	Herring	**Clu h 1**	IE, GF	(Kuehn et al., 2010)
	Horse mackerel	–	GF, RP-HPLC	(Hamada et al., 2004; Guo et al., 2012)
	Indian mackerel	**Ras k 1**	ASP	(Ruethers et al., 2018)
	Pacific mackerel	–	GF, RP-HPLC	(Hamada et al., 2004; Guo et al., 2012; Kobayashi et al., 2016b)
	Pangasius	–	IE, GF	(Ebo et al., 2010)
	Ocean perch	**Seb m 1**	IE, GF	(Kuehn et al., 2010)
	Rainbow trout	**Onc m 1**	IE, GF	(Kuehn et al., 2010)
	Whiff	**Lep w 1**	IE	(Perez-Gordo et al., 2011)
	Wolf-herring	–	IE	(Mohammadi et al., 2017)
	Yellowfin tuna	**Thu a 1**	IE, GF	(Ebo et al., 2010; Kuehn et al., 2010)
	Yellowtail	–	ASP, IE	(Beale et al., 2009)
β-enolase	**Atlantic cod**	**Gad m 2**	IE, GF	(Kuehn et al., 2013)
	Atlantic salmon	**Sal s 2**	IE, GF	
	Yellowfin tuna	**Thu a 2**	IE, GF	
Aldolase A	**Atlantic cod**	**Gad m 3**	IE, GF	
	Atlantic salmon	**Sal s 3**	IE, GF	
	Yellowfin tuna	**Thu a 3**	IE, GF	
Tropomyosin	**Mozambique tilapia**	**Ore m 4**	IE, RP-HPLC	(Liu et al., 2013)
Collagen	**Bigeye tuna**	–	Alkaline, Acid	(Hamada et al., 2001)
	Pacific mackerel	–	Alkaline, Acid	(Kobayashi et al., 2016b)
	Redbelly tilapia	–	Alkaline, butyl alcohol, Acid	(Pan et al., 2012)
Gelatin*	**Tuna**	–	Boil, Acid, Salt P	(Sakaguchi et al., 2000)
	Mackerel	–	Boil, Acid, Salt P	
	Salmon	–	Boil, Acid, Salt P	
	Saurel	–	Boil, Acid, Salt P	

The structures of other fish allergens are yet to be determined. The crystal structures of aldolase, β-enolase and collagen (potential allergen) from other species are available; hence those with the highest sequence identity to available fish allergen sequences are depicted as representative proteins to show the morphology in Fig. 5. The structure of tropomyosin is well conserved across phylogenetically distant

Table 9
Natural WHO/IUIS-registered and reported crustacean allergens in the literature. The purified natural crustacean allergens from respective sources are summarised with purification methods used. The WHO/IUIS name and accession number to either the nucleotide or amino acid sequence are provided where available. Note: ASP = ammonium sulfate precipitation, GF = gel filtration chromatography, IE = ion-exchange chromatography, IEP = isoelectric precipitation, RP-HPLC = reversed-phase high-performance liquid chromatography and '-' = no details available.

Protein	Crustacean species	Allergen	Purification method	References
Tropomyosin	**Black tiger prawn**	**Pen m 1**	ASP, IEP, RP-HPLC; IEP, ASP	(Shiomi et al., 2008; Wang et al., 2012; Lasekan et al., 2017)
	Kuruma prawn	–	IEP, ASP, IE	(Usui et al., 2013)
	White-leg shrimp	**Lit v 1**	IEP, ASP	(Liu et al., 2011a)
	Razor mud shrimp	–	IEP, ASP	(Gamez et al., 2015)
	American lobster	**Hom a 1**	IEP, ASP, RP-HPLC	(Motoyama et al., 2007)
Arginine kinase	**Red swamp crayfish**	–	ASP, IE	(Chen et al., 2013b)
	Mud crab	–	ASP, IE	(Shen et al., 2011)
	Black tiger prawn	**Pen m 2**	ASP, IE	(Yu et al., 2003)
	Craw fish	–	ASP, IE	(Yu et al., 2003)
	White-leg shrimp	**Lit v 2**	ASP, GF, IE	(Garcia-Orozco et al., 2007)
Myosin light chain	**Red swamp crayfish**	–	IEP, ASP, IE, GF	(Zhang et al., 2015)
Sarcoplasmic calcium-binding protein	**Red swamp crayfish**	–	ASP, IE, GF; GF	(Chen et al., 2013a; Rohrback et al., 2015)
	Black tiger prawn	**Pen m 4**	ASP, RP-HPLC	(Mita et al., 2013; Morii et al., 2013)
	Mud crab	–	ASP, IE, GF	(Hu et al., 2017)
Troponin	**Kuruma prawn**	–	ASP, IEP, ASP, IE	(Ojima et al., 1995)

species; hence, pig tropomyosin is depicted in Fig. 6A.

8.2. *Available structures of **shellfish allergens***

Structural data is available for three of the shellfish allergens, arginine kinase, triosephosphate isomerase and hemocyanin, all resolved by X-ray crystallography (summarised in Table 12; images in Fig. 6). Arginine kinase structures are available from horseshoe crab and white-leg shrimp (Fig. 6B) and depict structures in the substrate-free open state (Niu et al., 2011; Lopez-Zavala et al., 2012) and in complex with ligands (closed state), including structures of the transition state. (Lopez-Zavala et al., 2013). These different structures have helped in elucidating the conformational changes that occur to allow the transfer of phosphates. Arginine kinase is mainly α-helical and contains two domains; a small N-terminal domain and a larger C-terminal domain, which both close upon substrate binding (substrate induced fit) (Yousef et al., 2003; Niu et al., 2011; Lopez-Zavala et al., 2012). An Mg^{2+} ion is bound adjacent to the active site to facilitate the binding of ATP.

Table 10
Natural WHO/IUIS-registered and reported mollusc allergens in the literature. The purified natural crustacean allergens from respective sources are summarised with purification methods used. The WHO/IUIS name and accession number to either the nucleotide or amino acid sequence are provided where available. Note: AP = acetone precipitation, ASP = ammonium sulfate precipitation, GF = gel filtration chromatography, HIC = hydrophobic interaction chromatography, IE = ion-exchange chromatography, IEP = isoelectric precipitation, P = precipitation, RP-HPLC = reversed-phase high-performance liquid chromatography, UF = ultrafiltration and '-' = no details available.

Protein	Mollusc species	Allergen	Purification method	References
Tropomyosin	**Squid**	**Tod p 1**	ASP, IE, GF	(Miyazawa et al., 1996; Nakamura et al., 2006; Jin et al., 2015)
	Pacific oyster	**Cra g 1**	GF, IE, RP-HPLC	(Ishikawa et al., 1997; Ishikawa et al., 1998b)
	Turbo cornutus	–	GF, IE, RP-HPLC	(Ishikawa et al., 1998a)
	Octopus	–	IE, RP-HPLC	(Ishikawa et al., 2001)
	Scallop	–	ASP, IE, UF	(Nakamura et al., 2005)
	Japanese flying squid	–	ASP, IEP, RP-HPLC	(Emoto et al., 2009)
	Snail	**Hel as 1**	AP, IEP, IE	(Asturias et al., 2002)
Arginine kinase	**Octopus**	–	ASP, IE, GF	(Chen et al., 2013b)
Paramyosin	**Abalone**	–	ASP, IE	(Suzuki et al., 2011, 2014)
Triosephosphate isomerase	**Octopus**	–	ASP, IE, GF	(Yang et al., 2017)

Table 11
Available structures of bony fish parvalbumins. Crystal- or NMR-defined structures of bony-fish parvalbumins currently available (March 2018) in the Protein Data Bank (PDB - www.rcsb.org) are summarised. Information is given for the structural resolving method, resolution and special features for each of the structures. Note: Ca^{2+} = calcium, Cd^{2+} = cadmium, Mg^{2+} = magnesium, NH_4^+ = ammonium, Mn^{2+} = manganese, calc = calculation, sub = submitted and '-' = no details available.

Fish species	Allergen	PDB accession	Resolving method	Resolution	Features to note	Year of release	References
Atlantic cod	Gad m 1	2MBX	Solution NMR	200 conformers calc. 20 conformers sub.	Ca^{2+}-bound	2014	(Moraes et al., 2014)
Common carp	Cyp c 1	1B8C	X-ray diffraction	2 Å	Mutant, homodimer, Mg^{2+}-bound	1999	(Cates et al., 1999)
		1B8L		1.7 Å	Triple mutant		
		1B8R		1.9 Å	Recombinant (*E. coli*), Ca^{2+}-bound		
		1B9A		2.0 Å	Mutant, Recombinant (*E. coli*), Ca^{2+}-bound		
		4CPV		1.5 Å	Homodimer	1990	(Kumar et al., 1990)
		1CDP		1.6 Å	Homodimer, Cd^{2+}-bound		(Swain et al., 1989)
		5CPV		1.6 Å	Homodimer, Ca^{2+}-bound		
Northern pike	–	1 PA L	X-ray diffraction	1.65 Å	Ca^{2+}-bound, NH_4^+-bound	1990	(Declercq et al., 1991)
		2 PA L		1.8 Å	Mn^{2+}-bound		
		3 PA L		2.4 Å	Ca^{2+}-bound, Mg^{2+}-bound		
		4 PA L		1.8 Å	Ca^{2+}-bound, Mg^{2+}-bound		
		2PAS	Solution NMR	9 conformers sub.	Ca^{2+}-bound	1994	–
		3PAT		1 conformer sub.	Ca^{2+}-bound	1994	–
		1PVA	X-ray diffraction	1.65 Å	α-parvalbumin, Ca^{2+}-bound	1995	–
		1PVB		1.7 Å	β-parvalbumin, Ca^{2+}-bound, NH_4^+-bound		(Declercq et al., 1996)
		2PVB		0.91 Å	Ca^{2+}-bound, NH_4^+-bound, formic acid-bound	1998	(Declercq et al., 1999)
Pacific mackerel	–	5XND	Solution NMR	100 conformers calc. 20 conformers sub.	Recombinant (*E. coli*), Ca^{2+}-bound	2017	(Kumeta et al., 2017)
Silver hake	–	1BU3	X-ray diffraction	1.65 Å	Ca^{2+}-bound	1998	(Richardson et al., 2000)
Whiting	–	1A75	X-ray diffraction	1.9 Å	Ca^{2+}-bound	1998	–

The available structure for triosephosphate isomerase is from white-leg shrimp (Fig. 6F). The enzyme is a dimer with the catalytic site situated at the interface of the dimers. There are four catalytic residues and all are contained on one of the subunits. It has an extremely fast reaction mechanism and requires no additional cofactors. The enzyme is referred to as 'evolutionarily perfect' as its rate is only limited by substrate diffusion (Wierenga et al., 2010; Lopez-Zavala et al., 2016).

The hemocyanin structure has been resolved from Californian spiny lobster (*Panulirus interruptus*) (Volbeda and Hol, 1989) (Fig. 6C). It is a hexamer that is formed by three tight dimers coming together. The centre of the hexamer is lined with residues that are conserved among arthropods. There are two copper binding sites at the centre that bind one oxygen.

The other shellfish allergens do not have structures available so in lieu of this we can look at the structures from other species to get an idea of what they might look like. Species were chosen based on the highest sequence identity from available data on the PDB. Tropomyosin (Fig. 6A, wild boar, *Sus scrofa*, PDB: 1C1G), an actin-binding protein, forms a stable coiled-coil dimer due to a combination of the hydrophobic and ionic interactions of tropomyosins' unique amino acid pattern. This unbroken pattern is a heptad repeat and follows a similar pattern to: H-P-P-H-C-P-C, where H is hydrophobic, P is polar and C is charged. According to the structure from the wild boar, tropomyosin has a length of 420 Å, appropriate for it to span seven actin monomers (Whitby and Phillips, 2000).

Other proteins shown are MLC from blood fluke (Fig. 6C, *Schistosoma mansoni*, PDB: 3JAX (Sulbaran et al., 2015)), SCBP from lancelet (Fig. 6D, Amphioxus, PDB: 2SAS (Cook et al., 1993)) and troponin C from giant water bug (Fig. 6E, *Lethocerus indicus*, PDB: 2JNF (De Nicola et al., 2007)).

9. Component-resolved diagnostics for seafood allergy

The wide diversity of seafood is poses a challenge for clinicians to identify the exact species and allergens involved in allergic reactions. As commercially available diagnostic methods do not reflect this diversity, avoidance of all seafood is often recommended (Lopata and Kamath, 2012). Current diagnosis of seafood allergy is based on clinical history, sensitisation tests such as allergen-specific IgE quantification, and SPT, as well as oral challenge tests if required. The SPT is usually

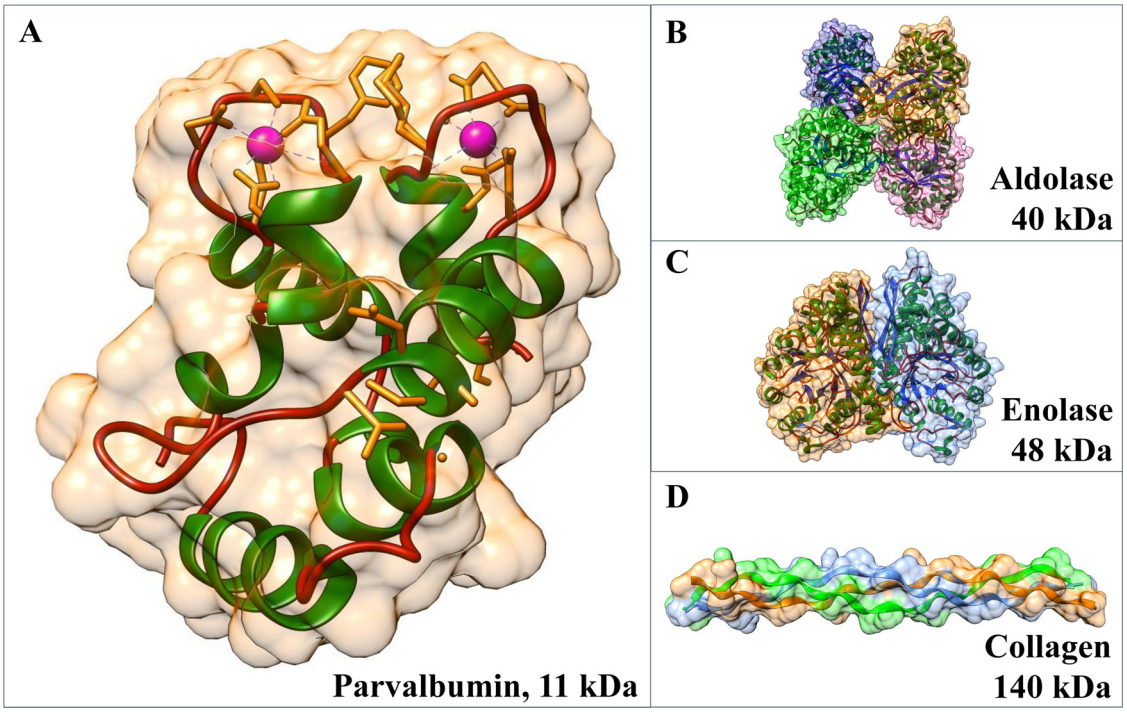

Fig. 5. Three-dimensional structural models of fish parvalbumin and representative proteins from non-fish species. Crystal structures of **A)** Parvalbumin from Northern pike (*Esox lucius*) (PDB: 2PVB) with two calcium ions (magenta) bound on the EF-hand domains, **B)** Fructose-1,6-(bis)phosphate aldolase (PDB: 1XFB) homotetramer from human, **C)** β-enolase (PDB:2XSX) homodimer from human, and **D)** Collagen-like peptides (PDB: 1CGD) shown in a triple helix conformation. The crystal structure of human brain fructose 1,6-(bis)phosphate Aldolase (C isozyme) (PDB: 1XFB) (Fig. 5B) was prepared based on a 56% identity match to Atlantic salmon fructose-bisphosphate aldolase A (UniProt: B5DGM7 ALDOA_SALSA), and human β-enolase (PDB: 2XSX) (Fig. 5C) was prepared based on an 85% identity match to Atlantic salmon β-enolase (UniProt: B5DGQ7 ENOB_SALSA). The homology search was conducted with Swissmodel (Arnold et al., 2006). The crystal structure of synthetic hydrated collagen peptides (PDB: 1CGD) (Fig. 5D) was chosen and prepared as it is the best representative exhibiting the unique triple helix of heterologous collagen chains. The α-helices are shown in green, β-sheets in blue and the loops in red. The protein surface is indicated in transparent colours, with each colour representing a monomer of the structure. The structures were built using UCSF Chimera 1.12 (Pettersen et al., 2004) and are not to scale (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 12
Available structures of crustacean allergens. Resolved crustacean allergen structures currently available (March 2018) in the Protein Data Bank (PDB - www.rcsb.org) are summarised. Information is given for the structural resolving method, resolution and special features for each of the structures. Note: ADP-Mg = adenosine-5′-diphosphate and magnesium ion, 2-PG = 2-phosphoglyceric acid, NO_3 = nitrate ion.

Allergen	Crustacean species	PDB accession	Resolution	Features to note	Year of release	References
Arginine kinase	White-leg shrimp	4BG4	1.6 Å	Ternary transition state analogue complex with arginine, ADP-Mg, NO_3	2013	(Lopez-Zavala et al., 2013)
		4BHL	1.9 Å	Binary complex with arginine		
		4AM1	1.25 Å	Unligated, monomeric	2012	(Lopez-Zavala et al., 2012)
Arginine kinase	Horseshoe crab	5J99, 5J9A	1.7 Å, 2.0 Å	Ambient temperature transition state	2016	(Godsey et al., 2016)
		4GVY, 4GVZ, 4GW0, 4GW2	2.1 Å, 3.0 Å, 2.5 Å, 2.2 Å	In complex with ADP, NO3 and various phosphagen analogs	2012	(Clark et al., 2012)
		3M10	1.7 Å	Substrate-free	2010	(Niu et al., 2011)
		1SD0	2.3 Å	Active site mutant	2004	(Gattis et al., 2004)
		1RL9	1.45 Å	Creatine-ADP ternary complex	2004	(Azzi et al., 2004)
		1P50, 1P52	2.8 Å, 1.9 Å	Transition state mutant	2003	(Pruett et al., 2003)
		1M15	1.2 Å	Transition state structure	2002	(Yousef et al., 2002)
		1BG0	1.9 Å	Transition state structure	1998	(Zhou et al., 1998)
Triosephosphate isomerase	White-leg shrimp	5EYW	1.7 Å	Homodimer, complexed with 2-PG inhibitor	2016	(Lopez-Zavala et al., 2016)

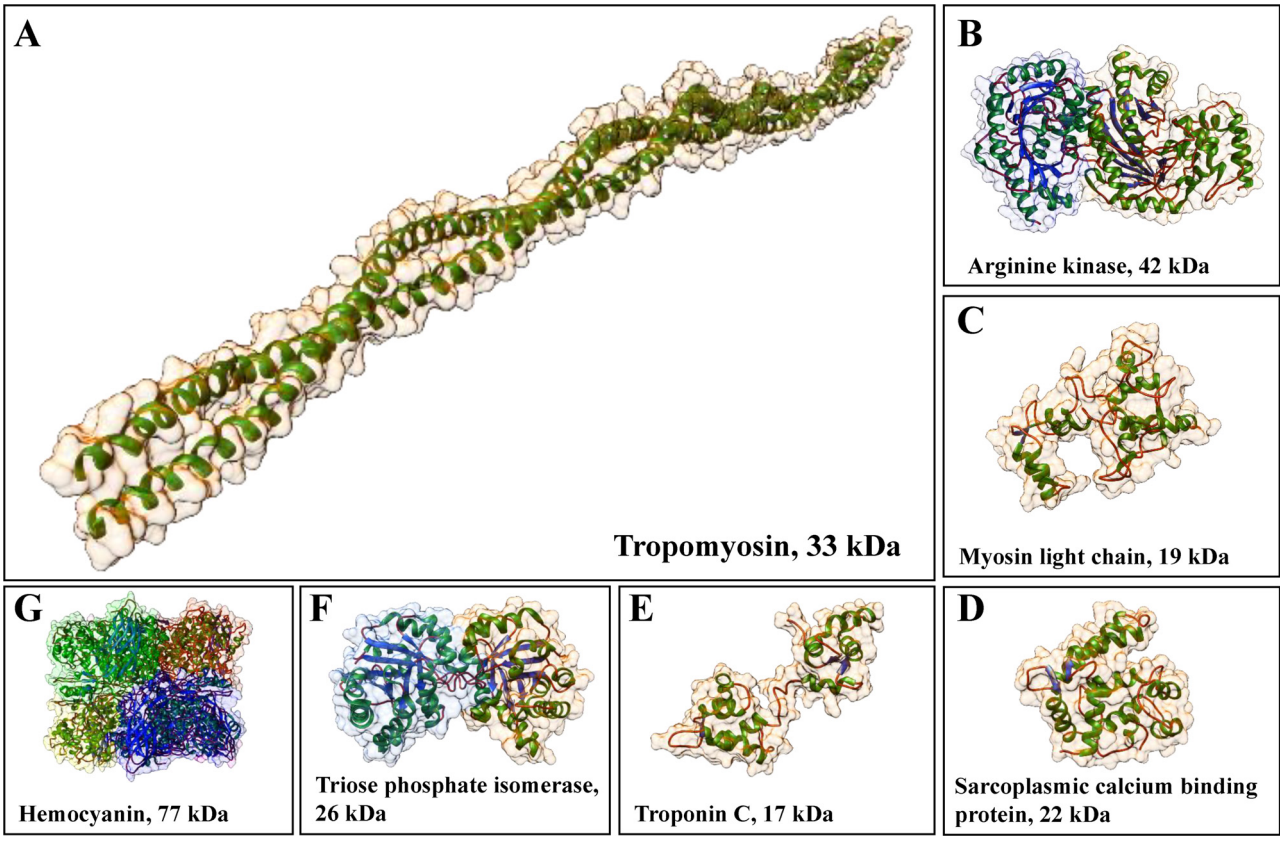

Fig. 6. Three-dimensional structural models based on existing crystal structures (RSCB Protein Data Bank) for major and minor shellfish allergens. The depicted models are based on crystal structures with the highest homology to prawn allergens (WHO/IUIS) predicted using the homology search function in Swissmodel (Arnold et al., 2006). The structures are shown in clockwise order following numeration of respective allergens by WHO/IUIS: **A)** Tropomyosin (PDB: 1C1G) dimer from pig, **B)** Arginine kinase (PDB: 4BG4) dimer from prawn, **C)** Myosin light chain (PDB: 3JAX) from blood fluke, **D)** Sarcoplasmic calcium binding protein (PDB: 2SAS) from lancelet, **E)** Troponin C (PDB: 2JNF) from waterbug, F) Triose phosphate isomerase (PDB: 5EYW) dimer from prawn, and **G)** Hemocyanin (PDB: 1HCY) hexamer from spiny lobster. The alpha-helices are shown in green, beta-sheets in blue and the loops in red. The protein surface is indicated in transparent colours, with each colour representing monomer of the structure. The structures were built using UCSF Chimera 1.12 (Pettersen et al., 2004) and are not to scale (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

performed with commercial preparations arriving from whole protein extracts with unknown protein composition, available from many different, often regional, manufacturers. In the case of patients with severe allergic reactions, *in vitro* IgE tests are preferred over SPT or oral food challenges to minimise the risk of severe allergic reactions.

Currently, *in vitro* IgE tests involve the use of whole natural extracts prepared from the offending source. The advantages of this approach are the ease of testing for sensitisation and the availability of commercial tests against a wide variety of seafood species on available platforms; for example, the ImmunoCAP® (Thermo Scientific), OPTIGEN®/CLA® (Hitachi Chemical Diagnostics), and Immulite® (Siemens) (Table 13). However, there are a few drawbacks. The use of natural extracts may not allow standardisation of allergen concentrations, and quantification of specific-IgE to less abundant allergens may not be accurate, leading to false-negative results. Current research is increasingly focused on the use of purified allergens for component-resolved diagnosis to overcome these drawbacks. The use of single allergens may improve the test sensitivity and specificity (markers of primary sensitisation and indicators of cross-reactivity) (Lopata et al., 2016). Developing component-resolved diagnosis for seafood allergy may require knowledge on the natural abundance of the allergens, its location in the organism, as well as its cross-reactive potential with other related allergen sources. Table 13 provides an overview of allergen sources and components available on the most common platforms for *in vitro* diagnostics. Notably, most of the species available for diagnostics are native to the Northern hemisphere. Species from the Southern Hemisphere seem to contain considerably different allergens and isoforms (Liang et al., 2017; Ruethers et al., 2018), posing a challenge for reliable diagnostics.

The commercially available component-resolved diagnostics ImmunoCAP® platforms allow the determination of the level of specific-IgE to recombinantly expressed parvalbumin from carp (rCyp c 1) and cod (rGad c 1). However, this does not represent the wide diversity of allergenic β-parvalbumins elaborated in Section 4.1. Cyp c 1 and Gad c 1 share a sequence identity of only 59–90 and 46–68%, respectively, with other WHO/IUIS-registered fish parvalbumins.

Due to the high abundance of tropomyosin in edible crustaceans and molluscs, specific-IgE to tropomyosin is easily detected with high sensitivity. rPen a 1 from brown shrimp (*P. aztecus*) on the ImmunoCAP® and rPen m 1 from black tiger prawn (*P. monodon*) on the Immulite® systems are commercially available for testing. Due to the highly cross-reactive nature of tropomyosin among crustaceans, molluscs, dust mites, and other invertebrate species, there might be an issue of false-positive detection. Testing against purified tropomyosin from diverse species may provide more information on the primary sensitiser and cross-reactivity to other seafood sources. Several studies have demonstrated the use of specific-IgE to tropomyosin to predict clinical reactivity to shrimps in preference to shrimp SPT and/or specific-IgE to whole shrimp extract (Yang et al., 2010; Gamez et al., 2011). In addition to tropomyosin (Pen m 1), other shrimp allergens such as arginine kinase (Pen m 2) and SCBP (Pen m 4) are available on the microarray platform called ImmunoCAP® ISAC (Thermo Scientific). Interestingly, a recent study has demonstrated the use of specific-IgE to tropomyosin and SCBP in predicting clinical reactivity to shrimps (Pascal et al.,

Table 13
Allergens commercially available for *in vitro* tests in March 2018. Allergens available for component-resolved diagnostics are either recombinantly expressed (r) or purified from natural sources (n). No mollusc allergens are available for component-resolved diagnostics. The allergens available for Siemens Immulite® have been retrieved from the 3gAllergy™ catalogue for liquid allergens. Note: PV = parvalbumin, TM = tropomyosin, AK = arginine kinase and SCBP = sarcoplasmic-binding protein.

Allergen sources available for seafood allergy diagnostics			
Platform	Fish	Crustacean	Mollusc
Phadia, ImmunoCAP® Rapid	**28** species	**5** species	**9** species
Siemens Immulite®	**18** species	**3** species	**8** species
Hitachi OPTIGEN®/CLA®	**3** species	**2** species	**1** species

Seafood allergens available for component-resolved diagnostics				
Platform	Fish		Crustacean	
	Allergen	Species	Allergen	Species
Phadia ImmunoCAP® Rapid	rCyp c 1 (PV)	Carp (*C. carpio*)	rPen a 1 (TM)	Brown shrimp (*P. aztecus*)
	rGad c 1 (PV)	Cod (*G. morhua*)		
Phadia, ImmunoCAP® ISAC	rGad c 1 (PV)	Cod (*G. callarias*)	nPen m 1 (TM)	Black tiger prawn (*P. monodon*)
			nPen m 2 (AK)	
			nPen m 4 (SCBP)	
Siemens Immulite®	Not available		rPen m 1 (TM)	Black tiger prawn (*P. monodon*)

2015). However, further investigation is required into the utility of specific-IgE quantification of one or more component allergens as biomarkers for clinical reactivity to crustaceans.

Currently, mollusc allergens have not been investigated for use in component-resolved diagnostics.

Detailed diagnostics are needed to specifically determine the often very narrow range of species a patient may react to, so that specific products can be avoided, improving the safety of the patient and eliminating the need to abstain from seafood entirely.

10. Therapeutic development for seafood allergy - vaccine candidates, hypoallergenic derivatives and clinical trials

Current treatment strategies for seafood allergy are mostly palliative in nature, along with complete avoidance of the offending source, underlining the need for labelling food and detecting seafood allergens. The major challenge in the development of seafood immunotherapy, using either whole extracts or natural purified allergens, is the risk of severe allergic reactions upon administration of the dose via oral, sublingual, intradermal or subcutaneous route in seafood allergic patients. Therapeutic strategies for seafood allergy, and in general for food allergy, are still in the developmental phase. Approaches for fish and shellfish allergy are fundamentally different and are discussed separately. To our best knowledge, all approaches for shellfish allergy are limited to crustaceans.

Alternative treatments of seafood allergy include the use of probiotics and Chinese herbal medicines as shown for shrimp (Schiavi et al., 2011) and fish (Srivastava et al., 2012) allergy in murine models. Interestingly, fish oil has been reported to be useful for allergy prevention (Calder, 2013).

10.1. Vaccine candidates, hypoallergenic derivatives and clinical trials for fish allergy

To date, the most developed immunotherapy for fish allergy is the use of hypoallergenic proteins for antibody binding to mimic their natural counterparts. The expression of recombinant Cyp c 1 that contained IgE epitopes from other fish species was first described in 2002 (Swoboda et al., 2002a). This was followed by a study in 2007, which expressed recombinant Cyp c 1 with mutations in either one or both of the CD- or EF-calcium-binding sites, demonstrating that these mutations could reduce the binding to patients IgE by as much as 95% (Swoboda et al., 2007). These mutations inhibited the association of Ca^{2+} due to the conformational alteration in the binding sites (Bugajska-Schretter et al., 2000), which resulted in the loss of the IgE epitope. The allergenicity of the protein was also diminished, confirmed by *in vivo* SPT and *in vitro* basophil histamine release, while maintaining its immunogenic capability to raise IgG to block the subsequently introduced parvalbumin from binding to IgE (Swoboda et al., 2007). The first comprehensive study using a murine model of fish allergy also demonstrated a reduction of the Th2-response by recombinant Cyp c 1 (van der Ventel et al., 2011). Since 2008, this approach using the mutated hypoallergenic parvalbumin has gained more attention and been further explored for its potential. The European Union-funded Food-Allergy Specific ImmunoTherapy (FAST) project is a multi-national, multi-institutional project that aims to combat food allergy by developing safe and efficacious allergen-specific subcutaneous immunotherapies using recombinant hypoallergenic proteins instead of food extracts. As a pre-clinical stage study, the efficacy and reduced allergenicity of recombinant mutated Cyp c 1 were tested *in vivo* using mice and rabbits, and *in vitro* using fish-allergic patients' peripheral blood mononuclear cells, and finally its safety confirmed according to OECD guidelines (Zuidmeer-Jongejan et al., 2015). The first-in-man subcutaneous immunotherapy showed a promising outcome and hence Phase I/IIa clinical trials were conducted with 15 participants in Denmark, 2013 (NCT number: NCT02017626), followed by Phase IIb clinical trial with 45 participants across six countries in 2015 (NCT number: NCT02382718).

More recently, the blocking antibodies produced by immunisation of a murine fish allergy model with the recombinant Cyp c 1 mutant was found to protect from allergic symptoms when used as a passive immunisation (Freidl et al., 2017). The only other candidature for

immunotherapy is based on parvalbumin-specific mimotopes, mimicking the epitope of the respective allergen (Untersmayr et al., 2006). However, to our best knowledge, this approach has not been investigated extensively.

*10.2. Vaccine candidates and hypoallergenic derivatives for **crustacean allergy***

Several studies have focused on the elucidation of B-cell and T-cell epitopes of crustacean allergens. Knowledge on these epitopes is central to the development of peptide- or protein-based vaccines which have abolished or reduced IgE reactivity, and hence no IgE-mediated side effects, but an ability to induce a strong T-cell response. B- and T-cell epitope studies and immunotherapy in animal models are mainly focused on shrimp tropomyosin due to its high clinical relevance. T-cell epitopes on shrimp tropomyosin have been elucidated and experimentally validated using computer algorithms (Subba Rao et al., 1998) or overlapping peptide libraries (Wang et al., 2012). T-cell reactivity has been extensively characterised for heated and raw crustacean species (Abramovitch et al., 2017). Hypoallergenic tropomyosin has been generated with reduced IgE binding through site-directed mutagenesis or truncation/removal of IgE binding epitopes (Reese et al., 2005; Wai et al., 2014), while leaving the T-cell epitopes intact, and tested for desensitising potential in a tropomyosin-sensitised mouse model. T-cell epitope-based peptides have also been investigated as novel immunotherapeutic candidates, demonstrating efficiency in reducing allergic responses towards shrimp tropomyosin in animal studies (Wai et al., 2016). In addition, it has been demonstrated that a low-dose administration of wild-type purified recombinant shrimp tropomyosin can favour a regulatory T-cell response in animal studies as compared to a high-dose regimen (Leung et al., 2017). The efficacy and therapeutic potential of recombinant wild-type and hypoallergenic crustacean allergens have yet to be investigated in clinical trials.

11. Future directions

The identification of allergy-inducing seafood species is often difficult due to the very limited availability of diagnostic methods, complicated by the large diversity of species and limited knowledge of allergens and cross-reactivities. To improve the diagnostics, and subsequent patient care and management, further research needs to be conducted. All clinically relevant allergens from a wide global diversity of species need to be identified and their cross-reactivities need to be investigated. With this gained knowledge, better component-resolved diagnosis based *in vitro* methods can be developed, allowing reliable and precise diagnostics, leading to urgently needed individual management and treatment, including component-resolved immunotherapy.

Recent advances in the field of genomics, bioinformatics, and proteomics have unlocked new opportunities for targeted high-throughput screening and identification of proteins based on amino acid sequence identities, resulting in direct comparative analysis of similar allergens from comprehensive allergen databases. Thus, the combinatorial approach using a specific organism's genome data and comparison with a growing number of comprehensive allergen databases, immune-reactivity, and high throughput protein sequence analysis can overcome the current limitations of allergen identification. This advanced, comprehensive strategy will allow for the development of more sensitive and specific molecular tools for the diagnosis and quantification of seafood allergens. Furthermore, epitope-specific binding, rather than allergen-specific IgE measurements, needs to be considered as it seems to better correlate with clinical reactivity. The current efforts in developing immunotherapeutic approaches for fish and shellfish allergy need to seriously consider the far greater molecular diversity of allergens compared to other food groups.

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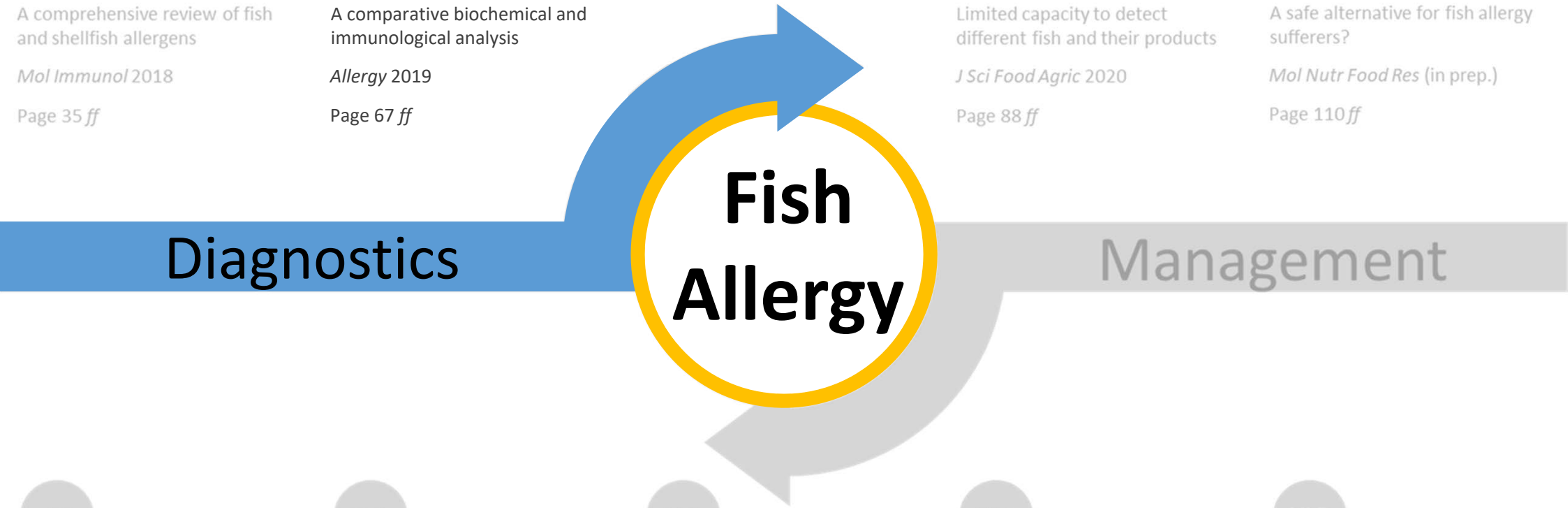

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Chapter II – Skin prick test preparations

Skin Prick Test preparations for fish allergy – A comparative biochemical and immunological analysis

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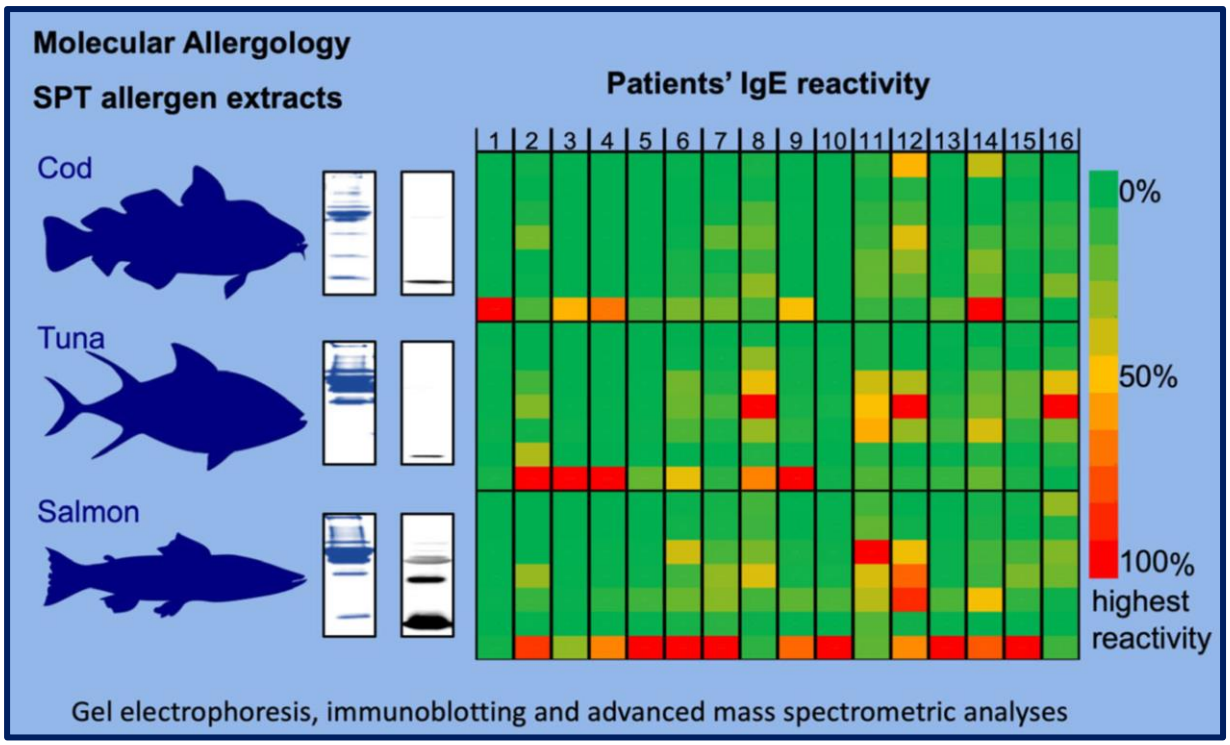

Graphical abstract

Contributions:

TR performed the study and wrote the manuscript. AL led the study. TR, AT, SK, SM, DC and AL designed the study. SM and DC recruited the patients and acquired the commercial fish extracts. TR, RN, TC, MK, SN, SO and NW contributed significantly to the generation of the data. All co-authors were substantially involved in the analyses and interpretation of all data and critically reviewed the manuscript.

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ORIGINAL ARTICLE

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Variability of allergens in commercial fish extracts for skin prick testing

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Abstract

Background: Commercial allergen extracts for allergy skin prick testing (SPT) are widely used for diagnosing fish allergy. However, there is currently no regulatory requirement for standardization of protein and allergen content, potentially impacting the diagnostic reliability of SPTs. We therefore sought to analyse commercial fish extracts for the presence and concentration of fish proteins and in vitro IgE reactivity using serum from fish-allergic patients.

Methods: Twenty-six commercial fish extracts from five different manufacturers were examined. The protein concentrations were determined, protein compositions analysed by mass spectrometry, followed by SDS-PAGE and subsequent immunoblotting with antibodies detecting 4 fish allergens (parvalbumin, tropomyosin, aldolase and collagen). IgE-reactive proteins were identified using serum from 16 children with confirmed IgE-mediated fish allergy, with focus on cod, tuna and salmon extracts.

Results: The total protein, allergen concentration and IgE reactivity of the commercial extracts varied over 10-fold between different manufacturers and fish species. The major fish allergen parvalbumin was not detected by immunoblotting in 6/26 extracts. In 7/12 extracts, five known fish allergens were detected by mass spectrometry. For cod and tuna, almost 70% of patients demonstrated the strongest IgE reactivity to collagen, tropomyosin, aldolase A or β-enolase but not parvalbumin.

Conclusions: Commercial fish extracts often contain insufficient amounts of important allergens including parvalbumin and collagen, resulting in low IgE reactivity. A comprehensive proteomic approach for the evaluation of SPT extracts for their utility in allergy diagnostics is presented. There is an urgent need for standardized allergen extracts, which will improve the diagnosis and management of fish allergy.

KEYWORDS
allergy diagnostics, fish allergy, IgE reactivity, parvalbumin, skin prick test

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Table S1: Details of analysed 26 Skin Prick Test fish extracts from 5 different manufacturers.

		Manufacturer				
		1 - Greer USA	2 - ALK-Abello USA	3 - Inmunotek	4 - Alyostal	5 - ALK-Abello Spain
Family/Tribe	Gadidae	**Codfish**	Atlantic **cod fish**	**Cod**	**Cod**	Atlantic **cod**
		Gadus spp.	*Gadus morhua*			*Gadus morhua*
	Thunnini	**Tuna**	**Tuna**	**Tuna**	**Tuna**	
		Thunnus sp.	*Thunnus spp.*			
	Salmonidae	**Salmon**	Atlantic **salmon**	**Salmon**		
		Salmo salar	*Salmo salar*			
	Pleuronectidae	**Flounder**	**Flounder** Atlantic **halibut**			
		Platichthys sp.	*Pleuronectes spp.* *Hippoglossus hippoglossus*			
	Scombrini Scomberomorini	**Mackerel**				Atlantic **mackerel**
		S. cavalla				*Scomber scombrus*
	Salmonidae	**Trout**				
		O. mykiss				
	Merlucciinae			**Hake**		
	Clupeidae					Atlantic **herring**
						Clupea harengus
	Ictaluridae	**Catfish**				
		Ictalurus spp.				
	Serranidae	**Bass** **Perch**				
		Centropristis striata *Serranus scriba*				
		Fish mix	**Fish, mixed**			**Fish mix II**
		Cod, flounder, halibut, mackerel, tuna	Flounder, cod, halibut			Cod, sole, sea bass, hake

Note: Common and scientific names of fish species are listed according to manufacturer's information where available. Classification of family/tribe is according to the UniProt database (www.uniprot.org).

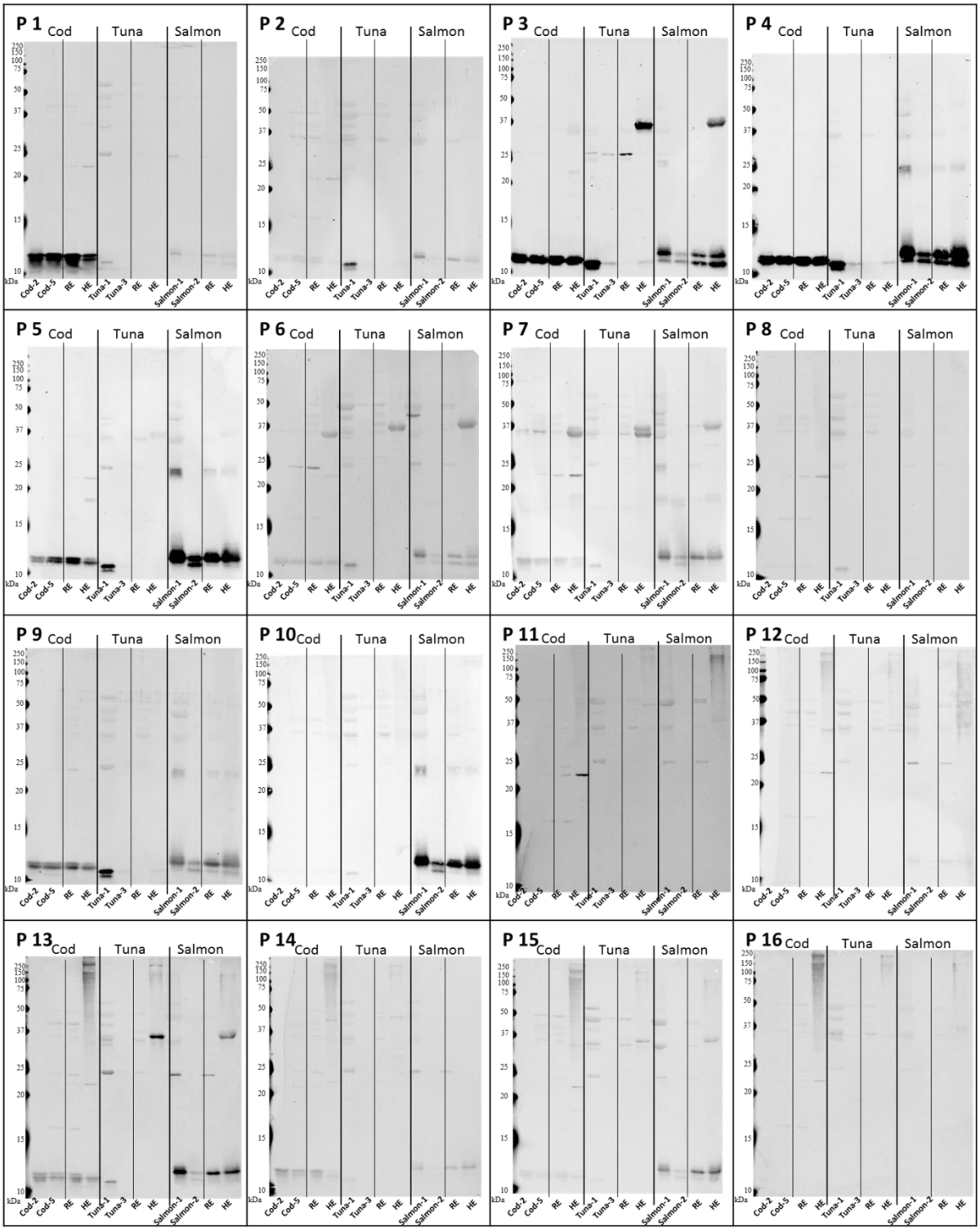

Figure S1: IgE reactivity of 16 fish-allergic patients to cod, tuna, and salmon extracts. The two SPT extracts with the lowest protein concentration (cod-2, tuna-3, salmon-2) and the highest (cod-5, tuna-1, salmon-1) were compared with in-house prepared raw (RE) and heated (HE) protein extracts from the corresponding species. The numbers in superscript on the common species names represent the different manufacturers listed in Table S1. The patient number corresponds to Table 1. Note: P, Patient.

Further results and discussion not included in the published manuscript

The mass spectrometric data, generated after tryptic digestion in-solution, were further analysed using MaxQuant as elaborated in **Chapter V A**. This allowed to estimate the relative abundance of all currently WHO/IUIS-registered Atlantic salmon allergens in the three above investigated salmon SPT preparations (see **Figure II 1 A**) and corresponding (putative) allergens in five cod SPT preparations (see **Figure II 1 B**). Some (putative) allergens, including enolase, aldolase, tropomyosin and collagen, were not detected in up to three of the eight preparations. The relative abundance of parvalbumin ranged from 2% to 20% in the salmon preparations and 30% to 84% in the cod preparations. Heat-stable tropomyosin and acid-soluble collagen had a low relative abundance of 0% to 1% in all eight preparations. Heat-labile enolase, aldolase, creatine kinase and triosephosphate isomerase had an abundance of 0%-25% in the salmon preparations and 0%-13% in the cod preparations. It is noteworthy that pyruvate kinase, lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase made up 28%-37% and 3%-15% of proteins in the salmon and cod preparations, respectively. These three heat-labile proteins are likely to exhibit IgE-binding based on *in silico* analyses performed after Nugraha *et al.* with results confirmed by AllerCatPro, and are registered as allergens with the WHO/IUIS for *Pangasius*/catfish (basa) (**Chapter V A**).[1,2] In summary, the further analyses of the generated data underlines that multiple (potential) allergens are under-represented in several commercial fish preparations analysed in this study.

As part of initial investigations for this study, nine shellfish preparations for SPT from two manufacturers were evaluated for their total protein and tropomyosin content as well as IgE-binding capacity (see poster in **Appendix B 4.2**). IgE-binding proteins were identified by mass spectrometric analyses after in-gel tryptic digest. Twenty-five shellfish SPT preparations from

six further manufacturers were acquired since and their analyses with an approach similar to the one presented in this Chapter is in progress. The total protein content in all 25 shellfish preparations has been estimated, ranging from 0.2 to 2.8 mg/ml. All preparations underwent tryptic in-solution digest followed by mass spectrometric analyses. Preliminary data suggest a similar high variance in protein and allergen content as discovered for fish preparations in this study. Many shellfish allergens appear to be under-represented in commercial shellfish preparations for SPT.

Since this study on fish SPT preparations was accepted for publication in January 2019, numerous commercial preparations used for allergy diagnostics and immunotherapy were analysed in multiple other studies. Many allergens were found to be under-represented and a great variance in *in vitro* and/or *in vivo* reactivity was discovered. Analysed preparations include those made from dog (fur and saliva), German cockroach, mites, mouse (urine), peach and peanut.[3–10] Consequently, it is often suggested to perform SPT with fresh allergen sources/foods.[11]

In this Chapter, the urgent need for improved fish allergy diagnostics was highlighted. Nevertheless, improved diagnostics can only lead to improved management if affected consumers have access to safe and correctly labelled food. In Australia and many other countries worldwide, food products are tested for their allergen content utilising commercial test kits. In 2018, three commercial ELISA kits for detecting fish were available worldwide and employed by the Australian National Measurement Institute (NMI) to monitor compliance with legislations. Subsequently, their utility to detect a wide variety of fish species is evaluated in **Chapter III A**.

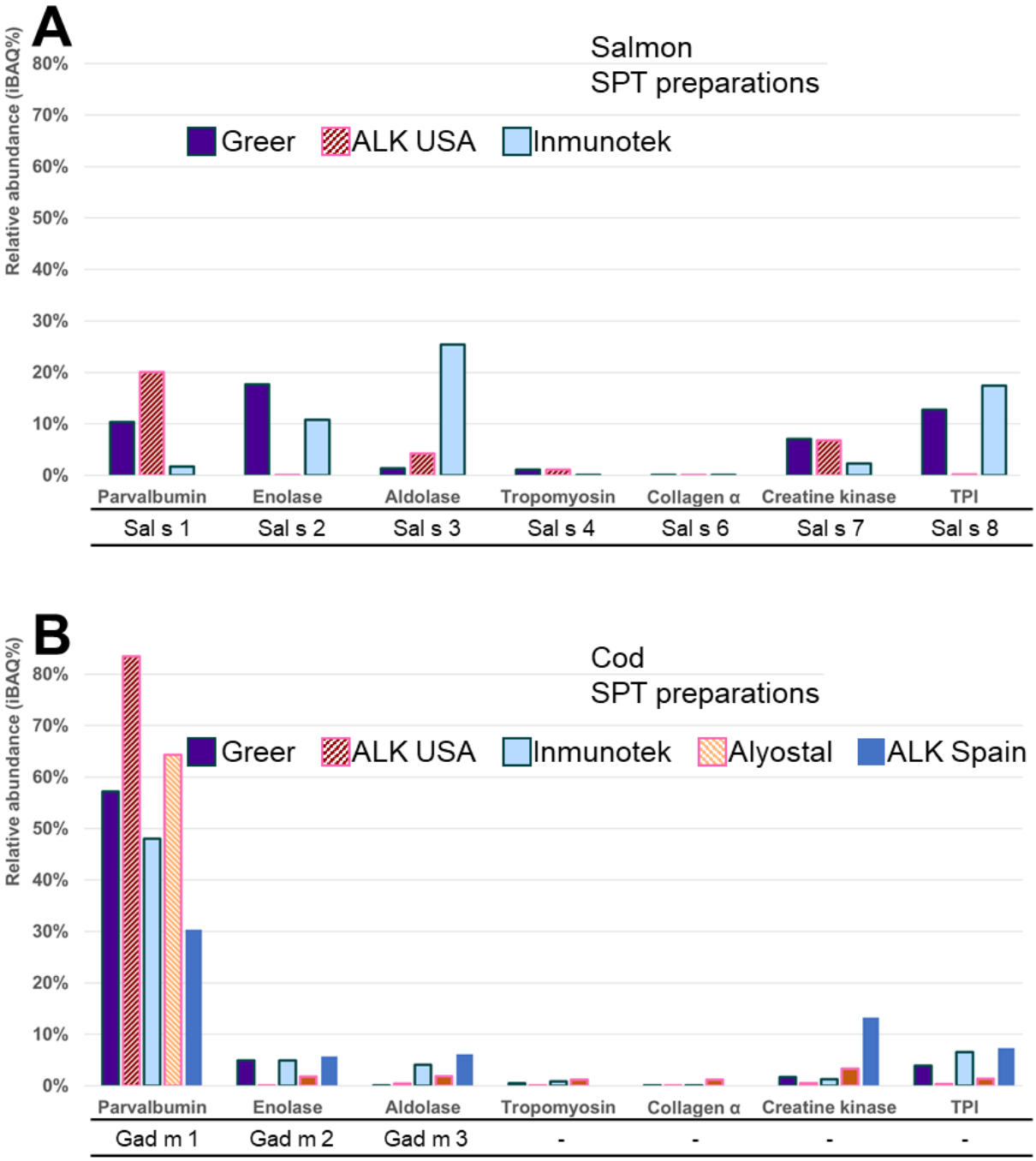

Figure II 1: Relative abundance (iBAQ%) of WHO/IUIS-registered Atlantic salmon allergens in commercial salmon SPT preparations for Skin Prick Testing (SPT) from three manufacturers (**A**) and corresponding (putative) allergens in commercial cod SPT preparations from five manufacturers (**B**). Proteins were digested with trypsin and analysed by mass spectrometry. The iBAQ% value is an indicator of the relative abundance of each protein group and is based on analyses with MaxQuant. The WHO/IUIS-name is based on the corresponding database accessible under www.allergen.org. Note: TPI=triosephosphate isomerase.

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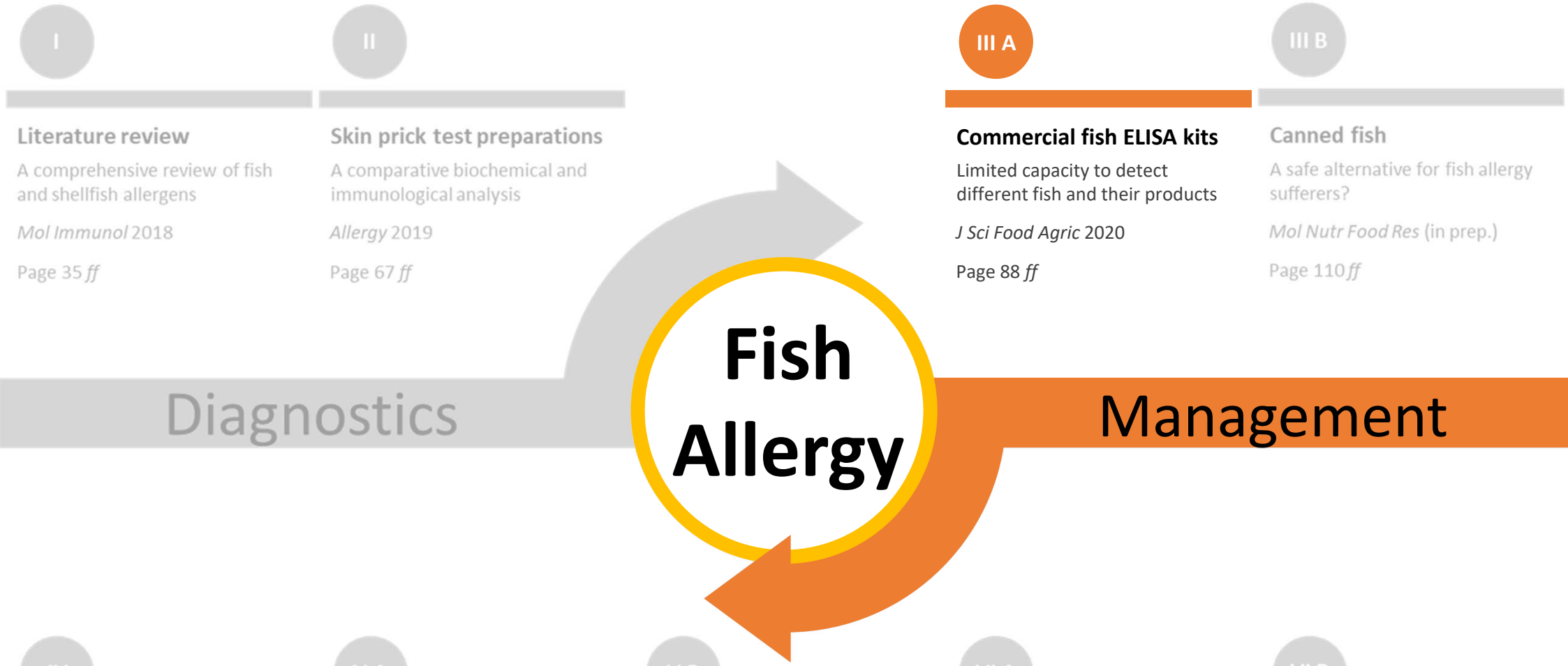
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Chapter III A – Commercial fish ELISA kits

Commercial fish ELISA kits have a limited capacity to detect different fish species and their products

Manuscript published as

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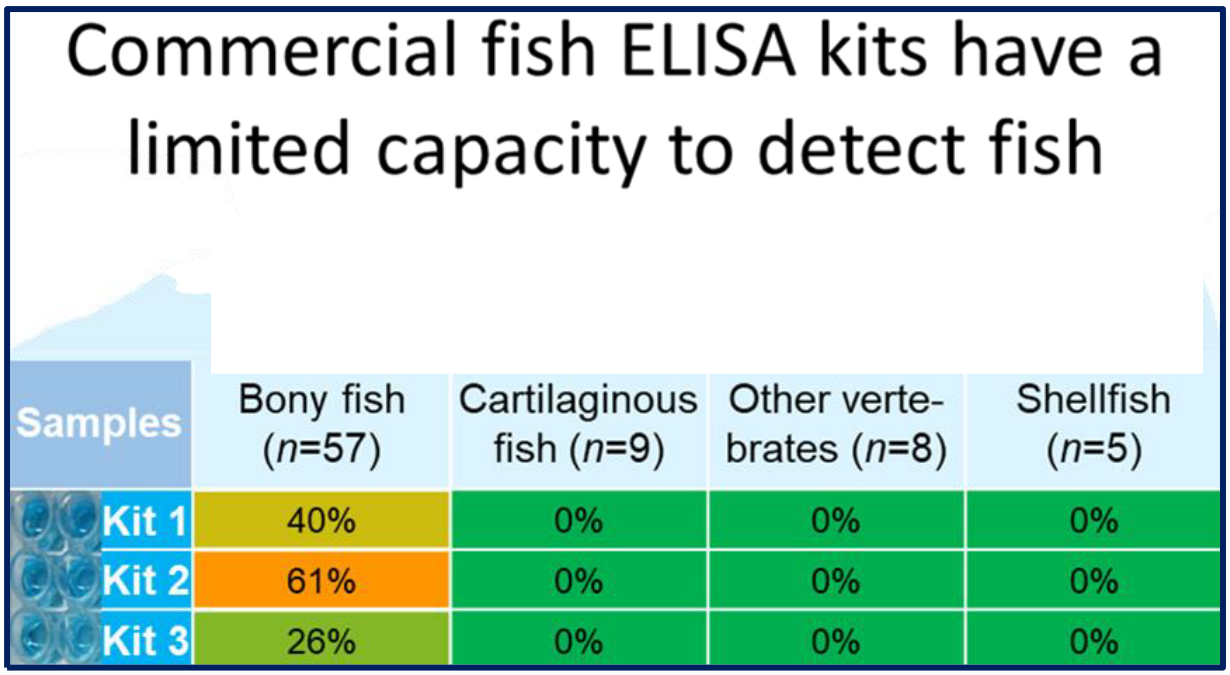

Graphical abstract

Contributions:

TR and MK performed the study and wrote the manuscript. TR, MK and AL designed the study. AC and CH assisted with sample preparations. All co-authors were involved in the analyses and interpretation of data and critically reviewed the manuscript.

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Commercial fish ELISA kits have a limited capacity to detect different fish species and their products

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Abstract

BACKGROUND: Fish is a major food and allergen source, requiring safety declarations on packages. Enzyme-linked immunosorbent assays (ELISAs) are often used to ensure that the product meets the required standards with regard to the presence of allergens. Over 1000 different fish species are traded and consumed worldwide, and they are increasingly provided by aquaculture. Up to 3% of the general population is at risk of sometimes fatal allergic reactions to fish, requiring strict avoidance of this commodity. The aim of this study is to evaluate the capacity of three commercially available ELISA tests to detect a wide variety of bony and cartilaginous fish and their products, which is essential to ensure reliable and safe food labeling.

RESULTS: The detection rates for 57 bony fish ranged from 26% to 61%. Common European and North American species, including carp, cod, and salmon species, demonstrated a higher detection rate than those from the Asia-Pacific region, including pangasius, and several mackerel and tuna species. Among the 17 canned bony fish products, only 65% to 86% were detected, with tuna showing the lowest rate. None of the cartilaginous fish ($n = 9$), other vertebrates ($n = 8$), or shellfish ($n = 5$) were detected.

CONCLUSIONS: We demonstrated that three commercial fish ELISA kits had a limited capacity to detect fish and their products. The complexity of fish as a protein source that is increasingly utilized means that there is an urgent need for improved detection methods. This is crucial for the food industry to provide safe seafood products and comply with international legislation.
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Supporting information may be found in the online version of this article.

Keywords: bony fish; cartilaginous fish; fish allergy; fish cross-reactivity; food labeling; food safety

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Table S1: Details of analyzed canned fish.

sample	detection by kit 1	kit 2	kit 3	product name	species labeled on the product	country of origin
salmon 1	■	■	■	pink salmon	not stated	USA
salmon 2	■	■	■	wild caught pink salmon	not stated	Canada
salmon 3	■	■	■	wild Alaskan pink salmon	not stated	USA
salmon 4	■	■	■	wild Alaskan red salmon	not stated	USA
salmon 5	■	■	■	wild Alaskan pink salmon	not stated	USA
salmon 6	■	■	■	wild Alaskan red salmon	not stated	USA
salmon 7	■	■	■	tempers salmon, spring water	not stated	Thailand
salmon 8	■	■	■	premium salmon in spring water	not stated	Thailand
tuna 1			■	tuna chunks in spring water	*Katsuwonus pelamis*	Thailand
tuna 2	■		■	tuna chunks in spring water	*Katsuwonus pelamis*	Thailand
tuna 3	■			yellowfin tuna in spring water	*Thunnus albacares*	Thailand
tuna 4			■	chunk style tuna in spring water	*Katsuwonus pelamis*	Thailand
tuna 5				tuna chunks in spring water	*Katsuwonus pelamis*	Thailand
tuna 6	■	■		tuna	*Thunnus spp.*	Indonesia
tuna 7	■		■	responsibly fished tuna	*Katsuwonus pelamis*	Thailand
sardine 1		■		sardine in spring water	not stated	Thailand
sardine 2	■	■	■	wild sardines in spring water	not stated	Canada

Note: The grey shaded boxes visualize that the specific kit detected the corresponding extract, while the white box marks no detection.

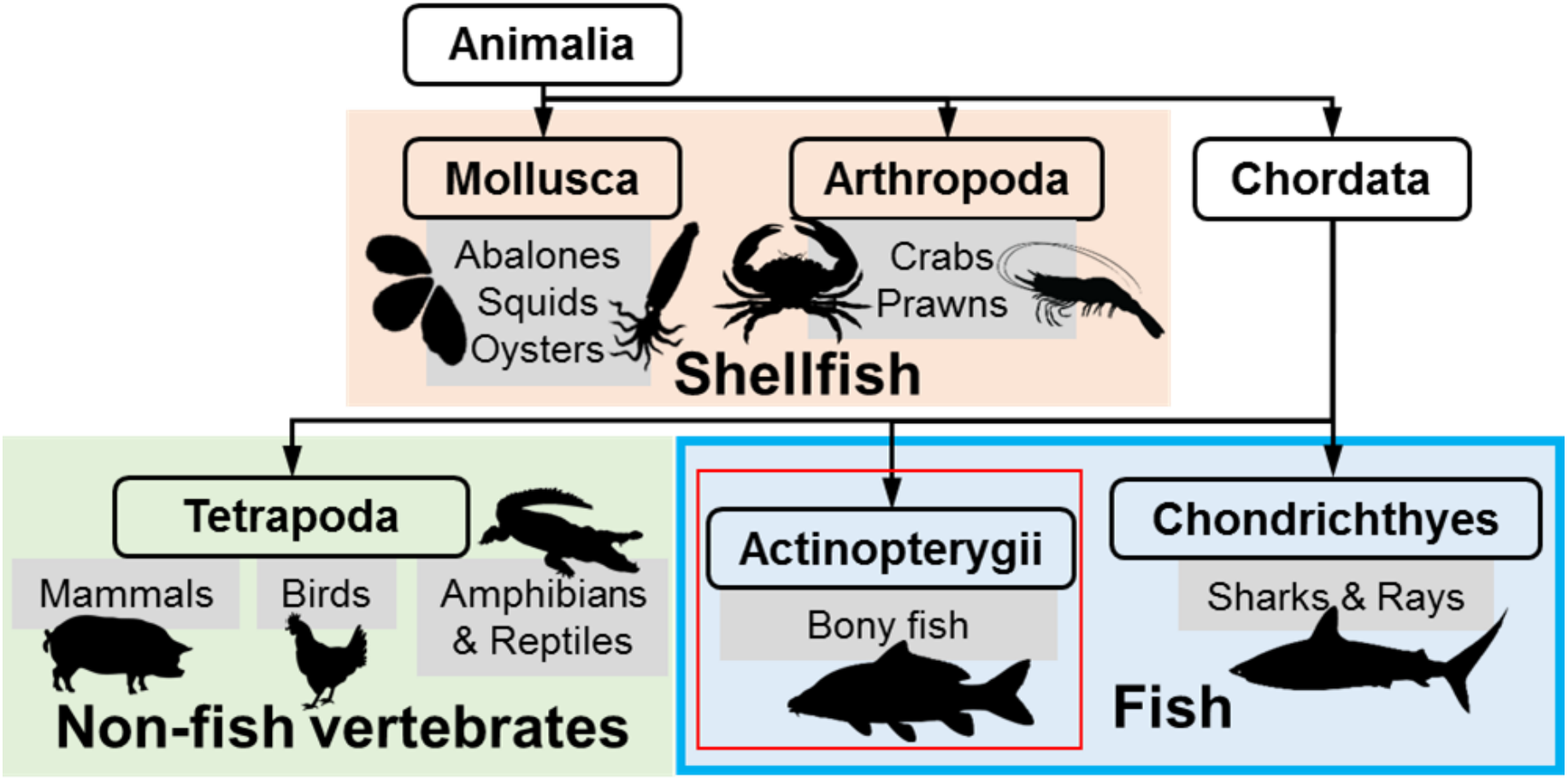

Figure S1. Taxonomic relationship of investigated species.

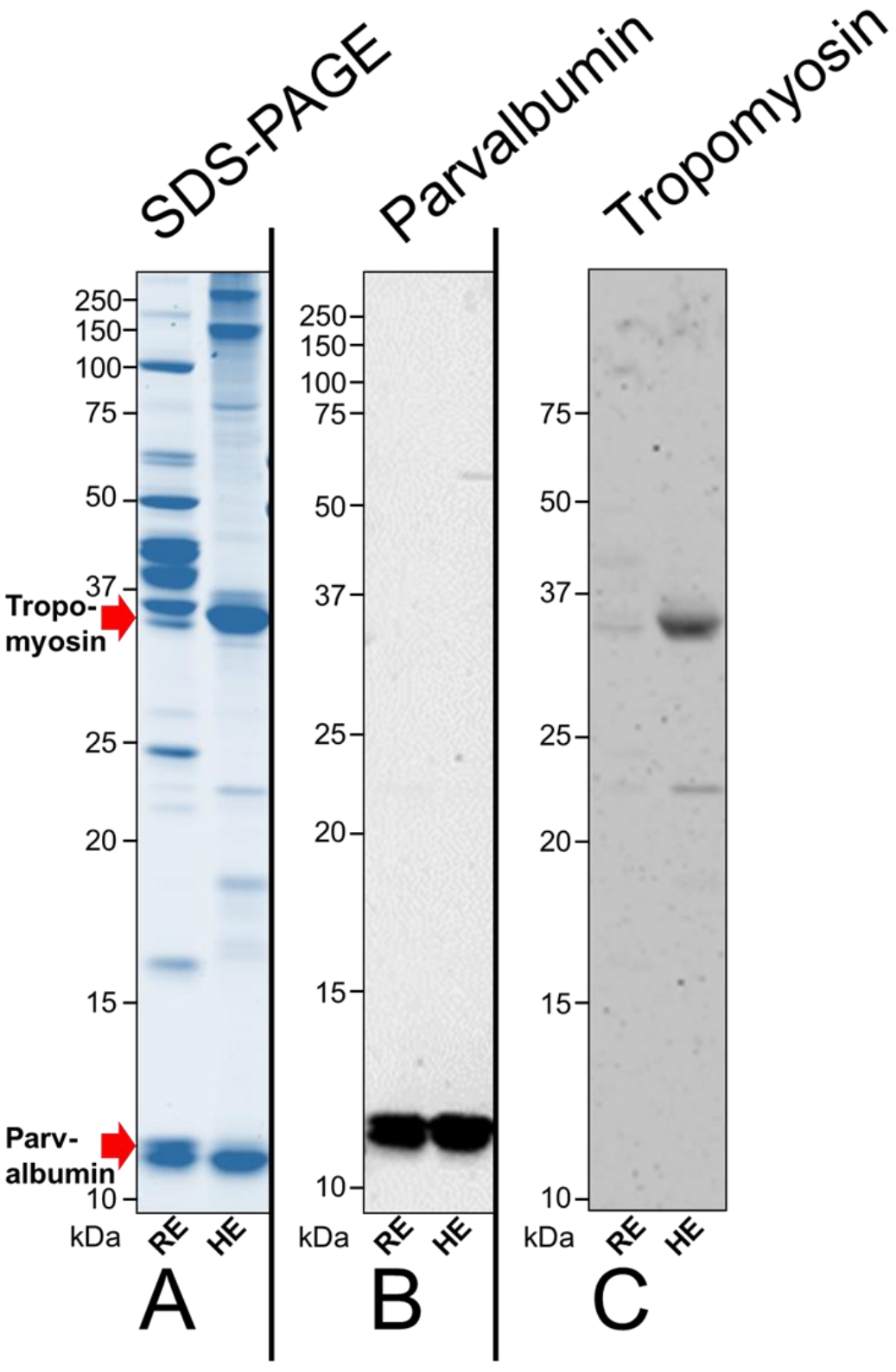

Figure S2. Protein profile of cod extracts used as references and controls. **A** shows the SDS-PAGE profile of raw (RE) and heated (HE) extracts from Atlantic cod (*Gadus morhua*). **B** and **C** show corresponding immunoblots using a parvalbumin- and tropomyosin-specific antibody, respectively.

Further results and discussion not included in the published manuscript

In this manuscript, a limited utility of commercial fish ELISA kits was demonstrated.

In **Chapter IV**, **V A** and **VI B**, it is demonstrated that in-house generated antibodies seem to detect a larger variety of fish species than the antibodies utilised in the three investigated commercial ELISA kits. **Figure III 1** below demonstrates the utility of a polyclonal rabbit antibody raised against barramundi (Asian seabass, *Lates calcarifer*) as described by Sharp *et al.* for detecting monomeric parvalbumin in heated extracts of all 31 tested species, except those from three tuna species for which the signal was very weak.[1] However, the antibody titre of the in-house produced anti-rabbit antibodies was too low for utilising them in improved methods for fish detection. Therefore, purified parvalbumins from Atlantic salmon (*Salmo salar*) and barramundi as well as Atlantic cod (*Gadus morhua*) were used to generate polyclonal antibodies in sheep for allergen detection. The utilised parvalbumins were purified as described in **Chapter IV** while the antibody production was performed by Osenses in South Australia (www.osenses.com). The specificity of the antibody was pre-evaluated using sheep serum in immunoblotting. Heated protein extracts from 30 fish species and four other vertebrates were separated by SDS-PAGE followed by transfer onto nitrocellulose and sequential incubation with sheep serum and infra-red labelled anti-goat antibody. At least one protein band in the molecular weight range of monomeric parvalbumin (10-15 kDa) was detected in 27 out of 30 extracts using the non-purified anti-salmon/barramundi or anti-cod parvalbumin antibodies (see **Figure III 2**). The latter demonstrated a lower titre and signal intensity. In heated extracts from other vertebrate species, a parvalbumin band was only detected in the crocodile extract, which is analysed and discussed in detail in **Chapter VI B**. Fish extracts in which no band was recognised by these antibodies did not show a prominent band in the molecular weight range of monomeric parvalbumin (see **Figure III 1**).

The here evaluated ELISA test kits are designed to detect fish also after heat-processing. However, extensive heat-processing of fish (muscle) tissue appears to alternate the repertoire of proteins and their IgE-binding capacity.[2–4] Parvalbumin, for example, was shown to lose its IgE-binding capacity when heated under pressure at 140°C.[5] Clinicians frequently recommend fish-allergic patients to try the implementation of canned fish in their diet. In **Chapter III B**, this recommendation is evaluated by investigating the protein repertoire and IgE-binding capacity of 17 canned fish products.

A

#	Species name	PV on gel	Signal PV Ab
1	Swamp eel	7680	26180
2	Orange roughy	5040	1636
3	Sardines	2320	5328
4	Common carp	5920	23590
5	Atlantic cod	1180	692.649
6	Hoki	1400	2282
7	Ling	5750	4487
8	Nile tilapia	150	n.d.
9	King George whiting	4070	15995.66
10	Yellowtail kingfish	448	1983.81
11	**Asian seabass**	6610	
12	Redthroat emperor	1390	8325.5
13	Murray cod		16497.6
14	King salmon	4270	3669.67
15	Cobia	2390	6150.253
16	Skipjack tuna	240	157.859
17	Indian mackerel	1470	8289.485

#	Species name	TM on gel	Signal TM Ab
18	Spanish mackerel	248	1880.503
19	Albacore tuna	111	545.604
20	Yellow Fin tuna	-2.17	1.723
21	(Pink) snapper	3020	2980
22	Silver perch	230	18096.44
23	Yellowbelly flounder	5140	2703
24	Rainbow trout	998	1824
25	Atlantic salmon	653	1128
26	Dusky flathead	3770	99.423
27	Pangasius catfish	6850	19010
28	John dory	2870	1146
29	Elephant shark	740	654.732
30	Bluespotted maskray	3720	173.91
31	Gummy shark	552	61.046
32	Chicken	73.5	1.427
33	Beef	110	1.5091
34	Pig	50.1	6.47

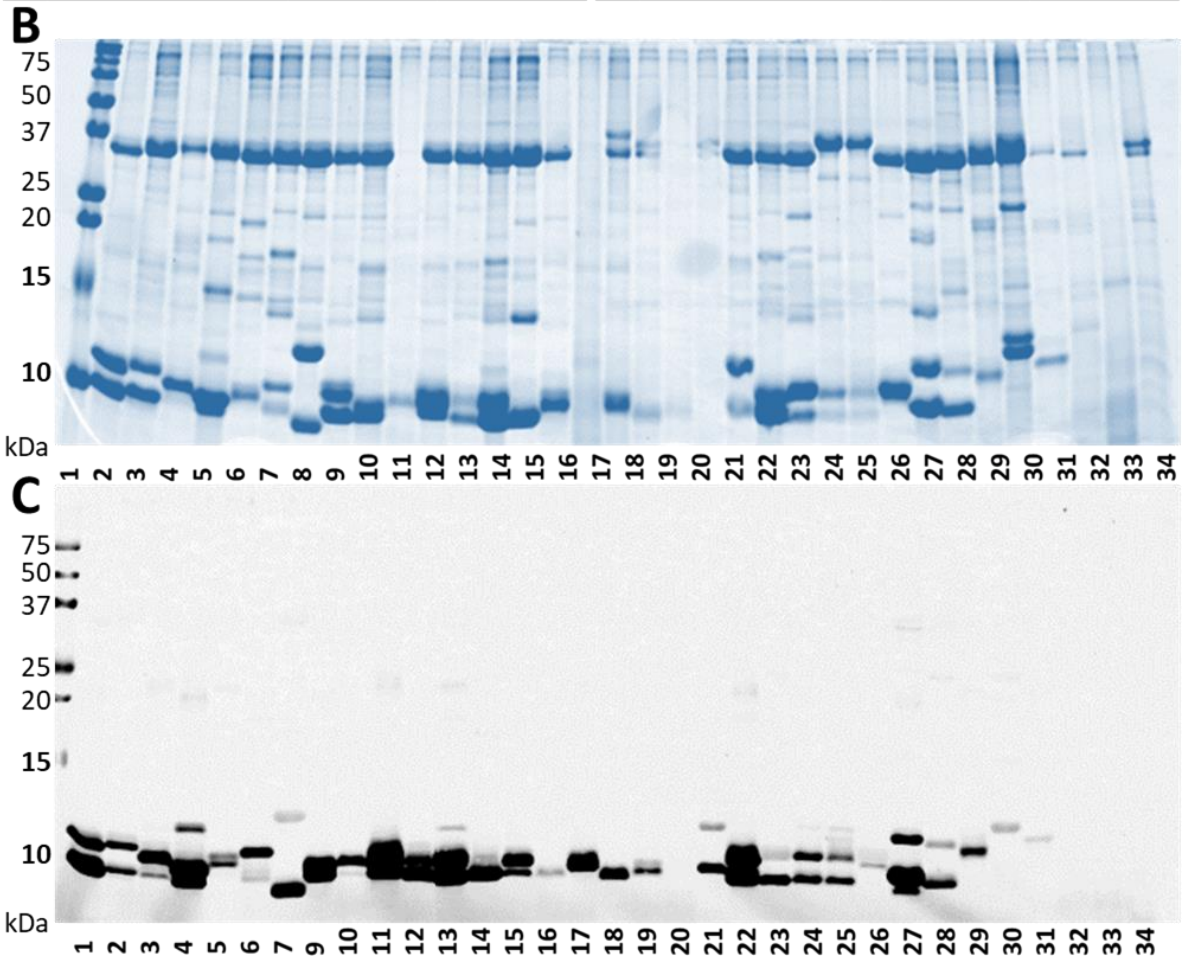

Figure III 1: Heated extracts from 30 fish and four other vertebrate species (**A**) were separated by SDS-PAGE (**B**) and transferred onto a nitrocellulose membrane. Parvalbumin (PV) monomers and dimers were detected using a polyclonal rabbit antibody raised against parvalbumin from Asian seabass (barramundi) (**C**). The monomeric PV bands and the signals to them were quantified (**A**) and compared with the signal to barramundi PV set to 100%. The species names refer to Table 1 in Chapter III A. Note: n.d.=not determined.

A

#	Species name	Signal Cod PV Ab	Signal Salmon/Barra Ab
1	Swamp eel	122000	403000
2	Sardines	34000	219000
3	Common carp	128000	305000
4	Pacific cod	132000	13200
5	**Atlantic cod**	158000	11300
6	Hoki	106000	59500
7	Milk fish	114000	315000
8	Ling	76500	154000
9	Nile tilapia	130000	240000
10	Yellowtail kingfish	14200	46100
11	**Asian seabass**	61600	761000
12	Redthroat emperor	65600	291000
13	Murray cod	108000	177000
14	King salmon	48000	40300
15	Cobia	80100	229000
16	Indian mackerel	47800	77100
17	Spanish mackerel	20700	57200
18	Albacore tuna	17200	14000
19	(Pink) snapper	70300	23100
20	Silver perch	134000	396000
21	Yellowbelly flounde	61600	30300
22	Rainbow trout	75700	364000
23	**Tas. Atl. salmon**	29800	461000
24	pangasius	92900	220000
25	John dory	70300	60200
26	Crocodile	10800	27800
27	Chicken	103	160
28	Beef	74.8	146
29	Pig	357	4740
30	Orange roughy	210000	91700
31	Longtail tuna	2210	2530
32	Elephant shark	1090	4840
33	Bluespotted maskra	4340	10900
34	Yellow Fin tuna	11000	2220

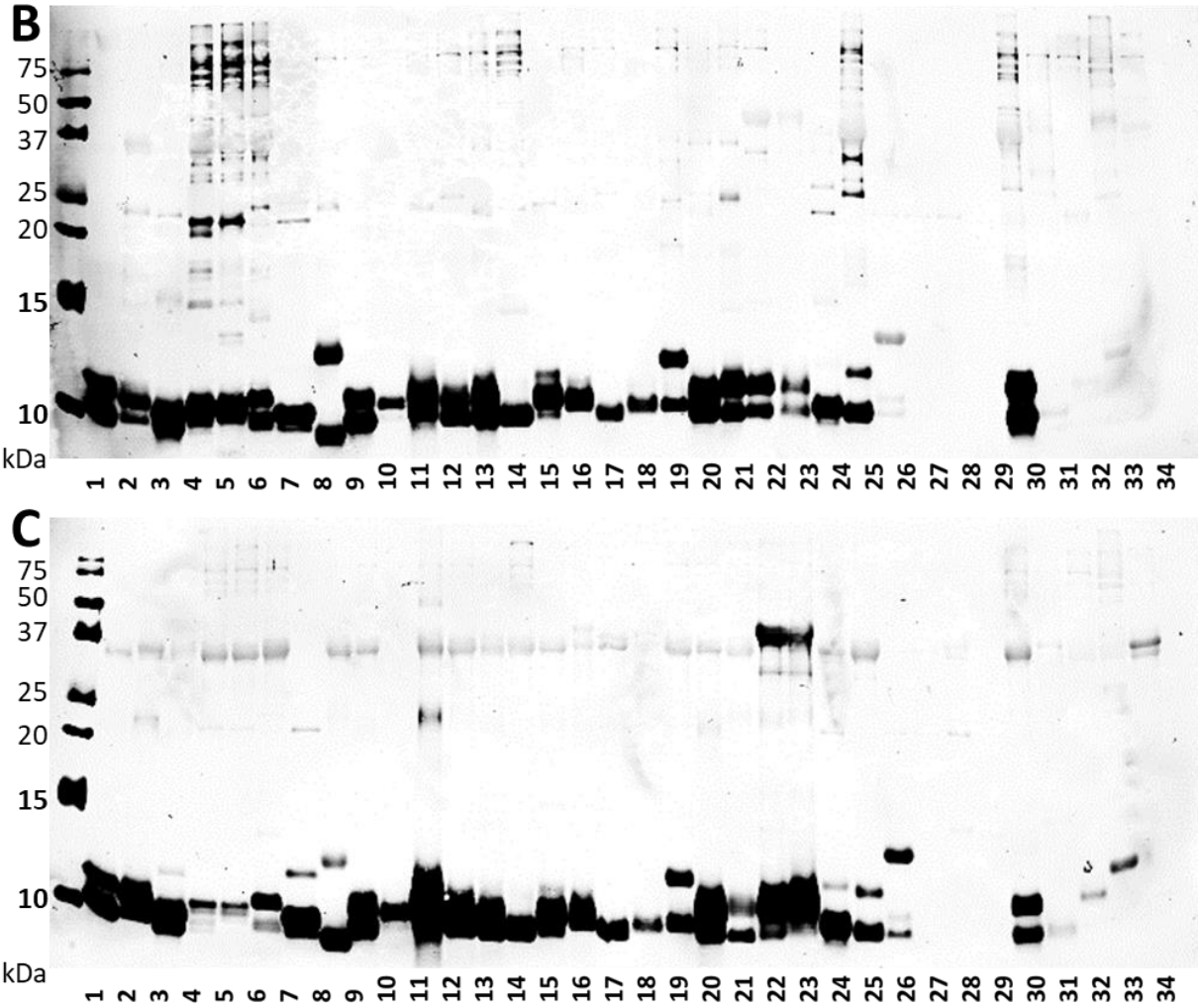

Figure II 2: Heated extracts from 30 fish and four other vertebrates species (**A**) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Parvalbumin (PV) monomers and multimers were detected using a polyclonal sheep antibody raised against parvalbumin from Asian seabass (barramundi, barra) and salmon (**B**) and cod (**C**). The signal to the monomers was quantified (**A**) and compared with the signal to barra PV set to 100%. The species names refer to Table 1 in Chapter III A.

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Chapter III B – Canned fish

Fish allergy: Is canned fish really safe? – Evaluation of IgE-binding proteins in 17 commercial canned fish products

Manuscript in preparation for submission to ***Molecular Nutrition & Food Research*** as Research article

Is canned fish safe for fish-allergic patients?

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Graphical Abstract

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Abstract

Scope: Major fish allergens, including the muscle protein parvalbumin (PV), are heat stable and can withstand extensive cooking processes and therefore, the management of fish allergy relies on complete avoidance of fish. Recommendations to consume canned fish are frequently advised by clinicians to maintain a healthy dietary intake, however, no comprehensive molecular analysis of canned fish has been performed to support such advice. This study investigates the IgE-binding proteins in canned fish products using comprehensive molecular approaches.

Methods and results: Seventeen commonly consumed canned fish products (salmon *n*=8, tuna *n*=7, and sardine *n*=2) from nine different manufacturers were evaluated for their PV contents and binding by two PV-specific antibodies, determined by immunoblotting. Sera from fish-allergic pediatric patients (*n*=53) were investigated to compare the *in vitro* binding of sIgE to cooked and canned fish protein extracts. The IgE-binding proteins and abundance of each detected protein in canned fish were identified by mass spectrometry after in-gel and in-solution tryptic digestion. Canned fish showed a markedly reduced content of PV and lost binding capacity of PV-specific antibodies compared to conventionally cooked fish. However, PV and other heat-stable fish proteins, including tropomyosin and collagen, maintained their IgE-binding capacity. Canned sardine demonstrated the highest IgE-binding capacity followed by canned salmon with serum from fish-allergic children. Patient IgE-binding to tuna is much lower but appears species-specific.

Conclusion: We showed that canned fish products might not be safe for all fish-allergic patients. Before canned fish can be considered into the diet of individuals with fish allergy, detailed *in vitro* diagnostics to several heat-stable allergens and/or incremental food challenges should be conducted.

Keywords: Canned fish; Fish allergy; Food allergy; IgE binding; Food allergy management; Parvalbumin; PARV-19; Tropomyosin

1. Introduction

IgE-mediated fish allergy is becoming a worldwide concern that affects both adults and children.[1,2] Fish allergy can cause life-threatening anaphylaxis and there is currently no effective therapy to mediate the disease beside the use of emergency medicine such as EpiPen®.[3] The current management of fish allergy relies on complete avoidance of implicated fish in the diet of affected individuals. The prevalence of fish allergy in countries frequently consuming seafood can be as high as 5% amongst children, such as in Finland and 1.5% in Vietnam.[4,5] Fish allergy, similar to nuts, are among the most common causes of anaphylaxis and death from food allergy.[6–8] Fish allergy is thought to persist for life in up to 90% of patients, with a similar trend also observed in peanut allergy.[9–11]

Some fish-allergic individuals can tolerate fish in canned form, although the exact mechanisms for this tolerance remain unknown.[12] Decreased allergenicity could be explained by the extreme thermal treatment during the canning process, resulting in some conformational changes in allergenic proteins.[13,14] Indeed, a reduction in IgE-binding in canned products has been demonstrated for tuna.[15] Recommendations to consume canned fish are frequently advised by clinicians and medical practitioners to maintain a healthy dietary intake, since the nutrient contained in fish, such as omega-3 fatty acids and minerals, are known for their beneficial properties.[16–20] However, no comprehensive molecular and immunological analyses of allergens in canned fish products has ever been performed to support such advice. Numbers of previous studies on canned fish are extremely limited and they often lack to investigate a variety of fish species and comparison of canned products. The potential of some fish species or canned fish products to maintain their allergenicity cannot be dismissed as allergic reactions to canned fish have been reported.[12] In fact, canned fish has been used to commit suicide by a man with known fish allergy.[21]

In the present study, we conducted a comprehensive biochemical analysis of a large panel of canned fish products of three different fish species, supported by a large cohort of fish allergic individuals. Furthermore, to evaluate the recommendations on the safe consumption of canned fish for IgE-mediated fish-allergic patients, we investigated IgE binding to proteins in 17 canned fish products (salmon, tuna and sardine) commercially available in Australia and worldwide. The well-studied major fish allergen, parvalbumin (PV), is a heat stable protein (10-14 kDa) and present in many different fish species and can withstand extensive cooking processes.[22,23] Other fish proteins, such as tropomyosin (TM) and collagens, were only recently confirmed to be allergenic, and demonstrated to be present in fish species such as barramundi/Asian seabass, *Pangasius*/catfish, salmon and tuna (see **Chapter V A** and **B**).[24] Using immunoblotting and mass spectrometry, we analysed the major fish allergen PV contents by measuring the binding capacity of three PV-specific antibodies. Sera from 54 paediatric patients with clinically-confirmed fish allergy were used to compare *in vitro* IgE binding capacity to cooked and canned fish, and identify the putative allergens.

2. Experimental section

2.1 Patient recruitment

Clinically confirmed fish-allergic paediatric patients (*n*=52; 1-18 years-old; see **Table 1**) were recruited at the Children's Hospital at Westmead, Australia, based on a convincing recent clinical history of IgE-mediated adverse reactions to any fish. Sensitisation to fish was confirmed by skin prick testing (SPT), and the presence of specific sIgE (ImmunoCAP; Phadia-Thermo Fisher Scientific, Uppsala, Sweden) to cod (*Gadus morhua*; f3), tuna (*Thunnus albacares*; f40), or salmon (*Salmo salar*; f41). Serum from non-seafood-allergic, atopic patients (*n*=4) served as negative controls. The study was approved by the Sydney Children's Hospital Network (approval number: LNR-14/SCHN/185). Parents of all participants gave written informed consent and patient anonymity was preserved.

2.2 Canned fish products

Canned fish products (*n*=17) from three fish, i.e., salmon (*n*=8), tuna (*n*=7) and sardine (*n*=2), were purchased from major supermarket retailers in Australia. These 17 canned fish products were produced by nine different manufacturers, from four countries (USA, Canada, Thailand and Indonesia), and are all prepared in salt-water. The details for each canned product are provided in **Table 2**.

2.3 Preparation of fish protein extracts

Protein extracts from all canned fish products were generated, as well as cooked salmon, tuna and sardine extracts (denoted as CE), and purified PVs from fresh salmon and cod, which were used as internal controls. Fish was purchased from fish retailers across Australia. The authenticity of specimens was confirmed using species identification guides and consulting

fishery experts at Centre for Sustainable Tropical Fisheries and Aquaculture (James Cook University, Townsville, Australia). Atlantic cod (*Gadus morhua*) was sourced from Norway (provided by Berit Bang; The Arctic University of Norway, Tromsø, Norway). Muscle tissues (30 g) from Atlantic salmon (*Salmo salar*), yellowfin tuna (*Thunnus albacares*), and sardine (*Sardina pilchardus*) were cooked in phosphate-buffered saline (PBS; 10 mM phosphate, pH 7.2) for 20 min at 95-100°C then homogenised with a rotor-stator homogeniser (5 min at 13,000 rpm, on ice) in PBS given at 2 mL per gram of tissue. Canned fish (30 g) were prepared in the same way without the cooking step. After gentle agitation overnight at 4°C, soluble proteins were collected by centrifugation (20,000 *x g*) and filtration through 0.45 μm membranes. PVs were purified from the CE of Atlantic salmon and Atlantic cod by ammonium sulphate fractionation method with subsequent dialyses in 100 mM ammonium bicarbonate as previously described.[25](**Chapter IV**) The extracts and purified PVs were stored at -80°C until further use. The protein concentration was estimated using the Pierce™ bicinchoninic acid assay (BCA) or 660nm Protein Assay (Thermo Fisher Scientific, USA) with bovine serum albumin used as standard.

2.4 Immunoblot analysis

All protein samples were resolved based on their molecular weights under reducing condition using either a Protean™ SDS-PAGE system or a Criterion™ SDS-PAGE system (Bio-Rad) as described by Laemmli.[26] Separated proteins were visualised by Coomassie Brilliant Blue R-250 (CBB) staining. All detection antibody incubations were performed for 1 h at room temperature. The antibody binding complexes were visualised using the enhanced chemiluminescence (ECL) method with horseradish peroxidase (HRP)-labelled detection antibodies. The Odyssey® Imaging Systems (LI-COR®) was used for imaging the immunoblot

membranes when infrared (IR)Dye 800CW antibodies were used. Densitometric analyses were conducted utilising the Image Studio Version 5.2 (LI-COR®).

2.5 Immunoblot analysis with parvalbumin-specific antibodies

For the detection of PV by immunoblotting, proteins (10 μg/lane) from all fish extracts and purified PVs from salmon and cod in various amounts (0.5, 1 and 2 μg) were resolved by SDS-PAGE (16%) and transferred onto an activated PVDF membrane (GE Lifesciences). The membrane was blocked in 5% de-fatted milk in PBS/0.05% Tween-20 for 1 h. PV was detected using a monoclonal anti-PV antibody (PARV-19; 1:3,000; Sigma-Aldrich), and either the in-house generated polyclonal rabbit antibodies against salmon PV or Asian seabass PV.[27] For the subsequent detection of PARV-19, the membranes were further incubated with HRP-labelled rabbit anti-mouse IgG antibody (1:20,000, Sigma-Aldrich). PARV-19 anti-frog PV antibody is a monoclonal antibody commercially available and used frequently in studies of parvalbumins.[25,28–31](see **Chapter IV** for 25) The in-house generated polyclonal antibodies were detected with HRP-labelled goat anti-rabbit IgG antibody (1:10,000, Promega). Antibodies were diluted in 1% de-fatted milk in PBS/0.05% Tween-20 and the exposure time for the ECL filming adjusted for each membrane.

2.6 Immunoblot analysis with sera from fish-allergic children

For the initial characterisation of IgE-binding proteins from canned fish extract, pooled serum of five selected patients was used for immunoblotting. Patients were selected based on their clinical history and/or sIgE-binding (salmon, tuna, sardine) or positive SPT (see **Table 1** and **Chapter V A**). Proteins from canned fish extracts (10 μg/lane) and CE from salmon (1 μg), tuna (10 μg) and sardine (5 μg) were resolved by SDS-PAGE (16%) and transferred onto activated

PVDF membranes. Different amounts of proteins were loaded for the CE to correct the dynamic range of detectable bands. The membrane was blocked with 5% de-fatted milk in PBS/0.05% Tween-20 and incubated with the pool of patient sera (diluted 1:20 in 1% de-fatted milk in PBS/0.05% Tween-20) overnight at 4°C. IgE binding was detected using rabbit anti-human IgE antibody (1:10,000; DAKO) followed by subsequent detection with HRP-labelled goat anti-rabbit antibody.

Five canned products were subsequently selected based on their strong IgE-binding patterns (see **Table 2**), to investigate the individual IgE binding of 52 patients. Furthermore, the IgE-binding profiles of individual patients were compared against the three fish CE. Two canned salmons (I-2 and I-7), both Pink salmon of different manufactures and origins were selected based on IgE binding to proteins in the 10-15 kDa region, possible representing PV. Two canned tuna (T-1 and T-3), skipjack and yellowfin tuna respectively, were selected based on IgE-binding to only the latter species. For canned sardine, the product (S-2) demonstrating the higher IgE-binding capacity was selected for this study. Proteins (100 μg) of each extract were resolved by SDS-PAGE (16%) and transferred onto a nitrocellulose membrane (BioRad). The membrane was dried, rehydrated with PBS and blocked with 1 X casein blocking solution (Sigma-Aldrich) for 1 h, and placed into the surf-blot manifold (Idea scientific). The patient sera (diluted 1:10 in 0.2 X casein blocking solution) was added to the membrane overnight at 4 °C with gentle rocking. IgE binding was detected using a rabbit anti-human IgE antibody, followed by subsequent probing with IRDye 800CW donkey anti-rabbit antibody (1:10,000; LI-COR®). Antibodies were diluted in PBS containing 0.1 X casein blocking solution and 0.05% Tween-20.

2.7 Identification of IgE-binding proteins by mass spectrometry

The prominent IgE-binding bands from select canned fish (I-2, I-7 and S-2; diluted to 200 µg/ml), and from respective CEs, as well as selected extracts from canned fish products were subjected to mass spectrometric analysis after tryptic digestion as previously detailed (see **Chapter V A**).[32,33](see **Chapter II** for 32) In brief, the extracted peptides were analysed 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-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 240k resolution followed by collision-induced dissociation (CID) fragmentation. Twenty of the most intense peptide ions with charge states ≥ 2 were isolated and fragmented using normalised collision energy of 35 and activation Q of 0.25 (CID).

Results were analysed using MaxQuant (v. 1.6.10.43), against an NCBI database containing amino acid sequences of all proteins from the corresponding species or closest higher classification with at least three annotated genomes/transcriptomes (July 2020). The relative protein abundance is expressed in relative intensity-based absolute quantification (iBAQ%) value.[34] Fixed modification carbamidomethyl-C and variable modifications of deamidation N, deamidation Q and oxidation of M were selected. Identified protein groups with at least 1 unique peptide and a minimum of 2 razor/unique peptides were included in the analysis.

2.8 *In silico* conservation analysis of fish parvalbumin and tropomyosin

PV and TM sequences, identified in canned salmon and sardine products by mass spectrometric analyses after in-solution digestion, were analysed and compared for amino acid sequence identities using Clustal Omega.[35]

3. Results

3.1 Detection of parvalbumin in canned fish products

The protein composition of all canned fish extracts differed greatly to that of the corresponding in-house generated CE protein extract (**Figure 1A**). The protein profile of all canned fish products consistently extended over the complete protein range, including undefined bands (smear), suggesting the presence of extensive protein aggregates and/or degradation. All CEs contained prominent band/s at approximately 35-40 kDa, which appeared as the most abundant proteins in all extracts. In contrast, this particular band was observable only in all canned salmon and one canned sardine (S-2). There were no distinct differences in the protein composition for canned salmon, independent of species or manufacturers. The differences between species were more evident in canned tuna. Skipjack tuna (T-1, 2, 4, 5, 7) demonstrated a slightly higher abundance of smaller proteins while yellowfin tuna (T-3, 6) showed abundance of larger proteins. The two canned sardines (S-1 and S-2) showed large protein profiles unique to each other, probably due to a similar species coming from two different origins (Thailand and Canada).

PARV-19 was able to detect the presence of PVs in CEs of salmon and sardine as well as purified salmon and cod PV in the expected size range of 12-14 kDa, however, not in any of the canned fish products (**Figure 1B**). Using our in-house generated anti-salmon and anti-Asian seabass PV-specific antibodies, the detection of PV did however not improve (**Figure 1C**). PV was not detected in any of the canned fish products nor cooked tuna extract. In in-house cooked salmon and sardine, at least two isoforms of PVs were observed.

3.2 Detection of IgE-binding proteins in canned fish products

The sera from five selected paediatric patients were pooled and used to identify IgE-binding proteins in different canned fish products and compared to in-house prepared cooked fish extracts (**Figure 2**). While the protein composition of canned fish was very similar within the three fish species analysed (**Figure 2A**), the sIgE binding profile was unique for each species (**Figure 2B**). Two of the canned salmon (I-2 and I-7) demonstrated IgE binding to proteins at the molecular weight appropriate for PV. Importantly, these bands were not detected by PARV-19 or any of the in-house anti-PV antibodies. Different IgE-binding profiles were observed in canned salmon independent of specific species, origin or the manufacturer. This is in contrast to tuna where yellowfin tuna demonstrated considerably more IgE-binding than skipjack tuna. The canned sardines from Canada (S-2) demonstrated more IgE-binding bands than the sardine from Thailand.

IgE binding to protein in the range of PV was the strongest in cooked salmon and sardine, while no binding was seen for tuna. In addition, strong IgE binding was seen in the 30-40 kDa region for tuna and sardine, but not salmon.

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

The bands of prominent IgE-binding protein from the CEs, and the gel sections (not bands, due to the smearing effect) of canned fish extracts were excised from the acrylamide gels and subjected to in-gel tryptic digestion for mass spectrometry analyses (see **Figure 3**). The 12 kDa protein from canned sardine (S-2) was confirmed to be PVs, and the proteins of approximately 38-42 kDa, particularly prominent in I-2, I-7 and S-2, were identified to be TMs (see **Supplementary Table 1** and **2**). The two most prominent IgE-binding proteins of 10-12 kDa and 36-42 kDa from all cooked fish were also identified to be PV and TM, respectively. The

adjacent bands in these molecular weight ranges were found to be different isoforms of PV and TM.

Type I collagen α-1 was the largest IgE-binding proteins with over 250 kDa in size identified for canned salmon, while a larger size protein, myosin heavy chain, was also detected but throughout the 60-155 kDa region despite its estimated size of 222 kDa. The presence of myosin heavy chain was similarly detected in the same region for canned sardine. Interestingly, TM was consistently identified in IgE-binding gel sections excised between 50-100 kDa regions for all canned fish products.

An approach was taken to determine the abundance of IgE-binding proteins presented in canned fish by in-solution digestion followed by mass spectrometry. TM was detected at markedly high level of abundance in all three canned fish products (I-2, I-7 and S-2) analysed, followed by myosin light chain, myosin heavy chain and actin (**Figure 5**). PV was high in abundance for canned sardine (S-2). In contrast, PV was of low abundance in both canned salmon, which was similar for most heat-labile proteins, including aldolase and enolase. Another heat-labile protein, glyceraldehyde-3-phosphate dehydrogenase, was detected from excised gels/section of both canned salmon extracts, however, its abundance was only 0.3-0.5% in-solution.

3.4 IgE-binding profiles of individual patients to canned fish products

The IgE-binding to cooked salmon, tuna and sardine extract was observed in 46.2%, 19.2% and 67.3% of 53 patients, respectively (see **Supplementary Figures 1-3** for the corresponding IgE immunoblots). In general, the IgE-binding capacity and frequency of proteins in the in-house cooked fish extracts were much higher than that of proteins in canned fish.

For both canned and cooked salmon and sardine, 11-12.5 kDa proteins, in the same molecular weight range as PV, showed the strongest and most frequent IgE binding within this cohort (**Figure 5**). The second strongest IgE-binding, at a moderate binding frequency, was to 35-40 kDa protein bands, the molecular weight range of TM. In contrast, IgE binding to PV bands in tuna (both canned and cooked) was considerably lower compared to salmon or sardine. Cooked tuna showed the strongest and most frequent IgE-binding to proteins of 35 kDa and 150 kDa, while moderate IgE-binding was observed for smaller proteins of 11-12 kDa.

In general, IgE-binding was also observed in the undefined protein band region between 35-100 kDa of all canned fish products. Interestingly, a larger protein of approximately 150 kDa showed IgE-binding in cooked salmon (19.2%), tuna (17.3%), and sardine 13.5%. This region approximates the size of collagen, identified as an IgE-binding protein in this cohort through in-gel mass spectrometry analysis. Only canned sardine also demonstrated IgE-binding in the same region (by 5.8% of patients).

In cooked salmon, two possible isoforms of PV, at 11.1 kDa and 11.7 kDa, bound sIgE of 40.4% and 46.2%, respectively. Similarly, possible multiple isoforms were also detected in canned salmon. Two possible monomers of PV isoforms of 11 kDa and 11.2 kDa were demonstrated in cooked tuna with IgE-binding frequencies of 9.6% and 11.5%, respectively. Only the larger PV isoform was detected in canned tuna (T-3) by 7.7% of patients. Sardine also contained several potential isoforms of PVs, estimated to be about 11.1, 11.7 and 12.2 kDa. IgE binding to sardine PVs was also the strongest and most frequent among the three fish species, with IgE from 67.3% and 44.2% of patients binding to two smaller PV isoforms from cooked sardine. Canned sardine also contained two similar-sized PVs with the frequencies of IgE-binding at 13.5% and 36.5% in this cohort. TMs, found in two isoforms, were the second most reactive protein in both cooked (21.2% and 9.6%) and canned sardines (3.8% and 5.8%).

3.5 Amino acid sequence conservation of fish allergens

Sequence identity matrix analysis was performed for PVs and TMs identified in the canned salmon and sardine products by mass spectrometry after in-solution tryptic digestion. TM was determined to be more conserved across the two species, salmon and sardine, as compared to PV. TM of two α-isoforms of sardine and two α- and one β-isoforms of salmon were 84-90% similar to each other while the majority of β-PV isoforms were only up to 80% similar, including within each of the analysed fish species.

4. Discussion

A great variance in the protein composition was demonstrated for 17 canned fish products. The IgE-binding capacity was patient-, species- as well as product-specific. Generally, the lowest frequency and intensity of IgE-binding was discovered for canned tuna. A low abundance and resistance to degradation of tuna proteins might explain the lower IgE-binding capacity. The only clinical study ever conducted for canned fish, nearly 30 years ago, demonstrated tolerance to canned tuna by 45 orally challenged children and adults, including 18 fish-allergic patients.[20] This report might be the foundation for the current recommendation of implementing canned fish in the diet of fish-allergic patients.

However, our study demonstrated considerable IgE-binding to canned salmon, sardine and even tuna, suggesting that they could trigger allergic reactions in fish-allergic patients. However, there seem to be considerable differences in the type and abundance of different fish allergens. The major fish allergen PV was not detected in any of the canned fish by the commercial or in-house anti-PV antibody, however these allergens were detected by mass spectrometry. Furthermore, PVs in canned salmon, sardine and tuna demonstrated IgE-binding in a similar frequency as the PVs in the corresponding in-house cooked fish. The in-house cooked fish represents the conventional heating preparation at home, and seems to be much less destructive on protein structures, in contrast to the much harsher canning process (retorting) of fish. Within all canned fish products, canned salmon had the highest frequency of strong IgE-binding to PV. The observed IgE-binding to PV in our study differs from previous reports for mackerel PV, suggesting species-specific stability. Kubota *et al.* described a 50% decrease of IgE-binding after heat treatment at 80 °C and complete loss at 140 °C.[23] The latter was also reported by Sletten *et al.* for yellowfin tuna, which appears to be different for skipjack tuna, the tuna species most frequently used for canning.[15]

Both allergens, parvalbumin and tropomyosin, appear to be the major IgE-binding proteins in canned fish as suggested by IgE-immunoblotting and subsequent mass spectrometric analyses. Tropomyosin was recently identified in our studies as major fish allergen of unrecognised importance (see **Chapter V A**). Importantly, TM demonstrated more frequent and stronger IgE binding in tuna extracts as compared to PV, highlighting its relevance also for tuna-allergic individuals.

Other proteins identified in this study with potential relevance for IgE-binding and resulting clinical reactivity include myosin heavy chain, myosin light chain and collagen.

5. Concluding remarks

We demonstrate in our comprehensive study for the first time that canned fish may not be safe to all fish allergic patients. Canned fish and its components should be further investigated and implemented in *in vitro* and *in vivo* diagnostic tools. An oral food challenge should be considered before incorporating canned fish into the diet of individuals with fish allergy.

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Table 1. Demographic and clinical characteristics of 53 fish-allergic patients recruited for the study.

Patient			sIgE level (kU/l)			Skin prick test (mm)			Clinical history	
ID	Sex	Age (yrs)	cod	tuna	salm-on	cod	tuna	salm-on	Implicated fish	Symptoms
1*	F	7	0.05	1.27	0.58	0.0	0.0	0.0	snapper, tuna	AE, GIS, U
2	F	5	-	-	-	6.0	0.0	5.5	salmon	GIS, U
3*	M	4	-	-	-	5.0	3.0	4.0	trout, tuna	AN, OAS
4	M	10	3.79	1.17	8.09	8.5	4.5	11.0	catfish, salmon, tuna	RD, U
5	M	7	-	-	-	4.0	3.5	3.0	salmon, white fish	AE, C, RD
6	M	5	-	-	-	0.0	4.0	0.0	bream	AE, U
7	F	5	-	-	-	4.5	3.5	5.5	snapper	AE, U
8	M	15	-	-	-	7.0	0.0	3.0	white fish	AE, U
9	F	4	-	-	-	4.0	5.0	5.0	salmon	U
10	F	8	-	-	-	6.5	0.0	4.0	catfish	U, OAS
11	M	18	-	-	-	5.5	0.0	4.5	catfish, white fish	GIS
12	M	14	1.29	1.53	3.96	13.5	0.0	0.0	snapper	AE, OAS
13*	M	5	-	-	-	10.5	5.0	8.5	salmon, sardine	AN, U
14	M	9	-	-	-	0.0	0.0	0.0	unknown	AE, U
15	F	7	-	-	-	5.0	6.0	3.0	catfish	GIS, U
16	M	9	-	-	-	12.5	0.0	6.5	unknown, white bait, white fish	AE, C, U
17	M	10	-	-	-	4.0	4.5	5.0	ling	AE, OAS
18	M	1	-	-	-	7.0	3.0	6.0	Asian seabass, salmon, white fish	AE, OAS, U
19*	M	5	-	-	-	6.5	0.0	5.0	bream, cod	U
20*	M	5	-	-	-	4.5	4.5	4.5	cod, white fish	AN, GIS, RD
21	F	13	-	-	-	3.5	4.5	0.0	Asian seabass, catfish	GIS, RD, U
22	F	10	-	-	-	0.0	0.0	0.0	salmon, white fish	AE, GIS, U
23	M	11	88.50	4.83	25.30	8.5	4.0	12.0	tuna, white fish	AE, GIS, OAS, U
24	M	14	-	-	-	5.5	6.0	0.0	Asian seabass	AN
25	F	5	-	-	-	6.0	0.0	5.5	salmon	GIS, U
26	M	9	-	-	-	5.5	2.0	7.5	salmon, unknown	U
27	M	10	-	-	-	4.5	3.0	4.0	white fish	AN, RD, U

28	F	8	-	-	-	3.5	0.0	2.5	unknown	GIS, U
29	M	10	<0.01	0.01	<0.01	0.0	2.5	0.0	Asian seabass	OAS, U
30	M	7	2.29	0.10	0.64	8.0	0.0	5.0	white fish	OAS, U
31	M	13	2.99	1.99	1.24	8.0	2.5	4.5	white fish	OAS, U
32	M	14	0.10	0.28	0.21	2.0	2.5	0.0	salmon	AE
33	M	3	0.02	0.03	0.03	0.0	4.0	0.0	catfish	AE, U
34	F	12	2.66	1.41	3.43	13.5	4.0	11.0	white fish	OAS, U
35	F	2	0.14	0.78	0.60	0.0	0.0	3.5	salmon	AE, U
36	M	1	-	-	-	2.5	0.0	4.5	catfish	AE
37	M	12	10.00	5.91	9.01	9.5	9.0	8.0	Asian seabass, salmon	U
38	M	9	7.25	2.15	5.33	10.0	5.5	3.0	trevally	AN, RD, U
39	M	11	0.11	0.14	0.11	3.5	0.0	0.0	white fish	AE, AN, RD, U
40	F	1	5.47	5.14	5.58	6.5	0.0	3.0	Asian seabass	AE, RD, U
41	M	11	92.10	39.50	66.10	14.0	5.0	4.5	salmon	AE
42	M	8	11.50	4.67	9.80	4.5	4.5	2.0	salmon, tilapia	OAS
43	M	15	18.70	12.10	17.60	15.0	5.5	8.5	ling	AE, OAS
44	F	2	90.40	31.00	73.40	13.5	5.5	13.5	cod	AE, U
45	M	8	75.00	21.00	70.50	14.0	6.5	12.0	white fish	GIS, RD, U
46	F	10	16.50	7.33	9.08	7.0	2.0	3.5	white fish	AE, OAS, RD
47	F	6	-	-	-	8.0	3.0	4.5	salmon, white fish	AE
48	M	9	0.13	0.06	0.46	3.0	2.0	6.5	croaker	AE
49	M	2	-	-	-	7.5	4.0	7.5	salmon	U
50	F	10	0.21	0.15	0.14	3.5	0.0	0.0	catfish, white fish	GIS, RD, OAS
51	M	5	10.20	6.78	11.50	6.5	5.0	6.0	leather jacket	AE
52	M	7	1.81	0.70	7.63	4.5	0.0	6.0	trout	AE, OAS, U
53	F	7	2.87	1.19	3.08	4.5	2.5	0.0	white fish	U

Note: Symptoms are listed in abbreviations. A = asthma; AN = anaphylaxis; AE = angioedema (lip swelling); E = erythema; GIS = gastrointestinal syndrome (vomiting); H = hypotension (low blood pressure); OAS = oral allergy syndrome (itchy mouth or swelling throat); R = rhinitis (irritation inside nose, sneezing); RD = respiratory distress (breathing difficulty); U = urticaria (hives, rash). "–" = Not performed. *Patients selected for pooled serum.

Table 2. List of 17 analysed canned fish products from nine different manufactures.

Code	Labelled fish	Species (labelled)	Species (speculated)	Product name	Brand	Processed type	Product origin	Fish caught origin
I-1	Pink salmon	-	*Oncorhynchus gorbuscha*	Pink Salmon	Homebrand	Salt	USA	-
I-2*	Pink salmon	-	*Oncorhynchus gorbuscha*	Wild caught Pink Salmon	Select	Salt	Canada	-
I-3	Pink salmon	-	*Oncorhynchus gorbuscha*	Wild Alaskan pink salmon	John West	Salt	USA	Alaska
I-4	Red Salmon	-	*Oncorhynchus nerka*	Wild Alaskan red salmon	John West	Salt	USA	Alaska
I-5	Pink salmon	-	*Oncorhynchus gorbuscha*	Wild Alaskan pink salmon	Paramount	Salt	USA	Alaska
I-6	Red Salmon	-	*Oncorhynchus nerka*	Wild Alaskan red salmon	Paramount	Salt	USA	Alaska
I-7*	Pink salmon	-	*Oncorhynchus gorbuscha*	Tempers Salmon, springwater	John West	Springwater, salt, natural color (paprika oleoresins)	Thailand	-
I-8	Salmon	-	-	Premium Salmon in springwater	Safcol	springwater, salt, natural color (160c)	Thailand	-
T-1*	Skipjack tuna	*Katsuwonus pelamis*	-	Tuna chunks in Springwater	Homebrand	Springwater	Thailand	The Western Pacific Ocean
T-2	Skipjack tuna	*Katsuwonus pelamis*	-	Tuna chunks in Springwater	Select	Springwater	Thailand	The South-West Atlantic Ocean or Western Pacific Ocean
T-3*	Yellowfin tuna	*Thunnus albacares*	-	Yellowfin tuna in Springwater	Woolworth	Springwater, salt	Thailand	-
T-4	Skipjack tuna	*Katsuwonus pelamis*	-	Chunk style tuna in springwater	John West	Springwater, salt	Thailand	-

T-5	Skipjack tuna	*Katsuwonus pelamis*	-	Tuna chunks in Springwater	Greenseas	Springwater, salt	Thailand	The Western Central Pacific Ocean
T-6	Tuna	Thunnus	*Thunnus* spp.	Tuna	Sirena	Springwater	Indonesia	
T-7	Skipjack tuna	*Katsuwonus pelamis*	-	Responsibly fished Tuna	Safcol	Springwater	Thailand	The Western Pacific Ocean
S-1	Sardine	-	*Sardinella* spp.	Sardine in Spring Water	Homebrand	Springwater, salt	Thailand	-
S-2*	Sardine	-	*Clupea harengus*	Wild Sardines in spring water	Pure Canadian Brunswick	-	Canada	-

Note: All the information listed were extracted from the product labels. "–" = No information provided. * Canned fish products selected for further analyses.

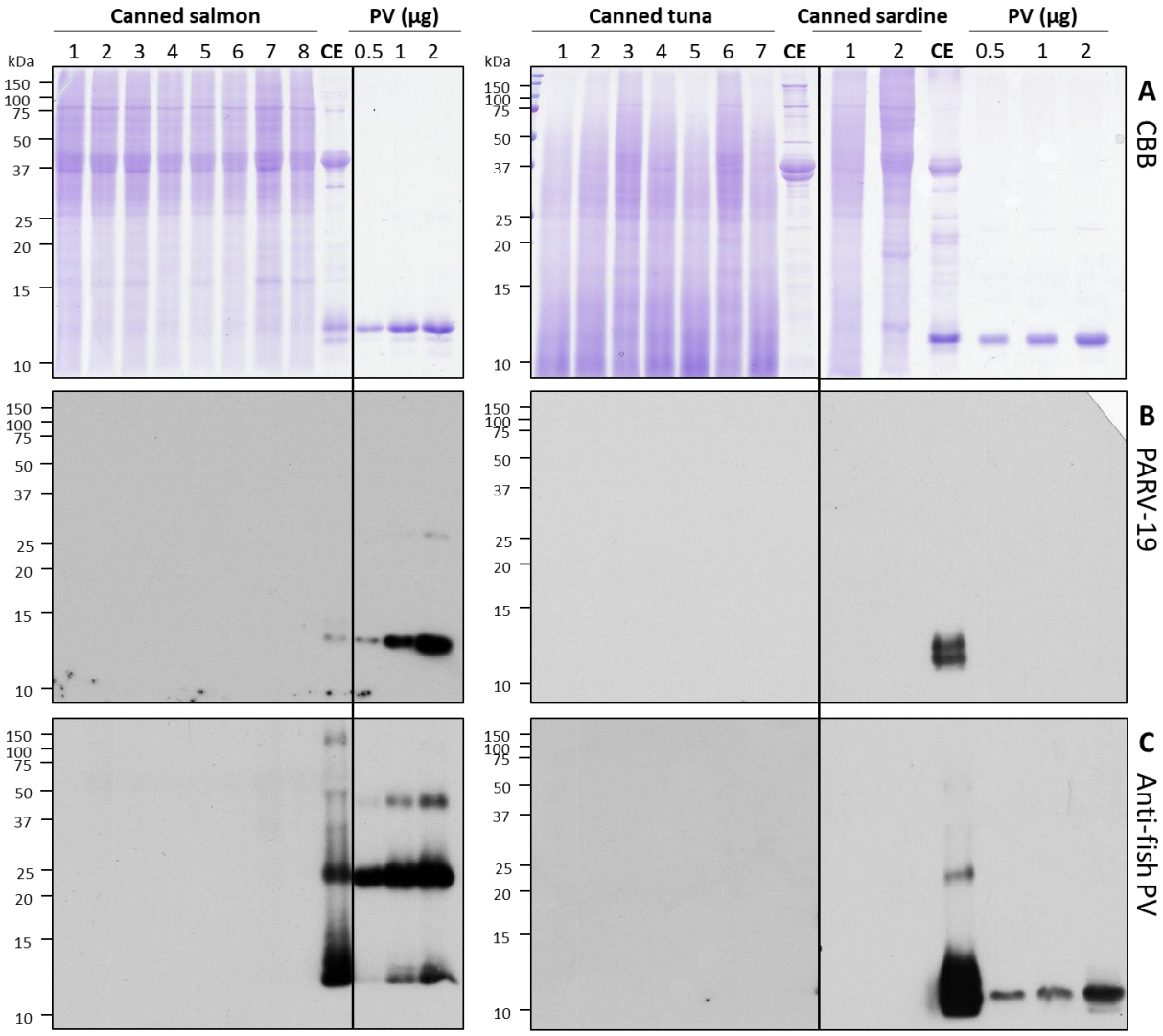


Figure 1. SDS-PAGE protein profile and parvalbumin-specific antibody reactivity of 17 different canned fish extracts. The canned fish extracts from three fish groups were resolved by SDS-PAGE (16%; panel **A**) to determine the protein profiles and analysed for the presence of parvalbumin (PV) by immunoblotting with monoclonal PARV-19 antibody (panel **B**) and rabbit anti-salmon PV or anti-Asian seabass PV antibodies (panel **C**). Cooked fish extracts (CE) from each Atlantic salmon, yellowfin tuna and sardine were used as references. Purified PVs from Atlantic salmon (against canned salmon) and Atlantic cod (against canned tuna and sardine) were applied as positive and quantitative controls in three incrementing amounts (0.5, 1 and 2 µg). The exposure time for the enhanced chemiluminescence (ECL) detection was adjusted for each membrane. The numbers represent the different canned fish products listed in Table 2.

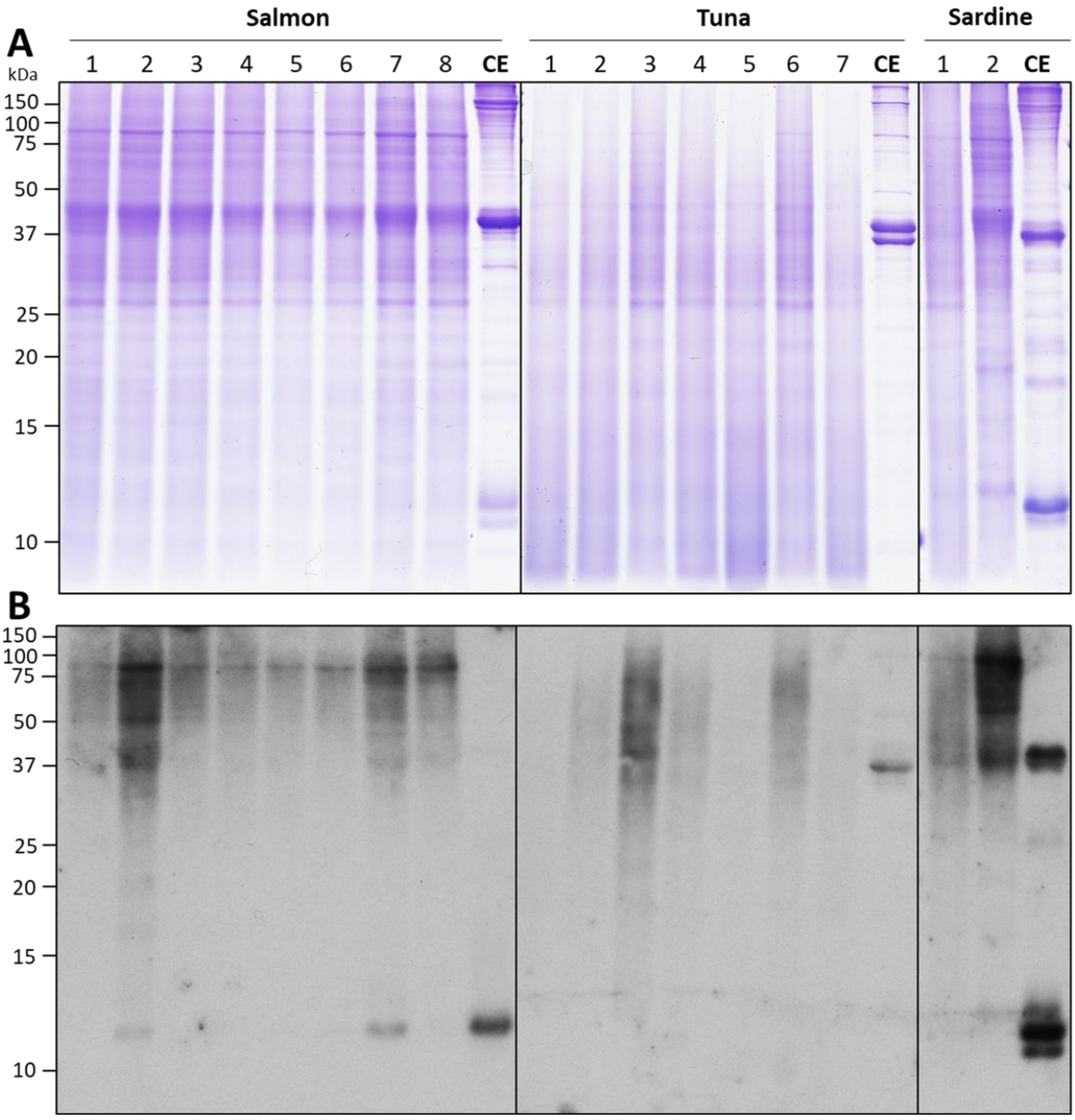


Figure 2. IgE-binding of pooled patient sera to canned and cooked fish proteins. The extracts from 17 canned fish and three cooked fish (CE) were resolved by SDS-PAGE (16%; panel **A**) and the IgE-binding of five patients (pooled sera) analysed by immunoblotting (panel **B**). The numbers represent the different canned fish products listed in Table 2.

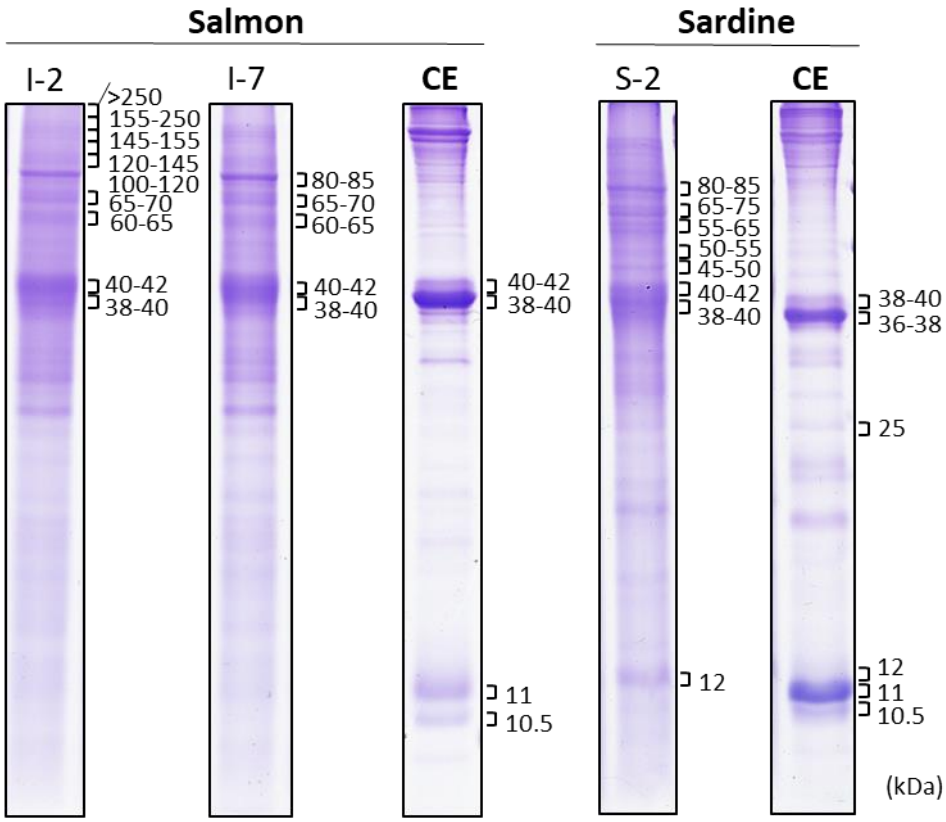


Figure 3. The identification of IgE-binding salmon and sardine proteins by mass spectrometry analysis. The extracts from canned and cooked salmon (I-2, I-7 and CE) and sardine (S-2 and CE) were resolved by SDS-PAGE (16%) and the identity of IgE-binding proteins determined by mass spectrometric analyses after tryptic digestion of the corresponding bands from the immunoblots. The excised bands or sections are indicated with molecular weights on the right hand side. The numbers above the lanes represent the different canned fish products listed in Table 2.

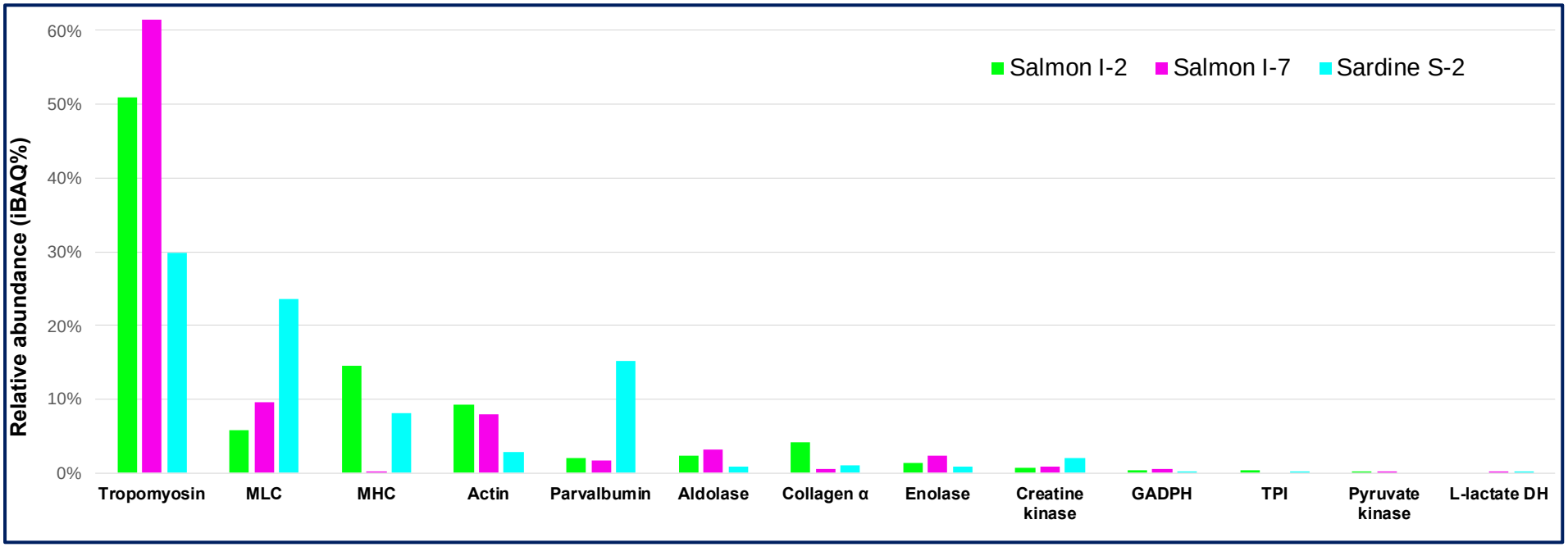


Figure 4. The in-solution mass spectrometric analysis of relative protein abundance in three select canned fish extracts. The relative protein abundance (iBAQ%) in tryptic-digested canned salmon (I-2 and I-7) and canned sardine (S-2) extracts was analysed by mass spectrometry. The iBAQ% value represents the relative abundance of each protein including several isoforms. The relative abundance was determined for proteins which demonstrated IgE-binding. Note: DH = dehydrogenase; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; MLC = myosin light chain; MHC = myosin heavy chain; TPI = triosephosphate isomerase.

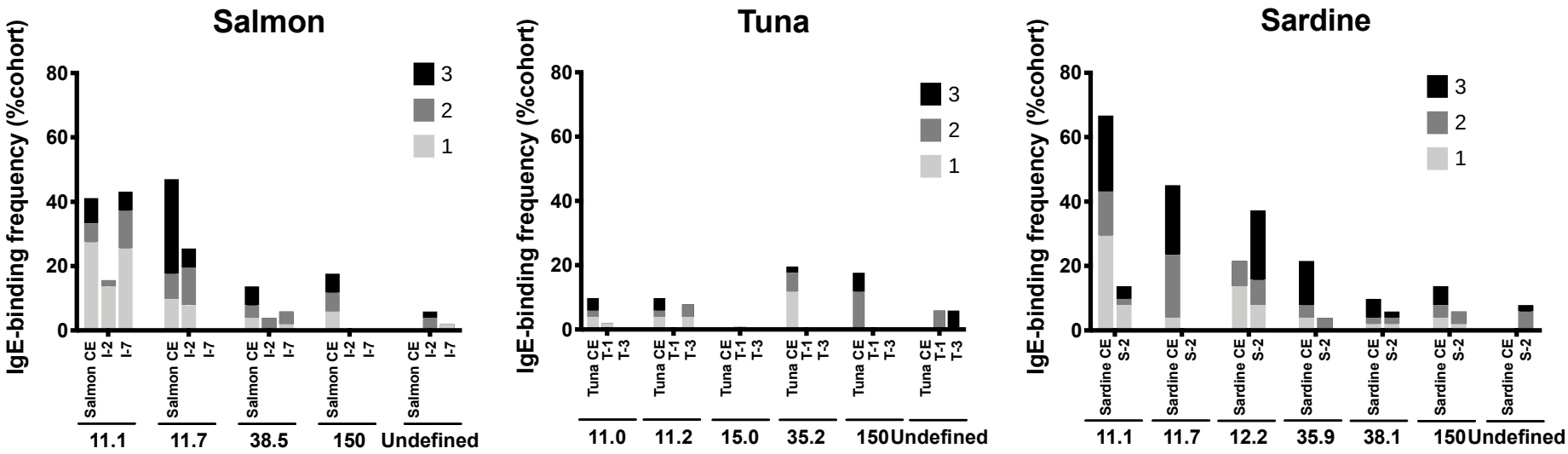


Figure 5. Characterisation of IgE binding of 53 fish-allergic patients to canned and cooked fish proteins. The distribution (%) of patients with different IgE-binding intensity (1 = weak; 2 = moderate; 3 = strong, classified based on densitometric analysis) to each of the major IgE-binding protein bands, based on molecular weight. The canned fish products were selected based on IgE binding intensity of pooled serum samples (see also Figure 2; I-2, I-7, T-1, T-3 and S-2) and compared to in-house generated cooked fish extracts (salmon, tuna and sardine). See Supplementary Figures 1 to 3 for the corresponding IgE immunoblots.

A - Parvalbumin (PV)

Accession I.D.	PV	Canned	#	1					
XP_029500831.1	β-1	Salmon	1	100	2				
XP_031435403.1	β	Sardine	2	70	100	3			
XP_012694365.1	β-2	Sardine	3	72	73	100	4		
XP_029540956.1	β-2	Salmon	4	70	71	75	100	5	
CBA73697.1	β-2	Sardine	5	71	72	77	81	100	6
XP_012694367.1	β-2	Sardine	6	72	71	74	78	80	100

B - Tropomyosin (TM)

Accession I.D.	TM	Canned	#	1					
XP_031441857.1	α-4	Sardine	1	100	2				
XP_012678334.1	α-3	Sardine	2	74	100	3			
XP_020360961.1	α-3	Salmon	3	75	90	100	4		
XP_012697822.1	α-1	Sardine	4	72	84	87	100	5	
XP_029523883.1	α-1	Salmon	5	72	84	89	92	100	6
XP_021457286.1	β	Salmon	6	73	84	87	86	89	100

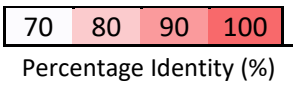


Figure 6. Conservation analysis of fish parvalbumin and tropomyosin. Sequence identity matrix was performed for parvalbumin and tropomyosin isoforms identified in the canned salmon and sardine products by mass spectrometry analysis after in-solution tryptic digestion.

Supplementary Table 1. Identity of IgE-binding proteins in canned and heated salmon extracts based on mass spectrometric analyses.

Extract	MW	Hit rank	NCBI ID	Protein	iBAQ%	MW (kDa)
I-2	40-42	1	XP_024234568.1	**troponin T, fast skeletal muscle isoforms-like isoform X4**	**56.5%**	27
I-2	40-42	2	XP_024244208.1	actin, aortic smooth muscle-like	23.0%	47
I-2	40-42	3	XP_020336021.1	tropomyosin alpha-1 chain-like isoform X3	5.9%	33
I-2	38-40	1	XP_031641992.1	**glyceraldehyde-3-phosphate dehydrogenase**	**54.7%**	36
I-2	38-40	2	BAC00871.1	myosin heavy chain	15.0%	222
I-2	38-40	3	XP_024244208.1	actin, aortic smooth muscle-like	12.0%	47
I-2	80-85	1	XP_024244208.1	**actin, aortic smooth muscle-like**	**83.7%**	47
I-2	80-85	2	CDQ67811.1	myosin-6	8.4%	217
I-2	80-85	3	XP_024293365.1	beta-enolase	5.4%	47
I-2	65-70	1	XP_024244208.1	**actin, aortic smooth muscle-like**	**29.0%**	47
I-2	65-70	2	XP_029493065.1	myosin light chain 1, skeletal muscle isoform-like	27.5%	21
I-2	65-70	3	BAC00871.1	myosin heavy chain	13.6%	222
I-7	60-65	1	XP_024244208.1	**actin, aortic smooth muscle-like**	**26.0%**	47
I-7	60-65	2	XP_029493065.1	myosin light chain 1, skeletal muscle isoform-like	23.0%	21
I-7	60-65	3	BAC00871.1	myosin heavy chain	15.3%	222
I-7	> 250	1	XP_031689743.1	**collagen alpha-1(I) chain-like**	**22.7%**	137
I-7	> 250	2	BAC00871.1	myosin heavy chain	20.5%	222
I-7	> 250	3	XP_031644762.1	collagen alpha-2(I) chain isoform X3	18.2%	125
I-7	155-250	1	XP_024244208.1	**actin, aortic smooth muscle-like**	**52.9%**	47
I-7	155-250	2	XP_031641992.1	glyceraldehyde-3-phosphate dehydrogenase	33.8%	36
I-7	155-250	3	XP_029493065.1	myosin light chain 1, skeletal muscle isoform-like	10.2%	21
I-7	145-155	1	BAC00871.1	**myosin heavy chain**	**36.8%**	222
I-7	145-155	2	XP_031689743.1	collagen alpha-1(I) chain-like	23.3%	137
I-7	145-155	3	XP_029493065.1	myosin light chain 1, skeletal muscle isoform-like	20.1%	21

I-7	120-145	1	XP_024244208.1	**actin, aortic smooth muscle-like**	**29.2%**	47
I-7	120-145	2	XP_031689743.1	collagen alpha-1(I) chain-like	25.7%	137
I-7	120-145	3	XP_029493065.1	myosin light chain 1, skeletal muscle isoform-like	17.7%	21
I-7	100-120	1	BAC00871.1	**myosin heavy chain**	**20.8%**	222
I-7	100-120	2	XP_031644762.1	collagen alpha-2(I) chain isoform X3	14.4%	125
I-7	100-120	3	XP_031689743.1	collagen alpha-1(I) chain-like	13.1%	137
I-7	65-70	1	BAC00871.1	**myosin heavy chain**	**30.1%**	222
I-7	65-70	2	XP_031689743.1	collagen alpha-1(I) chain-like	15.4%	137
I-7	65-70	3	XP_031644762.1	collagen alpha-2(I) chain isoform X3	14.1%	125
I-7	60-65	1	BAC00871.1	**myosin heavy chain**	**27.3%**	222
I-7	60-65	2	XP_024244208.1	actin, aortic smooth muscle-like	25.9%	47
I-7	60-65	3	XP_021457286.1	tropomyosin beta chain isoform X1	9.6%	33
I-7	40-42	1	XP_021457286.1	**tropomyosin beta chain isoform X1**	**47.0%**	33
I-7	40-42	2	XP_021419210.1	troponin T, cardiac muscle-like isoform X6	15.4%	28
I-7	40-42	3	BAC00871.1	myosin heavy chain	14.3%	222
I-7	38-40	1	XP_031641992.1	**glyceraldehyde-3-phosphate dehydrogenase**	**62.4%**	36
I-7	38-40	2	XP_021464133.1	myosin light chain 3, skeletal muscle isoform-like	21.5%	17
I-7	38-40	3	XP_024244208.1	actin, aortic smooth muscle-like	7.9%	47
Salmon CE	10.5	1	CBA35349.1	**parvalbumin beta-1**	**60.4%**	12
Salmon CE	10.5	2	Q91483.3	parvalbumin beta 2	21.8%	11
Salmon CE	10.5	3	ACH70862.1	parvalbumin 2	6.6%	12
Salmon CE	11	1	Q91483.3	**parvalbumin beta 2**	**94.6%**	11
Salmon CE	11	2	CBA35349.1	parvalbumin beta-1	5.0%	12
Salmon CE	11	3	XP_014005667.1	ATP synthase-coupling factor 6, mitochondrial-like	0.2%	12
Salmon CE	38-40	1	NP_001117128.1	**tropomyosin alpha-1 chain**	**96.7%**	32
Salmon CE	38-40	2	XP_013979548.1	fast myotomal muscle troponin-T-1 isoform X3	2.3%	27
Salmon CE	38-40	3	XP_014059932.1	collagen alpha-1(I) chain	0.6%	137

Salmon CE	40-42	1	NP_001117128.1	**tropomyosin alpha-1 chain**	**99.8%**	32
Salmon CE	40-42	2	XP_014032072.1	troponin T, fast skeletal muscle isoforms-like	0.1%	22
Salmon CE	40-42	3	XP_014036616.1	leukocyte elastase inhibitor-like	0.1%	43

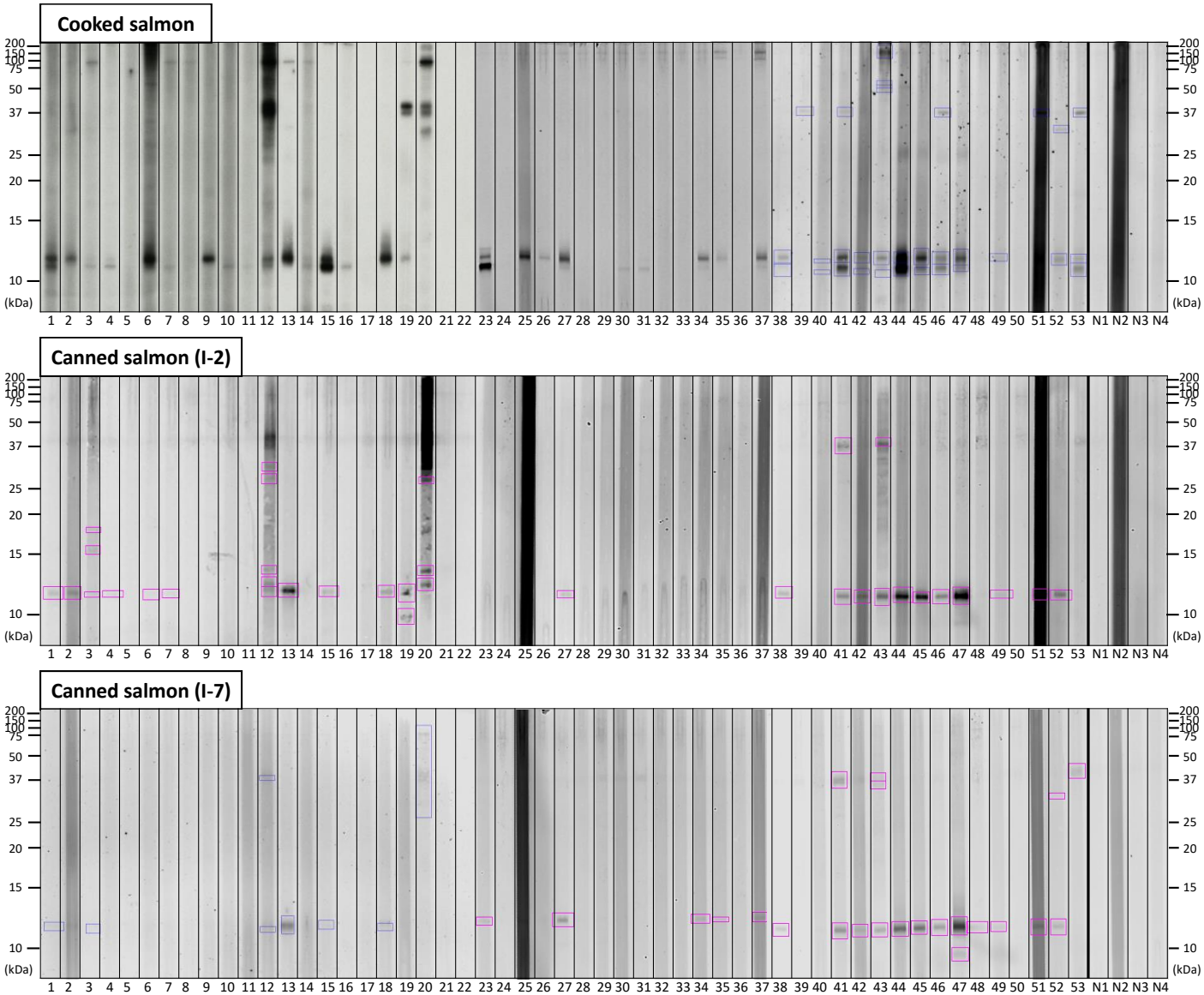
Note: CE = cooked extract, MW = molecular weight of excised protein bands. iBAQ% is an indicator for the realtive abundance of the identified protein. NCBI = National Center for Biotechnology Information. IDs are accessable via www.ncbi.nlm.nih.gov/protein

Supplementary Table 2. Identity of IgE-binding proteins in canned and heated sardine extracts based on mass spectrometric analyses.

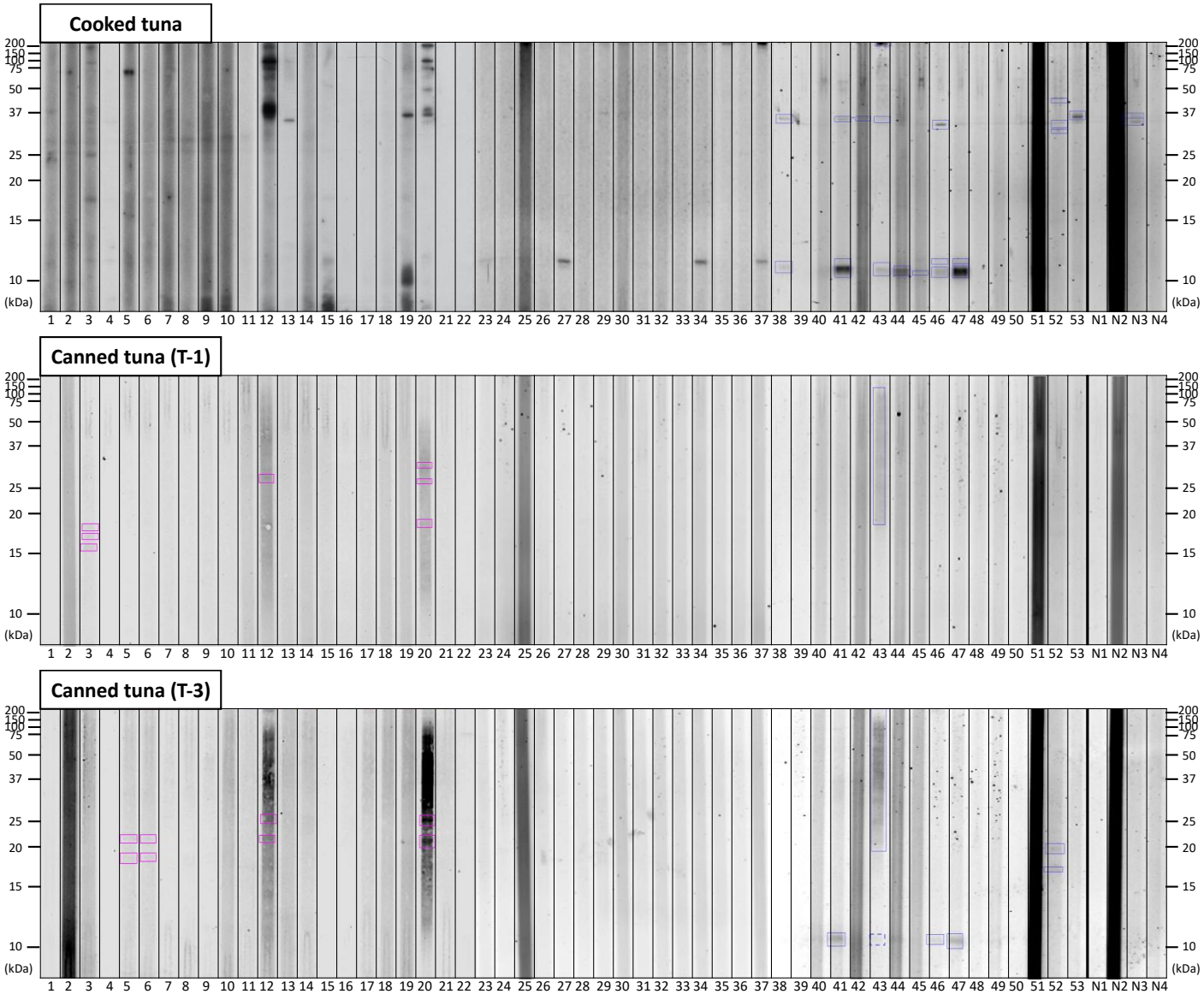
Extract	MW (range)	Hit rank	NCBI ID	Protein	iBAQ%	MW (kDa)
S-2	12	1	XP_012694365.1	**parvalbumin beta 2 isoform X1**	**84.6%**	12
S-2	12	2	XP_012691903.1	myosin light chain 1/3, skeletal muscle isoform	4.2%	22
S-2	12	3	CBA73697.1	parvalbumin beta-2	3.1%	12
S-2	80-85	1	XP_031417638.1	**myosin heavy chain, fast skeletal muscle**	**33.3%**	398
S-2	80-85	2	XP_012694365.1	parvalbumin beta 2 isoform X1	13.4%	12
S-2	80-85	3	XP_031439915.1	myosin-7-like	10.3%	223
S-2	65-75	1	XP_031417638.1	**myosin heavy chain, fast skeletal muscle**	**28.0%**	398
S-2	65-75	2	XP_012697822.1	tropomyosin alpha-1 chain	16.4%	33
S-2	65-75	3	XP_031439915.1	myosin-7-like	14.1%	223
S-2	55-65	1	XP_031417638.1	**myosin heavy chain, fast skeletal muscle**	**21.4%**	398
S-2	55-65	2	XP_012694365.1	parvalbumin beta 2 isoform X1	16.4%	12
S-2	55-65	3	XP_031439915.1	myosin-7-like	12.6%	223
S-2	40-42	1	XP_012697822.1	**tropomyosin alpha-1 chain**	**78.2%**	33
S-2	40-42	2	XP_031417638.1	myosin heavy chain, fast skeletal muscle	5.4%	398
S-2	40-42	3	XP_031421293.1	troponin T, fast skeletal muscle isoforms-like isoform X6	3.8%	30
S-2	38-40	1	XP_012697822.1	**tropomyosin alpha-1 chain**	**73.8%**	33
S-2	38-40	2	XP_012678334.1	tropomyosin alpha-3 chain isoform X5	5.7%	33
S-2	38-40	3	XP_031417638.1	myosin heavy chain, fast skeletal muscle	4.8%	398
S-2	50-55	1	XP_012697822.1	**tropomyosin alpha-1 chain**	**20.9%**	33
S-2	50-55	2	XP_012694365.1	parvalbumin beta 2 isoform X1	19.9%	12
S-2	50-55	3	XP_031417638.1	myosin heavy chain, fast skeletal muscle	13.4%	398
S-2	45-50	1	XP_012697822.1	**tropomyosin alpha-1 chain**	**30.0%**	33
S-2	45-50	2	XP_031421293.1	troponin T, fast skeletal muscle isoforms-like isoform X6	22.7%	30
S-2	45-50	3	XP_031417638.1	myosin heavy chain, fast skeletal muscle	10.9%	398

Sardine CE	10.5	1	XP_012694365.1	**parvalbumin beta 2 isoform X1**	**67.5%**	9
Sardine CE	10.5	2	CAQ68366.1	parvalbumin	32.5%	12
Sardine CE	10.5	3	XP_012695337.1	ATP synthase subunit alpha, mitochondrial	0.0%	60
Sardine CE	11	1	CAQ68366.1	**parvalbumin**	**99.9%**	12
Sardine CE	11	2	XP_028827318.1	actin, alpha skeletal muscle 2-like	0.0%	51
Sardine CE	11	3	BAA95140.1	myosin light chain 2	0.0%	19
Sardine CE	12	1	CAQ68366.1	**parvalbumin**	**98.4%**	12
Sardine CE	12	2	XP_028838185.1	creatine kinase M-type	0.4%	43
Sardine CE	12	3	BAA95139.1	myosin light chain 3	0.3%	18
Sardine CE	25	1	BAA95138.1	**myosin light chain 1**	**72.4%**	22
Sardine CE	25	2	XP_012683438.1	myosin light chain 4	10.7%	21
Sardine CE	25	3	CBE67080.1	parvalbumin beta-1, partial	6.9%	9
Sardine CE	36-38	1	XP_028856060.1	**tropomyosin alpha-4 chain isoform X3**	**56.7%**	33
Sardine CE	36-38	2	XP_028827277.1	tropomyosin beta chain isoform X1	20.2%	33
Sardine CE	36-38	3	XP_028814825.1	tropomyosin alpha-1 chain isoform X1	11.8%	33
Sardine CE	38-40	1	XP_028856060.1	**tropomyosin alpha-4 chain isoform X3**	**78.7%**	33
Sardine CE	38-40	2	XP_031420331.1	tropomyosin alpha-1 chain-like isoform X6	11.6%	33
Sardine CE	38-40	3	XP_028827277.1	tropomyosin beta chain isoform X1	4.0%	33

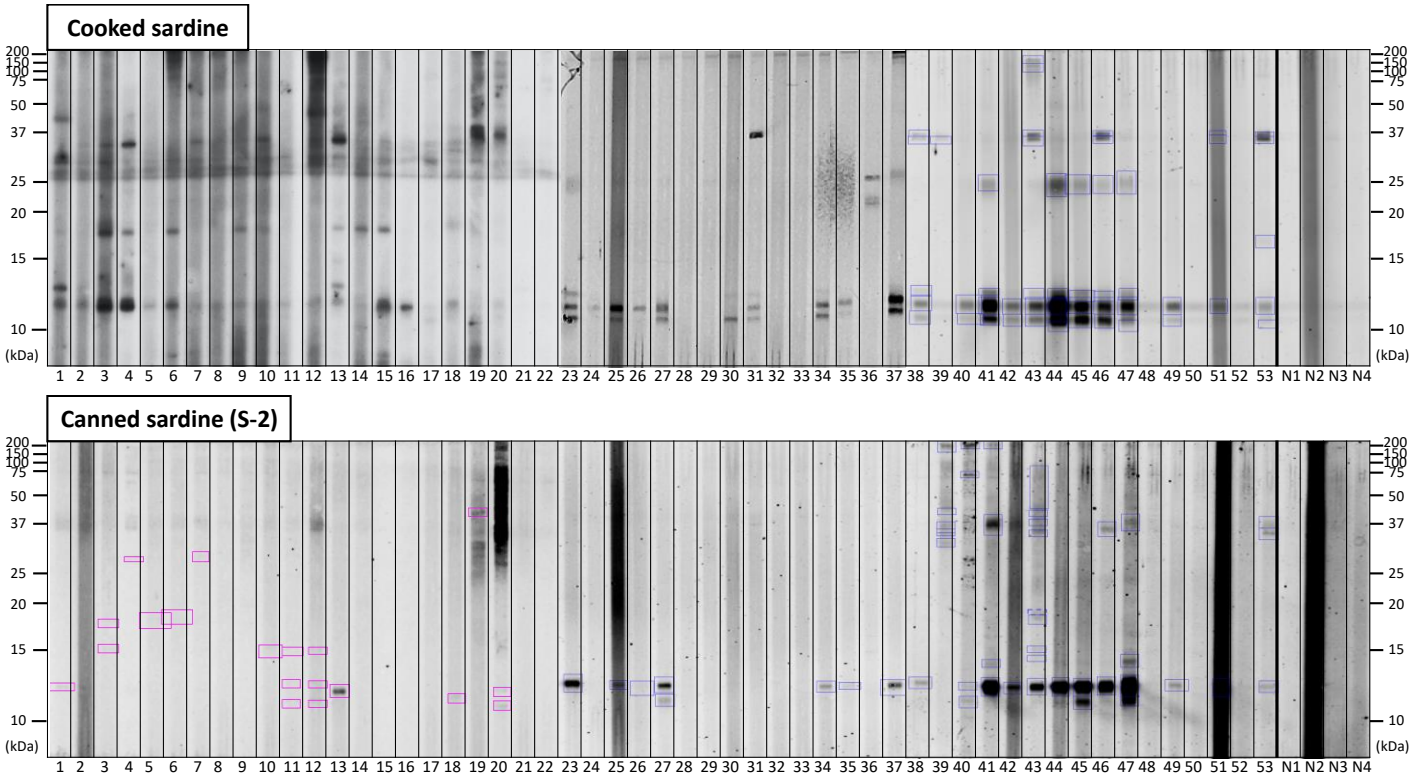
Note: CE = cooked extract, MW = molecular weight of excised protein bands. iBAQ% is an indicator for the realtive abundance of the identified protein. NCBI = National Center for Biotechnology Information. IDs are accessable via www.ncbi.nlm.nih.gov/protein



Supplementary Figure 1. IgE binding profile of 52 fish-allergic patients to selected canned and cooked salmon fish extracts.
See Table 1 for patient's characterstics.



Supplementary Figure 2. IgE binding profile of 52 fish-allergic patients to selected canned and cooked tuna fish extracts.
See Table 1 for patient's characterstics.



Supplementary Figure 3. IgE binding profile of 52 fish-allergic patients to canned and cooked sardine fish extracts.
See Table 1 for patient's characterstics.

Further results and discussion not included in the manuscript in preparation

In the above manuscript, 17 canned fish products were investigated for their protein content and IgE-binding capacity using serum from 53 fish-allergic patients. The observed high IgE-binding capacity of some products suggests that canned fish is not generally safe for fish-allergic individuals.

However, further analyses are required to demonstrate cellular and *in vivo* reactivity of canned fish. In addition, correlations between observed IgE-binding and patients' detailed clinical history, including reported tolerance to specific fish species and preparations (see **Appendix C Table 1**), should be identified. This might assist in the development of tools to predict tolerance to selected canned fish products.

A complete loss of IgE-binding was previously reported for Pacific mackerel parvalbumin when heated under pressure at 140°C, which is simulating treatment during the canning process.[1] In canned products of closely related tuna species, IgE-binding to parvalbumin was rare in the current study. In contrast, salmon and sardine parvalbumin appeared to retain their IgE-binding capacity after extensive processing. However, the relative abundance of parvalbumin was significantly lower in canned salmon and sardine extracts (1.7-15.2%) as compared to conventional heated/cooked fish extracts (14.3-88.1%, see **Chapter V A**). Importantly, tropomyosin was the most abundant protein in canned salmon and sardine. Both tropomyosin and parvalbumin seem to form stable aggregates/amyloids during processing. A high degradation resistance and retained immunoreactivity was recently suggested for fish parvalbumin aggregates/amyloids.[2,3]

In addition to the above presented mass spectrometric analyses for salmon and sardine preparations, preliminary data were generated for tuna. However, the utilised database for all bony fish (www.ncbi.nlm.nih.gov/protein), containing 5,233,788 amino acid sequences, results in a potential lack of unique peptides, which is needed for reliable results. **Figure III B 1** below shows the predicted relative abundance of putative fish allergens in two canned tuna products. Importantly, no parvalbumin was detected, which might explain the observed absence of binding by parvalbumin-specific and patient IgE antibodies. In both extracts, the highest relative abundance was observed for glyceraldehyde-3-phosphate dehydrogenase, which is considerably different from findings for salmon and sardine in the above manuscript (see **Figure 4**). This difference could originate from the use of the above-mentioned database containing sequences of all bony fish, which is not sufficient for tuna. The generated mass spectrometric data will be reanalysed once a suitable tuna-specific database is available. Dr Adam Ciezarek (Earlham Institute, Norwich, UK) recently provided me with the assembled transcriptomes from all tuna species, which are yet to be annotated.

This study suggests that both tropomyosin and parvalbumin are the major allergens in canned fish products. The importance of tropomyosin as a fish allergen is discussed in detail in **Chapter V A**. In the preceding **Chapter IV**, parvalbumin is identified and characterised in muscle tissue from 33 Asia-Pacific fish species with global relevance.

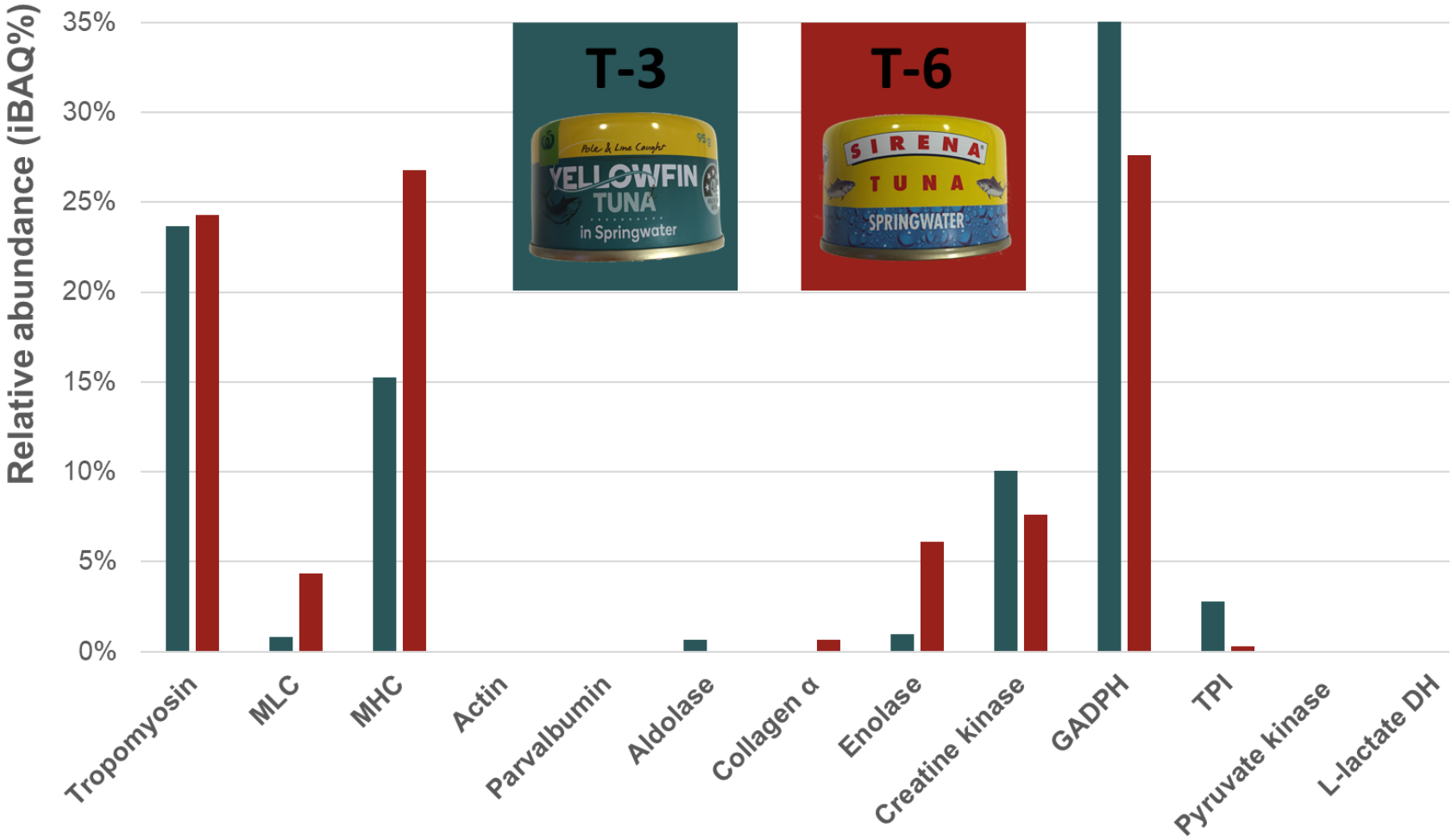


Figure III B 1: Predicted relative protein abundance (iBAQ%) in two canned tuna extracts (T-3 (left) and T-6 (right) in Table 2 of the above manuscript) based on preliminary data from mass spectrometric analysis after in-solution tryptic digestion. The iBAQ% value is based on analyses with MaxQuant and represents the relative abundance of each protein, including several isoforms. The relative abundance was determined for proteins which demonstrated IgE-binding in previous studies. The order of protein listing corresponds to Figure 4 of the above manuscript. Note: DH, dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MLC, myosin light chain; MHC, myosin heavy chain; TPI, triosephosphate isomerase.

References for further results and discussion

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I

Literature review

A comprehensive review of fish and shellfish allergens

Mol Immunol 2018

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II

Skin prick test preparations

A comparative biochemical and immunological analysis

Allergy 2019

Page 67 *ff*

III A

Commercial fish ELISA kits

Limited capacity to detect different fish and their products

J Sci Food Agric 2020

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III B

Canned fish

A safe alternative for fish allergy sufferers?

Mol Nutr Food Res (in prep.)

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Diagnostics

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IV

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Characterisation of parvalbumin in 33 Asia-Pacific fish

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V A

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V B

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J Allergy Clin Immunol Pract 2019

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VI A

Tolerance to sharks/rays

Bony fish-allergic patients can tolerate cartilaginous fish

J Allergy Clin Immunol Pract 2018

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VI B

Cross-reactivity to reptiles

Parvalbumin is also an allergen in amphibians and reptiles

Allergy (in prep.)

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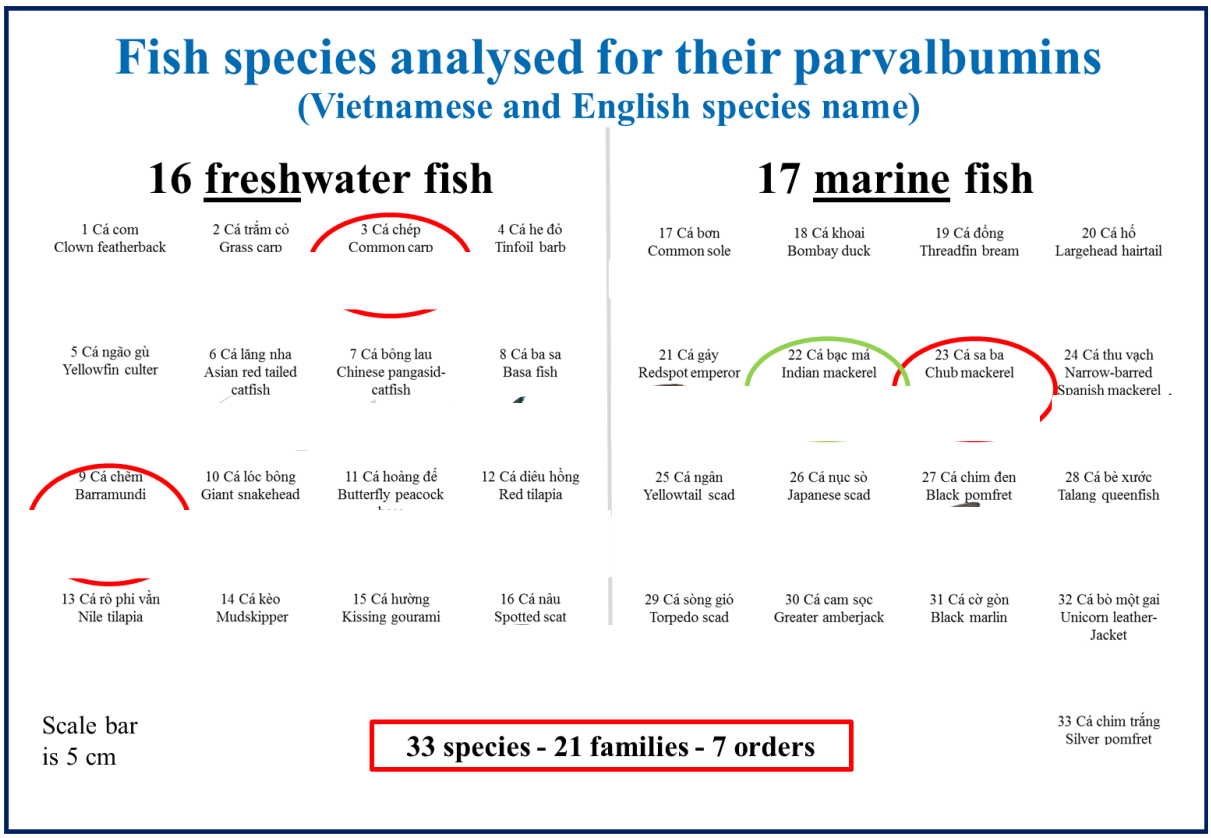
Chapter IV – Major fish allergen

Characterisation 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

Manuscript published as

T Ruethers, M Raith, MF Sharp, M Koeberl, JN Stephen, R Nugraha, TT Le, S Quirce, HX Nguyen, SD Kamath, SS Mehr, DE Campbell, CR Bridges, AC Taki, I Swoboda, AL Lopata; **2018**; Characterisation 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; ***Clinical and Experimental Allergy***, 48 (4), 452-463 (IF 5.1).



Graphical abstract

Contributions:

TR performed the study and wrote the manuscript. TR, IS and AL designed the study. SQ, SM and DC recruited the patients. MR, MS and MK contributed to the generation of the data. All co-authors were involved in the analyses and interpretation of data and critically reviewed the manuscript.

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ORIGINAL ARTICLE

WILEY

Allergens

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

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Summary

Background: Fish is a well-recognized cause of food allergy and anaphylaxis. The evolutionary and taxonomic diversity of the various consumed fish species pose a challenge in the identification and characterization of the major fish allergens critical for reliable diagnostics. Globally, fish is a rising cause of food allergy complicated by a large under-investigated variety of species as well as increasing global tourism and trade. This is the first comprehensive study on allergen profiles of heat-processed fish from Vietnam.

Objective: The aim of this study was to identify the major heat-stable allergens from frequently exported Asia-Pacific freshwater and marine fish and to characterize the major allergen parvalbumin (PV) from one of the most consumed and exported fish species from Asia, the Indian mackerel (*Rastrelliger kanagurta*).

Methods: Heated protein extracts from 33 fish species were separated by gel electrophoresis. PV isoforms were identified by immunoblotting utilizing 3 different PV-specific monoclonal and polyclonal antibodies and further characterized by mass

spectrometry. IgE reactivity was investigated using sera from 21 patients with confirmed fish allergy.

Results: Heat-stable IgE-reactive PVs, with up to 5 isoforms per species, were identified in all 33 analysed fish species. In the Indian mackerel, 7 PV isoforms were identified by 2D-gel electrophoresis combined with mass spectrometric analyses. The amino acid sequence deduced from cDNA of the most expressed isoform showed a high identity (>90%) to PVs from 2 other mackerel species.

Conclusions and Clinical Relevance: Different PVs were identified as the major heat-stable allergens in all 33 analysed freshwater and marine fish species from Vietnam, many of which are exported world-wide and 21 species that have never been investigated before. The Indian mackerel PV represents a novel fish allergen, now officially registered as Ras k 1. Improved diagnostics for fish allergy against Asia-Pacific species should be developed with focus on PV.

KEYWORDS

allergens and epitopes, Asia-Pacific, fish allergy, food allergy, IgE, immunologic tests, paediatrics, parvalbumin, prevention

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Supplementary information:

Table S1: Characteristics of the Australian patient cohort

			Skin Prick Test results (wheal diameter in mm)								Clinical history	
			Commercial preparation					Fresh fish				
#	Sex	Age (years)	Cod	Tuna	Salmon	Mackerel	Fish mix	Asian seabass	Basa fish	Indian mackerel	Symptoms	Implicated fish
1	M	7	8	0	5	6	11	18.5	19	9	OAS, U	White fish
2	M	13	8	2.5	4.5	7	8.5	21	12.5	n.d.	OAS, U	White fish
3	M	14	2	2.5	0	4	3	10.5	4.5	n.d.	AE	Salmon
4	M	3	0	4	0	4	2	5	4	n.d.	AE, U	Basa fish
5	F	12	14	4	11	5.5	10	19	15.5	n.d.	OAS, U	White fish
6†	M	12	9.5	9	8	7	11	16.5	17	10	U	Asian seabass
7	M	9	10	5.5	3	8	8	15.5	10	18	AN, RD, U	Trevally
8	F	1	6.5	0	3	4	0	15.5	13.5	15	AE, RD, U, hypotension	Asian seabass
9†	M	11	14	5	4.5	6	11.5	21	23	n.d.	AE	Salmon
10	M	8	4.5	4.5	2	6	8	7	7.5	n.d.	OAS	Salmon
11†	M	15	15	5.5	8.5	10.5	13.5	20	14.5	n.d.	AE, OAS	Ling fish
12†	F	2	14	5.5	13.5	9	11	18.5	15.5	n.d.	E, U	Cod fish
13†	M	8	14	6.5	12	14	16.5	19	16	n.d.	GIS, RD, U	White fish
14†	F	6	8	3	4.5	4	8	19	12.5	n.d.	AE	White fish
15	M	9	3	2	6.5	3.5	4	6.5	6	1.5	AE	Croaker
16	M	2	7.5	4	7.5	3.5	3	4.5	5.5	0	E, U	Salmon
17	F	7	4.5	2.5	0	4	0	16	9.5	8.5	U	White fish
18	F	8	6.5	0	4	4.5	7	15.5	13	n.d.	E, OAS, U	Basa fish, Salmon

Note: Fresh fish was prepared by homogenizing one part of muscle tissue with one part of Hank's Balanced Salt Solution (Thermo Fisher Scientific, Sweden). Indian mackerel tissue was cooked prior homogenization. Preparations were stored at -80°C until single use. M=male; F=female; n.d.=not determined; AN=anaphylaxis; AE=angioedema (lip swelling); E=erythema; GIS=gastrointestinal syndrome (vomiting); OAS=oral allergy syndrome (itchy mouth or swelling throat); RD=respiratory distress; U=urticaria (hives, rash). Serum from patients marked with † was reactive to PV dimer and pooled for further analyses.

Table S2: PV isoforms with similarity to identified peptides.

Prominent PV bands from extracts of 11 fish species were excised, digested and analysed by mass spectrometry followed by Mascot search. Similar protein sequence hits with the highest three scores are listed.

Fish species	ID kDa	Protein	Uniprot ID	Species	Score	Cov-erage
Clown featherback (*C. ornata*)	1 11.1	PV 4	R9R015	Silver carp (*H. molitrix*)	2772	69%
		PV 1b	W5ZMY4	Elephantfish (*C. compressirostris*)	2551	66%
		PV	C0LEL5	Largemouth bass (*M. salmoides*)	2217	55%
Chinese pangasid-catfish (*P. krempfi*)	7 10.6	PV β	E3TBW7	Blue catfish (*I. furcatus*)	579	34%
		PV1b	W5ZMY4	Elephantfish (*C. compressirostris*)	383	46%
		PV β1	E0WD92	Common carp (*C. carpio*)	383	46%
		PV 3	Q7ZT36	Zebrafish (*D. rerio*)	369	38%
	7 12.1	PV-7	E3TEC5	Channel catfish (*I. punctatus*)	597	15%
		PV β	E3TBW7	Blue catfish (*I. furcatus*)	219	25%
		UP	A0A087XJ36	Amazon molly (*P. formosa*)	204	8%
		UP	W5KAT3	Mexican tetra (*A. mexicanus*)	204	8%
Basa fish (*P. bocourti*)	8 11.0	PV β	E3TBW7	Blue catfish (*I. furcatus*)	798	43%
		PV 4	R9R015	Silver carp (*H. molitrix*)	753	34%
		PV	F1T2N9	Crimson seabream (*D. tumifrons*)	532	47%
	8 13.7	PV-7	E3TEC5	Channel catfish (*I. punctatus*)	694	25%
		PV β	E3TBW7	Blue catfish (*I. furcatus*)	511	67%
		PV 2	E3TGD0	Channel catfish (*I. punctatus*)	313	39%
Giant snakehead (*C. micropeltes*)	10 10.4	PV	B6UV97	Silver carp (*H. molitrix*)	430	63%
		PV 1b	W5ZMY4	Elephantfish (*C. compressirostris*)	416	49%
		PV 2 II	F8U036	Longtooth grouper (*E. bruneus*)	408	68%
	10 11.7	PV β-3	E0WDA1	Atlantic herring (*C. harengus*)	82	9%
		PV β-1	E0WDA2	Rainbow trout (*O. mykiss*)	56	7%
		PV 4	R9R015	Silver carp (*H. molitrix*)	35	10%
Red tilapia (*O.mossambicus x O. niloticus*)	12 10.4	UP	I3KV10	Nile tilapia *(O. niloticus)*	1933	74%
		PV 2	Q6B4H7	Mangrove rivulus (*K. marmoratus*)	1778	64%
		PV β1	Q5IRB2	Asian seabass (*L. calcarifer*)	1391	57%
	12 11.2	UP	I3J1U3	Nile tilapia *(O. niloticus)*	1498	89%
		PV 4	R9R015	Silver carp (*H. molitrix*)	1245	51%
		PV	C0LEL5	Largemouth bass (*M. salmoides*)	1211	54%
Mudskipper (*P. elongates*)	14 10.4	PV β1	E0WD92	Common carp (*C. carpio*)	656	47%
		UP	I3KV10	Nile tilapia *(O. niloticus)*	585	47%
		PV β1	Q5IRB2	Asian seabass (*L. calcarifer*)	527	47%
	14 11.6	PV 4	R9R015	Silver carp (*H. molitrix*)	747	51%
		PV	C0LEL5	Largemouth bass (*M. salmoides*)	709	60%
		PV β2	E0WD93	Common carp (*C. carpio*)	621	48%
Kissing gourami (*H. temminckii*)	15 10.8	PV 3	D2KQG2	Mandarin fish (*S. chuatsi*)	101	14%
		PV	D0VB96	Gilt-head bream (*S. aurata*)	79	14%
		PV β-2 I	F8U035	Longtooth grouper (*E. bruneus*)	65	14%
	15 13.0	PV β	E0WD95	Atlantic mackerel (*S. scombrus*)	169	23%
		PV	F1T2N9	Crimson seabream (*D. tumifrons*)	85	15%
		PV	T1E4U7	Timber rattlesnake (*C.horridus*)	66	17%

Largehead hairtail (*T. lepturus*)	20 10.1	PV	C6GKU3	Yellowfin tuna (*T. albacares*)	472	37%
		PV 2	D5LIS2	Orange-spotted grouper (*E. coioides*)	418	40%
		PV	Q0R3Z9	Mozambique tilapia (*O. mossambicus*)	416	40%
	20 12.2	PV 1	A0A1A8G5F7	Korthaus' killifish (*N. korthausae*)	119	20%
		PV β	P05941	Oyster toadfish (*O. tau*)	92	10%
		UP	A0A087XJ36	Amazon molly (*P. formosa*)	77	17%
Indian mackerel (*R. kanagurta*)	22 11.2	PV	D0VB96	Gilt-head bream (*S. aurata*)	1364	54%
		PV	A0A1B1V0G7	Indian mackerel (*R. kanagurta*)	1316	92%
		PV	B5TTU7	Bighead carp (*H. nobilis*)	1241	51%
Yellowtail scad (*A. mate*)	25 10.5	PV β1	E0WD92	Common carp (*C. carpio*)	949	57%
		PV 1	A0A1A8ARN4	Turquoise killifish (*N. furzeri*)	931	56%
		PV β1	Q5IRB2	Asian seabass (*L. calcarifer*)	901	54%
Japanese scad (*D. maruadsi*)	26 10.4	PV	D0VB96	Gilt-head bream (*S. aurata*)	511	34%
		DM PV	Q3C2C4	Japanese horse mackerel (*T. japonicus*)	428	28%
		PV	B5TTU7	Bighead carp (*H. nobilis*)	387	50%

Note: ID refers to number in Table 1 and lane number in Figure 1 or Figure2. The molecular weights are indicated in kDa. Score refers to Mascot search; PV=parvalbumin; UP=uncharacterised protein; DM=dark muscle.

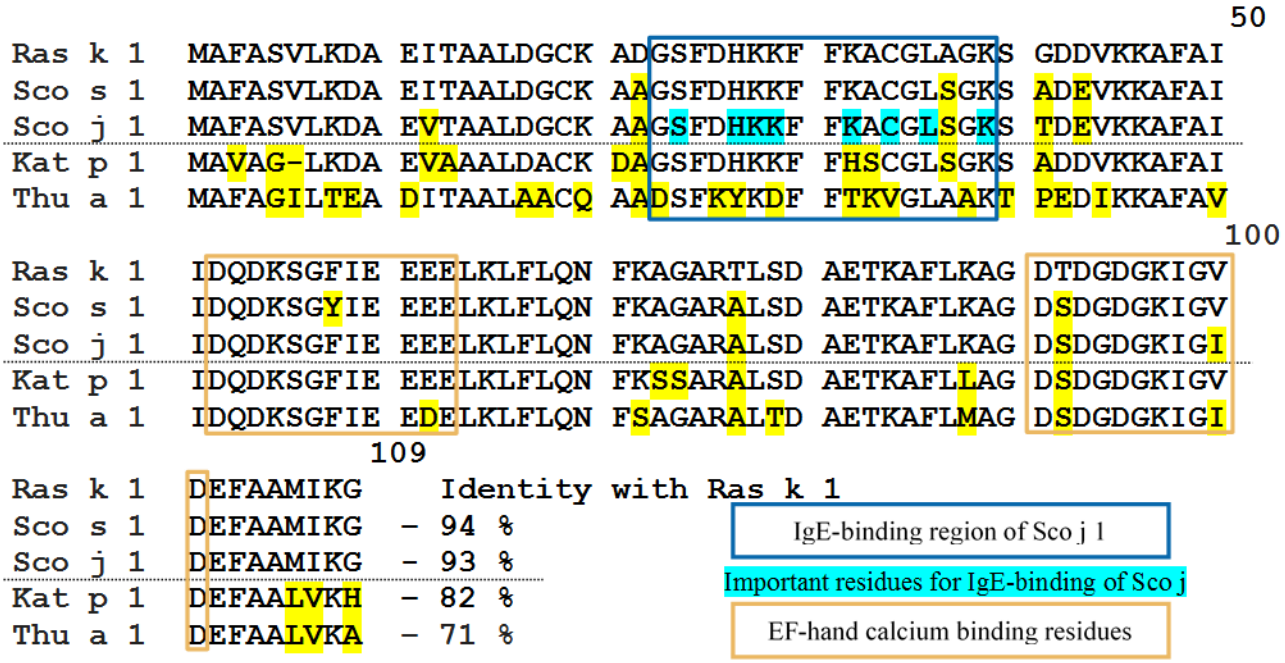

Figure S1: Complete amino acid **sequence alignment of PVs** from species of the family **Scombridae**. PV sequences with decreasing sequence identity to Indian mackerel PV are shown in top-down order: PVs from Atlantic mackerel (Sco s 1, UniProt-ID: D3GME4) and chub mackerel (Sco j 1, P59747) as well as, separated with a dotted line, skipjack (Kat p 1, 9ZTF1) and yellowfin tuna (Thu a 1, C6GKU3). The two Ca^{2+}-binding sites are boxed in orange. The IgE-binding region of Sco j 1 is boxed in blue and the important amino acids for IgE-binding of Sco j 1 are shaded cyan (after [23]). Residues that differ from the sequence of PV from Indian mackerel are shaded in yellow.

Further results and discussion not included in the published manuscript

In the above manuscript, parvalbumin isoforms from 33 fish species were identified and characterised, which provides the knowledgebase for further investigations and improved fish allergy diagnostics with focus on the Asia-Pacific region.

A method for the purification of different parvalbumin isoforms was established as demonstrated above for Indian mackerel parvalbumin (Ras k 1). Subsequently, parvalbumins from various fish species were purified by ammonium sulphate precipitation as described in the published manuscript. After the above manuscript was published in *Clinical and Experimental Allergy* in November 2017, Keshavarz *et al.* utilised a similar approach for parvalbumin purification and published the corresponding manuscript in *Food Chemistry* in November 2018.[1]

As part of further improving diagnostics and management of fish allergy, parvalbumins from barramundi/Asian seabass (*Lates calcarifer*), Nile tilapia (*Oreochromis niloticus*), Atlantic cod (*Gadus morhua*) and Atlantic salmon (*Salmo salar*) were purified for antibody production as described in **Chapter III A**. The latter two were utilised as reference material for the study described in **Chapter II** along with purified parvalbumin from yellowfin tuna (*Thunnus albacares*). In addition, parvalbumin from *Pangasius*/catfish (basa, *Pangasius hypophthalmus*) was purified for **Chapter V A** and from gummy shark (*Mustelus antarcticus*) and multiple other species for **Chapter VI A**. Parvalbumin from saltwater crocodile (*Crocodylus porosus*) was purified for **Chapter VI B** and compared with purified parvalbumins from multiple fish species. Purified parvalbumins from barramundi, salmon and tuna were also utilised in **Chapter V B**. Further species, from which parvalbumins were purified, include green-eyed dogshark (*Squalus megalops*), red emperor (*Lutjanus sebae*), Patagonian toothfish (*Dissostichus eleginoides*), King George whiting (*Sillaginodes punctatus*), ocean jacket (*Nelusetta ayraudi*),

skipjack tuna (*Katsuwonus pelamis*), longtail tuna (*Thunnus tonggol*), albacore tuna (*Thunnus alalunga*), zebra shark (*Stegostoma fasciatum*) and elephant/ghost shark (*Callorhinchus milii*), as well as salmon and tuna from canned products (samples S-2 and T-3 in **Chapter III B**). Most of these purified parvalbumins are yet to be utilised in comparative studies. **Figure IV 1 A** below shows the SDS-PAGE profile of purified parvalbumins along with the corresponding heated extracts from Atlantic salmon, Atlantic cod and Nile tilapia. The purified parvalbumins demonstrated a purity of over 95% based on densitometric analyses after SDS-PAGE. Parvalbumin monomers, dimers and trimers were detected by multiple parvalbumin-specific antibodies (see **Figure IV 1 B**). The established method was also utilised to attempt the purification of parvalbumin from silver perch (*Bidyanus bidyanus*), orange roughy (*Hoplostethus atlanticus*), rainbow trout (*Oncorhynchus mykiss*) and chicken (*Gallus gallus domesticus*) (data not shown). However, the resulting purity was below 90% and further purification steps are required prior to conducting further studies.

Some of these purified parvalbumins were subsequently utilised for detailed structural analyses. The corresponding manuscript is currently in preparation for submission to the *Journal of Biological Chemistry.* Parvalbumins from barramundi, catfish, cod, salmon, tilapia and gummy shark parvalbumin were subjected to circular dichroism (CD) spectroscopy and size exclusion chromatography immediately preceding small-angle X-ray scattering (SEC-SAXS). The X-ray source used was the SAXS/WAXS beamline at the Australian synchrotron.[2] **Figure IV 2** below shows the CD spectrum for gummy shark parvalbumin, demonstrating its heat stability and indicating an increase of β strands with rising temperature.

This study focused on the major fish allergen parvalbumin and no other IgE-binding proteins were investigated. However, previous studies have indicated that there are many more IgE-binding proteins which were discovered in various fish species (e.g., see Table 1 in **Chapter 1**). The aim of the subsequent study (**Chapter V**) is therefore to expand our knowledge about the allergen repertoire in numerous fish species while focussing on two heavily farmed fish, Atlantic salmon and *Pangasius*/catfish (sold as basa in Australia).

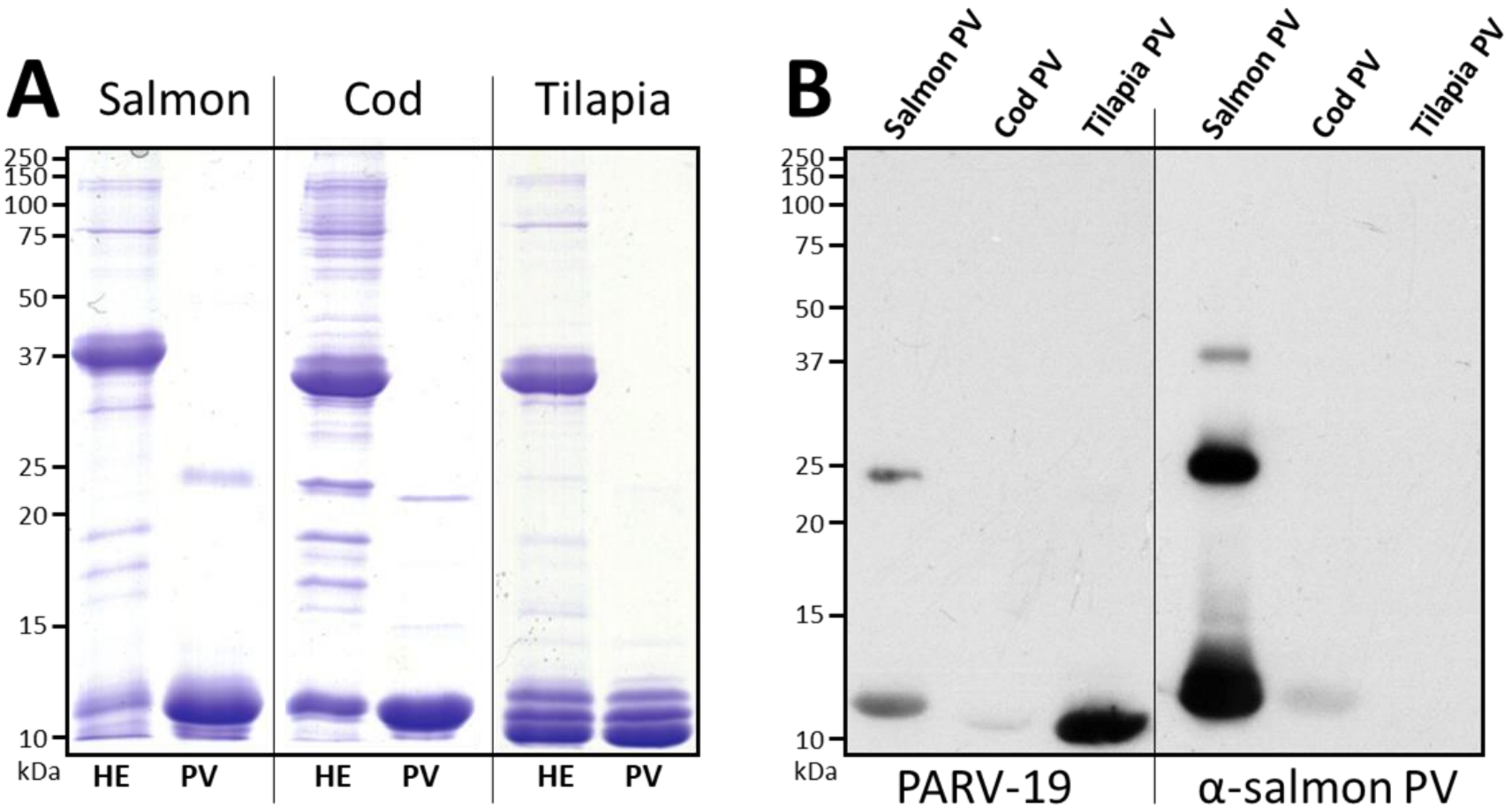

Figure IV 1: SDS-PAGE protein profiles of heated extracts (HE) and purified parvalbumin (PV) from Atlantic salmon, Atlantic cod and Nile tilapia (**A**). The purity of PVs is over 95% based on densitometric analyses. PV monomers and multimers were detected using the commercial monoclonal antibody PARV-19 (raised against frog PV) and an in-house polyclonal antibody (raised against salmon PV)[3] (**B**).

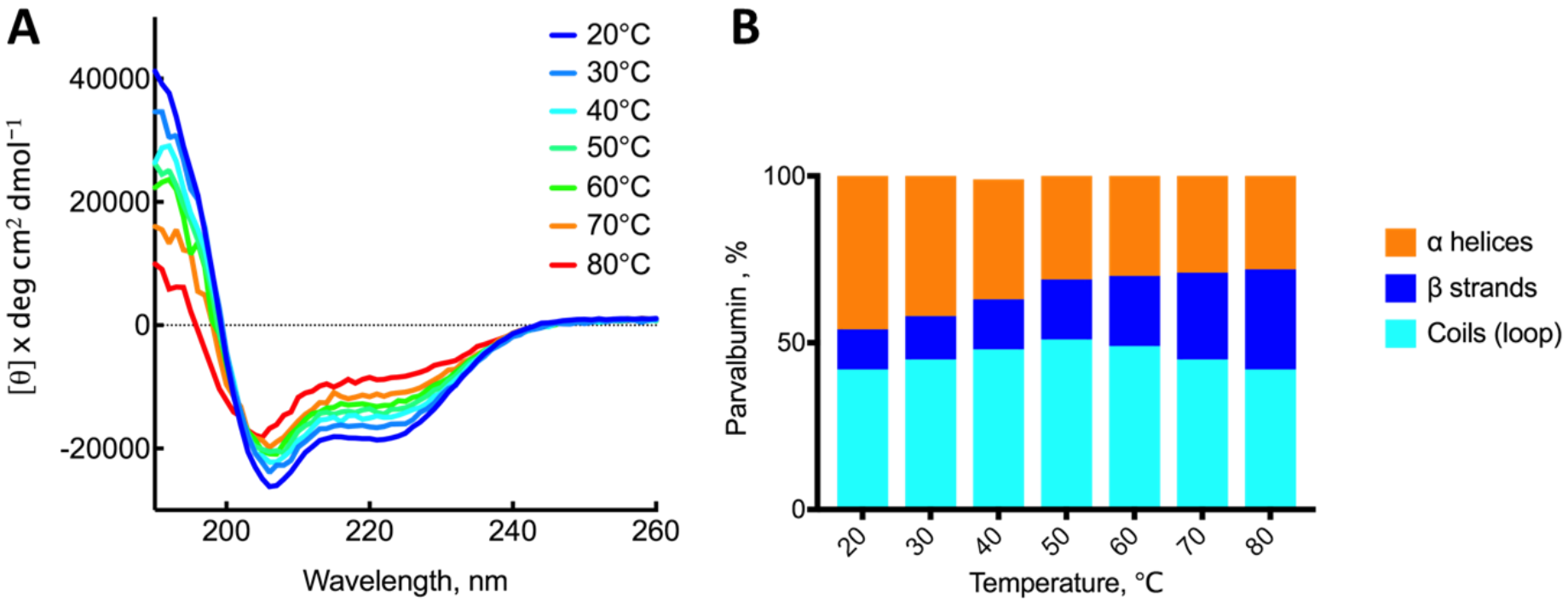

Figure IV 2: Circular dichroism spectrum of gummy shark parvalbumin (**A**) and its secondary structure at different temperatures (**B**). Data generated by Dr Aya C. Taki.

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I

Literature review

A comprehensive review of fish and shellfish allergens

Mol Immunol 2018

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II

Skin prick test preparations

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Allergy 2019

Page 67 *ff*

III A

Commercial fish ELISA kits

Limited capacity to detect different fish and their products

J Sci Food Agric 2020

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III B

Canned fish

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Mol Nutr Food Res (in prep.)

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IV

Major fish allergen

Characterisation of parvalbumin in 33 Asia-Pacific fish

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V A

Novel putative fish allergens

Allergen repertoire of Atlantic salmon and *Pangasius*/catfish

Allergy (in revision)

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V B

Collagen, a novel allergen

Collagen - an important fish allergen for improved diagnosis

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VI A

Tolerance to sharks/rays

Bony fish-allergic patients can tolerate cartilaginous fish

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VI B

Cross-reactivity to reptiles

Parvalbumin is also an allergen in amphibians and reptiles

Allergy (in prep.)

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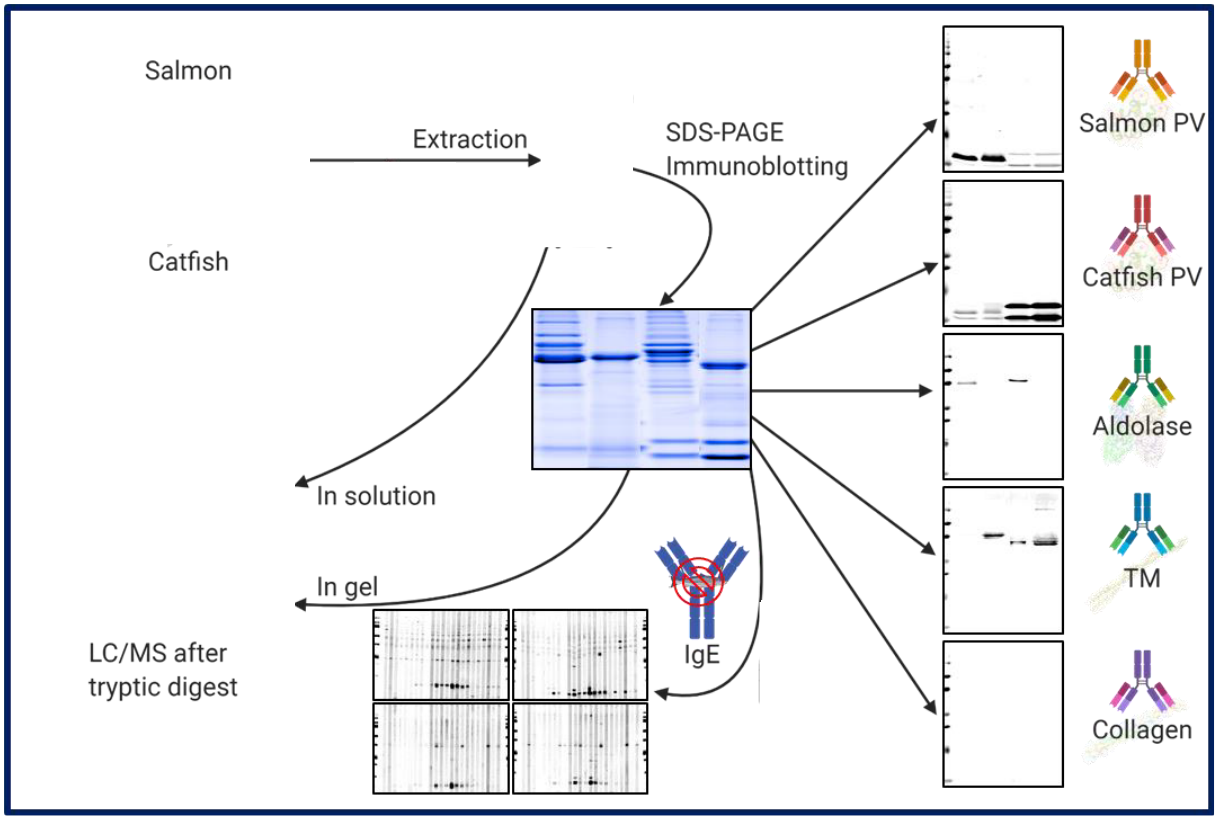
Chapter V A – Novel putative fish allergens

Expanding the allergen repertoire of salmon and catfish

Manuscript in revision with ***Allergy*** (IF 8.7) as

T Ruethers, AC Taki, SV Karnaneedi, S Nie, T Kalic, M Leeming, S Daduang, D Dai, NA Williamson, H Breiteneder, SS Mehr, SD Kamath, DE Campbell, AL Lopata; Expanding the allergen repertoire of salmon and catfish.



Graphical abstract

Contributions:

TR performed the study and wrote the manuscript. TR, AT, HB, DC and AL designed the study. DD, SM and DC recruited the patients. AT, SK, SN, TK, ML, SD, NW and SK contributed to the generation and/or anlayses of the data. All authors critically reviewed the manuscript.

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Expanding the allergen repertoire of salmon and catfish

Short title: Allergen repertoire of salmon and catfish

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ABSTRACT

Background: Diagnostic tests for fish allergy are hampered by the large number of under-investigated fish species. Four salmon allergens are well-characterized and registered with the WHO/IUIS while no catfish allergens have been described so far. In 2008, freshwater-cultured catfish production surpassed that of salmon, the globally most-cultured marine species. We aimed to identify, quantify and compare all IgE-binding proteins in salmon and catfish.

Methods: Seventy-seven pediatric patients with clinically confirmed fish allergy underwent skin prick tests to salmon and catfish. The allergen repertoire of raw and heated protein extracts was evaluated by immunoblotting using five allergen-specific antibodies and patients' serum followed by mass spectrometric analyses.

Results: Raw and heated extracts from catfish showed a higher IgE-binding capacity than those from salmon (77% versus 70% and 64% versus 53%, respectively). The major fish allergen parvalbumin demonstrated the highest IgE-binding capacity (10-49%), followed by triosephosphate isomerase (TPI; 19-34%) in raw, and tropomyosin (6-32%) in heated extracts. Six previously unidentified fish allergens, including TPI, were registered with the WHO/IUIS. Creatine kinase from salmon and catfish was detected by IgE from 14% and 10% of patients, respectively. Catfish L-lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, and glucose-6-phosphate isomerase showed IgE-binding for 6-13% of patients. In salmon, these proteins could not be separated successfully.

Conclusions: We detail the allergen repertoire of two highly farmed fish species. IgE-binding to fish tropomyosins and TPIs was demonstrated for the first time in a large patient cohort. Tropomyosins, in addition to parvalbumins, should be considered for urgently needed improved fish allergy diagnostics.

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HIGHLIGHTS:

- Raw and heated extracts from catfish demonstrated a higher prevalence of IgE-binding as compared to those from salmon (77% vs 70% and 64% vs 53%, respectively).
- Tropomyosin was the second most abundant protein, after parvalbumin, in heated extracts and up to 36% of patients with clinically confirmed fish allergy (*n*=77) demonstrated IgE-binding.
- Twelve new catfish and three new salmon IgE-binding proteins were registered with the WHO/IUIS, including three tropomyosin and two triosephosphate isomerase isoforms.

KEYWORDS

Fish allergy diagnosis, *Pangasianodon hypophthalmus*, *Salmo salar*, triosephosphate isomerase, tropomyosin

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1 | BACKGROUND

Fish allergy is associated with high rates of anaphylaxis[1] and affected patients often suffer for a lifetime[2,3]. The prevalence is region-specific and has been reported to be as high as 3% in the general population.[4] A higher prevalence of up to 8% has been reported among fish-processing workers.[5,6] In countries with high seafood consumption, fish is the second most common trigger of food allergy, following crustaceans.[7]

The production and consumption of fish is continuously increasing,[8] making adverse reactions to fish, including IgE-mediated fish allergy, a growing health burden with a negative impact on the quality of life.[9] However, diagnostics and management of species-specific fish allergy are hampered by the lack of studies on the large number and variety of under-investigated fish species[10] and the current limited availability of *in vivo*[11] and *in vitro*[12,13] diagnostic tests,[14] as well as reliable detection methods.[15] The increasing demand for fish as a valuable protein source[16] can only be satisfied by sustainable aquaculture,[17] and therefore heavily farmed fish species require evaluation of their allergen content.

The most commonly cultured marine fish species is the Atlantic salmon (*Salmon salar*).[8] Four well-investigated salmon allergens are registered with the World Health Organisation and International Union of Immunological Societies (WHO/IUIS; www.allergen.org): β-parvalbumin (Sal s 1), β-enolase (Sal s 2), aldolase A (Sal s 3), and collagen alpha (Sal s 6). Four additional salmon and other fish proteins with reported IgE-binding are listed by other databases (i.e. www.allergome.org), suggesting a broader repertoire of salmon allergens with potential clinical relevance. Recently, we underlined the importance of salmon collagen, tropomyosin and aldolase as IgE-binding proteins in commercial skin-testing preparations.[11]

Since 2008, the freshwater *Pangasius*/catfish surpasses salmon in global production, and since 2007, each of the two species surpass Atlantic cod (Figure S1). Two previous case reports

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described IgE-binding proteins in *Pangasius*/catfish, referred to as pangasius.[18,19] One patient showed IgE-binding possibly to parvalbumins, in contrast to the other patient who was not parvalbumin-sensitized, however, none of the IgE-binding proteins were identified.

The aim of this study was to identify candidates for urgently needed component-resolved diagnostics (CRDs). We therefore investigated the detailed repertoire and abundance of IgE-binding proteins in both the Atlantic salmon and *Pangasius*/catfish.

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2 | METHODS

2.1 In-house extracts

Whole specimen of Atlantic salmon (*Salmo salar*) and *Pangasius*/catfish (*Pangasianodon hypophthalmus*), referred to as catfish here, as well as for experimental controls, Asian seabass (*Lates calcarifer*), Atlantic cod (*Gadus morhua*), Nile tilapia (*Oreochromis niloticus*), and yellowfin tuna (*Thunnus albacares*), were sourced from local fishermen, retailers or fellow researchers. Muscle tissue samples were taken from the center of the fillets for consistency and stored at -80°C until further use.

Proteins were extracted as previously described.[11] In short, tissue was homogenized, extracted overnight in phosphate-buffered saline and after filtration stored at -20°C (raw protein extracts). For the heated extracts, tissue was heated at 95-100°C in PBS for 20 min before extraction in the same buffer.

The catfish preparation for skin prick testing (SPT) was generated by homogenizing minced raw muscle tissue with one part (w/v) Hanks' Balanced Salt Solution (Gibco®, ThermoFisher Scientific) as above. Aliquots were stored at -80°C until single use.

Collagens were extracted as experimental controls from the muscle tissue of Asian seabass, Atlantic salmon, and yellowfin tuna as described previously with modifications.[20] In short, muscle tissues were washed with water, 0.1 M NaOH, and 10% butyl alcohol followed by extraction with 0.5 M acetic acid.

2.2 Patients

Seventy-seven children (1-18 years, interquartile range (IQR) 6-13 years) with clinically confirmed allergy and history of IgE-mediated symptoms after ingesting fish underwent allergy skin prick tests (SPT) with commercial salmon and an in-house catfish preparation, as

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previously described.[11] Serum from all patients was obtained for *in vitro* analyses (*n*=77), while sIgE levels were determined for the available commerical salmon ImmunoCAP (Thermo Fisher Scientific, f41) for 43 patients (see Table S1 for demographic and clinical data). Parents gave written informed consents, and ethical approval was obtained from the Sydney Children's Hospitals Network (LNR-14/SCHN/185). Sera from two non-atopic and two atopic fish-tolerant donors were used as negative controls.

2.3 Protein concentration and SDS gel-electrophoresis

The protein concentration for all extracts was estimated using the Pierce™ BCA Protein Assay kit (Thermo Scientific) with bovine serum albumin as standard. All whole protein extracts were diluted to the same total protein concentration.

Proteins were separated according to their molecular weights using a Criterion™ SDS-PAGE system (Bio-Rad) or Dual Double Wide Mini Vertical System (C.B.S. Scientific). Proteins were visualized by Coomassie Brilliant Blue R-250 (CBB) staining and identified by subsequent immunoblotting with allergen-specific antibodies or patient serum IgE.

2.4 Immunoblotting

The separated proteins were transferred onto a nitrocellulose membrane. Subsequently, the fish allergens, parvalbumin,[21] aldolase,[22] tropomyosin,[23,24] and collagen[25] as well as patients' IgE-binding were detected as described previously.[11] In brief, membranes were blocked with casein and incubated with in-house generated polyclonal antibodies raised in rabbits against parvalbumin from Atlantic salmon and catfish and tropomyosin from shrimp,[26,27] commercial antibodies raised against rabbit aldolase (100-1141 by Rockland Immunochemicals) and tuna collagen (ab23730 by Abcam), and patients' sera. Patient blots were further incubated with a

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monoclonal mouse anti-human IgE antibody (sc-53346 by Santa Cruz) before all blots were developed with a corresponding infra-red-labelled antibody (DyLight anti-mouse/rabbit 4xPEG by Thermo Scientific or IR-Dye anti-goat by LI-COR®).

The Surf-Blot Antibody Screening System by Idea Scientific was used to investigate serum IgE-binding from all patients to the same extract. Densitometric analyses were conducted utilizing Image Studio Version 5.2 (LI-COR®) allowing sensitive and semi-quantitative evaluation of signals. The densitometric analyses utilizing this system is independent of background, contrast or other settings often used for best visualization of the immunoblot. Antibody-binding intensities were determined in comparison to negative controls and other patients as well as signals to other proteins.

2.5 Mass spectrometry analysis

Whole protein extracts, as well as IgE-binding bands, were digested with trypsin and analyzed by mass spectrometry as described previously.[11,28] Results were analyzed using both Mascot (v. 2.4) search engine and MaxQuant (v. 1.6.2.3), against an NCBI database containing amino acid sequences of all salmon or catfish proteins (July 2019). The relative protein abundance is expressed in relative intensity-based abosolute quantification (iBAQ%) value.[29] Identified protein groups with at least 1 unique peptide and a minimum of 2 razor/unique peptides were included in the analysis.

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3 | RESULTS

3.1 SDS-PAGE and the detection of previously recognized fish allergens

The protein composition of raw and heated extracts from both salmon and catfish was compared by SDS-PAGE and subsequent densitometric analyses (Figure 1). While the protein concentrations were adjusted for all extracts, the raw and heated extracts from catfish showed a higher number of protein bands than those obtained from salmon. In both raw and heated extracts, the most abundant protein bands were between 35-50 kDa and 11-12 kDa.

Using allergen-specific antibodies, the four WHO/IUIS-registered fish allergens parvalbumin, aldolase, tropomysoin, and collagen could be identified in most extracts (Figure 2). Two parvalbumin bands were detected for each species, with a higher signal intensity in the heated extracts as compared to the raw extracts. The anti-salmon parvalbumin antibody detected the 12 kDa band in salmon with the highest intensity, followed by an 11 and 12 kDa band in catfish, and the weakest intensity to an 11 kDa band in salmon. The anti-catfish parvalbumin antibody detected both 11 and 12 kDa bands in catfish with equally high intensity, while the 11 and 12 kDa band in salmon demonstrated a much lower binding capacity.

Aldolase was detected with higher intensity at 40 kDa in catfish (raw) as compared to the 37 kDa band in salmon (raw). No aldolase was detected in any heated extracts.

Tropomyosin was detected with similar intensity in heated extracts from salmon (at 37 kDa), catfish (at 35 and 36 kDa) and tilapia (at 36 kDa). A weak signal was observed for the corresponding band in the raw extract from catfish, but not from salmon.

Collagen was detected only in salmon heated extract; however the corresponding antibody demonstrated binding to purified collagen from salmon, seabass, and tuna.

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3.2 Patient characterization and *in vivo* reactivity

Twenty patients had a history of an allergic reaction to salmon (26% of cohort) and eight to catfish (10%) (Table S1). Among all 77 pediatric patients with a convincing clinical history of IgE-mediated fish allergy, the median wheal diameters for salmon and catfish were 4.5 mm (IQR; 0-6.5 mm) and 9.5 mm (5.5-14.5 mm) with 69% and 88% of patients with a SPT ≥3 mm to salmon and catfish, respectively. Lessof *et al.*[30] and Peters *et al.*[31] suggested a higher threshold to reduce the number of possible false-positive results. 43% and 78% had a positive skin reaction to salmon and catfish based on a threshold of ≥5 mm, respectively (Figure 3A). Five of the 20 salmon-allergic patients (25%) had a salmon SPT result of <3 mm, while the median for the remaining 15 patients was 7 mm (IQR; 5-8 mm). Among eight catfish-allergic patients, the median catfish SPT results was 7 mm (IQR; 4-9 mm). In summary, patients seem to demonstrate larger SPT wheal diameter to catfish, while over 10% had negative SPT results. The median sIgE level for salmon was 3.2 kU/l (n=43; IQR; 0.5-10.7 kU/l), while four patients had <0.01 kU/l. Eighty-seven percent of patients had a sIgE level of above 0.1 kU/l, of whom 40% had a low-moderate level (ImmunoCAP class I-II) and 47% a high-very high level (class III-V) (Figure 3B). For eight salmon-allergic patients, the median sIgE level was 5.7 kU/l (IQR 0.5-23.4 kU/l); all but one patient had an elevated sIgE level. In summary for salmon, an overall positive correlation between SPT and sIgE level was observed (r_s=0.74, p<0.0001), while the SPT was negative in 18 patients (<5 mm) with elevated sIgE levels.

3.3 Serum IgE-reactivity of salmon and catfish proteins

Serum from all 77 patients and controls (n=4) was analyzed for IgE–binding to heat-labile and heat-stable salmon and catfish proteins (Figure S2). All IgE-binding protein bands, with at least five patients, are indicated by an arrow and the corresponding molecular weight in Figure 1:

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Seven and 12 bands in raw, and two and five bands in heated salmon and catfish extracts, respectively (Table S2). In addition, IgE-binding to bands with less than five patients was observed (Table S3) and their identity has not been further investigated. Nineteen IgE-binding bands were evaluated for protein identity and relative abundance by advanced mass spectrometric analyses (Table S4 and S5). The majority of detected peptides (73-100%) corresponded to up to three major isoforms of one protein each in 18 bands (Table S4 and S5). In 17 bands, other proteins with valid hits had a relative abundance of up to 9%, but were more abundant in other bands not showing IgE-binding by the same patient. This enabled us to exclude these proteins. We therefore associated IgE-binding to one protein each for 17 IgE-binding bands. The other two analyzed IgE-binding bands were from raw salmon extract and contained considerable amounts of multiple proteins. The 37 kDa band contained both aldolase and glyceraldehyde-3-phosphate dehydrogenase (GADPH), while pyruvate kinase and glucose-6-phosphate isomerase were detected in the 65 kDa band. Table 1 summarises the prevalence of IgE-binding to all these proteins along with their respective allergen names where appropriate. Three salmon and 12 catfish proteins were registered as new fish allergens with the WHO/IUIS.

Parvalbumins (Sal s 1 and Pan h 1) were the only proteins binding IgE in both raw and heated extracts, and the proteins with the highest IgE-binding capacity (49% each) followed by triosephosphate isomerase (TPI; 34% to Sal s 8.0101, 14% to Pan h 8.0101) in raw extracts and tropomyosin (13% to Sal s 4.0101, 6% to Pan h 0201, and 32% to Pan h 4.0101) in heated extracts. Among all 77 patients, 70% and 77% showed IgE-binding to the raw extract from salmon and catfish, respectively, which decreased to 53% and 64% for the corresponding heated extracts.

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No IgE-binding was observed in serum from the control patients nor seven fish-allergic patients, who were therefore excluded from further analyses. Five of those patients had a clinical history of an allergic reaction to salmon; three had a salmon SPT result of 0 mm while the other two had 3 and 7.5 mm.

The remaining 70 fish-allergic patients were grouped based on the species implicated in the reported clinical allergic reaction and their salmon sIgE level (Table S6). Species-specific IgE-binding to parvalbumin was observed in eleven patients (salmon 9% and catfish 7%). Two of eight patients with a history of allergic reaction to catfish showed IgE-binding to catfish parvalbumin, but not to salmon parvalbumin. Monosensitivity to only one of the two catfish parvalbumins was observed in 39% of the 70 patients, while 6% (*n*=70) showed IgE-binding exclusively to parvalbumins. In contrast, 37% (*n*=70) showed no IgE-binding to parvalbumin, but to other proteins.

Forty-nine and 53 of 70 patients showed IgE-binding to parvalbumin, tropomyosin and/or TPI from salmon (70%) and catfish (76%), respectively (Figure 4). Five and seven patients (7% and 10%, respectively), demonstrated IgE-binding only to aldolase, enolase and/or GADPH. All but two patients demonstrated IgE-binding to any of these six proteins or creatine kinase from salmon and/or catfish or pyruvate kinase from catfish. The IgE from the remaining two patients showed binding only to a 28 kDa band in the heated extract or a 30 kDa band in the raw extract from catfish. These two patients had no clinical history of an allergic reaction to salmon or catfish but to croaker and white fish, respectively.

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3.4 The relative abundance of IgE-binding proteins

The relative abundance of the above described major IgE-binding proteins was evaluated by mass spectrometric analyses and totaled to 74-86% (Figure 5). Raw extracts showed a higher diversity of proteins as compared to heated extracts. Parvalbumins were the most abundant proteins in both raw (30 and 36%) and heated extracts (54 and 57%) from both species (salmon and catfish, respectively). The second most abundant proteins in raw salmon and catfish extracts were enolase (14%) and creatine kinase (12%), respectively. Tropomyosin was the second most abundant protein in heated extracts from both salmon and catfish (24 and 9%, respectively). The relative protein abundance was 80- and 46-fold higher compared to the raw extract. Collagen demonstrated low abundance (4%) in the salmon heated extract as compared to less than 0.5% in the other three extracts.

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4 | DISCUSSION

This is the first study to analyse the detailed allergen repertoire of two highly consumed fish species – Atlantic salmon and *Pangasius*/catfish. The latter is one of the most consumed freshwater fish species, traded worldwide under many names including pangasius, pangas, basa, catfish, swai, tra, sutchi, haiwels, cobbler, grey sole, Pacific dory, iridescent shark or freshwater fillet.

Based on the current study, the WHO/IUIS now lists twelve *Pangasius*/catfish proteins and three additional Atlantic salmon proteins (www.allergen.org) as allergens, including six fish allergens registered for the first time. However, the exact molecular properties and clinical relevance of these IgE-binding proteins require further investigations. The clinical relevance could be clarified with cell-based assays and basophil activation tests.

This study describes the identification of novel fish allergens using a well characterized cohort of fish-allergic pediatric patients by investigating the sensitization patterns to salmon and catfish. Fish allergy is a life-long condition and often starts in the early stages of life, and our patient cohort addressed this age group. A caveat of this study was the lack of comparative analysis of sensitization patterns with fish-allergic adults. To our knowledge, only two studies directly compared sensitization patterns between numerous fish-allergic children and adults. James et al. reported similar IgE-binding to parvalbumins from catfish, cod, and snapper (exact species unknown) for five children and five adults.[32] Similarly, Sharp et al. investigated IgE binding to Asian seabass parvalbumin among six children and ten adults.[33] Further comparative analysis with larger fish-allergic cohorts of different age-groups are required to investigate the role of specific fish allergens in early age sensitization.

We aimed to expand our understanding of the allergen repertoire in fish and identify suitable candidates for much-needed CRDs. The *in vitro* IgE-binding to raw and heated protein extracts

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from salmon and catfish depended on the presence of specific allergens and differed between patients and fish species. Importantly, the salmon sIgE level (ImmunoCAP) was not a good indicator for IgE-binding to extracts generated in-house, except for patients with a high to very high sIgE level (*n*=20) of which 95% showed IgE-binding to parvalbumin from salmon and/or catfish. The majority of patients with a negative or moderate salmon sIgE level demonstrated IgE-binding to proteins other than heat-stable parvalbumin and tropomyosin, suggesting that heat-labile proteins are under-represented in the utilized salmon ImmunoCAP.

However, there was a positive correlation between SPT results and sIgE level for salmon. Furthermore, we demonstrated a positive correlation in the SPT outcomes for salmon and catfish. It is noteworthy that the wheal diameter was overall greater for catfish compared to salmon and many patients with a negative salmon SPT had a positive catfish SPT. Currently, there are no commercial SPT preparations available for catfish and many other highly consumed fish species.Parvalbumin, the well-recognized major fish allergen,[9] was the protein with the highest IgE-binding capacity, possibly also due to its abundance in all extracts. However, the prevalence of IgE-binding to any salmon or catfish parvalbumin was only 57%, while in comparison previous studies state prevalences of 70-95% among fish-allergic patients.[34] This highlights the importance of additional fish allergens as also suggested previously for fish SPT diagnostics.[11] The observed limited IgE-binding of both salmon and catfish parvalbumins can partially be explained by amino acid sequence differences. Salmon parvalbumin Sal s 1.0101 has a rather low sequence identity of 66% and 57% with catfish parvalbumins Pan h 1.0101 and Pan h 1.0201, respectively. The latter two are only 57% identical, possibly resulting in the different IgE-binding observed (44% versus 10%). Similarly

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differences in amino acids sequences and IgE-binding capacity of parvalbumin isoforms were previously demonstrated for Asian seabass.[33]

Tropomyosin was the second most abundant protein after parvalbumin in heated extracts. We demonstrated for the first time IgE-binding to fish tropomyosin in a large patient cohort. Previous reports of IgE-binding to fish tropomyosin are rare and include two case reports[24,35], a description of 19 patients with undefined adverse reactions after fish intake,[36] and one study with ten presumably fish-allergic patients who additionally suffered from inflammatory bowel disease or shrimp allergy.[23] We demonstrated IgE-binding to one salmon tropomyosin and two catfish tropomyosins in 6% to 32% of our patients. The differential IgE-binding capacity of the three fish tropomyosins can be explained to some extend by amino acid sequence differences. Catfish tropomyosin Pan h 4.0101 demonstrated the highest IgE-binding capacity and shares 83% and 80% of its sequence with catfish tropomyosin Pan h 4.0201 and salmon tropomyosin Sal s 4.0101, respectively. The latter two are 93% identical. All three tropomyosin are 82-95% identical with the only other WHO/IUIS-registered tropomyosin Ore m 4.0101 from tilapia. Future research should focus on clinical cross-reactivity between various tropomyosin isoforms.

Recently, we identified heat-stable collagens as novel allergens in three fish species, including salmon.[37] In this study, however, only low quantities of collagen were detected in the PBS-based fish extracts. Collagen is generaly insoluble in neutral aqueous solutions, resulting in subsequent underrepresentation in extracts, as recently demonstrated in for commercial SPT preparations.[11]

While most fish are consumed after heat-treatment, heat-labile allergens seem to be of condiserable importance as demonstrated for aldolase and enolase - their implementation in CRDs can be useful.[38] In the current study, we reported IgE-binding to both allergens in catfish

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and registered their full sequence (Pan h 3.0101 and Pan h 2.0101, respectively). The utilization of these and other heat-labile allergens in CRDs could lower the rate of false-negative test results.

In addition, we demonstrated an even higher prevalence in our cohort for IgE-binding to TPI, which is a glycolytic enzyme found in nearly every organism and a registered allergen in arthropods[9]. IgE-binding fish TPI is distinguishable from other heat-labile proteins by its low molecular weight of 25 kDa and was previously reported in amago salmon,[39] mackerel,[40] silverside,[41] sole,[42] and swordfish.[43] It is to note that salmon TPI (34%; Sal s 8.0101) showed more frequent IgE-binding compared to catfish TPI (19%; Pan h 8.0101), possibly associated with the low sequence identity of 85% and different protein abundances.

The enzyme GADPH was identified as an IgE-binding protein in catfish (Pan h 13.0101), but not in salmon as it was not distinguishable from aldolase. IgE-binding GADPH has previously been reported in pilchard.[44]

We registered heat-labile creatine kinases from salmon and catfish as novel IgE-binding proteins, Sal s 7.0101 and Pan h 7.0101, respectively. IgE-binding to fish creatine kinase has previously been associated with occupational allergy[45] and allergy to bream[46] and tuna[47] but creatine kinase was not characterized and registered as an allergen.

To our knowledge, this is the first report of IgE-binding to catfish glucose-6-phosphate isomerase (Pan h 12.0101) and L-lactate dehydrogenase (Pan h 10.0101), and the second report for fish pyruvate kinase (Pan h 9.0101)[43]. However, these proteins were not separated successfully or of low abundanance in the salmon raw extract. All three allergens are now listed on www.allergen.org.

In summary, this study details the repertoire of IgE-binding proteins from two highly farmed and consumed fish, marine Atlantic salmon and freshwater *Pangasius*/catfish, and demonstrated more IgE-binding allergens in catfish compared with salmon. Future research should provide additional information on clinical cross-reactivity and the implementation of parvalbumins as well as tropomyosins and selected heat-sensitive allergens in CRDs.

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TABLE 1: *In vitro* IgE-binding capacity of salmon and catfish proteins in raw and heated extracts.

Raw salmon				Raw catfish			
MW	Protein(s) in band	IUIS name	*n=77*	MW	Protein(s) in band	IUIS name	*n=77*
65	Pyruvate kinase G-6-PI	-	8%	65	Pyruvate kinase	Pan h 9.0101	6%
60	n.d.	-	6%	60	G-6-PI	Pan h 11.0101	8%
48	beta-enolase	Sal s 2	34%	50	beta-enolase	Pan h 2.0101	21%
43	creatine kinase	Sal s 7.0101	14%	43	creatine kinase	Pan h 7.0101	10%
37	aldolase A GAPDH	Sal s 3.0101 -	26%	40	aldolase A	Pan h 3.0101	21%
				36	GAPDH	Pan h 13.0101	6%
				34	L-lactate DH	Pan h 10.0101	13%
				30	n.d.	-	14%
				27	n.d.	-	6%
25	TPI	Sal s 8.0101	34%	25	TPI	Pan h 8.0101	19%
12	PV	Sal s 1	**49%**	12	PV 2	Pan h 1.0201	10%
				11	PV 1	Pan h 1.0101	**42%**
any other band			1%	any other band			17%
Patients reactive to any band above			**70%**	Patients reactive to any band above			**77%**

Heated salmon				Heated catfish			
MW	Protein(s) in band	IUIS name	*n=77*	MW	Protein(s) in band	IUIS name	*n=77*
37	TM	Sal s 4.0101	13%	36	TM 2	Pan h 4.0201	6%
				35	TM 1	Pan h 4.0101	32%
				28	n.d.	-	21%
12	PV	Sal s 1	**49%**	12	PV 2	Pan h 1.0201	14%
				11	PV 1	Pan h 1.0101	**44%**
any other band			12%	any other band			13%
Patients reactive to any band above			**53%**	Patients reactive to any band above			**64%**

Note: The proteins refer to the bands in Figure 1. Results are based on IgE immunoblots (Figure S2) and conforming densitometric analyses (Table S2). The identity of the proteins in the band corresponds to mass spectrometric analyses (Table S4 and S5). The WHO/IUIS name refers to the corresponding database accessible under www.allergen.org. Abbreviations: MW, molecular weight in kDa; n.d., not determined; G6-PI, glucose-6-phosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DH, dehydrogenase; TPI, triosephosphate isomerase; PV, parvalbumin; TM, tropomyosin.

FIGURE 1: SDS-PAGE profiles and densitometric analyses of raw (A, B) and heated (C, D) extracts from salmon (A, C) and catfish (B, D). Bands with IgE-binding by at least five fish-allergic patients are indicated with an arrow and their observed molecular weight is provided.

FIGURE 2: Detection of registered fish allergens in raw (R) and heated (H) extracts from salmon (S) and catfish (C). Proteins were separated by SDS-PAGE and allergens identified by immunoblotting using antibodies specific to the respective fish allergens. Parvalbumin was detected using antibodies raised against parvalbumin (PV) from salmon (A) and catfish (B). The raw extracts from Atlantic cod containing Gad m 3.0101 and yellowfin tuna containing Thu a 3.0101 were loaded as a reference for the detection of aldolase (C). The reference for tropomyosin was heated extract from Nile tilapia, a closely related species to Mozambique tilapia, the only fish species for which tropomyosin (Ore m 5.0101) was recognized by the WHO/IUIS at the time of the study (D). Purified collagens from salmon, cod, and tuna were used as a reference for collagen detection (E).

FIGURE 3: Comparison of *in vivo* reactivity of 77 fish-allergic patients to salmon and catfish (A). The Skin Prick Test (SPT) wheal diameter is given in mm. For 43 patients the salmon sIgE level and corresponding ImmunoCAP class were determined and compared with the corresponding SPT result (B). The sIgE level was below 0.1 kU/l (class 0) for six patients who had SPT of 0 mm. A positive correlation is indicated by a curve of best fit.

FIGURE 4: IgE-binding of 64 fish-allergic patients to two heat-stable allergens (parvalbumin (PV) and tropomyosin (TM)) and four heat-labile allergens (triosephosphate isomerase (TPI), enolase (Eno), aldolase (Ald) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) from salmon (A) or catfish (B).

FIGURE 5: Relative protein abundance in raw and heated extracts from salmon and catfish. The extracts were digested with trypsin and analyzed by mass spectrometry. The iBAQ% value is an indicator of the relative abundance of each protein including several isoforms and is based on analyses with MaxQuant. Note: The relative abundance is only given for proteins for which IgE-binding with at least five patients was demonstrated. The WHO/IUIS-name is based on the corresponding database accessible under www.allergen.org. TPI=triosephosphate isomerase, DH=dehydrogenase, G6-PI=glucose-6-phosphate isomerase, GAPDH=glyceraldehyde-3-phosphate dehydrogenase.

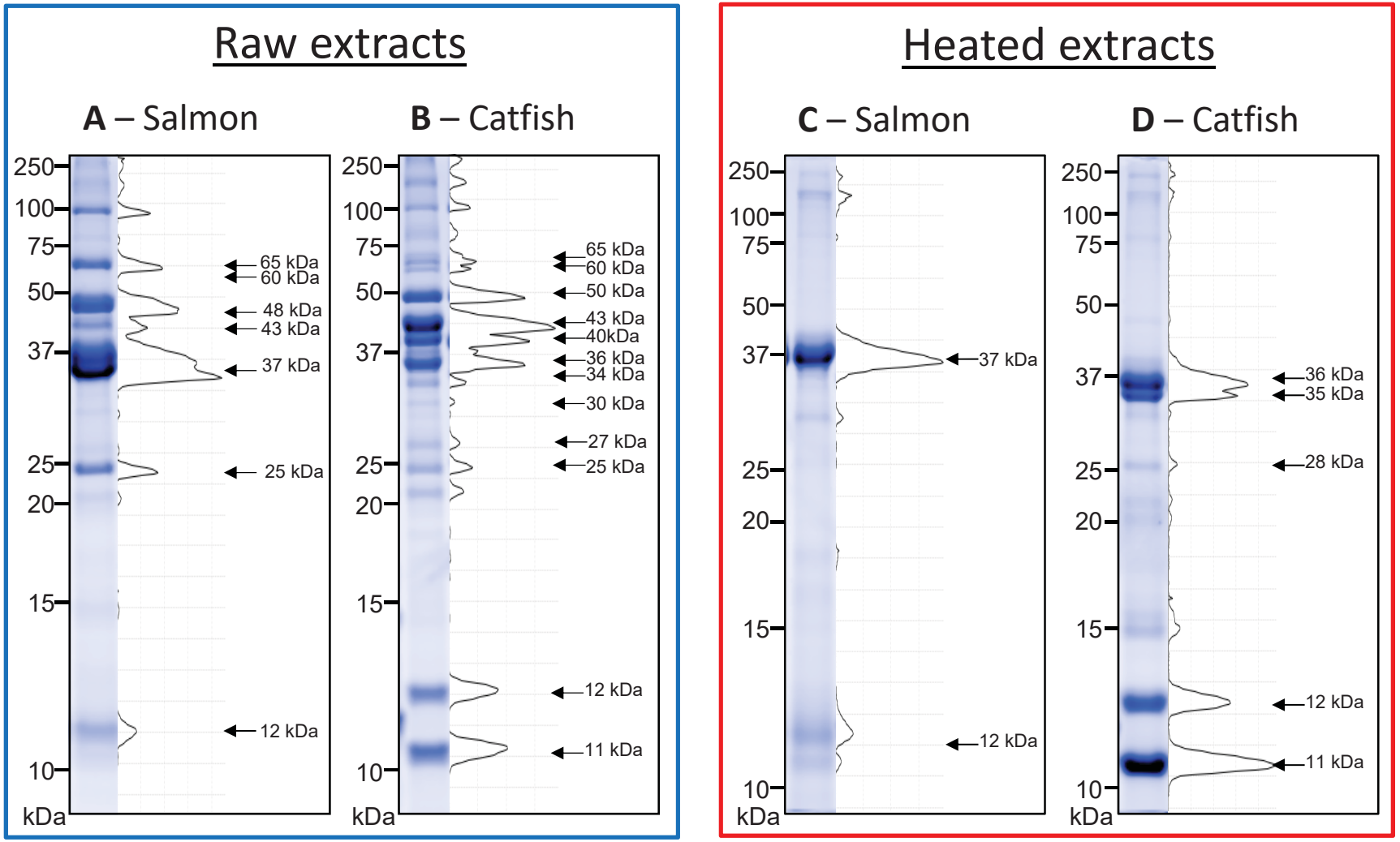

Raw extracts
A – Salmon
B – Catfish
Heated extracts
C – Salmon
D – Catfish
250
100
75
50
37
25
20
15
10
kDa
65 kDa
60 kDa
48 kDa
43 kDa
37 kDa
25 kDa
12 kDa
50 kDa
40kDa
36 kDa
34 kDa
30 kDa
27 kDa
11 kDa
35 kDa
28 kDa

FIGURE 1

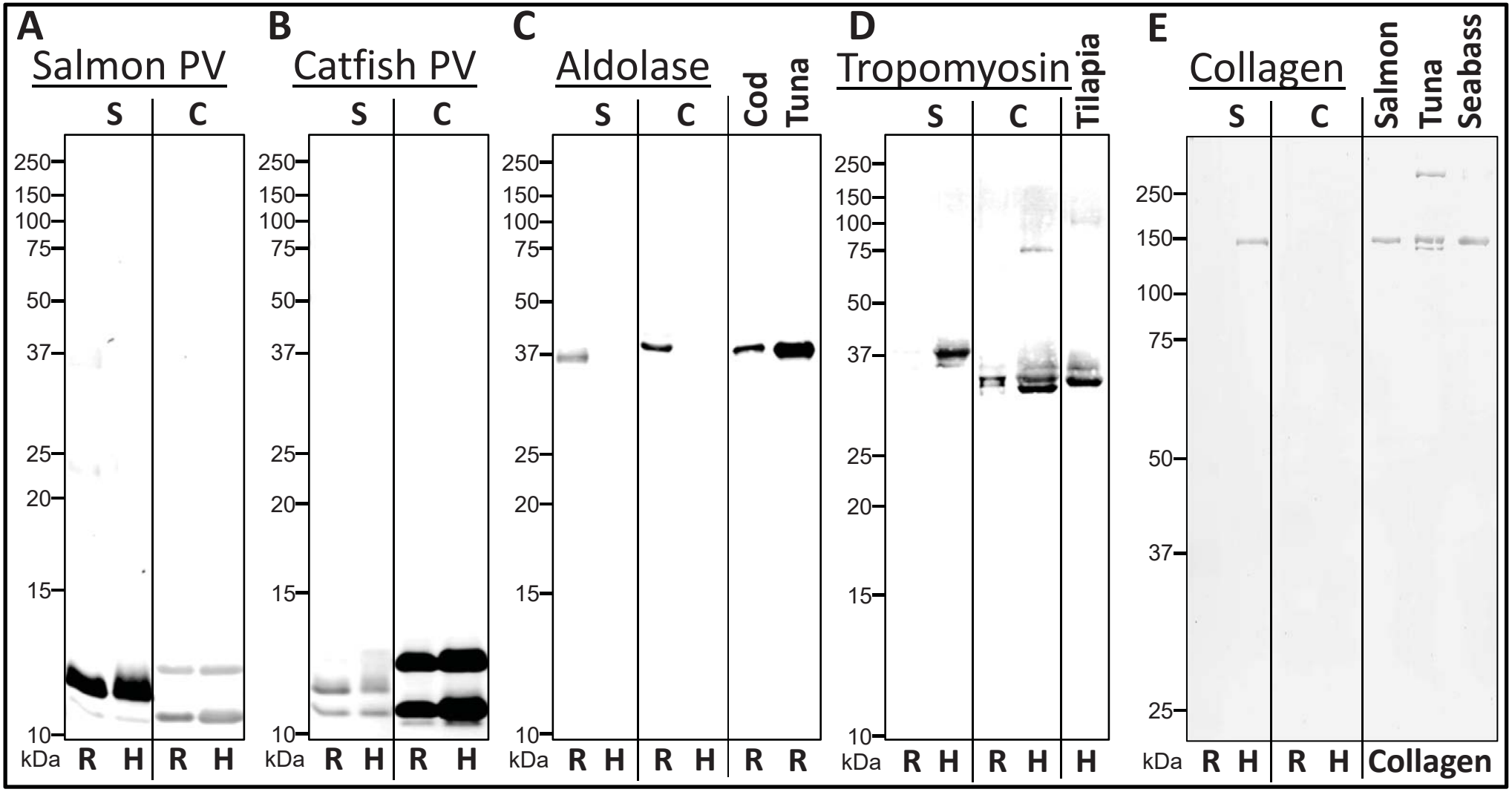

A
Salmon PV
S
C
B
Catfish PV
S
C
C
Aldolase
S
C
Cod
Tuna
D
Tropomyosin
S
C
Tilapia
E
Collagen
S
C
Salmon
Tuna
Seabass
250
150
100
75
50
37
25
20
15
10
kDa
R
H
R
H
Collagen

FIGURE 2

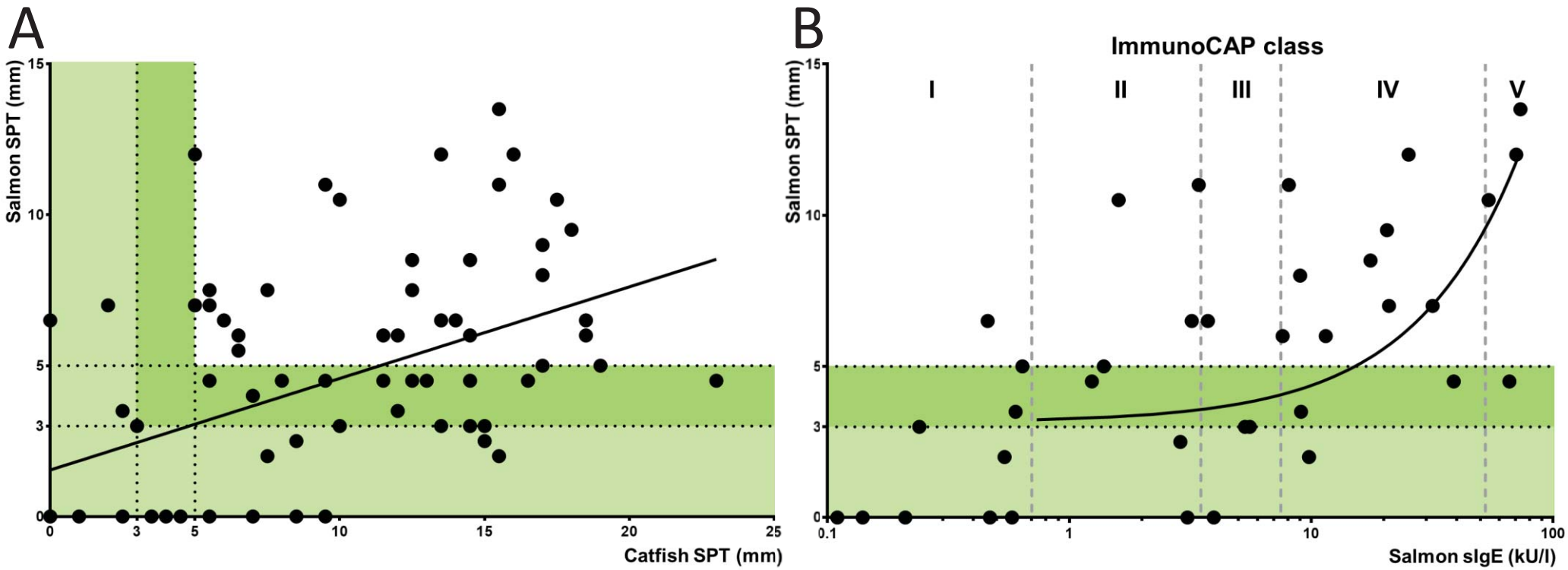

A
Salmon SPT (mm)
15
10
5
3
0
0
3
5
10
15
20
25
Catfish SPT (mm)
B
ImmunoCAP class
I
II
III
IV
V
Salmon SPT (mm)
15
10
5
3
0
0.1
1
10
100
Salmon sIgE (kU/l)

FIGURE 3

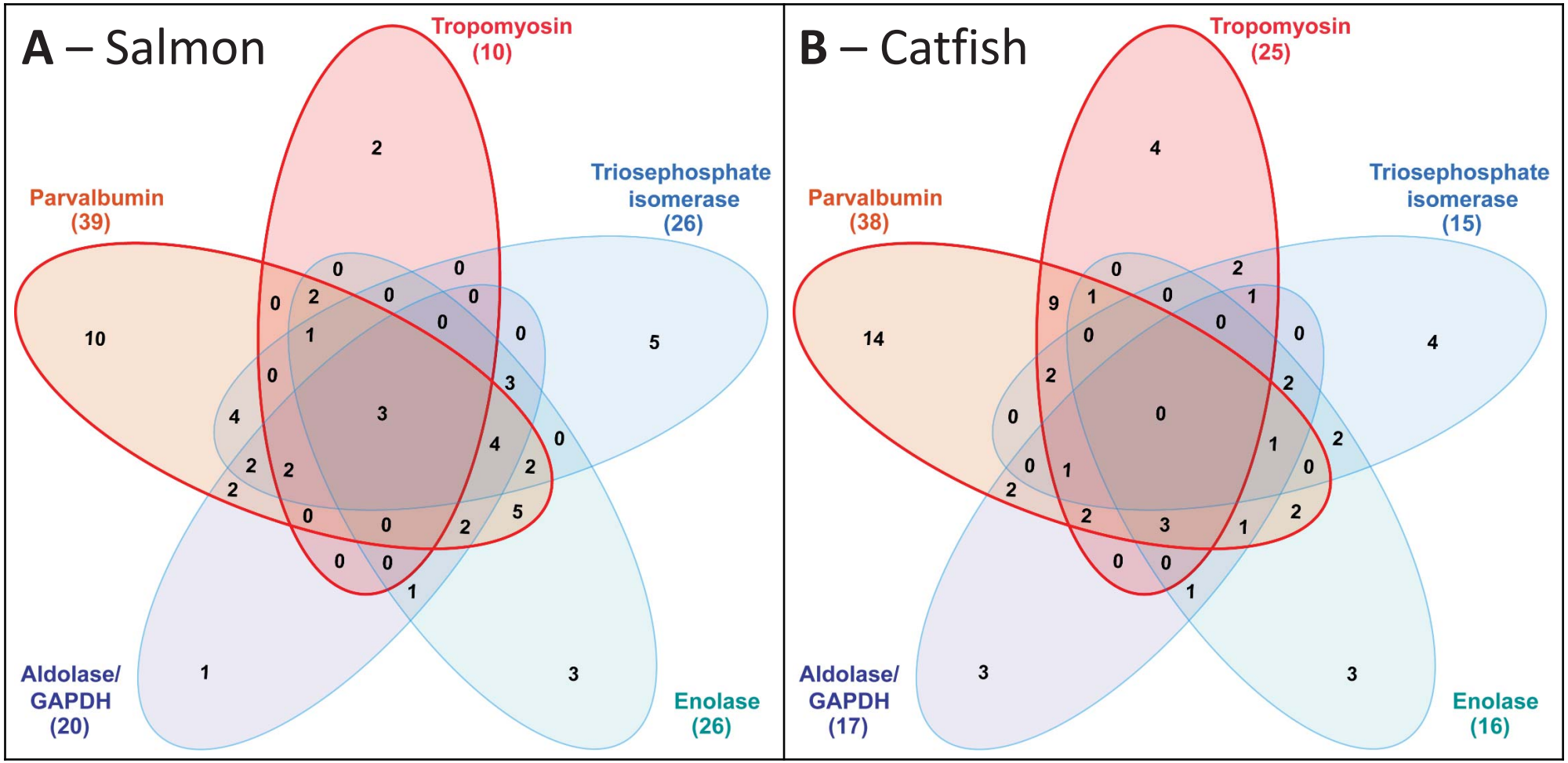

A – Salmon
Tropomyosin (10)
Parvalbumin (39)
Triosephosphate isomerase (26)
Aldolase/ GAPDH (20)
Enolase (26)
2
10
5
3
1
3
B – Catfish
Tropomyosin (25)
Parvalbumin (38)
Triosephosphate isomerase (15)
Aldolase/ GAPDH (17)
Enolase (16)
4
14
4
0
3
3

FIGURE 4

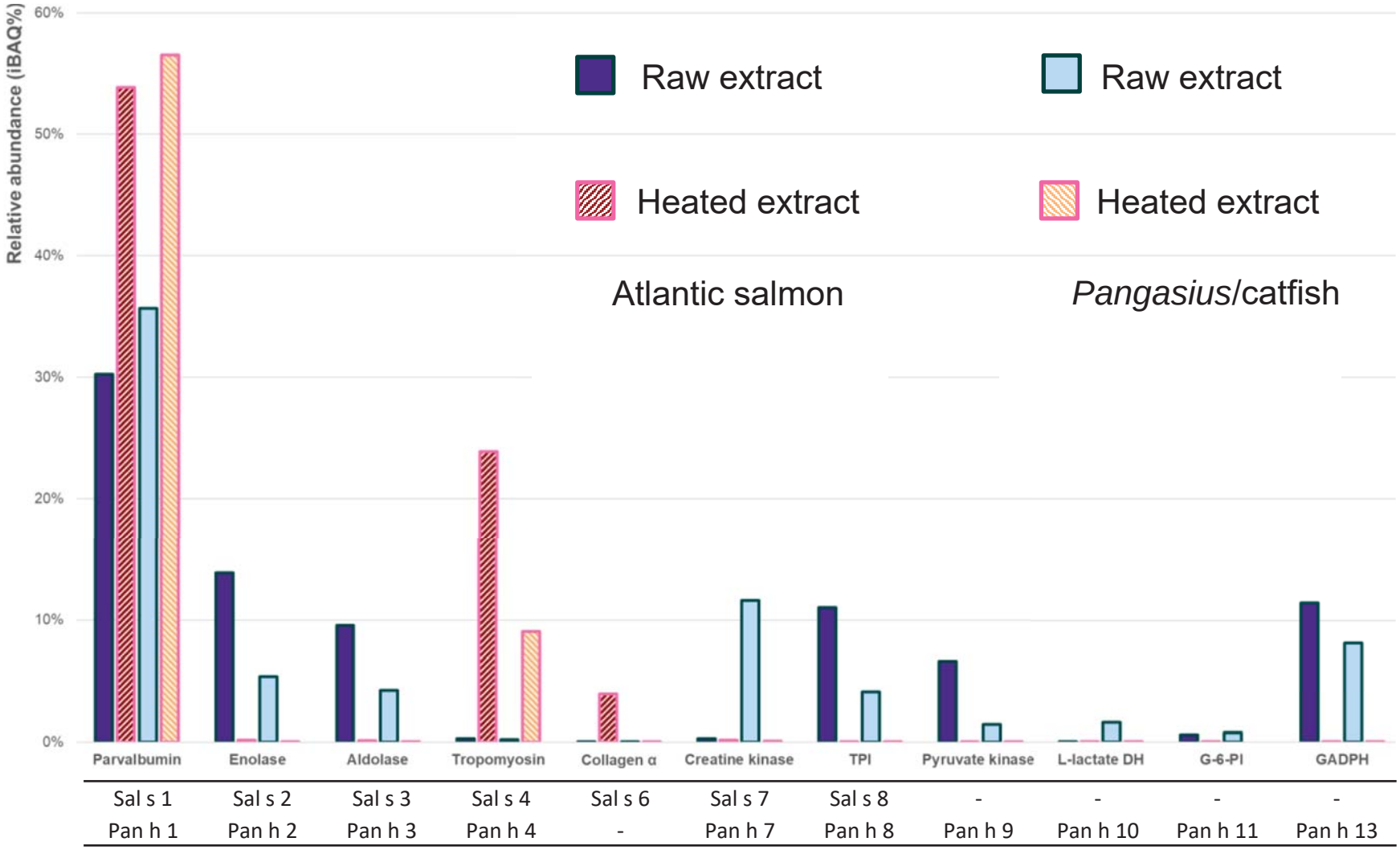

Relative abundance (iBAQ%)
60%
50%
40%
30%
20%
10%
0%
Raw extract
Heated extract
Atlantic salmon
Raw extract
Heated extract
Pangasius/catfish
Parvalbumin
Enolase
Aldolase
Tropomyosin
Collagen α
Creatine kinase
TPI
Pyruvate kinase
L-lactate DH
G-6-PI
GADPH
Sal s 1
Pan h 1
Sal s 2
Pan h 2
Sal s 3
Pan h 3
Sal s 4
Pan h 4
Sal s 6
-
Sal s 7
Pan h 7
Sal s 8
Pan h 8
-
Pan h 9
-
Pan h 10
-
Pan h 11
-
Pan h 13

FIGURE 5

TABLE S1: Clinical characteristics of 77 patients recruited for this study and their skin reactivity to salmon and catfish.

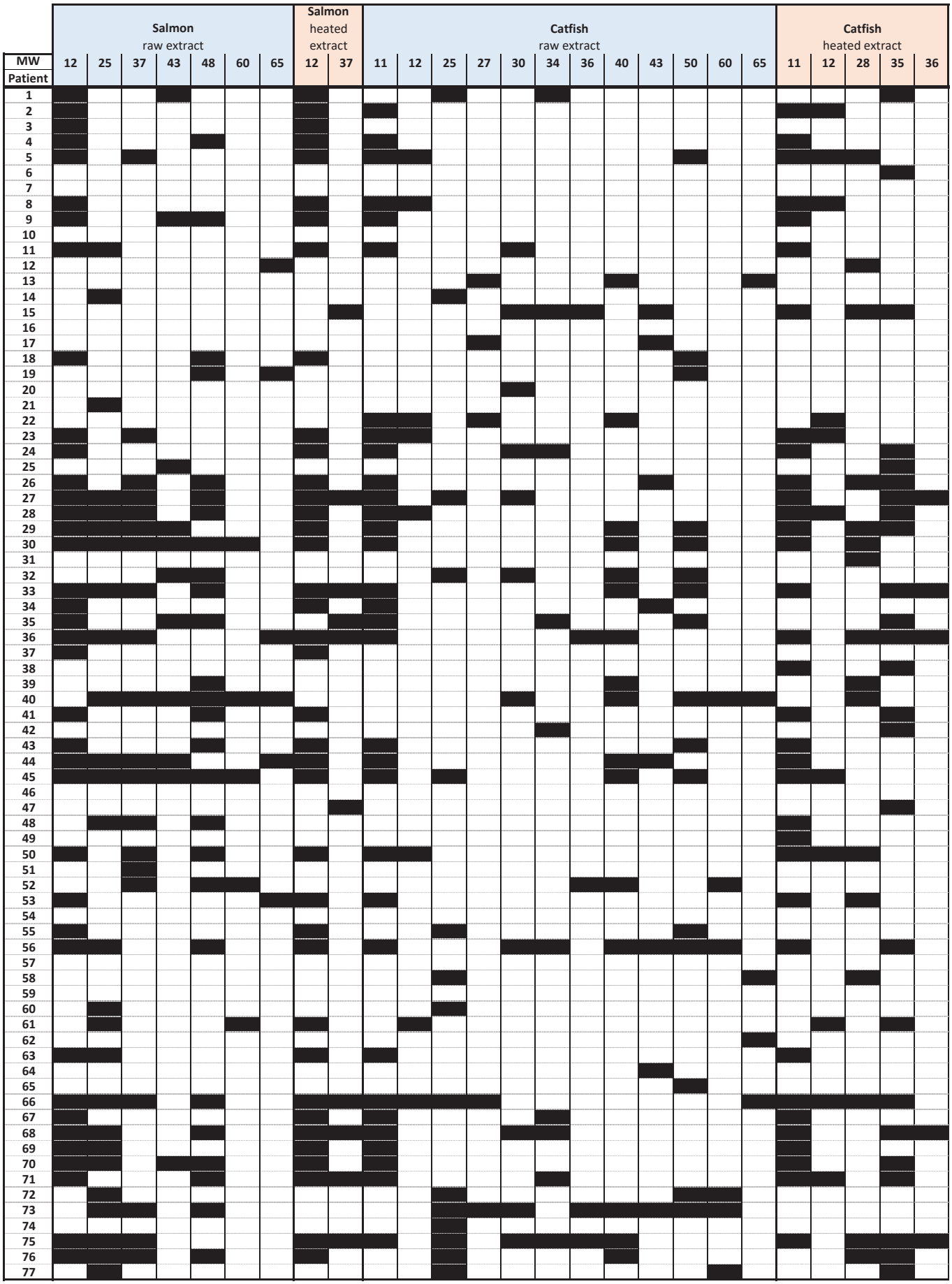
Patients			sIgE	Skin prick test results			
#	Sex	Age (yr)	Salmon (kU/L)	Salmon (mm)	Catfish (mm)	Implicated fish*	Symptoms
1	M	14	3.96	0	5.5	Snapper	AE, OAS
2	M	5	-	8.5	12.5	Salmon, sardine	AN, U
3	M	9	-	6.5	18.5	White bait	AE, C, U
4	M	1	-	6	18.5	Asian seabass, salmon, white fish	AE, OAS, U
5	M	5	-	4.5	13	Murray cod	AN, GIS, RD
6	F	13	-	0	0	Asian seabass, catfish	GIS, RD, U
7	F	10	-	0	4	Salmon, white fish	AE, GIS, U
8	M	11	25.3	12	13.5	Tuna, white fish	AE, GIS, OAS, U
9	F	5	-	5.5	6.5	Salmon	GIS, U
10	M	9	-	7.5	7.5	Salmon	U
11	F	7	-	9	17	Salmon, tilapia, tuna, yellowtail kingfish	AE, OAS, U
12	M	10	-	4	7	White bait	AN, RD, U
13	M	10	<0.01	0	4	Asian seabass	OAS, U
14	M	7	0.64	5	19	White fish	OAS, U
15	M	13	1.24	4.5	12.5	White fish	OAS, U
16	M	14	0.21	0	4.5	Salmon	AE
17	M	3	0.03	0	4	Catfish	AE, U
18	F	12	3.43	11	15.5	White fish	OAS, U
19	F	13	0.01	0	8.5	Murray cod or tuna	U
20	M	4	<0.01	0	4.5	White fish	U
21	F	2	0.6	3.5	2.5	Salmon	AE, U
22	M	1	-	4.5	5.5	Catfish	AE
23	M	12	9.01	8	17	Asian seabass	U
24	M	9	5.33	3	10	Trevally	AN, RD, U
25	M	11	0.11	0	1	White fish	AE, AN, RD, U
26	F	1	5.58	3	13.5	Asian seabass	AE, RD, U
27	M	8	9.8	2	7.5	Salmon	OAS

28	F	2	73.4	13.5	15.5	Cod	AE, U
29	M	8	70.5	12	16	White fish	GIS, RD, U
30	F	6	-	4.5	12.5	White fish	AE
31	M	9	0.46	6.5	6	Croaker	AE
32	F	10	0.14	0	4	Catfish	GIS, RD, OAS
33	M	5	11.5	6	11.5	Leather jacket	AE
34	M	7	7.63	6	6.5	Trout	AE, OAS, U
35	F	7	3.08	0	9.5	White fish	U
36	M	4	21	7	5	White fish	AP, U
37	M	14	1.6	10.5	10	Salmon	OAS, U
38	M	7	0.54	2	15.5	Tilapia	C, GIS
39	F	4	0.47	0	8.5	Catfish	AE, E, U
40	F	15	1.39	5	17	Asian seabass	AE, U
41	M	18	3.21	6.5	14	Tuna	RD, U
42	M	12	0.24	3	15	Unknown	RD, U
43	M	9	3.74	6.5	13.5	Silver perch	AE
44	F	2	-	7	5.5	Salmon, yellowtail kingfish	U
45	F	12	38.9	4.5	11.5	Flathead	AE
46	M	1	<0.01	0	0	Salmon	AE, U
47	F	3	-	0	0	Hake	AN, U
48	F	11	0.04	0	7	Silver bream	AE, AN, OAS, RD
49	F	17	-	0	8.5	Catfish	GIS
50	M	8	-	4.5	13	Cod, white fish	AN, R, RD, U
51	M	20	-	3	13.5	White fish	AE, U
52	F	13	20.6	9.5	18	Salmon	U
53	M	1	-	6	14.5	Tilapia, yellowtail kingfish	AE, U
54	M	5	-	3	14.5	White fish	AE, OAS, U
55	M	15	-	12	5	Milkfish	U
56	M	10	-	4.5	9.5	Perch	U

57	M	8	-	6.5	0	Bream	AE, AN, RD
58	M	16	-	4.5	8	Snapper	U
59	M	11	-	3	3	Salmon, white fish	AE, C, RD
60	M	15	-	2.5	8.5	Asian seabass, salmon	AP, GIS, RD
61	M	18	-	4.5	14.5	Catfish, white fish	GIS
62	F	17	2.88	2.5	15	Asian seabass, croaker	OAS, R
63	M	14	8.09	11	9.5	Catfish	RD, U
64	F	13	-	0	3.5	White fish	OAS, U
65	M	14	-	0	9.5	Asian seabass	AN
66	M	11	66.1	4.5	23	Salmon	AE
67	M	15	17.6	8.5	14.5	Ling	AE, OAS
68	F	10	9.08	3.5	12	White fish	AE, OAS, RD
69	M	2	-	7.5	5.5	Salmon	U
70	M	12	31.8	7	2	Salmon, tuna	E, U
71	M	7	-	7.5	12.5	Ling	AE, U
72	F	21	0.58	0	2.5	Snapper, tuna	AE, GIS, U
73	F	17	-	0	0	White fish	AE, AN, GIS, RD
74	M	18	-	6	12	Salmon, silver perch, shark	AE, AN, C, RD
75	M	10	54.2	10.5	17.5	Asian seabass, tuna	AE
76	F	15	-	5	19	Salmon	E, U
77	F	13	-	4.5	16.5	Milk fish, tilapia, white fish	C, RD, U

Note: M=male; F=female; '-'=not determined; AE, angioedema (lip/face swelling); AN, anaphylaxis; AP, abdominal pain; C, conjunctivitis; E, eczema; GIS, gastrointestinal syndrome (vomiting); OAS, oral allergy syndrome (itchy mouth or swelling throat/tongue); R, rhinitis (iriitation inside nose, sneezing); RD, respiratory distress; U, urticaria (erythema, hives, rash).
*Fish species which was associated with clinical reaction upon ingestion.

TABLE S2: Densitometric analyses of IgE surf immunoblots with both raw and heated extracts from salmon and catfish (Figure S2).



Note: The patient number corresponds to Table S1. A black box indicates IgE-binding. Bands with IgE-binding by less than five patients are not presented.
MW, molecular weight of the IgE-reactive band in the corresponding extract.

TABLE S3: Protein bands with IgE-binding for less than five patients in raw and heated extracts from salmon and catfish.

	MW of band with IgE reactivity in extract			
	Salmon		**Catfish**	
Patient	**RE**	**HE**	**RE**	**HE**
1		130		
5		70+	55	30, 45, 75+
15				70
28		23	20-25	18, 20
29				20
32			63	
33			15	
35			100+	
36	18	18,25,30		20
37			75	
38			75	
39			75	
44		75		
45		50		
50		18,75+		75+
64			35	
67				75+
68				70
69			63	
73			55, 63, 100+	
75		25,53	15	70
76				45
77			150	

Note: The patient number corresponds to Table S1. MW, molecular weight in kDa; RE, raw extract; HE, heated extract. The molecular weight is given in kDa. The '+' indicates that IgE-binding to several bands at and above the molecular weight was observed.

TABLE S4: Identity of IgE-binding salmon proteins based on mass spectrometric analyses.

extract	MW of band	hit	protein ID (NCBI)	protein name	proteins in group	relative abundance (iBAQ%)	coverage (%)	mass (kDa)	mascot score	empai value
RE	65 kDa	1	ACH70965.1	**pyruvate kinase**	3	86.3	82	59	4,266	175
		2	XP_013982708.1	**glucose-6-phosphate isomerase-like**	7	6.1	62	63	1,847	31
		3	ACN58696.1	pyruvate kinase muscle isozyme, partial	12	0	39	58	1,418	5
		4	ACN10472.1	pyruvate kinase muscle isozyme	12	0	42	59	1,390	5
RE	48 kDa	1	ACH70930.1	**enolase 3-1**	7	89.3	90	47	6,062	2,568
		2	B5DGQ7.1	beta-enolase	4	8.8	92	47	5,241	2,182
		3	B5DGM7.1	fructose-bisphosphate aldolase A	16	0.7	60	40	633	7
		4	ACH70965.1	pyruvate kinase	3	0.3	64	58	600	5
		5	ACH70902.1	aldolase a, fructose-bisphosphate 2	4	0	58	40	524	4
RE	43 kDa	1	ACH70914.1	**creatine kinase-2**	7	69.5	78	43	1,201	36
		2	ACH70916.1	creatine kinase-3	2	0	76	43	1,186	34
		3	B5DGQ7.1	beta-enolase	4	0	81	48	1,126	16
		4	ACH70930.1	enolase 3-1	9	3.2	78	48	1,079	17
		5	ACH70839.1	creatine kinase	6	7.9	56	43	942	20
		6	ACH70913.1	creatine kinase-1	6	7.9	58	43	934	17
RE	37 kDa	1	B5DGM7.1	**fructose-bisphosphate aldolase A**	16	36	80	40	3,064	46
		2	ACH70902.1	**aldolase a, fructose-bisphosphate 2**	4	6.4	77	40	2,755	49
		3	CBL79147.1	aldolase A	4	6.4	77	40	2,261	36
		4	ACN10292.1	**glyceraldehyde-3-phosphate dehydrogenase**	14	51.4	83	36	1,687	30
		5	ACH85353.1	aldolase a, fructose-bisphosphate, partial	8	0	75	27	1,378	42
		6	ACN10700.1	fructose-bisphosphate aldolase A	4	0	55	40	966	2
RE	25 kDa	1	ACM09737.1	**triosephosphate isomerase**	5	95.8	93	27	5,743	2,543
		2	ACI70188.1	triosephosphate isomerase	1	0	91	25	5,720	3,053
		3	ACN10206.1	triosephosphate isomerase	5	1.5	32	27	931	1
HE	37 kDa	1	NP_001117128.1	**tropomyosin alpha-1 chain**	24	99.7	95	33	4,228	6,954
		2	XP_013982753.1	tropomyosin alpha-1 chain-like isoform X1	33	0	95	33	2,610	493
		3	XP_013982754.1	tropomyosin alpha-1 chain-like isoform X2	6	0	79	28	1,888	52
		4	XP_014056639.1	tropomyosin alpha-3 chain-like isoform X1	20	0	58	34	1,258	9
		5	ACM08443.1	tropomyosin-1 alpha chain	8	0	61	33	814	19
		6	ACI33578.1	tropomyosin-1 alpha chain	9	0	52	33	810	28
HE	12 kDa	1	CBA35349.1	**parvalbumin beta-1**	1	99.5	78	12	589	44
		2	ACI68103.1	parvalbumin beta 1	22	0	83	12	578	57
		3	ADM16103.1	parvalbumin beta 2	13	0	28	11	86	1

Note: RE=raw extract, HE=heated extract.

TABLE S5: Identity of IgE-binding catfish proteins based on mass spectrometric analyses.

extract	MW of band	hit	protein ID (NCBI)	protein name	proteins in group	relative abundance (iBAQ%)	coverage (%)	mass (kDa)	mascot score	empai value
RE	65 kDa	1	XP_026775867.1	**pyruvate kinase PKM-like**	4	91.7	83	59	2,483	48
		2	XP_026769191.1	pyruvate kinase PKM isoform X1	5	0	25	58	684	1
RE	60 kDa	1	XP_026782721.1	**glucose-6-phosphate isomerase**	2	72.7	69	62	3,068	113
		2	XP_026775172.1	glucose-6-phosphate isomerase-like	1	0	23	62	847	1
		3	XP_026775867.1	pyruvate kinase PKM-like	1	4	58	59	742	4
RE	50 kDa	1	XP_026777250.1	**beta-enolase**	1	92.6	89	37	7,776	6,557
		2	XP_026776343.1	alpha-enolase isoform X2	2	1.7	78	47	3,754	53
		3	XP_026777316.1	enolase-like	2	0	52	47	3,238	12
		4	XP_026777571.1	creatine kinase M-type-like	2	0.4	55	43	844	23
RE	43 kDa	1	XP_026777571.1	**creatine kinase M-type-like**	2	25.7	85	43	5,294	20,522
		2	XP_026780620.1	**creatine kinase M-type-like**	1	61.7	85	43	4,909	60,653
		3	XP_026771637.1	fructose-bisphosphate aldolase A	3	7	81	40	1,758	132
		4	XP_026796505.1	creatine kinase B-type isoform X1	3	0	41	43	676	2
RE	40 kDa	1	XP_026771637.1	**fructose-bisphosphate aldolase A**	3	88.7	86	40	3,870	2,033
		2	XP_026777571.1	creatine kinase M-type-like	2	3.6	80	43	1,883	106
		3	XP_026780620.1	creatine kinase M-type-like	1	4.9	80	43	1,431	62
		4	XP_026777250.1	beta-enolase	1	0.9	36	37	737	7
		5	XP_026782131.1	glyceraldehyde-3-phosphate dehydrogenase	2	0.2	38	36	540	3
RE	36 kDa	1	XP_026782131.1	**glyceraldehyde-3-phosphate dehydrogenase**	1	73.9	90	36	3,631	3,045
		2	XP_026777250.1	beta-enolase	1	8.3	86	38	1,628	80
		3	XP_026777571.1	creatine kinase M-type-like	2	1.7	74	43	816	17
		4	XP_026780620.1	creatine kinase M-type-like	1	9.3	72	43	693	10
RE	34 kDa	1	XP_026774991.1	**L-lactate dehydrogenase A chain**	2	94.8	63	36	1,448	27
		2	XP_026777250.1	beta-enolase	1	1.9	71	37	909	12
		3	XP_026778582.1	L-lactate dehydrogenase B chain	1	0.1	35	36	585	1
		4	XP_026775475.1	L-lactate dehydrogenase B-B chain	2	94.8	16	39	542	0
		5	XP_026780620.1	creatine kinase M-type-like	1	0.5	49	43	369	4
		6	XP_026782131.1	glyceraldehyde-3-phosphate dehydrogenase	2	0.2	55	36	361	4
RE	25 kDa	1	XP_026795867.1	**triosephosphate isomerase**	1	90.1	82	26	2,296	44

TABLE S5 continued

extract	MW of band	hit	protein ID (NCBI)	protein name	proteins in group	relative abundance (iBAQ%)	coverage (%)	mass (kDa)	mascot score	empai value
HE	36 kDa	1	XP_026775428.1	**tropomyosin alpha-1 chain isoform X1**	7	97.2	95	33	2,235	3,110
		2	XP_026775435.1	tropomyosin alpha-1 chain isoform X8	7	97.2	73	38	1,618	359
		3	XP_026786514.1	tropomyosin alpha-1 chain-like	4	1.1	95	33	1,407	447
		4	XP_026775431.1	tropomyosin alpha-1 chain isoform X4	1	0	79	33	1,086	161
		5	XP_026798115.1	tropomyosin alpha-3 chain isoform X5	5	0	83	33	909	83
		6	XP_026781482.1	tropomyosin alpha-1 chain-like isoform X1	4	0.1	86	33	698	47
HE	35 kDa	1	XP_026781482.1	**tropomyosin alpha-1 chain-like isoform X1**	4	97	97	33	1,305	598
		2	XP_026781484.1	tropomyosin alpha-4 chain-like isoform X3	4	97	81	29	722	125
		3	XP_026775428.1	tropomyosin alpha-1 chain isoform X1	7	1.8	71	33	422	19
		4	XP_026786514.1	tropomyosin alpha-1 chain-like	4	0.3	64	33	295	5
		5	XP_026786233.1	tropomyosin alpha-4 chain isoform X3	2	0	59	29	149	3
		6	XP_026780620.1	creatine kinase M-type-like	1	0.2	30	43	104	0
HE	12 kDa	1	XP_026803769.1	**parvalbumin-7**	1	97.1	85	12	1,029	52
		2	XP_026772003.1	parvalbumin beta	2	0.8	46	12	126	2
HE	11 kDa	1	XP_026772003.1	**parvalbumin beta**	2	95.5	51	12	277	7
		2	XP_026770387.1	parvalbumin alpha	1	0.3	65	11	188	1
		3	XP_026803769.1	parvalbumin-7	1	4.2	57	12	154	2
		4	XP_026771441.1	parvalbumin-2	-	0	21	12	78	1
		5	XP_026774930.1	troponin I, fast skeletal muscle-like	4	0	17	20	55	0

Note: RE=raw extract, HE=heated extract

TABLE S6: Analyses of *in vitro* IgE-binding among patient groups with different clinical history and salmon sIgE levels.

		Patients with IgE reactivity	Implicated species				S sIgE level for salmon (class)		
			S	C	S or C	not S or C	Negative	Low to moderate (I-II)	High to very high (III-V)
	n =	70	15	8	23	47	5	16	20
Reactivity to specific proteins	**Any PV**	**63%**	**73%**	**50%**	**65%**	**62%**	**20%**	**38%**	**95%**
	C PV1 but not C PV2	36%	47%	25%	39%	34%	20%	19%	60%
	C PV2 but not C PV1	3%	-	25%	9%	-	-	-	-
	S PV but not C PV	9%	13%	-	9%	9%	-	13%	5%
	C PV but not S PV	7%	-	25%	9%	6%	20%	13%	-
	PV but no other	6%	7%	13%	9%	4%	-	-	5%
	Any TM	**36%**	**27%**	**25%**	**26%**	**40%**	**-**	**38%**	**60%**
	S TM	14%	13%	-	9%	17%	-	13%	30%
	C TM 1	**36%**	**27%**	**25%**	**26%**	**40%**	**-**	**38%**	**60%**
	C TM 2	7%	7%	-	4%	9%	-	-	25%
	TM but not PV	7%	-	13%	4%	9%	-	13%	-
Reactivity to TM or PV	**Any TM or PV**	**70%**	**73%**	**63%**	**70%**	**70%**	**20%**	**50%**	**95%**
	Or TPI	**83%**	**93%**	**75%**	**87%**	**81%**	**20%**	**81%**	**95%**
	Or Ald/GADPH or Eno	91%	100%	88%	96%	89%	60%	88%	100%

Note: The patients were grouped based on *in vivo* and *in vitro* reactivity. Patients with no IgE-binding to any protein band, are excluded from this table. The implicated species refers to the reported allergic reaction upon ingestion. Abbreviations: SPT, skin prick test; S, salmon; C, catfish; RE, raw extract; HE, heated extract; PV, parvalbumin; TM, tropomyosin; TPI, triosephosphate isomerase; GADPH, glyceraldehyde-3-phosphate dehydrogenase; Ald, aldolase; Eno, enolase.

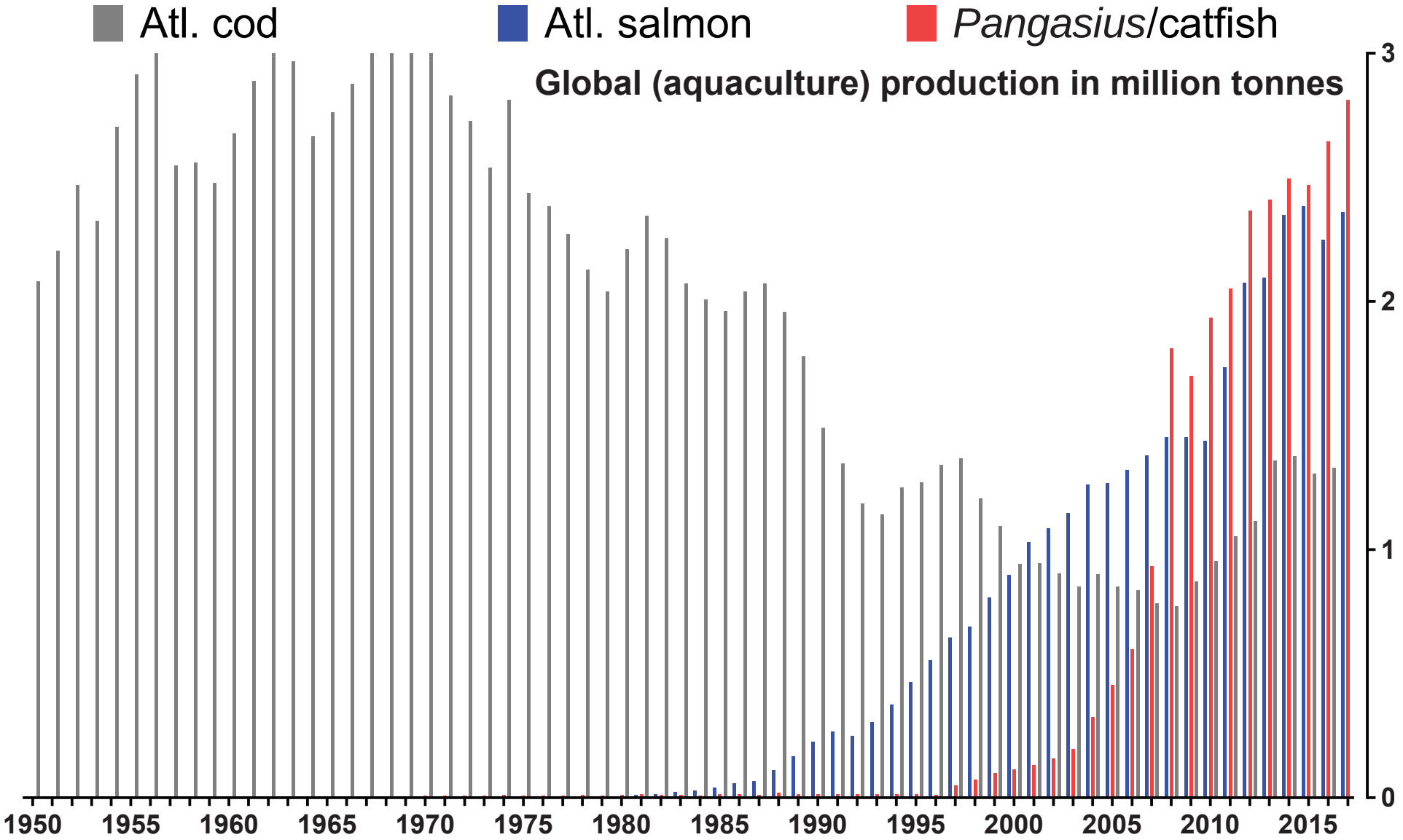


FIGURE S1: Global aquaculture production of Altantic cod (grey), Atlantic salmon (blue), and *Pangasius*/catfish (red) in million tonnes from 1950 till 2017 according to FAO FishStatJ accessible under www.fao.org.

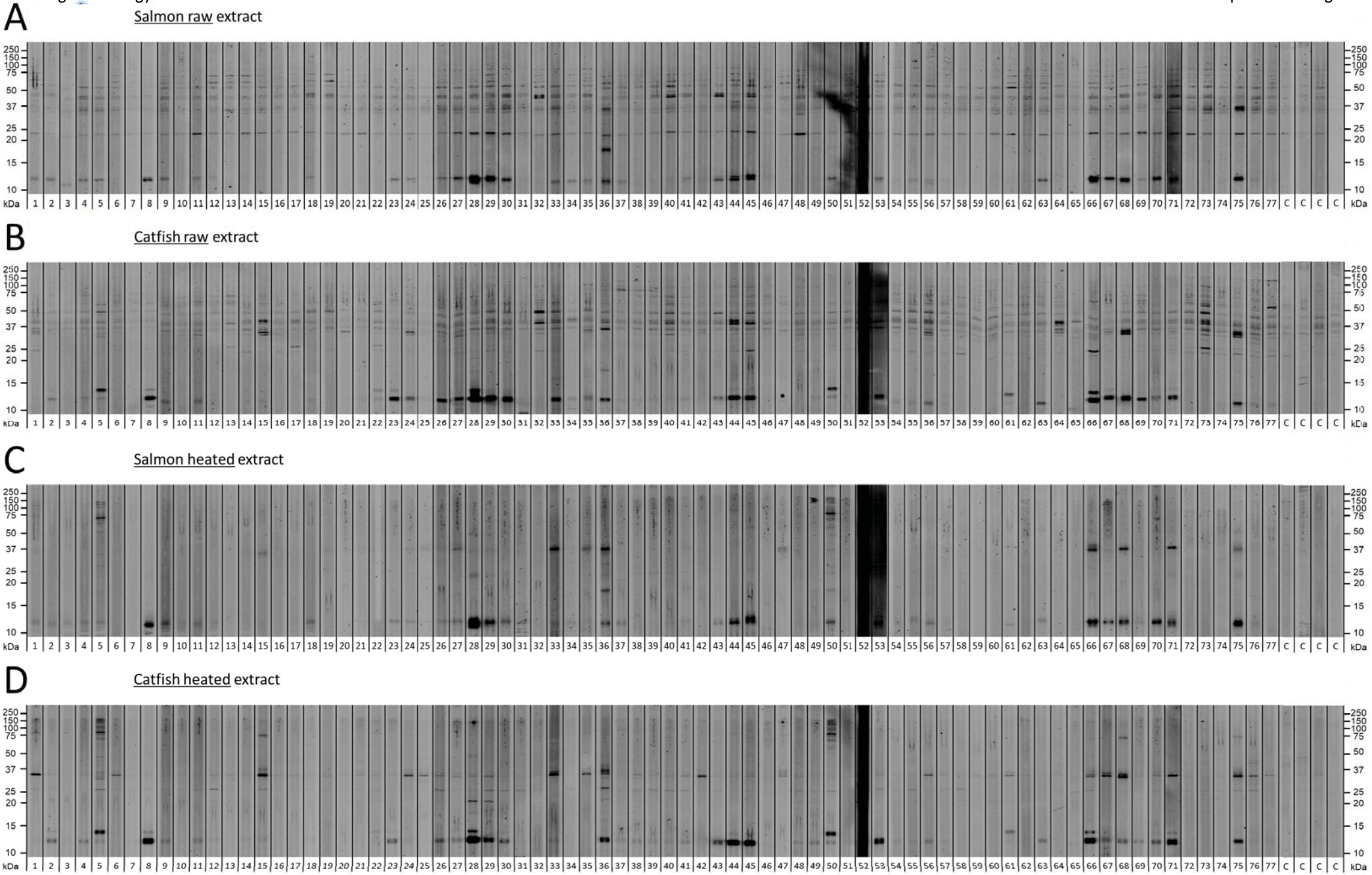


FIGURE S2: IgE-immunoblots with raw (A, B) and heated (C, D) extracts from salmon (A, C) and catfish (B, D) using serum from 77 patients. The patient number on the bottom refers to Table S1. 'C'=controls.

Further results and discussion not included in the manuscript in revision

The above manuscript focused on Atlantic salmon and *Pangasius/*catfish (basa) for which overall 15 proteins were now registered as new fish allergens with the WHO/IUIS based on this study.

Similar results were generated for up to 64 other fish species. In total, raw and heated extracts from 66 fish species were prepared (see **Table V A 1** below and **Table 1** in **Chapter III A** for details and **Appendix B 4.5/4.6** for images of each species) and their total protein content was estimated as described above. The protein concentration ranged from 1.4 to 15.9 mg/ml in raw and 1 to 5.1 mg/ml in heated extracts.

Protein extracts were separated by SDS-PAGE as detailed in the manuscript (see **Figure V A 1**). The raw extracts exhibited more protein bands as compared to heated extracts. Major differences in the abundance of protein bands in the molecular weight range of parvalbumin (10-15 kDa), tropomyosin (32-37 kDa) and collagen (120 kDa and above) were observed in the heated extracts.

Previously reported allergens were detected by immunoblotting using 7 different commercial and in-house antibodies (**Appendix C Figure 1**). In addition to the 5 antibodies described in the manuscript, in-house generated polyclonal antibodies raised in rabbits against parvalbumin from Asian seabass (barramundi, *Lates calcarifer*) and sardine (pilchard, *Sardinops sagax*) were utilised.[1] Densitometric analyses were performed to quantify the signal intensity to the monomers of each of the four allergens (see **Table V A 1**). The signal intensity was species- and antibody-specific. Heat-labile aldolase was detected in all but two raw extracts. Parvalbumin and collagen bands were detected in about 85% of the heated extracts. Notably, the detection rate was the lowest among cartilaginous fish, sharks and rays (species 58-66). In over 90% of heated extracts, the anti-tropomyosin antibody detected at least one

band, with a higher detection rate for cartilaginous as compared to bony fish (species 1-57). The detection of the allergens correlates to the abundance of the corresponding protein bands in most cases.

Among the 77 fish-allergic patients described in the manuscript, 56 patients were not known to be shellfish-allergic (group 1). The other 21 patients have a clinical history of both fish and shellfish allergy (group 2). The most common fish implicated in the allergic reaction were white fish, salmon and Asian seabass/barramundi (reported by 25, 22 and 11 patients, respectively). In addition, the characteristics and serum from a further 10 patients were analysed. Four of these patients avoided fish due to sensitisation to fish, determined by SPT (group 3). The other 6 patients have a clinical history of an IgE-mediated allergic reaction to shellfish and are not known to be allergic to fish (group 4). Shrimp/prawn was the implicated shellfish species in 77% of the 31 patients with shellfish allergy (group 2, 3 and 4 combined).

All 87 patients underwent SPT with 5 commercial fish preparations and 3 in-house fish preparations as well as commercial preparations for shrimp/prawn and 2 species of house dust mite (*Dermatophagoides farinae* and *D. pteronyssinus*). The SPT response to the in-house fish preparations (mean of 9 mm) was significantly higher compared to the commercial fish preparations (mean of 4.5 mm) as evaluated for the 56 fish-allergic patients (p=0.0275 (nested t test, t=2.895) – see **Figure V A 2**). The median wheal diameter was about two times lower for shark (5.5 mm) than for seabass (12 mm) and catfish (9.5 mm). All patient characteristics are provided in **Appendix C Table 1**. sIgE levels for cod (f3), tuna (f40) and salmon (f41) were determined for 47 patients by ImmunoCAP. Forty-three of these patients were fish-allergic. Among those, 6 patients had a sIgE level below 0.1 kU/l for all three tested fish, which is considered negative (ImmunoCAP class 0). The median sIgE level for the other 37 patients was 3.4 kU/l for cod, 2.0 kU/l for tuna and 4.0 kU/l for salmon. There was an overall positive

correlation between SPT and sIgE level for cod and tuna for 36 patients with a positive sIgE level (r_S=0.596, p=0.0001 and r_S=0.571, p=0.0003, respectively - see **Figure V A 3**), which was also described for salmon in the manuscript.

IgE-binding bands in raw Asian seabass/barramundi extract and heated extracts from 10 fish species (in addition to salmon and catfish) were identified by immunoblotting using the Surf-Blot Antibody Screening System by Idea Scientific (**Appendix C Figures 2.1-2.15**). Detection patterns for each patient and extract were evaluated based on detailed densitometric analyses and are provided in **Appendix C Table 2**. Protein bands which demonstrated IgE-binding by at least 5 fish-allergic patients were excised and underwent tryptic digest. Their composition was evaluated by mass spectrometric analyses as described in the manuscript. In barramundi raw extract, parvalbumin was detected by 35% of 77 fish-allergic patient, lactate dehydrogenase by 8% for, GADPH by 19%, aldolase/creatine kinase by 26%, enolase by 34% and glucose-6-phosphate isomerase/pyruvate kinase by 14%r. In addition, a 15 kDa band demonstrated weak IgE-binding for 52% of patients. Its composition and relevance are currently under investigation.

In all heated fish extracts, parvalbumin and tropomyosin were detected by IgE antibodies from 22-49% and 12-32% of fish-allergic patients, respectively (see **Table V A 2**). No IgE-binding to any fish parvalbumin or tropomyosin was observed in 30% of fish-allergic patients. Notably, 49% showed IgE-binding to at least one fish tropomyosin and 22-69% of patients with IgE-binding to tropomyosin did not detect parvalbumin from the same species. This highlights the possible high importance of heat-stable tropomyosin isoforms for component-resolved fish allergy diagnostics.

In cod and hoki, a 24/25 kDa band demonstrated IgE-binding by 69% and 73% of fish-allergic patients. This was 21% to 36% for ling, tilapia, catfish, John Dory and gummy shark, and 10%

or less for the other 5 investigated fish species. This band was also detected using serum from up to 3 controls. Those bands contained apolipoprotein, myosin light chain and parvalbumin according to mass spectrometric analyses, and should be investigated further.

No IgE-binding to any of the extracts was observed for 2 patients (6 and 59 in **Appendix C Table 1**). Both patients had negative SPT results (<5 mm) to any of the 8 tested fish preparations.

Fish tropomyosin was identified as a major IgE-binding protein for fish-allergic patients with possible relevance for shellfish-allergic patients. To allow further investigations and determine their cellular reactivity, fish tropomyosins have to be isolated in solution. A few studies are published, which describe the purification of fish tropomyosin.[2–10] However, the resulting purity was below 95% and/or not sufficiently demonstrated or the method was not feasible to be performed in the Molecular Allergy Research Laboratory in Townsville, Australia. Therefore an advanced method was established in-house. In brief, heated fish extract underwent ammonium sulphate precipitation. The 30-40% fraction was dialysed against PBS and tropomyosin was further enriched by 4.6 isoelectric point precipitation using HCl. A purity of over 95% (based on densitometric analyses after SDS-PAGE) was achieved by fast protein liquid chromatography using first an anion-exchange column (Bio-Scale™ Mini Macro-Prep® High Q Cartridge, Bio-Rad) with a 0-1 M NaCl linear gradient in 25 mM Tris HCl (pH 6.5) and then a ceramic hydroxyapatite column (Bio-Scale™ Mini Cartridge, Type I, 40 µm Media, Bio-Rad) with a 10-250 mM $NaPO_4$ linear gradient in 1 M NaCl (pH 7). **Figure V A 4 A** below shows the SDS-PAGE profile of purified tropomyosin and heated extract from catfish. Tropomyosin monomers and dimers were detected as described above (see **Figure V A 4 B**). Mass spectrometric analyses are in progress to confirm the purity of the catfish tropomyosin preparation.

Raw and heated extracts from all 10 fish species, analysed for their IgE-binding proteins, (in addition to salmon and catfish) underwent tryptic digest in-solution. **Figure V A 5** below shows the relative abundance of 10 (putative) allergens, which are registered for catfish with the WHO/IUIS based on this study, and collagen in barramundi, common carp, Atlantic cod and Nile tilapia extracts. Parvalbumin was the most abundant protein in both raw and heated extracts from barramundi, cod and tilapia (36%-49% and 53%-88%, respectively). In carp raw extract, parvalbumin was most abundant (14%) followed by aldolase (13%), while it was tropomyosin (40%) followed by parvalbumin (17%) in the heated extract. In general, tropomyosin and collagen were more abundant in heated than in raw extracts (0.0%-1.2% versus 3.0%-39.7%). Collagen had a relative abundance of below 1% in all extracts except cod heated extract (1.9%). The other 7 (putative) allergens were most abundant in the raw extracts (0.0% versus 0.4%-13.3%). In the heated extracts, these proteins collectively made up 0.1%-0.5% of proteins. Overall, these results align with findings presented in the manuscript and highlight the abundance of parvalbumin and tropomyosin in heated fish extracts and resulting importance for the development of improved component-resolved fish allergy diagnostics.

In summary, the comparative abundance of 4 known fish allergens was estimated in 66 fish species and 6 novel fish allergens were discovered and registered with the WHO/IUIS for the first time. The repertoire of confirmed IgE-binding proteins in 12 fish species was expanded, which provides the knowledgebase for the development of component-resolved fish allergy diagnostics. However, analyses in this chapter are limited to phosphate-buffered saline- (PBS-) derived protein extracts, in which selected proteins are likely to be under-represented, as demonstrated in **Chapter II** for collagen. **Chapter V B** therefore focuses on acid-derived protein extracts, in which above mentioned collagen is more abundant.

Table V A 1. Details of investigated species and densitometric analyses of immunoblots using antibodies specific to aldolase, parvalbumin (PV), tropomyosin (TM) and collagen. The corresponding immunoblots are shown in Appendix C Figure 1.

			raw extract, **antibody** against		**heated** extracts, **antibody** against						**taxonomic classification**		
	common name(s)	**scientific name**	rabbit **aldolase**	seabass **PV**	seabass **PV**	salmon **PV**	catfish **PV**	sardine **PV**	prawn **TM**	tuna **collagen**	**family**	**order**	**class**
1	Asian swamp eel	*Monopterus albus*									Synbranchidae	Anguilliformes	Actinop-terygii
2	splendid alfonsino	*Beryx splendens*									Berycidae	Beryciformes	
3	orange roughy†	*Hoplostethus atlanticus*									Trachichthyidae		
4	European pilchard†	*Sardina pilchardus*									Clupeidae	Clupeiformes	
5	European carp†	*Cyprinus carpio*									Cyprinidae	Cypriniformes	
6	Pacific cod	*Gadus macrocephalus*									Gadidae	Gadiformes	
7	Atlantic cod†	*Gadus morhua*											
8	Southern blue whiting	*Micromesistius australis*											
9	blue grenadier	*Macruronus novaezelandiae*									Merlucciidae		
10	milkfish	*Chanos chanos*									Chanidae	Gonorynchiformes	
11	flathead grey mullet	*Mugil cephalus*									Mugilidae	Mugiliformes	
12	ling/pink cusk-eel	*Genypterus blacodes*									Ophidiidae	Ophidiiformes	
13	Chinese icefish	*Neosalanx tangkahkeii*									Salangidae	Osmeriformes	
14	Nile tilapia	*Oreochromis niloticus*									Cichlidae	Perciformes	
15	king George whiting	*Sillaginodes punctatus*									Sillaginidae		
16	sand whiting	*Sillago ciliata*											
17	yellowtail kingfish/amberjack	*Seriola lalandi*									Carangidae		
18	blue eye trevalla/bluenose warehou	*Hyperoglyphe antarctica*									Centrolophidae		
19	mahi mahi/dolphinfish	*Coryphaena hippurus*									Coryphaenidae		
20	barramundi/Asian seabass	*Lates calcarifer*									Latidae		
21	grass emperor	*Lethrinus laticaudis*									Lethrinidae		
22	redthroat/trumpet emperor	*Lethrinus miniatus*											
23	spangled emperor	*Lethrinus nebulosus*											
24	longface/-nose emperor	*Lethrinus olivaceus*											
25	green jobfish	*Aprion virescens*									Lutjanidae		
26	crimson snapper	*Lutjanus erythropterus*											
27	Malabar blood/ saddletail snapper	*Lutjanus malabaricus*											
28	emperor red snapper	*Lutjanus sebae*											
29	goldband(ed) jobfish/snapper	*Pristipomoides multidens/typus*											
30	Patagonian toothfish	*Dissostichus eleginoides*									Nototheniidae		
31	murray cod	*Maccullochella peelii*									Percichthyidae		
32	blue/fourfinger threadfin	*Eleutheronema tetradactylum*									Polynemidae		
33	king threadfin	*Polydactylus macrochir*											
34	cobia	*Rachycentron canadum*									Rachycentridae		

Table V A 1 continued.

	common name(s)	scientific name	raw extract, antibody against rabbit aldolase	raw extract, antibody against seabass PV	heated extracts, antibody against seabass PV	heated extracts, antibody against salmon PV	heated extracts, antibody against catfish PV	heated extracts, antibody against sardine PV	heated extracts, antibody against prawn TM	heated extracts, antibody against tuna collagen	taxonomic classification family	order	class
35	parrot fish	*Scarus spp.*									Scaridae		
36	skipjack tuna†	*Katsuwonus pelamis*									Scombridae		
37	Indian mackerel	*Rastrelliger kanagurta*											
38	Spanish mackerel	*Scomberomorus commerson*											
39	broad-barred king/ grey mackerel	*Scomberomorus semifasciatus*											
40	albacore tuna†	*Thunnus alalunga*											
41	yellowfin tuna†	*Thunnus albacares*											
42	longtail tuna	*Thunnus tonggol*											
43	orange-spotted grouper	*Epinephelus coioides*									Serranidae		
44	giant grouper	*Epinephelus lanceolatus*											
45	blacksaddled coralgrouper	*Plectropomus laevis*											
46	coral trout /leopard coralgrouper	*Plectropomus leopardus*											
47	king soldier/frypan bream/snapper	*Argyrops spinifer*									Sparidae		
48	pink snapper/silver seabream	*Pagrus auratus*											
49	silver perch	*Bidyanus bidyanus*									Terapontidae		
50	swordfish†	*Xiphias gladius*									Xiphiidae		
51	yellowbelly flounder	*Rhombosolea leporina*									Pleuronectidae	Pleuronectiformes	
52	rainbow trout†	*Oncorhynchus mykiss*									Salmonidae	Salmoniformes	
53	Atlantic salmon†	*Salmo salar*											
54	dusky flathead	*Platycephalus fuscus*									Platycephalidae	Scorpaeniformes	
55	pangasius/striped catfish	*Pangasianodon hypophthalmus*									Pangasiidae	Siluriformes	
56	ocean (Chinaman-leather)jacket	*Nelusetta ayraud*									Monacanthidae	Tetraodontiformes	
57	John Dory†	*Zeus faber*									Zeidae	Zeiformes	
58	elephant/ghost shark	*Callorhinchus milii*									Callorhinchidae	Chimaeriformes	Holocephali
59	Australian sharpnose shark	*Rhizoprionodon taylori*									Carcharhinidae	Carcharhiniformes	Elasmo-branchii
60	longsnout dogfish	*Deania quadrispinosa*									Centrophoridae	Squaliformes	
61	bluespotted stingray/maskray	*Neotrygon kuhlii*									Dasyatidae	Myliobatiformes	
62	longtail butterfly ray	*Gymnura poecilura*									Gymnuridae		
63	saw shark	*Pristiophorus spp.*									Pristiophoridae	Pristiophoriformes	
64	shortnose spurdog	*Squalus megalops*									Squalidae	Squaliformes	
65	zebra shark	*Stegostoma fasciatum*									Stegostomatidae	Orectolobiformes	
66	gummy shark	*Mustelus antarcticus*									Triakidae	Carcharhiniformes	

Note: All fish species, except Atlantic cod, are consumed in the Asia-Pacific region and most are traded globally. Fish species with a cross '†' are native to Europe/North America. The black bars indicate the intensity of the signal to the monomeric respective protein band(s). The strongest signal (100%) is indicated with a red box.

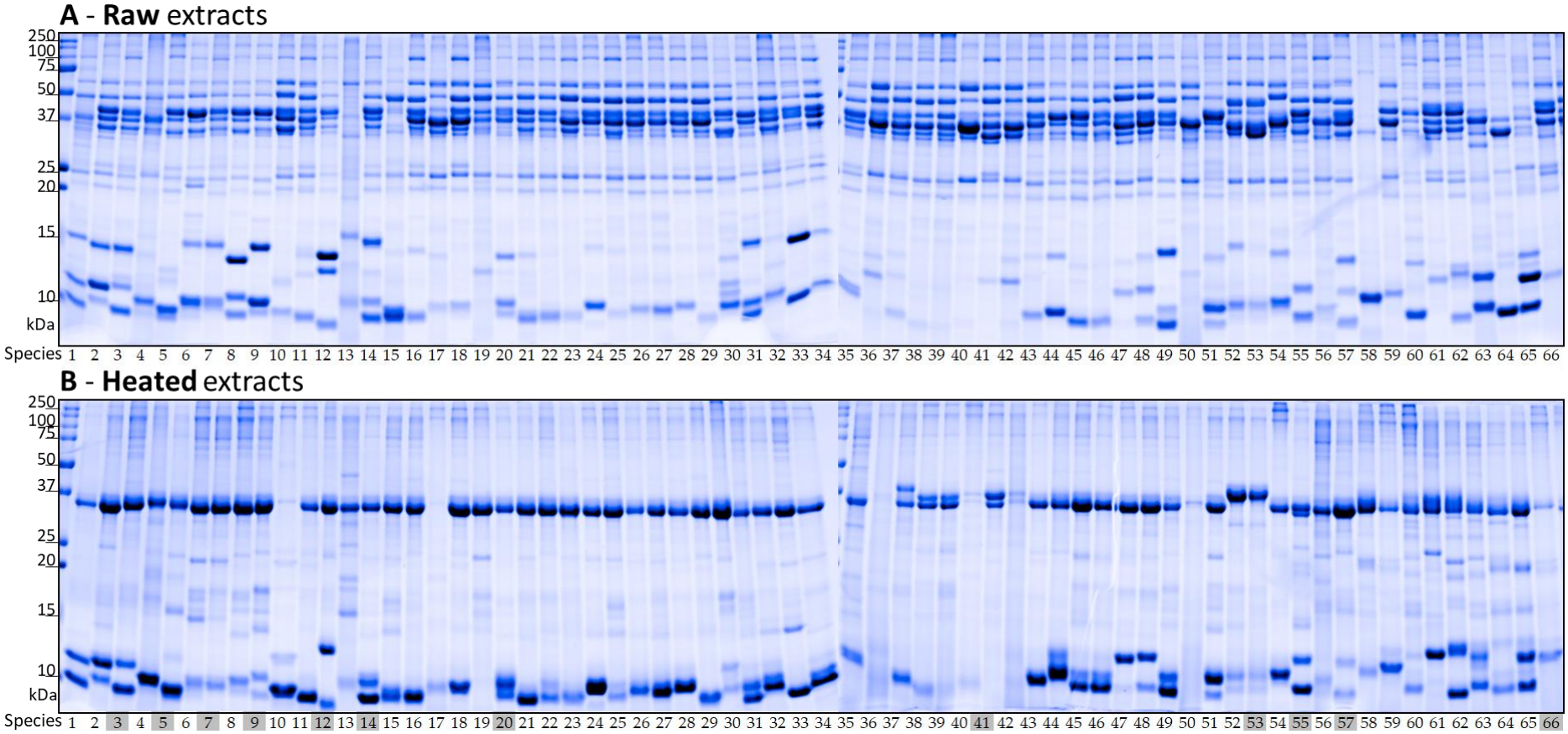


Figure V A 1: SDS-PAGE protein profiles of raw (**A**) and heated (**B**) extracts from 66 fish species. The species number refers to Table V A 1 above. IgE-binding proteins were identified in the heated extracts from species with their number shaded in grey.

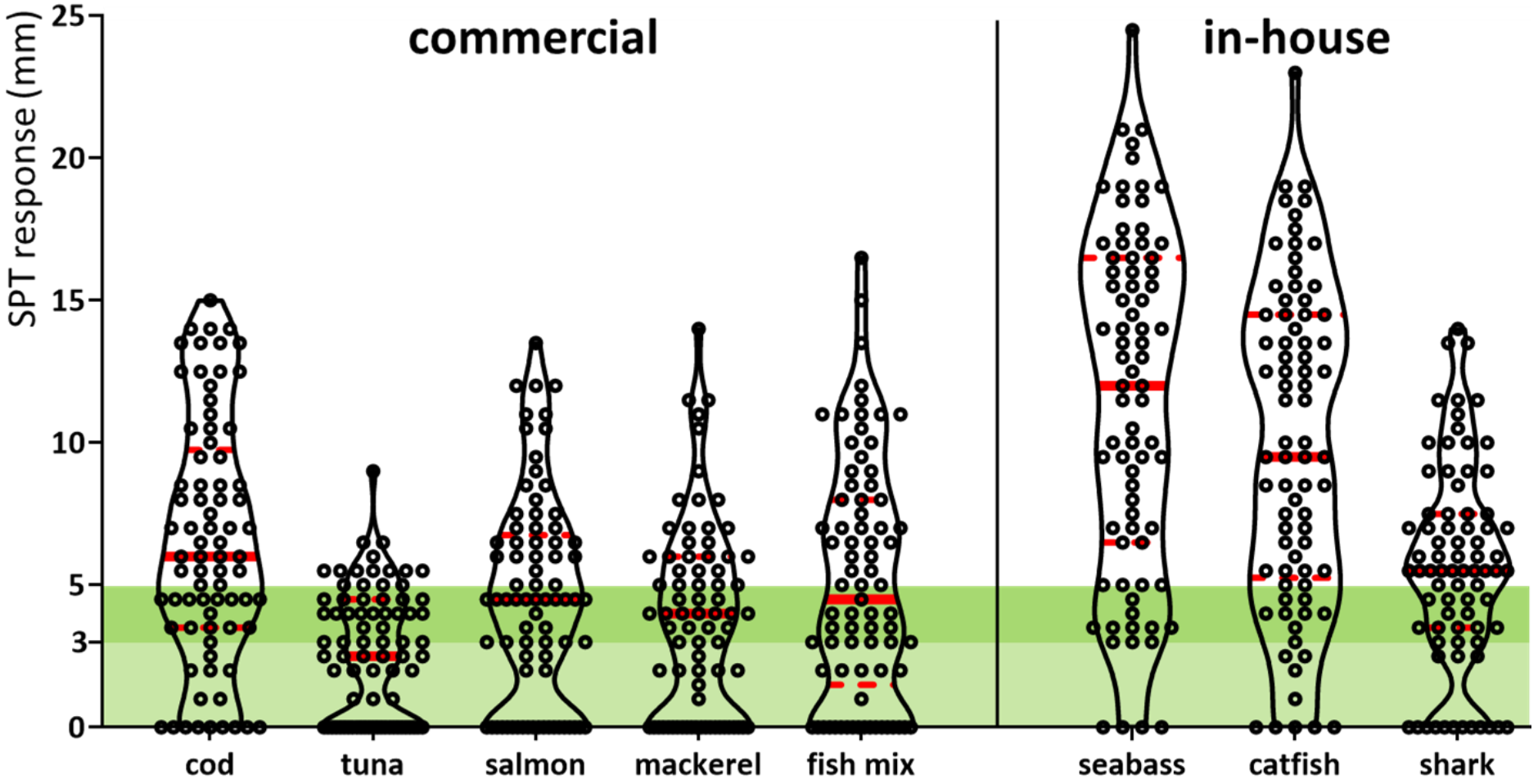


Figure V A 2: Skin Prick Test (SPT) response to 5 commercial and 3 in-house fish preparations by 77 fish-allergic patients described in Appendix C Table 1. An SPT is considered positive if the wheal diameter is at least 3 mm (light green) or 5 mm depending (dark green) on different clinical practice. The median is indicated with a red line and the interquartile range with dotted red lines. Note: G., Gummy

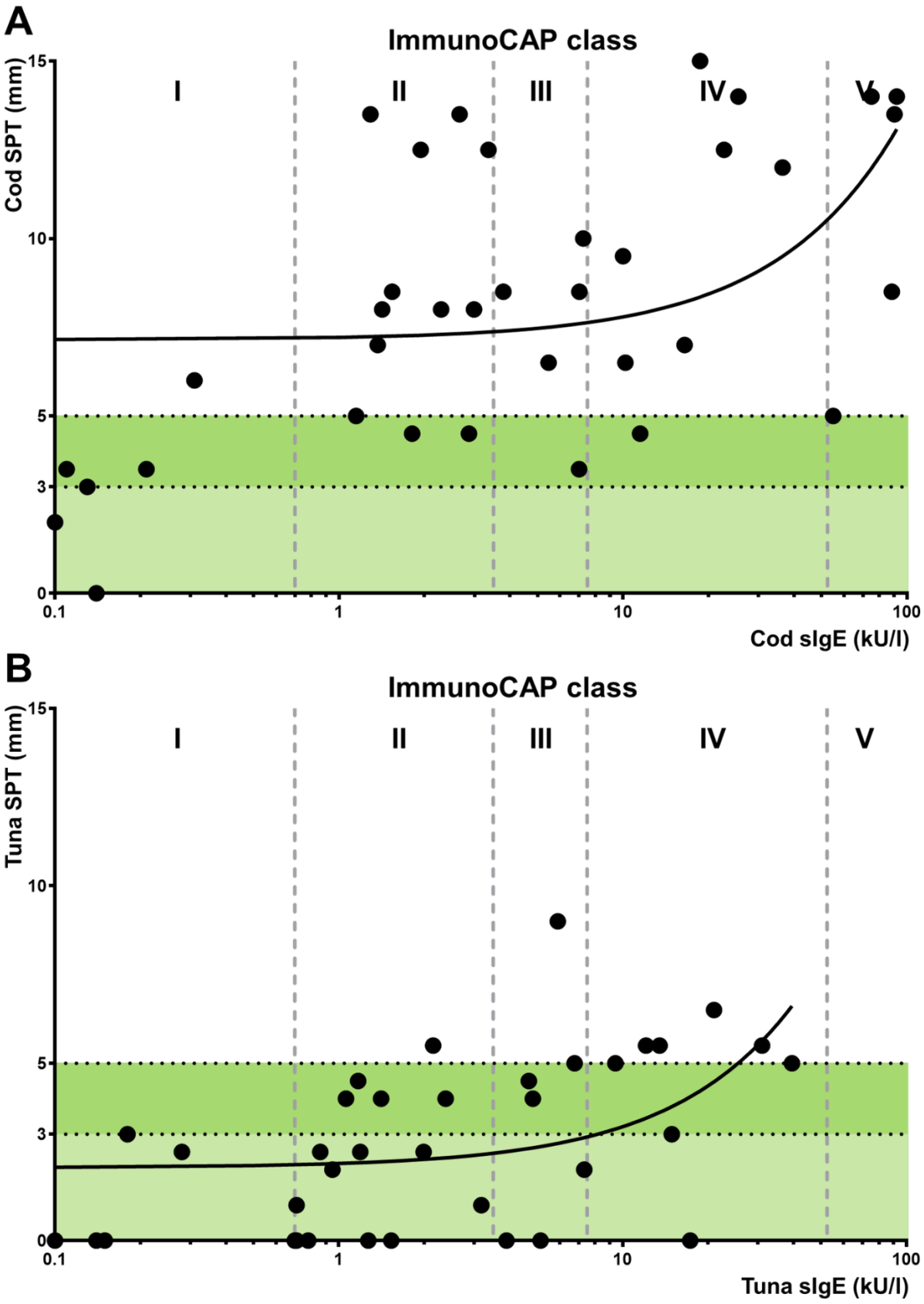


Figure V A 3: Comparison of *in vivo* reactivity of 36 fish-allergic patients to cod (**A**) and tuna (**B**) and corresponding sIgE level and ImmunoCAP class. The *in vivo* reactivity was determined by skin prick testing (SPT) using commercial fish preparations. Specific values are provided in Appendix C Table 1.

Table V A 2: sIgE-binding to tropomyosin and parvalbumin from 12 fish species.

species	paediatric patients (*n*=77) with IgE-binding to		IgE-binding to **tropomyosin** but **not** **parvalbumin**
	tropomyosin	**parvalbumin**	
55 catfish	32%	47%	32%
14 Nile tilapia	31%	44%	38%
20 Asian seabass	30%	43%	22%
57 John dory	29%	38%	41%
41 yellowfin tuna	26%	25%	55%
66 gummy shark	25%	25%	68%
12 ling	18%	22%	64%
9 hoki	18%	35%	43%
3 orange roughy	17%	22%	69%
5 common carp	16%	39%	42%
53 Atlantic salmon	13%	49%	30%
7 Atlantic cod	12%	31%	44%

Note: The number in front of the fish species corresponds to Table V B 1 and Figure V B 1 above.

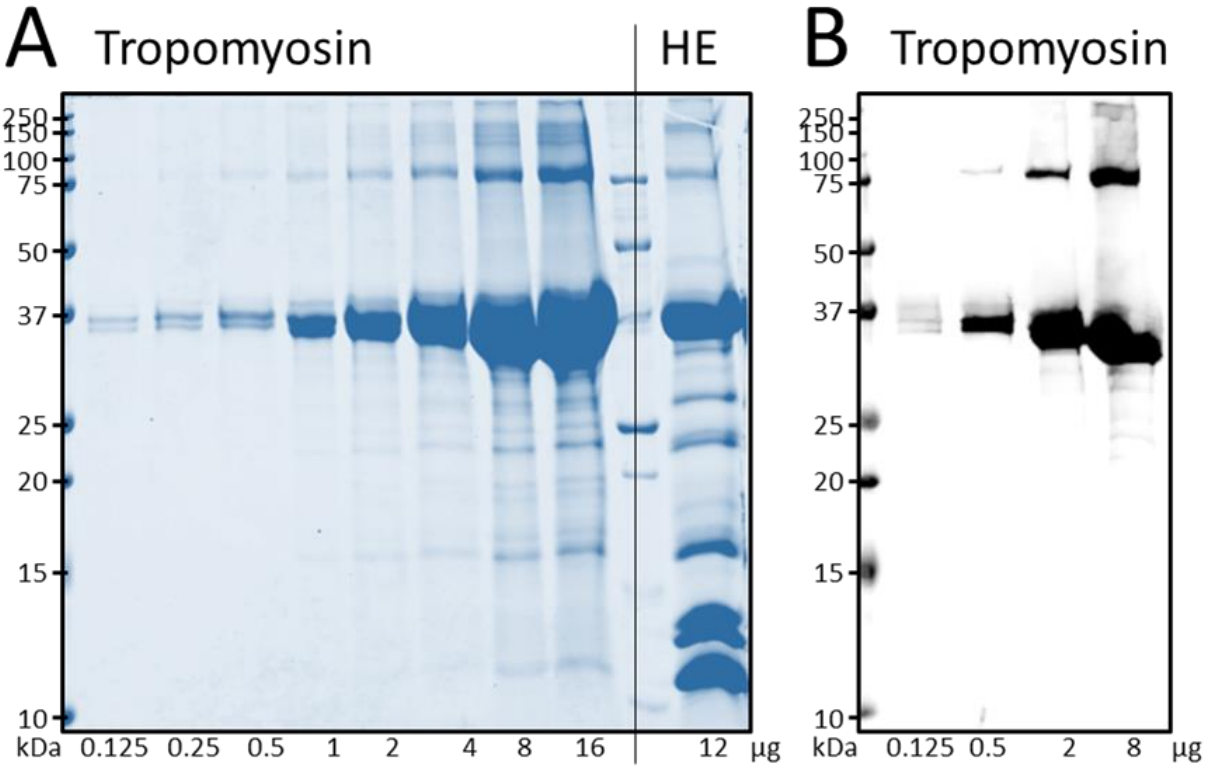


Figure V A 4: SDS-PAGE protein profiles of purified tropomyosin and heated extract (HE) from catfish (**A**). The purity of tropomyosin is over 95% based on densitometric analyses. Tropomyosin monomers and dimers were detected using an in-house polyclonal antibody as described in the above manuscript (**B**).[11]

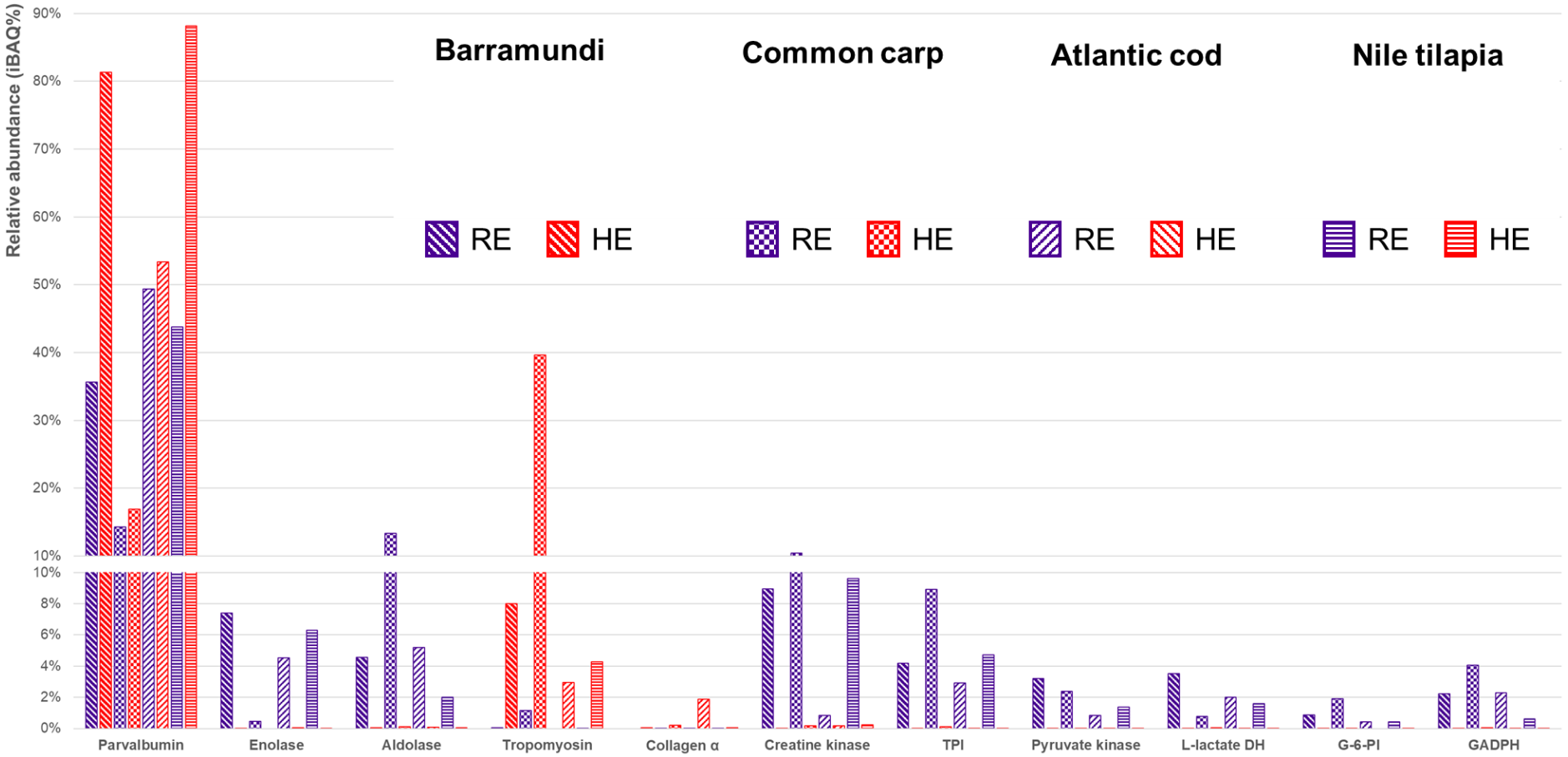


Figure V A 5: Relative abundance (iBAQ%) of (putative) allergens in raw (RE, blue) and heated (HE, red) protein extracts from barramundi, common carp, Atlantic cod and Nile tilapia. Proteins were digested with trypsin in-solution and analysed by mass spectrometry. The iBAQ% value is an indicator of the relative abundance of each protein group and is based on analyses with MaxQuant. Note: TPI=triosephosphate isomerase, DH=dehydrogenase, G6-PI=glucose-6-phosphate isomerase, GAPDH=glyceraldehyde-3-phosphate dehydrogenase.

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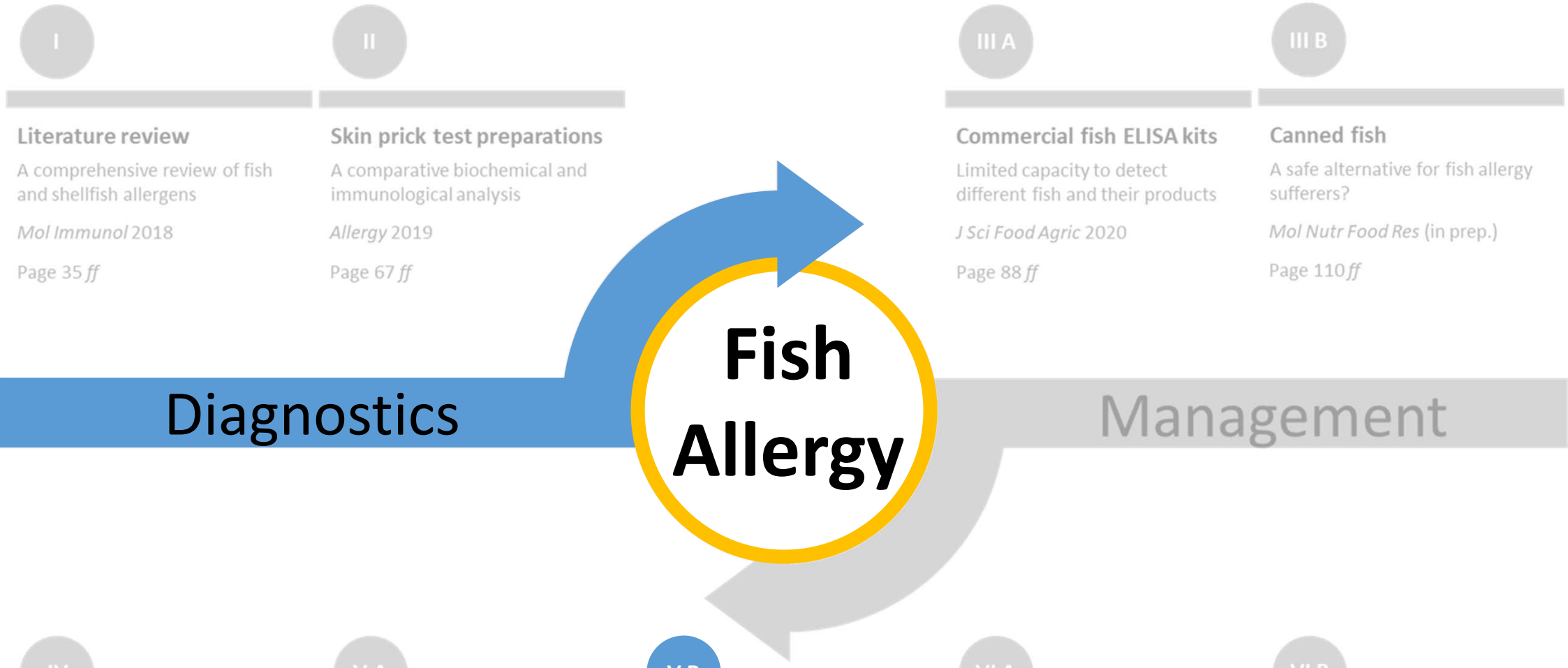
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Chapter V B – Collagen, a novel fish allergen

Collagen - an important fish allergen for improved diagnosis

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Contributions:

TK, SK, TR, DC, HB and AL designed the study. TL, DH, JR, RO, DD and DC recruited the patients. TK, SK and TR generated the data. AT, RN, PH and NW assisted with data generation. TC led the manuscript writing.

Original Article

Collagen—An Important Fish Allergen for Improved Diagnosis

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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 patients. Inclusion of relevant collagen allergens in routine diagnosis is indicated to improve the capacity to accurately diagnose fish allergy. **© 2020 American Academy of Allergy,**

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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

Asthma & Immunology (J Allergy Clin Immunol Pract 2020;■:■-■)

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 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

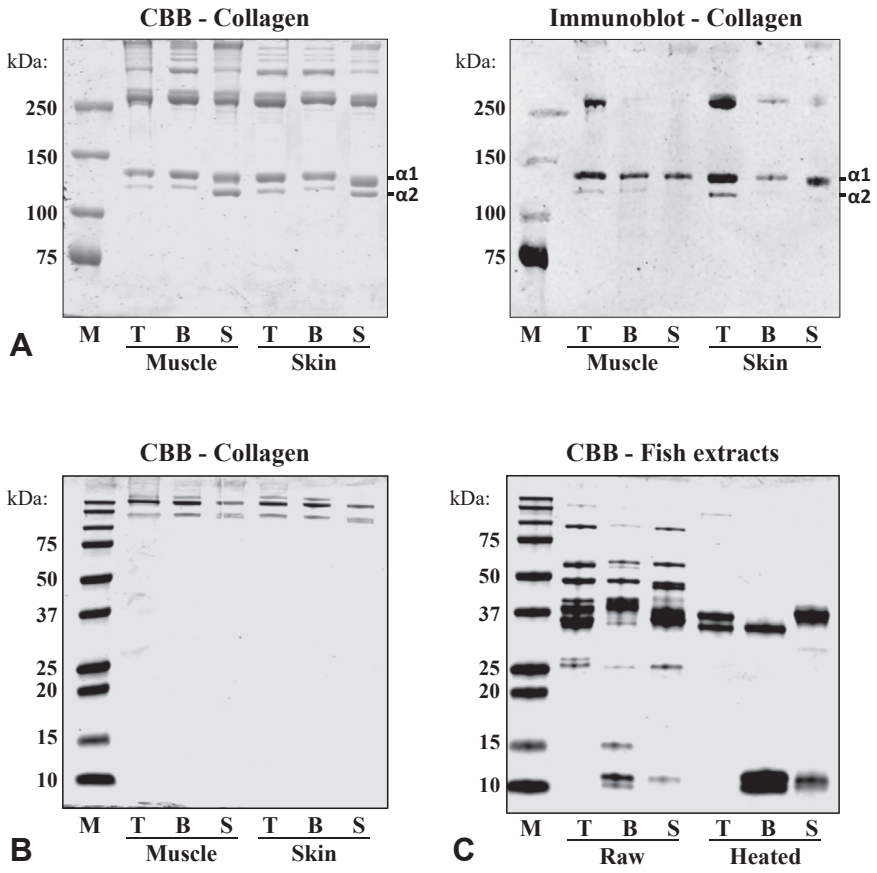

FIGURE 1. (**A**) Collagen extracted from fish muscle and skin, visualized by CBB staining of 8% gels and immunoblotting with a collagen-specific antibody. (**B**) CBB staining of purified collagen demonstrating absence of low-molecular-weight proteins (gradient gels). (**C**) Low abundance of collagen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

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.

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.

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).

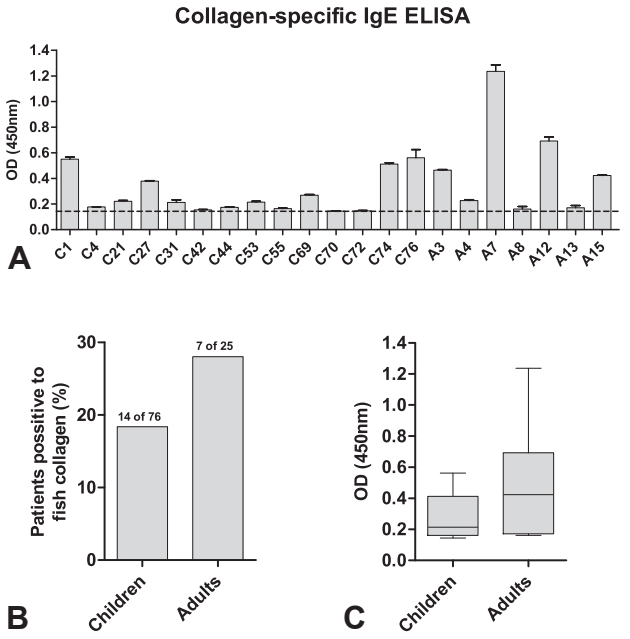

FIGURE 2. (**A**) IgE binding to a pool of purified collagens from muscle and skin of yellowfin tuna, barramundi, and Atlantic salmon. Results for collagen-positive (21 of 101) patients are shown. The threshold for positivity (dashed line) was calculated using the average signal from 5 negative controls + 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.

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 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

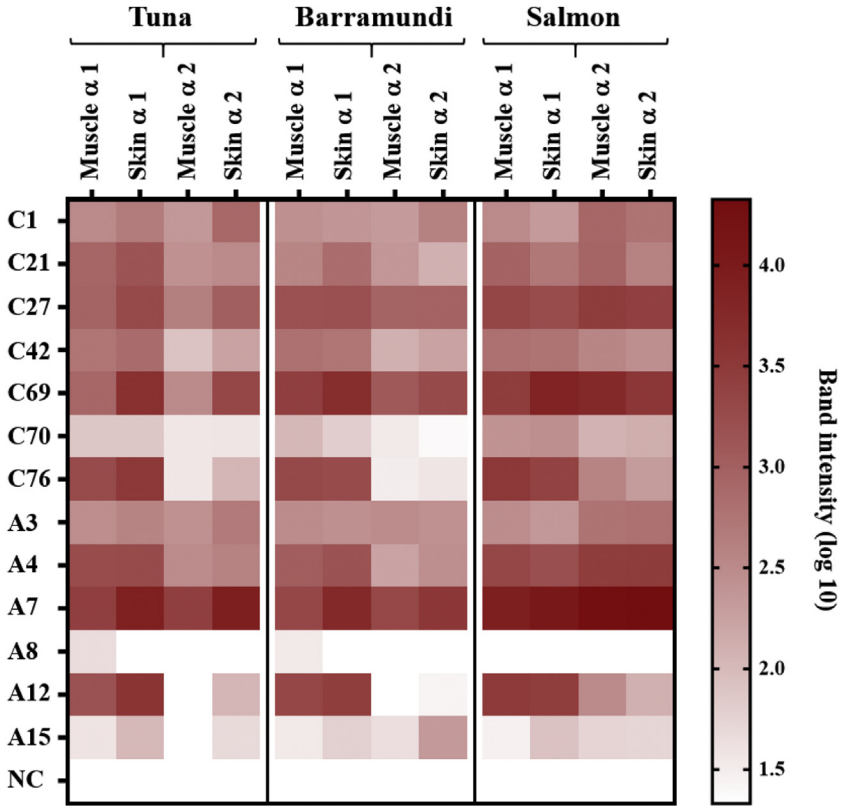

FIGURE 3. Densitometric analysis of IgE-binding intensity to collagen α1 and α2 chains obtained by IgE immunoblotting using sera of collagen-sensitized patients. *A*, Adults; *C*, children; *NC*, immunoblot using pooled sera from 5 negative controls.

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-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.

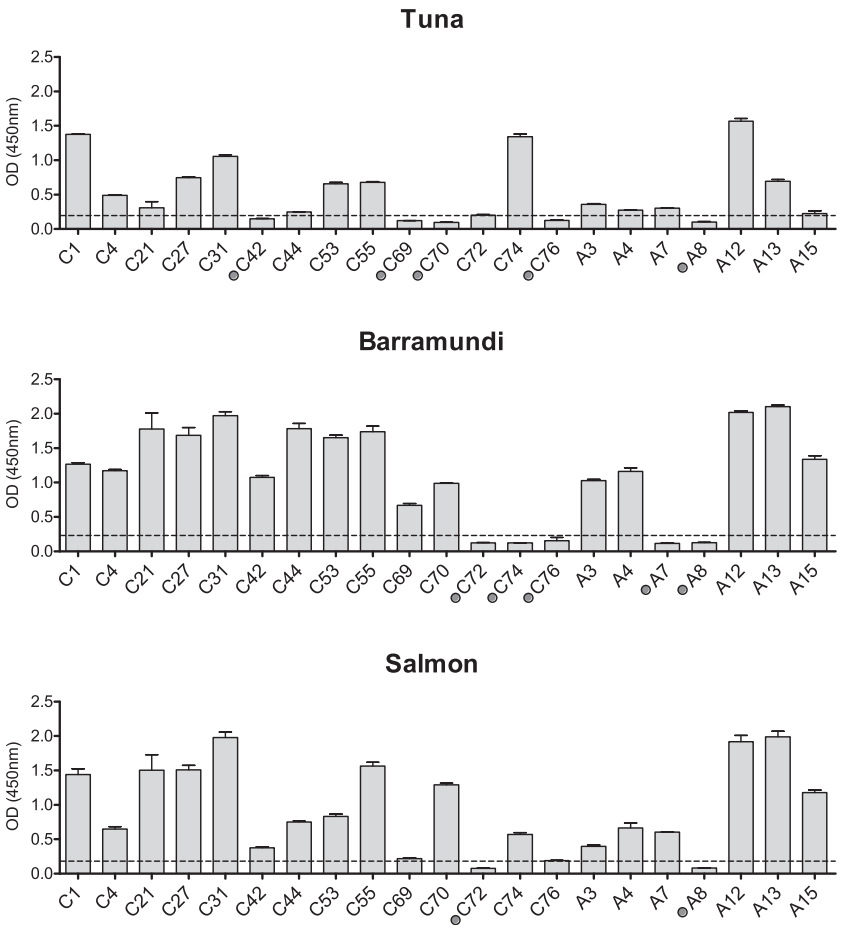

FIGURE 4. Binding to fish parvalbumins by IgE from collagen-sensitized patients' sera. Patients with sensitization to collagen but absence of IgE to parvalbumin from the corresponding species are depicted with gray circles. The threshold for positivity (dashed line) was calculated using the average signal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

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 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

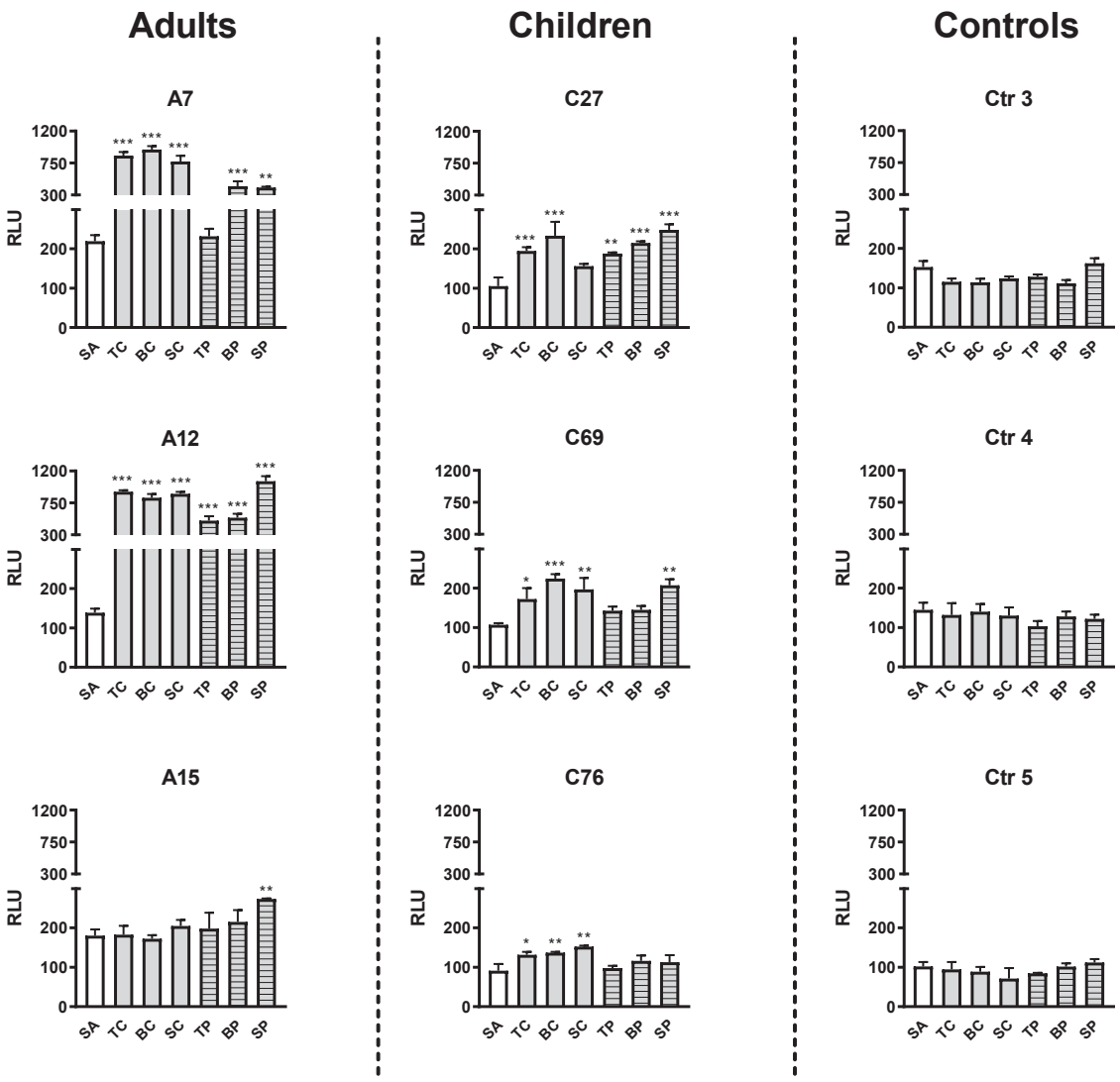

FIGURE 5. Activation of RS-ATL8 cells sensitized by sera of 3 collagen-sensitized children, 3 adults, and 3 negative controls, upon exposure to collagens and parvalbumins from tuna, barramundi, and salmon. *A*, Adult; *BC*, barramundi collagen; *BP*, barramundi parvalbumin; *C*, children; *Ctrl*, control; *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).

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 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.

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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 ($\sim$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

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.

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
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

(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
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.

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.

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.

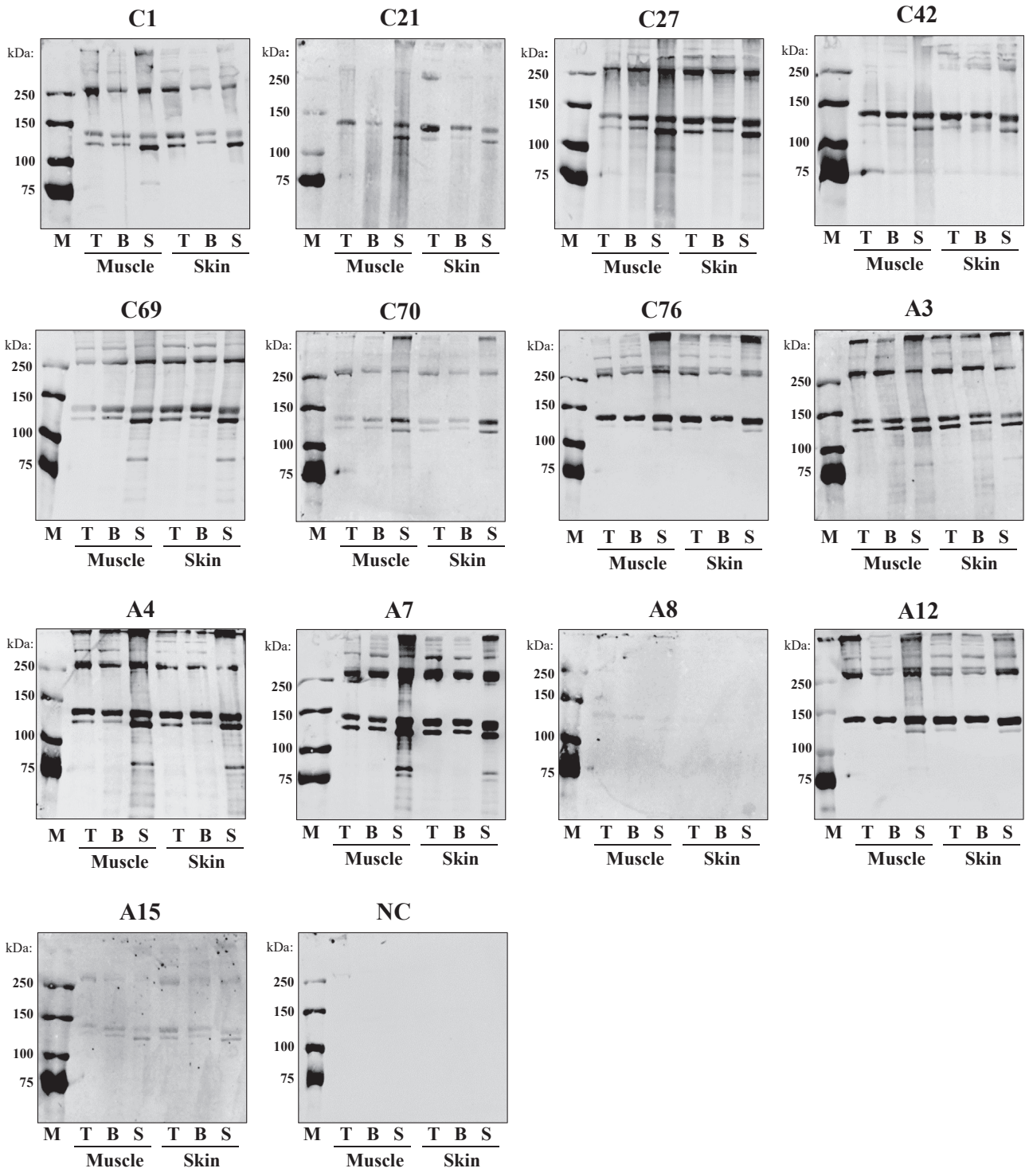

FIGURE E1. IgE sensitization to fish collagens demonstrated by immunoblots using purified collagens from muscle and skin tissues of yellowfin tuna, barramundi, and Atlantic salmon. *A*, Adults; *B*, barramundi; *C*, children; *M*, protein marker; *NC*, negative control; *S*, salmon; *T*, tuna.

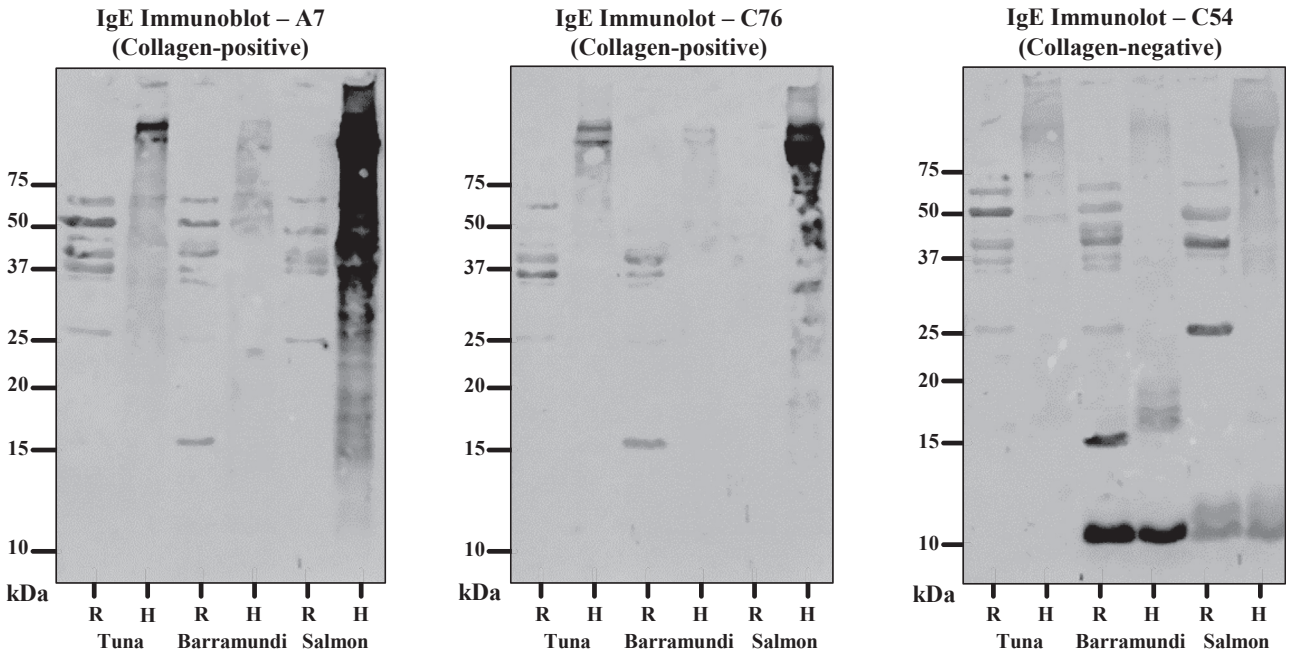

FIGURE E2. IgE binding to proteins from whole-fish extracts detected by immunoblots. *A*, Adults; *C*, children; *H*, heated; *R*, raw.

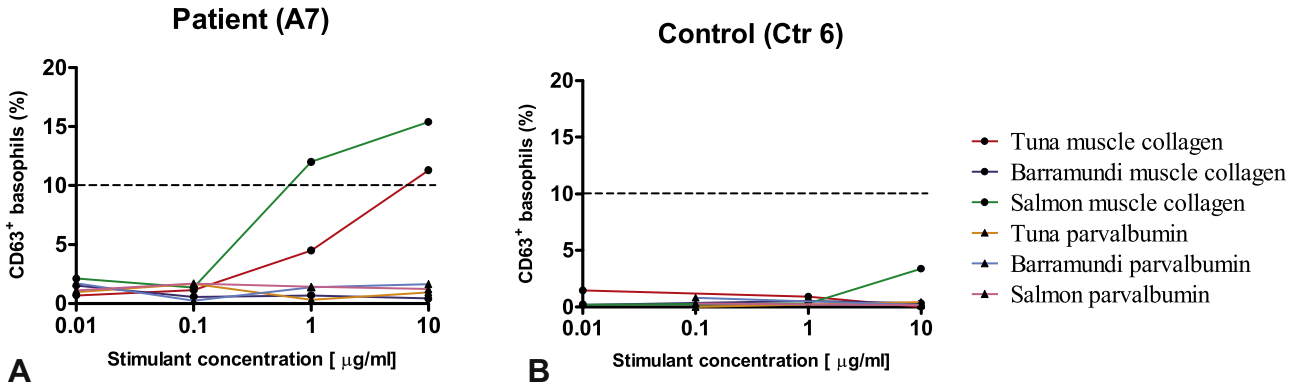

FIGURE E3. BAT using fresh blood of a fish-allergic patient (A7) and an atopic control (Ctr 6). Data indicate percentage of $CD63^+$ basophils in response to stimulation with collagens and parvalbumins from yellowfin tuna, barramundi, and Atlantic salmon. Dashed line indicates threshold for positive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

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Further results and discussion not included in the published manuscript

The importance of collagen as a fish allergen and its cellular reactivity has been demonstrated in the above manuscript. However, the investigations were limited to three fish species. Collagen was described in detail in Atlantic salmon (*Salmo salar*) and Asian seabass (barramundi, *Lates calcarifer*) and registered with the WHO/IUIS as Sal s 6 and Lat c 6, respectively. At the time of this study, the amino acid sequence for collagen from yellowfin tuna (*Thunnus albacares*) could not be retrieved. Recently, a transcriptome assembly for this species was kindly provided by Dr Adam Ciezarek (Earlham Institute, Norwich, UK) which allowed to predict the sequence of tuna collagen isoforms.[1] Mass spectrometric analyses are in progress to evaluate the sequence and abundance of these predicted isoforms.

Patients sensitised to collagen from one bony fish species appear to demonstrate IgE binding to collagen from most or all bony fish. This is indicated in the above manuscript and literature.[2–5] However, this has not been analysed in a comparative study. To confirm or refute this hypothesis and expand our knowledge about fish collagens, acid-derived extracts were also generated from Atlantic cod (*Gadus morhua*) and *Pangasius*/catfish (basa, *Pangasianodon hypophthalmus*) muscle and skin tissue. In addition to PBS-derived raw and heated muscle extracts (described in **Chapter III A** and **V A**), corresponding extracts were also produced from the skin to evaluate if collagen can be extracted from fish skin using traditional buffers. The total protein content was estimated for all extracts as described in the manuscript. PBS-derived heated skin extracts contained more proteins as compared to raw skin extracts (28-42 mg/ml versus 3-6 mg/ml, respectively). In contrast, raw PBS-derived muscle extracts contained more proteins as compared to heated muscle extracts (1-5 mg/ml versus 1-16 mg/ml, respectively) as described and discussed in **Chapter V A.**

Subsequently, all extracts were evaluated for their protein composition by SDS-PAGE and immunoblotting. Protein bands, which were detected by the anti-collagen antibody, were excised and analysed by mass spectrometry after tryptic digestion in-gel. In the high molecular weight range (>75 kDa), collagen α-1 and α-2 bands were observed at ~140 kDa and ~130 kDa, respectively, in all acid-derived extracts (see **Figure V B 1**). Collagen β and γ were identified at ~250 kDa and >250 kDa, respectively. Prominent collagen bands were observed in PBS-derived heated muscle extracts from cod and salmon but not in corresponding extracts from any of the other three investigated species, which is consistent with results from mass spectrometric analyses presented in **Chapter V A**. No collagen was detected in any of the PBS-derived raw muscle extracts. Acid-derived skin extracts demonstrated similar SDS-PAGE and antibody-detection patterns as corresponding muscle extracts. In contrast, PBS-derived muscle and skin extracts differed greatly. A prominent collagen band was detected in the raw salmon skin, but not other raw skin extracts. All PBS-derived heated skin extracts were very similar to acid-derived skin extracts in SDS-PAGE profile and antibody detection pattern.

Outside the molecular weight range of collagen and below 75 kDa, all acid-derived extracts and PBS-derived heated skin extracts did not show many protein bands as also described in the manuscript (see **Figure V B 2**). In contrast, PBS-derived heated muscle extracts showed most protein bands at 30-50 kDa and 10-15 kDa (molecular weight range of parvalbumin) as previously described and discussed in detail (**Chapter V A**). Raw skin extracts did not contain considerable protein bands at 10-15 kDa and overall, not many protein bands were visible.

The observed difference between muscle and skin extracts can partially be explained by an about 70-fold higher relative abundance of collagen in the skin as compared to the muscle of fish.[6] The higher abundance of collagen in heated extracts compared to raw extracts appears to derive from the gelatinisation of collagen, which increases its solubility.[4,7–10]

The published manuscript and further preliminary data from IgE-immunoblots suggest that collagen-sensitised patients exhibit isoform-specific patterns. Notably, collagen α-1 was detected by IgE from all patients. Collagen α, β and γ from cod and catfish were therefore partially separated by size-exclusion chromatography (HiPrep™ 16/60 Sephacryl™ S-300 HR, GE Healthcare) with 20 mM sodium acetate (pH 4.8) as buffer (see **Figure V B 3**). Collagen α-1 from cod was subsequently isolated by cation-exchange chromatography (Bio-Scale™ Mini Macro-Prep® High S Cartridge, Bio-Rad) with a 0-1 M NaCl gradient in 50 mM sodium acetate buffer (pH 4.7) (see **Figure V B 4**). Mass spectrometric data after tryptic digestion in-solution are in progress and will determine the purity of cod collagen α-1, which is >95% based on SDS-PAGE analysis. Purified cod collagen α-1 could be used in studies to evaluate collagen-sensitisation in other cohorts and to develop improved component-resolved diagnostics.

In summary, the importance of collagen for the development of improved fish allergy diagnostics was demonstrated in this Chapter. However, all analyses were limited to bony fish. In one study from Japan, less frequent binding to cartilaginous fish collagens as compared to bony fish collagens was suggested.[2] An overall lower allergenicity of cartilaginous fish has been suggested (see the review of the literature in **Chapter I** and [11](**Appendix D 3**), and **Chapter V A**). Subsequently, cartilaginous fish is evaluated as a safe alternative for bony fish-allergic patients in **Chapter VI A**, with focus on thornback ray (*Raja clavata*) and gummy shark (*Mustelus antarcticus*) and their parvalbumin isoforms.

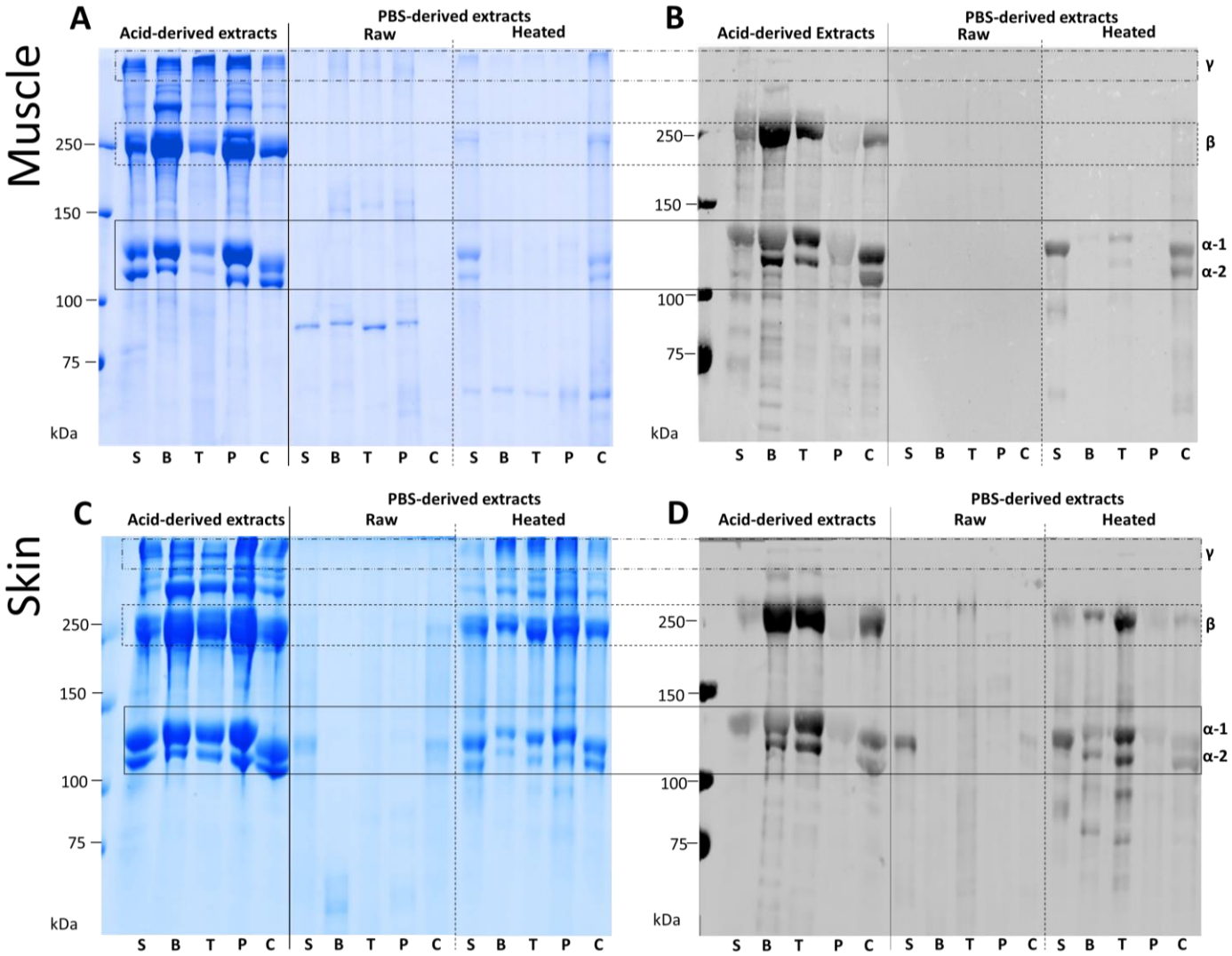

Figure V B 1: SDS-PAGE (8%) profiles of acid- and PBS-derived protein extracts from muscle (**A**) and skin (**C**) tissue from salmon (S), barramundi (B), tuna (T), *Pangasius*/catfish (P) and cod (C). Collagen bands (α-1, α-2, β and γ) were identified using a collagen-specific antibody in immunoblotting (**B** and **D**) and mass spectrometric analyses as described in the manuscript. Mr Lirui Sun assisted in data generation and analyses.

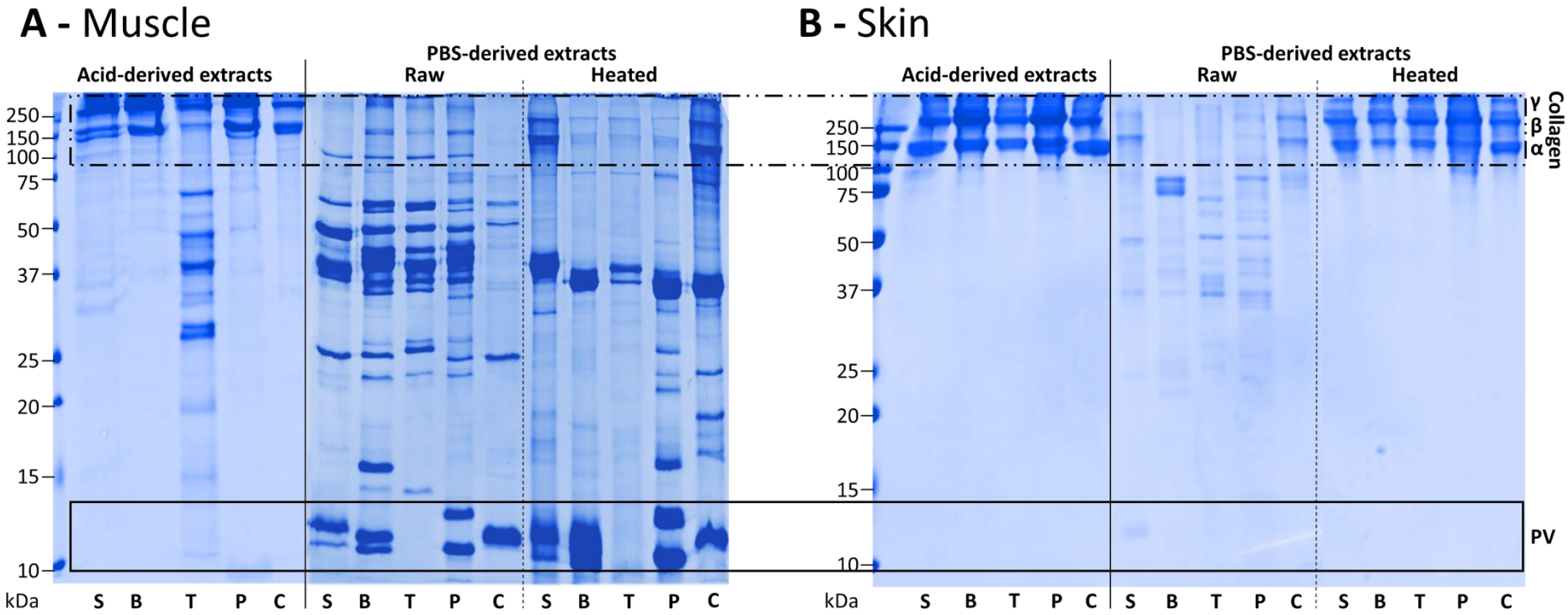

Figure V B 2: SDS-PAGE (14%) profiles of acid- and PBS-derived protein extracts from muscle (**A**) and skin (**C**) tissue from salmon (S), barramundi (B), tuna (T), *Pangasius*/catfish (P) and cod (C). Note: It is assumed that an incorrect sample was loaded as acid-derived muscle tuna extract because the SDS-PAGE profile is different to all other acid-derived extracts and the corresponding profile in the published manuscript (Figure 1 B). Mr Lirui Sun assisted in data generation and analyses.

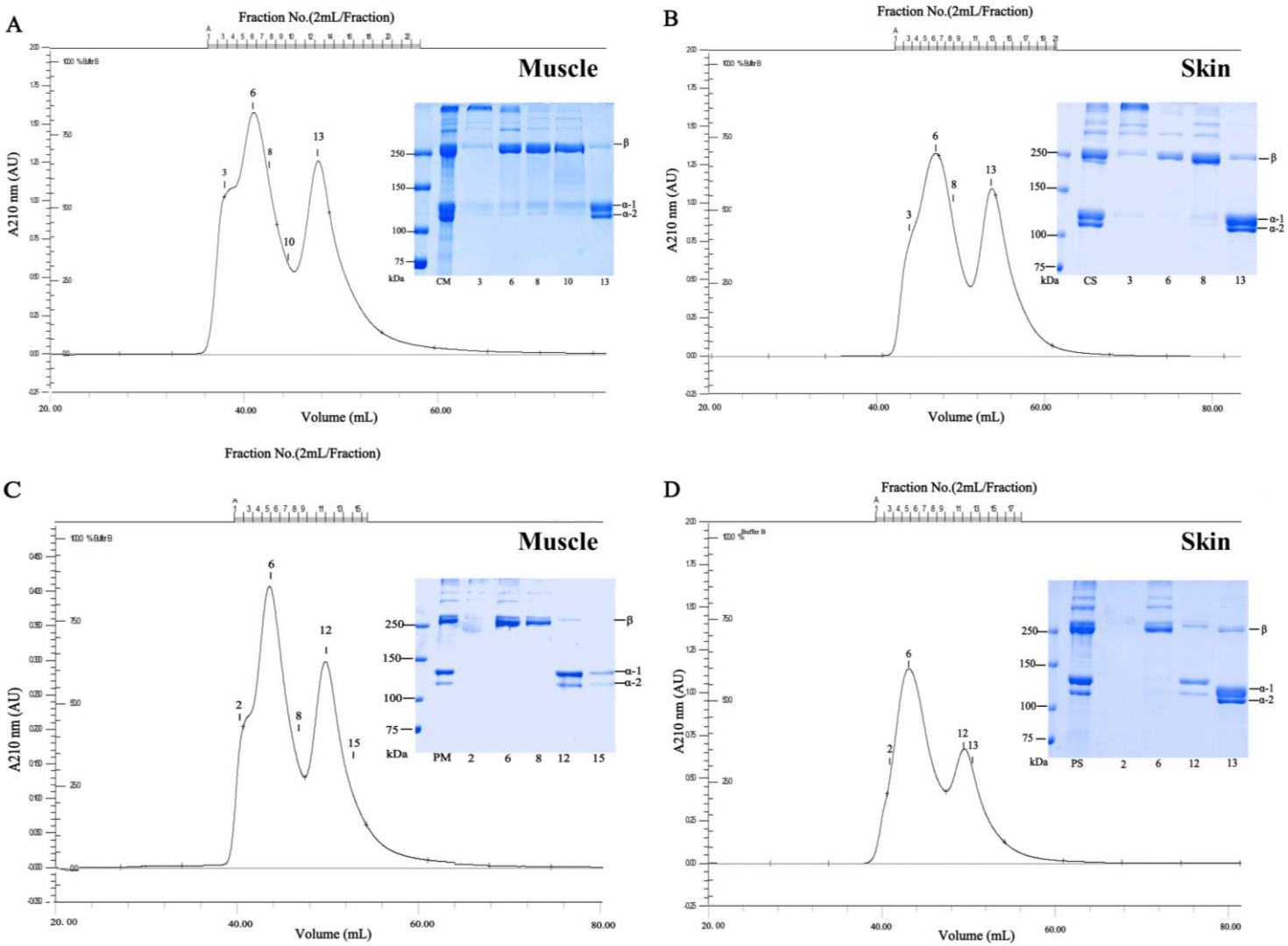

Figure V B 3: Size exclusion chromatogram and SDS-PAGE of selected fractions of acid-derived extracts from cod muscle (CM, **A**) and skin (CM, **B**) tissue and respective *Pangasius* extracts (PM, **C** and PS, **D**, respectively). The numbers at the bottom of the SDS-PAGE gels align with the numbers in the corresponding chromatogram. Mr Lirui Sun assisted in data generation and analyses.

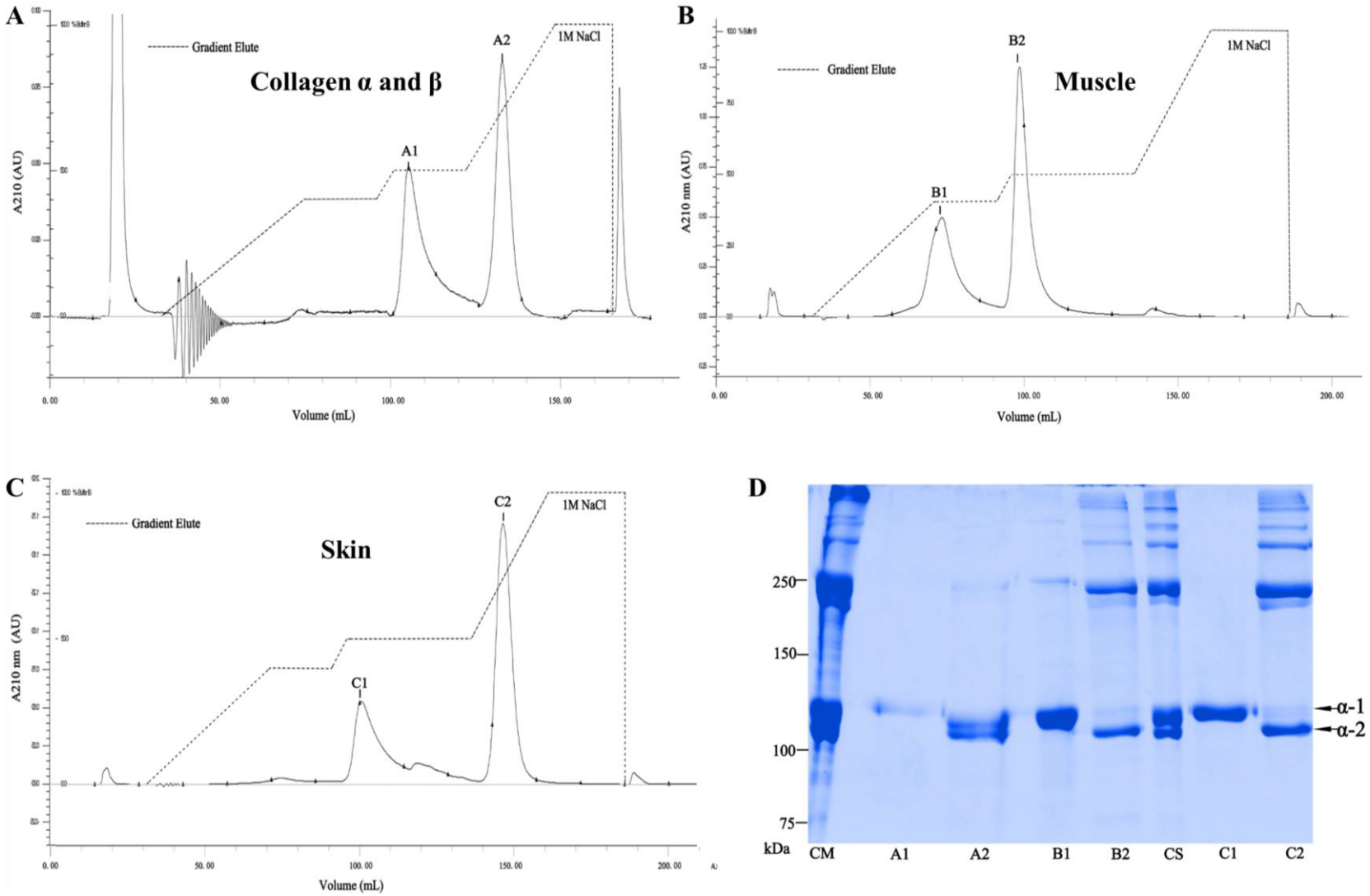

Figure V B 4: Cation-exchange chromatogram of pre-purified collagen α and β fractions (No. 13 from Figure V B 3 A) (**A**) and acid-derived cod muscle (CM, **B**) and skin (CS, **C**) extracts. The SDS-PAGE profile of selected fractions is shown in **D**, demonstrating that collagen α-1 was isolated. The samples A1, A2, B1, B2, C1 and C2, separated by SDS-PAGE, align with the corresponding fractions in the chromatogram. Mr Lirui Sun assisted in data generation and analyses.

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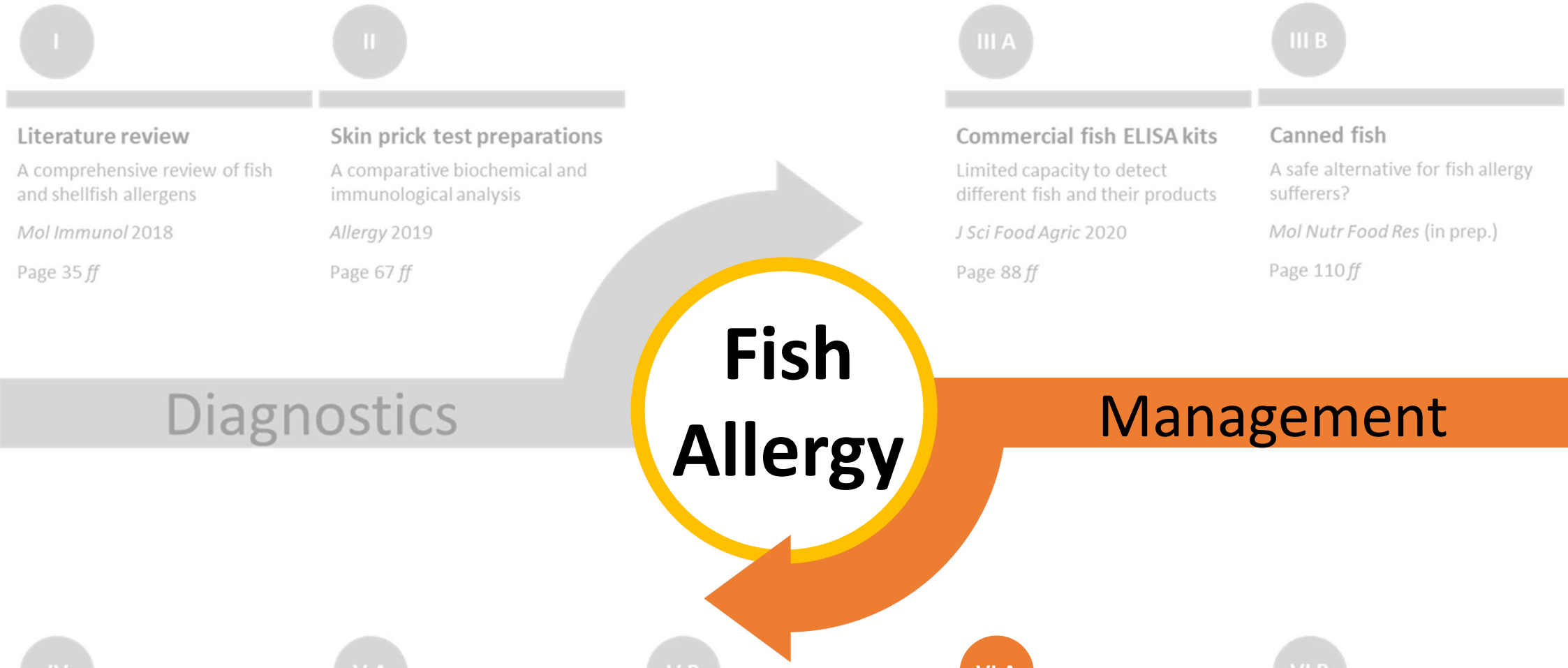
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Chapter VI A – Tolerance to sharks/rays

Patients allergic to fish tolerate ray based on the low allergenicity of its parvalbumin

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Contributions:

TK, CR, TR, IS, AL, MM, HM and AK designed the study. FM, MO, CH and MM recruited the patients. TK, CR and TR generated the data. AT, CH and KH assisted with data generation and analyses. TC led the manuscript writing.

Original Article

Patients Allergic to Fish Tolerate Ray Based on the Low Allergenicity of Its Parvalbumin

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What is already known about this topic? Patients allergic to fish are generally advised to avoid all types of fish. Most of these patients are sensitized to bony fish and their major allergen parvalbumin. The allergenicity of cartilaginous fish, a potential dietary alternative, is not well understood.

What does this article add to our knowledge? We demonstrated tolerance of ray, a cartilaginous fish, by patients with allergy to bony fish. Furthermore, ray parvalbumin showed lower allergenicity than did the parvalbumins from bony fish.

How does this study impact current management guidelines? Current diagnosis of fish allergy focuses on bony fish. Inclusion of cartilaginous fish and their parvalbumins in routine diagnosis of fish allergy may prevent unnecessary food restrictions.

BACKGROUND: Clinical reactions to bony fish species are common in patients with allergy to fish and are caused by parvalbumins of the β-lineage. Cartilaginous fish such as rays and sharks contain mainly α-parvalbumins and their allergenicity is not well understood.

OBJECTIVE: To investigate the allergenicity of cartilaginous fish and their α-parvalbumins in individuals allergic to bony fish.

METHODS: Sensitization to cod, salmon, and ray among patients allergic to cod, salmon, or both (n = 18) was explored by prick-to-prick testing. Clinical reactivity to ray was assessed in 11 patients by food challenges or clinical workup. IgE-binding to β-parvalbumins (cod, carp, salmon, barramundi, tilapia) and α-parvalbumins (ray, shark) was determined by IgE-ELISA. Basophil activation tests and skin prick tests were performed with β-parvalbumins from cod, carp, and salmon and α-parvalbumins from ray and shark.

RESULTS: Tolerance of ray was observed in 10 of 11 patients. Prick-to-prick test reactions to ray were markedly lower than to bony fish (median wheal diameter 2 mm with ray vs 11 mm with cod and salmon). IgE to α-parvalbumins was lower (median, 0.1 kU/L for ray and shark) than to β-parvalbumins (median, ≥1.65 kU/L). Furthermore, α-parvalbumins demonstrated a significantly reduced basophil activation capacity compared with β-parvalbumins (eg, ray vs cod, $P < .001$; n = 18). Skin prick test further demonstrated lower reactivity to α-parvalbumins compared with β-parvalbumins.

CONCLUSIONS: Most patients allergic to bony fish tolerated ray, a cartilaginous fish, because of low allergenicity of its α-parvalbumin. A careful clinical workup and *in vitro*

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Abbreviations used
BAT- basophil activation test
PPT- prick-to-prick test
SPT- skin prick test

IgE-testing for cartilaginous fish will improve patient management and may introduce an alternative to bony fish into patients' diet. **© 2018 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 2019;7:500-8)**

Key words: *Parvalbumin; Fish allergy; Cod; Ray; Food challenge; Basophil activation; Skin prick test; IgE*

INTRODUCTION

Fish allergy is typically a life-long disease with symptoms of varying severity including life-threatening anaphylaxis.[1,2] During the evolution of jawed vertebrates, 2 classes of fish developed, cartilaginous (Chondrichthyes) and bony fish (Osteichthyes).[3] Although most of the studies focusing on fish allergy have described bony fish as the primary allergen source, the allergenicity and cross-reactivity of cartilaginous fish is not well understood.[4-7]

The prevalence of fish allergy ranges from 0.2% to 3% in the general population and up to 8% in occupational settings.[8-10] Increasing rates of allergic sensitization to fish may be linked to the increasing worldwide production and consumption of fish.[11] As of 2015, annual per capita fish consumption in the European Union was about 25 kg, while in the Asia-Pacific region over 100 kg was reached.[12,13]

The most frequently consumed fish species belong to the bony fish. In the European Union, the top 5 consumed species in 2014 were tuna, cod, salmon, Alaska pollock, and herring.[13] In the United States, the most commonly consumed species include anchovy, Alaska pollock, and herring.[13] In Asia, the variety of eaten species is enormous and often include tilapia, carp, and barramundi.[12] Cartilaginous fish, including rays and sharks, are also commonly traded and consumed worldwide.[10,14] In Europe, shark meat is commonly consumed in Spain and Italy.[15]

The diversity of consumed fish species and the increase in their global availability pose a challenge for accurate diagnosis and management of patients with fish allergy. Diagnostic tests for fish allergy use a limited number of only bony fish species. They do not include cartilaginous fish.[16] The potential risk of reacting to multiple fish species, along with the lack of comprehensive and accurate diagnostic products, often results in the recommendation of complete avoidance of all fish to patients presenting to the clinic with fish allergy.[17,18] This may adversely affect the nutritional requirements and the quality of life of affected individuals.[19]

The major fish allergen parvalbumin is a heat-stable, intracellular EF-hand calcium-binding protein of low molecular weight (10-14 kDa).[20,21] It is abundant in fast-twitch white muscle where it is involved in muscle relaxation.[22] In addition, minor allergens such as aldolase A and β-enolase have been identified and were demonstrated to be heat-sensitive.[23] Parvalbumins are present in all vertebrates and are divided into 2 evolutionary sublineages, α and β, which have different biochemical properties.[21] Although β-parvalbumins are abundant in bony fish, α-parvalbumins are mainly found in cartilaginous fish and higher vertebrates.[4] Bony fish β-parvalbumins are predominant sensitizers and often cross-reactive.[4,24] Regarding the allergenicity of fish α-parvalbumins, there is only 1 report demonstrating binding of patients' IgE to α-parvalbumin from red stingray.[25]

An in-depth investigation of the allergenicity of cartilaginous fish and their parvalbumins has not been performed yet. We conducted a comprehensive study characterizing the clinical reactivity of patients with bony fish allergy to ray, a cartilaginous fish. Furthermore, we analyzed patients' IgE reactivity to parvalbumins from both bony and cartilaginous fish using ELISA, basophil activation test (BAT), and skin prick test (SPT).

METHODS

Study subjects

Eighteen individuals with fish allergy were recruited from the Centre Hospitalier de Luxembourg (Table I). Criteria for study participation included a documented clinical history of fish allergy and positive ImmunoCAP (Thermo Fisher Scientific, Waltham, Mass) for cod, salmon, or both. Total IgE was measured using ImmunoCAP. The study was approved by the National Committee for Medical Research Ethics in Luxembourg (Ref. 201307/04). Seven individuals with allergies other than to fish were used as controls in ELISA and BAT (Table E1 in this article's Online Repository at www.jaci-inpractice.org). Informed written consent was obtained from all participants or their legal representatives.

Prick-to-prick testing and clinical reactivity to bony and cartilaginous fish

All patients allergic to fish were subjected to prick-to-prick tests (PPTs) with boiled (20 minutes) bony (Atlantic cod, salmon, or both) and cartilaginous fish (thornback ray) meat. Glycerin-containing saline and 0.1% histamine dihydrochloride were used as negative and positive controls, respectively. An average wheal diameter of greater than or equal to 3 mm compared with negative control was rated positive.

Information about clinical reactivity to cod and salmon, based on history of allergic symptoms after eating specific fish, was obtained from all patients. Clinical reactivity to ray by oral exposure was assessed according to the procedure described in Figure E1 in this article's Online Repository at www.jaci-inpractice.org. Briefly, PPT with ray was performed in all 18 patients. Eleven patients (10 negative and 1 positive in PPT with ray) agreed to be tested for potential tolerance of ray either by food challenge or by following recommendations to introduce eating ray at home. Of these, 8 patients consumed a serving dose of ray (200 g for adults, 100 g for children) at least twice. In addition, 2 patients (P4 and P14) were subjected to open food challenges because they were apprehensive to directly introducing ray in the diet. One patient (P18), with a positive PPT result to ray, was subjected to a double-blind placebo-controlled food challenge performed according to previously published procedures.[26]

TABLE I. Demographic and clinical characteristics of patients allergic to fish

Patient	Age (y)/sex	Fish allergy symptoms	Total IgE (kU/L)	ImmunoCAP (kU_A/L)		PPT (mm)			Clinical reactivity		
				Cod	Salmon	Cod	Salmon	Ray	Cod	Salmon	Ray
1	19/F	A, AE, U	294	1.5	3.1	15	10	2	+	+	ND
2	7/M	U, OAS	2244	0.2	7.9	0	10	0	−	+	−
3	12/F	AE, U, OAS, V	352	5.9	5.4	8	10	2	+	+	−
4	8/M	A, U	828	9.8	5.0	10	12	3	+	+	*−
5	13/M	AE, AP, U, V	2005	1.6	0.9	22	21	0	+	+	ND
6	35/M	A, U	46	12.0	8.5	17	4	2	+	+	−
7	16/F	U, V	1184	1.5	6.5	11	15	0	+	+	−
8	35/M	OAS, U	48	0.5	1.3	8	8	2	+	+	−
9	16/F	AD, A	660	23.0	22.0	10	11	2	+	+	ND
10	35/M	U, A	380	48.0	56.0	23	18	5	+	+	ND
11	9/M	U, OAS	1455	0.8	0.3	11	0	0	+	−	−
12	9/M	AE, AP	745	6.4	8.0	24	13	1	+	+	−
13	11/M	AP, OAS	116	7.7	9.4	8	12	2	+	+	ND
14	14/M	C, U	345	0.8	1.1	11	12	0	+	+	*−
15	15/M	U, OAS	1218	2.0	1.1	18	4	0	+	+	ND
16	10/M	U, OAS	1432	4.0	6.5	28	ND	0	+	+	−
17	11/M	A, U	2297	>100.0	>100.0	9	ND	6	+	+	ND
18	12/M	A, AE, AP	723	>100.0	>100.0	18	ND	15	+	+	†+
Median	NA	NA	734	4.9	6.5	11	11	2	NA	NA	NA

A, Asthma; *AD*, atopic dermatitis; *AE*, angioedema; *AP*, abdominal pain; *C*, conjunctivitis; *F*, female; *M*, male; *NA*, not available; *ND*, not determined; *OAS*, oral allergy syndrome; *U*, urticaria; *V*, vomiting.

Reactivity to bony and cartilaginous fish was explored by PPT with cooked fish and the wheal size (mm) is indicated. Information about clinical reactivity is based on self-report or diagnostic food challenges.

*Open food challenge.

†Double-blind placebo-controlled food challenge.

Purification and characterization of the parvalbumins

Purified parvalbumins used in this study are listed in Table II. Detailed purification and characterization procedures are available in this article's Method's section in the Online Repository at www.jaci-inpractice.org.

ELISA

Quantification of parvalbumin-specific serum IgE in patients allergic to fish and controls was performed by ELISA as previously described.[23] Five β-parvalbumins and 3 α-parvalbumins were used. Specific IgE values of greater than 0.1 kUA/L were rated positive. Negative values were rated as 0.1 kUA/L.

Inhibition ELISA

Cross-reactivities between β-parvalbumins and α-parvalbumins were analyzed by inhibition ELISA. Plates were coated with 1 μg/mL cod β-parvalbumin. Sera from 10 individuals allergic to fish were pre-incubated with β-parvalbumins from cod (self-inhibition) or salmon, or with ray α-parvalbumin at serial dilutions (0.01-100 μg/mL). Binding of serum IgE to coated cod parvalbumin was determined as described.[23]

Basophil activation test

Basophil activation on stimulation with increasing concentrations of parvalbumins was assessed using the Flow-CAST kit (Bühlmann Laboratories AG, Schönenbuch, Switzerland) according to the manufacturer's protocol. The gating strategy is shown in Figure E2 in this article's Online Repository at www.jaci-inpractice.org. Detailed methods of the BAT are specified in this article's Methods section in the Online Repository.

Skin prick testing with purified parvalbumins

SPT was performed in patients allergic to fish using purified natural parvalbumins (0.1, 1, 10, and 50 μg parvalbumin/mL) diluted in saline containing 0.03% human serum albumin (ALK, Inc., Hørsholm, Denmark). β-Parvalbumins from cod, carp, and salmon and α-parvalbumins from ray and shark were used. In the children with a clinical history of severe allergic reactions to fish, the number of parvalbumins was reduced by excluding β-parvalbumins from carp, salmon, or both. Skin test results were rated positive when the average wheal diameter was greater than or equal to 3 mm compared with that achieved with saline.

RESULTS

Demographic and clinical characteristics of study subjects

Eighteen individuals with fish allergy (mean age, 16 years) and 7 controls (mean age, 29 years) were included in this study. Total serum IgE level ranged from 46 to 2297 kU/L for individuals with fish allergy and from 59 to 1121 kU/L for controls (Table I and Table E1). Severity of the clinical symptoms of fish allergy varied, including asthma, urticaria, and gastrointestinal symptoms (Table I). Sensitization to cod and salmon was confirmed by ImmunoCAP with the respective fish extracts. Specific IgE titers for cod ranged from 0.2 kUA/L to more than 100 kUA/L (median, 4.9 kUA/L). IgE specific to salmon ranged from 0.3 to more than 100 kUA/L (median, 6.5 kUA/L) (Table I). All patients were positive for both cod and salmon in ImmunoCAP according to cutoff of 0.1 kUA/L.

TABLE II. Parvalbumins investigated in the study

Species (common name)	Species (scientific name)	Allergen name	Parvalbumin lineage	Parvalbumin used in ELISA and BAT	Parvalbumin used in SPT
Atlantic cod	*Gadus mohrua*	Gad m 1	β	n	n
Common carp	*Cyprinus carpio*	Cyp c 1	β	n	n
Atlantic salmon	*Salmo salar*	Sal s 1	β	n	n
Atlantic salmon	*Salmo salar*	NA	α	r	—
Barramundi	*Lates calcarifer*	Lat c 1	β	n	—
Nile tilapia	*Oreochromis niloticus*	NA	β	n	—
Thornback ray	*Raja clavata*	NA	α	n	n
Gummy shark	*Mustelus antarcticus*	NA	α	n	n

n, Natural; *NA*, not available; *r*, recombinant; —, not used.

Low *in vivo* reactivity to ray

To explore the reactivity of patients allergic to fish to ray, a cartilaginous fish, PPTs with cooked cod, salmon, and ray were performed (Table I). All patients reacted to at least 1 bony fish (17 of 18 for cod, median wheal diameter, 11 mm; 14 of 15 for salmon, median wheal diameter, 11 mm). In contrast, PPTs with ray demonstrated positive reactions in only 4 of 18 patients, with a median wheal diameter of 2 mm for all patients (Table I). For the group of positive patients in PPT with ray (P4, P10, P17, and P18), the mean wheal diameter was 7 mm. In case of the other 14 patients, negative in PPT with ray, the mean wheal diameter was 1 mm.

Clinical reactivity to bony fish correlated with PPT results, with 16 of 18 patients being positive to both cod and salmon, whereas P2 and P11 reacted only to salmon or cod, respectively. Clinical reactivity on ingestion of ray was assessed in 11 patients, of which 10 were negative and tolerated this fish. The only patient showing allergic symptoms after ingestion of ray (double-blind placebo-controlled food challenge) was P18, who had a positive result in PPT with ray (average wheal diameter, 15 mm) (Table I).

Characterization of the parvalbumins

Eight fish parvalbumins (5 β and 3 α) were purified and used in the study (Table II). Their purity was demonstrated by Coomassie brilliant blue staining of SDS gels (see Figure E3, *A*, in this article's Online Repository at www.jaci-inpractice.org). Protein identity was confirmed by Western blotting with anti-parvalbumin antibodies (Figure E3, *B*). All parvalbumins demonstrated the expected α-helical secondary structure, as determined by circular dichroism spectroscopy (Figure E3, *C*). Membership to the α or β lineage of parvalbumins was confirmed by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry (data not shown).

Low IgE titers to α-parvalbumins from cartilaginous fish

IgE titers for α-parvalbumins from cartilaginous fish (ray, shark) were significantly lower compared with IgE titers for any of the tested β-parvalbumins (eg, cod vs shark, $P < .05$; tilapia vs ray, $P < .0001$; Figure 1, *A*; see Table E2 in this article's Online Repository at www.jaci-inpractice.org). No significant difference between IgE titers for different β-parvalbumins was observed (Table E2). IgE levels to salmon α-parvalbumin were lower than to β-parvalbumins, but higher than to cartilaginous fish α-parvalbumins (median IgE to β-parvalbumins, ≥1.6 kUA/L; salmon α-parvalbumin, 0.2 kUA/L; ray and shark α-parvalbumins, 0.1 kUA/L; Figure 1, *A*; see Table E3 in this article's Online Repository at www.jaci-inpractice.org).

All 18 patients exhibited positive IgE titers (0.2-72 kUA/L) to at least 1 of the β-parvalbumins (Figure 1, *B*; Table E3). Seventeen of 18 patients had IgE for multiple β-parvalbumins, while P2 was monosensitized to salmon β-parvalbumin, which was in accordance with the clinical reactivity of this patient (Figure 1, *B*, and Table I). Positive IgE titers to ray and shark parvalbumin were found in 2 and 6 patients, respectively, and were generally lower compared with IgE titers to β-parvalbumins in corresponding patients (Figure 1, *B*, and Table E3).

Specific IgE levels to all tested parvalbumins were below the detection limit in control subjects (data not shown).

Weak IgE cross-reactivity between cod β-parvalbumin and ray α-parvalbumin

IgE cross-reactivity between cod β-parvalbumin and salmon β-parvalbumin or ray α-parvalbumin was analyzed. The concentration of cod parvalbumin required to reach 50% inhibition of IgE-binding to cod parvalbumin was as low as 0.01 μg/mL for 7 of 10 subjects. The highest tested concentration of the inhibitor (100 μg/mL) resulted in an inhibition of more than 90% of IgE-binding in all tested individuals (Figure 2, *A*).

The cross-reactivity between cod and salmon β-parvalbumins was patient-dependent. To inhibit IgE-binding to cod parvalbumin by 50%, 0.01 μg/mL salmon β-parvalbumin was required for P1, P7, and P13. An inhibitor concentration of 0.1 μg/mL inhibited binding to cod parvalbumin in 3 additional patients (P3, P10, and P12). The highest tested inhibitor concentration resulted in more than 50% inhibition in 9 of 10 patients. For P9, inhibition did not reach 50% in the tested range of inhibitor concentrations (Figure 2, *B*).

IgE cross-reactivity between cod and ray parvalbumins was very low. Ray parvalbumin induced more than 50% inhibition only when used in the highest concentration (100 μg/mL) in 8 of 10 subjects. For P9 and P13, the inhibition did not reach 50% in the tested range of inhibitor concentrations (Figure 2, *C*).

Low basophil activation in response to fish α-parvalbumins

α-Parvalbumins from ray, shark, and salmon demonstrated lower capacity to activate basophils than did β-parvalbumins from cod, carp, and salmon (Figure 3). On stimulation with 100 ng/mL parvalbumins, the median amount of $CD63^+$ basophils was 13.9%

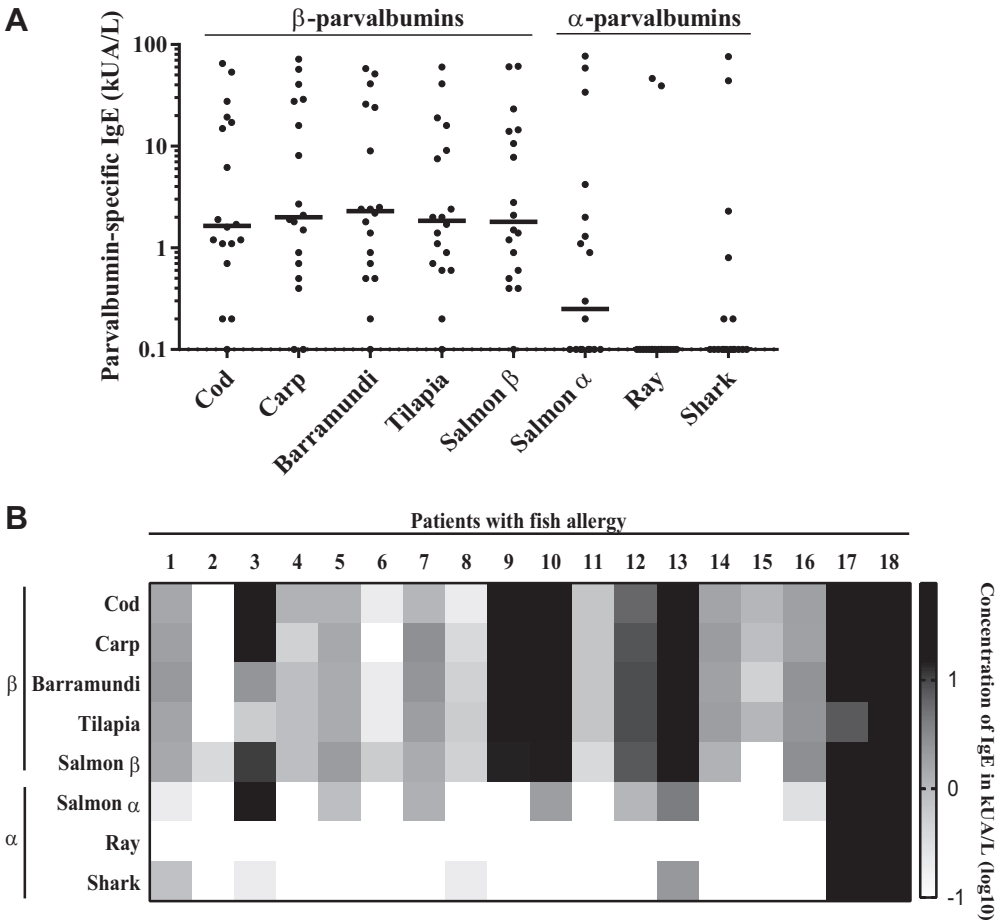

FIGURE 1. IgE levels to α-parvalbumins are significantly lower than IgE levels to β-parvalbumins. **A,** IgE titers (kUA/L) to α-parvalbumins and β-parvalbumins among 18 patients allergic to fish were determined by ELISA. **B,** Patient-specific IgE recognition patterns to α-parvalbumins and β-parvalbumins. The signal intensity of parvalbumin-specific IgE is represented in a grading log10 scale for concentrations of measured parvalbumin-specific IgE (kUA/L).

to 16.2% with various β-parvalbumins compared with less than 1.2% with α-parvalbumins (Figure 3; see Table E4 in this article's Online Repository at www.jaci-inpractice.org). The difference in basophil activation capacity between α-parvalbumins from cartilaginous fish and any of the tested β-parvalbumins was statistically significant (eg, ray vs cod, $P < .001$; shark vs cod, $P < .01$; n = 18) (see Table E5 in this article's Online Repository at www.jaci-inpractice.org). Response to salmon α-parvalbumin was significantly lower than to cod and salmon β-parvalbumins but not when compared with carp parvalbumin (Table E5). Fourteen of 18 patients demonstrated positive reactions to at least 1 of the parvalbumins. All 14 patients were positive to β-parvalbumins, whereas only 2 (P12 and P18) reacted to α-parvalbumins as well when proteins were used at concentrations up to 100 ng/mL (Table E4; see Figure E4 in this article's Online Repository at www.jaci-inpractice.org). Furthermore, the concentration of α-parvalbumins required to elicit a positive response was higher than that of β-parvalbumins.

All patients responded positively to the stimulation with an anti-FcεRI mAb, whereas 16 of 18 patients responded to formyl-methionyl-leucyl-phenylalanine stimulation (Figure E5, available in this article's Online Repository at www.jaci-inpractice.org). None of the controls demonstrated a positive basophil activation in response to stimulation with parvalbumins in a concentration of up to 10 μg/mL (data not shown).

Weak skin prick reactivity to α-parvalbumins in patients allergic to bony fish

β-Parvalbumins from cod, carp, and salmon induced positive skin reactions in 94%, 78%, and 80% of the tested patients, respectively (Figure 4). In contrast, each of the α-parvalbumins induced positive skin reactions in only 3 of 16 patients. Furthermore, the parvalbumin concentration needed to induce positive skin reactions was 100- to 500-fold higher for α-parvalbumins than for β-parvalbumins (Figure 4; see Table E6 in this article's Online Repository at www.jaci-inpractice.org). Detailed results of the SPT for patients allergic to fish are presented in Table E6. Negative control (saline) did not induce any skin reaction.

DISCUSSION

Fish-allergic individuals are generally advised to avoid all fish due to the potential risk of reacting to a wide variety of species in addition to the primary sensitizing source.[18,27] Although many studies have described different species of bony fish as highly

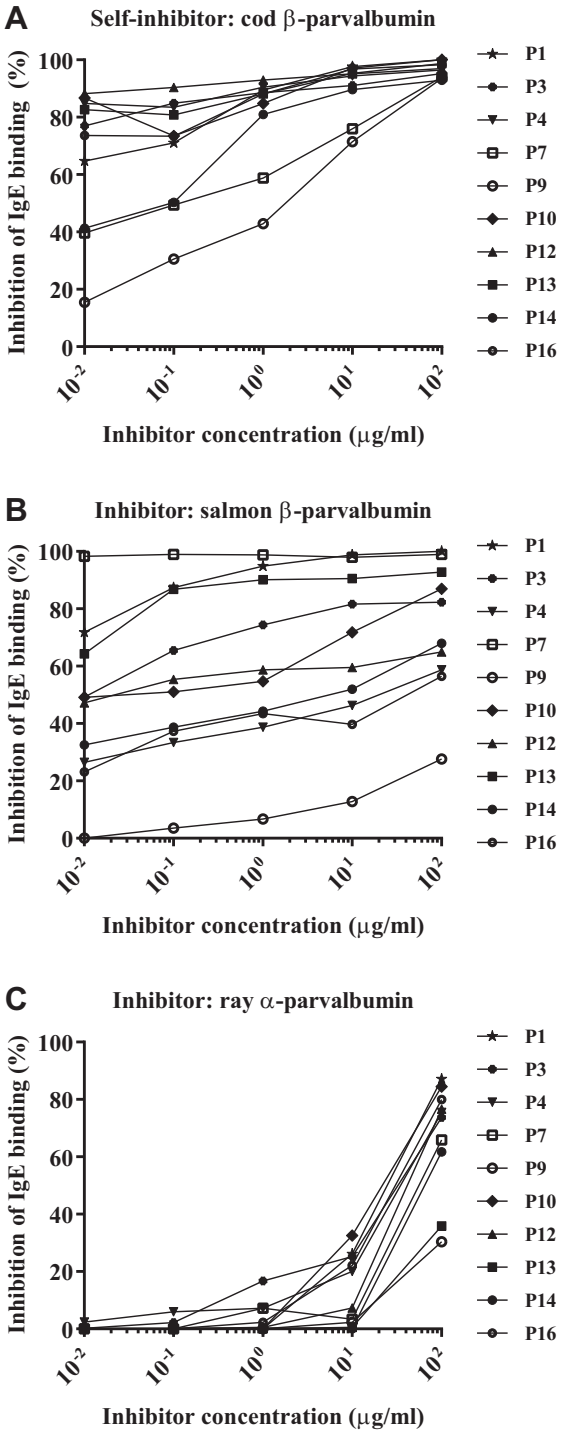

FIGURE 2. IgE antibody cross-reactivity of cod β-parvalbumin to salmon (β) and ray (α) parvalbumin determined by inhibition ELISA in patients allergic to bony fish (n = 10). Microtiter plates were coated with cod β-parvalbumin and sera preincubated with cod β-parvalbumin (**A**), salmon β-parvalbumin (**B**), or ray α-parvalbumin (**C**) at increasing concentrations.

allergenic and cross-reactive, reports of allergy to cartilaginous fish are rare and contradictory.[5-7] Because of the lack of knowledge about the allergenicity of alternative fish sources such as cartilaginous fish, patients may be subjected to unnecessary food restrictions, possibly influencing their dietary requirements and quality of life.[19,28]

Most patients with fish allergy are sensitized to β-parvalbumins present in the muscle of bony fish.[24,29] Parvalbumins of the α-lineage are abundant in muscle tissue of cartilaginous fish and higher vertebrates.[24] Chicken and frog α-parvalbumins were shown to be responsible for IgE cross-reactivity to bony fish β-parvalbumins in some of the patients allergic to fish.[30,31] However, only 1 study explored the sensitization to an α-parvalbumin from cartilaginous fish.[25] In this study, Cai et al[25] demonstrated IgE-binding to parvalbumin from red stingray in patients allergic to bony fish by IgE immunoblotting but no functional assays or *in vivo* studies were performed.

Here, we analyzed the allergenicity of cartilaginous fish and their parvalbumins in patients with allergy to bony fish. We were able to demonstrate low reactivity to ray, a cartilaginous fish, by PPT (median wheal diameter, 2 mm; n = 18). Furthermore, 11 patients were tested for tolerance of ray, 10 of which were able to consume a serving dose of ray without developing allergic symptoms (Table I). Only P18 had a positive reaction to ray on double-blind placebo-controlled food challenge, which was in accordance with his skin reaction in PPT with cooked ray. In addition, P10 and P17 demonstrated positive skin reactions to cooked ray. However, these 2 patients did not agree to undergo a food challenge and their clinical reactivity to ray could not be confirmed.

To our knowledge, besides the study from Cai et al,[25] only 2 other studies explored the IgE reactivity to cartilaginous fish. Calderon-Rodriguez et al[5] demonstrated low allergenicity of cooked dogfish sharks in 34 patients with fish allergy. However, PPT result with raw dogfish was positive in 6 patients, possibly demonstrating sensitization to fish allergens that are not heat-stable. A study from Koyama et al[7] that examined patients' IgE-binding to raw extracts from 43 fish species demonstrated a low IgE recognition of cartilaginous compared with bony fish.[7]

To dissect the molecular basis of the observed tolerance to cartilaginous fish in patients with confirmed allergy to bony fish, we explored IgE reactivity to fish α-parvalbumins and β-parvalbumins in our patient cohort. Levels of IgE specific to α-parvalbumins from cartilaginous fish were significantly lower than to bony fish β-parvalbumins (Figure 1 and Tables E2 and E3). Previous studies demonstrated high cross-reactivity of β-parvalbumins from different fish species; however, the cross-reactivity between fish β-parvalbumins and α-parvalbumins has not been explored.[27,32,33] We demonstrated patient-dependent IgE cross-reactivity between cod and salmon β-parvalbumins by inhibition ELISA. Interestingly, the cross-reactivity between parvalbumins from cod (β) and ray (α) was strikingly low (Figure 2).

BATs further confirmed the low IgE reactivity of α-parvalbumins in our patient cohort (Figure 3). Overall, 14 of 18 patients demonstrated positive reactions with any of the tested parvalbumins. All 14 patients were positive to β-parvalbumins and only 3 reacted to α-parvalbumins when tested up to 1000 ng/mL. Four patients did not respond to purified parvalbumins in BAT possibly due to the low parvalbumin-specific IgE levels compared with the total IgE levels in these patients (P2, P6, P11, and P15). Furthermore, these patients may have been sensitized to other fish allergens such as aldolase A, β-enolase, or collagen.[23,34] Previous studies have tested the capacity of BAT to diagnose food allergy and have shown that it has superior specificity and comparable sensitivity compared

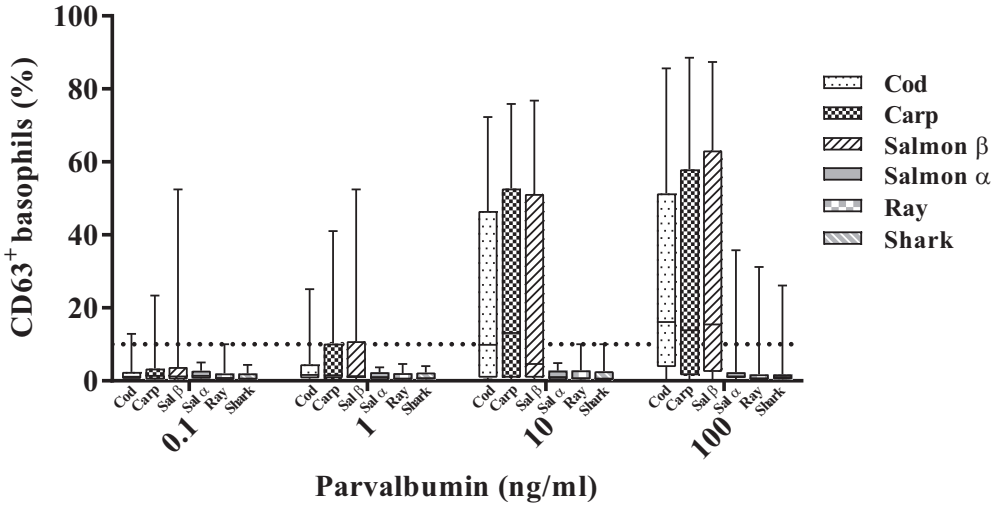

FIGURE 3. Basophil response to α-parvalbumins was lower than to β-parvalbumins. Data indicate basophil activation (measured as percentage of $CD63^+$ basophils) in response to stimulation with different doses of fish parvalbumins in patients allergic to fish (n = 18).

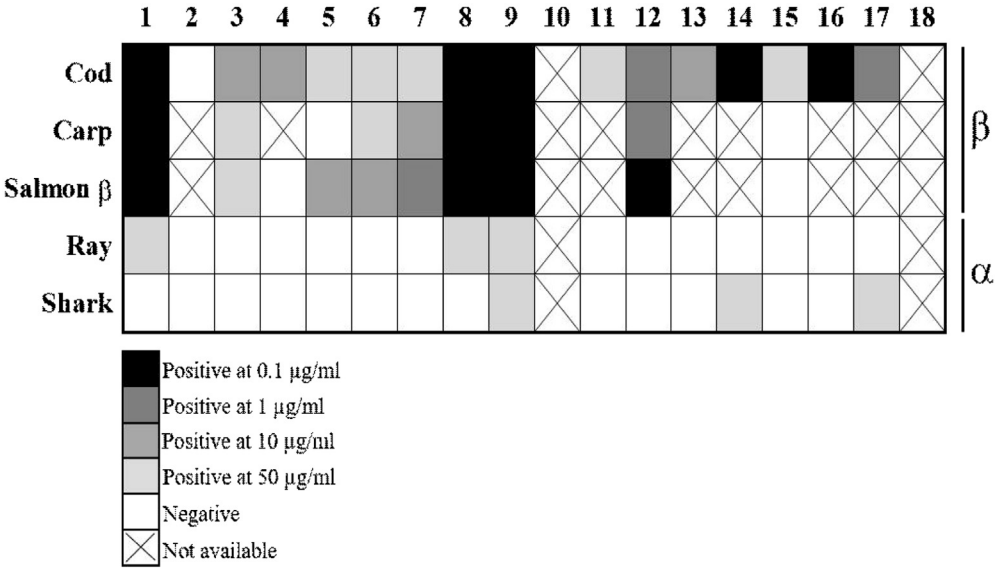

FIGURE 4. α-Parvalbumins show lower capacity than β-parvalbumins to induce skin reactions in patients allergic to fish. SPTs were performed with different doses of natural purified fish parvalbumins. Parvalbumins were used in concentrations ranging from 0.1 µg/mL to 50 µg/mL. Average wheal diameter of greater than or equal to 3 mm compared with negative control was rated as a positive response. (X) indicates that SPT was not performed.

with measuring specific IgE titers and SPT.[35] However, BAT has never been investigated as an additional diagnostic tool for fish allergy. In our study, parvalbumin reactivity in BAT was comparable to reactivity in ELISA and SPT in case of β-parvalbumins for all 14 patients who reacted positively in BAT. However, quantitatively, the Spearman correlation test did not demonstrate a significant correlation between the titer of parvalbumin-specific IgE (ELISA) and reactivity in BAT (see Table E7 in this article's Online Repository at www.jaci-inpractice.org).

Lower reactivity to α-parvalbumins compared with β-parvalbumins was further confirmed by SPT with purified natural parvalbumins (Figure 4). Only 5 of 16 patients were positive to ray or shark parvalbumin, and the concentration of α-parvalbumins needed to elicit positive reactions was in all cases higher than the concentrations of β-parvalbumins in the corresponding patients.

The observed difference in allergenicity between fish β-parvalbumins and α-parvalbumins may be explained by low sequence identity between these 2 parvalbumin lineages (<50% sequence identity between α-parvalbumin from leopard shark or thornback ray and β-parvalbumins from cod, carp, or salmon).[4,16] Interestingly, sequence identity between fish β-parvalbumins and α-parvalbumin from frog and chicken is higher than 50%, possibly contributing to occasional cross-reactivities between these species.[4,36]

Most commonly used *in vitro* diagnostic platforms for fish allergy such as the ImmunoCAP Rapid, ImmunoCAP ISAC (Thermo Scientific), and MADx (Macro Array Diagnostics, Vienna, Austria) are based on quantification of IgE against extracts from a total of 28 different fish species and 2 recombinant β-parvalbumins (cod and carp).[16] Cartilaginous fish, such as different species of rays and sharks, are not included in these tests. Cartilaginous fish have been described as equal in nutritional value to bony fish and have multiple health benefits.[37,38] The inclusion of specific cartilaginous fish species and their parvalbumins in the current diagnostic assays would be essential to confirm the tolerance among patients allergic to fish, eventually resulting in less stringent curtailment of the diet of individuals allergic to fish.

Our study demonstrated that IgE reactivity to parvalbumin may predict the clinical reactivity to specific fish species. However, it is important to note that some patients may be sensitized to other, minor fish allergens such as aldolase A, β-enolase, or collagen.[23,34] Because cartilaginous fish is most commonly consumed cooked, we performed IgE immunoblots with extracts of raw and cooked ray (see the Methods section and Figure E6 in this article's Online Repository at www.jaci-inpractice.org). Only 1 of 14 patients tested was found to be positive to ray enolase and 2 patients to ray aldolase when tested on an extract of raw ray. However, these 2 patients were negative to these 2 allergens when tested on extract of cooked ray (Figure E6, *E*). None of the patients showed IgE-binding to ray collagen (expected molecular weight of collagen α-chain, ∼110 kDa) (Figure E6, *E*).

Currently, diagnostic testing (PPT, food challenges, or both) for several cartilaginous fish species can be recommended to explore the possibility of tolerance of cartilaginous fish in individuals sensitized to bony fish. A limitation of this study is the low number of patients tested for tolerance to ray using food challenges, as well as the number of explored cartilaginous fish species. Future studies investigating the potential tolerance of several cartilaginous fish species in a larger patient cohort will help to develop a diagnostic workup of patients with fish allergies and improve patient management.

In summary, our study demonstrated the low allergenicity of ray and fish α-parvalbumins in patients sensitized and allergic to bony fish. Inclusion of cartilaginous fish in routine diagnosis of fish allergy may prevent unnecessary food restrictions. This approach represents a first step toward precision medicine in patients allergic to fish leading to improved quality of life of affected individuals.

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ONLINE REPOSITORY

METHODS

Purification and characterization of the study parvalbumins

Natural β-parvalbumins were purified from Atlantic cod (*Gadus morhua*), common carp (*Cyprinus carpio*), Atlantic salmon (*Salmo salar*), barramundi (Asian sea bass, *Lates calcarifer*), and Nile tilapia (*Oreochromis niloticus*). Natural α-parvalbumins were purified from thornback ray (*Raja clavata*) and gummy shark (*Mustelus antarcticus*). To explore the allergenicity of an α-parvalbumin from bony fish and to compare it with the β-counterpart from the same species, a recombinant α-parvalbumin from Atlantic salmon (Uniprot ID: C0HAT9) was expressed in *Escherichia coli* and purified.

Natural cod and ray parvalbumins were purified from extracts of muscle tissue of the respective fish species using sequential ion-exchange and size exclusion chromatography following the methods described in previous studies.[E1,E2]

For purification of carp parvalbumin, a protein extract was prepared by stirring homogenized carp muscle tissue in 3 volumes of PBS overnight at 4°C. The extract was centrifuged to remove cell debris and heated to 95°C for 30 minutes. Precipitated proteins were removed by centrifugation and the supernatant dialyzed against 20 mM Bis-Tris buffer (pH 6.5) and applied to a Q Sepharose ion-exchange column (GE Healthcare, Chicago, Ill). Bound proteins were eluted from the column by a linear salt gradient from 0% to 50% elution buffer (20 mM Bis-Tris, 1 mol NaCl, pH 6.5). Fractions containing carp parvalbumin were loaded onto HiPrep 26/60 Sephacryl S-200 High Resolution Column (GE Healthcare) equilibrated with PBS. Low-molecular-weight fractions containing carp parvalbumin were dialyzed against 20 mM Bis-Tris, pH 5.5, and loaded onto a Mono Q 5/50 GL Tricon column (GE Life Science). Pure carp parvalbumin was eluted as a single peak from the column by a linear salt gradient from 0% to 35% elution buffer (20 mM Bis-tris, 1 mol NaCl, pH 5.5).

Natural parvalbumins from salmon (β), barramundi, tilapia, and gummy shark were isolated using ammonium sulfate precipitation as previously described for mackerel.[E3] Briefly, fish muscle tissue was heated in PBS at 95°C for 20 minutes. After homogenization, overnight stirring at 4°C, and centrifugation, the parvalbumins were purified from the supernatant by ammonium sulfate precipitation followed by dialyses against ammonium bicarbonate buffer and subsequently PBS.

Recombinant salmon α-parvalbumin was expressed and purified according to previously published procedures.[E4]

Purified parvalbumins were visualized by Coomassie brilliant blue staining of the 15% SDS gels. Protein identity was confirmed by Western blotting using 2 antiparvalbumin antibodies (Swant, 235, an mAb raised against β-parvalbumin, and Abcam, ab11427, a polyclonal antibody raised against α-parvalbumin). Secondary structure of the purified parvalbumins was determined by circular dichroism spectroscopy. Circular dichroism spectra were measured from 190 to 250 nm using Jasco J-810 spectropolarimeter (Jasco International Co., Hachioji, Tokyo), and 5 separate acquisitions for each protein were averaged. Presence of specific lineage (α or β) and isoforms of natural purified parvalbumins was determined using full-length proteins and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometer (Microflex, Bruker Daltonics, Bremen, Germany).

Basophil activation test

BAT was performed using the Flow-CAST kit (Bühlmann Laboratories AG, Schönenbuch, Switzerland), according to the protocol recommended by the manufacturer. Fresh blood samples from 18 patients allergic to fish and 7 controls were tested with β-parvalbumins from cod, carp, and salmon, and α-parvalbumins from salmon, ray, and shark. Blood samples were incubated with 10-fold serial dilution of allergens. For patients allergic to fish, parvalbumins were initially tested in dilutions between 0.1 and 100 ng/mL. Because for some of the patients 100 ng/mL of the parvalbumins was not sufficient to reach maximum basophil activation, patients were re-tested wherever possible and parvalbumins in a concentration up to 1000 ng/mL were used. In control subjects, concentration of parvalbumins used in BAT was up to 10,000 ng/mL. The percentage of activated ($CD63^+$) basophils on stimulation was determined by flow cytometry. CCR3 was used as a marker for basophils. Stimulation buffer without parvalbumins was used as a negative control. Formyl-methionyl-leucyl-phenylalanine and anti-FcεRI antibody were used as positive controls. Data acquisition and analysis were performed using BD FACSDiva (BD Biosciences) and Kaluza (Beckman Coulter, Brea, Calif) softwares, respectively. The gating strategy is represented in Figure E5. The response was considered positive if the parvalbumin induced an activation of more than 10% of the basophils.

Detection of minor fish allergens in ray extract

To explore the relevance of fish allergens other than parvalbumins in cartilaginous fish, Western blots with antiparvalbumin antibody as well as with antienolase and antialdolase antibodies were performed with extracts from ray filet cooked for 5, 10, or 20 minutes, according to previously published protocols.[E2] Uncooked fish was used as a control. Furthermore, IgE immunoblots with extracts from raw and cooked (10 minutes) ray filet were performed using sera of 14 patients, to analyze whether IgE-binding bands were visible in the molecular weight ranges of β-enolase, aldolase A, or collagen.[E5]

Statistical analysis

The nonparametric paired Friedman test was used for comparisons between the responses to different parvalbumins in ELISA and BAT. Multiple comparisons were performed using Dunn posttest. Data are expressed as medians. For the correlation between parvalbumin-specific IgE and percentage of activated basophils for each parvalbumin, the Spearman correlation test was used, and *P* values adjusted using the Bonferroni correction for multiple comparisons. The analyses were performed using GraphPad Prism software version 7 (GraphPad Software, La Jolla, Calif).

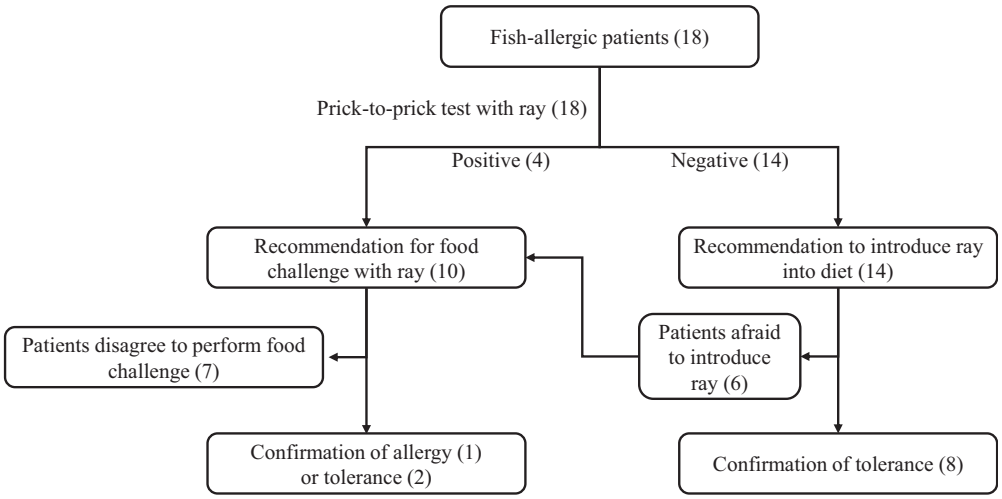

FIGURE E1. Diagnostic flowchart for assessing tolerance of ray. Numbers in parentheses represent the numbers of subjects for each step.

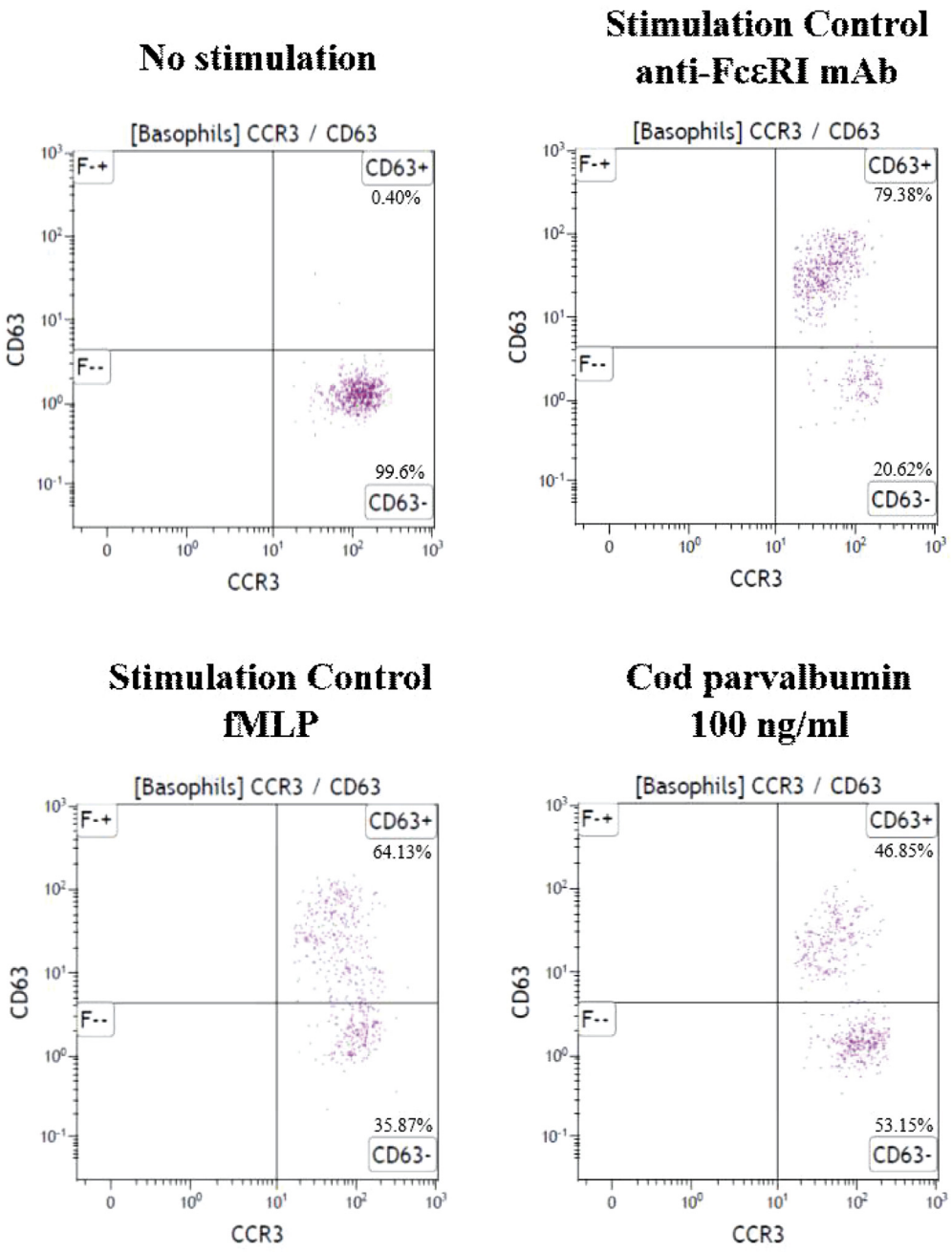

FIGURE E2. Gating strategy in BAT. Basophilic cells were selected from whole blood based on $CCR3^{high}/SSC^{low}$. Activation of basophils was determined by expression of an activation marker CD63. Expression of CD63 for unstimulated control, 2 stimulation controls (anti-FCεRI mAb and fMLP), and stimulation with 100 ng/mL cod parvalbumin is demonstrated for P1 as an example. *fMLP*, Formyl-methionyl-leucyl-phenylalanine.

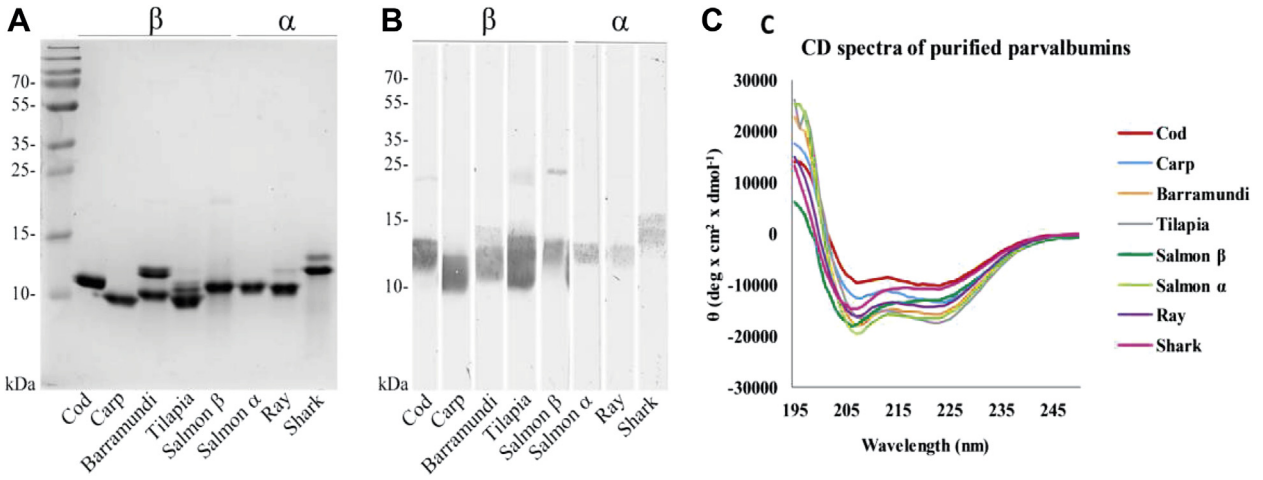

FIGURE E3. Characterization of parvalbumins from bony and cartilaginous fish species. **A,** Coomassie-stained SDS-PAGE gel of purified parvalbumins. **B,** Western blot using commercial antiparvalbumin antibodies. **C,** Circular dichroism (CD) spectra of purified parvalbumins.

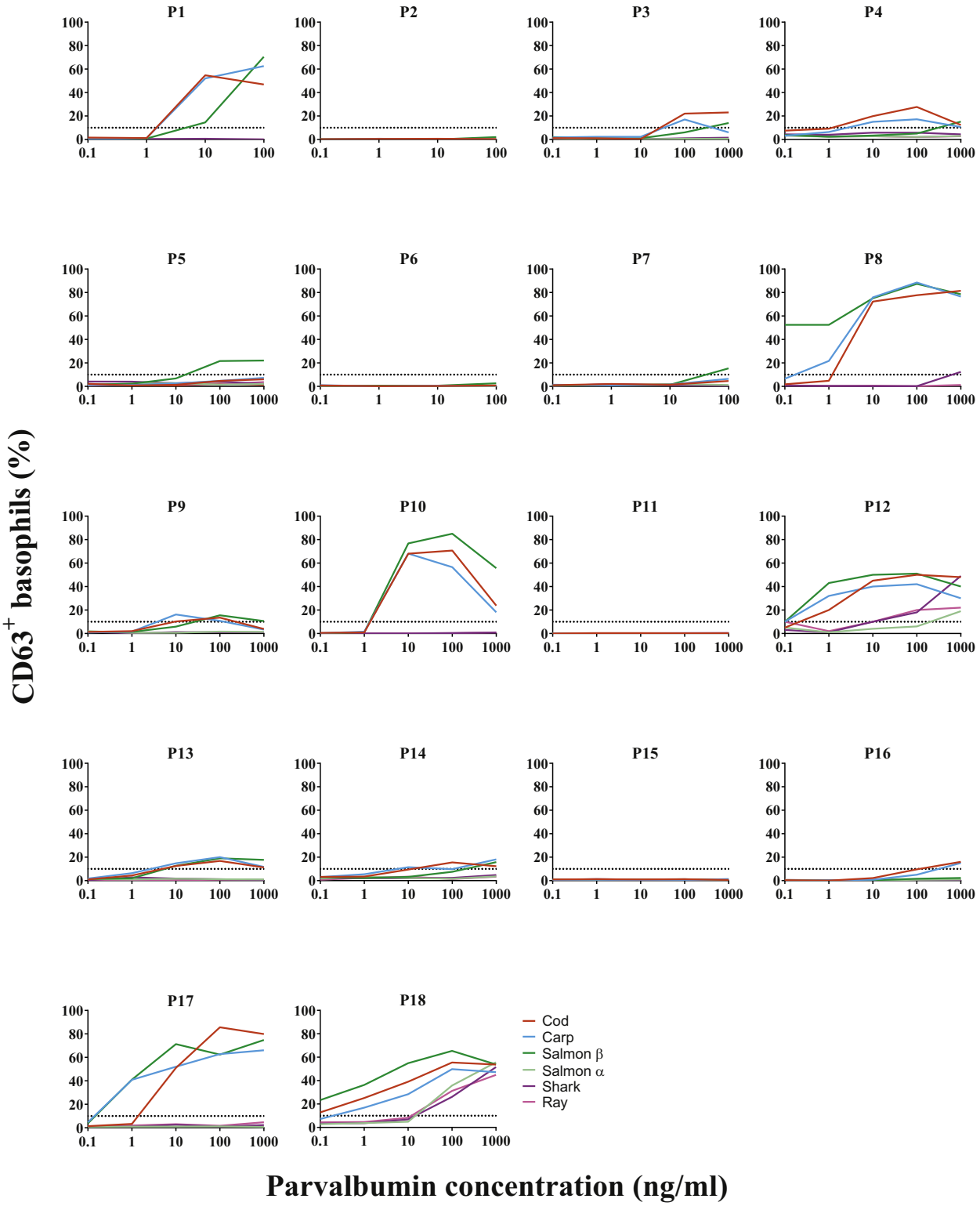

FIGURE E4. BAT. Percentages of $CD63^{+}$ basophils (y-axes) at different concentrations of β-parvalbumins and α-parvalbumins are represented for individual subjects allergic to fish (P1-P18).

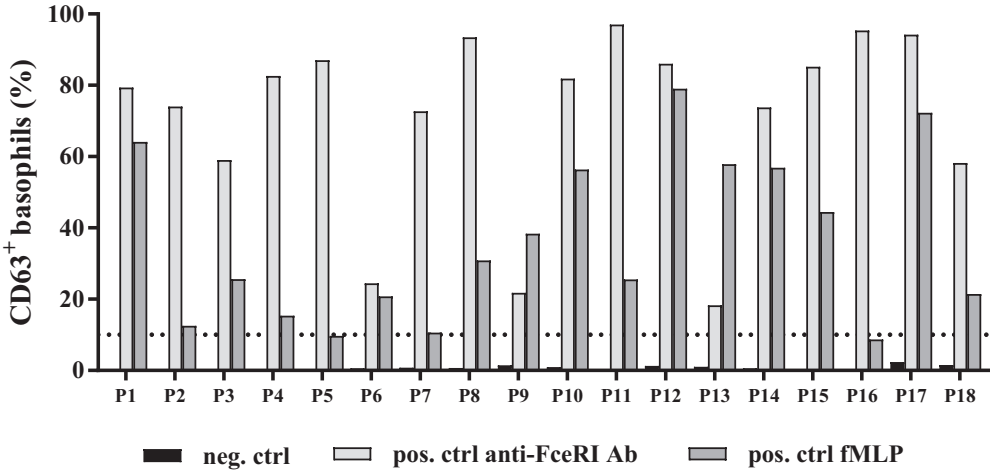

FIGURE E5. Response to positive (anti-FcεRI and fMLP) and negative (stimulation buffer) controls in BAT for subjects with fish allergy. *Ctrl*, Control; *fMLP*, formyl-methionyl-leucyl-phenylalanine; Neg, negative; *pos*, positive.

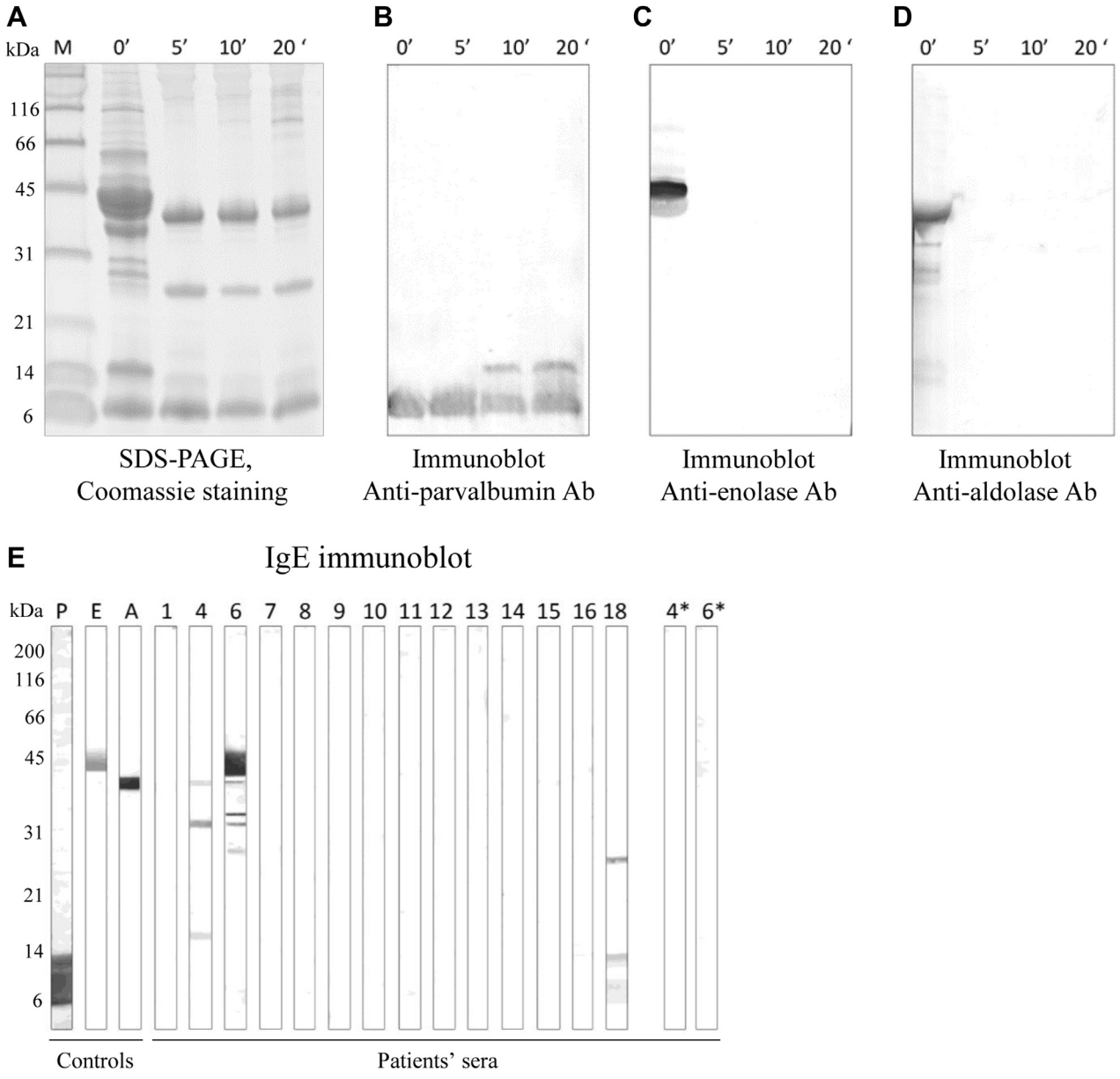

FIGURE E6. Detection of fish allergens in ray extract. **A,** Coomassie-stained SDS-PAGE of extract of raw ray filet and ray filet cooked for 5, 10, or 20 minutes. **B-D,** Immunoblots with ray extract using antiparvalbumin antibody (Fig E6, *B*), antienolase antibody (Fig E6, *C*), and antialdolase antibody (Fig E6, *D*). **E,** IgE immunoblots using extract of raw ray filet. P, E, and A stand for controls parvalbumin, enolase and aldolase, respectively. P4 and P6 were additionally tested on extract of cooked ray (4* and 6*). *Ab,* Antibody.

TABLE E1. Demographic and clinical characteristics of control subjects

Control no.	Sex	Age (y)	Total IgE (kU/L)	Allergies
C1	M	17	1121	Peanut
C2	F	45	59	Pollen
C3	M	14	16	Hazelnut
C4	M	12	913	Peanut
C5	M	48	86	Wasp venom
C6	F	28	533	Grass pollen
C7	F	42	123	House dust mite

F, Female; *M*, male.

TABLE E2. Comparison (Friedman test with Dunn posttest) of IgE levels specific to different purified parvalbumins*

	Parvalbumin						
Parvalbumin	**Cod**	**Carp**	**Barramundi**	**Tilapia**	**Salmon β**	**Salmon α**	**Ray**
Carp	NS						
Barramundi	NS	NS					
Tilapia	NS	NS	NS				
Salmon β	NS	NS	NS	NS			
Salmon α	NS	NS	*	NS	NS		
Ray	†	‡	‡	‡	§	NS	
Shark	*	§	§	‡	†	NS	NS

NS, Nonsignificant.
Refer to Figure 1 and Table E3 for IgE quantification data.
*$P < .05$.
†$P < .01$.
‡$P < .001$.
§$P < .0001$.

TABLE E3. Quantification of parvalbumin-specific IgE antibodies (kU_A/L) in sera of fish-allergic subjects using direct ELISA*

Patient no.	**Cod**	**Carp**	**Barramundi**	**Tilapia**	**Salmon β**	**Salmon α**	**Ray**	**Shark**
P1	1.6	1.9	2.2	1.7	1.5	0.2	0.1	0.8
P2	0.1	0.1	0.1	0.1	0.4	0.1	0.1	0.1
P3	17.1	27.7	2.4	0.6	10.6	33.9	0.1	0.2
P4	1.2	0.5	0.9	0.9	0.9	0.1	0.1	0.1
P5	1.2	1.5	1.4	1.4	2.1	0.9	0.1	0.1
P6	0.2	0.1	0.2	0.2	0.6	0.1	0.1	0.1
P7	1.1	2.7	2.4	2.0	1.4	1.3	0.1	0.1
P8	0.2	0.4	0.5	0.6	0.5	0.1	0.1	0.2
P9	19.3	29	24.0	16.0	14	0.1	0.1	0.1
P10	14.9	16	26.0	19.0	14.5	2	0.1	0.1
P11	0.7	0.7	0.7	0.7	0.4	0.1	0.1	0.1
P12	6.2	8.1	9.0	9.1	7.8	1.1	0.1	0.1
P13	27.6	40.6	41.0	41.0	23.3	4.2	0.1	2.3
P14	1.7	2.1	1.8	2.0	1.2	0.1	0.1	0.1
P15	1.1	0.9	0.5	1.1	0.1	0.1	0.1	0.1
P16	1.9	1.8	2.5	2.4	2.8	0.3	0.1	0.1
P17	65.1	72	51.5	7.5	60.5	77	46.5	76
P18	53.4	57	58	60	61	58.8	39.2	44
Average	11.9	14.6	12.5	9.2	11.3	10.0	4.8	6.9
Median	1.6	2	2.3	1.8	1.8	0.2	0.1	0.1
Positive patients (%)	94.4	88.9	94.4	94.4	94.4	55.6	11.1	33.3

*Values above 0.1 kU_A/L were regarded as positive.

TABLE E4. Percentage of $CD63^+$ basophils in blood of patients allergic to fish after stimulation with 100 ng/mL parvalbumins

Patient no.	Cod	Carp	Salmon β	Salmon α	Ray	Shark
P1	46.8	62.5	70.4	ND	0.1	0.1
P2	0.4	0.1	2.1	ND	0.1	0.1
P3	22.0	17.0	6.0	0.6	0.2	1.0
P4	27.7	17.2	5.0	2.2	2.0	5.8
P5	4.7	4.7	21.6	2.4	4.0	1.6
P6	0.8	0.6	2.6	ND	0.6	0.2
P7	4.6	6.4	15.3	ND	0.6	1.2
P8	77.7	88.5	87.4	ND	0.2	0.2
P9	13.6	10.8	15.5	ND	1.0	1.6
P10	70.7	56.5	85.1	ND	0.4	0.4
P11	0.2	0.2	0.2	0.1	0.2	0.4
P12	50.0	42.0	51.0	6.0	20.0	18.0
P13	16.8	20.0	19.1	1.2	0.8	1.2
P14	15.5	9.8	7.6	1.6	1.4	2.4
P15	1.2	0.4	1.2	1.0	1.2	0.4
P16	9.6	5.0	1.6	0.8	0.6	0.6
P17	85.6	62.8	62.4	1.2	1.7	1.1
P18	55.4	49.8	65.4	35.8	26.1	31.2
Average	28.0	25.2	28.9	4.8	3.4	3.7
Median	16.2	13.9	15.4	1.2	0.7	1.1
Positive patients (%)	61.1	55.6	55.6	9.1	5.6	5.6

ND, Not determined.

TABLE E5. Comparison (Friedman test with Dunn posttest) of basophil response to stimulation with 100 ng/mL parvalbumins*

	Parvalbumin				
Parvalbumin	Cod	Carp	Salmon β	Salmon α	Ray
Carp	NS				
Salmon β	NS	NS			
Salmon α	†	NS	‡		
Ray	§	†	||	NS	
Shark	†	‡	§	NS	NS

NS, Nonsignificant.
*Refer to Figure 3, Table E4, and Figure E2 for BAT data.
†$P < .05$.
‡$P < .01$.
§$P < .001$.
||$P < .0001$.

TABLE E6. SPT with purified parvalbumins*

Parvalbumin	μg/mL	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18
Cod	0.1	3	0	0	0	1	0	0	3	4	ND	0	2	0	4	0	6	1	ND
	1	3	0	0	1	2	0	2	ND	ND	ND	0	5	2	ND	2	ND	7	ND
	10	ND	0	7	4	2	2	2	ND	ND	ND	2	ND	3	ND	2	ND	ND	ND
	50	ND	0	ND	ND	4	3	7	ND	ND	ND	4	ND	ND	ND	7	ND	ND	ND
Carp	0.1	3	ND	0	ND	1	0	0	4	3	ND	ND	2	ND	ND	0	ND	ND	ND
	1	7	ND	0	ND	2	0	1	ND	ND	ND	ND	3	ND	ND	0	ND	ND	ND
	10	ND	ND	2	ND	ND	0	5	ND	ND	ND	ND	ND	ND	ND	0	ND	ND	ND
	50	ND	ND	11	ND	ND	3	ND	ND	ND	ND	ND	ND	ND	ND	0	ND	ND	ND
Salmon β	0.1	3	ND	0	ND	1	0	2	3	5	ND	ND	3	ND	ND	0	ND	ND	ND
	1	10	ND	0	1	1	2	3	ND	ND	ND	ND	ND	ND	ND	0	ND	ND	ND
	10	ND	ND	2	2	4	6	ND	ND	ND	ND	ND	ND	ND	ND	0	ND	ND	ND
	50	ND	ND	7	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0	ND	ND	ND
Ray	0.1	0	0	0	0	0	ND	ND	0	1	ND	0	0	0	0	0	2	0	ND
	1	2	0	0	0	0	ND	ND	0	1	ND	0	0	0	0	0	0	0	ND
	10	1	0	0	0	0	1	0	1	0	ND	0	0	0	2	0	0	0	ND
	50	3	0	0	1	0	0	0	3	3	ND	0	0	0	2	0	0	2	ND
Shark	0.1	0	0	0	0	0	ND	ND	0	0	ND	0	0	0	0	0	0	0	ND
	1	0	0	0	0	0	ND	ND	0	0	ND	0	0	0	0	0	0	0	ND
	10	0	0	0	0	0	1	0	2	1	ND	2	0	0	1	0	0	0	ND
	50	1	0	1	1	0	2	0	1	3	ND	ND	2	0	3	0	0	3	ND

ND, Not determined.
*Numbers indicate average wheal diameter (mm).

TABLE E7. Spearman correlation test for the amount of parvalbumin-specific IgE (ELISA) and the percentage of activated basophils with 100 ng/mL parvalbumins (BAT)*

Parvalbumin	Spearman *r*	Corrected *P* value
Cod	0.5974	0.0516
Carp	0.5266	0.1398
Salmon β	0.5658	0.0833
Salmon α	0.2378	0.9791
Ray	0.4367	0.3530
Shark	0.0012	1

**P* values listed in the table were obtained using the Bonferroni correction for multiple comparisons ($P_{corrected} = 1 - (1 - P_{uncorrected})$,[6] where 6 is the number of independent comparisons.

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Further results and discussion not included in the published manuscript

The above manuscript resulted from a large multinational collaboration. All eighteen fish-allergic patients were recruited in Luxembourg, where they also underwent SPT and oral food challenges (OFCs). Notably, the investigated thornback ray (*Raja clavata*) is popular in the Atlantic and Southwest Indian Ocean, however its conservation status is near threatened (population decreasing).[1] Consequently, the sustainability of thornback ray fisheries is questionable.[2–4] Nevertheless, this study indicated that cartilaginous fish can be a safe food alternative for bony fish-allergic patients.

As part of further investigations, Australian fish-allergic patients were skin-tested with locally available and sustainably-sourced gummy shark (flake, *Mustelus antarcticus*). Twenty-nine (38%) out of 77 patients had a negative SPT result (<5 mm) to raw gummy shark preparation (**Figure V A 2** in **Chapter V A** and **Appendix C Table 1**). However, cartilaginous fish is very unlikely to be consumed raw and therefore further investigations focussed on heat-stable putative allergens. Parvalbumin and tropomyosin were each recognised by a quarter of the seventy-seven patients (**Table V A 2** in **Chapter V A**). IgE-binding bands were identified by mass spectrometric analyses and three main types of IgE-binding pattern were observed: (1) detection of monomeric parvalbumin, (2) detection of monomeric and dimeric parvalbumin along with tropomyosin and (3) detection of monomeric parvalbumin isoforms together with collagen (see **Figure VI A 1 A**). Sequence and structure analyses of the major parvalbumin isoform suggest a cross-reactive confirmational IgE epitope between bony and cartilaginous fish in the calcium-binding EF-loop (see **Figure VI A 1 B** and **C**).

To further investigate cartilaginous fish as a safe food alternative, a clinical study is commencing soon at the Epworth hospital (Melbourne, Australia) in collaboration with Dr Sam Mehr. I sourced muscle tissue from five shark and ray species, which derive from sustainable fishery:[5] Gummy shark, elephant/ghost shark (*Callorhinchus milii*), common sawshark (*Pristiophorus cirratus*), Soutern sawshark (*Pristiophorus nudipinnis*) and Eastern shovelnose ray (*Aptychotrema rostrata*). The first two species were investigated for their detection by commercial test kits in **Chapter III A** and for their putative allergens in **Chapter V A** and **VI B**. Authentic samples from the other three species were sourced for the first time for this clinical study. Preparations for skin-testing were generated from heat-treated muscle tissue from all five species as described in **Chapter VI B**. As soon as COVID-19 restrictions allow, at least 30 well-characterised fish-allergic children will be skin-tested to the mentioned sharks and rays as well as five bony fish, shrimp/prawn and house dust mite. Subsequently, OFCs with highly standardised cooked gummy shark fillets will be performed. Serum will be taken from all patients for *in vitro* analyses. The study is approved by the responsible ethics committee. Publication of the outcomes is expected in 2022.

In summary, cartilaginous fish can be a safe food alternative for some fish-allergic patients. *In vitro* tests should be developed to allow reliable diagnosis before recommending OFCs with cartilaginous fish. Our understanding of the allergen repertoire of multiple shark and ray species, which derive from sustainable fishery, should be expanded. Nevertheless, cartilaginous fish is only one of many possible food alternatives to bony fish. The allergenicity/allergen repertoire of many vertebrate species, including amphibians, reptiles, birds and higher vertebrates, is however under-investigated. Selected non-fish vertebrate

species were therefore evaluated as a food alternative for fish-allergic patients in **Chapter VI B**, with focus on saltwater crocodile (*Crocodylus porosus*) and its parvalbumin isoforms.

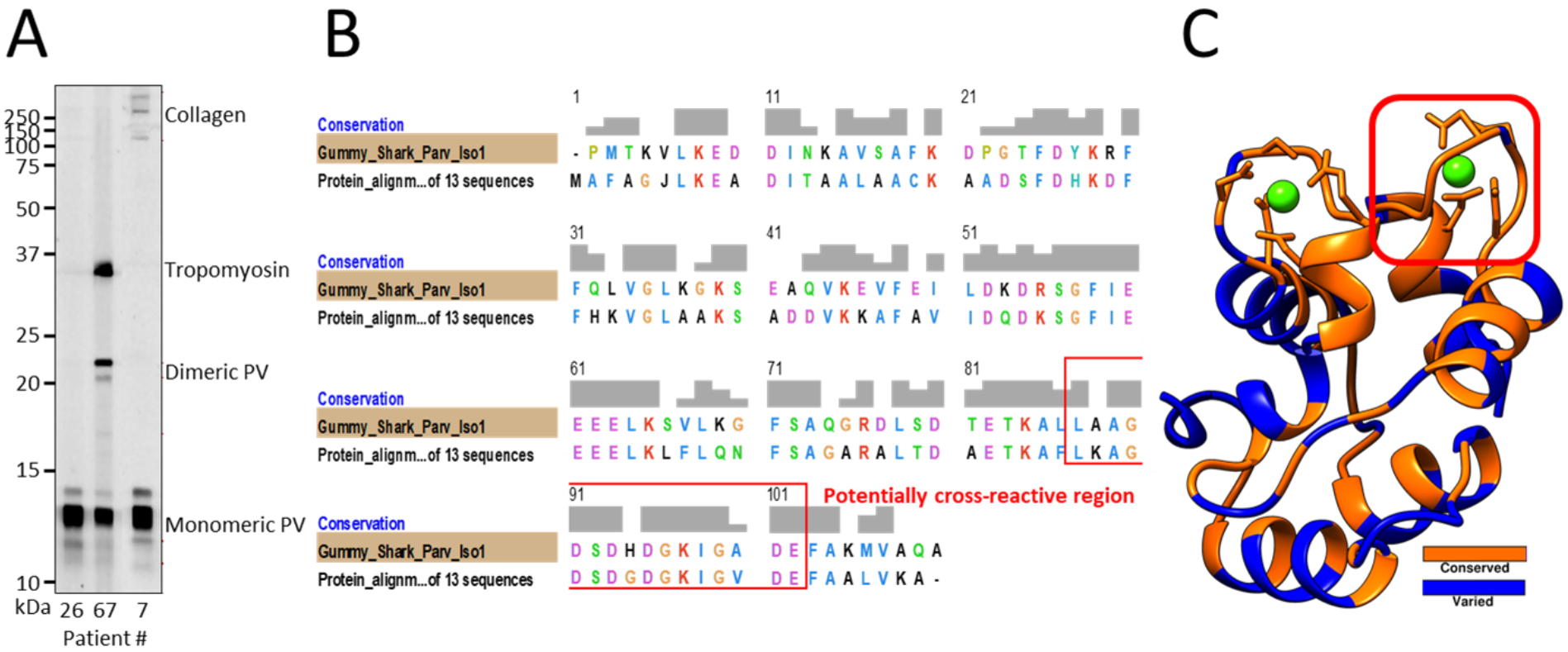

Figure VI A 1: IgE-surfblots with a heated extract from gummy shark using serum from three fish allergic patients (**A**). See Appendix C Table 1 for patients' characteristics. The identification of IgE-binding bands is based on mass spectrometric analyses after tryptic digestion in-gel. The sequence of the major parvalbumin (PV) isoform was determined *de novo* and compared with the sequences from 13 WHO/IUIS-registered bony fish parvalbumins (**B**). The identified potentially cross-reactive region is in the calcium-binding EF-loop, which is highlighted in the 3D model (**C**). Data were partially generated by Dr Aya C. Taki.

References for further results and discussion

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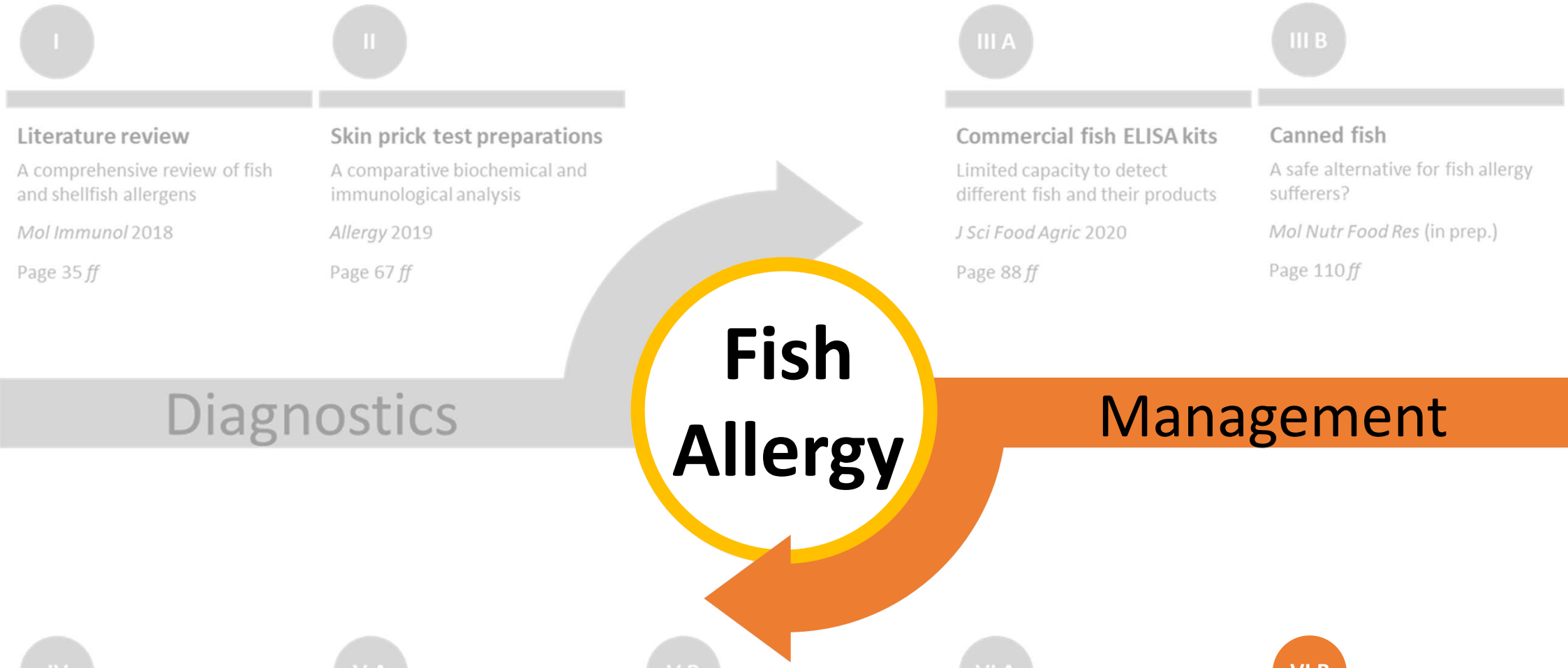
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Chapter VI B – Cross-reactivity to reptiles

The major fish allergen parvalbumin is also a major allergen in reptiles – the fish-reptile syndrome

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Graphical abstract

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Abstract

Background: Cross-reactivity between bony and cartilaginous fish as well as chicken has been previously demonstrated for fish-allergic patients. However, there is a wide variety of under-investigated vertebrates consumed worldwide. Recent reports of anaphylaxis upon consumption of crocodile meat initiated the evaluation of the allergenicity of crocodilians and several other vertebrates in a comparative study with a focus on the major fish allergen parvalbumin.

Methods: Proteins were extracted from muscle tissue of four cartilaginous, five bony fish, four reptiles/amphibians and four other vertebrates. Previously reported allergens and other IgE-binding proteins were identified and quantified by SDS-PAGE, mass spectrometric analyses and immunoblotting using commercial and in-house antibodies as well as patients' serum. A large cohort of fish-allergic pediatric patients (*n*=86) was skin-tested to eight fish preparations and a sub-cohort (*n*=21) to crocodile.

Results: Tropomyosin and parvalbumin were the most abundant proteins in heated extracts. Parvalbumin was most abundant (85%) in heated crocodile preparation, which triggered a positive skin reaction (≥3 mm) in 57% of 21 fish-allergic patients. Seventy-two per-cent of 46 parvalbumin-sensitized patients demonstrated IgE-binding to crocodile parvalbumin. Two crocodile parvalbumin isoforms were purified and characterized biochemically. IgE-binding to parvalbumin was species-specific and most frequent for bony fish, followed by reptiles/amphibians and the least for cartilaginous fish. *In vitro* reactivity to other vertebrates was rare.

Conclusions: Crocodile β- and α-parvalbumin were registered as the first reptile allergens with the WHO/IUIS based on this study (Cro p 1 and Cro p 2, respectively). The observed cross-binding can partially be explained by comprehensive parvalbumin sequence comparisons. We advise that fish-allergic individuals should refrain from consuming reptiles/amphibian meat unless tolerance is confirmed by an oral food challenge.

Keywords: Allergy diagnosis, allergy management, fish allergy, parvalbumin, *Crocodylus porosus*

Short title:

Cross-reactivity of fish and other vertebrates

HIGHLIGHTS:

- Every second fish-allergic patient might be at risk of severe allergic reactions upon consumption of amphibian/reptile meat due to high IgE cross-binding to β-parvalbumins.
- Crocodile β- and α-parvalbumin are the first reptile allergens registered with the WHO/IUIS.
- Crocodile β-parvalbumin is 10 - 20-times less abundant than α-parvalbumin but demonstrates much higher IgE-binding intensity and frequency (63% versus 39%).

Abbreviations

CBB - Coomassie Brilliant Blue

MLC – Myosin light chain

NCBI – National Center for Biotechnology Information

PBS – Phosphate-buffered saline

SPT – Skin prick test(ing)

WHO/IUIS – World Health Organization and the International Union of Immunological Societies

1 | BACKGROUND

Fish is an often life-long disease, which frequently results in anaphylaxis and affects up to 3% of the general population.[1–3] The complexity of the food commodity 'fish' poses a major challenge for diagnostics and management.[4(**Chapter I**)] The medical practitioner therefore often recommends avoiding all fish and fish products once diagnosed with an allergy to any fish species.[5] Fish often refers to bony fish, of which over 1,000 different species are consumed worldwide. The major allergen in bony fish is β-parvalbumin, to which up to 95% of fish-allergic patients show IgE-binding.[6] However, patients often demonstrate sensitization to a narrow range of fish, which is often difficult to predict because of a diagnostic capacity limited to a few well studied species.[4,7–11(see **Chapter I** for 4)]

Diagnostics and management are further complicated by a range of immunological and/or clinical cross-reactivities reported to other vertebrates. The evolutionary relationship between animal groups indicates that cartilaginous fish, including sharks and rays, are followed by bony fish, amphibians, reptiles, birds and other vertebrates, including mammals.[12] Most fish allergy research is however limited to bony fish while our recent investigations support the view that some cartilaginous fish can be safely consumed by bony fish-allergic patients.[13–17(see **Appendix D 3** for 13)] The underlying reasons is most likely that cartilaginous fish contain mostly α-parvalbumin which is considered less allergenic.[18(**Chapter V A**)]

Clinical cross-reactivity has been demonstrated among amphibians; only two frog species (edible frog (*Rana*/*Pelophylax* e*sculentus*) and bullfrog (*R./Lithobates catesbeianus*)) are reported to trigger allergic reactions in fish-allergic patients, predominantly due to IgE-binding to β-parvalbumins.[19–21] Furthermore, there are three reports of anaphylaxis upon consumption of reptile meat. One patient had IgE-mediated symptoms upon consuming soft-shelled turtle (*Pelodiscus sinensis*), which was assumed to be the primary sensitizer through

inhalation of cooking fumes.[22] Parvalbumin was determined as the only IgE-binding protein, but not further characterized. In 2017, two reports of anaphylaxis upon consumption of crocodile meat in one fish- and one chicken-allergic patient were linked to IgE-binding of α-parvalbumin.[23,24] The exact species of crocodile is unknown and both allergic patients tried crocodile meat as an alternative to fish/chicken, which was strictly avoided.

Meat from amphibians and reptiles is generally considered as exotic food and consumption is often restricted to cultural occasions or associated with international travelling.[25] Crocodilians, including alligators and crocodiles, are the most consumed reptiles worldwide. Crocodilian meat predominately derives from farms since 1985 and is most widely consumed in Africa, the US and Asia while it is widely available in many EU Member States.[26] The global annual trade in crocodilian meat peaked in 2007 with approximately 1,000 t and is at least 600 t since 2012.[27]

Crocodilians are the closest living relatives of birds.[28] Birds, including chicken, duck and turkey, can be primary sensitizer of food allergies and rare cross-reactivity between fish and chicken has been reported with α-parvalbumin as cross-allergen.[29–33] Allergies to other non-fish vertebrates, i.e., mammals, is predominantly related to exposure to their dander or milk, but not their meat.[34,35] Primary mammal meat allergies have been reported but are very rare and not directly to meat proteins.[36–38]

Fish, on the other hand, is a common trigger of life-threatening anaphylaxis.[39] The capacity to trigger an allergic reaction appears to be patient- and species-specific.[40,41] However, our detailed knowledge is limited to a few fish species and the ability of fish allergic individuals to ingest meat from other vertebrates has never been investigated.

We therefore sought to investigate the presence and capacity of IgE-binding to parvalbumins and other putative allergens in non-fish vertebrates with a focus on crocodile, which was also tested *in vivo*.

2 | METHODS

2.1 | In-house extracts and purification of parvalbumins

Muscle tissue was collected from the 17 vertebrate species listed in **Table 1**, including four cartilaginous fish (Chondrichthyes), five bony fish (Osteichthyes), one amphibian (Amphibia), three reptiles (Reptilia), one bird (Aves) and three mammals (Mammalia). Whole specimens were sourced and samples collected from the middle of the fillets where applicable. All samples were stored at -80°C until use.

Proteins were extracted in phosphate-buffered saline (PBS) as described previously.[42](**Chapter III B**) In short, tissue was homogenized, extracted overnight and after filtration stored at -20°C (raw protein extracts). For the heated extracts, tissue was heated at 95-100°C in PBS for 20 min before extraction in the same buffer.

Preparation for skin prick testing (SPT) were generated from gummy shark, barramundi and catfish by homogenizing minced raw muscle tissue with one part (w/v) Hanks' Balanced Salt Solution (Gibco®, ThermoFisher Scientific) as described previously (see **Chapter V A**). A crocodile SPT preparation was prepared the same way with heating for 20 minutes at 95-100°C prior to homogenizing. Aliquots were stored at -80°C until single use.

Crocodile parvalbumin was purified by ammonium sulphate precipitation as described previously.[43](**Chapter IV**)

2.2 | Patients

Seventy-seven paediatric patients with a history of clinically confirmed IgE-mediated symptoms after fish consumption were recruited (see **Chapter IV A** for details). All patients underwent SPT to five commercial fish preparations (cod, tuna, salmon, mackerel and fish

mix) and the three above mentioned in-house fish preparations. A sub-cohort (*n*=12) and four shellfish-allergic patients were also skin-tested with the heat-treated crocodile preparation. The sIgE levels for cod (f3), tuna (f40) and salmon (f41) were determined for 43 patients by ImmunoCAP (Thermo Fisher Scientific). All patient characteristics and results from *in vivo* and *in vitro* testing are provided in the supplement (**Appendix C Table 1**). The sensitization pattern of this large cohort of fish-allergic patient was described previously for salmon and catfish proteins (see **Chapter V A**). In addition, nine fish-allergic patients, who were not investigated for *in vitro* reactivity, were skin-tested with the fish and crocodile preparations.

Serum from all patients was obtained for *in vitro* analyses and parents gave written informed consents. Sera from two non-atopic donors were used as negative controls. Ethical approval was obtained from the Sydney Children's Hospitals Network (LNR-14/SCHN/185).

2.3 | Protein concentration, SDS gel-electrophoresis and immunoblotting

The protein concentration for all extracts, including purified parvalbumin, was estimated using the Pierce™ BCA Protein Assay kit (Thermo Scientific) with bovine serum albumin as standard. All whole protein extracts were diluted to the same total protein concentration. Proteins were separated according to their molecular weights using a Criterion™ SDS-PAGE system (Bio-Rad) or Dual Double Wide Mini Vertical System (C.B.S. Scientific). Crocodile parvalbumin isoforms were separated by their isoelectric point using ReadyStripTM IPG Strips (11 cm, pH 3-6) in a PROTEAN IEF cell (Bio-Rad) prior to SDS-PAGE as described previously.[43(**Chapter IV**)] Proteins were visualized by Coomassie Brilliant Blue (CBB) R-250 staining.

For subsequent immunoblotting, proteins were transferred onto a nitrocellulose membrane and incubated with antibodies or serum as described previously (see **Chapter VA** for further

details).[44](**Chapter II**) Parvalbumins were detected using the commercial monoclonal PARV-19 antibody raised against frog α-parvalbumin and a polyclonal in-house anti-fish β-parvalbumin antibody raised against barramundi parvalbumin.[45] Densitometric analyses were conducted utilizing Image Studio Version 5.2 (LI-COR®), allowing sensitive and semi-quantitative evaluation of signals.

2.4 | **Mass spectrometry analysis**

Selected whole protein extracts, as well as IgE-binding bands, were digested with trypsin and analyzed by mass spectrometry as described previously (see **Chapter V A**).[44,46](see **Chapter II** for 44) Results were analyzed using both Mascot (v. 2.4) search engine and MaxQuant (v. 1.6.10.43), against an NCBI database containing amino acid sequences of all proteins from the corresponding species or closest higher classification with at least three annotated genomes/transcriptomes (May 2020). The relative protein abundance is expressed in relative intensity-based absolute quantification (iBAQ%) value.[47] Identified protein groups with at least 1 unique peptide and a minimum of 2 razor/unique peptides were included in the analysis.

3 | RESULTS

3.1 | Protein composition of heated vertebrate extracts

Heated extracts from 17 vertebrate species (**Table 1**) were separated by SDS-PAGE and proteins visualized by CBB (**Figure 1 A**). Every extract was different in number and abundance of protein bands. Up to four parvalbumin bands were identified by immunoblotting and mass spectrometric analyses in each extract at 10-15 kDa, except in cattle (**Figure 1 B** and **C**). Monoclonal antibody PARV-19, raised against α-parvalbumin, detected most parvalbumin bands. The in-house antibody, raised against β-parvalbumin, detected bands with a high signal intensity in bony fish and amphibians/reptiles and with lower intensity in cartilaginous fish. Bands in other vertebrates, including chicken, were detected by PARV-19 but not the in-house antibody. In the crocodile extract, two major parvalbumins were identified, the more abundant isoform (13 kDa) belongs to the α-lineage (XP_019397705.1 in NCBI database available under https://www.ncbi.nlm.nih.gov/protein) while the other (11 kDa) is a β-parvalbumin isoform (XP_019400389.1). All bony fish parvalbumin bands contained mostly β-parvalbumins, while it was α-parvalbumins for cartilaginous fish. Parvalbumins from both lineages were detected in amphibian/reptiles. In other vertebrates, including chicken, only α-parvalbumins were detected.

Tropomyosin bands (34-38 kDa) were present in all extracts with the lowest abundance in gummy shark, crocodile and cattle. Bands at about 24 kDa were most abundant in amphibians/reptiles followed by cartilaginous fish and other vertebrates, and least abundant in bony fish. These bands contained apolipoprotein, myosin light chain (MLC) and parvalbumin as determined by mass spectrometric analyses. In the higher molecular weight range (>100 kDa), bands were most abundant in other vertebrates, followed by cartilaginous fish, bony fish and amphibians/reptiles, and contained both myosin heavy chain and collagen.

3.2 | Patients' characteristics and *in vivo* reactivity to crocodile

All recruited patients underwent SPT with seven bony fish and one cartilaginous fish preparation. Barramundi and catfish induced the largest *in vivo* skin reaction and tuna the lowest (**Figure V A 2** in **Chapter V A**). Seventy-five out of the 77 fish-allergic patients (97%) had an SPT result of 5 mm or larger to at least one of these preparations (**Appendix C Table 1**). Two patients had a wheal diameter of 0 mm to all eight fish preparations. The sIgE level was elevated for all three tested fish species (cod, tuna and salmon) in 35 of the 43 tested patients (82%). Two patients (5%) had an elevated level for salmon and cod or tuna only. Six patients (14%) had a sIgE level of <0.1 kU/l to all three tested fish, which is considered negative.

Six out of twelve fish-allergic patients who were also skin-tested to crocodile, had a wheal diameter of or above 5 mm (**Appendix C Table 1**). Two patients had a skin reaction of 3 and 3.5 mm. The nine fish-allergic patients, who were not investigated for *in vitro* reactivity, had a median wheal diameter of 2.5 mm to crocodile; five patients had a negative SPT result (<3 mm), one 3.5 mm and three 8 mm or above. Notably, the median wheal diameter for crocodile (3.5 mm) was greater than the one for the commercial tuna (2.0 mm) and fish mix (3.3 mm) (**Figure 2**). Four fish-allergic patients as well as the four tested shellfish-allergic patients had a negative SPT result (<3 mm).

Based on our previous study, we confirmed that 22-49% of the 77 here investigated fish-allergic patients have IgE-binding to parvalbumin depending on the fish species (**Chapter V A**). 60% of patients showed IgE-binding to at least one parvalbumin from any of the twelve investigated fish species.

3.3 | Frequent IgE-binding to crocodile and toad parvalbumin

Among the 31 patients with no IgE-binding to any fish parvalbumin, two patients (49 and 77 in **Appendix C Table 1**) showed IgE-binding to crocodile parvalbumin (**Appendix C Figure 3** and **Appendix C Table 3**). Among 46 patients with IgE-binding to at least one fish parvalbumin, 33 patients (72%) showed binding to crocodile parvalbumin; 14 (30%) to both α- (13 kDa) and β- (11 kDa), 15 (33%) only to β-, and four (9%) only to α-parvalbumin (**Figure 3 A**). Importantly, crocodile parvalbumin showed a higher frequency of IgE-binding than most investigated fish species, including carp, cod, shark and tuna. All four patients with a crocodile SPT result above 5 mm and one patient with 3 mm showed IgE-binding to crocodile β-parvalbumin. All other patients with a SPT result of or below 5 mm (*n*=7) did not show any IgE-binding to crocodile heated extract.

Among all 77 fish-allergic patients, eight (10%) showed binding to a 25 kDa band, three (4%) to tropomyosin, three (4%) to an unidentified 65 kDa band and seven (9%) to other bands. None of the four tested shellfish-allergic patients and two controls showed IgE-binding to heated crocodile extract.

Seventeen fish-allergic patients with IgE-binding to crocodile parvalbumin as well as three shellfish-allergic patients and two controls were tested for IgE-binding to heated extracts from elephant shark, toad and chicken (**Appendix C Figure 4** and **Appendix C Table 4**). IgE-binding was observed to a 24 kDa band in all three extracts by most fish-allergic patients (47-76%), one shellfish-allergic patient and one control. None of the shellfish-allergic patients or controls showed IgE-binding to any other band. Fifteen fish-allergic patients (88%, *n*=17) showed IgE-binding to toad parvalbumin (**Figure 3 B**). Two and one patient also detected parvalbumin from elephant shark and chicken, respectively. At least one tropomyosin band was recognized by IgE from two to three patients in each extract.

3.4 | IgE-binding capacity of vertebrate parvalbumins

Five patients with IgE-binding to crocodile parvalbumin were further investigated for IgE-binding to heated extracts from all 17 vertebrate species (**Appendix C Figure 5**). IgE from all patients and controls recognized a band at about 24 kDa. No other bands showed binding using serum from controls. Two patients demonstrated weak IgE-binding to selected tropomyosin bands. For each patient, considerable IgE-binding was observed to multiple parvalbumin bands. **Figure 3** shows the IgE-binding pattern of two representative patients to the parvalbumin monomers, highlighting a general pattern of IgE-binding capacity for fish-allergic patients: Bony fish parvalbumins showed the highest IgE-binding capacity and frequency, followed by amphibians/reptiles and rare binding to cartilaginous fish and chicken. No IgE-binding to parvalbumins from higher vertebrates (kangaroo, cattle and pig) was observed.

3.5 | Abundance and characteristic of crocodile parvalbumins

Based on mass spectrometric results, parvalbumin is the eighth-most abundant protein in the raw crocodile extracts (4.7%). More abundant proteins are pyruvate kinase (17.7%), adenylate kinase (7%), phosphoglycerate kinase/mutase (6.7/6.5%), glyceraldehyde-3-phosphate dehydrogenase (6.3%) and aldolase (6.1%). In contrast, in the heated extract, parvalbumin is the most abundant protein (85%), followed by troponin (5%). α-parvalbumin constitutes 91% and 97% of all-parvalbumins in the raw and heated extract, respectively. The SDS-PAGE profiles of these extracts and purified crocodile parvalbumin are shown in **Figure 4 A**, which reflect mass spectrometric results. In the purified parvalbumin, the α-parvalbumin band (13 kDa) was about 20-times more abundant and demonstrated a signal with the PARV-19 antibody 20-times stronger compared to β-parvalbumin (**Figure 4 B**). The signal intensity to the two isoforms was similar using the in-house anti-β-parvalbumin antibody (**Figure 4 C**). Using patients' serum, the signal to the β-parvalbumin was up to 500-times stronger compared to α-parvalbumin (**Figure 4 D**). The two major parvalbumin isoforms were further separated by their isoelectric point (**Figure 4 E**), demonstrating their difference in both molecular weight and isoelectric point. According to mass spectrometric analyses, 90.5% of proteins are α-parvalbumin and 6.8% β-parvalbumin in the purified crocodile parvalbumin preparations (**Figure 4 F**), suggesting a collective purity of over 95% as also suggested by SDS-PAGE. The third-most abundant protein is acyl-CoA-binding protein (0.7%). It is to note that the observed molecular weight of α-parvalbumin is 13-14 kDa while its predicted molecular weight is 11.9-21.1 kDa. Mass spectrometric analyses did not indicate any difference between eight excised α-parvalbumin bands, which was the same for the six excised β-parvalbumin bands. Two bands of different molecular weight were visible for β-parvalbumin while both containing only one parvalbumin isoform (XP_019400389.1).

4 | DISCUSSION

Fish-allergic patients who are sensitized to the major allergen parvalbumin appear to be at high risk of an allergic reaction upon consumption of amphibian and reptile meat. Seventy-two per-cent of 46 fish parvalbumin-sensitized patients demonstrated IgE-binding to crocodile parvalbumin; 63% to β- and 39% to α-parvalbumin. Over half (57%) of 21 tested fish-allergic patients had a positive SPT result to the heated crocodile preparation (≥3 mm), constituting about 85% of parvalbumin protein. Consequently, saltwater crocodile β- and α-parvalbumin were registered as the first reptile allergens with the WHO/IUIS as Cro p 1 and Cro p 2, respectively (www.allergen.org). Overall, bony fish-allergic patients showed the highest frequency and intensity of IgE-binding to parvalbumins from bony fish, followed by amphibians/reptiles and cartilaginous fish.

In contrast, IgE-binding to chicken parvalbumin was observed for only one out of 77 fish-allergic patients. Previously, IgE-binding to chicken parvalbumin was reported for 20 out of 29 patients with both chicken and fish allergy.[33] In our cohort, no patient reported an allergy to chicken. The only patient with IgE-binding to chicken parvalbumin had a clinical history of both fish and shellfish allergy as well as to hen's egg, soy, tree nuts and wheat. Accordingly, it appears very rare for fish-allergic patients to be sensitized to chicken parvalbumin.

Apart from parvalbumin, several other proteins from multiple vertebrate species demonstrated IgE-binding. A 24/25 kDa band demonstrated most frequent binding by both patients and controls. We are therefore suspecting non-specific binding and/or that the observed IgE-binding does not correlate to clinical relevance. MLC, along with apolipoprotein and parvalbumin, was detected in this particular band. MLC was recently described in detail

as a chicken allergen in a cohort of 28 patients allergic to chicken meat.[32] The relevance of the observed IgE-binding in our cohort should be clarified in further investigations.

We observed IgE-binding to vertebrate tropomyosins, which was recently also reported by us and others for fish.[44,48–50](see **Chapter II** for 44) It has been hypothesized that vertebrate tropomyosins are allergens; however, this has never been evaluated *in vitro* or *in vivo*.[51,52] A recent study among shellfish-allergic patients demonstrated IgE-binding to chicken tropomyosin, which did not correspond to cellular reactivity.[53] IgE-binding to collagen was not observed in this study, likely due to its low abundance in the investigated PBS-derived extracts as demonstrated by us recently.[54](**Chapter V B**)

We demonstrate here that the major fish allergen parvalbumin appears to be a pan-allergen among vertebrates, excluding mammals, as suggested by observed IgE-binding patterns of fish-allergic patients. In crocodile, two distinct parvalbumin isoforms demonstrate different IgE-binding capacity. While α-parvalbumin (Cro p 2) is the most abundant isoform, the β-isoform (Cro p 1) demonstrates the much higher IgE-binding capacity. Contrarily, the two reports of anaphylaxis upon consumption of crocodile meat suspected α-parvalbumin to have caused the allergic reaction, probably due to the much higher abundance.[23,24] For frog, β-parvalbumin showed more frequent IgE-binding compared to α-parvalbumin.[20,21] Amino acid sequence comparisons between parvalbumins from Crocodilians, including highly consumed alligator and crocodile species as well as critically endangered gharial, exhibit a high conservation (95-100% sequence identity) among α- and β-parvalbumin within the order of Crocodilia (see poster in **Appendix B 4.7**). Accordingly, all crocodilians appear to have the same likelihood to trigger an allergic reaction in fish-allergic patients. α- and β-parvalbumin from crocodilians share 54-57% of their amino acid sequence. It is to note that there are two β-parvalbumins predicted for saltwater crocodile based on genomic data.[55] However, only

one β-parvalbumin (Cro p 1) was detected in the investigated extracts and its sequence was confirmed by mass spectrometric analyses. Amino acid sequence comparisons for all WHO/IUIS-registered parvalbumins (see poster in **Appendix B 4.7**) can explain some of the observed IgE-binding and limited cross-reactivity between α- and β-parvalbumins. However, only detailed sequence and structure comparisons and the identification of potential epitopes along with comprehensive *in silico*, *in vitro* and *in vivo* analyses, will allow to predict the allergenicity of non-fish vertebrate species for fish-allergic patients.

For crocodile, we demonstrated *in vivo* reactivity by SPT (>3 mm) in 57% of our patients and there are previous reports of anaphylaxis after consumption of crocodile meat as mentioned above. In addition, we are aware of one case each in Australia and New Zealand of a fish-allergic patient developing IgE-mediated symptoms upon consumption of crocodile meat (under further investigation). The clinical relevance of IgE-binding to non-fish vertebrate parvalbumins should be further investigated. However, previous reports of allergic reactions upon consumption of amphibian or reptile meat suggest a high clinical relevance.[19–24]

In summary, a significant proportion of fish-allergic patients may be at risk of severe allergic reactions upon consumption of crocodilian meat due to high cross-binding of crocodile β-parvalbumin (fish-crocodile syndrome). In addition, other reptiles and amphibians seem to pose a similar risk for fish-allergic patients (fish-reptile syndrome). Fish-allergic individuals should avoid the consumption of meat from reptiles and amphibians unless a tolerance is confirmed by a controlled oral food challenge. Further research should assist with the implementation of reptile and amphibian allergens in improved component-resolved diagnostics and *in vivo* SPT to avoid accidental allergic reactions.

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TABLE 1 Details of investigated vertebrate species in descending order of approximate evolutionary age

#	Common name	Scientific name	Parv-albumin*	Taxonomic classification		
				Family	Order	Class
1	Gummy shark	*Mustelus antarcticus*		Triakidae	Carcharhiniformes	Chondrich-thyes (cart. fish)
2	Shortnose spurdog	*Squalus megalops*		Squalidae	Squaliformes	
3	Bluespotted stingray/maskray	*Neotrygon kuhlii*		Dasyatidae	Myliobatiformes	
4	Elephant/Ghost shark	*Callorhinchus milii*		Callorhinchidae	Chimaeriformes	
5	Atlantic cod	*Gadus morhua*	Gad m 1	Gadidae	Gadiformes	Osteich-thyes (bony fish)
6	European carp	*Cyprinus carpio*	Cyp c 1	Cyprinidae	Cypriniformes	
7	Atlantic salmon	*Salmo salar*	Sal s 1	Salmonidae	Salmoniformes	
8	Nile tilapia	*Oreochromis niloticus*		Cichlidae	Perciformes	
9	Barramundi/Asian seabass	*Lates calcarifer*	Lat c 1	Latidae		
10	Cane toad	*Rhinella marina*		Bufonidae	Anura	Amphibia
11	Snake	*Python spp.*		Pythonidae	Squamata	Reptilia
12	Turtle	*Myuchelys latisternum*		Chelidae	Testudines	
13	Saltwater crocodile	*Crocodylus porosus*	**Cro p 1/2**	Crocodylidae	Crocodylia	
14	Chicken	*Gallus gallus*	Gal d 8	Phasianidae	Galliformes	Aves
15	Kangaroo	*Macropus spp.*		Macropodidae	Diprotodontia	Mammalia
16	Cattle	*Bos taurus*		Bovidae	Artiodactyla	
17	Pig	*Sus scrofa*		Suidae		

Note: The colour-code is based on observed IgE-binding capacity and cross-reactivity predicted for bony fish-allergic patients. *The parvalbumin names correspond to entries in the WHO/IUIS database (www.allergen.org). The crocodile parvalbumin names (Cro p 1 and Cro p 2) were assigned based on this study. The taxonomic classification refers to the Catalogue of Life (www.catalogueoflife.org). Note: cart., cartilaginous

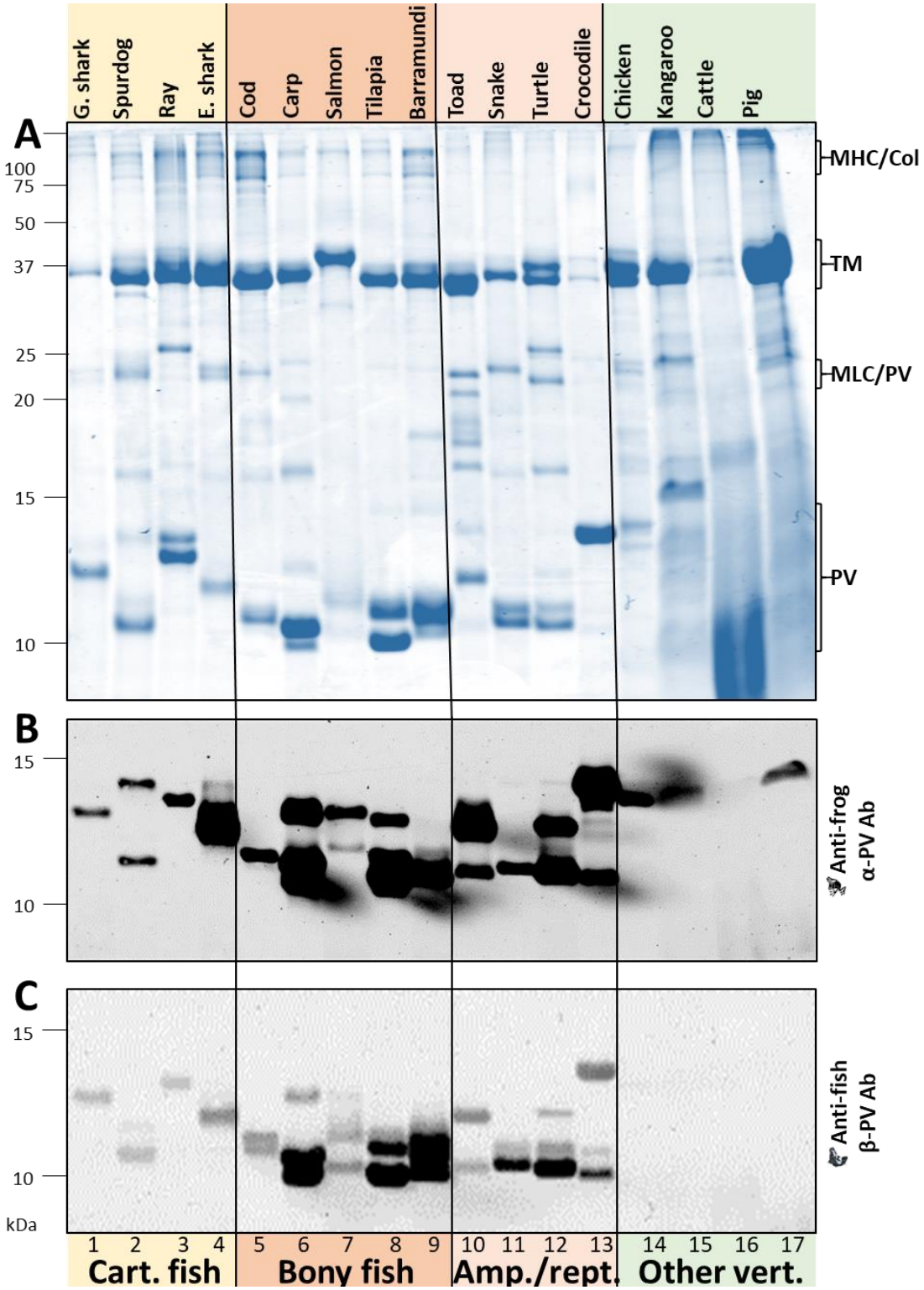


FIGURE 1 SDS-PAGE protein profiles of heated extracts from the muscle tissue of 17 vertebrates (**A**) and immunoblots using parvalbumin-specific antibodies (**B** and **C**). See Appendix C Figure 5 for the full image of the immunoblots. The identification of protein bands is based on mass spectrometric analyses. The numbers at the bottom refer to the species in Table 1. Note: G., Gummy; E., Elephant; MHC, myosin heavy chain; Col, collagen; TM, tropmyosin; MLC, myosin light chain; PV, parvalbumin; Cart., cartilaginous; Amp., amphibians; rept.; reptiles; vert., vertebrates

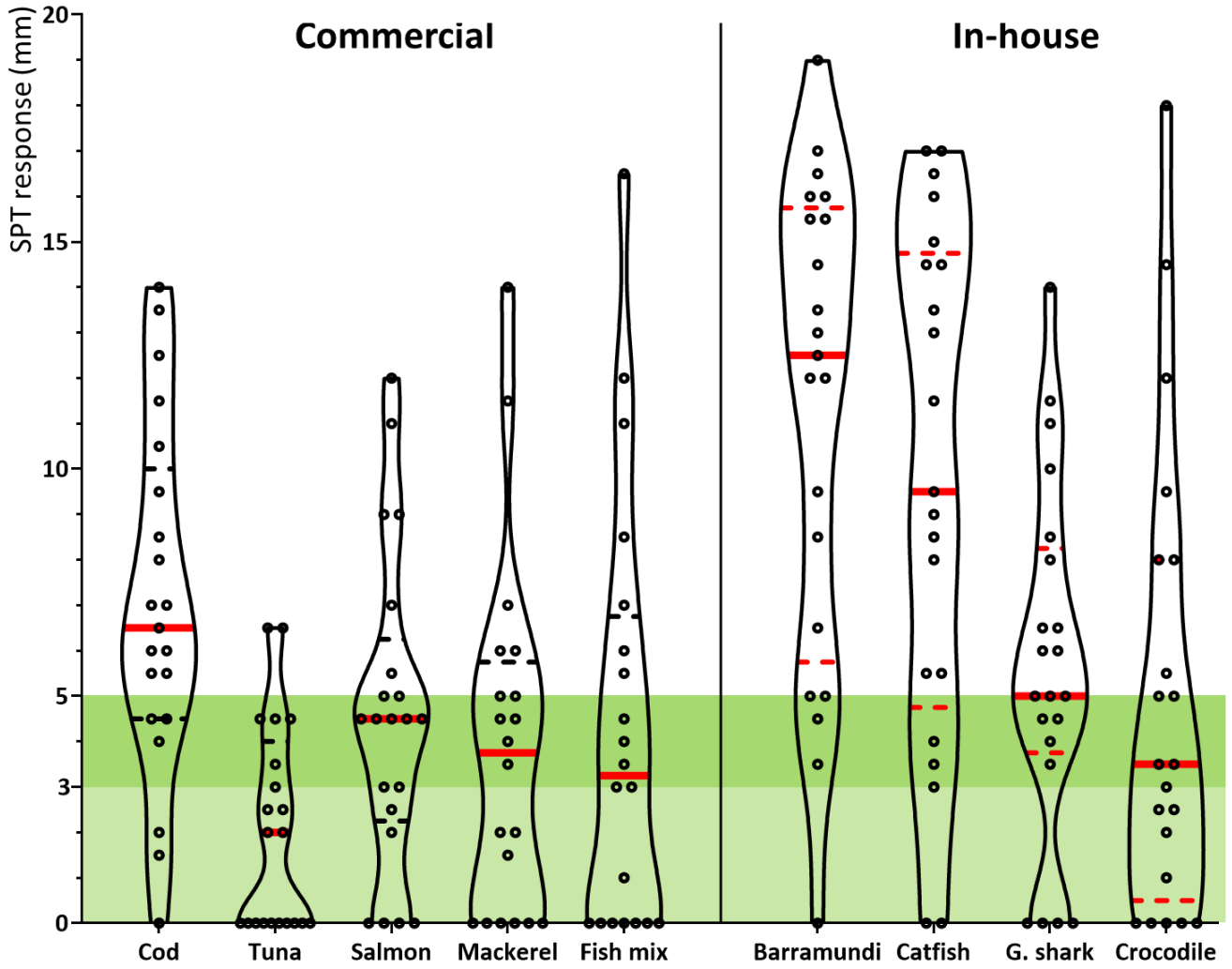


FIGURE 2 Skin Prick Test (SPT) response to five commercial and four in-house fish/crocodile preparations by 21 fish-allergic patients described in Appendix C Table 1. An SPT is considered positive if the wheal diameter is at least 3 mm (light green) or 5 mm (dark green) depending on different clinical practice. The median is indicated with a red line and the interquartile range with dotted red lines. Note: G., Gummy

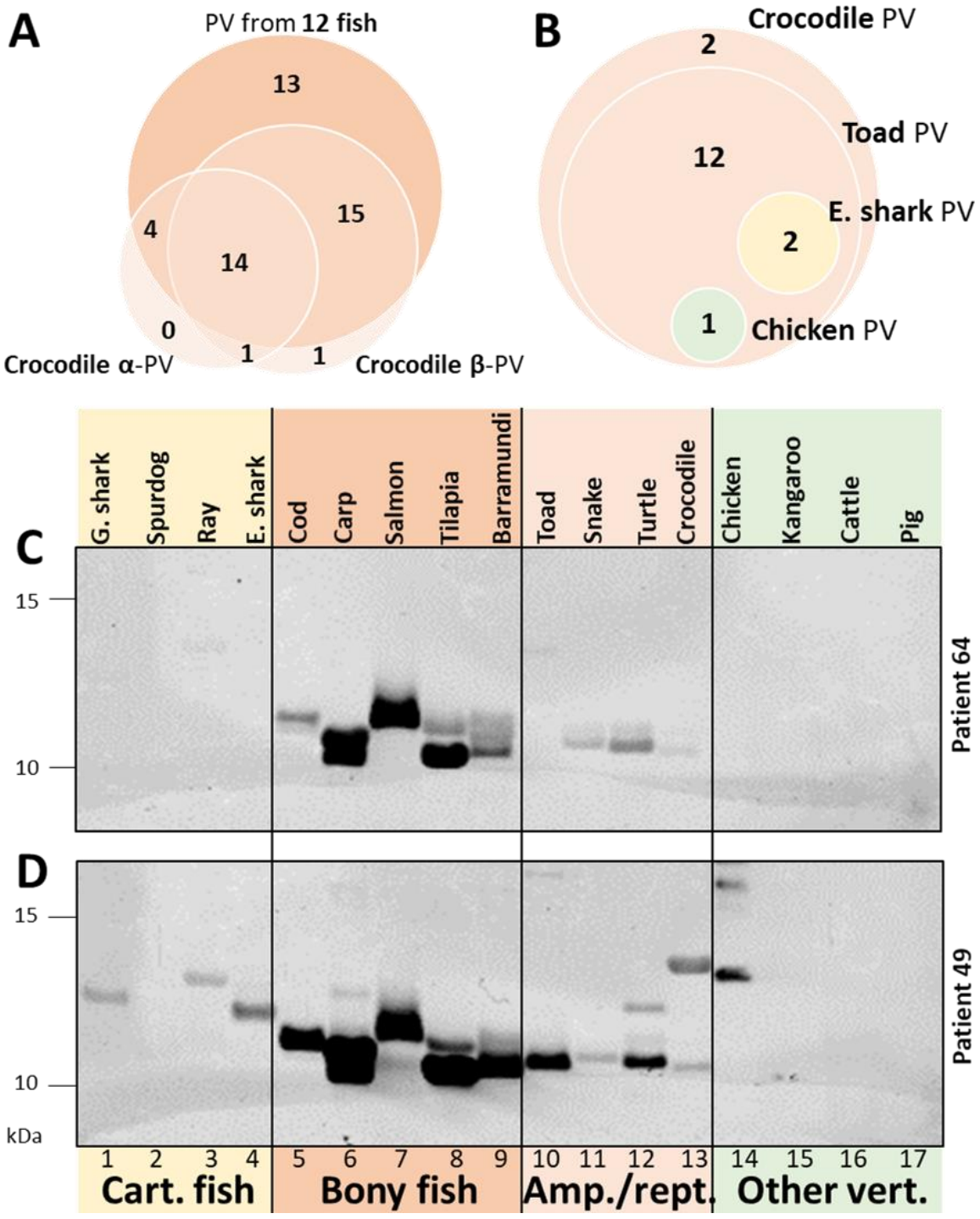
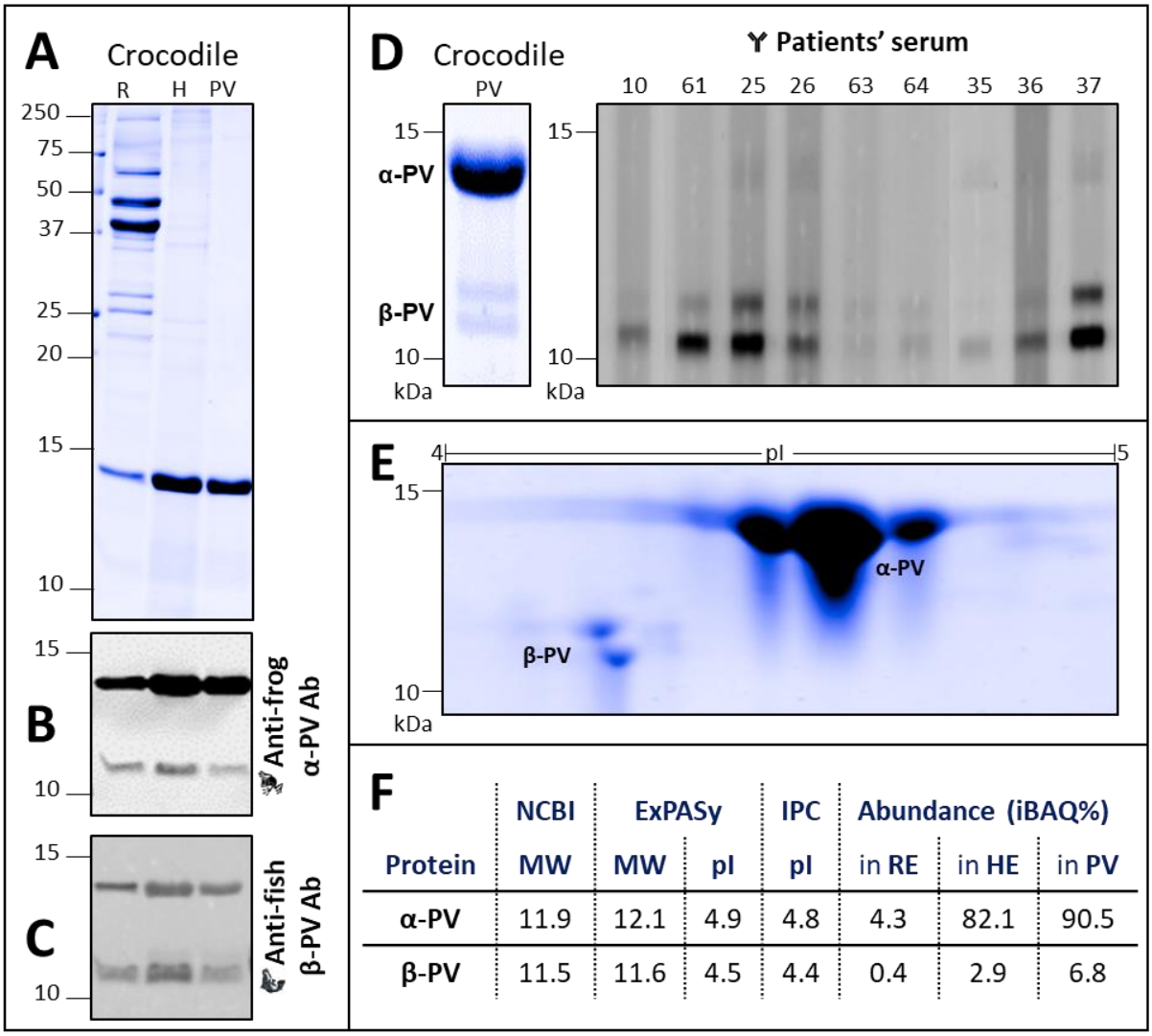


FIGURE 3 Frequency of IgE-binding to crocodile α- and β-parvalbumin (PV) by 46 fish-allergic patients with IgE-binding to at least one PV from 12 fish species (**A**). Frequency of IgE-binding to toad, shark and chicken (PV) by 17 patients with IgE-binding to at least one PV from crocodile (**B**). The corresponding IgE-immunoblots and densitometric analyses are shown in Appendix C Figure 4 and Appendix C Table 4, respectively. Representative IgE-immunoblots with heated extracts from 17 vertebrate species (Figure 2) using patients' serum (**C** and **D**). See Appendix C Figure 5 for the full image of the immunoblots. The patient's number refers to Appendix C Table 1. The numbers at the bottom refer to Table 1. Note: G., Gummy; E., Elephant; Cart., cartilaginous; Amp., amphibians; rept.; reptiles; vert., vertebrates



Protein	NCBI MW	ExPASy MW	ExPASy pI	IPC pI	Abundance (iBAQ%) in RE	Abundance (iBAQ%) in HE	Abundance (iBAQ%) in PV
α-PV	11.9	12.1	4.9	4.8	4.3	82.1	90.5
β-PV	11.5	11.6	4.5	4.4	0.4	2.9	6.8

FIGURE 4 SDS-PAGE profiles of crocodile raw (R, RE) and heated (H, HE) extracts and purified parvalbumin (PV) (**A**). Immunoblots were performed using PV-specific antibodies (**B** and **C**) and patients' serum (**D**). The numbers above the immunoblot image refer to patients in Appendix C Table 1. Purified PV was further separated by isoelectric focusing in 2-D gel electrophoresis (**E**). No bands/signals above 15 kDa or below 10 kDa were visible for the purified PV. PV isoforms were identified by mass spectrometric analyses (**F**). α- and β-PV refers to XP_019397705.1 and XP_019400389.1, respectively, accessible under www.ncbi.nlm.nih.gov/protein (NCBI). The molecular weight (MW) was calculated using the NCBI platform and ExPASy accessible under www.expasy.org/compute_pi. The isoelectric point (pI) was calculated using ExPASy and isoelectric point calculator (IPC) accessible under www.isoelectric.org/calculate.php. iBAQ% refers to the relative abundance of the protein, based on mass spectrometric analyses using MaxQuant

Supplementary tables and figures

- Patients characteristics (**Appendix C Table 1**)
- Surf blot images and corresponding densitometric analyses – crocodile heated extract (**Appendix C Figure 3** and **Appendix C Table 3**, respectively)
- Surf blot images and corresponding densitometric analyses – shark, toad and chicken heated extracts (**Appendix C Figure 4** and **Appendix C Table 4**, respectively)
- Further patient blots with heated extracts from 17 vertebrate species and full images of the immunoblots shown above (**Appendix C Figure 5**)

Further results and discussion not included in the manuscript in preparation

The IgE-binding capacity of proteins in heated extracts from 17 vertebrate species was evaluated in the above manuscript.

Corresponding raw extracts were generated in parallel and analysed. The total protein content was determined and proteins separated by SDS-PAGE (data not shown). However, the extracts were not further characterised because exposure to raw meat from vertebrates is rare in the investigated paediatric patient cohort. As highlighted in **Chapter V A**, there are likely many more IgE-binding proteins in raw as compared to heated extracts, while their clinical reactivity is often unknown.

As part of initial investigations, muscle tissue was collected from further vertebrate species, including those frequently consumed in indigenous communities in Australia (emu, lizard and multiple turtle species at different stages of life), and proteins extracted without and with heat treatment. **Figure VI B 1** below shows the SDS-PAGE and immunoblots with the heated extracts. Parvalbumin bands were detected as described in the above manuscript. Immunoblots using serum from a parvalbumin-sensitised fish-allergic patient (#25 in **Appendix C Table 1**) are shown in **Figure VI B 1 D**. No parvalbumin was detected in the extracts from emu and lizard, which were therefore excluded from further analyses. IgE-binding parvalbumin was detected in turtle hatchling and juvenile but not egg and embryo. Only the turtle juvenile extract was described in the manuscript as it is the closest to what humans may consume. It is to note that the chicken extract characterised in the manuscript was generated from the drumstick dark muscle tissue. Initially, the chicken extract was made from breast white muscle tissue, which did not contain a considerable parvalbumin band (see **Figure VI B 1**). This has been reported previously for chicken and the tissue specificity of parvalbumin content is well known for fish.[1–5] The tissue-specific variance in parvalbumin

content is likely the reason for the absence of a parvalbumin band in the beef/cattle, emu and lizard extract. The exact origin of the muscle tissue from these species is unknown.

Prior to having access to muscle tissue from a range of non-fish vertebrates, investigations were limited to nine fish species and crocodile. Purified parvalbumins, generated as described in **Chapter IV**, were compared for their binding by commercial PARV-19 and in-house anti-β-parvalbumin antibody as well as patients' serum (see poster in **Appendix B 4.7**). The purified parvalbumins should be further compared using (inhibition) ELISA and the clinical reactivity should be investigated as described for collagen in **Chapter V B** and parvalbumin in **Chapter VI A**.

An important finding is that crocodile α-parvalbumin is much more abundant (10-20 times) than β-parvalbumin while its IgE-binding capacity is almost neglectable. To date, crocodile is the second species, after edible frog (*Rana*/*Pelophylax* e*sculentus*), for which both α- and β-parvalbumin are registered with the WHO/IUIS. However, IgE-binding to frog α- and β-parvalbumin was only shown for three and eleven patients, respectively.[6,7] In the above manuscript, IgE-binding to the corresponding crocodile parvalbumins was demonstrated for 19 and 31 fish-allergic patients, respectively. For further investigations, we sought to resolve the crystal structure. The crystals, generated from the provided purified crocodile parvalbumin, did not diffract and therefore both α- and β-parvalbumin are currently getting expressed recombinant by collaborators in the US. Purified crocodile α- and β-parvalbumin in-solution will also enable the investigation of cellular reactivity in detail.

Overall, the risk of an allergic reaction for bony fish-allergic individuals upon the consumption of amphibian and reptile meat was demonstrated. In comparison, the risk is lower for cartilaginous fish and almost neglectable for birds and other vertebrates.

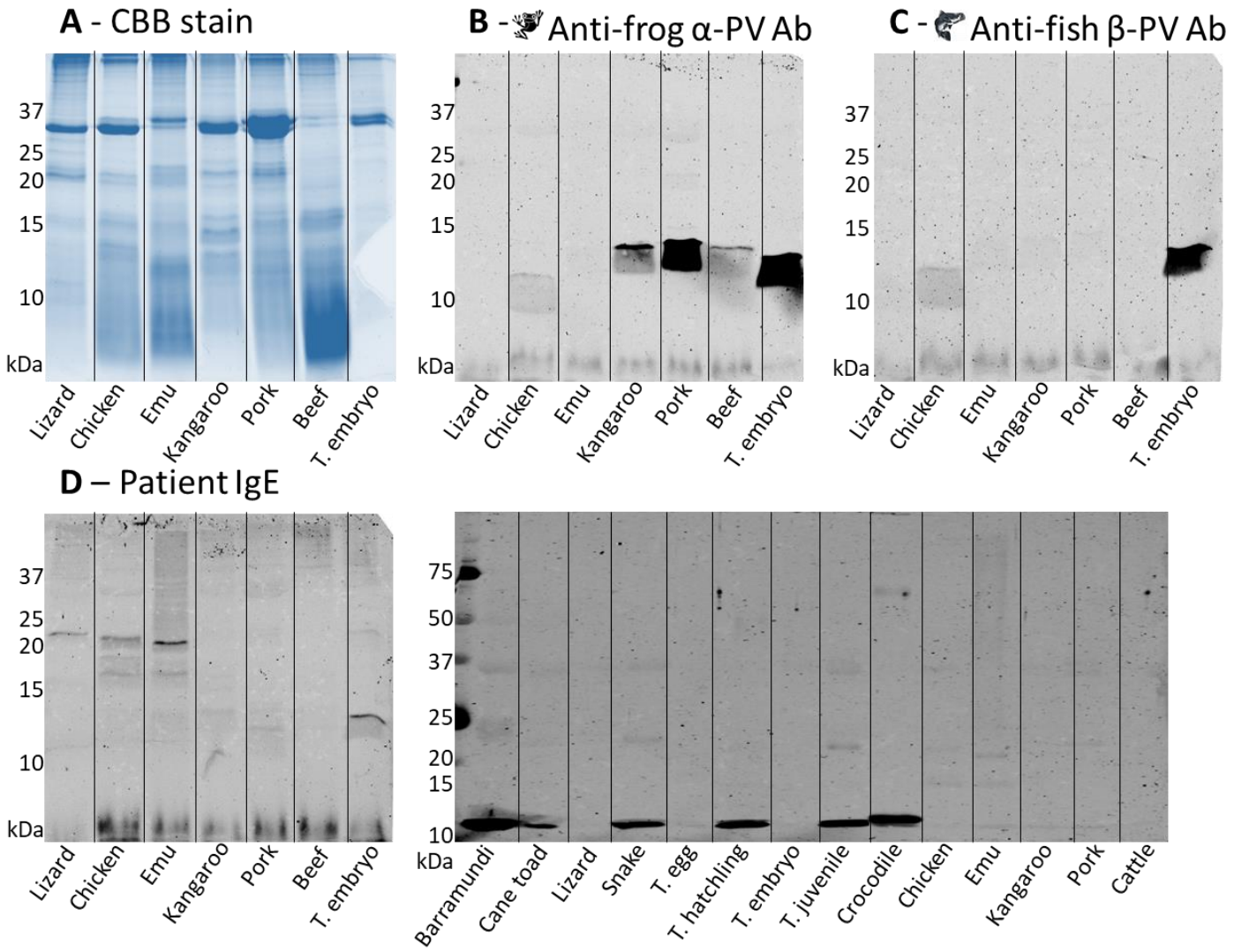


Figure VI B 1: SDS-PAGE with heated extracts from the muscle tissue of multiple vertebrate species (**A**) and immunoblotting utilising the commercial PARV-19 antibody (**B**) and the in-house anti-barramundi β-parvalbumin antibody (**C**) as well as serum from one parvalbumin-sensitised fish-allergic patient (#25 in Appendix C Table 1) (**D**). Note: CBB, Coomassie brilliant blue; Ab, antibody; T, Turtle.

References for further results and discussion

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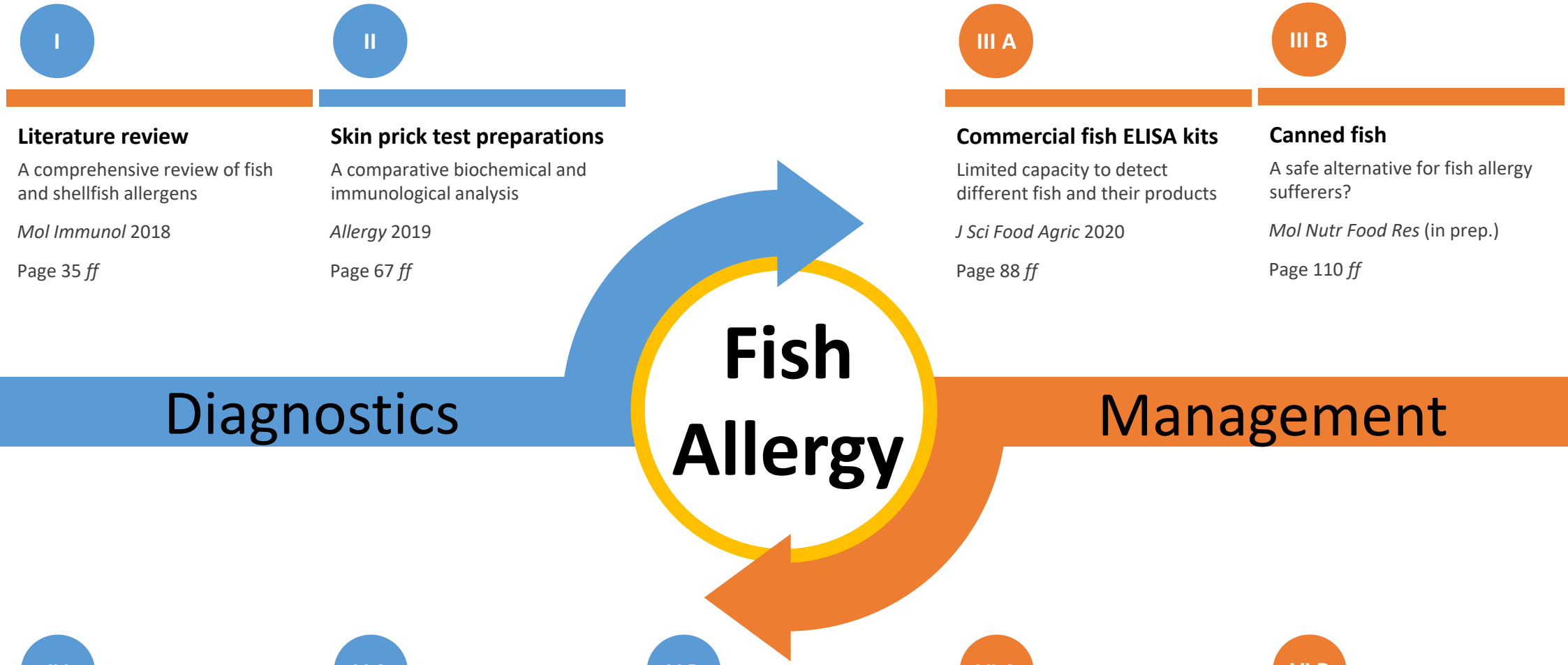
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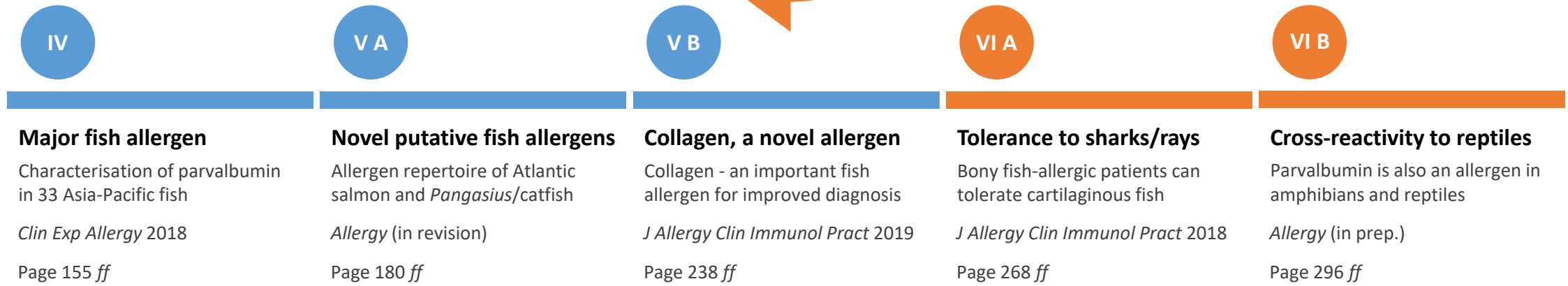
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General discussion and future directions: **Tackling fish allergy**

1. Problem statement

Fish is nutritious and a key factor for sustainable food security. However, fish is also a major trigger of frequent life-threatening allergic reactions. The diagnosis and management of fish allergy are limited and can be unreliable, which is hampering the utility of fish as a food source and puts lives at risk.

2. Thesis outcomes and implications for fish allergy diagnostics and management

This thesis reviews the literature on fish allergy, evaluates current diagnostic and management tools, and provides the foundation for their improvement.

In **Chapter I** and the **General introduction**, the importance of fish as food and allergen source was highlighted.[1] Characteristics of IgE-mediated fish allergy were elaborated and compared to other adverse reactions and allergies to other food groups including shellfish. Shellfish, i.e., crustacean and molluscs, are often combined with fish as seafood. The current knowledge about seafood allergens was reviewed in detail. Knowledge gaps, which hamper the utility and improvement of diagnostics and management, were identified. Fish allergy is species- and patient-specific, which is not sufficiently represented in commonly used *in vitro* and *in vivo* tests. Over 1,000 fish species are consumed worldwide, but patients currently can get tested for their sensitisation to less than 30 species and only five of those are relevant in Australia. As a consequence, there is a high risk of false-negative test results, putting lives at risk, and the management of fish allergy is limited to strict avoidance of all fish and fish products, which relies on correct food labelling.

The overarching aims of the subsequent data **Chapters** were to evaluate current diagnostic and management tools (**II**, **III A** and **B**), expand the allergen repertoire of fish (**IV**, **V A** and **B**) and identify potential food alternatives to bony fish (**VI A** and **B**), whilst investigating the sensitisation pattern of a large cohort of fish-allergic patients (*n*=77).

In **Chapter II**, 28 commercial fish skin prick test (SPT) preparations were evaluated for their utility to determine sensitisation to fish *in vivo*.[2] SPT is the most commonly employed diagnostic test in Australia and many other countries worldwide. This study was initiated by the observation of inconsistencies in SPT results with clinical history and a lack of correlation with *in vitro* test results and outcomes from oral food challenges, the gold standard of diagnostics. A high variance in total protein and allergen content, as well as IgE-binding capacity, was discovered. Most preparations contained insufficient amounts of important allergens, posing the risk of false-negative test results. Consequently, commercial fish preparations are often not reliable and SPT has a limited utility as *in vivo* diagnostic tool. Preparations from cod (most often Atlantic cod, *Gadus morhua*) appeared most reliable in regards to their allergen content, but are of limited utility for Australia as this fish is not available in the Asia-Pacific region. However, a commercial cod preparation demonstrated the highest median SPT result for 77 fish-allergic patients in comparison to four other commercial preparations, indicating its possible utility for diagnosing fish allergy in Australia. Nevertheless, in-house preparations, generated from fish commonly consumed in Australia, appeared to be most useful for *in vivo* diagnostics by SPT as indicated in **Chapter V A**.

In **Chapter III A**, commercial enzyme-linked immunosorbent assay (ELISA) kits, widely employed for essential detection of fish in food products, were evaluated for their utility to detect a wide variety of fish species.[3] The commercial kits detected 26-61% of 57 bony fish species, 65-86% of 17 canned fish products, no cartilaginous fish species and no non-fish vertebrates or seafood invertebrates, which would represent non-specific binding. Consequently, there is a high rate of false-negative test results for numerous fish species, of which many are frequently used in the food industry and consumed in the Asia-Pacific region. The demonstrated limited utility of the three tested ELISA kits, on which regulatory bodies such as the National Measurement Institute (NMI) rely, implies that food products are often incorrectly labelled in term of fish content. This puts the life of fish-allergic consumers at risk as safe fish allergy management currently depends on the strict avoidance of all fish and fish products. Accordingly, there is an urgent need for the development of improved methods for the detection of fish, which is a highly complex task. The utility of antibodies directed to parvalbumin from fish other than cod for improved detection capacity was demonstrated in this chapter (Further results and discussion of **Chapter III A**).

In **Chapter III B**, above-mentioned 17 canned fish products were evaluated for their protein content and IgE-binding capacity. Canned fish is often considered and recommended by medical practitioners as a safe alternative for fish-allergic patients. However, this recommendation is not based on scientific evidence and the repertoire of IgE-binding proteins in canned fish was previously unknown. The IgE-binding capacity of canned fish products differed between fish family, species and manufacturer. Tropomyosin was the most abundant protein in the investigated products and remained IgE-binding capacity even after extensive processing. IgE-binding parvalbumin was detected in most products, but with a much lower

relative abundance as compared to conventional heated protein extracts from the muscle tissue (95°C for 20 min). Furthermore, species-specific stability of parvalbumin was suggested and aggregation of both parvalbumin and tropomyosin isoforms was observed. Overall, this study suggests that canned fish is not generally safe for fish-allergic individuals and tools should be developed to predict tolerance prior to introducing canned fish in the diet.

In **Chapter IV**, the major fish allergen parvalbumin was identified and characterised 33 fish species, which are harvested in the Asia-Pacific region and consumed worldwide.[4] The heat stability of parvalbumin was highlighted and multiple parvalbumin isoforms identified and quantified in the muscle tissue from each species. Parvalbumin from Indian mackerel (*Rastrelliger kanagurta*) was characterised in detail and registered with the WHO/IUIS as the 15th fish parvalbumin (Ras k 1) based on this study. It was highlighted that the presence and IgE-binding capacity of parvalbumin is species-specific and different in species from the Asia-Pacific region compared to Europe and North America, following the large diversity of this major fish allergen. Bombay duck (*Harpadon nehereus*) was the only fish of which parvalbumin did not show IgE-binding. This fish species is therefore a potentially safe alternative for fish-allergic patients and should be further investigated. A parvalbumin purification protocol was established and parvalbumin subsequently purified from the muscle tissue of about twenty fish species and crocodile (Further results and discussion of **Chapter IV**). Purified parvalbumins were utilised in all other chapters of this thesis and provide the foundation for downstream analyses.

In **Chapter V A**, the protein profile and abundance of four different allergens in the muscle tissue of 66 fish species was determined. Each fish exposed a unique allergen repertoire and some fish exhibited a low content of specific allergens, which may result in their possible tolerance for selected fish-allergic patients. In addition, some species could be grouped based on the discovered allergen profile, allowing streamlined development of diagnostic tools. The detailed repertoire of IgE-binding proteins was investigated in twelve most frequently consumed fish. The focus was on two of the worldwide most farmed fish, Atlantic salmon (*Salmo salar*) and *Pangasius*/catfish (*Pangasianodon hypophthalmus*) for which three and twelve proteins, respectively, were registered as new allergens with the WHO/IUIS based on this study. Six of these fish proteins were previously unidentified as fish allergens and their discovery doubles the number of WHO/IUIS-recognised fish allergens. The WHO/IUIS listed twelve fish proteins as allergens in July 2020 (www.allergen.org). IgE-binding to fish parvalbumin was less frequent than suggested previously (22-46% versus 70-95%).[5] In comparison, IgE-binding to tropomyosin was also very frequent (12-32%) and demonstrated for the first time in a large patient cohort (*n*=77). Overall, this study suggests the implementation of a variety of both parvalbumin and tropomyosin isoforms in component-resolved diagnostics (CRDs).

In **Chapter V B**, acid-soluble fish collagen was discovered as a fish muscle and skin allergen of under-recognised importance.[6] Fish collagen is frequently used in food, cosmetics and medicine. In addition to IgE-binding capacity, IgE crosslinking and basophil activation was demonstrated for collagen. Collagen is under-represented in protein extracts investigated in all other Chapters and preparations utilised for *in vitro* and *in vivo* diagnostic tests (e.g. SPT), which therefore may show false-negative results. In this study, 21% of 101 fish-allergic

patients demonstrated IgE-binding to collagen, highlighting its relevance for fish allergy diagnostics. Collagen α-1 was identified as the most suitable candidate for the development of CRDs. Importantly, 38% of the twenty-one collagen-sensitised patients did not demonstrate IgE-binding to parvalbumin from the corresponding fish species. Collagen was registered with the WHO/IUIS as an allergen (as Lat c 6 for barramundi and Sal s 6 for salmon) for the first time based on this study.

In **Chapter VI A**, cartilaginous fish was identified as a safe alternative for some fish-allergic patients based on low IgE-binding capacity and low cellular reactivity of α-parvalbumin.[7] The α-lineage of parvalbumin is predominant in cartilaginous fish, while it is the β-lineage for bony fish.[8](**Appendix D 3**) Oral food challenges confirmed the predicted tolerance to thornback ray for fish-allergic patients. Subsequently, parvalbumin from this ray was implemented in the novel ALEX^{2}® *in vitro* diagnostic platform (www.macroarraydx.com/alex). However, this ray species is not available in Australia and the utility of this diagnostic platform is currently unknown for Australian patients. A clinical trial with fish-allergic patients is therefore commencing soon (at the Epworth hospital (Melbourne, Australia) in collaboration with Dr Sam Mehr) to investigate the safety of consuming meat from selected sustainably-sourced cartilaginous fish, which are often sold as 'flake' and commonly used in popular fish and chips. The aim is to develop a diagnostic test to predict tolerance to those selected cartilaginous fish species, including gummy shark, elephant fish/shark, sawsharks and Eastern shovelnose ray.

In **Chapter VI B**, alternative food sources for fish-allergic patients were evaluated for their safety, including crocodile and other non-fish vertebrates. Based on this study, parvalbumin is suggested as a pan-allergen among vertebrates, excluding mammals, for fish-allergic patients. Every second fish-allergic patient demonstrated IgE-binding to crocodile parvalbumin (*n*=77) and over half of the tested patients (*n*=21) had a positive SPT to crocodile, demonstrating cellular reactivity. Fish-allergic patients might therefore be at risk of severe allergic reactions upon consumption of reptile/amphibian meat (termed fish-reptile syndrome in this study). In contrast, meat from cartilaginous fish and birds, including chicken, appeared to be safe for most fish-allergic patients. No IgE-binding to proteins from mammal meat was observed. Consequently, parvalbumin-sensitised individuals should be advised to refrain from consuming reptile/amphibian meat unless tolerance is confirmed by an oral food challenge. This recommendation was recently implemented for crocodile in the guidelines of the not-for-profit organisation 'Allergy & Anaphylaxis Australia Allergy' (www.allergyfacts.org.au, see **Appendix B 5**). Crocodile β- and α-parvalbumin were registered with the WHO/IUIS as the first reptile allergens (as Cro p 1 and Cro p 2, respectively), based on this study. A large difference in abundance and IgE-binding capacity of these two parvalbumin isoforms was discovered. Further comparative *in vitro* and *in silico* analyses are currently in progress.

The fish-reptile syndrome may also apply to food allergies in companion animals. Food allergies in veterinary patients can be similar to those in humans.[9] For dogs, crocodile meat is often considered hypoallergenic. However, for dogs a fish-chicken syndrome was recently suggested and based on this thesis it appears likely that, in comparison, a fish-reptiles syndrome is prevailing in dogs.[10]

Overall, the global health burden of fish allergies was tackled from the molecular to the global level. It was demonstrated that the diagnosis and management of fish allergy (in Australia) are hampered by limited knowledge about species-, preparation- and patient-specific allergenicity. Based on this thesis, it appears that individuals with a clinically confirmed allergy to one bony fish have a ~50% risk to also react to other bony fish and reptiles/amphibians, but only a ~20% and ~10% risk to react to cartilaginous fish and birds, respectively, upon consumption of their meat (see **Figure X** below). This predicted clinical cross-reactivity requires further investigations and seems to be linked to the evolution of vertebrate species. Furthermore, a limited utility of commercially available diagnostic and detection tests was discovered. Advanced *in vitro* diagnostic tests are urgently needed to provide safe and reliable CRDs. Better management of fish allergy can be enhanced by species-specific recommendations and improved detection methods to ensure a nutritious and safe diet for fish allergy sufferers. This thesis provides a foundation for the development of improved diagnostics and management of fish allergy worldwide.

Clinical cross-reactivity

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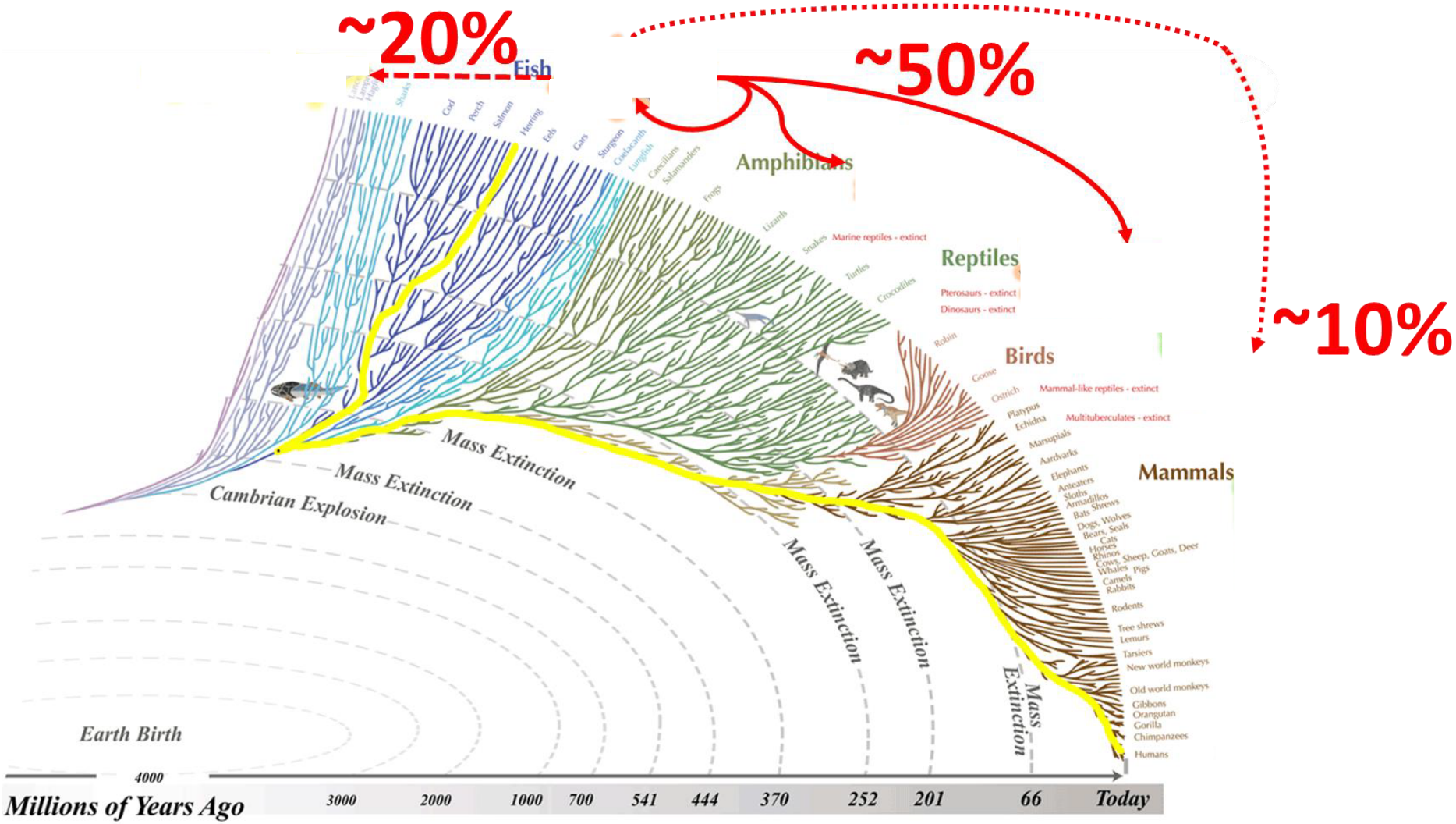

Figure X: Predicted clinical cross-reactivity among vertebrates for bony fish-allergic patients based on this thesis. The taxonomic tree is modified after [11].

3. Future directions

This thesis provides a knowledgebase for the development of urgently needed improved diagnostics and management of fish allergy in Australia, the Asia-Pacific and the world. The investigated patient cohort will be further monitored to provide an insight into the persistence of fish allergy, which is thought to be 90% while it has not been investigated in detail.[12–14]

3.1 Discovery of fish allergens and determining their abundance

Streamlined approaches for the discovery of allergens in fish were presented. Increasingly available genomic and transcriptomic data will enhance the identification and characterisation of the detailed allergen repertoire in multiple fish species as recently also suggested for shellfish and food allergy research in general.[15–17](see **Appendix D 4** for 17) In July 2020, the genome of 317 bony and ten cartilaginous fish species were publicly available, of which most (55%) were published in 2018 and 2019 (www.ncbi.nlm.nih.gov/genome). Identifying allergens should focus on fish species deriving from sustainable aquaculture to support the protection of dwindling wild-caught species.

The abundance of allergens depends on the fish species as well as the location of muscle tissue as comprehensively investigated for parvalbumin.[18–25] In addition, differences between specimens of the same age/size and species were hypothesised. Two studies, both investigating salmonids, did not discover any difference between anadromous (migrating) and landlocked or growth hormone-transgenic and non-transgenic specimens.[26,27] In contrast, recently generated preliminary data from comparative analyses of the protein composition in muscle tissue from 28 wild and farmed barramundi growing in fresh-, brackish- or saltwater, suggest significant differences in the abundance of IgE-binding parvalbumin and

tropomyosin (data not shown). Furthermore, considerable differences in the abundance of the putative allergens actin, myosin light chain and myosin heavy chain were observed.

3.2 Evaluation of cross-binding and clinical relevance of fish allergens

In this thesis, the detailed *in vitro* and *in vivo* sensitisation was analysed for 77 fish-allergic paediatric patients. However, the further correlation of clinical data with the observed sensitisation patterns requires comprehensive statistical analyses and may reveal suitable molecular markers needed for CRDs. Nevertheless, most investigations were limited to evaluating IgE-binding capacity. Future studies will need to clarify the clinical relevance of newly discovered fish allergens. The required purification of crocodile parvalbumin, catfish tropomyosin and cod collagen α-1 is part of this thesis and their evaluation for cellular reactivity will be an important contribution to further publications in this field.

Comprehensive *in silico* analyses of parvalbumins would allow predicting IgE epitopes and cross-reactivities as recently demonstrated for invertebrates.[28](**Appendix D 5**) A verification of predictions is possible through already generated *in vitro* data. However, potential IgE epitopes should subsequently be further discovered by *in vitro* epitope mapping as recently performed for barramundi parvalbumin (see **Appendix D 6**).

3.3 Development of improved diagnostics

In vivo SPT diagnostics could be improved through region-specific standardised fish preparations. In addition, preparations from canned fish and muscle tissue of non-fish vertebrates, including crocodile, should be considered. However, the diagnostic utility of SPT is also limited by the number of preparations which can be skin-tested simultaneously, not allowing to determine sensitisation to a comprehensive panel of fish species as required.

The development of reliable *in vitro* species-specific IgE CRDs is therefore key to ensure safe management of fish allergy. CRDs should implement heat-stable selected parvalbumin, tropomyosin and collagen isoforms. For collagen, it might be sufficient to implement cod collagen α-1. Tropomyosin appears to be higher conserved among bony fish as compared to parvalbumin and it therefore appears adequate to focus only on few tropomyosin isoforms (**Appendix C Figure 6**). For parvalbumin, IgE-binding to synthetic peptides, designed by detailed *in silico* and *in vitro* analyses, may allow predicting patient-specific reactivity or tolerance for a wide variety of fish and other vertebrates. Prior to implementation of any heat-labile allergens, their clinical relevance should be further clarified.

Ultimately, candidates for CRD should be analysed in a comparative study evaluating their efficiency to predict clinical reactivity with an approach similar to what has been previously conducted for prawn/shrimp allergy.[29,30] It appears that allergens other than parvalbumin will have a higher utility for diagnosing patients who are allergic to a narrow range of fish species.[31] The above mentioned growing number of transcriptomic and genomic data (see 3.1) will additionally contribute to the development of personalised species-specific diagnostics and potentially immunotherapies.[32,33]

3.4 Development of tools and raising awareness for improved management

Improved tools for the detection of fish in food products could be based on traditional antibodies as demonstrated in this thesis or advanced antibodies such as human single chain antibodies.[34] Another approach could be the development of aptamers as recently shown for the detection of shellfish tropomyosin.[35]

Furthermore, it is important that all consumers are aware of food allergies. For fish, it is crucial to understand the limitations of current diagnostic and detection results while strictly

avoiding all fish and derived products unless a tolerance to some fish (products) was demonstrated. In future, affected consumers should have access to reliable species-specific diagnostics to ensure a healthy fish-rich diet.

3.5 Approaches for the development of immunotherapies

The treatment of allergies with a 'vaccine' or immunotherapy is the aim of ongoing research worldwide and roadmaps have been suggested for their development.[36–38] For fish allergy, most efforts are linked to a hypoallergenic variant of carp parvalbumin (mCyp c 1). However, prior to the development of immunotherapy, it is important to understand the sensitisation phase of fish allergy. Sensitisation to fish most commonly occurs via the gastrointestinal tract.[39] However, sensitisation via skin contact or inhalation of aerosolised allergens appears to contribute to a higher prevalence among fish-processing workers.[40–42] Aerolised parvalbumin was recently detected in 13-58% of workplaces on fish trawlers for the first time (see **Appendix D 7**). The internalisation of intact parvalbumin by bronchial epithelial cells and the role of low molecular weight components were demonstrated and may play a critical role in the sensitisation phase and subsequent immunotherapy of fish allergy.[43](**Appendix D 8**) The identification of low molecular weight components can also provide an important contribution to the development of fish allergy immunotherapies. Further research may also help to prevent the development of allergies among fish-processing workers through prophylactic measures.

3.6 Overarching future direction

Fish is a key factor for sustainable food security. The diagnostics and management of fish allergies urgently require improvement to enable a balanced diet for affected consumers. Future developments in CRDs and management tools can advance how we cope with the increasing burden of fish allergies and allow reliable and comprehensive diagnostics and management.

Keep eating fish; it's the best way to feed the world!

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Appendix

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GRANTS

2015-2019	**Full-time postgraduate scholarship** Centre for Food and Allergy Research (CFAR), a Centre of Research Excellence by the Australian National Health and Medical Research Council (NHMRC)	87,500 AUD
2019	**Congress travel grant** Asia Pacific Association of Pediatric Allergy, Respirology and Immunology (APAPARI)	~1,000 AUD
2019	**Allergy school travel grant** European Academy of Allergy and Clinical Immunology (EAACI)	~420 AUD
2019	**Travel grant** Robert Logan Memorial Bursary	2,750 AUD
2019	**Congress travel grant** European Academy of Allergy and Clinical Immunology (EAACI)	~2,200 AUD
2019	**Doctoral completion grant** James Cook University (JCU)	3,000 AUD
2018	**Publication grant** James Cook University (JCU)	3,000 AUD
2018	**Travel grant** Australian and New Zealand Society for Immunology (ASI)	3,000 AUD
2018	**Congress travel grant** Asia Pacific Association of Allergy, Asthma and Clinical Immunology (APAAACI)	~1,030 AUD
2018	**Travel grant** Centre for Food and Allergy Research (CFAR), a Centre of Research Excellence by the Australian National Health and Medical Research Council (NHMRC)	2,000 AUD
2017	**Travel grant** James Cook University (JCU)	3,000 AUD
2016	**Travel grant** Centre for Food and Allergy Research (CFAR), a Centre of Research Excellence by the Australian National Health and Medical Research Council (NHMRC)	1,000 AUD
2016	**Travel grant** James Cook University (JCU)	2,025 AUD

AWARDS and PRIZES

2019	**Best oral presentation in the session** Annual congress, European Academy of Allergy and Clinical Immunology (EAACI)	~340 AUD
2019	**National Finalist** **State Finalist** FameLab An international live science communication competition by the British Council	-
2018	**Winner 'My Research Rules' Oral Competition** **Best postgraduate student Poster** Science Research Festival, James Cook University (JCU)	2,500 AUD 300 AUD
2018	**Queensland's fastest in tip racking** Interpath Services	~750 AUD
2017	**Best oral presentation in the session** Annual congress, European Academy of Allergy and Clinical Immunology (EAACI)	~340 AUD
2017	**Winner 'So you think you can research' Oral Competition** Science Research Festival, James Cook University (JCU)	2,500 AUD
2017	**Winner 'My Research in 8 Minutes'** Scientific retreat, Australian Institute of Tropical Health and Medicine (AITHM)	50 AUD
2016	**Allergy award** (best presentation) Annual conference, Australasian Society of Clinical Immunology and Allergy (ASCIA)	1,000 AUD
2016	**People's Choice award** Reperio – Innovation Pitching Competition, Townsville	500 AUD
2016	**Runner up 'My Thesis in 3 Minutes'** Scientific retreat, Centre for Biodiscovery and Molecular Development of Therapeutics (CBMDT)	-
2016	**Poster and oral presentation awards** Student conference, James Cook Uinversity (JCU)	200 AUD

SCIENCE COMMUNICATION

Selected presentations

1) Invited speaker at
- **R-Biopharm AG** Darmstadt, Germany, Jan **2020**
- **University Medical Center** Amsterdam, Netherlands, Dec **2019**
- **Institute for Prevention and Occupational Medicine** Bochum, Germany, Dec **2019**
- **Charité – Berlin University of Medicine** Berlin, Germany, Jun **2019**
- **Starship Children's and General Hospital** Auckland, New Zealand, Mar **2019**
- **Khon Kaen University** Khon Kaen, Thailand, Nov **2018**
- **Siriraj Hospital, Mahidol University** Bangkok, Thailand, Nov **2018**

2) **FameLab**, a live science communication competition by the British Council Perth, Australia, May **2019**
Finalist at the state and national level ➢ Watch the corresponding clip Brisbane, Australia, Mar **2019**

3) **Science with Sushi** seminar in conjunction with CSIRO Townsville, Australia, May **2019**
Molecular evolution of food allergens – from sharks and barras to crocs and chicken

4) **Pint of Science** Australia, supported by CSIRO Townsville, Australia, May **2018**
Are you food allergy aware?

5) Reperio - **Innovation Pitching** competition Townsville, Australia, Sep **2016**
➢ **People's Choice Award**

6) James Cook University **Science Research Festival** Australia, Nov **2015**, Jul **2016**, Sep **2017**, and **2018**
in conjunction with the Townsville Health Research Showcase
Oral and poster presentations ➢ Awards for **best presentations** (see **Appendix 4.1**)

Further science communication includes internal and external articles as well as radio and TV interviews

KEY AFFILIATIONS/MEMBERSHIPS

Molecular Allergy Research Laboratory (MARL)

Centre for Food and Allergy Research (CFAR), a Centre of Research Excellence by the Australian National Health and Medical Research Council (NHMRC)

Australian Institute of Tropical Health and Medicine (AITHM), James Cook University (JCU)

Centre for Molecular Therapeutics (CMT), JCU

Centre for Tropical Bioinformatics and Molecular Biology (CTBMB), JCU

Centre for Sustainable Tropical Fisheries and Aquaculture (CSTFA), JCU

World Allergy Organization (WAO)

Australasian Society of Clinical Immunology and Allergy (ASCIA)

European Academy of Allergy and Clinical Immunology (EAACI)

American Academy of Allergy, Asthma and Immunology (AAAAI)

Asia Pacific Association of Allergy, Asthma and Clinical Immunology (APAAACI)

Asia Pacific Association of Pediatric Allergy, Respirology and Immunology (APAPARI)

Australian and New Zealand Society for Immunology (ASI)

Allergy & Anaphylaxis Australia (A&AA)

*in*VIVO Planetary Health Network (*in*VIVO)

CONFERENCE PRESENTATIONS

Selected conference presentations as lead author

1) Annual Meeting of the Philadelphia, USA, Sep **2019**
American Academy of Allergy Asthma and Immunology (AAAAI)
Poster presentation (see **Appendix 4.6**)

2) **Allergy School on Food Allergy**: Trends and Novelties by the Paris, France, Dec **2019**
European Academy of Allergy and Clinical Immunology (EAACI)
Oral presentation ➢ **Travel grant**

3) Annual Congress by the Bali, Indonesia, Sep **2019**
Asia Pacific Academy of Pediatric Allergy, Respirology & Immunology (APAPARI)
Oral presentation delivered by R Nugraha ➢ **Congress award**

4) Annual Congress by the Lisbon, Portugal, Jun **2019**
European Academy of Allergy and Clinical Immunology (EAACI)
Oral and poster presentation ➢ Award for **best presentation in the session** (see **Appendix 4.5**)

5) **Food Allergen Management Symposium** (FAMS) Melbourne, Australia, May **2019**
Oral presentation delivered by AL Lopata

6) **International Conference on Food Analysis** (ICFA) Melbourne, Australia, Nov **2018**
Oral and poster presentation delivered by M Koeberl (see **Appendix 4.3**)

7) Annual Congress by the Bangkok, Thailand, Oct **2018**
Asia Pacific Association of Allergy, Asthma and Clinical Immunology (APAAACI)
Oral presentation ➢ **APAAACI Junior Member award**

8) **Centre for Food and Allergy Research** (CFAR) Symposium Canberra, Australia, Sep **2018**
Annual Conference of the **Australasian Society of Clinical Immunology and Allergy** (ASCIA)
Invited speaker at symposium and allergy update dinner; Poster presentation at the conference

9) Annual Congress by the Munich, Germany, May **2018**
European Academy of Allergy and Clinical Immunology (EAACI)
Poster presentation (see **Appendix 4.7**)

10) Annual Congress by the Helsinki, Finland, Jun **2017**
European Academy of Allergy and Clinical Immunology (EAACI)
Oral presentation delivered by AL Lopata ➢ Award for **best presentation in the session**

11) **International Conference Sustainable Agriculture and Environment** (ICSAE) Saigon, Vietnam, Dec **2016**
Oral presentation

12) Annual Conference of the Gold Coast, Australia, Sep **2016**
Australasian Society of Clinical Immunology and Allergy (ASCIA)
Oral and poster presentation ➢ **Allergy Award** (see **Appendix 4.2**)

13) **International Congress of Immunology** (ICI) Melbourne, Australia, Aug **2016**
Oral presentation

CONFERENCE PROCEEDINGS

Selected proceedings in the past five years:

1) **T Ruethers**, SD Kamath, AC Taki, TTK Le, S Karnaneedi, R Nugraha, TT Cao, S Nie, NA Williamson, SS Mehr, DE Campbell, AL Lopata; **2020**, **AAAAI**; Tropomyosin is a novel major fish allergen of unrecognized importance; *The Journal of Allergy and Clinical Immunology*, 145 (2), AB226.
2) **T Ruethers**, AC Taki, R Nugraha, S Karnaneedi, TT Cao, D Dai, T Shanmuganathan, M Leeming, S Nie, NA Williamson, SS Mehr, DE Campbell, AL Lopata; **2019**, **EAACI**; IgE-reactive proteins defined by 88 fish-allergic children, predicting the allergenicity of 66 Asia-Pacific fish species; *Allergy* 74 (S106), 109.
3) SD Kamath, **T Ruethers**, R Nugraha, T Kalic, O Mendozaporras, N Wade, TTK Le, RE O'Hehir, DE Campbell, BE Bang, T Sigsgaard, JH Bønløkke, AL Lopata; **2019**; **EAACI**; Differential IgE sensitization to the prawn allergen hemocyanin among food-allergic patients and seafood processing workers; *Allergy* 74 (S106), 257.
4) **T Ruethers**, AC Taki, TT Cao, R Nugraha, SS Mehr, DE Campbell, AL Lopata; **2018**; **ASCIA**-P6; Fish allergy among 88 Australian children and the allergenicity of 66 fish species; *Internal Medicine Journal* 48 (S6), 6.
5) **T Ruethers**, R Nugraha, AC Taki, SS Mehr, DE Campbell, AL Lopata; **2018**; **EAACI**; Crocodile parvalbumin is a new allergen - The fish-crocodile syndrome; *Allergy* 73 (S105), 493.
6) AC Taki, **T Ruethers**, R Nugraha, NA Williamson, J Stephen, SS Mehr, PA Ramsland, DE Campbell, AL Lopata; **2018**; **EAACI**; The risk of shark meat consumption for fish allergy patients and the implication of parvalbumin cross-reactivity; *Allergy* 73 (S105), 253.
7) T Kalic, C Radauer, **T Ruethers**, AC Taki, I Swoboda, F Morel, C Hilger, K Hoffmann-Sommergruber, C Hafner, AL Lopata, M Morisset, A Kuehn, H Breiteneder; **2018**; **EAACI**; Individuals allergic to bony fish tolerate cartilaginous fish due to the low allergenicity of their parvalbumins; *Allergy* 73 (S105), 255-256.
8) **T Ruethers**, AC Taki, R Nugraha, SD Kamath, EB Johnston, NA Williamson, C Ang, RE O-Hehir, SS Mehr, DE Campbell, AL Lopata; **2017**; **EAACI**; Skin prick test preparations for seafood allergy: a molecular and immunological assessment; *Allergy* 72 (S103), 70-71.
9) R Nugraha, SD Kamath, **T Ruethers**, EB Johnston, M Koeberl, JM Rolland, RE O'Hehir, KR Zenger, AL Lopata; **2017**; **EAACI**; Identification of novel oyster allergens using a combined transcriptomic and proteomic approach for improved diagnosis of oyster allergy; *Allergy* 72 (S103), 12.
10) **T Ruethers,** AC Taki, SD Kamath, R Nugraha, EB Johnston, SS Mehr, DE Campbell, AL Lopata, *et al.*; **2016**; **ASCIA**-P20; Skin prick test preparations for seafood allergy – A molecular and immunological assessment; *Internal Medicine Journal* 46 (S4), 10.
11) AC Taki, **T Ruethers**, DE Campbell, AL Lopata; **2016**; **ASCIA**-P22; Is canned fish really safe? - Evaluation of current food recommendation using an allergenomic approach; *Internal Medicine Journal* 46 (S4), 11. (see **Appendix 4.4**)
12) SD Kamath, M Thomassen, **T Ruethers**, R Nugraha, L Aasmoe, B Bang, AL Lopata ; **2016**; **ASCIA**-P38; An allergenomic approach for identification of novel air-borne allergens affecting crab-processing workers due to inhalational exposure; *Internal Medicine Journal* 46 (S4), 16-17.
13) R Nugraha, SD Kamath, **T Ruethers**, EB Johnston, M Koeberl, JM Rolland, RE O'Hehir, KR Zenger, AL Lopata; **2016**; **ASCIA**-P16; Identification of novel oyster allergens using a combined transcriptomic and proteomic approach for improved component resolved diagnosis; *Internal Medicine Journal* 46 (S4), 9.
14) **T Ruethers**, SD Kamath, R Nugraha, JN Stephen, SS Mehr, DE Campbell, AL Lopata; **2016**; **ICI**; Molecular and immunological characterisation of different seafood preparations for skin testing; *European Journal of Immunology* 46 (S1), 599.
15) J Stephen, M Sharp, **T Ruethers**, S Buddhadasa, S Kumaran, J Roberts, DE Campbell, AL Lopata ; **2015**; **ASCIA**-P33; Fish Allergy: From Cods and Sharks to Dinofish; *Internal Medicine Journal* 45 (S4), 11.

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BMDT AITHM

Why tackle fish allergy in Australian kids?

Allergic reactions to fish are species-specific and depend on individual sensitisation patterns

JAMES COOK UNIVERSITY AUSTRALIA 2016

Thimo Ruethers[a,b,c], Aya C. Taki[a,b], Juan N. Stephen[a,b], Dianne E. Campbell[c,d], Andreas L. Lopata[a,b,c]

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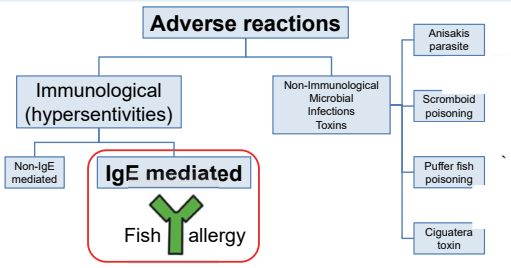
Background

- Eight food allergen groups account for 90% of allergic reactions to food; fish is one of the major groups[1].
- Food allergy is an adverse inappropriate immune response to food and often called as 'food hypersensitivity'[2].
- The increasing incidence of food allergy epidemic with 240 – 550 million people suffering worldwide, makes it a major and increasing health burden[3-5].
- Fish allergy is usually not outgrown, decreases the quality of life and has a high risk of severe reactions such as life-threatening anaphylaxis[6].
- There are several adverse reactions to fish other than actual fish allergy[7].



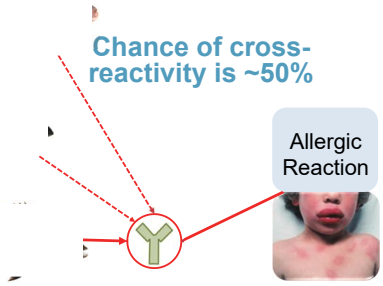
Fish allergens

- Fish allergy is commonly triggered by the recognition of allergens by specific immunoglobulin E (IgE) antibodies[10].
- *In vitro* diagnostics are based on the detection of such IgE antibodies in serum.
- >90% of allergic reactions to fish account to parvalbumin (PV), but there are minor allergens, too[11].

Fish allergen	Physiological function	Heat-stable	Molecular weight
Parv-albumin	Calcium-binding protein	Yes	10-13 kDa
Vitello-genin	Yolk protein	Yes	18 kDa
Tropo-myosin	Muscle protein	Yes	33 kDa
Aldolase	Enzyme in glycolysis	No	~40 kDa
β-enolase	Enzyme in glycolysis	No	~50 kDa
Collagen	Structural protein	Yes	>100 kDa

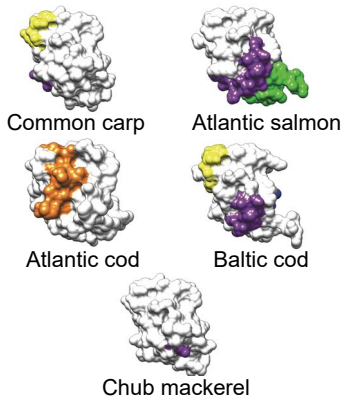
Cross-reactivity

- There are >32,000 fish species described worldwide and they are all very different from one and another[12].
- IgE antibodies are produced against allergens from one fish species upon initial exposure (sensitisation). These antibodies recognise only allergens from specific fish species upon subsequent exposure.
- For instance, there are patients that are only allergic to salmonides[13].
- Cross-reactivity to other species does occur and is depending on the specificity of IgE antibodies.



IgE-binding sites

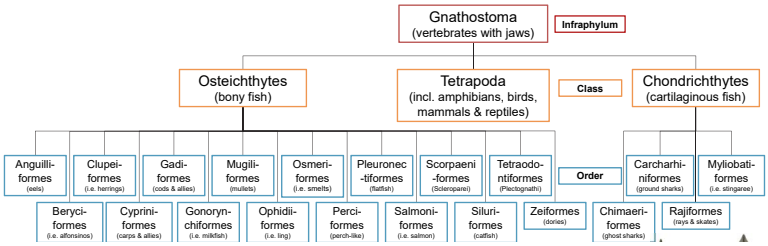
- IgE antibodies bind specific allergens such as PV in very specific sites.
- Linear IgE-binding sites of PV from five fish species have been elucidated, demonstrating very different regions and can explain some observed immunological cross-reactivities.



- Future research will need to address conformational epitopes.

Conclusions

- Fish allergy is species-specific and depends on individual sensitisation patterns.
- Effective management of fish allergy requires species-specific diagnostic methods.
- Current diagnostics in Australia are based on species consumed in the Northern hemisphere.
- Hence, there is a strong need for investigating allergens in fish consumed in Australia.
- A comprehensive study, identifying and characterising allergens from ~60 fish species - belonging to 20 orders - on a molecular and immunological level, is currently undergoing.



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Centre for Food & Allergy Research An NHMRC Centre of Research Excellence
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Skin prick test preparations for fish and shellfish allergy: A molecular and immunological assessment

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Background

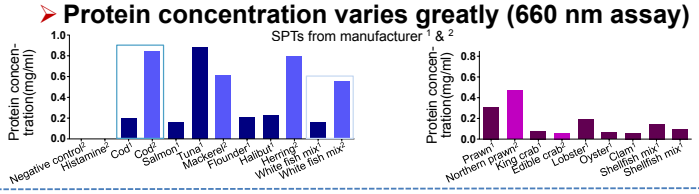
- Skin Prick Testing (SPT) is a common *in vivo* method to diagnose fish and shellfish allergy worldwide.
- The diagnostic utility of the SPT is limited by many factors.
- Suitable commercial SPT preparations are often not available due to regional variations in seafood species.
- Available SPT preparations may not be well standardised.
- **Hypothesis**: Protein & allergen composition vary greatly in fish & shellfish SPT preparations thereby impacting the diagnostic value.

Methods

- 19 fish and shellfish SPT preparations from two manufacturers (1 & 2) were analysed.
- This study focused particularly on the major allergen from fish and shellfish; parvalbumin (PV) and tropomyosin (TM) respectively.
- Protein concentration was measured. Protein composition was visualised (SDS-PAGE) and analysed with allergen specific antibodies and sera from patients with confirmed fish or shellfish allergy (immunoblotting & ELISA).
- Relevant proteins were identified by mass spectrometry (MS).

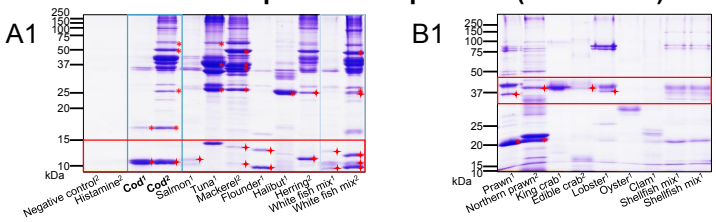
Results

Protein concentration varies greatly (660 nm assay)



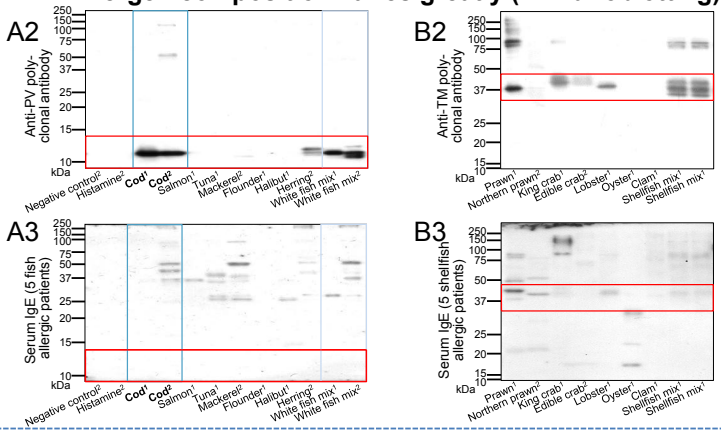
- Protein concentration of SPT preparations varied more than 10-fold - from <0.06 to 0.88 mg/ml.
- In general the protein concentration was higher in fish compared to shellfish preparations from both manufacturers.
- Protein concentration in **cod[1]** and **white fish mix[1]** was 4-fold lower than in **cod[2]** and **white fix mix[2]** respectively.

Differential protein composition (SDS-PAGE)



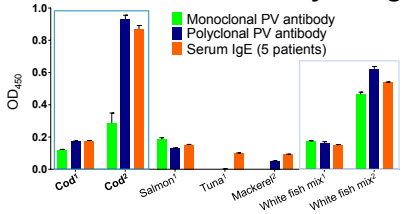
- Protein distribution of different preparations demonstrate great variation in number and intensity of bands. Differences are particularly prominent for bands suspected to be PV (A1) or TM (B1) according to molecular weight.
- Proteins highlighted with + are identified as PV **(A1)** or TM **(B1)** by MS. Proteins highlighted with * are reported allergens.
- In **cod[1]**, only parvalbumin and nucleoside disphosphate kinase were identified as known allergens, while in **cod[2]**, in addition α-enolase, pyruvate kinase and triosephosphate isomerase were identified.

Allergen composition varies greatly (immunoblotting)



- PV was identified using polyclonal antibody against barramundi PV in 5/10 fish SPT preparations **(A2)**.
- TM was detected using polyclonal antibody against black tiger prawn TM in 6/9 shellfish SPT preparations **(B2)**.
- IgE antibodies using serum pool of patients with confirmed fish allergy did not detect monomeric PV, but many other proteins which differed greatly in number and intensity of IgE-reactive bands **(A3)**. In **cod[1]** no IgE-reactive bands were found, while there were more than 3 different proteins in **cod[2]**.
- IgE antibodies using a serum pool of patients with confirmed shellfish allergy demonstrated moderate binding to shellfish preparations **(B3)**.

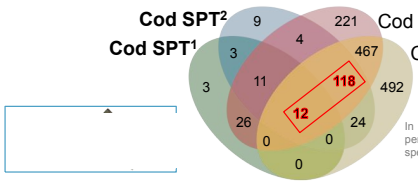
Differential antibody recognition to fish proteins (ELISA)



- Quantification of IgE reactivity to 7 different fish SPT preparations demonstrated great differences .
- Cod preparations from 2 manufactures demonstrated over 4-fold difference in patient IgE binding and PV specific antibody binding.

Great protein diversity between cod preparations

(profiling of protein composition by mass spectrometry)



In solution digestion with trypsin was performed and peptides identified by mass spectrometry followed by mascot search.

- Number of identified proteins varied greatly:
  Raw extract (1,113) > heated extract (859) > **SPT[2]** (181) > **SPT[1]** (55)
- 12 proteins were common in all four preparations.
- 118 proteins were identified in **cod SPT[2], heated and raw extract**, but not identified in **cod SPT[1]**.

Conclusions

- 19 SPT preparations for different fish and shellfish species were analysed and showed great variability in protein and allergen content.
- Different allergen content resulted in differential reactivity to allergen-specific antibodies and patient IgE antibody.
- SPT preparations for the same species vary greatly between manufacturers as demonstrated for cod and white fish.
- Some allergens were not represented in commercial SPT preparations.
- Variability limits the diagnostic utility of commercial SPTs.
- There is an urgent need for region-specific, standardised allergen preparations leading to component resolved diagnosis.

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AL & DC are holder of an NHMRC grant
TR is a scholar of CFAR

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Orange roughy | Sardine | Common carp | Atlantic cod | Hoki | Ling | Nile Tilapia | Yellowtail kingfish | Mahi mahi | Barramundi | Saddletail snapper | Red emperor | Blue salmon | King salmon | Cobia | Skipjack tuna

Spanish mackerel | Grey mackerel | Albacore tuna | Yellowfin tuna | Longtail tuna | Common coral trout | Frypan bream | Pink snapper | Swordfish | Rainbow trout | Tasmanian Atlantic salmon | Pangasius | John dory | Elephant shark | Bluespotted maskray | Gummy shark

Australian Government
Department of Industry and Science

National Measurement Institute

JAMES COOK UNIVERSITY AUSTRALIA

Detection of Asia-Pacific fish by commercial ELISA kits

Thimo Ruethers[1], Aya C. Taki[1], James Roberts[2], Saman Buddhadasa[2], Dean Clarke[2], Andreas L. Lopata[1], Martina Koeberl[2]

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Introduction

- Food allergy is an increasing health burden worldwide, with Australia having the highest prevalence (10%).
- Fish is among the eight food groups causing 90% of allergic reactions to food. Fish-allergic individuals often suffer a life-time from potentially life-threatening rapid allergic reactions. The management of fish allergy is limited to strict avoidance of all fish and fish products.
- Therefore, the detection and quantification of fish in food products is crucial for the health and safety of suffering individuals. To our knowledge, there are currently only three commercially available ELISA kits to detect and quantify fish allergens, manufactured by Romer Labs (Austria, UK) and Xema (Russia).
- However, they are primarily designed to detect fish from Europe/North America, which are significantly different to Asia-Pacific fish mainly consumed in Australia. We therefore aimed to evaluate those kits for their suitability to detect fish species relevant for the Australian market.

Methods

Fish preparation and extraction. Muscle tissue samples from 34 fish species, 6 canned fish products, chicken and prawn were collected (summarised in **Table 1**). **Figure 1** shows their taxonomic relationship. The sample tissues were boiled and proteins extracted in PBS (2 ml/g). After overnight incubation and centrifugation, the supernatant was used for the analyses.

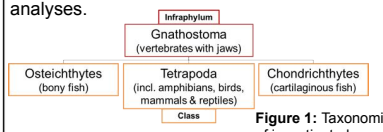

Figure 1: Taxonomic relationship of investigated species.

ELISA kits. AgraQuant Fish ELISA kit was provided by Romer Labs. Common Bone Fish Antigen EIA ELISA kit, version 803 as well as Common Bone Fish Antigen EIA ELISA kit, version 8032 was provided by Xema. The kits from Xema and Romer differ, including calibrants, extraction, antibodies and expressed units. The tests were preformed according to the manufactures protocol and instructions. The heat-treated cod sample was diluted post ELISA extraction to fall in the mid-high range of the standard curve. All other fish samples were diluted and tested accordingly, correlating the cod dilution. Canned fish extracts were tested undiluted. The obtained OD values for all samples were divided by the OD sample from standard 1 of specific ELISA kit.

Table 1: Details of investigated Asia-Parcific species/samples. Species with asterisks * are also common species in the Europe/North America.

No	Common name	Scientific name	Class	Order	Family
1	Black Tiger Prawn	*Penaeus monodon*	Malacostraca	Decapoda	Penaeidae
2	Chicken	*Gallus gallus*	Aves	Galliformes	Phasianidae
3	Elephant shark	*Callorhinchus milii*	Holocephali	Chimaeriformes	Callorhinchidae
4	Bluespotted maskray	*Neotrygon kuhlii*	Elasmobranchii	Myliobatiformes	Dasyatidae
5	Gummy shark	*Mustelus antarcticus*		Carcharhiniformes	Triakidae
6	Orange roughy*	*Hoplostethus atlanticus*	Actinopterygii	Beryciformes	Trachichthyidae
7	Sardine*	*Sardina pilchardus*		Clupeiformes	Clupeidae
8	Common carp*	*Cyprinus carpio*		Cypriniformes	Cyprinidae
9	Atlantic cod*	*Gadus morhua*		Gadiformes	Gadidae
10	Hoki	*Macruronus novaezelandiae*		Gadiformes	Merlucciidae
11	Ling	*Genypterus blacodes*		Ophidiiformes	Ophidiidae
12	Nile tilapia	*Oreochromis niloticus*		Perciformes	Cichlidae
13	Yellowtail kingfish	*Seriola lalandi*			Carangidae
14	Mahi mahi	*Coryphaena hippurus*			Coryphaenidae
15	Barramundi	*Lates calcarifer*			Latidae
16	Saddletail snapper	*Lutjanus malabaricus*			Lutjanidae
17	Red emperor	*Lutjanus sebae*			Lutjanidae
18	Blue salmon	*Eleutheronema tetradactylum*			Polynemidae
19	King salmon	*Polydactylus macrochir*			
20	Cobia	*Rachycentron canadum*			Rachycentridae
21	Spanish mackerel	*Scomberomorus commerson*			Scombridae
22	Grey mackerel	*Scomberomorus semifasciatus*			
23	Skipjack tuna	*Katsuwonus pelamis*			
24	Albacore tuna	*Thunnus alalunga*			
25	Yellowfin tuna	*Thunnus albacares*			
26	Longtail tuna	*Thunnus tonggol*			
27	Common coral trout	*Plectropomus leopardus*			Serranidae
28	Frypan bream	*Argyrops spinifer*			Sparidae
29	Pink snapper	*Pagrus auratus*			
30	Swordfish	*Xiphias gladius*			Xiphiidae
31	Rainbow trout*	*Oncorhynchus mykiss*		Salmoniformes	Salmonidae
32	Tasmanian Atlantic salmon*	*Salmo salar*			
33	Pangasius/basa	*Pangasius hypophthalmus*		Siluriformes	Pangasiidae
34	John dory	*Zeus faber*		Zeiformes	Zeidae
35	Canned Tuna 1 (Skipjack tuna)	*Katsuwonus pelamis*	Actinopterygii	Perciformes	Scombridae
36	Canned Tuna 2 (Skipjack tuna)				
37	Canned Tuna 3 (Yellowfin tuna)	*Thunnus albacares*			
38	Canned Sardine 1	Unknown		Clupeiformes	Clupeidae
39	Canned Salmon 1	Unknown		Salmoniformes	Salmonidae
40	Canned Salmon 2				

Results

Establishment of ELISA kits

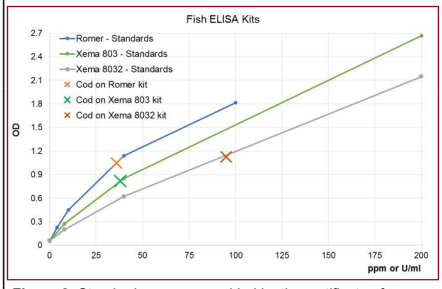

Figure 2: Standard curve as provided by the certificate of manufacturers and the obtained OD value of the diluted heat-treated cod sample.

Detection of fish samples

Figure 3 shows the results for all tested samples for each ELISA kit (Kit 1-3). Samples are grouped and coloured according to their taxonomy. The response of the diluted heat-treated cod is shown as purple lines, standards of the kit are shown as red lines. The Limit of Quantification (LOQ) correlates with Standard 1 of the ELISA kit, the Limit of Detection (LOD) was selected as half of standard 1 (**Figure 3**).

- All tested samples showed lower reactivity compared to the heat-treated cod, except 2 samples by kit 2.
- Kit 1 detected 15 out of 40 samples. Black Tiger Prawn was false positively detected.
- Kit 2 detected 17 out of 40 samples.
- Kit 3 detected 10 out of 40 samples.
- 5 fish species were detected by all 3 kits.
- All (2) undiluted canned salmon samples were detected by all 3 kits.
- 11 samples were detected by more than 1 kit.
- 16 fish species were not detected by any kit.
- Canned Sardine was only detected by kit 2.
- Canned Tuna 1 and 2, canned tuna 2 and 3 were detected by kit 1 and kit 3, respectively.

For this study, results between LOD and LOQ were considered inconclusive and not counted as detections.

Figure 2 shows the standard curve as provided by each manufacturer. The diluted heat-treated cod is also shown in **figure 2**, in the mid-high range of the relevant standard curve. The dilution factors for the ELISA kits vary.

The two Xema kits showed similar fish detection capability for the samples analysed (**Figure 2 and Figure 3**).

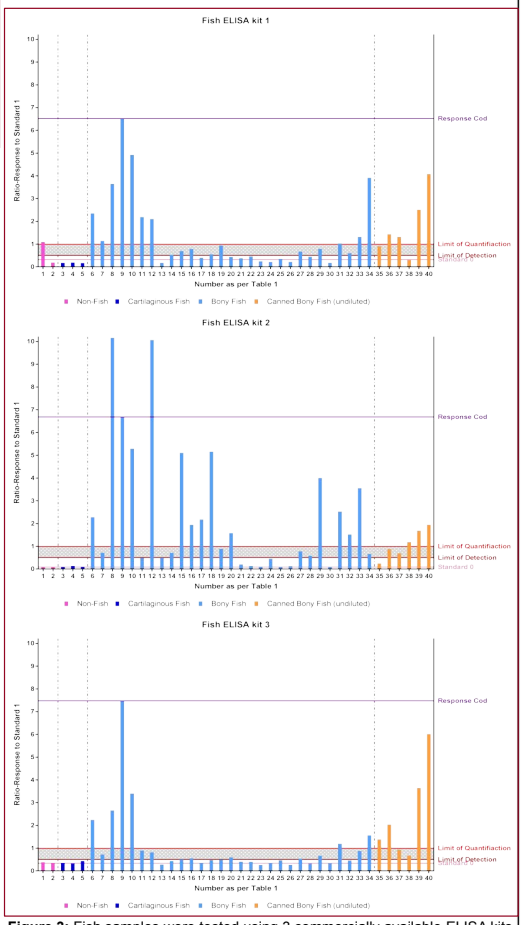

Figure 3: Fish samples were tested using 3 commercially available ELISA kits. The measured OD for each sample is shown as ratio to standard 1. Sample numbers refer to **table 1.**

Summary

- Out of 40 samples, only 7 **(5 fish species and 2 canned products) were detected by all three kits.**
- In contrast, **16 fish species were not detected** by any kits.
- 11 samples were detected by more than one kit.
- **1 false positive** was detected by one kit.
- In Australia most frequently consumed **fish species** (barramundi, pangasius/basa, salmon and tilapia) were detected only by kit 2.

Conclusion

- The suitability of commercial ELISA kits to detect Asia-Pacific fish consumed in Australia is limited.
- As the kits have different antibodies, calibrants, dilutions and units it is impossible to comprehensively compare them.
- There is a risk of false negative test results using these kits for detecting Asia-Pacific fish.
- Further evaluation using more species and preparations is needed.
- The relationship between heat-treated and raw fish needs to be investigated further.
- **Future direction:** Improved test kits are needed to allow **reliable detection of fish in Australia** and subsequently ensure safe management of fish allergy. A wider range of Asia-Pacific fish needs to be investigated.

Contact

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TR is a PhD full-time scholar of the Centre for Food and Allergy Research (CFAR, Australia). AL is holder of a National Health and Medical Research Council (NHMRC, Australia) grant.

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In relation to this presentation, the authors declare that there are no conflicts of interest

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IS CANNED FISH REALLY SAFE?- EVALUATION OF CURRENT FOOD RECOMMENDATION USING AN ALLERGENOMIC APPROACH

Aya C. Taki[1,2,3], Thimo Ruethers[1,2,3,4], Sam Mehr[4], Dianne E. Campbell[4,5], and Andreas L. Lopata[1,2,3,4]

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[4]Centre for Food and Allergy Research, Murdoch Children's Research Institute, Melbourne, Australia; [5]Department of Allergy and Immunology, Children's Hospital at Westmead, Sydney, Australia,

Background

FISH ALLERGY and CANNED FISH

- **IgE-mediated fish allergy** – risk of anaphylaxis and typically persists into adulthood
- The major fish allergen, **parvalbumin** (**PV**) - heat stable and can withstand the cooking process
- Some fish-allergic individuals are able to tolerate fish in canned form[1]
- However, the exact mechanisms for this remain unclear
- Decreased allergenicity may be due to the extreme thermal treatment during the canning process[2], resulting in conformational changes in proteins

CLINICAL IMPLICATION

- Recommendations to consume canned fish are frequently advised by physicians, so that patients with fish allergy can safely eat fish and maintain a healthier diet
- However no comprehensive molecular and immunological analysis of canned fish products has been performed to support such advice

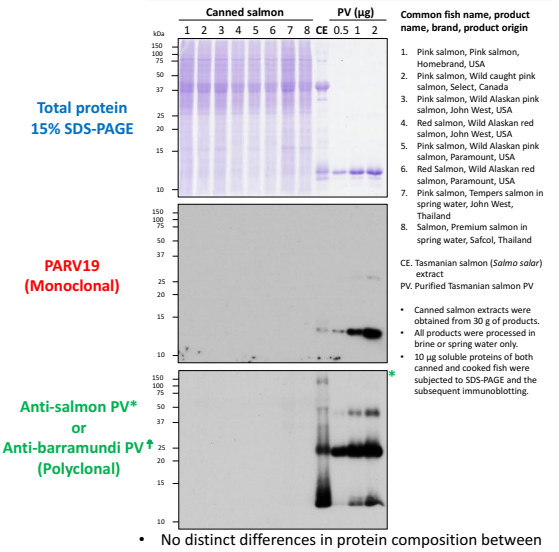
HYPOTHESIS

➢ **Canned fish contains IgE-reactive fish allergens**

Canning process reduces the concentration of major allergen Parvalbumin

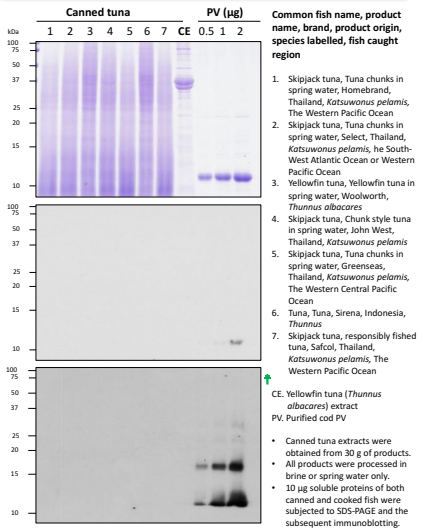
A panel of commonly consumed canned fish products in Australia was evaluated for its major allergen (PV) contents and its reactivity to PV-specific monoclonal and polyclonal antibodies.
Monoclonal anti-PV IgG: PARV19 (frog PV), Polyclonal anti-PV IgG: Anti-salmon PV, Anti-barramundi PV

Canned salmon



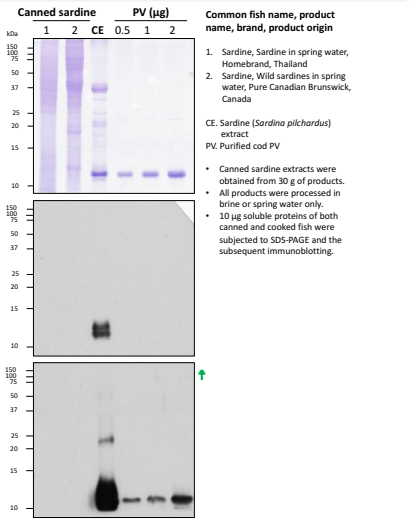
- No distinct differences in protein composition between products or species
- Protein composition similar to cooked extract (CE)
- No reactivity to neither mono- nor polyclonal antibodies

Canned tuna



- Protein composition varies slightly between species
- Yellowfin tuna has very different protein composition
- No reactivity to neither mono- nor polyclonal antibodies

Canned sardine



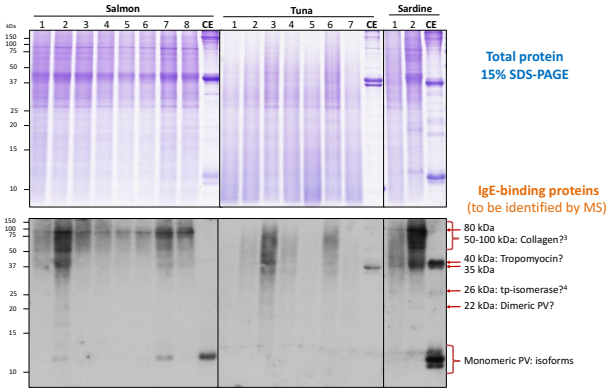
- Protein composition varies between products
- One product similar to cooked extract (CE)
- One product possibly contains one of the PV isoforms
- No reactivity to neither mono- nor polyclonal antibodies

Canned fish products contain IgE reactive allergens

Pooled serum from 5 pediatric patients with confirmed fish allergy detected IgE binding allergens

Patient number	Age (yo)	Gender	Skin Prick Test (mm)			Symptoms	Contact	Reactive history to fish	Canned fish tolerance
			Cod	Tuna	Salmon				
C1	10	M	6	5	5	OAS, GI	Oral	White fish	Salmon
C4	13	M	3	8	3	AN	Oral	Basa	
C"2	7	M	8	5	10	AN, OAS	Oral	Sardine, tuna, salmon	Tuna, salmon
C"8	7	M	5	2	8	AN, U	Oral	Bream, cod	Tuna
C"9	7	F	0	0	0	U	Oral	Australian cod	

AN, anaphylaxis; AE, angioedema; GIS, gastrointestinal syndrome; OAS, oral allergy syndrome; U, Urticaria



IgE binding proteins in all canned products

- **Canned salmon** – species, product origin, brand independent on IgE reactivity, all products reactive
- **Canned tuna** – seems species-specific (Yellowfin tuna)
- **Canned sardine** – one product more reactive, possibly proteins less denatured or due to different species

- **PV**: Reactivity to cooked salmon PV much higher than canned salmon PV

Conclusions

➢ Canned fish **seems to lose PV** compared to normally cooked fish
➢ However, PV allergen **maintains reactivity to IgE**
→ **Additional allergens seem to be present in canned fish**
➢ Tolerance may be based upon the reduced total amount of PV[5] within the canned food
➢ Extreme thermal processing may also reduce IgE reactivity to PV[6]

➢ **Canned fish may not be safe to all fish-allergic patients**

➢ Laboratory analysis and in-hospital observed incremental food challenge to canned fish should be considered before incorporation of canned fish into the diet of individuals with known fish allergy[1]

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TR is a scholar of CFAR.
Ethics approval: LNR-14/SCHN/185

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IgE-reactive proteins defined by 88 fish-allergic children, predicting the allergenicity of 66 Asia-Pacific fish species

Thimo Ruethers[1,2,3], Aya C. Taki[1,2,3], Roni Nugraha[1,3,4], Shaymaviswanathan Karnaneedi[1,2,3], Trúc T. Cao[1], Danyi Dai[5], Thayalini Shanmuganathan[5], Michael Leeming[6], Shuai Nie[6], Nicholas A. Williamson[6], Sam S. Mehr[2,5,7], Dianne E. Campbell[2,5,8], Andreas L. Lopata[1,2,3]

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Background → there are many under-investigated fish species

- Fish allergy is often a life-long disease with high frequencies of anaphylaxis.
- Diagnostics and management are hampered by a large number of under-investigated fish species.
- **Aim**: Quantification of 4 recognised allergens in 66 Asia-Pacific fish species and identification of all IgE-reactive proteins defined by 88 children with clinically confirmed fish allergy.

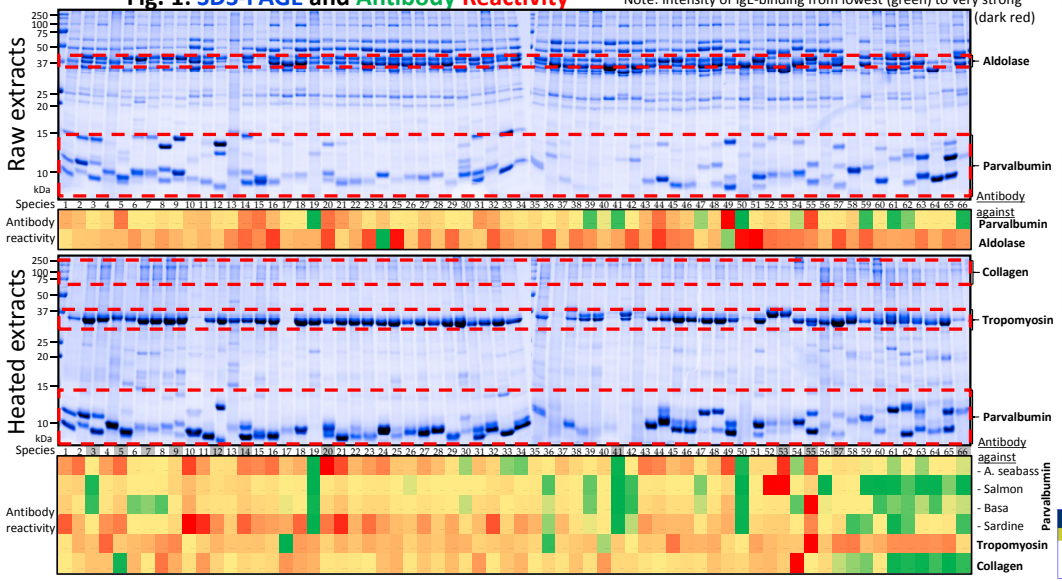
Methods → SDS-PAGE, (IgE-)immunoblotting & mass spectrometry

- Tissue samples from 66 fish species (shown in wave above) were collected.
- Proteins extracted from raw and heated tissue were separated by SDS-PAGE and allergens quantified by immunoblotting using 7 allergen-specific antibodies.
- 88 patients with confirmed IgE-mediated fish allergy were recruited (Ethics approval: LNR-14/SCHN/185), followed by immunoblot analyses.
- IgE-reactive proteins were identified by mass spectrometry.

Results → many novel IgE-reactive proteins were identified

- The fish allergens; parvalbumin, tropomyosin, collagen and aldolase A, were detected with large differences in allergen-specific antibody-binding in over 90% of the 66 species studied by immunoblotting (Fig. 1).
- Serum from 25-54 patients showed IgE reactivity to proteins in the heated extract from 12 selected fish species. The most, heat-stable IgE-reactive allergen was parvalbumin, followed by tropomyosin and collagen (Fig. 2).
- Several novel IgE-reactive proteins were identified in raw and heated extracts (XXXX).
- Parvalbumin and collagen were detected in much lower quantity in cartilaginous fish compared to bony fish.

Fig. 1: SDS-PAGE and Antibody Reactivity



Conclusions

- Frequently consumed species including Asian seabass, pangasius/basa, salmon and tilapia have a higher IgE reactivity compared to Atlantic cod (often used in diagnostics).
- Tropomyosin, collagen and many other proteins, showing considerable IgE reactivity, are identified by advanced mass spectrometric analyses.
- Patients can be sensitized to a narrow range of fish species/allergens and need to be diagnosed accordingly.

Clinical implication: Allergen extracts or purified allergens from a wide variety of species should be used for diagnostics.

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Acknowledgements

AL & DC are holders of a National Health and Medical Research Council (NHMRC, Australia) grant. TR is a PhD full-time scholar of the Centre for Food and Allergy Research (CFAR, Australia).

In relation to this presentation, the authors declare that there are no conflicts of interest

Fig. 2: sIgE-binding to 12 most consumed fish

Species	Number of patients with IgE reactivity to (*n*=88)			
	Any protein	Parvalbumin	Tropomyosin	Collagen
55 Pangasius/basa	54	46	32	5
20 Asian seabass	47	32	15	5
5 Commo	46	38	13	2
14 Nile tila	46	39	18	7
53 Tas. Atl. salmon	45	39	15	5
9 Hoki	42	33	9	6
57 John dory	40	27	18	2
3 Orange rougny	32	19	16	2
7 Atlanti	31	21	11	6
12 Ling	29	18	12	4
41 Yellow Fin tuna	28	21	16	4
66 Gumm	25	23	11	2

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Poster **731**

Tropomyosin Is A Novel Major Fish Allergen Of Unrecognized Importance

Thimo Ruethers[1,2,3], Sandip D. Kamath[1,2,3], Aya C. Taki[2,4], Thu T. K. Le[1,3], Shaymaviswanathan Karnaneedi[1,2,3], Roni Nugraha[1,5], Trúc T. Cao[1], Shuai Nie[6], Nicholas A. Williamson[6], Sam S. Mehr[2,7,8], **Dianne E. Campbell**[2,7,9], Andreas L. Lopata[1,2,3]

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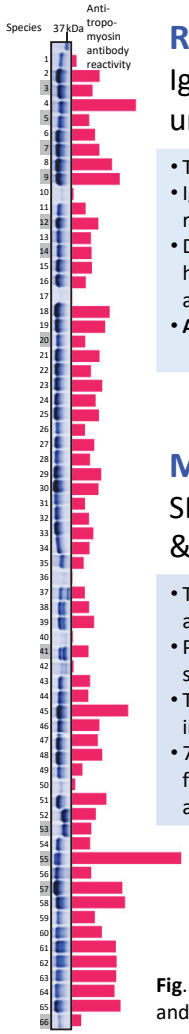

Rationale

IgE-binding to fish tropomyosins is under-investigated

- Tropomyosin is a well-known invertebrate pan-allergen.
- IgE-binding to vertebrate fish tropomyosins was reported, but never comprehensively analyzed.
- Diagnostics and management of fish allergy are hampered by a large number of under-investigated fish allergens and species.
- **Aim**: Evaluation of abundance and IgE-binding of fish tropomyosins.

Methods

SDS-PAGE, (IgE-)immunoblotting & mass spectrometry

- Tissue samples from 66 fish species (shown in wave above) were collected.
- Proteins extracted from raw and heated tissue were separated by SDS-PAGE.
- Tropomyosins and parvalbumins were detected by immunoblotting and mass spectrometry.
- 77 children and 12 adults with confirmed IgE-mediated fish allergy were recruited, followed by immunoblot analyses.

Fig. 1: SDS-PAGE of heated extracts from 66 fish species and anti-tropomyosin antibody reactivity.

Results

Fish tropomyosins are major IgE-binding proteins

- Tropomyosins were at least 10-fold more abundant in heated compared to raw extracts.
- A prominent tropomyosin band was observed in 89% of 66 heated extracts (Fig. 1).
- IgE-binding was patient- and species-specific (Tab. 1).
  12-32% of paediatric patients demonstrated tropomyosin-specific IgE antibodies.
  Only 22-49% showed IgE reactivity to parvalbumins (major allergen).
  22-69% patients with IgE-binding to tropomyosin did not detect parvalbumin from the same species.
- 67% of adult patients recognized recombinant tilapia tropomyosin.
- Catfish and salmon tropomyosins were registered with the WHO/IUIS (www.allergen.org) based on this study.

Tab. 1: **sIgE-binding** to **tropomyosin** & **parvalbumin** from **12 fish**.

Species	Paediatric patients (*n*=77) with IgE-binding to		IgE-binding to **tropomyosin** but **not parvalbumin**
	Tropomyosin	**Parvalbumin**	
55 Catfish	32%	47%	32%
14 Nile tilapia	31%	44%	38%
20 Asian seabass	30%	43%	22%
57 John dory	29%	38%	41%
41 Yellowfin tuna	26%	25%	55%
66 Gummy shark	25%	25%	68%
12 Ling	18%	22%	64%
9 Hoki	18%	35%	43%
3 Orange roughy	17%	22%	69%
5 Common carp	16%	39%	42%
53 Atlantic salmon	13%	49%	30%
7 Atlantic cod	12%	31%	44%

Conclusions

tropomyosins are major IgE-binding heatstable proteins.

The clinical relevance and cross-reactivity of tropomyosins require further investigations.

tropomyosins s be considered for component-resolved diagnostics.

Ethics approval: LNR-14/SCHN/185

AL & DC are holders of a National Health and Medical Research Council (NHMRC, Australia) grant. TR is a PhD full-time scholar of the Centre for Food and Allergy Research (CFAR, Australia). SK is an NHMRC Peter Doherty Early Career Research Fellow. The authors thank **Lirui Sun** for his assistance in data analyses.

In relation to this presentation, the authors declare that there are no conflicts of interest.

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Crocodile parvalbumin is a new allergen – The fish-crocodile syndrome

Thimo Ruethers[1,2,3,4], Aya C. Taki[1,2,3,4], Roni Nugraha[1,3,4,5], Trúc T. Cao[1], Sam Mehr[2,6,7], Dianne E. Campbell[2,6,8], Andreas L. Lopata[1,2,3,4]

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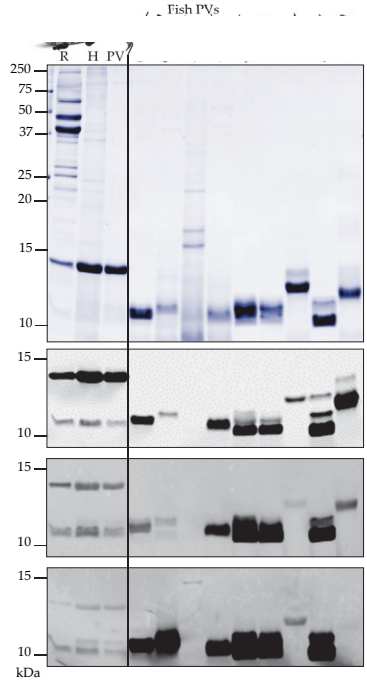
Background

- First two case reports of anaphylaxis upon ingestion of crocodile in 2017
- The suspected major cross-reactive allergen: α-parvalbumin (PV)
- α-PV: Allergen in chicken, frog, and cartilaginous fish
- β-PV: Major allergen in bony fish

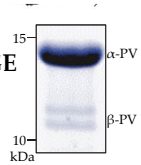
Materials and Methods

- 66 fish-allergic (FA) children underwent skin testing (SPT) to 7 fish species
- A sub-cohort (n=12) underwent SPT using in-house heat-treated Saltwater crocodile tissue
- Crocodile proteins were extracted in PBS and PVs were purified from the same fish species used for SPT plus tilapia, elephant shark and chicken protein extract (for comparison)
- Proteins were separated by SDS-PAGE and PVs were detected using commercial PARV19 and in-house anti-fish β-PV antibody; IgE reactivity was determined using serum from FA patients
- Proteins of interest were identified by mass spectrometry after tryptic digest

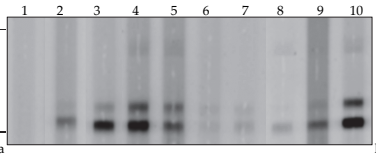
Results



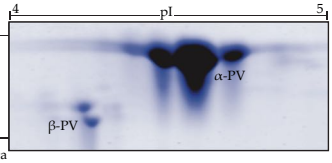
➢SDS-PAGE



➢Immunoblots with patients' serum



➢2D-gel



➢Immuno-blots using

- PARV-19
- Anti-fish β-PV Ab
- Patients' serum

- **7/12 FA children were positive on SPT to crocodile (clinical sensitisation)**
- PVs constituted to 90% of protein from crocodile heated extract
- Several PV isoforms were detected in raw (R) and heated (H) extracts from crocodile, and 8 of 9 purified fish PVs
- IgE reactivity to fish and crocodile PVs was confirmed
- α- and β-PV were identified by mass spectrometry with 99% and 99/91% coverage
- **37/66 FA children's serum showed IgE reactivity to crocodile β-PV and 27/66 to α-PV**
- α-PV was 10 times more abundant, but 100 times less IgE-reactive compared to β-PV
- PVs from all crocodilians have a high sequence identity (95-100%), suggesting a comparable allergenicity
- Crocodile β-PV **Cro p 1** has a sequence identity of 60-74% with allergenic fish β-PVs, explaining the observed cross reactivity
- Allergenic α- and β-PVs share only 50-66% of their amino acid sequence, likely explaining differences in IgE reactivity

Sequence comparisons

	Species	PV lineage									
α-PVs	S. crocodile	α	100	α							
	Gharial	α	97	100	α						
	A. alligator	α	97	98	100	α					
	C. alligator	α	96	97	99	100	β1				
β-PVs	S. crocodile	β1	66	65	65	65	100	β1			
	Gharial	β1	66	65	65	65	100	100	β2		
	S. crocodile	β2	54	53	54	54	74	74	100	β2	
	Gharial	β2	54	53	54	54	74	74	95	100	β
	A. alligator	β	57	56	56	56	76	76	95	95	100
	C. alligator	β	57	56	56	56	75	75	95	97	98

Crocodile Gharial Alligator Chicken (Gal d) Frog (Ran e)

% sequence identity

100 90 80 70 60 50

PV name	Ran e 1	Gal d 8	Cro p 2	Sal s 1	Lat c 1.02	Ras k 1	Gad m 1.02	Gad m 1.01	Thu a 1	Lat c 1.01	Ran e 2	Cro p 1
α-PVs	100											
	73	100										
	73	87	100									
β-PVs	50	52	53	100								
	47	53	55	61	100							
	50	56	55	65	76	100						
	48	53	55	59	63	76	100					
	51	53	55	66	65	70	72	100				
	50	56	54	68	65	75	79	77	100			
	50	54	60	70	68	75	79	77	88	100		
	51	55	57	58	60	62	68	62	66	68	100	
	59	63	66	60	62	64	65	67	70	72	74	100

Conclusions

- **Crocodile β-PV** and **α-PV** are now registered with WHO/IUIS as **Cro p 1** and **Cro p 2**
- **Cro p 1** is the **major crocodile allergen** and is 100 times more IgE-reactive than Cro p 2

The **fish-crocodile syndrome**:

A significant proportion of **fish allergic patients may be at risk of severe allergic reactions upon consumption of crocodilian meat** due to high cross reactivity of β-PVs

Ethics approval: LNR-14/SCHN/185 & JCU-H4313

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TR is a PhD full-time scholar of the Centre for Food and Allergy Research (CFAR, Australia). TR acknowledges the support of CFAR and James Cook University in the form of Travel Scholarships, which enabled him to attend this conference.

In relation to this presentation, the authors declare that there are no conflicts of interest

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FISH Allergen Card

Fish that may be used as an ingredient include*:

Anchovy
Barramundi
Bass
Bluefish
Bream
Carp
Catfish
Caviar
Char
Chub
Cisco
Cod
Eel
Fish
Flake
Flathead
Flounder
Grouper
Haddock
Hake
Halibut
Herring
Imitation crab
Mackerel
Mahi-mahi
Marlin
Monkfish
Orange Roughy
Perch
Pickerel
Pike
Plaice
Pollock
Pompano
Porgy
Rockfish
Salmon
Sardine
Shark
Smelt
Snapper
Sashimi
Surimi
Tilapia
Trout

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Tuna
White Fish
Whiting

Products which might contain fish include:

Antipasto
Asian dishes
Canned spreads
Caesar salad
Dips
Fish sauce
Fish stock
Gelatin
Greek dishes
Marshmallow
Pizza toppings
Salad dressings
Sauces (eg Worcestershire)
Soups
Sushi
Surimi

Please note: *that it is possible for an allergic reaction to occur from cooking odours or handling of fish. Food served in a seafood or other restaurant may be cross contaminated with fish or shellfish during storage, preparation and cooking.*

*This is not a complete or comprehensive list of ingredients to avoid but is intended as a helpful aid for living with fish allergy. It is NOT meant to replace medical advice given by your doctor.

Remember: Cosmetics, lotions, shampoos, moisturisers and the like can contain food allergens. Some medications (prescribed and over the counter) and alternate therapies can also contain food allergens.

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Allergy&Anaphylaxis Australia
Your trusted charity for allergy support

If you are allergic to fish, you are at high risk of being allergic to crocodile meat.

FOOD ALLERGY SAFETY

Avoidance of the food allergen is crucial if you are food allergic.

There are many strategies you can put in place to help reduce the risk of an allergic reaction including anaphylaxis. Strategies include:

- Plan ahead; educate those around you.
- Always read the food label, even if the product has been purchased and eaten safely before.
- Disclose your allergy when eating out.
- If there is no label and you cannot access clear information on food content, do not eat the food.
- Have an appropriate snack available when you go out. If you are hungry, you will be more tempted to take a risk with a non-labelled food purchase.
- Do not share straws, cups, cutlery and other food utensils.
- Call food manufacturers and ask about food labels if you are unsure about a product.
- Be extra careful when eating away from home. Whilst a particular food order may be fine to eat questions must be asked about the risk of cross-contamination during food storage, preparation and serving.
- Check medications, creams, cosmetics and all skin applications for the presence of food allergens.
- Check pet food for allergens (e.g. bird seed for nuts, cat food for fish)
- ALWAYS have your ASCIA Action Plan for Anaphylaxis and your adrenaline autoinjector (EpiPen®) with you. **No Adrenaline = No Eat!**

Food allergy is manageable if people are well informed. Australian labelling requires all major allergens to be clearly specified in the ingredient list. Note: Warning statements such as "May contain…" are voluntary.

MORE INFORMATION:

- **Food allergy management call 1300 728 000 or visit www.allergyfacts.org.au and www.allergy.org.au**
- **Australian food labelling: www.foodstandards.gov.au www.afgc.org.au and www.allergenbureau.net**
- **Dietitians Association of Australia www.daa.asn.au**
- **NSW Food Authority www.foodauthority.nsw.gov.au**

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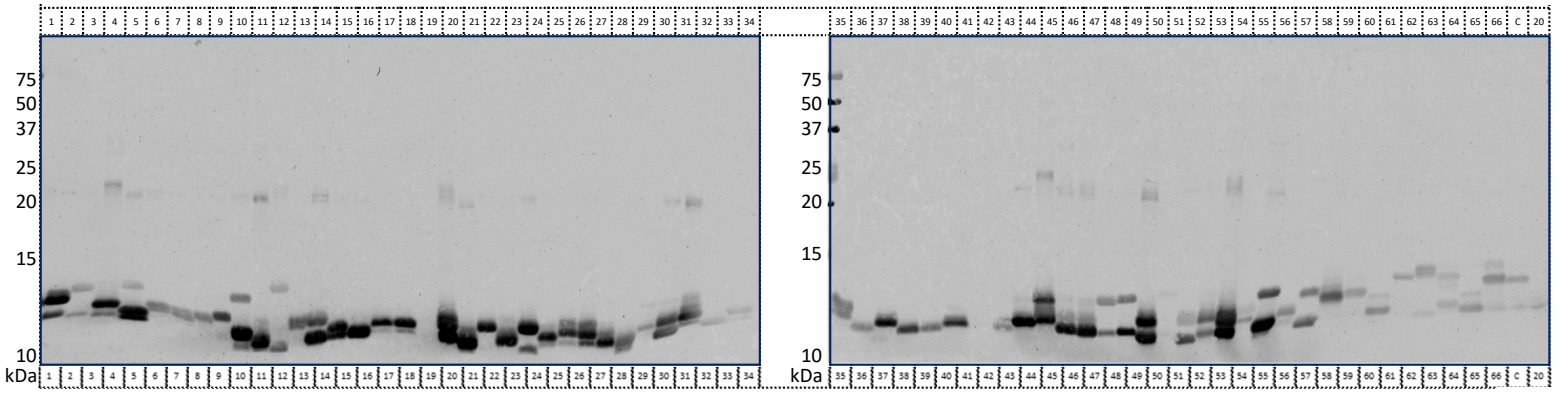
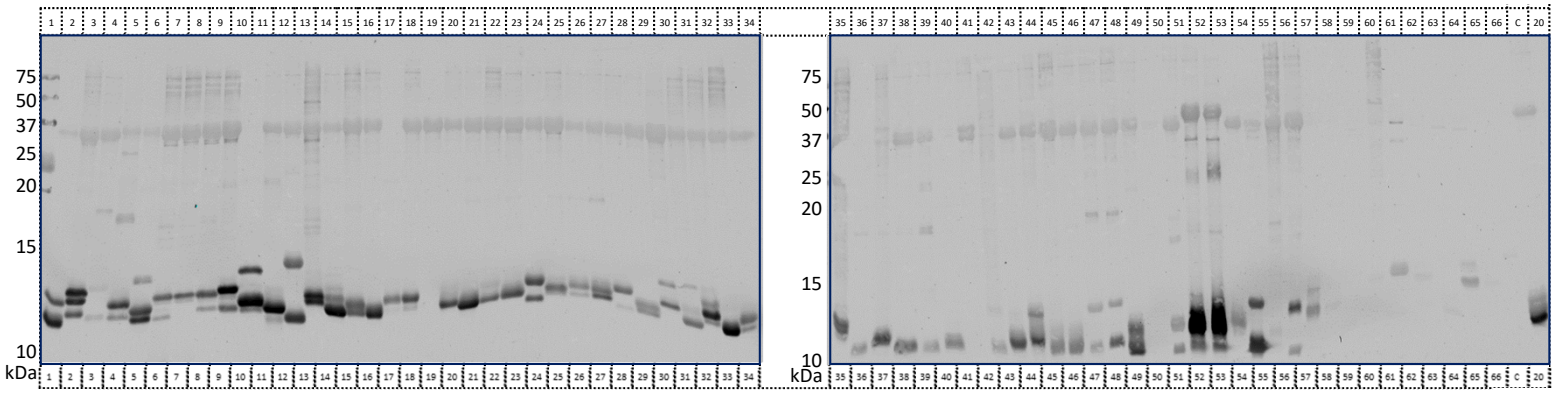
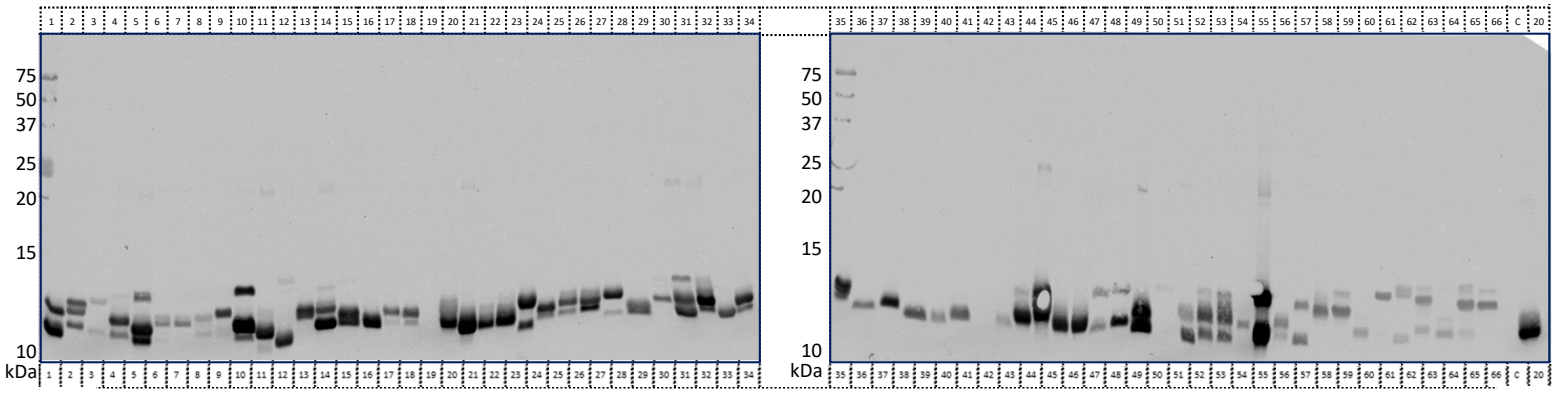
Anti-**barramundi parvalbumin** polyclonal antibody (in-house)



Anti-**salmon parvalbumin** polyclonal antibody (in-house)



Anti-**catfish parvalbumin** polyclonal antibody (in-house)



Anti-**pilchard parvalbumin** polyclonal antibody (in-house)

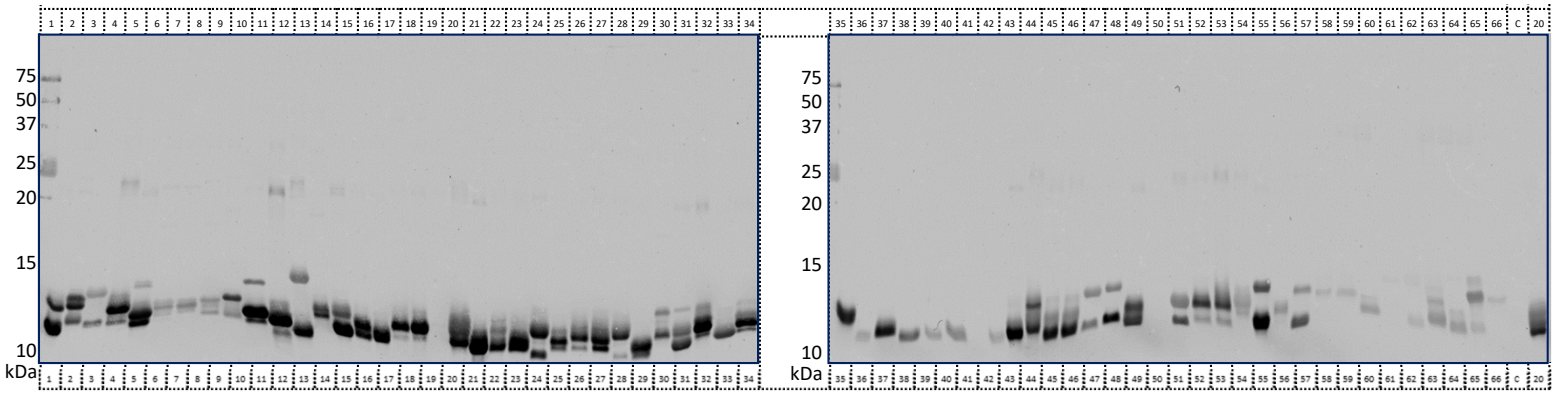
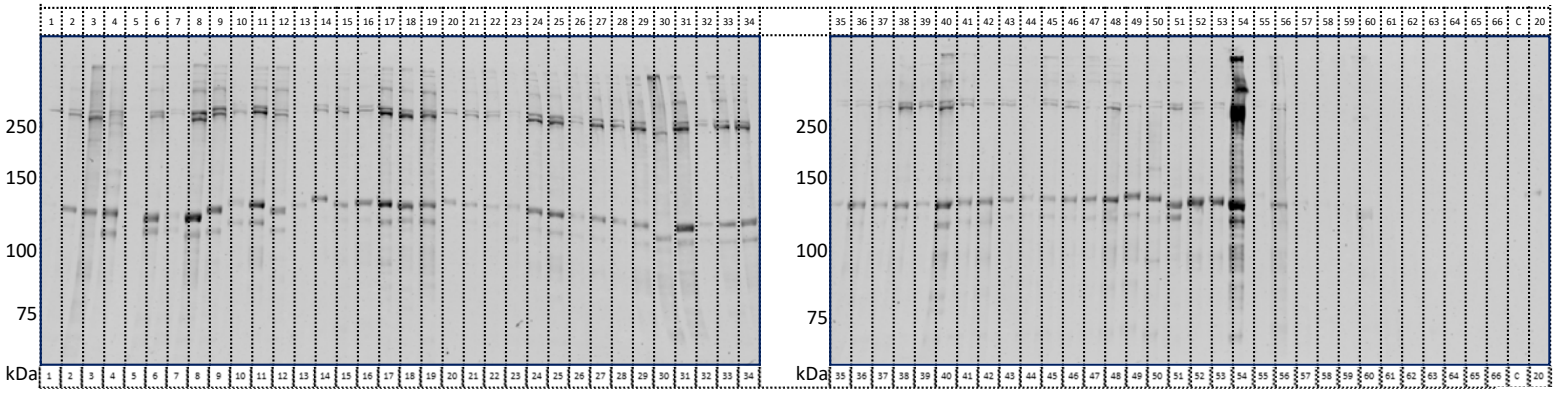
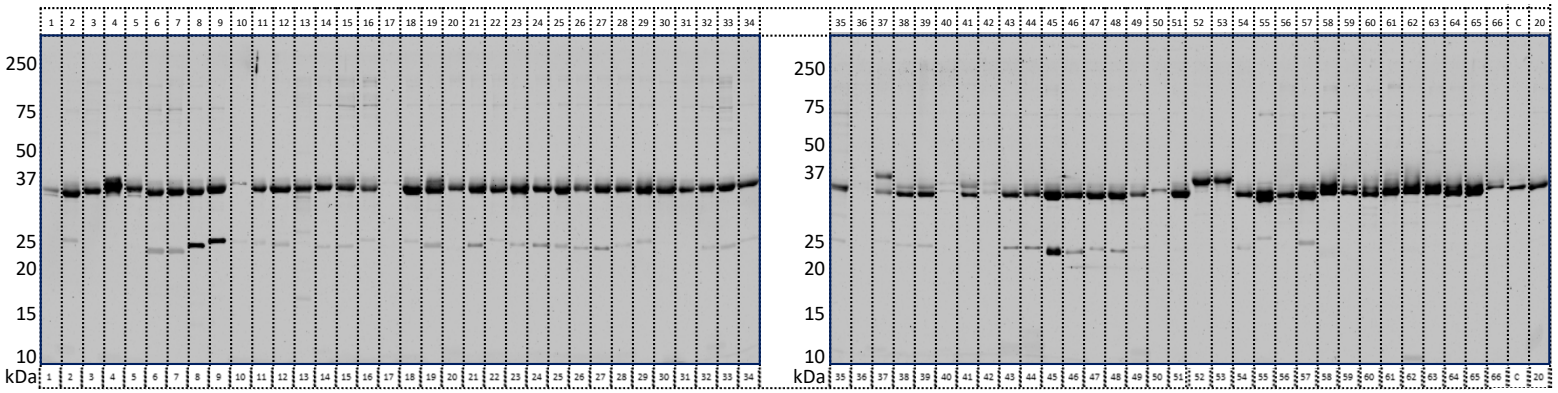


Figure 1 A: Immunoblots with heated muscle protein extracts from 66 fish species utilising four in-house antibodies raised against parvalbumin from different fish. See Chapter V A for methods, corresponding densitometric analyses and data interpretation. The numbers refer to the fish species in Table V A 1.

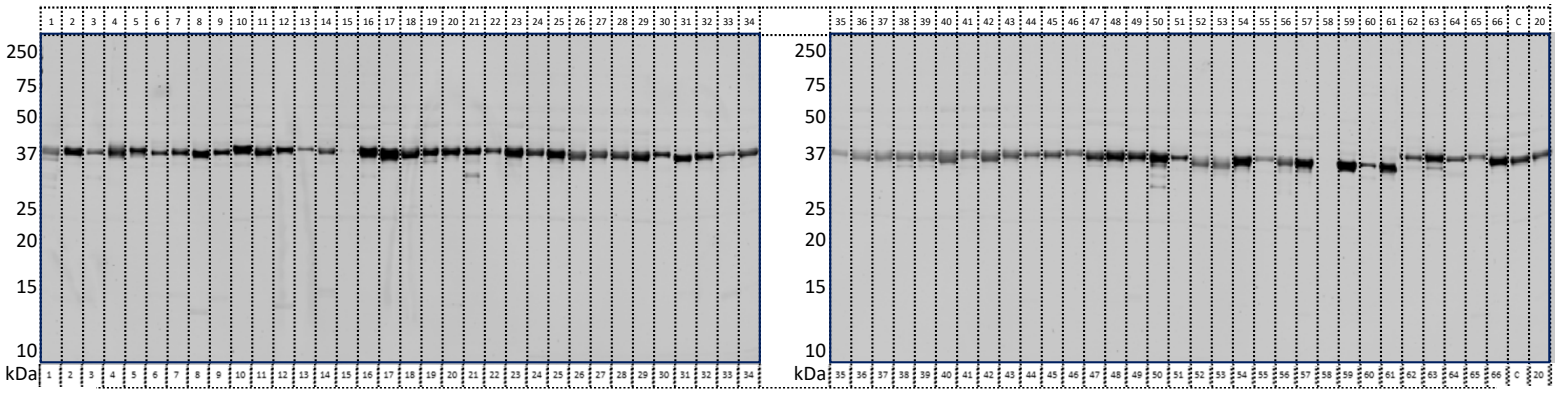
Anti-**tuna collagen** polyclonal antibody (commercial)



Anti-**prawn/shrimp tropomyosin** polyclonal antibody (in-house)



Anti-**rabbit aldolase** polyclonal antibody (commercial)



Anti-**barramundi parvalbumin** polyclonal antibody (in-house)

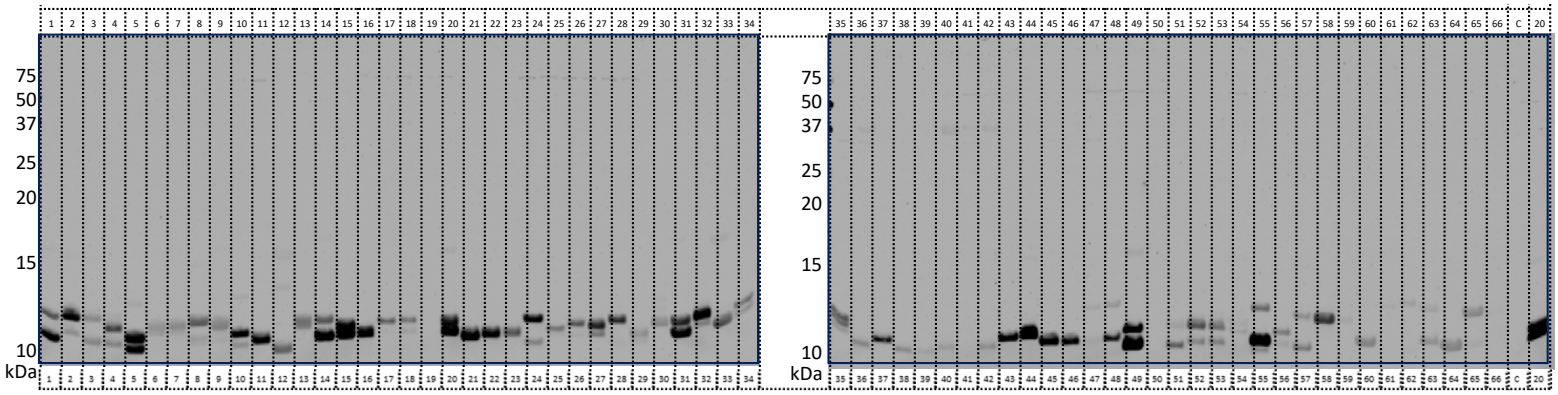


Figure 1 B: Immunoblots with heated muscle protein extracts from 66 fish species utilising antibodies raised against collagen and tropomyosin. Immunoblots with corresponding raw extracts were performed using antibodies raised against aldolase and parvalbumin. See Chapter V A for methods, corresponding densitometric analyses and data interpretation. The numbers refer to the fish species in Table V A 1.

Table 1: Clinical charactersistics of 87 patients recruited for the thesis-associated study.

patient					sIgE level (kU/l)			Skin Prick Test results (wheal diameter in mm)												implicated fish*	implicated shellfish*	symptoms	tolerated seafood
								commercial						HDM *D.*		in-house							
group	#	ID†	sex	age (yr)	cod	tuna	salmon	cod	tuna	salmon	mackerel	fish mix	prawn	*pter.*	*fari.*	seabass	shark	catfish	crocodile				
1	**1**	2	M	5	-	-	-	10.5	5.0	8.5	11.5	9.0	5.0	5.5	6.0	15.0	10.0	12.5	-	salmon, sardine	-	AN, U	canned salmon
1	**2**	5	M	9	-	-	-	12.5	0.0	6.5	11.0	11.0	0.0	11.5	11.5	15.0	10.0	18.5	-	unknown, white bait, white fish	-	AE, C, U	-
1	**3**	7	M	1	-	-	-	7.0	3.0	6.0	0.0	0.0	0.0	4.5	4.5	24.5	9.0	18.5	-	Asian seabass, salmon, white fish	-	AE, OAS, U	shrimp
1	**4**	9	M	5	-	-	-	4.5	4.5	4.5	0.0	2.0	0.0	4.0	5.0	10.0	0.0	13.0	-	cod, white fish	-	AN, GIS, RD	canned salmon and tuna
1	**5**	10	F	13	-	-	-	3.5	4.5	0.0	0.0	0.0	0.0	9.5	10.0	12.0	6.5	0.0	-	Asian seabass, catfish	-	GIS, RD, U	canned tuna and salmon
1	**6**	11	F	10	-	-	-	0.0	0.0	0.0	0.0	0.0	0.0	8.0	5.5	3.0	3.5	4.0	-	salmon, white fish	-	AE, GIS, U	Asian seabass, shark, shrimp, whiting
1	**7**	12	M	11	88.50	4.83	25.30	8.5	4.0	12.0	7.0	7.0	0.0	6.5	7.5	11.5	2.5	13.5	-	tuna, white fish	-	AE, GIS, OAS, U	-
1	**8**	14	F	5	-	-	-	6.0	0.0	5.5	0.0	7.0	0.0	8.0	8.0	7.0	7.0	6.5	-	salmon	-	GIS, U	canned salmon and tuna, shrimp
1	**9**	15	M	9	-	-	-	5.5	2.0	7.5	4.0	3.5	0.0	4.5	8.0	5.0	7.0	7.5	-	salmon, unknown	-	U	-
1	**10**	16	F	7	-	-	-	9.5	6.5	9.0	3.5	3.0	0.0	17.5	13.0	16.0	6.5	17.0	5.5	salmon, tilapia, tuna, yellowtail kingfish	-	AE, OAS, U	-
1	**11**	17	M	10	-	-	-	4.5	3.0	4.0	0.0	6.5	0.0	6.5	5.5	8.0	7.0	7.0	-	white fish	-	AN, RD, U	canned salmon and tuna
1	**12**	19	M	10	<0.01	0.01	<0.01	0.0	2.5	0.0	0.0	3.5	3.0	6.5	9.0	3.5	2.5	4.0	-	Asian seabass	-	OAS, U	canned salmon and tuna
1	**13**	20	M	7	2.29	0.10	0.64	8.0	0.0	5.0	6.0	11.0	0.0	6.0	0.0	18.5	5.5	19.0	-	white fish	-	OAS, U	canned salmon
1	**14**	21	M	13	2.99	1.99	1.24	8.0	2.5	4.5	7.0	8.5	6.5	5.0	3.5	21.0	6.0	12.5	-	white fish	-	OAS, U	-
1	**15**	22	M	14	0.10	0.28	0.21	2.0	2.5	0.0	4.0	3.0	-	10.5	6.0	10.5	7.0	4.5	-	salmon	-	AE	canned tuna

Table 1 continued.

patient					sIgE level (kU/l)			Skin Prick Test results (wheal diameter in mm) commercial								in-house						
group	#	ID†	sex	age (yr)	cod	tuna	salmon	cod	tuna	salmon	mackerel	fish mix	prawn	HDM *D. pter.*	HDM *D. fari.*	seabass	shark	catfish	crocodile	implicated fish*	implicated shellfish*	symptoms	tolerated seafood
1	**16**	23	M	3	0.02	0.03	0.03	0.0	4.0	0.0	4.0	2.0	0.0	8.0	8.5	5.0	5.0	4.0	-	catfish	-	AE, U	-
1	**17**	24	F	12	2.66	1.41	3.43	13.5	4.0	11.0	5.5	10.0	11.0	13.0	10.0	19.0	4.5	15.5	-	white fish	-	OAS, U	-
1	**18**	27	F	13	<0.01	0.02	0.01	2.0	2.5	0.0	1.0	4.5	0.0	7.5	9.5	9.5	5.5	8.5	-	cod or tuna	-	U	canned salmon and tuna, shark
1	**19**	32	F	2	0.14	0.78	0.60	0.0	0.0	3.5	0.0	0.0	4.0	0.0	0.0	0.0	3.0	2.5	-	salmon	-	AE, U	canned tuna
1	**20**	33	M	1	-	-	-	2.5	0.0	4.5	0.0	0.0	0.0	0.0	0.0	3.5	6.0	5.5	-	catfish	-	AE	-
1	**21**	34	M	12	10.00	5.91	9.01	9.5	9.0	8.0	7.0	11.0	3.5	13.0	11.0	16.5	4.5	17.0	-	Asian seabass, salmon	-	U	canned salmon and tuna
1	**22**	35	M	9	7.25	2.15	5.33	10.0	5.5	3.0	8.0	8.0	0.0	10.5	12.0	15.5	9.0	10.0	-	trevally	-	AN, RD, U	tuna, salmon
1	**23**	37	F	1	5.47	5.14	5.58	6.5	0.0	3.0	4.0	0.0	4.0	0.0	0.0	15.5	10.0	13.5	14.5	Asian seabass	-	AE, RD, U	canned salmon and tuna
1	**24**	39	M	8	11.50	4.67	9.80	4.5	4.5	2.0	6.0	8.0	5.5	5.0	4.5	7.0	5.5	7.5	-	salmon, tilapia	-	OAS	canned salmon and tuna
1	**25**	41	F	2	90.40	31.00	73.40	13.5	5.5	13.5	9.0	11.0	0.0	11.5	10.0	18.5	9.0	15.5	-	cod	-	AE, U	-
1	**26**	42	M	8	75.00	21.00	70.50	14.0	6.5	12.0	14.0	16.5	5.5	12.0	14.5	19.0	11.5	16.0	18	white fish	-	GIS, RD, U	canned tuna
1	**27**	45	M	9	0.13	0.06	0.46	3.0	2.0	6.5	3.5	4.0	4.5	7.0	8.0	6.5	6.0	6.0	-	croaker	-	AE	-
1	**28**	47	F	10	0.21	0.15	0.14	3.5	0.0	0.0	3.5	0.0	0.0	2.5	3.5	17.0	13.5	4.0	-	catfish, white fish	-	GIS, RD, OAS	canned tuna, salmon
1	**29**	48	M	5	10.20	6.78	11.50	6.5	5.0	6.0	1.5	5.0	9.0	19.0	13.5	12.5	9.0	11.5	-	leather jacket	-	AE	-
1	**30**	49	M	7	1.81	0.70	7.63	4.5	0.0	6.0	2.0	5.5	0.0	8.0	2.0	7.5	0.0	6.5	-	trout	-	AE, OAS, U	canned tuna

Table 1 continued.

patient					sIgE level (kU/l)			Skin Prick Test results (wheal diameter in mm) commercial								in-house						
group	#	ID†	sex	age (yr)	cod	tuna	salmon	cod	tuna	salmon	mackerel	fish mix	prawn	HDM *D. pter.*	HDM *D. fari.*	seabass	shark	catfish	crocodile	implicated fish*	implicated shellfish*	symptoms	tolerated seafood
1	**31**	52	M	14	1.42	0.71	1.60	8.0	1.0	10.5	5.0	3.0	3.0	10.5	6.0	9.5	2.5	10.0	-	salmon	-	OAS, U	-
1	**32**	53	M	7	1.37	0.95	0.54	7.0	2.0	2.0	5.0	6.5	0.0	7.0	4.0	17.5	7.5	15.5	-	tilapia	-	C, GIS	canned tuna
1	**33**	54	F	4	1.54	0.71	0.47	8.5	0.0	0.0	4.5	9.5	0.0	6.5	11.0	17.0	11.5	8.5	-	catfish	-	AE, E, U	-
1	**34**	55	F	15	1.94	0.86	1.39	12.5	2.5	5.0	5.0	11.0	0.0	14.0	10.0	16.5	0.0	17.0	3.5	Asian seabass	-	AE, U	canned tuna, cooked salmon
1	**35**	59	M	9	7.03	2.38	3.74	8.5	4.0	6.5	8.0	10.0	7.5	0.0	4.5	14.0	7.5	13.5	-	silver perch	-	AE	canned salmon
1	**36**	60	F	2	-	-	-	11.5	4.5	7.0	11.5	12.0	4.5	4.0	5.5	17.0	14.0	5.5	12.0	salmon, yellowtail kingfish	-	U	-
1	**37**	61	F	12	55.00	14.90	38.90	5.0	3.0	4.5	5.0	7.0	2.0	5.0	13.5	14.0	5.0	11.5	-	flathead	-	AE	canned salmon
1	**38**	65	M	1	<0.01	<0.01	<0.01	1.0	0.0	0.0	3.0	2.0	0.0	0.0	0.0	3.5	3.5	0.0	-	salmon	-	AE, U	canned tuna
1	**39**	69	F	3	-	-	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	hake	-	AN, U	-
1	**40**	70	F	11	0.02	0.02	0.04	0.0	5.5	0.0	4.5	3.5	0.0	7.5	11.5	4.0	5.5	7.0	-	silver bream	-	AN, AE, OAS, RD	-
1	**41**	72	F	17	-	-	-	6.0	4.0	0.0	0.0	7.5	0.0	9.0	6.5	7.0	0.0	8.5	-	catfish	-	GIS	-
1	**42**	73	M	8	-	-	-	4.5	4.5	4.5	0.0	2.0	0.0	4.0	5.0	10.0	0.0	13.0	-	cod, white fish	-	AN, R, RD, U	canned salmon and tuna
1	**43**	74	M	20	-	-	-	7.0	0.0	3.0	0.0	5.5	7.0	9.0	18.5	14.5	0.0	13.5	-	white fish	-	AE, U	canned tuna, crab, shrimp
1	**44**	77	F	13	22.70	9.43	20.60	12.5	5.0	9.5	3.0	10.5	5.0	0.0	4.0	15.5	7.0	18.0	-	salmon	-	U	-
1	**45**	78	M	1	-	-	-	1.0	1.0	6.0	6.5	4.0	1.0	1.0	1.0	12.0	5.5	14.5	-	tilapia, yellowtail kingfish	-	AE, U	-

Table 1 continued.

patient					sIgE level (kU/l)			Skin Prick Test results (wheal diameter in mm) commercial								in-house				implicated fish*	implicated shellfish*	symptoms	tolerated seafood
group	#	ID†	sex	age (yr)	cod	tuna	salmon	cod	tuna	salmon	mackerel	fish mix	prawn	HDM *D. pter.*	HDM *D. farі.*	seabass	shark	catfish	crocodile				
1	**46**	79	M	5	-	-	-	4.5	0.0	3.0	0.0	0.0	0.0	6.0	8.5	13.5	7.5	14.5	-	white fish	-	AE, OAS, U	-
1	**47**	80	M	15	-	-	-	11.0	4.0	12.0	6.0	9.0	1.0	14.5	10.0	19.0	13.5	5.0	-	milkfish	-	U	-
1	**48**	81	M	10	-	-	-	6.0	0.0	4.5	0.0	0.0	6.0	6.0	6.5	14.0	11.0	9.5	-	perch	-	U	-
1	**49**	82	M	8	-	-	-	7.0	0.0	6.5	6.5	15.0	6.5	10.5	11.0	20.5	7.0	0.0	-	bream	-	AN, AE, RD	canned tuna
1	**50**	87	M	16	-	-	-	3.5	5.5	4.5	8.0	6.5	0.0	10.5	12.0	17.0	4.5	8.0	-	snapper	-	U	canned tuna
1	**51**	92	M	11	-	-	-	4.0	3.5	3.0	0.0	0.0	0.0	5.5	3.5	3.5	4.5	3.0	1.0	salmon, white fish	-	AE, C, RD	shrimp
1	**52**	93	M	15	-	-	-	2.0	2.0	2.5	4.5	1.0	5.0	5.0	7.0	8.5	0.0	8.5	0.0	Asian seabass, salmon	-	AP, GIS, RD	-
1	**53**	96	M	18	-	-	-	5.5	0.0	4.5	2.0	4.0	4.0	9.0	7.0	5.0	4.0	14.5	2.5	catfish, white fish	-	GIS	canned salmon and tuna
1	**54**	97	F	17	1.15	3.18	2.88	5.0	1.0	2.5	5.5	2.0	1.5	7.5	8.5	11.5	4.0	15.0	-	Asian seabass, croaker	-	OAS, R	salmon, tuna
1	**55**	98	M	14	3.79	1.17	8.09	8.5	4.5	11.0	0.0	3.5	0.0	4.5	5.5	6.5	5.0	9.5	5.0	catfish, salmon, tuna	-	RD, U	-
1	**56**	100	F	13	-	-	-	4.5	0.0	0.0	0.0	0.0	0.0	15.0	21.5	5.0	0.0	3.5	0.0	white fish	-	OAS, U	canned salmon and tuna
2	**57**	1	M	14	1.29	1.53	3.96	13.5	0.0	0.0	7.0	8.5	11.5	15.0	14.0	13.0	8.5	5.5	5	snapper	unknown	AE, OAS	-
2	**58**	13	M	14	-	-	-	5.5	6.0	0.0	0.0	4.0	0.0	8.0	7.5	9.5	5.5	9.5	-	Asian seabass	calamari, shrimp	AN	canned salmon and tuna, shrimp
2	**59**	31	M	4	<0.01	<0.01	<0.01	0.0	0.0	0.0	2.0	3.0	0.0	0.0	0.0	3.0	3.5	4.5	-	white fish	shrimp	U	-
2	**60**	36	M	11	0.11	0.14	0.11	3.5	0.0	0.0	2.5	0.0	3.5	4.5	3.0	3.5	0.0	1.0	-	white fish	shrimp	AE, AN, RD, U	-

Table 1 continued.

patient					sIgE level (kU/l)			Skin Prick Test results (wheal diameter in mm) commercial								in-house				implicated fish*	implicated shellfish*	symptoms	tolerated seafood
group	#	ID†	sex	age (yr)	cod	tuna	salmon	cod	tuna	salmon	mackerel	fish mix	prawn	HDM *D. pter.*	HDM *D. fari.*	seabass	shark	catfish	crocodile				
2	**61**	38	M	11	92.10	39.50	66.10	14.0	5.0	4.5	6.0	11.5	3.5	4.5	3.0	21.0	5.5	23.0	-	salmon	shrimp	AE	-
2	**62**	40	M	15	18.70	12.10	17.60	15.0	5.5	8.5	10.5	13.5	0.0	10.0	9.0	20.0	10.0	14.5	-	ling	unknown	AE, OAS	-
2	**63**	43	F	10	16.50	7.33	9.08	7.0	2.0	3.5	-	3.0	0.0	7.0	5.0	9.0	0.0	12.0	-	white fish	shrimp	AE, OAS, RD	canned salmon and tuna
2	**64**	44	F	6	-	-	-	8.0	3.0	4.5	4.0	8.0	0.0	11.0	13.0	19.0	10.5	12.5	-	salmon, white fish	shrimp	AE	tuna
2	**65**	46	M	2	-	-	-	7.5	4.0	7.5	3.5	3.0	0.0	0.0	2.0	4.5	6.0	5.5	-	salmon	shrimp	U	canned tuna
2	**66**	50	F	7	2.87	1.19	3.08	4.5	2.5	0.0	4.0	0.0	2.5	8.5	15.0	16.0	6.5	9.5	-	white fish	crab	U	canned salmon and tuna
2	**67**	51	M	4	25.50	17.30	21.00	14.0	0.0	7.0	6.0	7.0	4.5	3.0	4.5	10.0	11.5	5.0	-	white fish	calamari, shrimp	AP, U	-
2	**68**	56	M	18	3.36	1.06	3.21	12.5	4.0	6.5	4.0	6.0	4.0	10.5	9.0	13.0	3.0	14.0	-	tuna	crab	RD, U	-
2	**69**	58	M	12	0.31	0.18	0.24	6.0	3.0	3.0	2.0	5.0	4.5	5.0	7.5	14.0	7.5	15.0	-	unknown	calamari	RD, U	-
2	**70**	66	M	12	7.01	3.90	31.80	3.5	0.0	7.0	3.0	5.0	5.0	8.5	8.5	3.0	3.5	2.0	-	salmon, tuna	shrimp	E, U	canned salmon and tuna
2	**71**	76	M	7	-	-	-	13.5	4.0	7.5	5.0	7.0	0.0	14.0	9.5	13.5	5.5	12.5	-	ling	unknown	AE, U	-
2	**72**	84	F	21	0.05	1.27	0.58	0.0	0.0	0.0	0.0	0.0	10.0	10.5	8.5	0.0	0.0	2.5	-	snapper, tuna	shrimp	AE, GIS, U	-
2	**73**	85	F	17	-	-	-	0.0	3.5	0.0	0.0	0.0	7.0	4.5	4.5	0.0	3.0	0.0	-	white fish	shrimp	AE, AN, GIS, RD	-
2	**74**	86	M	18	-	-	-	5.5	3.0	6.0	6.0	6.5	3.0	11.5	8.5	9.5	3.5	12.0	-	salmon, silver perch, shark	shrimp	AE, AN, C, RD	canned tuna
2	**75**	88	M	10	36.50	13.50	54.20	12.0	5.5	10.5	0.0	0.0	9.5	11.5	7.0	17.5	5.5	17.5	-	Asian seabass, tuna	shrimp	AE	-

Table 1 continued.

patient					sIgE level (kU/l)			Skin Prick Test results (wheal diameter in mm) commercial								in-house				implicated fish*	implicated shellfish*	symptoms	tolerated seafood
group	#	ID†	sex	age (yr)	cod	tuna	salmon	cod	tuna	salmon	mackerel	fish mix	prawn	HDM *D. pter.*	HDM *D. fari.*	seabass	shark	catfish	crocodile				
2	**76**	90	F	15	-	-	-	10.5	5.5	5.0	5.5	0.0	7.5	4.5	4.0	16.5	5.5	19.0	-	salmon	unknown	E, U	-
2	**77**	95	F	13	-	-	-	10.5	0.0	4.5	2.0	6.0	8.0	7.5	7.5	16.0	6.0	16.5	3.0	milkfish, tilapia, white fish	shrimp	C, RD, U	canned tuna
3	**78**	63	M	7	-	-	-	0.0	3.5	3.5	1.0	2.0	0.0	9.0	8.5	3.5	1.0	4.0	-	avoiding fish	shrimp	AN, RD, U	canned salmon and tuna
3	**79**	64	F	15	-	-	-	6.0	4.5	4.0	7.0	13.0	4.5	11.5	11.0	16.0	3.0	15.0	-	avoiding fish	shrimp	AE, U	-
3	**80**	68	M	9	-	-	-	8.0	0.0	4.5	5.0	4.5	7.0	6.0	7.0	10.0	0.0	11.0	-	avoiding fish	crab or shrimp	AE, E, U	canned salmon
3	**81**	89	M	11	-	-	-	7.0	0.0	9.0	0.0	6.0	11.5	9.0	10.5	9.5	5.5	10.0	-	avoiding fish	shrimp	OAS, AP	canned tuna
4	**82**	3	M	9	-	-	-	0.0	0.0	0.0	0.0	0.0	1.0	14.5	6.5	3.5	0.0	0.0	0	-	octopus, scallop, shrimp	AE, U	canned salmon and tuna, shark
4	**83**	25	M	10	0.07	0.23	0.03	0.0	2.0	0.0	3.0	0.0	4.0	8.0	5.0	5.0	4.5	4.5	-	-	shrimp	RD, U	-
4	**84**	26	M	13	<0.01	<0.01	<0.01	0.0	3.5	0.0	4.0	5.5	5.0	6.0	7.5	6.5	7.5	7.0	-	-	shrimp	OAS, U	canned salmon and tuna
4	**85**	28	F	10	0.21	0.34	0.11	2.0	0.0	2.0	-	0.0	13.0	4.0	8.5	0.0	0.0	0.0	-	-	shrimp	OAS	canned salmon and tuna, shark
4	**86**	30	M	15	0.01	0.02	0.02	0.0	5.5	0.0	4.5	4.5	5.5	10.5	10.5	8.5	8.0	8.0	-	-	shrimp	U	canned salmon and tuna, shark
4	**87**	99	F	11	-	-	-	0.0	0.0	0.0	0.0	0.0	6.0	4.5	2.5	3.0	4.0	4.5	-	-	shrimp	OAS, U	canned salmon and tuna, shark

Note: Patients are grouped based on their clinical history: 1=allergic to fish but not shellfish, 2=allergic to fish and shellfish, 3=allergic to shellfish and avoiding fish, 4=allergic to shellfish but fish; M=male; F=female; † refers to internal patient code C'; yr=years; sIgE levels were determined by ImmunoCAP; '-'=not determined; *Species which was associated with clinical reaction upon ingestion; AE, angioedema (lip/face swelling); AN, anaphylaxis; AP, abdominal pain; C, conjunctivitis; E, eczema; GIS, gastrointestinal syndrome (vomiting); OAS, oral allergy syndrome (itchy mouth or swelling throat/tongue); R, rhinitis (iriitation inside nose, sneezing); RD, respiratory distress; U, urticaria (erythema, hives, rash).

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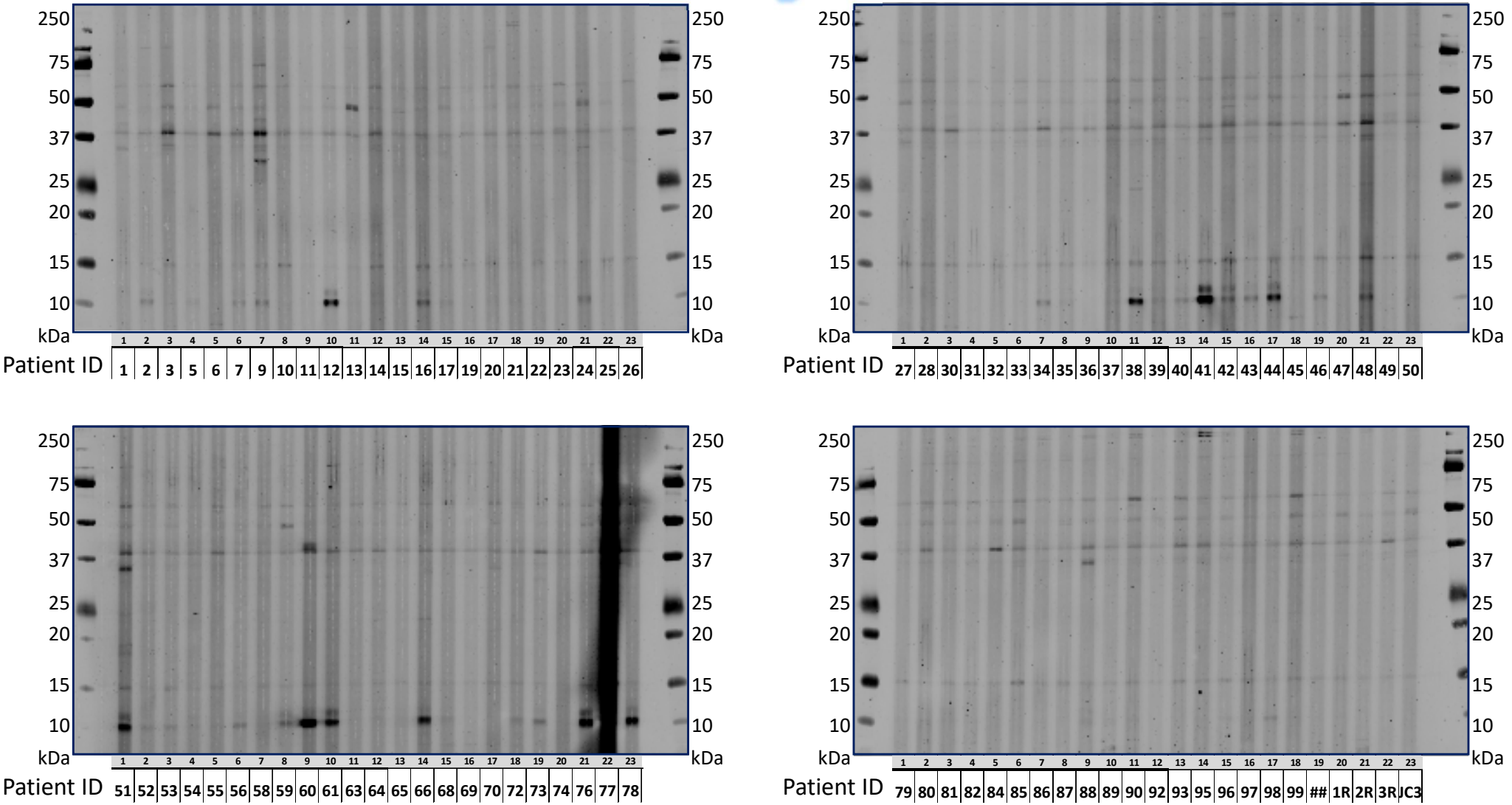


Figure 2.1: IgE-surfblots with barramundi/Asian seabass (*Lates calcarifer*) raw muscle protein extract (100 µg each).
See Appendix C Table 1 for patient details and Appendix Table 2 for corresponding densitometric analyses. See Chapter IV A for methods and data analyses.

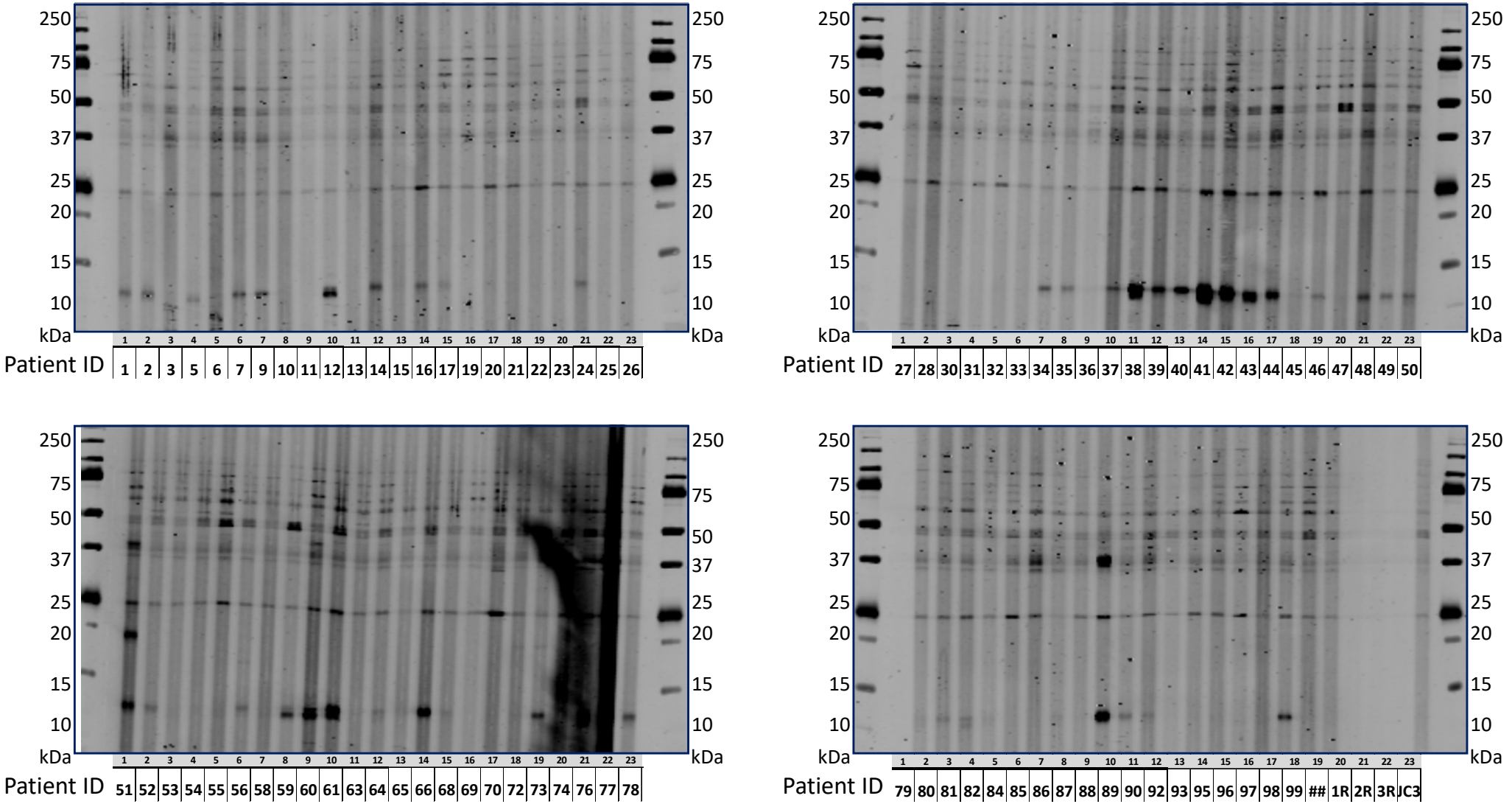


Figure 2.2: IgE-surfblots with Atlantic salmon (*Salmo salar*) raw muscle protein extract (100 µg each).
See Appendix C Table 1 for patient details and Appendix Table 2 for corresponding densitometric analyses. See Chapter IV A for methods and data analyses.

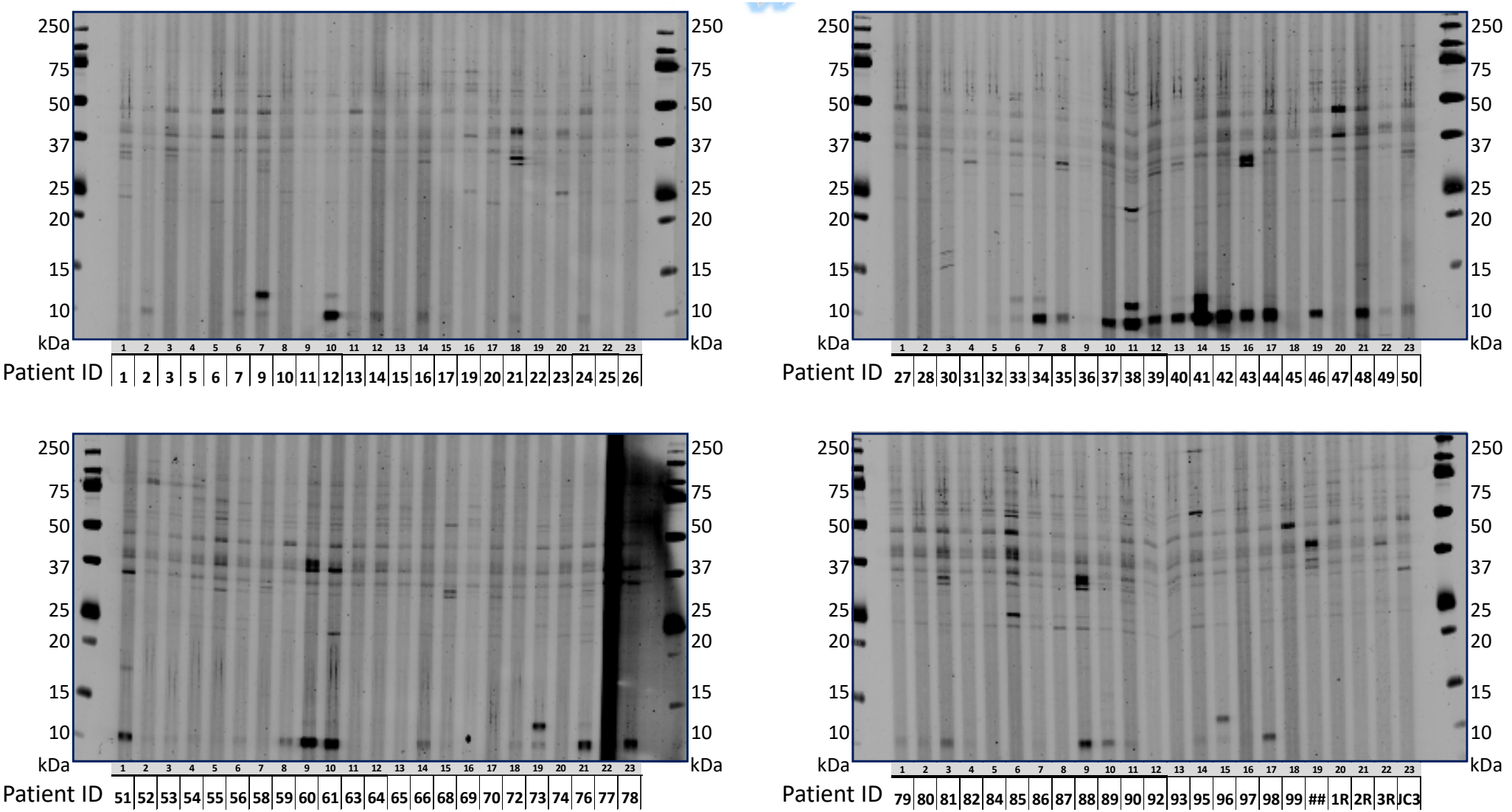


Figure 2.3: IgE-surfblots with <u>*Pangasisus*/catfish</u> (*Pangasianodon hypophthalmus*) <u>raw</u> muscle protein extract (100 µg each). See Appendix C Table 1 for patient details and Appendix Table 2 for corresponding densitometric analyses. See Chapter IV A for methods and data analyses.

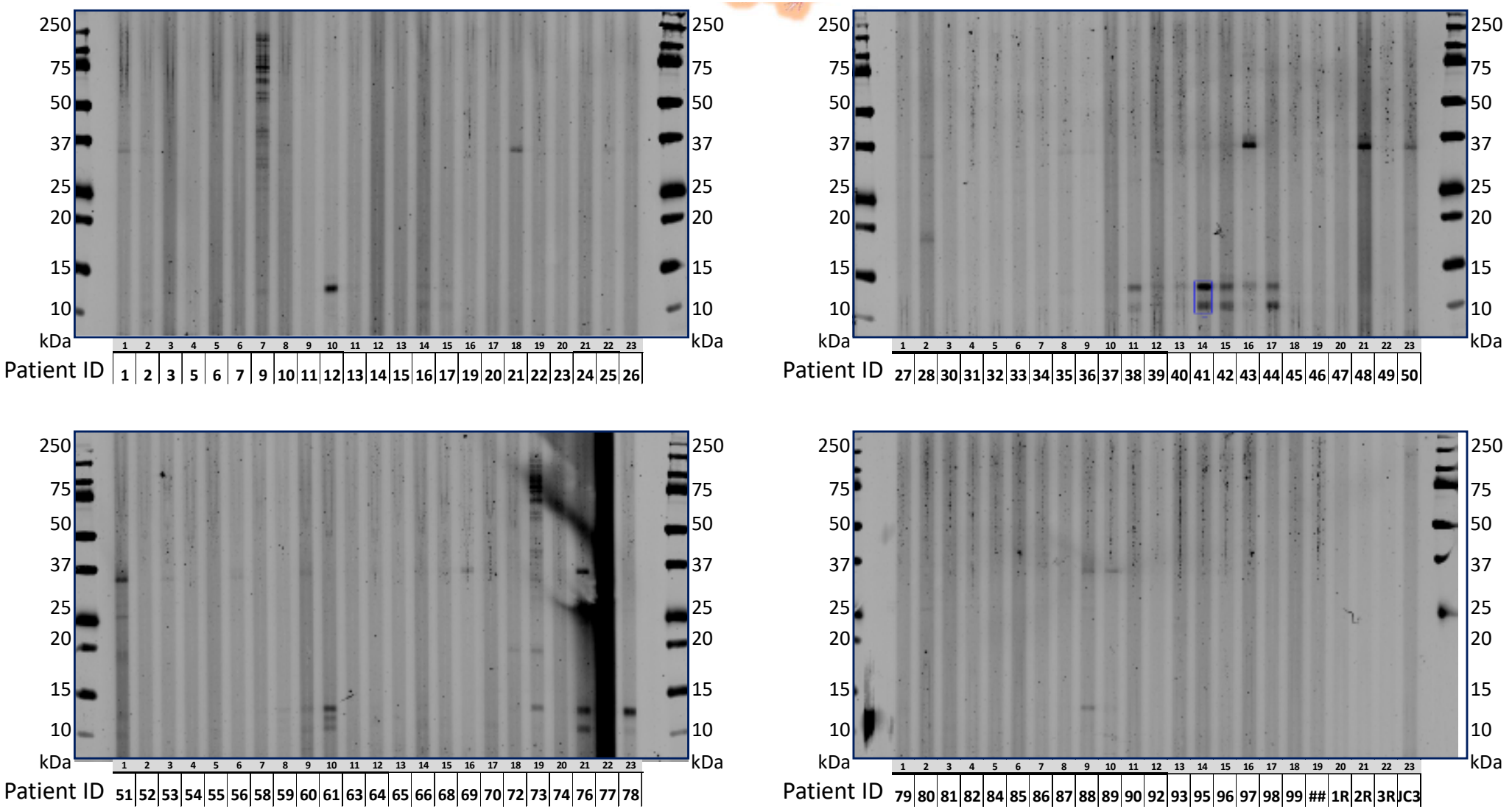


Figure 2.4: IgE-surfblots with orange roughy (*Hoplostethus atlanticus*) heated muscle protein extract (100 µg each).
See Appendix C Table 1 for patient details and Appendix Table 2 for corresponding densitometric analyses. See Chapter IV A for methods and data analyses.

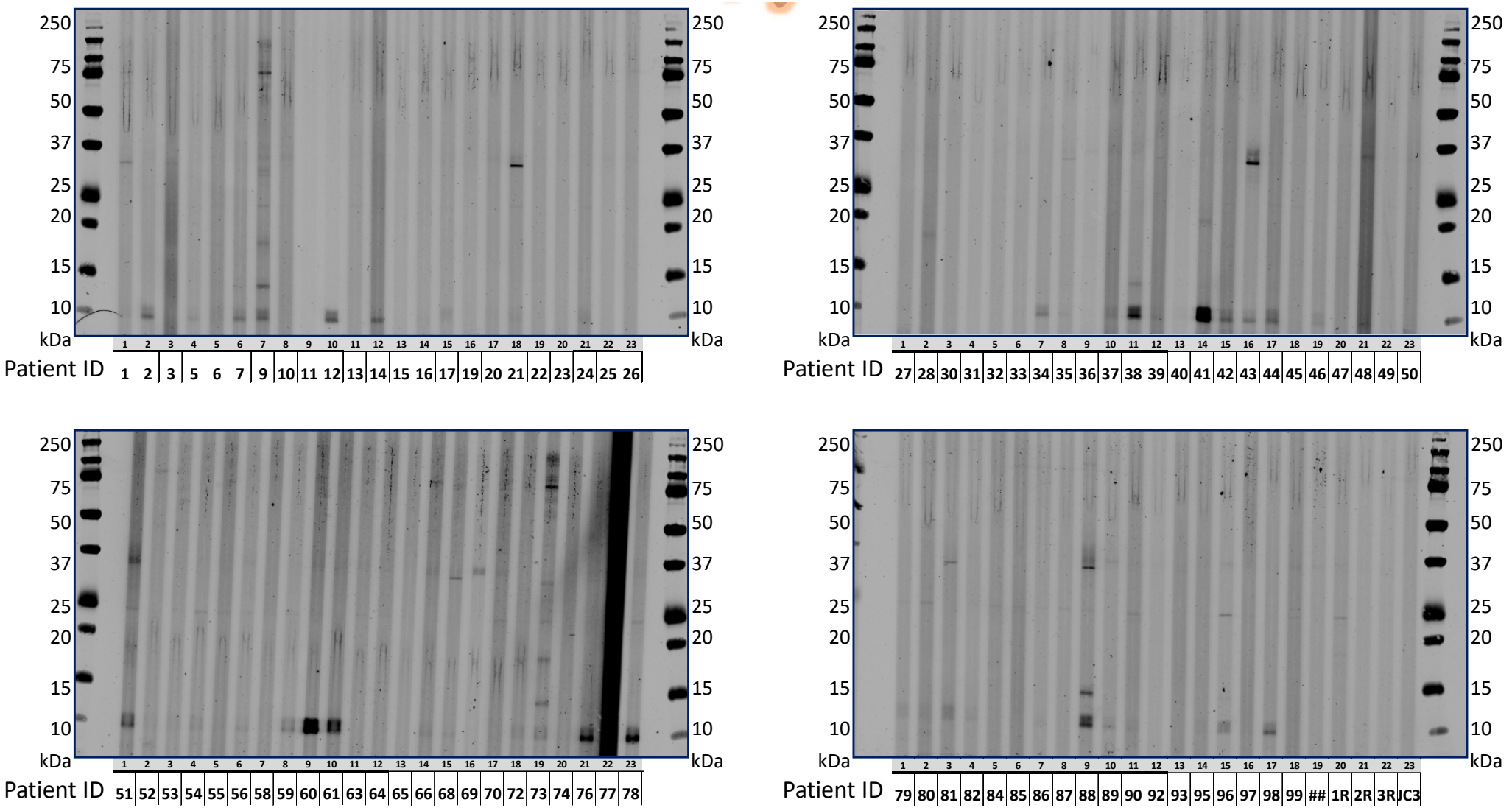


Figure 2.5: IgE-surfblots with common carp (*Cyprinus carpio*) heated muscle protein extract (100 µg each).
See Appendix C Table 1 for patient details and Appendix Table 2 for corresponding densitometric analyses. See Chapter IV A for methods and data analyses.

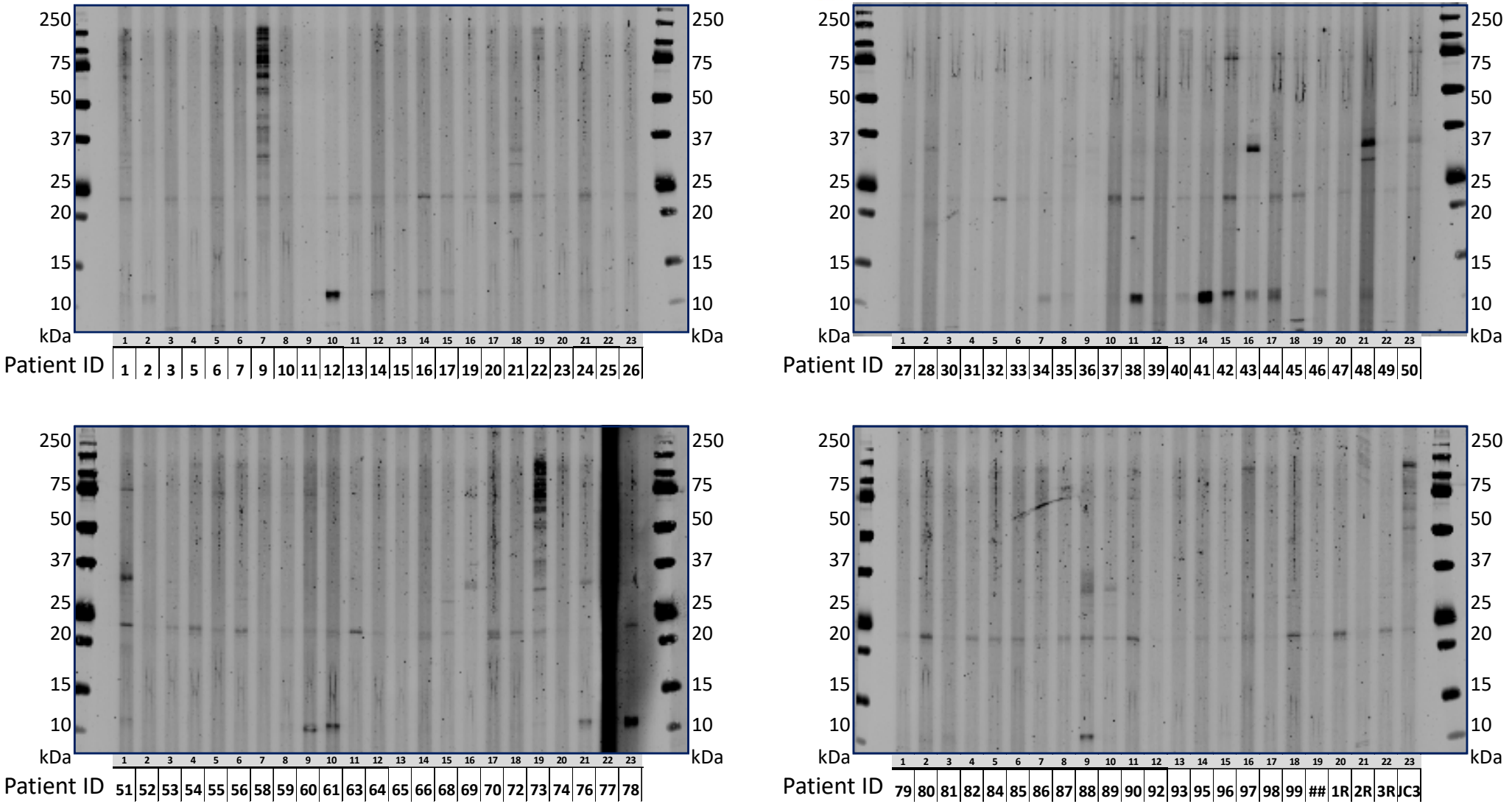


Figure 2.6: IgE-surfblots with Atlantic cod (*Gadus morhua)* heated muscle protein extract (100 µg each).
See Appendix C Table 1 for patient details and Appendix Table 2 for corresponding densitometric analyses. See Chapter IV A for methods and data analyses.

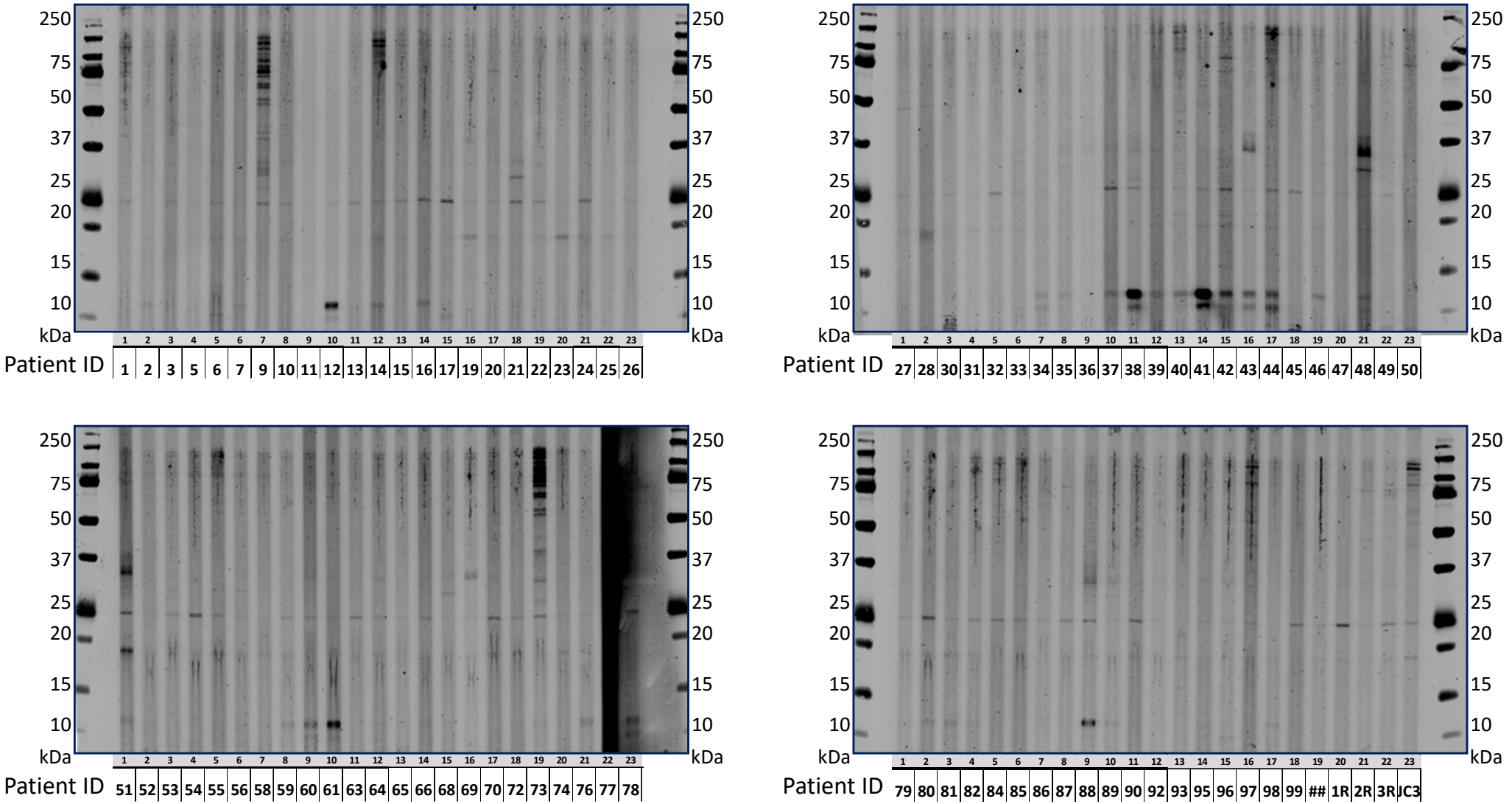


Figure 2.7: IgE-surfblots with <u>hoki/blue grenadier</u> (*Macruronus novaezelandiae*) <u>heated</u> muscle protein extract (100 µg each). See Appendix C Table 1 for patient details and Appendix Table 2 for corresponding densitometric analyses. See Chapter IV A for methods and data analyses.

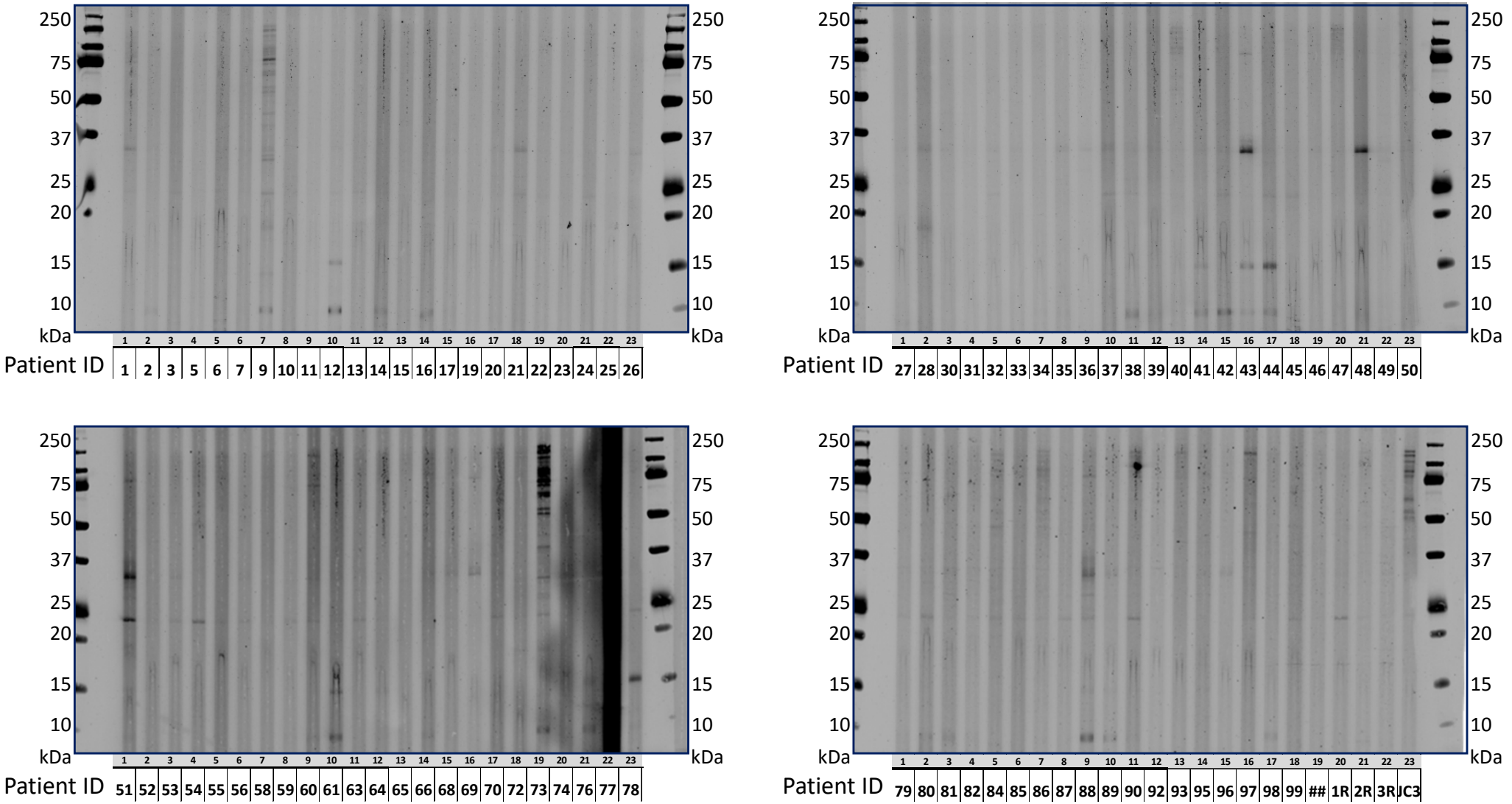


Figure 2.8: IgE-surfblots with ling/pink cusk-eel (*Genypterus blacodes*) heated muscle protein extract (100 µg each).
See Appendix C Table 1 for patient details and Appendix Table 2 for corresponding densitometric analyses. See Chapter IV A for methods and data analyses.

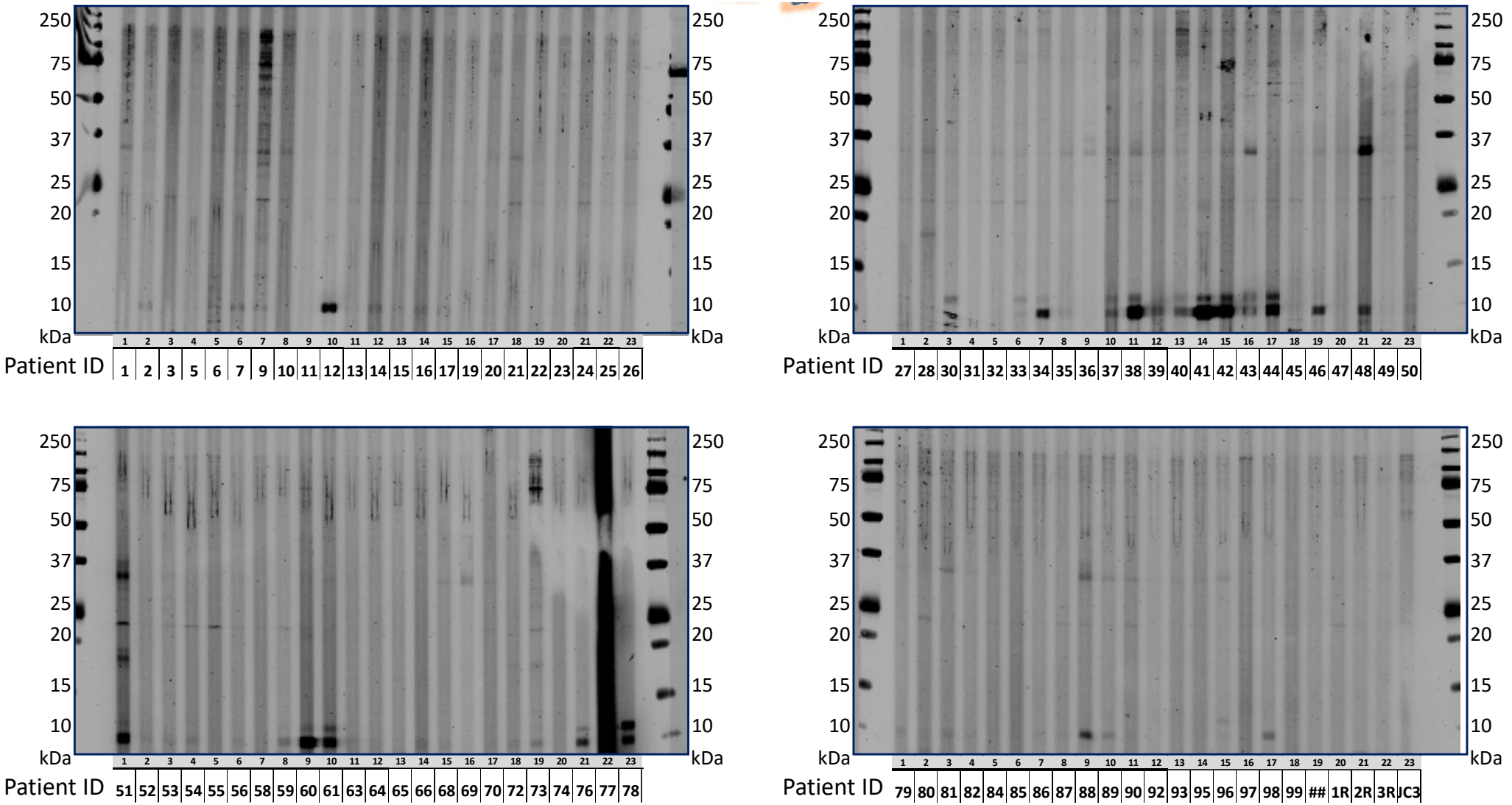


Figure 2.9: IgE-surfblots with Nile tilapia (*Oreochromis niloticus*) heated muscle protein extract (100 µg each).
See Appendix C Table 1 for patient details and Appendix Table 2 for corresponding densitometric analyses. See Chapter IV A for methods and data analyses.

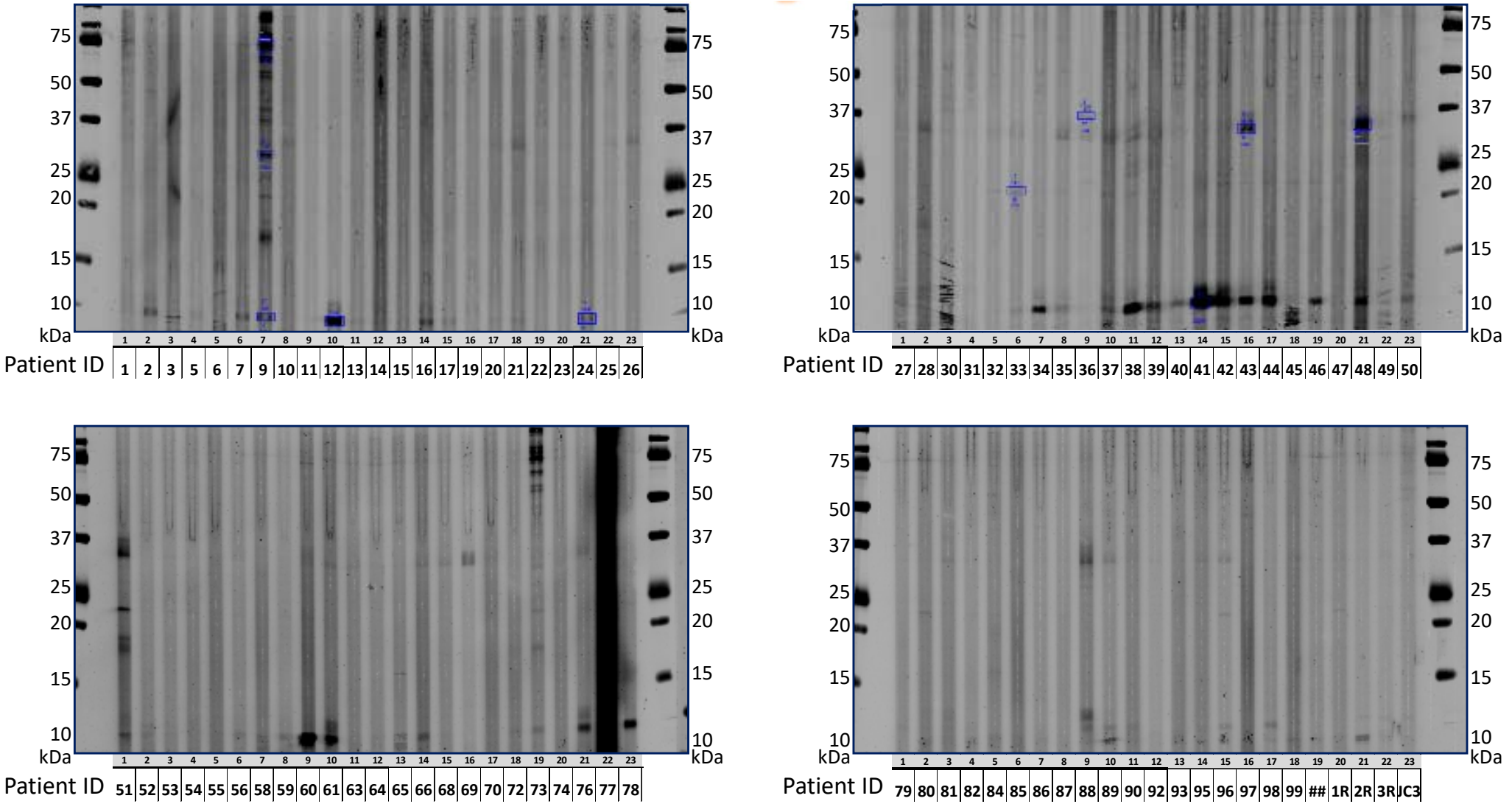


Figure 2.10: IgE-surfblots with barramundi/Asian seabass (*Lates calcarifer*) heated muscle protein extract (100 µg each). See Appendix C Table 1 for patient details and Appendix Table 2 for corresponding densitometric analyses. See Chapter IV A for methods and data analyses.

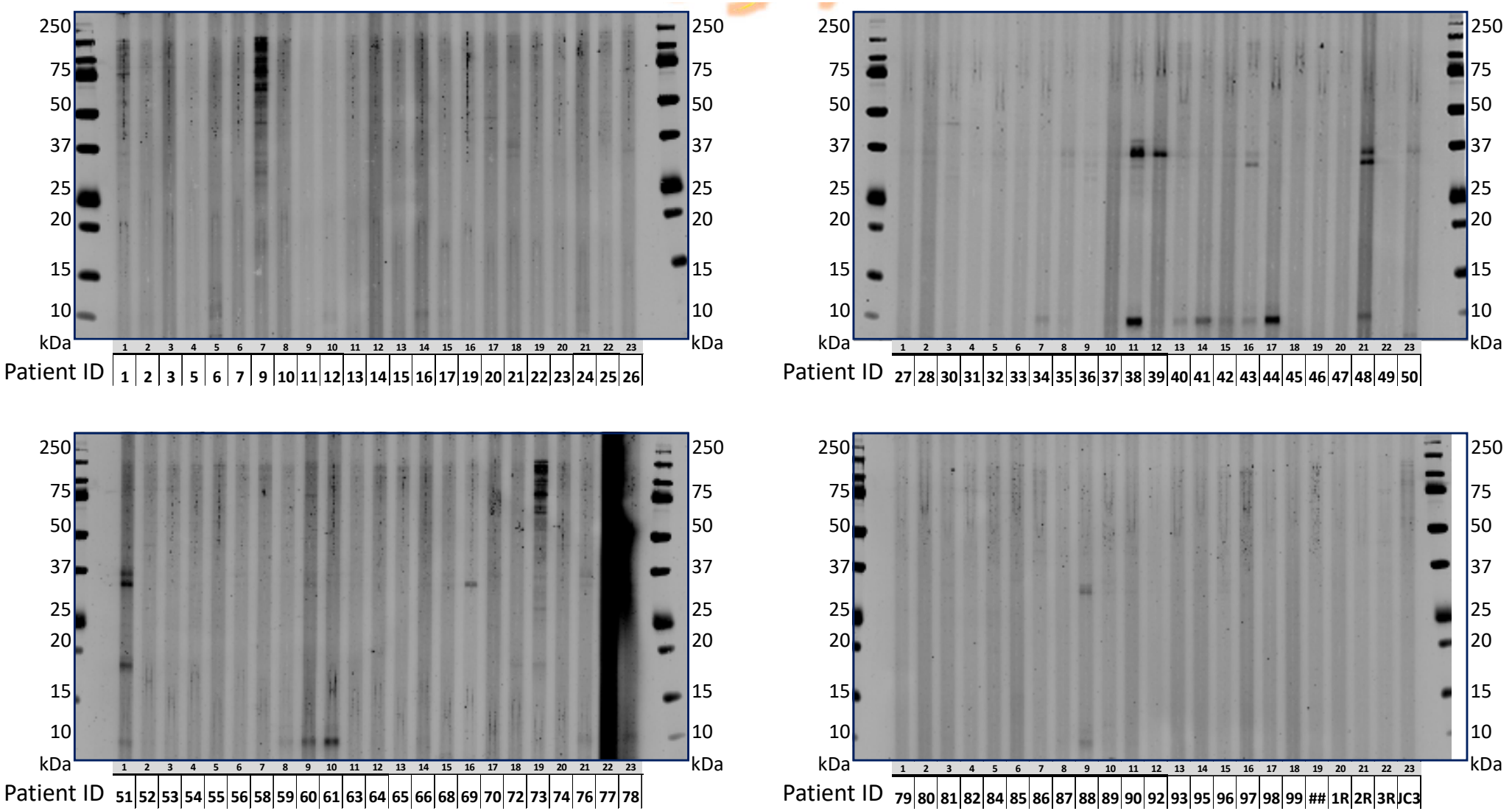


Figure 2.11: IgE-surfblots with yellowfin tuna (*Thunnus albacares*) heated muscle protein extract (100 µg each).
See Appendix C Table 1 for patient details and Appendix Table 2 for corresponding densitometric analyses. See Chapter IV A for methods and data analyses.

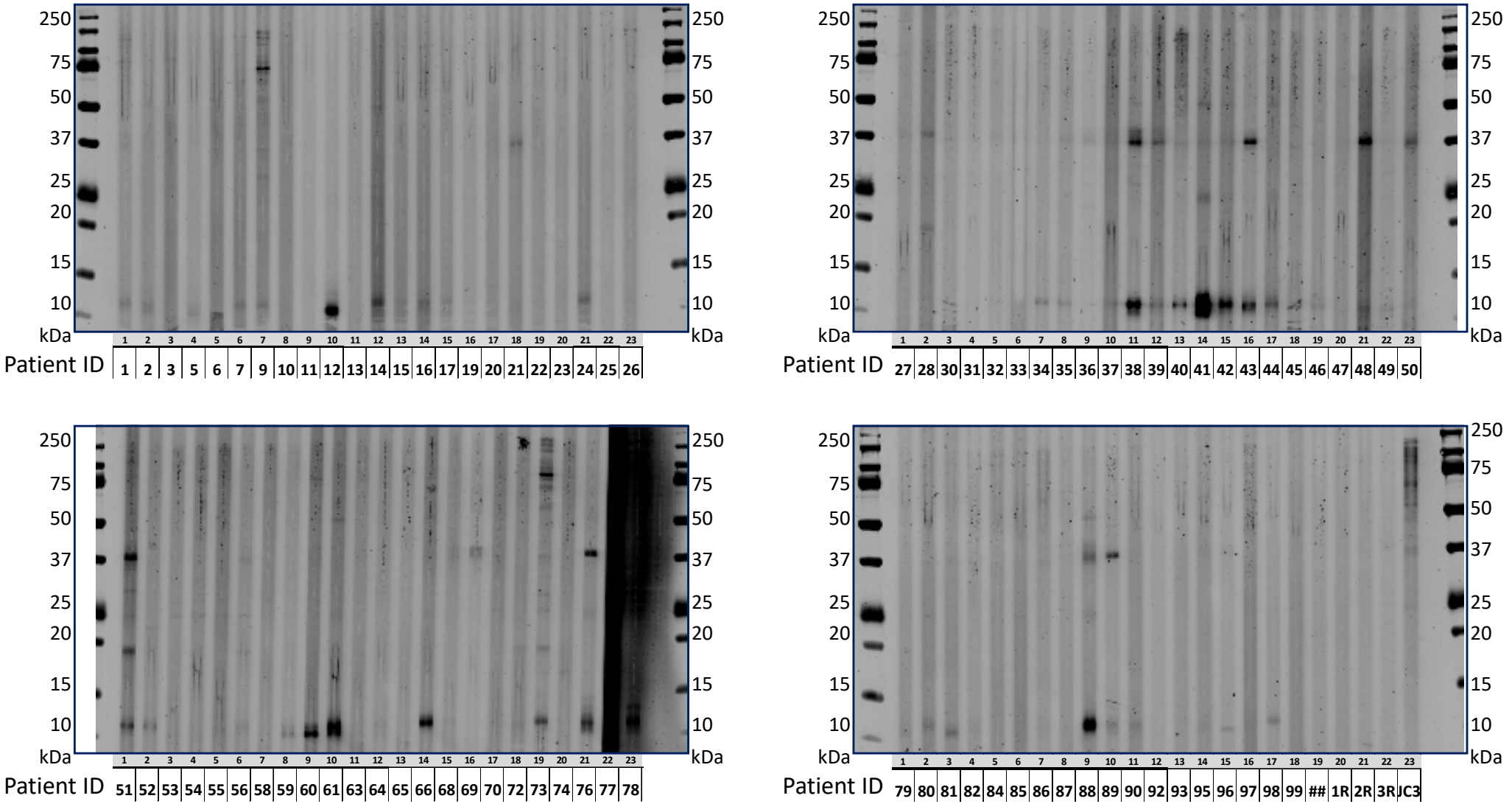


Figure 2.12: IgE-surfblots with Atlantic salmon (*Salmo salar*) heated muscle protein extract (100 µg each).
See Appendix C Table 1 for patient details and Appendix Table 2 for corresponding densitometric analyses. See Chapter IV A for methods and data analyses.

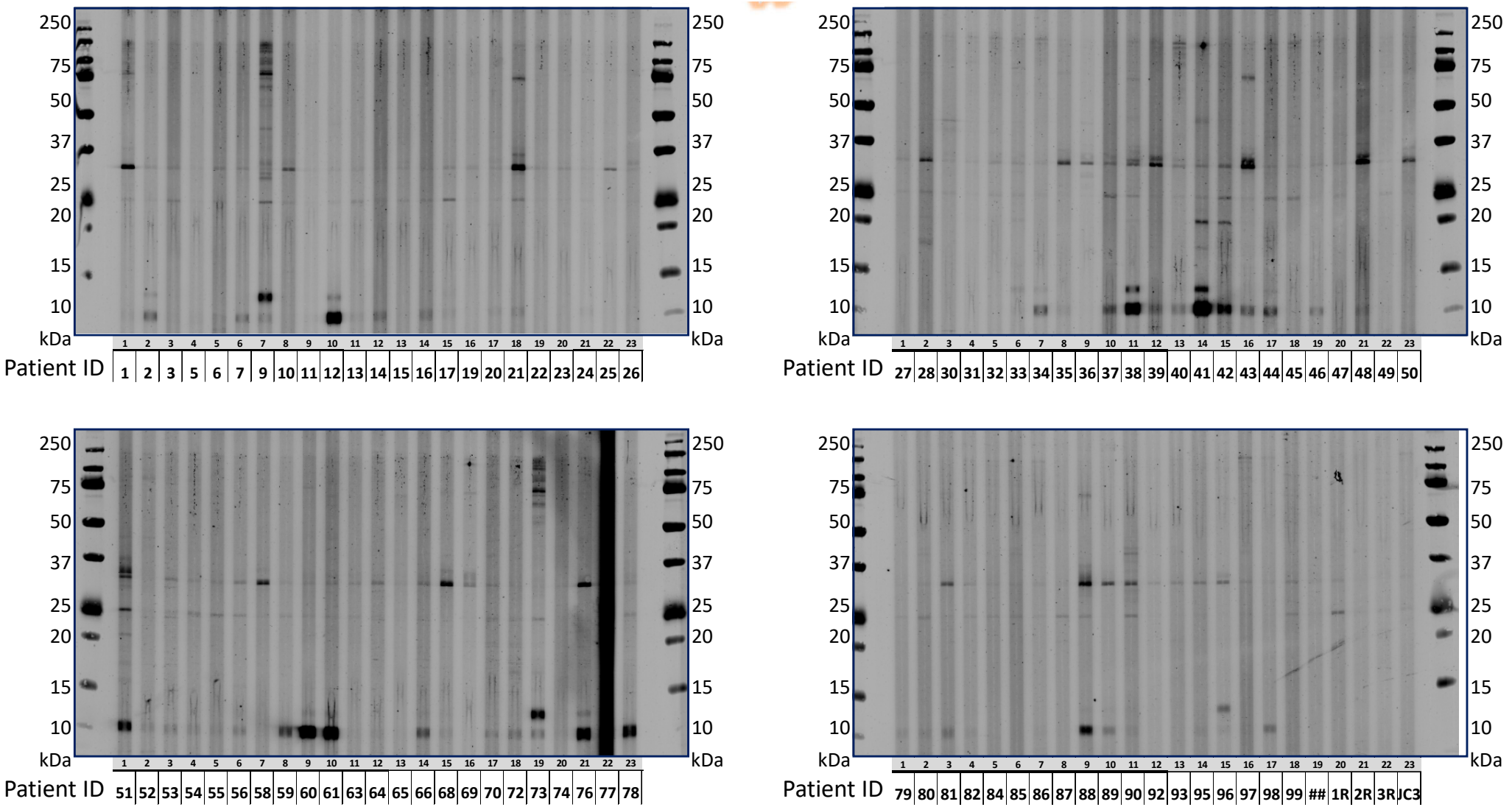


Figure 2.13: IgE-surfblots with <u>*Pangasisus*/catfish</u> (*Pangasianodon hypophthalmus*) <u>heated</u> muscle protein extract (100 µg each). See Appendix C Table 1 for patient details and Appendix Table 2 for corresponding densitometric analyses. See Chapter IV A for methods and data analyses.

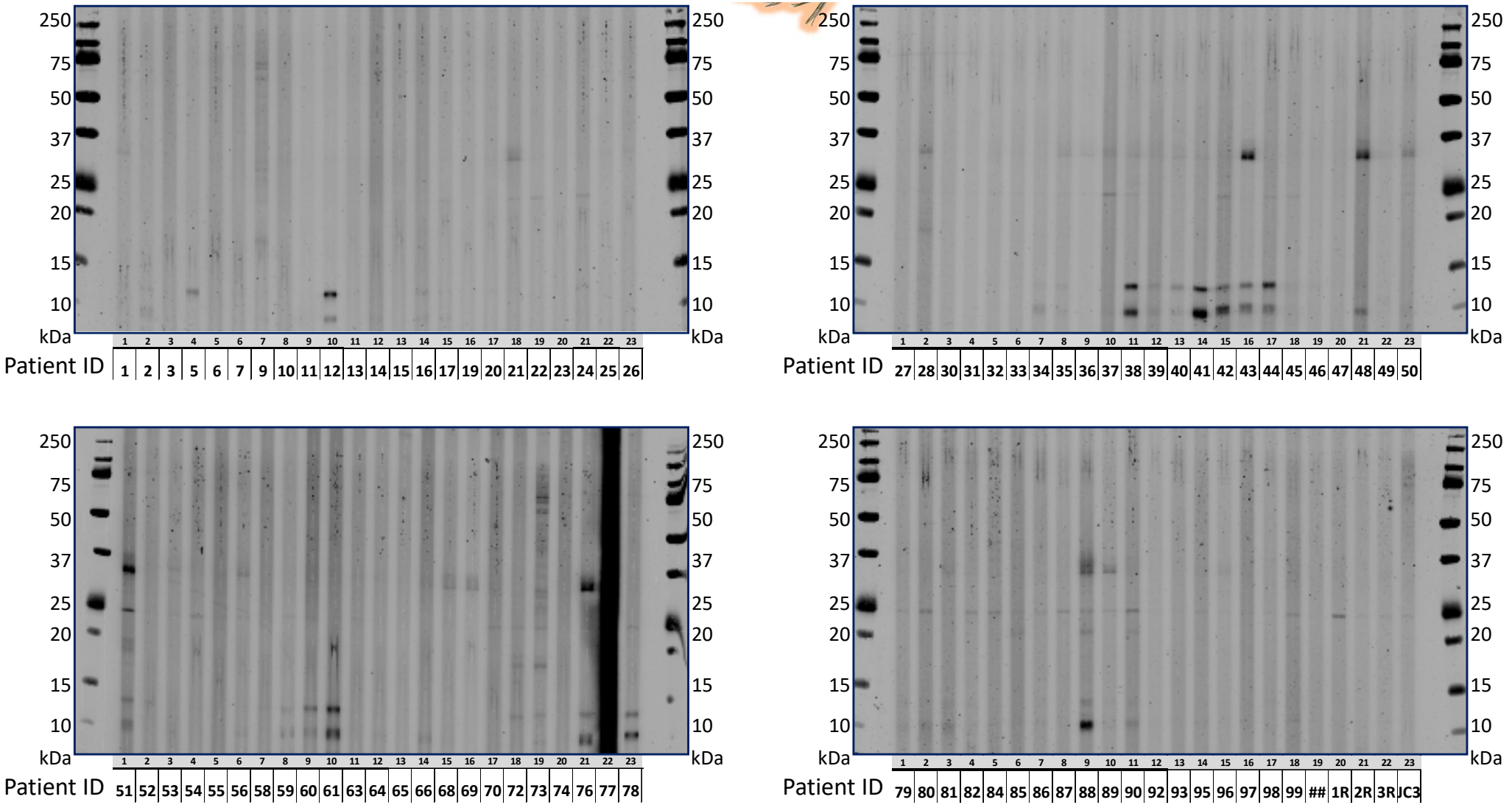


Figure 2.14: IgE-surfblots with John Dory (*Zeus faber*) heated muscle protein extract (100 µg each).
See Appendix C Table 1 for patient details and Appendix Table 2 for corresponding densitometric analyses. See Chapter IV A for methods and data analyses.

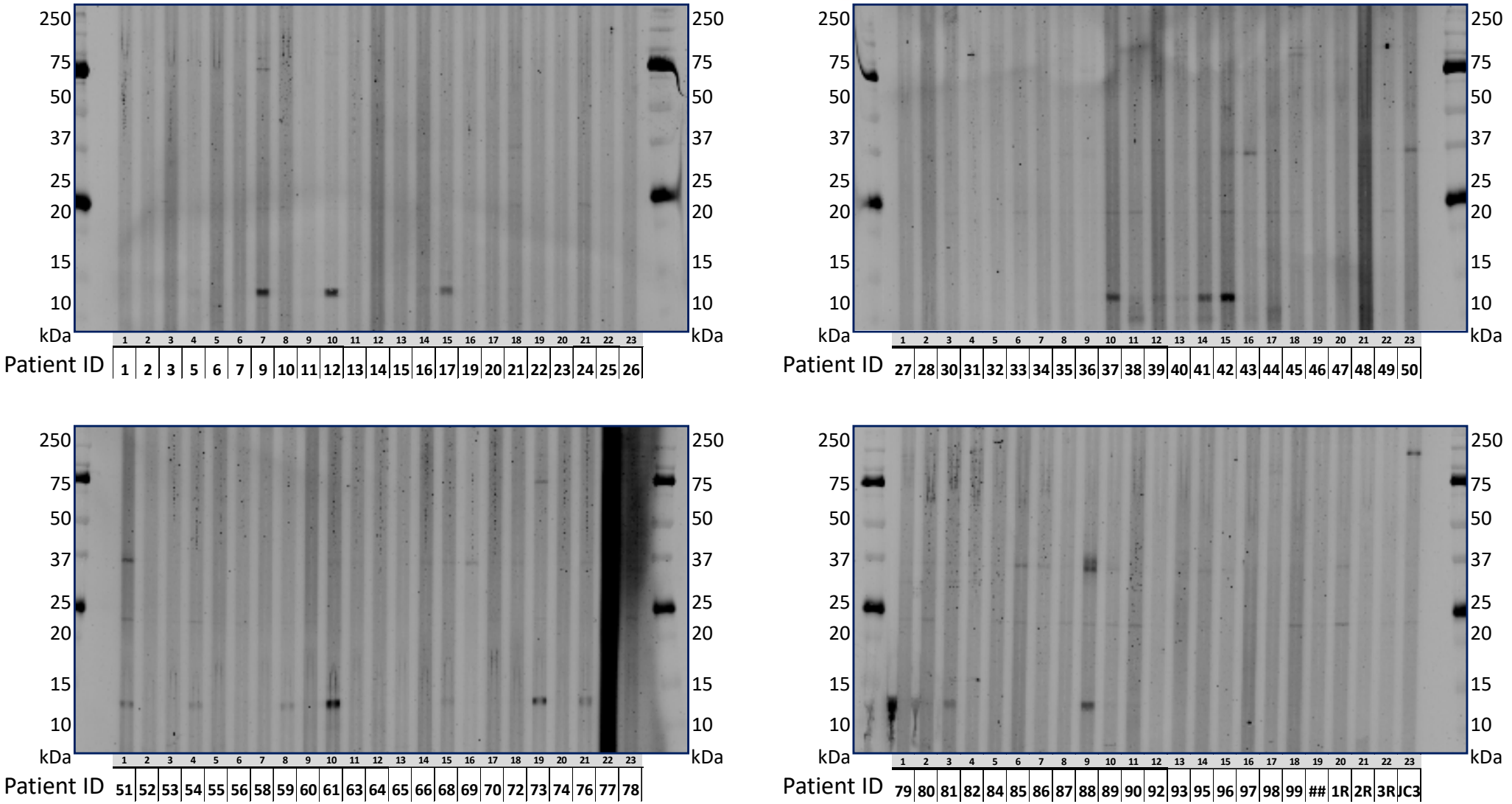
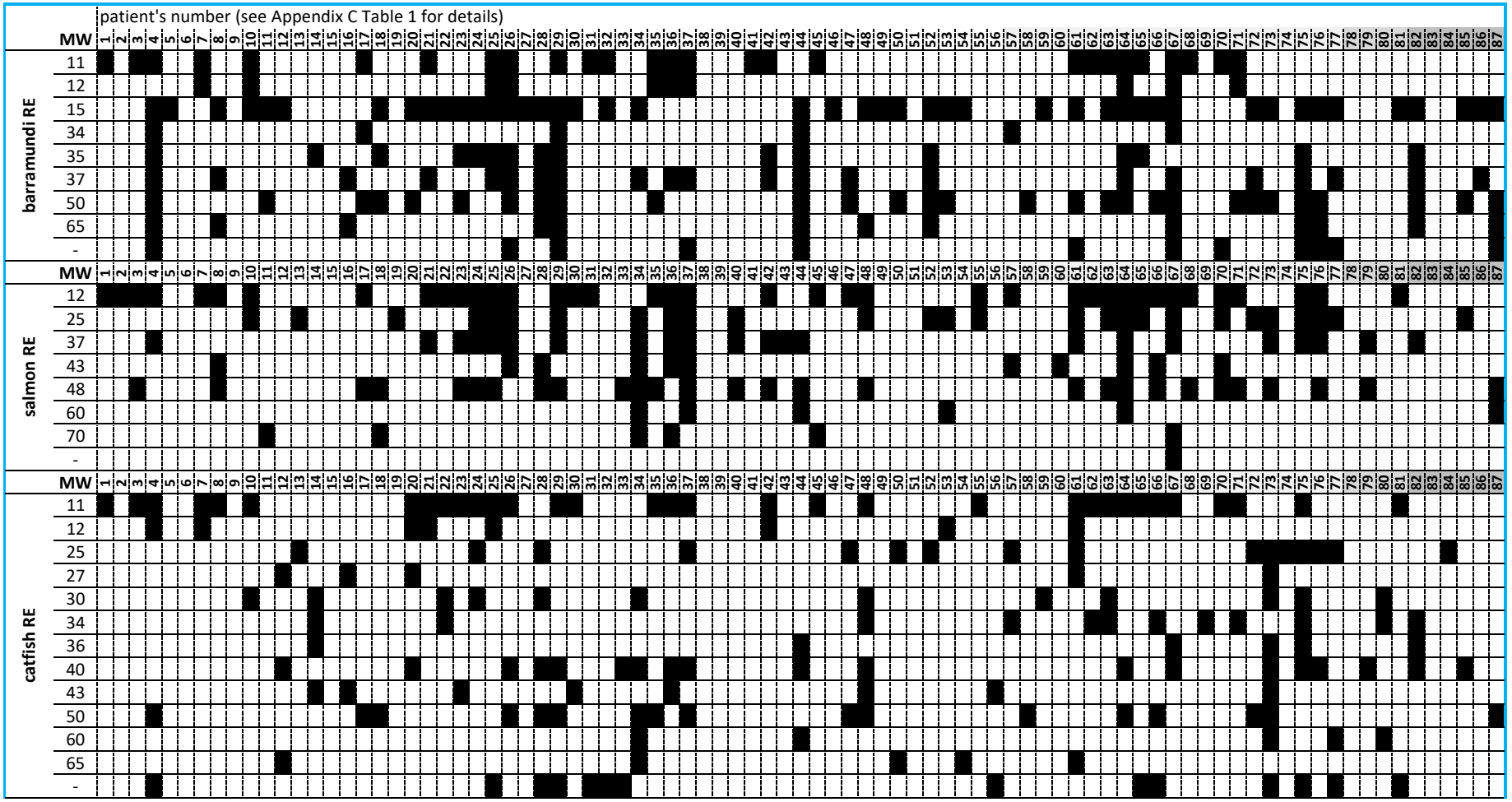


Figure 2.15: IgE-surfblots with gummy shark (*Mustelus antarticus*) heated muscle protein extract (100 µg each).
See Appendix C Table 1 for patient details and Appendix Table 2 for corresponding densitometric analyses. See Chapter IV A for methods and data analyses.

Appendix Table 2: Densitometric analyses of IgE surf immunoblots with raw (RE) and heated (HE) extracts (Appendix C Figures 2.1-15).
A black box indicates IgE-binding. MW, molecular weight of the IgE-binding band in the corresponding extract. '-', one or multiple other bands.



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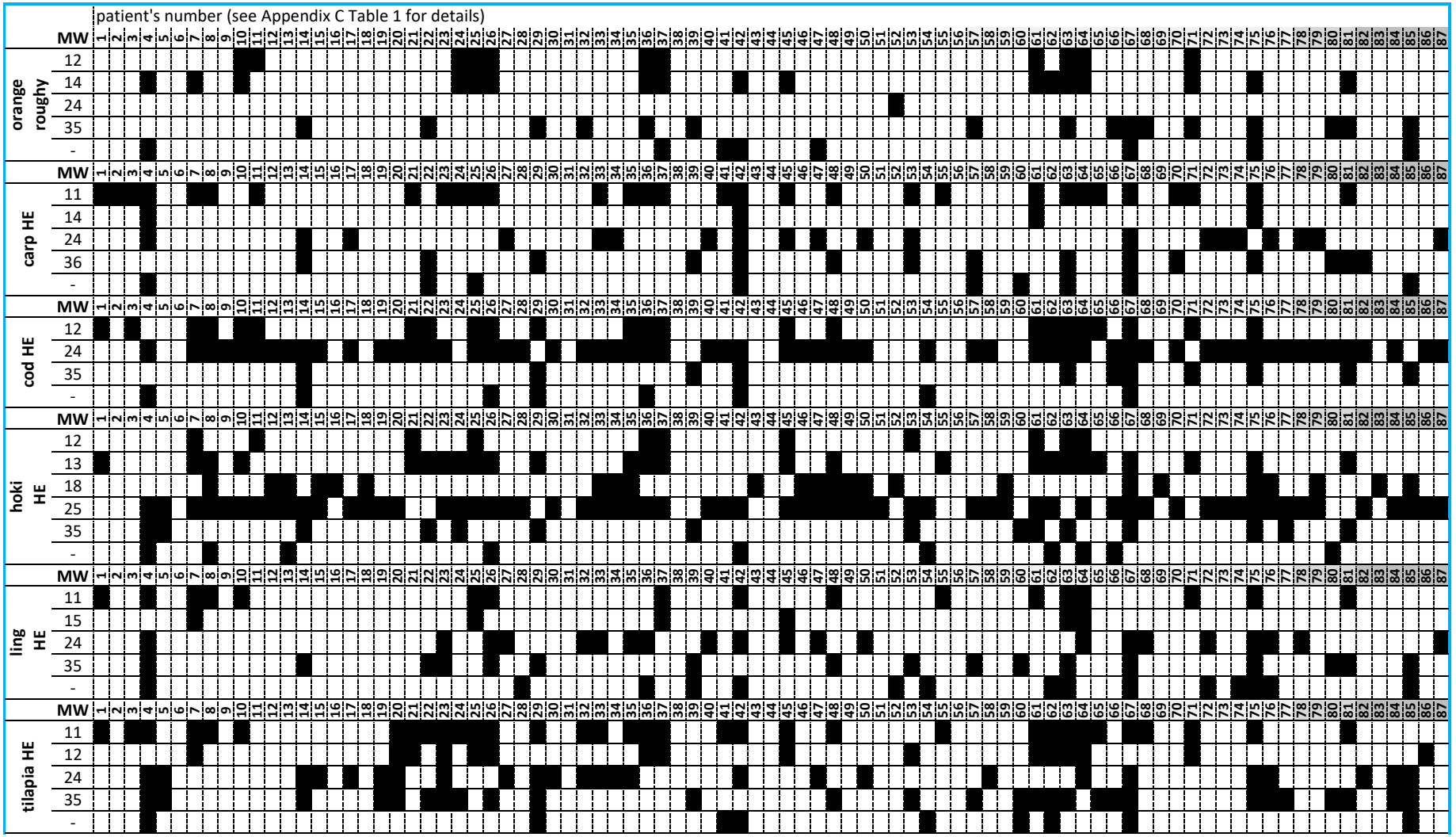

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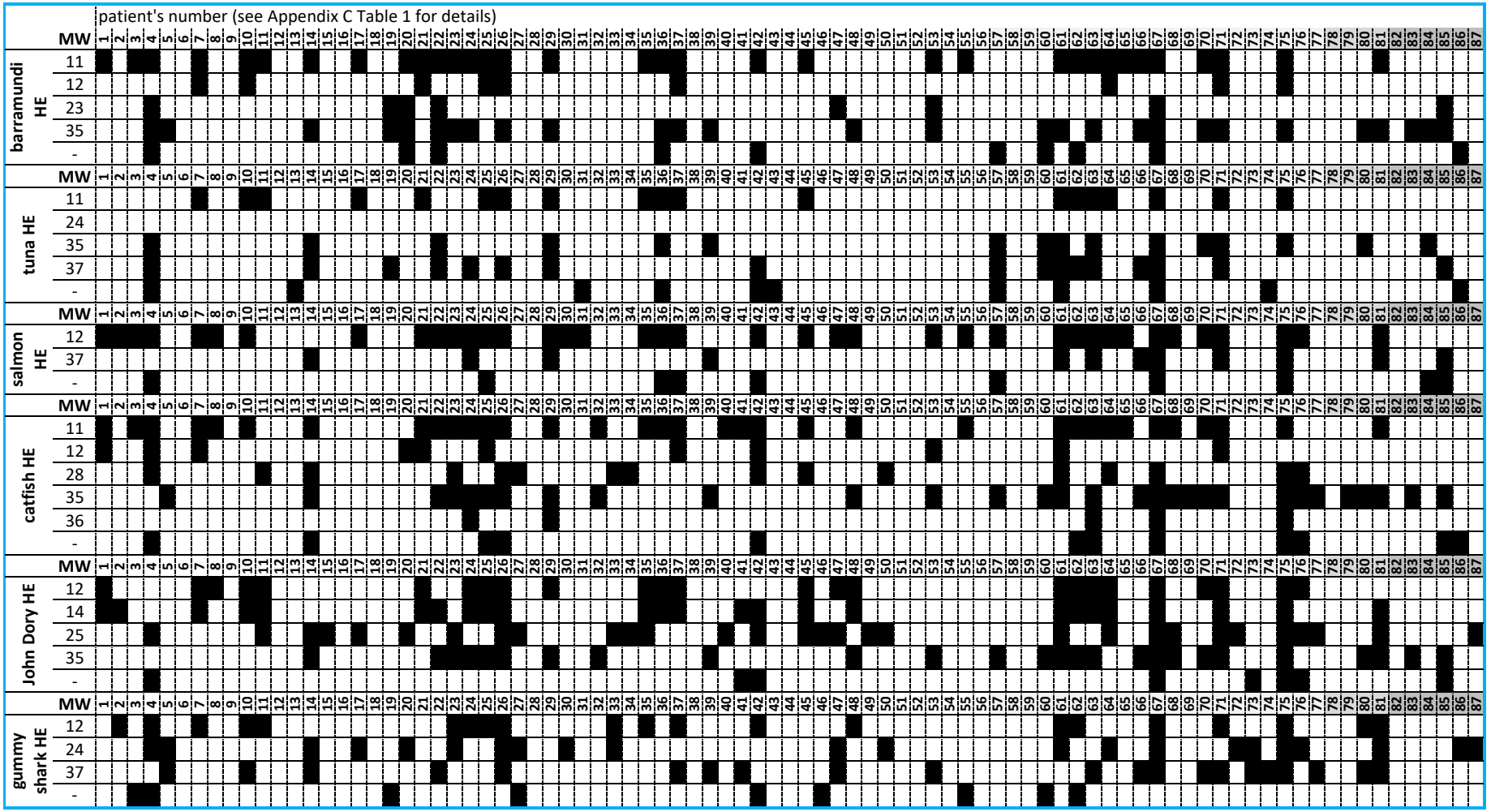
A black box indicates IgE-binding. MW, molecular weight of the IgE-binding band in the corresponding extract. '-', one or multiple other bands.



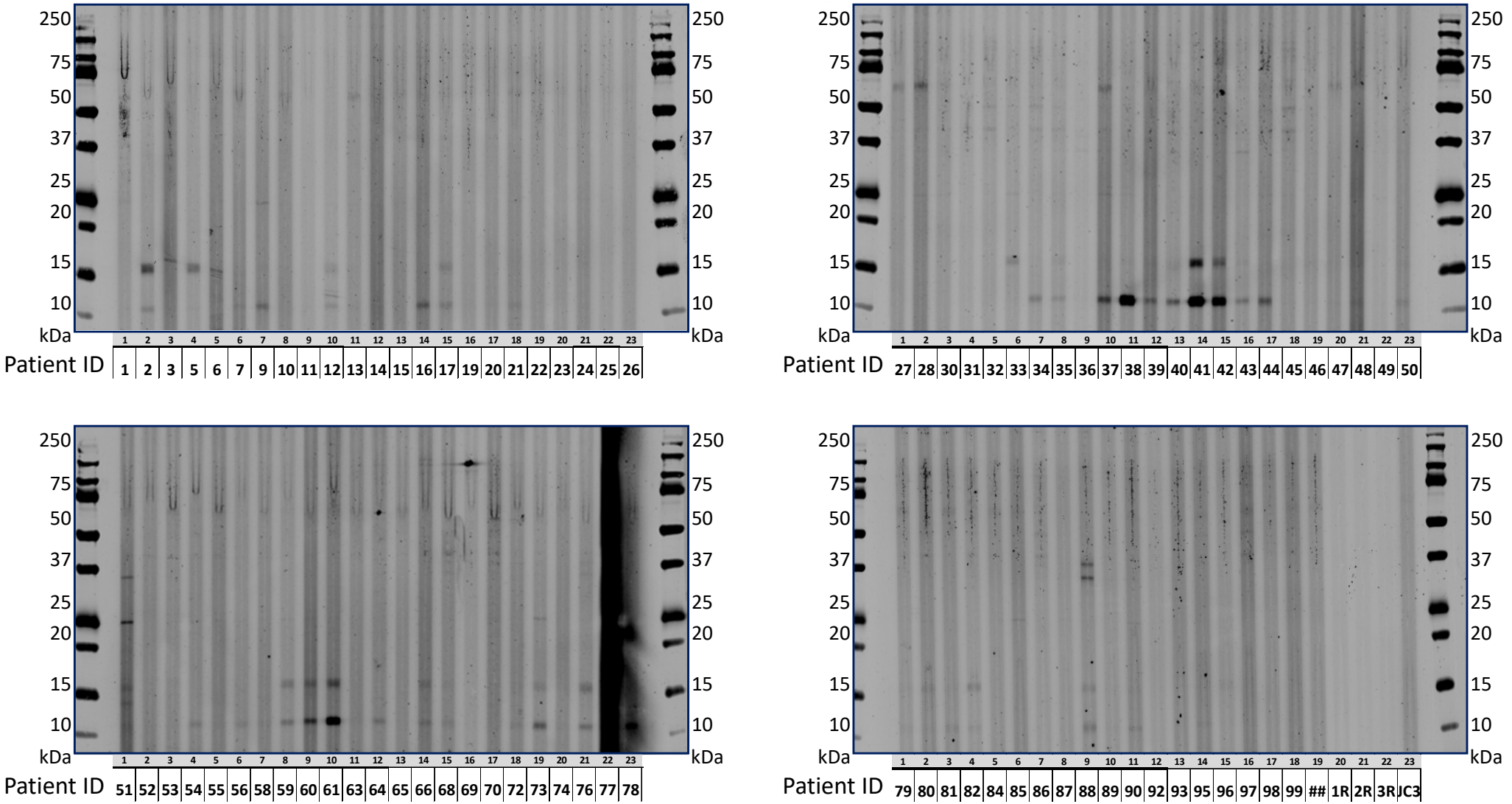
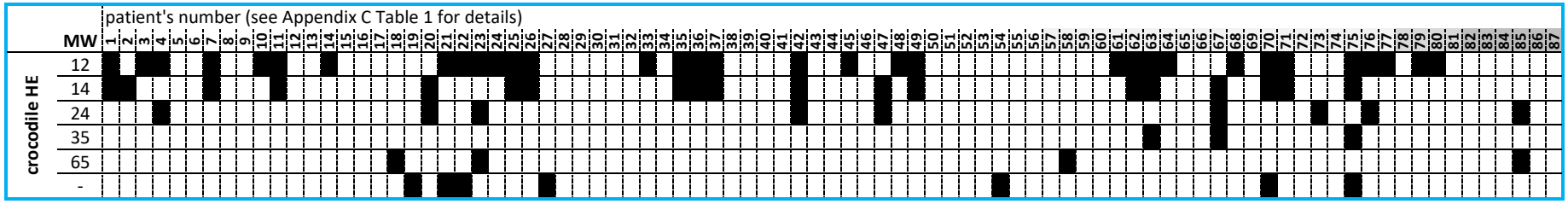


Figure 3: IgE-surfblots with saltwater crocodile (*Crocodylus porosus*) heated muscle protein extract (100 µg each).
See Appendix C Table 1 for patient details and Appendix Table 3 for corresponding densitometric analyses. See Chapter VI B for methods and data analyses.

Appendix C Table 3: Densitometric analyses of IgE surf immunoblots with heated (HE) extract from crocodile (Appendix C Figure 3).
A black box indicates IgE-binding. MW, molecular weight of the IgE-binding band in the corresponding extract. '-', one or multiple other bands.



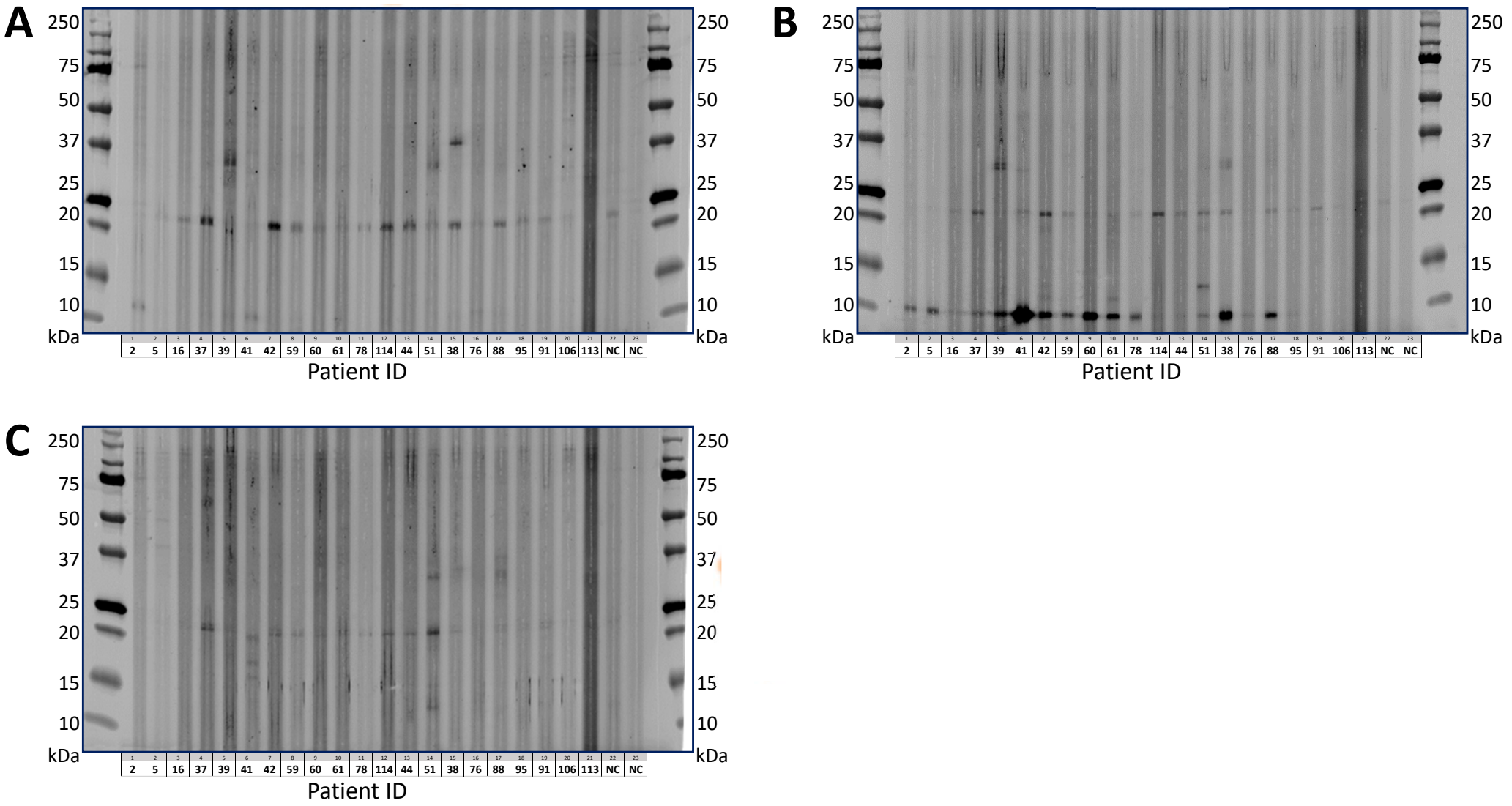
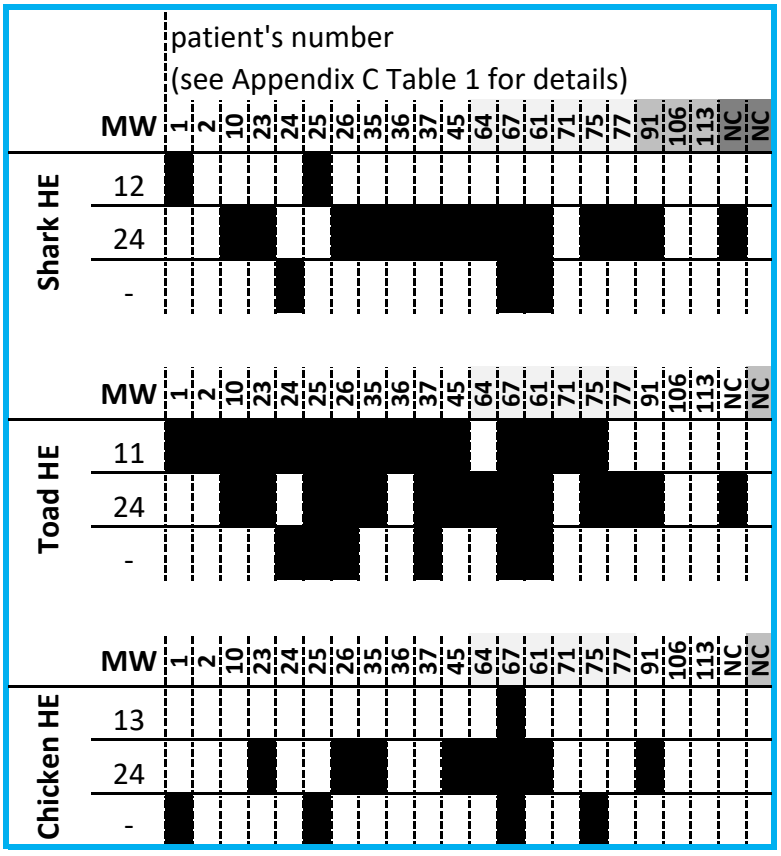


Figure 4: IgE-surfblots with heated muscle protein extract elephant/ghost shark (*Callorhinchus milii*, **A**), cane toad (*Rhinella marina*, **B**) and chicken (*Gallus gallus*, **C**) (100 µg each). See Appendix C Table 1 for patient details and Appendix Table 4 for corresponding densitometric analyses. See Chapter VI B for methods and data analyses.

Appendix C Table 4: Densitometric analyses of IgE surf immunoblots with heated extract (HE) from elephant shark (*Callorhinchus milii*), cane toad (*Rhinella marina*) and chicken (*Gallus gallus*) (Appendix C Figure 4).
A black box indicates IgE-binding. MW, molecular weight of the IgE-binding band in the corresponding extract. '-', one or multiple other bands. NC, negative controls with no history of any allergic disease.



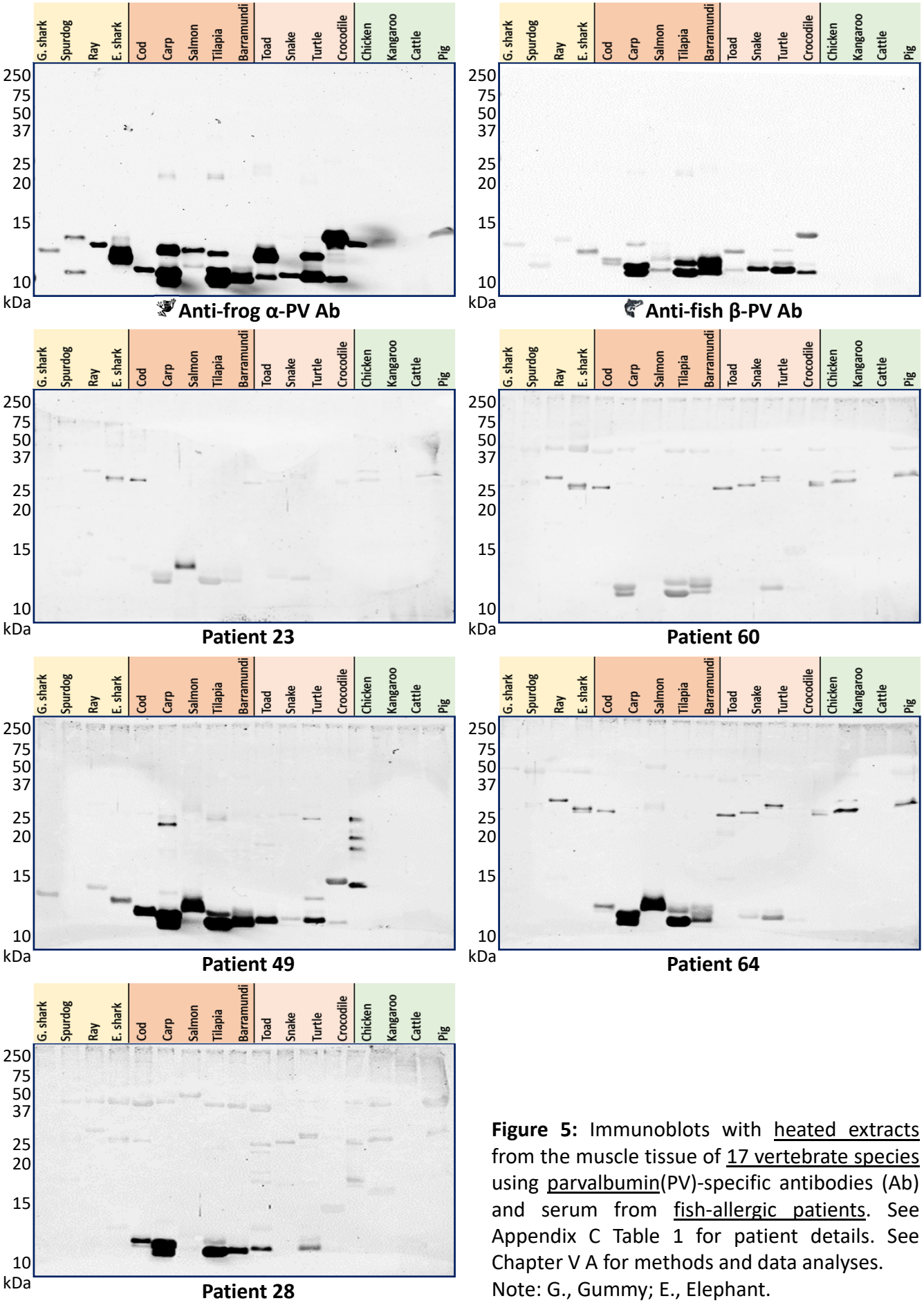


Figure 5: Immunoblots with heated extracts from the muscle tissue of 17 vertebrate species using parvalbumin(PV)-specific antibodies (Ab) and serum from fish-allergic patients. See Appendix C Table 1 for patient details. See Chapter V A for methods and data analyses.
Note: G., Gummy; E., Elephant.

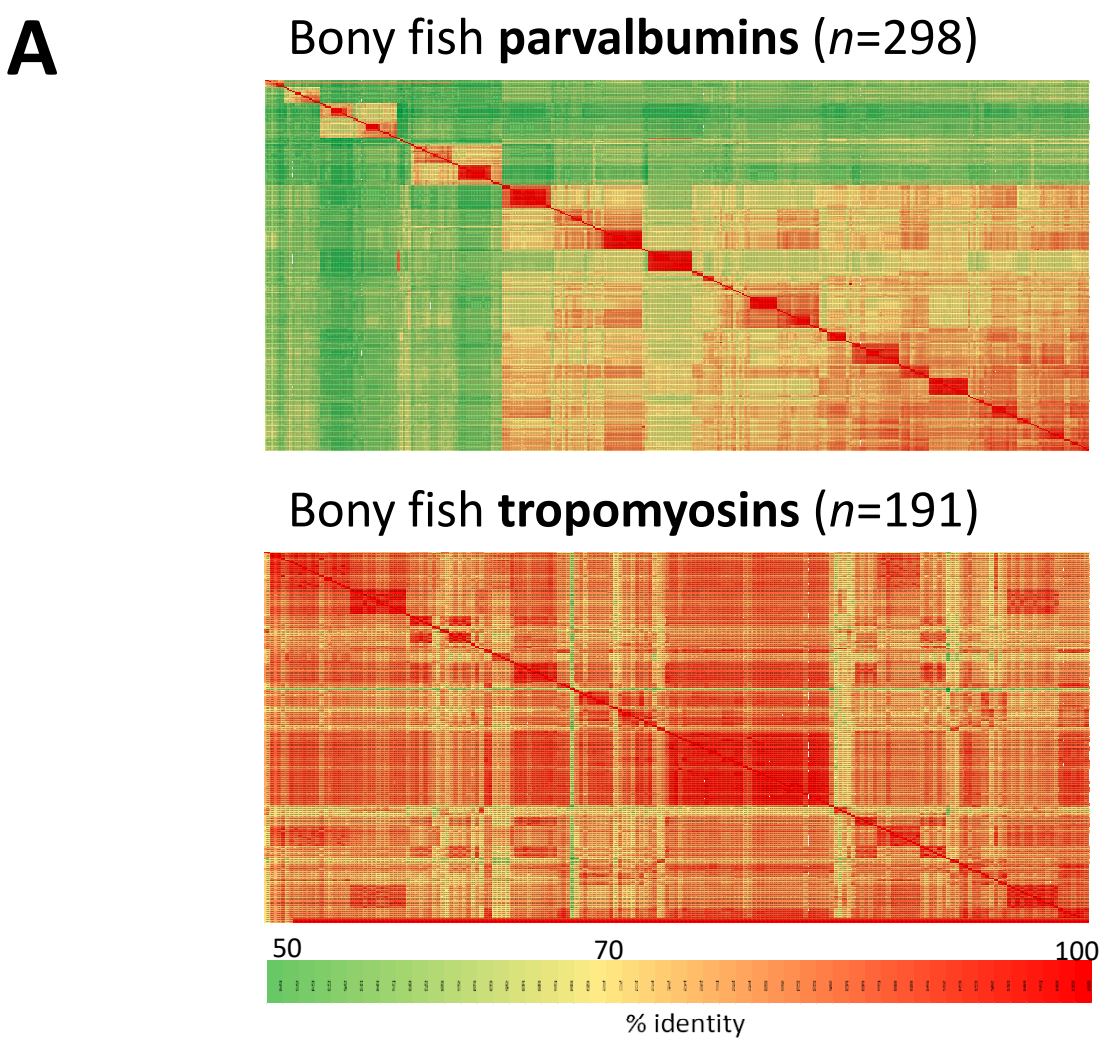


Figure 6: Conservation analyses for bony fish parvalbumin (PV) and tropomyosin (TM) isoforms. The sequence identity matrices (**A**) were generated using Clustal Omega. The bottom panel shows the colour scheme. The amino acid sequence of tropomyosin is more conserved in bony fish as compared to parvalbumin based on this analyses. Protein alignment and conservation analysis using Consurf demonstrated that tropomyosin has more conserved amino acids compare to parvalbumin (**B**). Data were generated together with Dr Roni Nugraha.

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ORIGINAL ARTICLE
Food Allergy & Anaphylaxis

WILEY

A cross-sectional, population-based study on the prevalence of food allergies among children in two different socio-economic regions of Vietnam

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Editor: Jennifer Koplin

Abstract

Background: There is a paucity of data on the prevalence of food allergy (FA) in Vietnam. A cross-sectional, population-based study was conducted to evaluate the current prevalence of FA among 2- to 6-year-old children in two different regions in Vietnam.

Method: A structured, anonymous questionnaire, modified from published FA epidemiologic studies and based on EAACI guidelines, was distributed to parents/guardians of participating children in Hue City (urban area) and Tien Giang Province (rural area). Data collected from the survey were statistically analyzed to generate the prevalence of self-reported and doctor-diagnosed FA and overarching pattern of food allergens.

Results: A total of 8620 responses were collected (response rate 81.5%). Children in Tien Giang reported more than twice the food-induced adverse reactions seen in children in Hue (47.8% *vs.* 20.5%). In contrast, children in Hue showed higher self-reported FA (9.8%) and doctor-diagnosed FA rates (8.4%) than children in Tien Giang (7.9% and 5.0%, respectively). Crustacean was the predominant allergy-inducing food in both areas (330 of 580 cases, 56.9%), followed by fish, mollusk, beef, milk, and egg. However, substantial variations of FA patterns were seen between the study sites. Geographic location and co-morbidities of other allergic diseases were key risk factors for FA ($P < 0.001$).

Conclusions: The prevalence of FA in Vietnamese children seems to be higher than previously reported from other Asian countries. Crustacean is the predominant allergy-inducing food among participating preschool children in Vietnam. The variation of reported food allergen sources across different socio-economic locations could imply different eating habits or the participation of indoor and outdoor allergen exposure.

KEYWORDS
children, crustacean allergy, fish allergy, food allergy, prevalence, Vietnam

Abbreviations: CI, confidence interval; EAACI, European Academy of Allergy and Clinical Immunology; FA, food allergy; IgE, immunoglobulin E; OFC, oral food challenge.

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Undeclared allergens in imported packaged food for retail in Australia

Michael John Sheridan [a], Martina Koeberl [b], Claire Elizabeth Hedges[b], Erik Biros [c], Thimo Ruethers [a], Dean Clarke[b], Saman Buddhadasa[b], Sandip Kamath [a,d] and Andreas Ludwig Lopata [a,d]

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ABSTRACT

The *Australia New Zealand Food Standards Code* (*the Code*) requires a declaration of the presence of 11 different allergens made through the label on a food product. Most food recalls in Australia are now due to undeclared allergens . This survey determined the extent of undeclared allergens in imported food products on the Asian retail market in Australia. A total of 50 imported packaged foods were selectively purchased from local Asian grocery retail stores in Melbourne and the presence of undeclared gluten, milk, peanut and egg determined. Analysis was performed using commercial enzyme-linked immunosorbent assay (ELISA) (R-Biopharm). Thirty-seven undeclared allergens (gluten n = 12, milk n = 12, peanut n = 6, and egg n = 7) were detected in 23 of the 50 products analysed (46%), with 18% containing multiple undeclared allergens. The high number of undeclared allergens is alarming and in line with the increasing number of food recalls and anaphylaxis recorded in Australia.

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Introduction

The prevalence of food allergy is increasing globally with Australia having one of the highest incidences of confirmed food allergy among children (Osborne et al. 2011). The HealthNuts study (Osborne et al. 2011) confirm challenge-proven IgE-mediated food allergy reaction to egg, peanut, sesame, shellfish, and cow's milk in 10% of children and constitute the majority of undeclared food allergens in processed food products (Osborne et al. 2011; Prescott and Allen 2011).

Food-induced anaphylaxis hospital admissions rose in Australia by approximately 350% between 1997 and 2005 (Liew et al. 2009). Mullins et al. found hospital admission food anaphylaxis rates increasing 1.5-fold over 7 years (2005/6 to 2011/12) (Mullins et al. 2015). In addition, Mullins et al. revealed a 10% per year increase in food anaphylaxis admissions in Australia from 1997 to 2013 (Mullins et al. 2016). Also, hospital fatal food anaphylaxis rates increased by 9.7% per year over the same period (Mullins et al. 2016).

There is no cure for food allergy, so management relies on strict food avoidance. Undeclared allergens in packaged food present a serious health risk to allergic consumers. Despite mandatory food-labelling laws, accidental ingestion of undeclared allergens in food products is common globally, causing frequent life-threatening and sometimes fatal reactions (Burks et al. 2012).

All food products sold in Australia must comply with the *Australia New Zealand Food Standards Code*, set by Food Standards Australia and New Zealand (FSANZ). According to *the Code*, all mandatory allergens must be labelled on food products. Accurate and complete food label information is a primary risk management control to assist allergic consumers in avoiding potentially contaminated foods (Gendel 2013). Consumer interpretation of label information has been associated with perceived level of risk and consumer behaviour (Hefle et al. 2007; Zurzolo et al. 2012). A study from Canada on 1,454 peanut allergic respondents demonstrated that 47% attributed accidental exposure to inappropriate labelling (Sheth et al.

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2010). Similarly, a survey of food allergic consumers performed by the German Allergy and Asthma Association found more than 40% of reactive respondents reported allergen presence, which was not declared on the label of food products (Schnadt and Pfaff 2016). A European Union prospective cohort study reported that 37% of patients attributing accidental allergic reactions to mandatory allergens not declared on the product label, including peanut, milk and sesame (Blom et al. 2018). In line with these findings, a recent Australian survey of allergy clinicians over a three-month period reported 14 incidents of anaphylaxis due to suspected consumption of packaged food containing undeclared allergens (Zurzolo et al. 2018).

In Australia, FSANZ also coordinates and monitors food recalls due to contamination (FSANZ 2017). Indications from recent Australian food recalls, often associated with high-risk food categories, continue to show that the majority of food recalls are due to undeclared allergens and are the most frequent cause for recalls (FSANZ 2017). Categorisation of food groups enables regulatory bodies, including FSANZ, to partition food recalls for better monitoring and reporting (Bucchini et al. 2016).

Koeberl et al points out that Australian regulatory agencies take a 'lightly regulated rather than strictly regulated approach', as a balance between the costs of regulatory burden and system efficiency (Koeberl et al. 2018). While food labelling appears well regulated in Australia and largely adopted by industry, this is less common in some Asian countries. Australian food imports continually increase, with the total share from the Association of Southeast Asian Nations (ASEAN) increasing from 18 to 23% from 2002 to 2012 (DAWR 2014). The aim of the current study was to determine the extent of compliant food products sold in Australia according to the *Australia New Zealand Food Standards Code*. This pilot study selectively purchased imported packaged food products, from a number of Asian countries, for sale in Asian retail grocery stores in Melbourne, Australia.

Materials and methods

Food selection

A total of 50 packaged food samples were purchased from six Asian retail grocery stores across Melbourne, Australia. Food products were selected according to the following criteria: (1) Products lacking evidence of labelling for the four allergens to be analysed and (2) packaged foods were selected based on the publicly available FSANZ guidance list of food recall categories: 'Mixed and/or Processed Foods' (n = 17), 'Bread and Bakery' (n = 14), 'Confectionery' (n = 13) and 'Non-Alcoholic Beverages' (n = 6). A detailed description of the selected food types per category is listed in Table 1.

Food samples that could be identified by name on the label were designated into one of these four categories. Food samples lacking label identification were given generic names based upon visual interpretation, if not evident from product label and/or contents. Grocery stores and food products (brands and identifying product names) have been de-identified for this study. All packaged food products were dry and shelf stable and transported and stored at ambient temperature in original packaging.

Sample analysis

Sample preparation and analysis were performed at the Food Allergens Laboratory of the National Measurement Institute (NMI), Analytical Services Branch, Port Melbourne, Australia. The ELISA test kits from R-Biopharm, utilised in this study, are validated and certified in many studies and the test for gliadin is a Codex Alimentarius Type I method, approved by the AACC and the AOAC. The aim of this study was not to compare the actual values of these allergens, but the simple detection of allergen in each of the analysed

Table 1. Food product categories.[a]

Food Categories	Examples of food types
Mixed and/or Processed Foods	Custard mix, custard powder and blanc mange powder, jelly, dairy and fat-based desserts, dips and snacks, sauces and toppings (including mayonnaises and salad dressings), soup bases, snack food including chips and crackers, frozen meals, canned products including spaghetti and baked beans.
Bread and Bakery	Breads and related products, including fancy breads, biscuits, cakes and pastries.
Confectionery	Lollies, jubes, gelatin-based candies.
Non-Alcoholic Beverages	Water including carbonated, mineralised and soda waters, water based flavoured drinks including fruit drink and soft drink, fruit and vegetable juices, coffee, coffee substitutes, tea, herbal infusions and similar products.

[a] FSANZ Guidance List – These definitions were obtained from FSANZ and are referred to on the Food Recall Statistics web page under the heading 'food categories associated with food recalls' (FSANZ 2017).

extracts (R-Biopharm AG, Darmstadt, Germany; RIDASCREEN®FAST Peanut R6202, RIDAS CREEN®FAST Ei/Egg 6402, RIDASCREEN®FAST Milk R4652, RIDASCREEN® Gliadin R7001, Immer et al. 2004; Weiss et al. 2014, 2016). Refer to Table 2 for R-Biopharm RIDASCREEN® LOD and LOQ.

Sample preparation and sample analysis were conducted according to the manufacturer's protocols. Each sample was analysed once, using duplicate wells for standards and samples. Tested samples with detected allergen were retested. Samples that resulted in a concentration higher than the standard curve were retested with dilutions to fall within the standard range of the respective ELISA kit. The result was multiplied by the dilution factor for final concentration of the sample. Conversion of whole peanut and egg results to equivalent protein (multiplication by 0.22 and 0.49, respectively) was in accordance with manufacturer instructions. ELISA systems are the most utilised technique for the detection of food allergens in various food matrices (Koeberl et al. 2018). However, ELISA kits have some disadvantages, as analytical tool because values calculated by one ELISA kit cannot be considered as absolute values. Nevertheless, one can still compare if a food sample is regarded positive by different ELISA kits (Senyuva et al. 2019).

Label verification

The information provided on the food-packaging label in the English language, including ingredient list, allergen warnings, manufacture and distributor information was recorded. Here, we define 'country of origin' as country of export, and assume significant ingredients originated and/or substantial manufacturing occurred in that country. Native speaking staff of the NMI translated label declarations and/or ingredient lists (in a language other than English) into English. Information provided in English and other languages were compared. This was performed to determine if the declaration was made in the original language and if any variation exists between labels.

Detected analytical results for each product sample were compared against each product food label for assessment of compliance with *the Code*, Standard 1.2.1–8(1)(d) for information required on a food label, including Standard 1.2.3–4 Mandatory Declaration Statements. In addition, assessment of English legibility requirements, Standard 1.2.1–24 (Australia New Zealand Food Standards Code 2017; Australia New Zealand Food Standards Code 2018) and for 'hidden allergens', *the Code*, Standard 1.2.4–4 (Australia New Zealand Food Standards Code 1.2.4 2015) were conducted.

Statistical analysis

The risk of undeclared allergens being detected in food products was determined using the generalised linear models (GLM) for binomial family. A country of origin was also included in models as a covariate. The results are presented as risk ratio (RR) with 95% confidence interval. Statistical significance was defined at the conventional 5% level. All computations were performed using the Stata/MP 13.1 statistical package (StataCorp LP, USA).

Results and discussion

The present study reveals a high percentage of imported packaged foods with undeclared allergen (46% for sale in Asian stores in Melbourne, Australia). The analytical detected allergens and their distribution amongst the four food product categories are shown in Figure 1. The highest

Table 2. R-Biopharm RIDASCREEN® analysis limits (lower limits) for limit of detection and quantification.[a.]

	Peanut (whole peanut) mg/kg	Gliadin (Gluten) mg/kg	Milk (Protein) mg/kg	Egg (whole egg powder) mg/kg
Limit of Detection (LOD)	0.13	0.5 mg/kg gliadin or to 1 mg/kg gluten	0.7	0.5
Limit of Quantification (LOQ)	2.5	2.5 mg/kg gliadin or to 5 mg/kg gluten	2.5	1.0

[a]Biopharm RIDASCREEN® (R-Biopharm AG, Darmstadt, Germany): R-Biopharm
RIDASCREEN® FAST Peanut Art. Nr. R6202 (20 March 2016), RIDASCREEN® FAST Ei/Egg Protein Art. Nr. 6402 (14 December 2015), RIDASCREEN® FAST Milk Art. Nr. R4652 (09thJuly 2015) and RIDASCREEN® Gliadin Art. Nr. R7001 (9 October 2015). Supplied by R-Biopharm Australia, 34 Woodfield Boulevard, Caringbah, NSW.

number of allergens was detected in the 'Bread and Bakery' category with 26 allergens in 14 products. This was followed by 'Confectionery' (8 in 13) and 'Mixed and/or Processed Foods' (3 in 17). No undeclared allergen was detected in six coconut drinks ('Non-alcoholic Beverages') analysed, which may reflect increased regulatory scrutiny due to a large number of recent food recalls from this category (FSANZ 2015). A total of 37 allergens detected in all, with 18% of all products contained multiple undeclared allergens.

The Code 1.2.3–4 does not allow for the presence of any foods or substances listed in the mandatory declaration requirements, excluding exemptions (Australia New Zealand Food Standards Code 1.2.3 2017). In Australia, Voluntary Incidental Trace Allergen Labelling (VITAL), an industry initiative to formalise Precautionary Allergen Labelling (PAL), performed dose-distribution modelling for PAL recommendations and proposed threshold reactions in 1% of the representative population (ED_{01}) (Allergen Bureau 2011). These recommendations include 0.2 mg for peanut protein, 0.03 mg for egg protein and 0.1 mg cow's milk protein (Allen et al. 2014). The 'Summary of the VITAL Scientific Expert Panel Recommendations' includes a wheat protein threshold of 1.0 mg (Allergen Bureau 2011). It was noted that for wheat-allergic consumers, foods containing <20 mg/kg, would be largely protective (Allergen Bureau 2011). In this current study, none of the samples with detectable peanut, egg or milk, was below the VITAL recommended threshold values. For this comparison, the results for whole peanut and whole egg were converted to equivalent protein. The concentration of some allergens, in particular milk, peanut and gluten was over 8,000 mg/kg. Figure 2 reveals some of the allergens were present in very high concentrations, with 75% of gluten containing products above 8,000 mg/kg and also one-third of milk containing products. The low levels of detected allergens can occur due to cross-contamination or sharing the same processing equipment. The high values detected represent percentages of the food product analysed, which are more likely to be intentionally added to the food product.

The highest number of undeclared allergens was in the category 'Bread and Bakery' products for gluten and milk (Figure 2), followed by egg and peanut. The overall risk of detecting undeclared allergens is shown in Table 3, with the highest risk for gluten with 2.19, followed by milk (1.95), peanut (1.35) and egg (1.22). However, the risk was the highest for undeclared gluten in this category with 4.3, followed by milk (3.44) and interestingly the lowest risk was for peanut (0.7) (Table 4).

These findings appear to somewhat contrast FSANZ food recall statistics (1 January 2007 and 31 December 2016) showing 'Mixed and/or Processed Foods' category with the highest number of recalls, more than double than in the category 'Bread and Bakery' (FSANZ 2017).

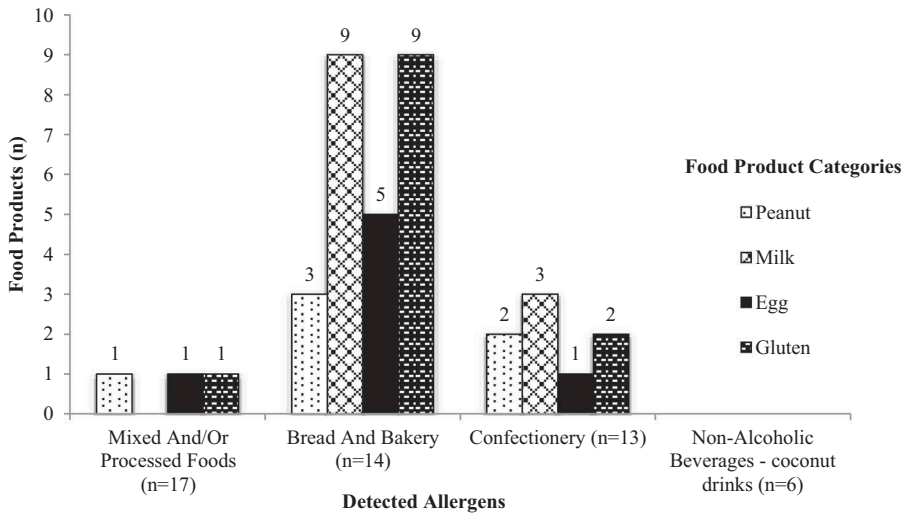

Figure 1. Distribution of detected allergens in food product categories.

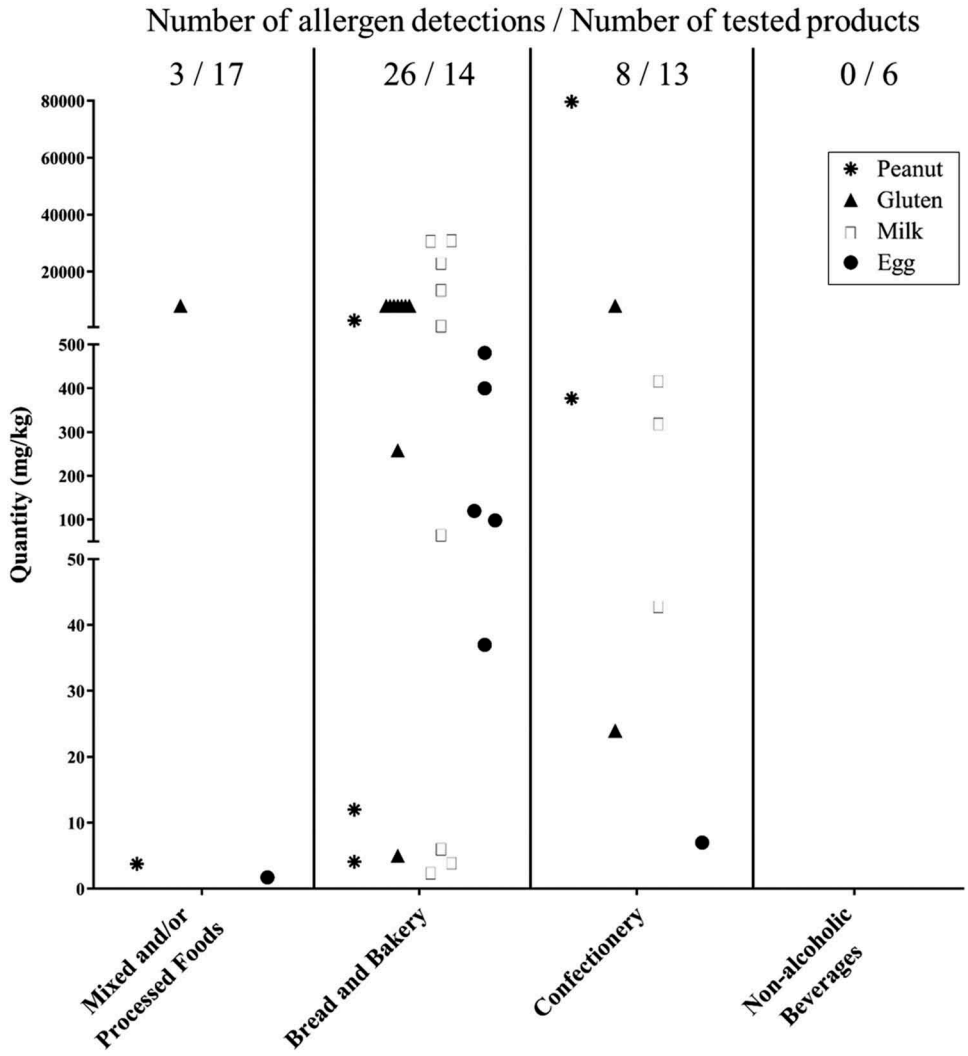

Figure 2. Distribution of detected food allergens within the four food product categories.

Table 3. Undeclared Allergen risk profile.

Allergen	Risk Ratio	P-value	95% Confidence Interval	
Peanut	1.35249	0.000	1.352168	1.352817
Gluten	2.19374	0.001	1.400028	3.437428
Milk	1.95228	0.001	1.337709	2.849213
Egg	1.21596	0.000	1.215677	1.216261

Note: Top 4 undeclared allergens most likely to cause food contamination are: gluten > milk > peanut > egg. The effect size (risk ratio) was determined using generalised linear models for binomial family.

Table 4. 'Bread & Bakery' undeclared allergen risk profile.

Allergen	Risk Ratio	P-value	95% Confidence Interval	
Peanut	0.705807	0.000	0.705638	0.705977
Gluten	4.311255	0.000	1.942099	9.570532
Milk	3.443748	0.002	1.568817	7.559455
Egg	1.378119	0.000	1.377788	0.222577

Note: The highest risk that the undeclared allergen is present in bakery products is for gluten > milk > egg > peanut, as determined using generalised linear models for binomial family.

Nevertheless, FSANZ statistics include all food recalls for the period, not only undeclared allergen recalls, thus not corresponding to our methodology and findings.

Studies of undeclared food allergens in Europe and the USA are numerous in contrast to Australian studies, yet each study differs in food category, labelling, allergen selection and methodology. In 2015 authorities in Denmark, Norway, Sweden, and Finland performed a survey of undeclared milk, egg, peanut, hazelnut, and gluten allergens. This extensive study on 351 imported packaged foods determined that 10% of products where not correctly labelled with the detected allergen (Bolin and Lindenberg 2016). A study by Pele et al. of 569 cookies and chocolates, for undeclared peanut and hazelnut, found that

chocolates were more likely to contain undeclared allergens than cookies (Pele et al. 2007).

A USA study of dark chocolate bars detected peanut at concentrations of 9 to 170 mg/kg^{-1}. 17% of these chocolates did not bear a label statement for peanut, while 33% contained milk at between 60 and 3,400 mg/kg^{-1} (Bedford et al. 2017). Similarly, Ford et al. found peanut more likely in products bearing a PAL statement (Ford et al. 2010). In addition, this study found egg, analysing 401 food products, in 1.8% of products with egg PAL statement and 2.6% without PAL. Furthermore, milk in 10.2% of products with milk PAL statement and 3% without, and peanut in 4.5% with PAL and nil without (Ford et al. 2010). A different study by Crotty and Taylor detected milk in 14 of 18 dark chocolate candy products bearing PAL statements, ranging in allergen levels from 3.7 to 15,000 mg/kg^{-1} (Crotty and Taylor 2010). It is noteworthy that method comparison studies can yield different results and this disparity must be considered by regulatory agencies if placing allergen thresholds on packaged foods or where quantification is cited (Senyuva et al. 2019).

In Australia there are very limited studies investigating high-risk food categories for mandatory undeclared allergens. In 2004–2005 the Western Australia (W.A.) Department of Health, Food Monitoring Program, surveyed tree nuts in 76 packaged foods for retail sale including biscuits, cakes, chocolates, convenience meals and ice cream, and found 55% (n = 76) of products with detectable allergens and 50% of these lacked label declarations (WAFMP 2006). In contrast a study by the Department of Primary Industries (DPI) Food Authority, New South Wales (N.S.W.) analysed various food categories for dairy, egg, gluten, peanuts, sesame, soy and tree nuts, and found only approximately 15% of products (n = 448) with detectable undeclared allergen not identified in the ingredient list (DPI 2018). Nevertheless, these studies differ considerably in the type of allergens and food categories analysed. This is expected because there is no single global list of mandated substances that must be declared on packaged food products. The World Health Organisation Codex Standard (STAN 1–1985) for food labelling prescribes nine foods and ingredients that are to be declared (WHO 2007). Australia's mandatory declared substances are inclusive of all nine including gluten, peanut, egg, and milk analysed in this study and in addition incorporate sesame and lupin (Australia New Zealand Food Standards Code 1.2.3 2017; FSANZ 2018).

Mandatory declarations

In Australia, food for sale is required to adhere to labelling provisions for substances that must be declared in accordance with *the Code* (Australia New Zealand Food Standards Code 1.2.3 2017). In this study, all samples were purchased selectively as described in the material and method section. Hence, all 23 products with detectable allergens did not display mandatory declaration(s) on the package labelling, non-compliant with *the Code*. *The Code* does not specify the format for displaying mandatory allergens on the product label, other than legibility requirements (AFGC 2007; Australia New Zealand Food Standards Code 1.2.1 2018). Therefore, mandatory substance(s) listed in the ingredient list fulfil *the Code* requirement regarding mandatory substance declarations.

This present study examined package labelling to determine whether products found to contain detectable allergen(s), declared these allergen(s) on the package label, in either English or in a language other than English. The language labelling for all products is summarised in Figure 3(a), while labelling for products found to contain detectable allergens are summarised in Figure 3(b). It is notable that a comparison between total products analysed and those found to contain detectable allergen showed the majority of products that did not possess a label in English were also found to contain detectable undeclared allergen; 35% of products did not possess a label in English and are thereby non-compliant with *the Code* mandate for a package label to be in English. Of the eight products with no English label, only one product failed to declare detectable peanut, yet declared detected milk, egg and gluten in the original language. This outcome suggests that the manufacturers of these products may have attempted to comply with legislation requirements. However, the importer, wholesaler or supplier may have failed to ensure entire label translation to English was applied to the package. Nevertheless, this product is still not compliant with *the Code*.

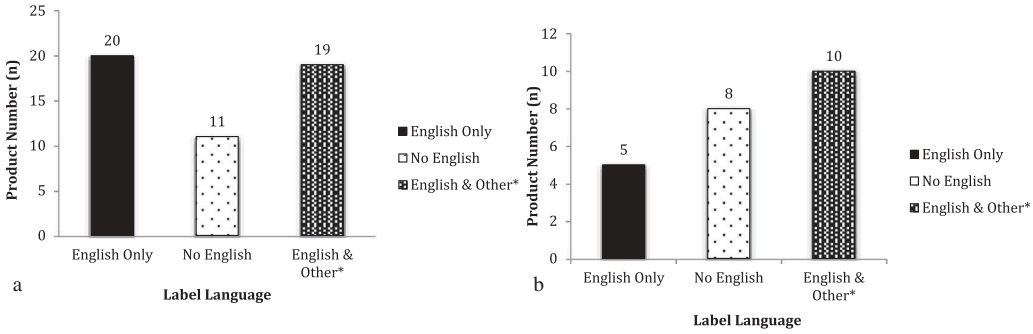

Figure 3. (a) Label language comparison for all samples (n = 50). Note: English & Other refers to a combination of both labelling in English and in a language other than English. (b) Label language comparison for positive samples (n = 23). Note: English & Other refers to a combination of both labelling in English and in a language other than English.

The Code also requires a food label statement of ingredients to identify each ingredient in accordance with Standard 1.2.4–4, which includes: "a name by which the ingredient is commonly known; or a name that describes the true nature of the ingredient; or a generic name for the ingredient that is specified … " (Australia New Zealand Food Standards Code 1.2.4 2015). Three products used subjective terminology. One contained detectable milk and declared 'butter' in both languages and one product contained detectable gluten, declared as 'flour' in both languages. In addition, one product contained detectable milk declared as 'sodium caseinate' in both languages. Consumers may be unaware that this scientific term refers to a dairy derivative.

Terminology used to describe mandatory allergen can be ambiguous leading to consumer confusion. In an attempt to mitigate this, the Australian food industry has produced guidance documents such as *The Food Industry Guide to Allergen Management and Labelling* to assist manufacturers in providing food labelling clarity (AFGC 2007) and *Unexpected Allergens in Food* to assist with identifying 'hidden allergens' (Steinman 1996; Allergen Bureau UAIF 2011; Zurzolo et al. 2012; Baker et al. 2018). Use of subjective terminology is regulated in Australia to prevent 'hidden allergens'. Packaged food containing allergenic substances that are not declared on the label, or are not legible, or provided in ambiguous or subjective terms, or in a language other than English, pose a significant health risk to allergic consumers. In this study, subjective terminology to describe a mandatory substance was discovered on both English labels and the corresponding translated labels, as well as with products that displayed a label in the original language only. This study shows that Asian packaged foods sold in Asian grocery stores in Australia possess often non-uniform presentation of labelling, resulting in products containing undeclared food allergens.

Precautionary Allergen Labelling (PAL)

Mandatory allergen labelling has improved the safety of food for allergic consumers. However, an additional form of voluntary labelling (termed Precautionary Allergen Labelling; PAL) has evolved to minimise the risk to consumers. The majority of studies in the literature focus on analysis of priority allergens in PAL-packaged foods from high-risk categories. Surveys of non-speciality supermarket packaged foods indicate that PAL statements are frequently encountered. In Australia, several studies have examined PAL products for sale, with one survey identifying that over 70% possessed PAL statements (Koplin et al. 2010). Another survey of 1,355 food products identified that 65% had a PAL advisory statement (Zurzolo et al. 2013b). In total, 128 PAL food samples from high-risk food categories, including muesli bars, sweet biscuits (cookies), savoury biscuits, chocolates and breakfast cereals were analysed for six allergens including peanut, hazelnut, milk, egg, soy and lupin. The authors found that only 7% contained detectable peanut. Since no other allergens were detected in PAL foods tested, the authors suggested that PAL packaged foods for retail sale in Australia pose a low risk to allergic consumers (Zurzolo et al. 2013a). Their findings were determined from packaged foods

sampled at major supermarkets only (Woolworths, Coles, Aldi), therefore, predominantly reflecting Australian products with a higher likelihood of scrutiny by the supermarket chains.

In contrast, our current study focused on imported foods from Asian countries for sale in Asian grocery stores in Melbourne, Australia. A very high proportion (46%) of analysed packaged foods from these stores was found to contain undeclared allergens. This study determined the number of PAL statements on product labels, although, not necessarily related to the allergen of study. PAL in the original language was not displayed on any product label in English and therefore, none of the 50 products sampled possessed a PAL statement in English. However, 22% possessed a PAL statement in a language other than English, indicating a very low level of PAL for packaged Asian food products. In comparison, of the 23 positive products 9 possessed PAL in a language other than English. Given the correlation between samples found to contain undeclared allergen and those with PAL, this finding may suggest manufacturers suspected cross-contamination. Low level PAL of Asian products suggests that food purchases from these stores pose a higher risk to allergic consumers than packaged foods purchased from major Australian supermarket chains. This is of particular importance as the food trade from Asia into Australia continues to increase by about 2.5% per annum (DAWR 2014).

Country of origin

The highest representation of food products analysed in this study came from China and Thailand followed by South Korea (Figure 4): 28% China, 26% Thailand, 14% South Korea, 10% India, Taiwan and Sri Lanka 8% respectively, 2% each for Malaysia, Japan and Vietnam. Likewise, products with the highest number of detectable undeclared allergens were imported from China (50%), followed by Thailand (39%) and South Korea (71%). However, there was no statistically significant association between country of origin and number of undeclared allergens in imported products.

Conclusion

In conclusion, this study confirms that undeclared allergens can be frequently detected in imported packaged foods from Asia for retail sale in Melbourne, Australia. In addition, these foods often contain multiple undeclared allergens, with gluten the most frequent. Those products containing undeclared allergen failed to provide mandatory allergen declarations on the label in English or in terminology consistent with regulations. Inconsistent and variable label terminology may be due to allowances in *the Code*. The difference between native language labelling in the "country of origin" and applied English labelling should be further investigated and may require greater governance.

Packaged food products lacking regulatory labelling compliance can be readily obtained from Asian grocery stores in Melbourne, Australia. Further targeted food category and alternate method studies are necessary to validate these findings, providing important information

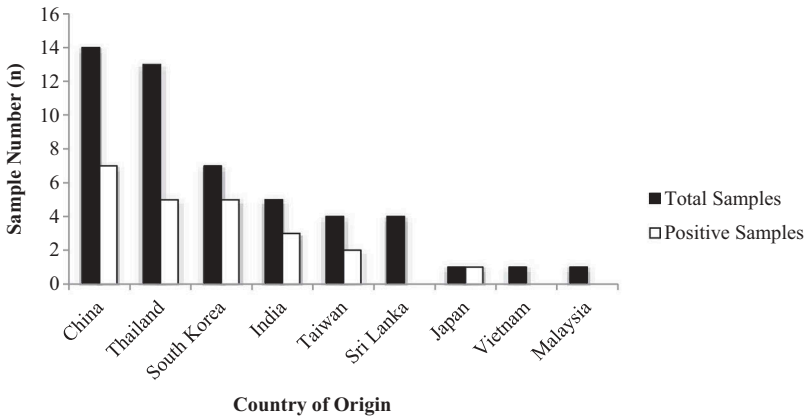

Figure 4. Comparison between total products sampled and products containing detectable allergens by country of origin.

for regulatory compliance agencies to protect allergic consumers.

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Disclosure Statement

No potential conflict of interest reported by the authors.

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REVIEW

Allergenicity of bony and cartilaginous fish – molecular and immunological properties

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Summary

Allergy to bony fish is common and probably increasing world-wide. The major heat-stable pan-fish allergen, parvalbumin (PV), has been identified and characterized for numerous fish species. In contrast, there are very few reports of allergic reactions to cartilaginous fish despite widespread consumption. The molecular basis for this seemingly low clinical cross-reactivity between these two fish groups has not been elucidated. PV consists of two distinct protein lineages, α and β. The α-lineage of this protein is predominant in muscle tissue of cartilaginous fish (Chondrichthyes), while β-PV is abundant in muscle tissue of bony fish (Osteichthyes). The low incidence of allergic reactions to ingested rays and sharks is likely due to the lack of molecular similarity, resulting in reduced immunological cross-reactivity between the two PV lineages. Structurally and physiologically, both protein lineages are very similar; however, the amino acid homology is very low with 47–54%. Furthermore, PV from ancient fish species such as the coelacanth demonstrates 62% sequence homology to leopard shark α-PV and 70% to carp β-PV. This indicates the extent of conservation of the PV isoforms lineages across millennia. This review highlights prevalence data on fish allergy and sensitization to fish, and details the molecular diversity of the two protein lineages of the major fish allergen PV among different fish groups, emphasizing the immunological and clinical differences in allergenicity.

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Running title: *Karnaneedi et al / Transcriptomic analysis of shrimp allergens*

Abstract

Shellfish allergy affects up to 2% of the world's population and persists for life in most patients. The diagnosis of a shellfish allergy, in particular shrimp, is however often challenging due to the similarity of allergenic proteins in other invertebrates. Despite the clinical importance, the complete allergen repertoire of allergy-causing shrimps remains unclear. Here we mine the complete transcriptome of five frequently consumed shrimp species to identify and compare allergens with all known allergen sources. The transcriptomes were assembled *de novo* from raw RNA-Seq data of the whiteleg shrimp (*Litopenaeus vannamei*), black tiger shrimp (*Penaeus monodon*), banana shrimp (*Fenneropenaeus merguiensis*), king shrimp (*Melicertus latisulcatus*), and endeavour shrimp (*Metapenaeus endeavouri*). Trinity was used to assemble the transcriptome, and Transrate and BUSCO applied to verify the assembly. Blast search with the two major allergen databases, WHO/IUIS Allergen Nomenclature and AllergenOnline, successfully identified all seven known crustacean allergens. Salmon was utilised to measure their relative abundance, demonstrating sarcoplasmic calcium-binding protein, arginine kinase and myosin light chain as highly abundant allergens. In addition, the analyses revealed up to 40 unreported allergens in different shrimp species, including heat shock protein (HSP), alpha-tubulin, chymotrypsin, cyclophilin, beta-enolase, aldolase A, and glyceraldehyde-3-phosphate dehydrogenase (G3PD). Multiple sequence alignment, conducted in Jalview 2.1 with Clustal Omega, demonstrated high homology with allergens from other invertebrates including mites and cockroaches. This first transcriptomic analyses of allergens in a major food source provides a valuable genomic resource for investigating shellfish allergens, comparing invertebrate allergens and developing improved diagnostics and novel immunotherapeutics for food allergy.

Keywords: Allergy; Prawn; Tropomyosin; Allergen; RNA-Seq

Introduction

Food allergy affects up to 10% of children and 10% of adults, and the prevalence is projected to be on the rise [1, 2]. Food allergy is caused through ingestion of food that contains allergenic proteins that triggers adverse reactions in sensitised individuals [3, 4]. The term “allergen” refers to a protein capable of inducing sensitisation and subsequent allergic immune responses through immunoglobulin E (IgE)-mediated type 1 hypersensitivity in patients [3-7].

Shellfish allergy is often lifelong, similar to peanut allergy, affects about 2% of the global population and appears to be highly prevalent in the Asia-Pacific region and other countries where seafood consumption is high [8-11]. A recent epidemiology study from Vietnam revealed that the prevalence of shellfish allergy is as high as 4.2% [11], while up to 3% of adults in the USA are sensitised to shellfish [1].

Among shellfish allergic individuals, shrimp allergy seems to be the most prominent crustacean allergy and remains to be difficult to diagnose and manage, for multiple reasons. Shrimp accounts for one of the most prevalent events of food derived anaphylactic reactions after peanuts and tree nuts [12-15].

The management of shrimp allergy is often challenging due to immunological cross-reactivity to molecularly similar allergens [12, 13, 16-22]. The similarity of shrimp allergens to proteins of other shellfish species, including crabs and lobsters, and other invertebrates such as house dust mites (HDM) and cockroaches, can induce unexpected allergic reactions [15-20, 23, 24]. Although this cross-reactivity has been observed in the clinical setting, the underlying molecular basis is not well understood.

Over the past decades, more than 2000 allergens are now well characterised and accessible via several databases, including the World Health Organization & International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature database (www.allergen.org) having the most stringent inclusion criteria [7, 25], and the highly peer-reviewed AllergenOnline: Home of the FARRP (Food Allergy Research and Resource Program) Allergen Protein database (www.allergenonline.org) [26, 27].

Allergen discovery is traditionally conducted using whole allergen sources and isolation of IgE antibody binding proteins [4, 28-31]. However, this approach has many

limitations, including low sensitivity and small patient cohorts that does not allow the detection of all possible allergenic proteins [32].

Here we report the first complete transcriptome analysis of shellfish food allergen source, with focus on shrimp allergens, the most common shellfish allergy. The transcriptomes of five most frequently consumed shrimp species were assembled *de novo* and screened for the presence of similar amino acid (AA) sequences to 2,172 allergens in the WHO/IUIS Allergen Nomenclature and AllergenOnline databases (Figure 1).

Results

Assessment of 15 assembled transcriptomes

Illumina HiSeq® 2500 (Illumina Australia and New Zealand, VIC, Australia) sequencing produced 125bp paired-end sequencing data with a total number of paired-end reads for each sample of approximately 20 million reads. The *de novo* assembly for 15 samples (three replicates each for five shrimp species) resulted in 28,101 to 42,510 contigs (Table 1). All 15 samples had more than 87% of read pairs that mapped back to the contigs within the assembled transcriptome. TransRate scores (assembly scores) for each of the 15 transcriptomes were approximately 0.4 (Table 1). BUSCO (Benchmarking Universal Single-Copy Orthologs) results, overall, had a complete genes (C) score ranging between 43% - 67%; fragmented genes (F) score ranging between 16% - 26%; and missing genes (M) score between 14% - 32% (Table 1). The transcriptomes of *P. monodon* and *F. merguiensis* had the highest values for complete BUSCO's (C scores) (Table 1). An observable pattern here is that both these shrimp species also had the highest number of contigs and assembly size.

Large numbers of allergens identified within the transcriptomes

After duplicate removal, the results yielded 40 unique allergen (AA sequences) identified in whiteleg shrimp (*L. vannamei*), 44 in black tiger shrimp (*P. monodon*), 42 in banana shrimp (*F. merguiensis*), 44 in king shrimp (*M. latisulcatus*), and 50 in endeavour shrimp (*M. endeavouri*) (Figure 2). Approximately two thirds of allergen AA sequences that matched with all five shrimp species' transcriptomes, belonged to shellfish, mites, and fungi species (Figure 2). The remaining allergen AA sequences belonged to plants, insects, fish and other species.

Known crustacean allergens identified

Contigs that matched with the major shrimp allergen tropomyosin (TM) were identified in all five species, with some species having more than one contig representing this allergen (Figure 3.A). *L. vannamei* shrimp's TM_Contig_1 has a 100% AA sequence identity with the previously recorded and IUIS registered Lit v 1 (ACB38288). This is a similar finding to *P. monodon*'s TM_Contig_1, which has a 100% sequence identity with Pen m 1 (AAX37288). Both TM_Contig_1's also match with a 100% similarity with each other (Figure 3.A). Overall, TM_Contig_1 of all five species showed a high

sequence similarity (pairwise identity of 99-100%) with Lit v 1 and Pen m 1, (Figure 3.A). However, TM_Contig_2 of *P. monodon* and *M. endeavouri* only showed a pairwise identity (PI) of 91% and 82%, respectively, with both Lit v 1 and Pen m 1 (Figure 3.A). The inclusion of HDM and cockroach tropomyosin allergens, Der p 10 (AAB69424), Bla g 7 (AAF72534), and Per a 7 (CAB38086), in the analyses of tropomyosin AA sequences revealed to have more than 70% PI with shrimp TM (Figure 3.A). Molecular phylogenetic tree analyses of previously published AA sequences of TM revealed that the crustacean TM's are not only very similar to each other, but also to insect and mite TM's. In comparison, molluscs, which are also grouped as "shellfish" with crustaceans, were found to be only distantly related in terms of TM AA sequence (Figure 3.B).

One contig in each of four shrimp species was identified as the arginine kinase (AK) allergen, while *M. endeavouri* had two contigs. In contrast to TM, all contigs were highly similar to each other and to the published AK allergens in *L. vannamei*, Lit v 2 (ABI98020), and *P. monodon*, Pen m 2 (AAO15713), with more than 95% PI (Figure 4.A). They were all also found to be more similar to the published cockroach AK allergens in *B. germanica*, Bla g 9 (ACM24358), and *P. americana*, Per a 9 (AAT77152), (83-84% identity) than the published HDM AK allergens in *D. pteronyssinus*, Der p 20 (ACD50950), and *D. farinae*, Der f 20 (AIO08850) (78-79% identity) (Figure 4.A). Similar to TM, published AA sequences of crustacean AK are more closely related to each other; and insects and mites as opposed to molluscs (Figure 4.B).

Only one contig each from all five shrimp species analysed matched myosin light chain (MLC), and demonstrated almost identical AA sequences. Interestingly, they were not at all similar to the published MLC allergens in *L. vannamei*, Lit v 3 (ACC76803), or *P. monodon*, Pen m 3 (ADV17342), with only 16-17% PI (Figure 5.A). Instead, they were found to be more similar to the *C. crangon* (North-sea shrimp) MLC allergen, Cra c 5 (ACR43477) with 86-87% PI (Figure 5.A). The contigs were also identified to be more closely related to the american HDM, *D. farinae*, MLC allergen, Der f 26, (51-54% identity) than the German cockroach, *B. germanica*, MLC allergen, Bla g 8 (18-19% identity) (Figure 5.A). Molecular phylogenetic tree analyses on the distance of MLC among edible crustaceans, molluscs and allergy causing mites confirmed that not all crustacean MLC are closely related to each other. For example, mud crab (*S.*

paramamosain) is more closely related to molluscs' MLC than shrimps and crayfish (Figure 5.B). Black tiger shrimp (*P. monodon*) and whiteleg shrimp (*L. vannamei*) contain MLC that are distantly related to kuruma shrimp (*M. japonicus*) and north-sea shrimp (*C. crangon*), but closely related to MLC from German cockroach (*B. germanica*) (Figure 5.B).

Four of the shrimp species had two contigs matching sarcoplasmic calcium-binding protein (SCBP), while *M. endeavouri*, only had one. SCBP_Contig_1 from all five shrimp species were highly similar to each other and also with the published SCBP allergen in *L. vannamei*, Lit v 4 (ACM89179) and *P. monodon*, Pen m 4 (ADV17343) with PI close to 100%, but only over 80% with the published SCBP allergen in *C. crangon*, Cra c 4 (ACR43475) (Figure 6.A). In contrast, SCBP_Contig_2 from the four species, except *M. endeavouri*, were only 82-84% identical to Lit v 4, Pen m 4 and Cra c 4, with the latter having a slightly higher match than the two former (Figure 6.A). Unlike MLC, but similar to TM and AK, the published AA sequences of SCBP in a phylogenetic tree analyses portrayed that all SCBP from edible crustaceans and molluscs are very closely related to other species within the same phylum, but distantly related between the phyla (Figure 6.B).

Seven contigs matched with Troponin C (TNC) in all five shrimp species, with *M. latisulcatus* and *M. endeavouri* having two contigs each whilst the other three shrimp species having only one each. All seven contigs were moderate to highly similar to each other and also with the published TNC allergens in *P. monodon*, Pen m 6 (ADV17344) and *C. crangon*, Cra c 6 (ACR43478), with PI ranging between 81-100% (Supplementary Figure 1). The PI of shrimp TNC with cockroach and storage mite TNC allergens ranged between 57-65% (Supplementary Figure 1). Meanwhile, only one contig from each shrimp species matched with Troponin I (TNI) allergen, and they were all highly identical to each other (PI: 87-99%), but were only moderately identical to the published TNI allergen in the narrow-clawed crayfish *P. leptodactylus*, Pon l 7 (P05547) (PI: 78-88%) (Supplementary Figure 2). Similarly, only one contig matched with Triosephosphate isomerase (TIM) allergen in each shrimp species and were all highly identical to each other and also with the published TIM allergen in *C. crangon*, Cra c 8 (ACR43476), (PI: 87-99%) (Supplementary Figure 3). However, they had lower PI to American HDM TIM allergens, Der f 25.01 (AGC56216) and Der f 25.02 (AIO08860), with PI values ranging between 66-69% (Supplementary Figure 3).

Abundance of known crustacean allergens varies between shrimp species

The average expression or mean abundance, measured in transcripts-per-million (TPM), of TM across all five species ranges from 10,000 – 15,000 TPM (Figure 7.A). Comparing the difference in abundance between the two tropomyosin contigs within the same species (*P. monodon* and *M. endeavouri*), TM_Contig_2 of *P. monodon* was found to be significantly lower than its counterpart, TM_Contig_1 (Figure 7.A). Meanwhile, there was no significant difference between TM_Contig_1 and TM_Contig_2 of *M. endeavouri* (Figure 7.A). With AK, the mean abundance was approximately 40,000 – 80,000 TPM in all five species (Figure 7.B). Comparing the abundance of the two AK contigs in *M. endeavouri*, AK_Contig_1 was significantly lower than AK_Contig_2 (Figure 7.B). The mean abundance of MLC was approximately 30,000 – 50,000 TPM in all species (Figure 7.C). Meanwhile, for SCBP, the mean abundance was between 40,000 and 90,000 TPM in all species (Figure 7.D). Interestingly, SCBP_Contig_1 of *L. vannamei, P. monodon, and F. merguiensis* were all significantly higher than their respective SCBP_Contig_2 (Figure 7.D). The same pattern could be visually observed on *M. latisulcatus* too but unfortunately, due to the presence of only two replicates instead of three (refer to: *Removal of inconclusive dataset* in Materials and methods section), the significance of this difference could not be statistically confirmed by T-test (GraphPad Prism (v7.03)). Similarly, one could not predict the significance of differences in the two TNC contigs of *M. latisulcatus*. However, for *M. endeavouri,* TNC_Contig_1 was found to be significantly higher than its TNC_Contig_2 (Figure 7.E). Overall, the mean abundance value for TNC was around 4,000 – 10,000 TPM for all five shrimp species (Figure 7.E). As for TNI and TIM, the mean abundance values for all five shrimp species were approximately 16,000 – 20,000 TPM (Figure 7.F) and 2,000 – 6,000 TPM respectively (Figure 7.G).

We then examined the difference in abundance of each allergen within individual shrimp species. We only took into account the contig with the highest PI value when there were more than one contig for that allergen. In all species, the top three most highly expressed allergens were SCBP, AK, and MLC (Figure 8). In fact, SCBP was the most highly expressed allergen in all species except *P. monodon*, where AK was higher (Figure 8.B). In descending order of abundance, these three allergens are followed by TNI, TM, TNC, and TIM (Figure 8). However, in *F. merguiensis*, TM was higher than TNI, TNC and TIM (Figure 8.C). In addition, only in *F. merguiensis,* TM's

abundance was not significantly different from all the three highly abundant allergens, namely, SCBP, AK and MLC (Figure 8.C).

Evolutionary relationship of shellfish allergens TM, AK, MLC, and SCBP

The evolutionary distance of shrimp TM, AK, MLC, and SCBP were analysed among other edible crustacean and mollusc species; and allergy causing mite and insect species. The generated molecular phylogenies of all four shrimp proteins showed close affinities to homologues of other crustaceans such as crab, lobster and crayfish. However, homologues of the other class of "shellfish", molluscs, have a distant relationship to shrimps. Molecular phylogenetic analyses of TM and AK revealed that allergy inducing mite and insect homologues are closer to shrimp TM and AK than molluscs. This observation is supported by a recent study by Nugraha et al. where IgE antibody binding epitopes demonstrated shared protein regions of clinical importance [33]. MLC of German cockroach is found to have a closer evolutionary relationship to the black tiger shrimp and whiteleg shrimp, whilst the MLC belonging to American house dust mite is closely related to MLC of a different subset of crustaceans, including, the north-sea shrimp, kuruma shrimp, and red swamp crayfish. Another interesting finding is that the crustacean MLC of mud crab have a closer evolutionary distance with homologues from the mollusca phylum, especially the pacific oyster, but not to those of the other crustaceans. Molecular phylogenetic analysis of SCBP shows a demarcated distance between the crustacean SCBP and mollusc SCBP. No insect or mite SCBP was included in this analyses as there were no AA sequence data available for insect or mite SCBP on NCBI Genbank or UniProt databases.

Discovery of unreported shrimp allergens

Apart from the previously established shellfish allergens that were confirmed in the five shrimps' transcriptomes, some of the allergens of non-shellfish species are identified to be standout candidates to be unreported allergens in shrimps due to their high % PI values (Table 2). These allergens are heat shock protein (HSP), alpha-tubulin, chymotrypsin, beta-enolase, glyceraldehyde-3-phosphate dehydrogenase (G3PD), cyclophilin and aldolase A (Table 2). The HSP70 (Tyr p 28, AOD75395) from the storage mite *T. putrescentiae* that matched with the shrimp transcriptomes has the highest PI values with all 5 shrimp species (>82%) (Table 2). Other allergen AA sequences that matched with a PI of more than 70% to all 5 shrimp species'

transcriptomes are alpha-tubulin (Der f 33, AIO08861) and chymotrypsin (Der f 6, AAP35065) of the american HDM *D. farinae*, beta-enolase (Sal s 2, ACH70932) of the atlantic salmon *S. salar*, and glyceraldehyde-3-phosphate dehydrogenase or G3PD (Tri a 34, CAZ76054) of wheat *T. aestivum* (Table 2). The allergen cyclophilin (Asp f 27, CAI78448) of the common mould *A. fumigatus,* only matched with a PI of more than 70% with the banana and king shrimps' transcriptomes. Meanwhile, the allergen Aldolase A (Thu a 3, CAX62602) of the yellowfin tuna *T. albacares* matched with a PI of more than 70% only with the banana and endeavour shrimps' transcriptomes (Table 2).

Discussion

Allergen discovery using traditional protein isolation and immunological assay methods, have identified and characterised seven shellfish allergens including the major allergen TM, in addition to AK, MLC, SCBP, TNC, TNI, and TIM. All seven allergens have been identified in various shrimp species except TNI. The increased reporting of allergic cross-reactivity in shrimp-allergic patients to non-shrimp sources demands a full analysis of allergenic proteins. This study utilised an advanced transcriptomic approach to discover the whole repertoire of shrimp allergens, both reported and unreported. Using this comprehensive approach, combining the generation of transcriptomes of five shrimp species and BLAST searching the transcriptomes with all known allergen AA sequences, we identified up to 50 allergens. The majority of identified allergens (45%) belong to the group of shellfish and mite allergens. This is not surprising as the shellfish group consists of crustacean (shrimp) and molluscs, which are often combined when analysing related allergens [15, 33, 34].

In line with existing studies, we confirmed the presence of TM in all five investigated shrimp species, however, the AA sequence was not always similar and the abundance varied significantly. The major allergen TM, a rod-shaped muscle protein (33 – 39 kDa), demonstrates 100% PI between the whiteleg and black tiger shrimp, as recently reported by Ruethers et al. [15], validating the *in silico* approach used in this study. Furthermore, we demonstrated for the first time that TM from banana and king shrimp also exhibited 100% PI to whiteleg and black tiger shrimp's TM. In contrast, known TM allergen from the king shrimp (Mel l 1; AGF86397) shares only 95% of AA identity with the other shrimp species, which was previously demonstrated to result in different allergenicity in patients [35]. This is an important finding which needs to be followed up in clinical studies. However, the identified TM in all five shrimps are very similar and therefore termed isoallergens. The IUIS Allergen Nomenclature identifies an isoallergen to be two proteins with the same biological function with more than 67% AA sequence identity and similar molecular size [36]. The high AA sequence identity (PI: >70%) of the house dust mite (HDM) and cockroach TM allergens (Der p 10, Bla g 7 and Per a 7) with all the analysed shrimp TM's indicate a likelihood of all these invertebrate allergens of being cross-reactive. As previously established, an AA

sequence identity of more than 70% would demonstrate a highly-likely possibility of cross-reactive IgE antibody binding to these allergens [32, 37]. Clinical studies have previously demonstrated a phenomenon named 'HDM-cockroach-shrimp' cross-reactivity [23, 38-42], and we provide here definitive molecular data on the AA sequence similarity of a major shrimp allergen with other invertebrate species.

AK, an important enzymatic protein which regulates the cellular ATP levels of invertebrates, is a heat labile protein (38 – 45 kDa) and highly concentrated in muscle tissue [43]. All five analysed shrimp species demonstrate very high AA sequence similarity with each other, indicating that shrimp allergic patients reacting to AK would most likely react to all five species. AK is considered a major allergen amongst insects and mites and potentially a pan-allergen implicated in cross-reactivity between invertebrate species [44-46]. All five shrimp AK identified in this study are highly-likely allergens with high AA sequence identity (>70%) to AKs from mites (Der p 20; Der f 20) and cockroaches (Bla g 9; Per a 9). Furthermore, the two almost similar AK contigs (PI: 99%) found in endeavour shrimp indicate that they are potential variants instead of isoforms [36], however, the significantly low abundance of AK_Contig_1 than AK_Contig_2 of endeavour shrimp reduces the likelihood of AK_Contig_1 having a role as an allergen.

MLC is part of a large macromolecular complex in muscle tissue consisting of two heavy and four light chains. There are two crustacean MLC allergens, the essential MLC1 (~18kDa) and the regulatory MLC2 (~20kDa) [47, 48]. MLC allergens have been identified in the whiteleg shrimp (Lit v 3) [49] and black tiger shrimp (Pen m 3) [28] – both MLC2 – but not in other shrimp species investigated in this study. While the AA sequences of MLC1 and MLC2 are very different from each other [50], the MLC contigs found in all 5 analysed shrimps are most likely MLC1. Furthermore, this study also suggests that HDM MLC (Der f 26) and cockroach MLC (Bla g 8) are most likely MLC1 and MLC2, respectively, explaining the close molecular phylogenetic relationship to crustacean, but not molluscs.

Another crustacean allergen involved in invertebrate muscle contraction is SCBP (20 – 24 kDa), through binding of calcium ions [51, 52]. We identified two different SCBP contigs for each shrimp species (except endeavour shrimp), with PI values between 81-85%, implicating the presence of SCBP isoallergens. However, the significantly low abundance of SCBP_Contig_2 diminishes its role as an allergen compared to SCBP_Contig_1.

Other muscle regulatory protein identified include troponin. This protein is composed of three subunits, suffixed C, I, and T, with Troponin C and I being registered as allergens. TNC has been identified as an allergen in various crustaceans [53-55], cockroaches [56] and the storage mite [57]. Meanwhile, TNI has only been identified in narrow-clawed crayfish [58]. TIM, an enzyme that is involved in glucose metabolism, is also a registered allergen in north-sea shrimp [53], red swamp crayfish [59], american HDM [60], octopus [61] and wheat [62]. TNC, TNI, and TIM are highly conserved among shrimp species with sequence homology higher than 80%, 78%, and 87%, respectively.

Having considered the different shrimp allergens, it is important to note that apart from allergen presence, the abundance of isoforms also needs to be taken into consideration. Correlation between protein abundance and RNA-Seq data has been established, with some post-transcriptional cellular processes affecting this interpretation [63]. A study on European HDM allergen transcript levels using RNA-Seq data concluded that allergens have a higher level of abundance than non-allergens [64]; and their results were found to be to relatively similar to homologues identified in american HDM from a different study [65]. In particular, these dust mite allergen studies indicate that there is substantial correlation between RNA-Seq dependent abundance levels and a protein's allergen status. Therefore, when there were more than one contig identified for any allergens in the five shrimps, we proceeded to analyse the isoform that had the highest abundance. Comparing known allergens within every shrimp species, SCBP, AK, and MLC were the most abundant in all five shrimps. Interestingly, TM was found to be significantly less abundant than SCBP (in king and endeavour shrimp); AK (in black tiger shrimp); and all three

allergens (in whiteleg shrimp). However, TM is the major and most recognised shrimp allergen, despite its relatively low abundance. The stronger allergenicity is possible due to being very heat stable and having linear IgE binding epitopes as compared to AK, SCBP, or MLC [8, 28, 33].

In addition to previously implicated crustacean allergens, this study also identified up to 38 previously unreported but likely allergens (>50% PI), including seven proteins which are very-likely allergens (>70% PI). Three of these proteins, HSP70, alpha-tubulin, and chymotrypsin, have very high matches to known mite allergens in this study. Additionally, these three proteins have been identified as allergens in different mite and insect species [25, 66-69]. Subsequently, clinical cross-reactivity has been reported as crustacean-mite-insect syndrome [20, 38, 40, 42, 70-72], and we report here the most likely allergens involved. Furthermore, this study identified for the first time very likely allergens, responsible for possible cross-reactivity between shrimp and fish. Beta-enolase and aldolase A, enzymatic proteins that play a role in the glycolytic pathway [15], have previously been identified as heat labile allergens in various fish species and also chicken [73, 74]. Our findings implicate the importance of both proteins as strong candidate allergens in shrimps. In addition, other proteins that are identified to be candidate allergens include cyclophilin and G3PD. Cyclophilin allergen is generally found in fungi, plants and dust mites, and has been shown to have high rates of IgE-binding [60, 75]. Meanwhile, G3PD, an enzymatic protein that is involved in the process of glycolysis similar to aldolase A and beta-enolase, has been identified as allergens in wheat [76], and recently in cockroach and fish [25].

In conclusion, this study accomplished the comparative analyses of all known shrimp allergens derived from five different shrimp species' transcriptomes assembled *de novo* from raw RNA-Seq data. The identification of previously known shrimp allergens validated the comprehensive approach utilised in this study. Moreover, over 30 additional proteins known for their allergenic properties in mite, fungi, plants, insect and fish were identified as candidate shrimp allergens. These includes HSP70, alpha-tubulin, chymotrypsin, beta-enolase aldolase A, cyclophilin and G3PD, which were further identified as very-likely candidate allergens of shrimps. Further immunological

studies would be required to confirm clinical allergenicity in patients. The findings of this study will enable improved diagnostics for shrimp allergy and future therapeutics for this lifelong disease.

Materials and methods

Sample selection

Specimen of the five species of shrimps (*Litopenaeus vannamei*, *Penaeus monodon Fenneropenaeus merguiensis, Melicertus latisulcatus,* and *Metapenaeus endeavouri*) were supplied by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) based in Queensland, Australia. *L. vannamei* and *P. monodon* samples originated from aquaculture farms whilst the other three species were caught as part of the CSIRO Northern Prawn Fishery Surveys from the benthic trawls in the Gulf of Carpenteria, Australia [77]. The shrimps were immersed in an ice-seawater slurry for a few minutes immediately after being caught, to be euthanized. Species-specific reference material were utilised to identify the species of shrimps [78]. Muscle tissue was then removed and stored in RNAlater™ (Invitrogen, Carlsbad, CA, USA) [79]. *P. monodon* samples were collected as described by Huerlimann et al (2018) [80]. Total RNA was extracted from the muscle tissue of three randomly selected adult shrimps of each of the five shrimp species (total of 15 samples) with an RNeasy Universal Extraction kit (QIAGEN) using manufacturer's instruction in an RNase-free laboratory [79]. RNA concentration, quality and purity was assessed using a Nanodrop UV spectrophotometer (Thermo Fisher Scientific) and Agilent Bioanalyzer (Agilent Technologies), before being selected for sequencing.

Illumina library preparation and RNA sequencing

All 15 samples were sequenced via Illumina HiSeq® 2500 System (Illumina Australia and New Zealand, VIC, Australia). Before sequencing, samples were quality checked with the Bioanalyzer RNA 6000 nano reagent kit (Agilent); and Illumina libraries were prepared using the TruSeq Stranded mRNA Library Preparation Kit (Illumina) according to established protocols at the Australian Genome Research Facility (AGRF). The resulting libraries were checked again with the TapeStation DNA 1000 TapeScreen Assay (Agilent). Cluster generation was performed immediately before sequencing on a cBot with HiSeq® PE Cluster Kit v4 – cBot. The sequencing was conducted using a HiSeq® SBS Kit on a HiSeq® 2500, operating with HiSeq Control Software v2.2.68 and base-calling with RTA v1.18.66.3. Raw RNA-Seq short read data for all samples are freely available on NCBI under BioProject PRJNA482687.

De novo transcriptome assembly and quality control

RNA-Seq reads for all 15 samples were corrected using the software Rcorrector (v1.0.2) [81]. Transcriptomes of all 15 samples were individually assembled from their RNA-Seq data, *de novo*. The assembly was carried out using Trinity (v2.4.0) [82, 83]. The quality of the *de novo* transcriptome assembly was assessed using TransRate (v1.0.3) [84] and BUSCO (Benchmarking Universal Single-Copy Orthologs) (v1.2) [85] using the arthropoda odb9 database [86]. The quality score, also known as the TransRate score, is a score between 0.0 – 1.0 that is obtained by multiplying the mean of individual contig scores by the proportion of read pairs (original sequencing reads) that supported the transcriptome [84, 87]. The results of BUSCO assessment are given in percentages of complete (C), fragmented (F) and missing (M) genes within the transcriptome [85]. Using *L. vannamei* as an example, stepwise methods of sample extraction, sequencing, *de novo* transcriptome assembly and quality check are summarised and schematically represented in Figure 1.A.

Removal of inconclusive dataset

Using the Rcorrected reads in an Assembly and Alignment-Free (AAF) method to create a phylogeny [88], it was discovered that one replicate of *M. latisulcatus* grouped with *M. endeavouri* rather than with the other two replicates of *M. latisulcatus*. To confirm the potentially misidentified sample, the assembled transcriptome was BLAST searched against the other *M. latisulcatus* and *M. endeavouri* transcriptomes, where the potentially misidentified sample also showed more similarity to *M. endeavouri*. Lastly, the transcriptomes were compared to known sequences of Enolase [89], which also confirmed that the misidentified sample is not *M. latisulcatus*.

Allergen reference database construction

Known allergen AA sequences were retrieved from two reputable and peer-reviewed online databases to construct a reference allergen database for this study. The first is the World Health Organization & International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature database (www.allergen.org) [25]. The second is the AllergenOnline: Home of the FARRP (Food Allergy Research and Resource Program) Allergen Protein database (v.17) (www.allergenonline.org) [26, 27]. At the time of retrieval, the WHO/IUIS Allergen Nomenclature database contained 875 allergen AA sequences while the AllergenOnline database contained 2,035 allergen

AA sequences [25-27]. After removing duplicates between the 2 databases, a total of 2,172 allergen AA sequences were compiled to form the reference allergen database for this study.

BLAST search for allergens

The allergen database and the assembled transcripts for all 15 samples were imported into the Geneious™ software (v8.1.9, Biomatters Limited, USA) [90]. In order to compare and search for transcripts which contain similar sequences to the allergen sequences compiled in the allergen database, blastx searches were carried out using the BLAST (Basic Local Alignment Search Tool) module within the Geneious™ software. The criteria for the search conducted are shown in Supplementary Table 1.

Refining the BLAST search results

The BLAST search results were filtered for matched sequences with a PI of 50% or more. Subject coverage (percentage of the allergen sequence that is covered by the matching transcript from the transcriptome) was manually calculated using the formula: Subject coverage = Sequence length / Subject length x 100%, where Sequence length is the length of the matched consensus sequence and the Subject length is the actual length of the allergen sequence from the constructed database. Results were then filtered again by selecting only sequences that have 90% or more subject coverage.

Duplicates of allergen sequences that aligned with contigs within the transcriptome were removed by keeping the top-matched allergen-transcript consensus sequence. The BLAST search results of 3 replicates of each species were then combined to form one list of allergens for every species and the duplicates (between replicates) were removed. Stepwise methods of allergen database construction and the processing of transcriptome data such as BLAST search, results refinement and removal of duplicates are schematically represented in Figure 1, using the three assembled transcriptome replicates of *L. vannamei* as an example.

Analysing the BLAST search results

For each shrimp species, the matched allergen AA sequences were grouped into: ‘Shellfish’, ‘Mites’, ‘Insects’, ‘Fungi’, ‘Plants’, ‘Fish’, and ‘Other’, based on the organism that the allergen was documented in. The proportion of allergen sequences belonging

to each group were graphed into a pie chart using GraphPad Prism version 7.03 for Windows [91] to show their distribution amongst different groups of allergen sources.

Multiple sequence alignment was conducted on all the contigs/transcripts that matched tropomyosin allergen in all five transcriptomes with shellfish tropomyosin allergens' sequences (as reference). Mites' and cockroaches' tropomyosin allergen sequences were also included in the multiple sequence alignment that was conducted in Jalview2.1 using Clustal Omega [92]. Comparative AA sequence identities were carried out between the contigs from all five shrimp species that matched with tropomyosin, and previously reported crustacean, mites, and cockroach tropomyosin allergens using Clustal Omega, EMBL-EBI [93]. The multiple sequence alignment and comparative sequence identities were carried out for other documented crustacean allergens: arginine kinase, myosin light chain, sarcoplasmic calcium-binding protein, troponin C, troponin I, and triosephosphate isomerase.

Non-crustacean allergens that have a PI value of more than 70% were shortlisted as highly likely candidates of unreported allergens in shrimp species. These unreported allergens were selected based on their match with the transcriptome of a minimum of 70% PI in at least one of the 5 shrimp species.

Measuring the abundance of allergen sequences

Abundance of each transcript/contigs within the transcriptomes, in transcript-per-million (TPM) values, was quantified using Salmon software [94]. Briefly, Salmon is a software that estimates the abundance of each contig by measuring the number of reads from the RNA-Seq data that align to the contig being measured [94]. Abundance estimation values for all known crustacean allergens were retrieved from all 15 samples. For each allergen in each sample, the estimated abundance value is the sum of all TPM values of all the contigs that matched with that allergen. The mean TPM values with standard deviation error bars for each allergen of the three replicates for each shrimp species are graphically represented in Figure 7 and 8. Standard deviation error bars were omitted from *M. latisulcatus* samples as only 2 replicates were investigated in this study. We first analysed the difference in abundance of all contigs representing a specific allergen, between the 5 shrimp species (Figure 7). In order to look for significant differences between two contigs representing the same allergen, we used unpaired *T-test* using GraphPad Prism version 7.03 for Windows [91]. Next,

we analysed the difference in abundance of allergens within each shrimp species (Figure 8). For this analyses, we only took into account the contig with the highest abundance, when there are more than one contig representing one allergen. To analyse significant differences between the seven crustacean allergens' abundance, we used One-way ANOVA test using GraphPad Prism version 7.03 for Windows [91].

Molecular phylogenetic tree building of TM, AK, MLC and SCBP

Published AA sequences of the four widely studied crustacean allergens, TM, AK, MLC and SCBP belonging to edible crustacean and mollusc species; and allergy causing mite and insect species were mined from NCBI Genbank and UniProt databases. The proteins which are not registered as an allergen in WHO/IUIS or AllergenOnline databases were also included. To determine the evolutionary distance between the same proteins from different species, molecular phylogenetic trees for each protein was built using MEGA X software (v10.0.5). The trees were constructed using the neighbour-joining method with the Poisson correction model. Hence, the branch lengths are the proportion of AA substitutions per site. Bootstrap test was also included (10,000 replicates) and the percentages are shown next to the branches. The gaps which occurred in alignment were treated as pairwise deletion.

Authors' contributions

EJ and AL conceptualized the research objectives. SK, RH, EJ, RN, AT, and AL designed the research methods. RH, NW, and DJ provided the shrimp samples, conducted the RNA extraction and funded the RNA sequencing. SK and RH carried out the *de novo* transcriptome assembly, transcriptome quality check, BLAST search and relative abundance estimation. SK and RN constructed the allergen reference database and the molecular phylogenetic tree of known shellfish allergens. SK, RN, EJ, TR, AT, SDK, and AL determined the relevant allergens identified within the shrimp transcriptomes and the downstream analyses of these potential allergens. SK, RN, TR, RH, and AL designed the figures and tables. SK and AL wrote the first draft. All authors contributed to manuscript editing and revision.

Competing interests

The authors have declared no competing interests.

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Figure legends

Figure 1: Schematic representation of (A) *de novo* transcriptome assembly and (B) transcriptomic analysis used in the identification of allergens in shrimps. The example shown here is for *L. vannamei* species. LV1, LV2, and LV3 represents the 3 biological replicates of *L. vannamei* samples. 'n' value refer to the number of allergens identified in *L. vannamei*. A total of 40 allergens were identified.

Figure 2: Total allergens identified from the transcriptomic analysis in each five shrimp species, distributed based on the matched allergen's source. The distribution amongst different groups of allergen sources are shown in percentages and arranged in a descending order.

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Figure 5: A. Comparison of amino acid sequence identities of (1-5) contigs from five shrimp species that matched with myosin light chain (MLC) allergen, (6-8) known shrimp MLC allergen and (9-10) house dust mite and cockroach MLC allergen. The sequence identities were calculated using multiple sequence alignment in Clustal Omega (EMBL-EBI). **B. Molecular phylogenetic tree based on published amino acid sequences of Myosin light chain (MLC) from edible crustacean and mollusc species; and allergy causing mite and insect species.** The branches consist of UniProt ID/Genbank Accesion ID, species name, and followed by common name in brackets. The numbers next to the branches indicate the bootstrap test percentage of 10,000 replicate trees.

Figure 6: A. Comparison of amino acid sequence identities of (1-9) contigs from five shrimp species that matched with sarcoplasmic calcium-binding protein (SCBP) allergen and (10-12) known shrimp SCBP allergen. The sequence identities were calculated using multiple sequence alignment in Clustal Omega (EMBL-EBI). **B. Molecular phylogenetic tree based on published amino acid sequences of Sarcoplasmic calcium-binding protein (SCBP) from edible crustacean and mollusc species; and allergy causing mite and insect species.** The branches consist of UniProt ID/Genbank Accesion ID, species name, and followed by common name in brackets. The numbers next to the branches indicate the bootstrap test percentage of 10,000 replicate trees.

Figure 7: Abundance estimation values in transcript-per-million (TPM) for contigs in the 5 analysed shrimp species that matched with shrimp allergens. **A**: tropomyosin, **B**: arginine kinase, **C**: myosin light chain, **D**: sarcoplasmic calcium-binding protein, **E**: troponin C, **F**: troponin I, and **G**: triosephophate isomerase. *T-test* was employed to measure the significance of difference between two contigs from the same species, if present (*: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****: $P \leq 0.0001$)

Figure 8: Abundance estimation values in transcript-per-million (TPM) for contigs that matched with shrimp allergens in the 5 analysed shrimp species. **A**: *L. vannamei*, **B**: *P. monodon*, **C**: *F. merguiensis*, **D**: *M. latisulcatus*, **E**: *M. endeavouri*. ANOVA test was employed to measure the significance of difference between the seven shrimp allergens. Only one contig with the highest Pairwise Identity with known shrimp allergens value was included where there was more than one contig for one allergen in each species. The contigs are arranged in descending order of on their abundance. Allergen abundance values with the same letter are not significantly different to each other.

Table 1: Results of Trinity transcriptome assembly, TransRate, and BUSCO. Shrimp species name (common name) and their 1-3 biological replicates are shown here with their transcriptomes' number of contigs and assembly size after assembly by Trinity. TransRate score and BUSCO scores (C: complete, F: fragmented, M: missing) of each transcriptome are also shown here.

Table 2: List of unreported allergens identified that have a minimum of 70% pairwise identity value in at least one species. List includes protein name, the common and scientific name of the allergen source, along with the allergen sequence's IUIS nomenclature. % Pairwise identity and E-values. Proteins with a % Pairwise identity of 70% or higher (highly likely to be allergenic) are highlighted in red.

Supplementary material

Supplementary Figure 1: Comparison of amino acid sequence identities of (1-7) contigs from five shrimp species that matched with Troponin C (TNC) allergen, (8-9) known shrimp TNC allergen, and (10-14) cockroach and storage mite TNC allergen. The sequence identities were calculated using multiple sequence alignment in Clustal Omega (EMBL-EBI).

Supplementary Figure 2: Comparison of amino acid sequence identities of (1-5) contigs from five shrimp species that matched with Troponin I (TNI) allergen and (6) known crayfish TNI allergen. The sequence identities were calculated using multiple sequence alignment in Clustal Omega (EMBL-EBI).

Supplementary Figure 3: Comparison of amino acid sequence identities of (1-5) contigs from five shrimp species that matched with triosephosphate isomerase (TIM) allergen, (6) known shrimp TIM allergen, and (7-8) house dust mite TIM allergen. The sequence identities were calculated using multiple sequence alignment in Clustal Omega (EMBL-EBI).

Supplementary Figure 4: Multiple sequence alignment of (1-2) known shrimp tropomyosin (TM) allergen, (3-9) contigs from five shrimp species that matched with TM allergen and (10-12) TM allergen sequences from house dust mite and cockroaches. Multiple sequence alignment was conducted in Jalview 2.1 using Clustal Omega.

Supplementary Figure 5: Multiple sequence alignment of (1-2) known shrimp arginine kinase (AK) allergen, (3-8) contigs from five shrimp species that matched AK allergen and (9-12) AK allergen sequences from house dust mites and cockroaches. Multiple sequence alignment was conducted in Jalview 2.1 using Clustal Omega.

Supplementary Figure 6: Multiple sequence alignment of (1-3) known shrimp myosin light chain (MLC) allergen, (4-8) contigs from five shrimp species that matched with MLC allergen and (9-10) house dust mite and cockroach MLC allergen. Multiple sequence alignment was conducted in Jalview 2.1 using Clustal Omega.

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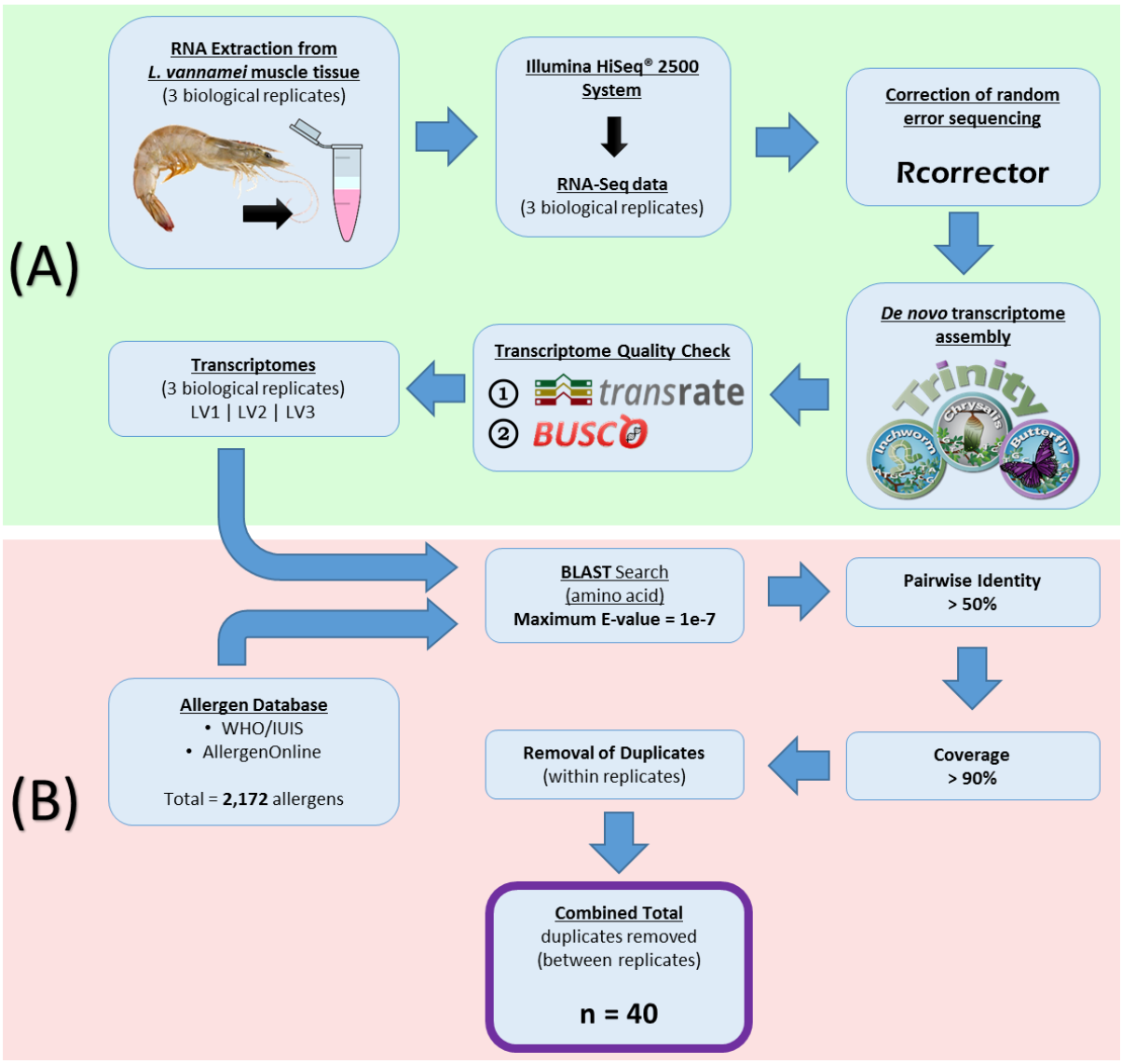
Supplementary Figure 8: Multiple sequence alignment of (1-2) known shrimp Troponin C (TNC) allergen, (3-9) contigs from five shrimp species that matched with TNC allergen and (10-14) TNC allergen sequences from house dust mites and cockroaches. Multiple sequence alignment was conducted in Jalview 2.1 using Clustal Omega.

Supplementary Figure 9: Multiple sequence alignment of (1) known crayfish Troponin I (TNI) allergen and (2-6) contigs from five shrimp species that matched with TNI allergen. Multiple sequence alignment was conducted in Jalview 2.1 using Clustal Omega.

Supplementary Figure 10: Multiple sequence alignment of (1) known shrimp Triosephosphate isomerase (TIM) allergen, (2-6) contigs from five shrimp species that matched with TIM allergen and (7-8) TIM allergen sequences from house dust mites. Multiple sequence alignment was conducted in Jalview 2.1 using Clustal Omega.

Supplementary Table 1: Criteria used in the BLAST search conducted. The criteria shown here are only for the BLAST search utility within the Geneious™ software. Additional search criteria (for this project) was later used in the refining process of the search results, e.g. Minimum % Pairwise Identity of 50%.

Figures



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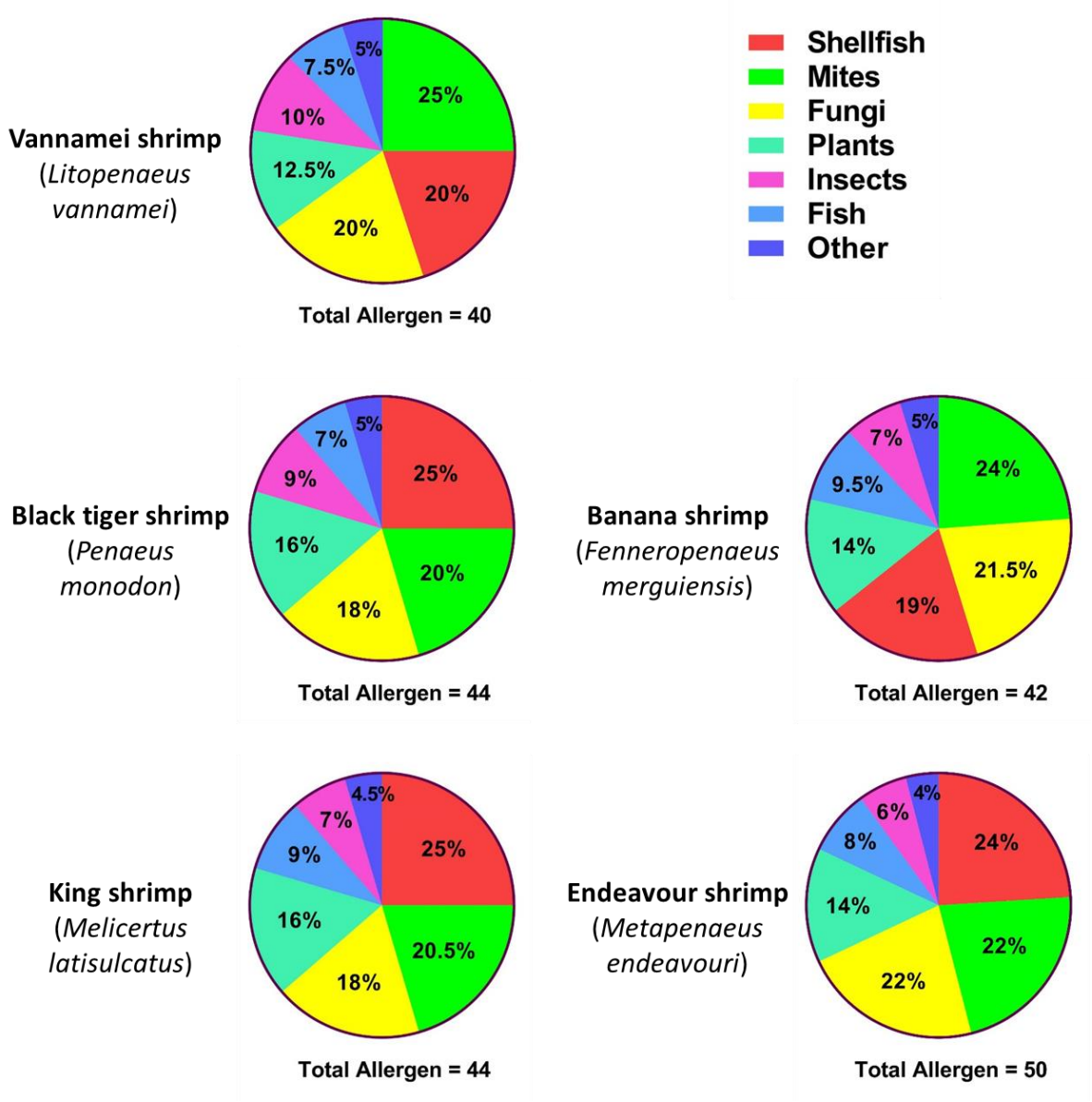

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A.

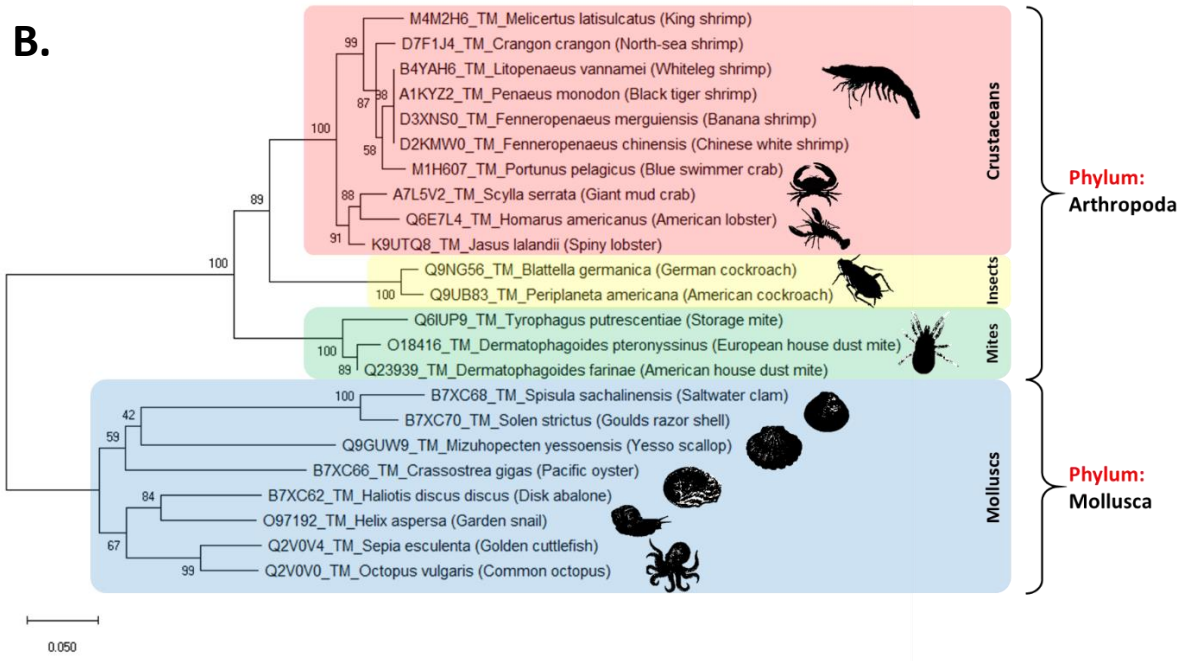
Tropomyosin

		Species	Contig ID/AccessionID	#	1	2	3	4	5	6	7	8	9	10	11	12
Contigs	Shrimp	*L._vannamei_TM_Contig_1*	TRINITY_DN8531_c1_g1_i23	1	100	100	91	100	100	99	82	100	100	81	82	82
		P._monodon_TM_Contig_1	TRINITY_DN9650_c0_g5_i8	2		100	91	100	100	99	82	100	100	81	82	82
		P._monodon_TM_Contig_2	TRINITY_DN10227_c1_g1_i21	3			100	91	91	91	87	91	91	78	81	81
		F._merguiensis_TM_Contig_1	TRINITY_DN9629_c3_g1_i18	4				100	100	99	82	100	100	81	82	82
		M._latisulcatus_TM_Contig_1	TRINITY_DN10005_c3_g3_i4	5					100	99	82	100	100	81	82	82
		M._endeavouri_TM_Contig_1	TRINITY_DN9495_c0_g1_i3	6						100	82	99	99	81	83	83
		M._endeavouri_TM_Contig_2	TRINITY_DN9495_c0_g1_i16	7							100	82	82	72	74	74
Allergen		*L._vannamei_TM_[Lit_v_1]*	ACB38288	8								100	100	81	82	82
		P._monodon_TM_[Pen_m_1]	AAX37288	9									100	81	82	82
	HDM	*D._pteronyssinus_TM_[Der_p_10]*	AAB69424	10										100	80	80
	Cockroach	*B._germanica_TM_[Bla_g_7]*	AAF72534	11											100	97
		P._americana_TM_[Per_a_7]	CAB38086	12												100

% Identity

50 55 60 65 70 75 80 85 90 95 100

B.



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A.

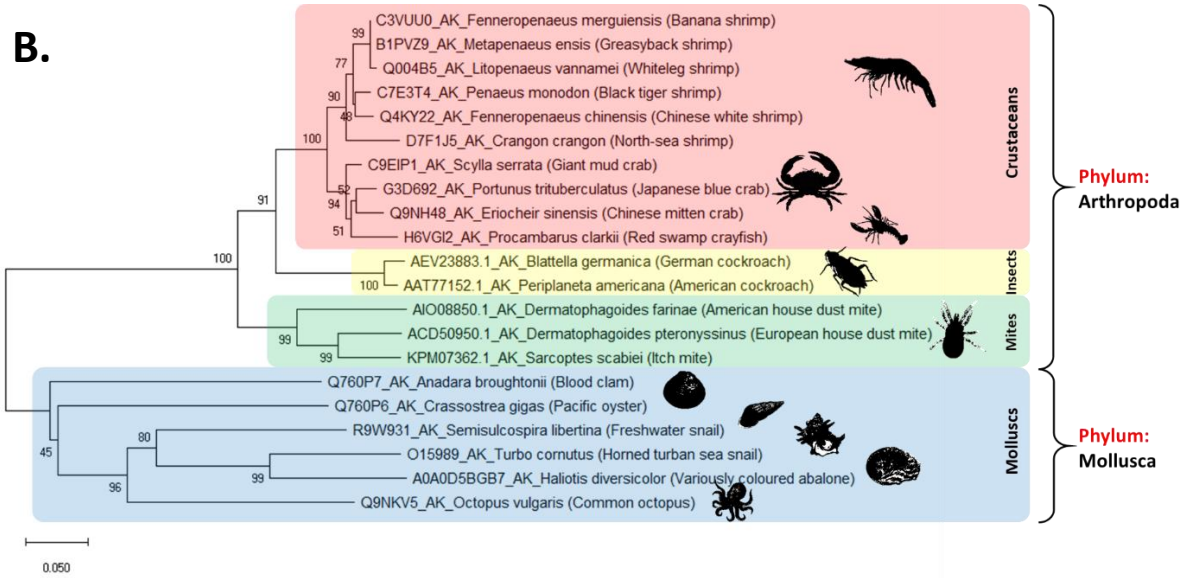
Arginine Kinase

		Species	Contig ID/Accession ID	#	1	2	3	4	5	6	7	8	9	10	11	12
Contigs	Shrimp	*L._vannamei_AK_Contig_1*	TRINITY_DN9209_c0_g2_i17	1	100	97	97	97	97	97	100	97	78	79	84	84
		P._monodon_AK_Contig_1	TRINITY_DN10454_c1_g1_i5	2		100	98	98	98	97	97	100	79	78	84	84
		F._merguiensis_AK_Contig_1	TRINITY_DN9637_c2_g2_i13	3			100	97	97	97	97	98	78	79	84	84
		M._latisulcatus_AK_Contig_1	TRINITY_DN10028_c2_g2_i10	4				100	97	97	96	98	79	78	83	83
		M._endeavouri_AK_Contig_1	TRINITY_DN9459_c1_g1_i7	5					100	99	97	98	78	78	84	84
		M._endeavouri_AK_Contig_2	TRINITY_DN9459_c1_g1_i10	6						100	97	97	79	79	84	84
Allergen		*L._vannamei_AK_[Lit_v_2]*	ABI98020	7							100	97	78	79	83	83
		P._monodon_AK_[Pen_m_2]	AAO15713	8								100	79	78	83	83
	HDM	*D._pteronyssinus_AK_[Der_p_20]*	ACD50950	9									100	84	75	75
		D._farinae_AK_[Der_f_20]	AIO08850	10										100	75	75
	Cockroach	*B._germanica_AK_[Bla_g_9]*	ACM24358	11											100	100
		P._americana_AK_[Per_a_9]	AAT77152	12												100

% Identity

50 55 60 65 70 75 80 85 90 95 100

B.



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A.

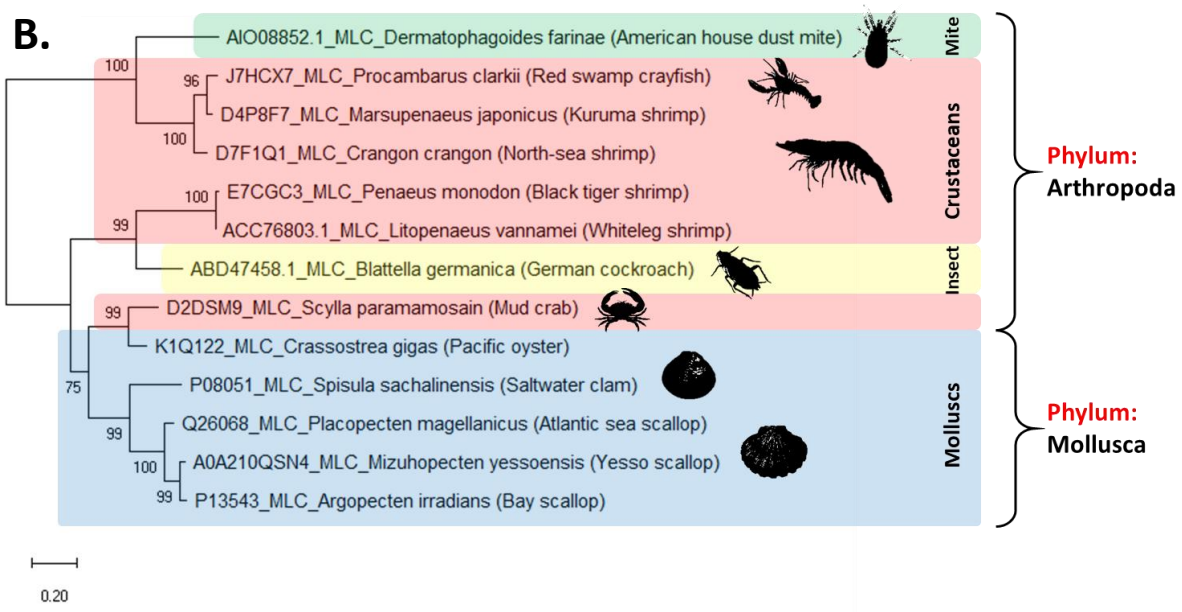
Myosin Light Chain

		Species	Contig ID/Accession ID	#	1	2	3	4	5	6	7	8	9	10
Contigs	Shrimp	*L._vannamei_MLC_Contig_1*	TRINITY_DN9011_c1_g2_i5	1	100	99	99	100	100	16	16	87	51	18
		P._monodon_MLC_Contig_1	TRINITY_DN10152_c4_g1_i1	2		100	99	99	99	16	16	86	51	18
		F._merguiensis_MLC_Contig_1	TRINITY_DN9676_c1_g1_i9	3			100	99	99	17	16	87	54	19
		M._latisulcatus_MLC_Contig_1	TRINITY_DN10011_c0_g1_i15	4				100	100	17	16	87	53	18
		M._endeavouri_MLC_Contig_1	TRINITY_DN8762_c0_g1_i11	5					100	16	16	87	51	18
Allergen		*L._vannamei_MLC_[Lit_v_3]*	ACC76803	6						100	98	18	18	55
		P._monodon_MLC_[Pen_m_3]	ADV17342	7							100	18	18	55
		C. crangon_MLC_[Cra_c_5]	ACR43477	8								100	49	21
	HDM	*D._farinae_MLC_[Der_f_26]*	AIO08852	9									100	21
	Cockroach	*B._germanica_MLC_[Bla_g_8]*	ABD47458	10										100

% Identity

10 25 40 55 70 85 100

B.



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A.

Sarcoplasmic Calcium-Binding Protein

		Species	Contig ID/Accession ID	#	1	2	3	4	5	6	7	8	9	10	11	12
Contigs	Shrimp	*L._vannamei_SCBP_Contig_1*	TRINITY_DN9179_c0_g1_i9	1	100	83	99	83	98	82	98	83	98	100	100	82
		L._vannamei_SCBP_Contig_2	TRINITY_DN9179_c0_g2_i2	2		100	82	97	81	96	84	96	82	83	83	84
		P._monodon_SCBP_Contig_1	TRINITY_DN9551_c2_g5_i3	3			100	82	98	82	97	82	98	99	99	81
		P._monodon_SCBP_Contig_2	TRINITY_DN10440_c3_g5_i1	4				100	82	99	84	97	82	83	83	84
		F._merguiensis_SCBP_Contig_1	TRINITY_DN9648_c3_g10_i1	5					100	81	96	82	97	98	98	80
		F._merguiensis_SCBP_Contig_2	TRINITY_DN9648_c3_g1_i4	6						100	83	97	81	82	82	83
		M._latisulcatus_SCBP_Contig_1	TRINITY_DN9900_c3_g1_i12	7							100	85	98	98	98	83
		M._latisulcatus_SCBP_Contig_2	TRINITY_DN7399_c0_g2_i5	8								100	83	83	83	84
		M._endeavouri_SCBP_Contig_1	TRINITY_DN8910_c1_g1_i8	9									100	98	98	82
Allergen		*L._vannamei_SCBP_[Lit_v_4]*	ACM89179	10										100	100	82
		P._monodon_SCBP_[Pen_m_4]	ADV17343	11											100	82
		C. crangon_SCBP_[Cra_c_4]	ACR43475	12												100

% Identity

60 65 70 75 80 85 90 95 100

B.

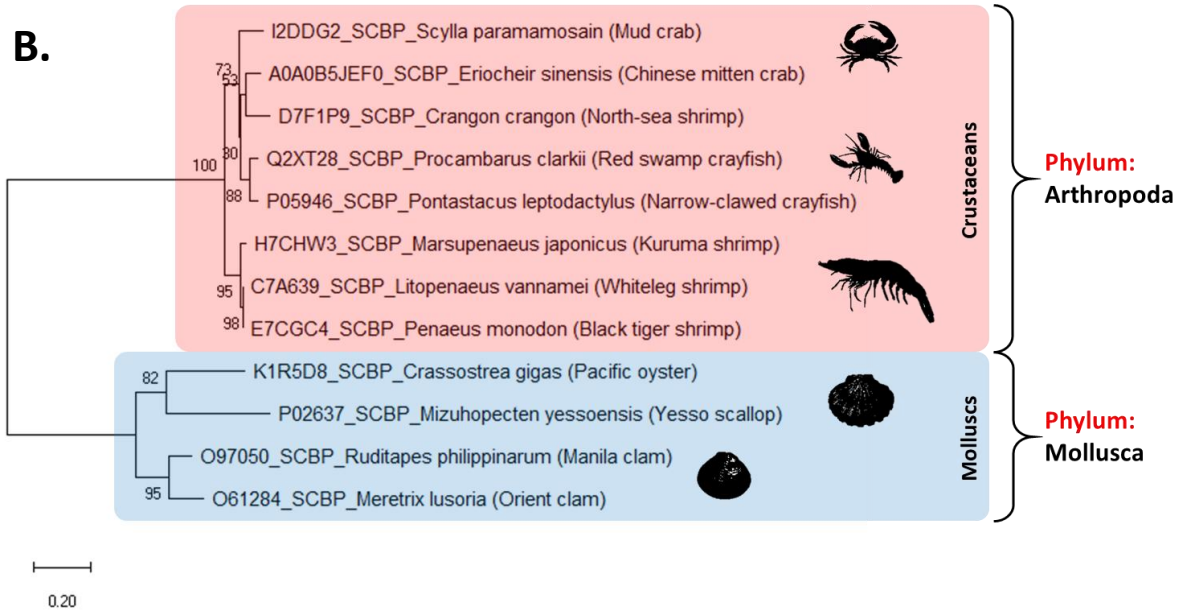

Figure 6: A. Comparison of amino acid sequence identities of (1-9) contigs from five shrimp species that matched with sarcoplasmic calcium-binding protein (SCBP) allergen and (10-12) known shrimp SCBP allergen. The sequence identities were calculated using multiple sequence alignment in Clustal Omega (EMBL-EBI). **B.** Molecular phylogenetic tree based on published amino acid sequences of Sarcoplasmic calcium-binding protein (SCBP) from edible crustacean and mollusc species; and allergy causing mite and insect species. The branches consist of UniProt ID/Genbank Accesion ID, species name, and followed by common name in brackets. The numbers next to the branches indicate the bootstrap test percentage of 10,000 replicate trees.

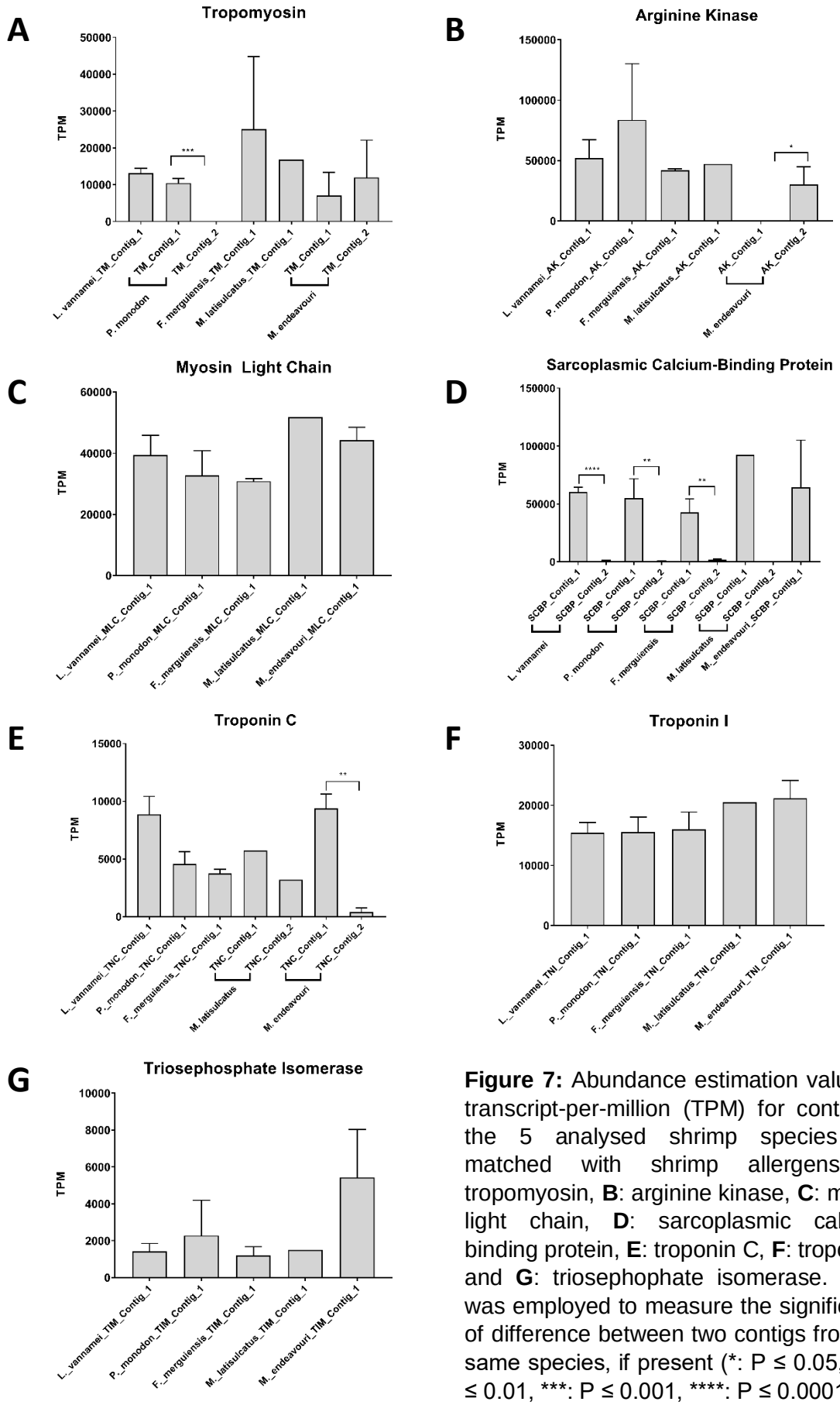

Figure 7: Abundance estimation values in transcript-per-million (TPM) for contigs in the 5 analysed shrimp species that matched with shrimp allergens. **A**: tropomyosin, **B**: arginine kinase, **C**: myosin light chain, **D**: sarcoplasmic calcium-binding protein, **E**: troponin C, **F**: troponin I, and **G**: triosephophate isomerase. *T-test* was employed to measure the significance of difference between two contigs from the same species, if present (*: P ≤ 0.05, **: P ≤ 0.01, ***: P ≤ 0.001, ****: P ≤ 0.0001)

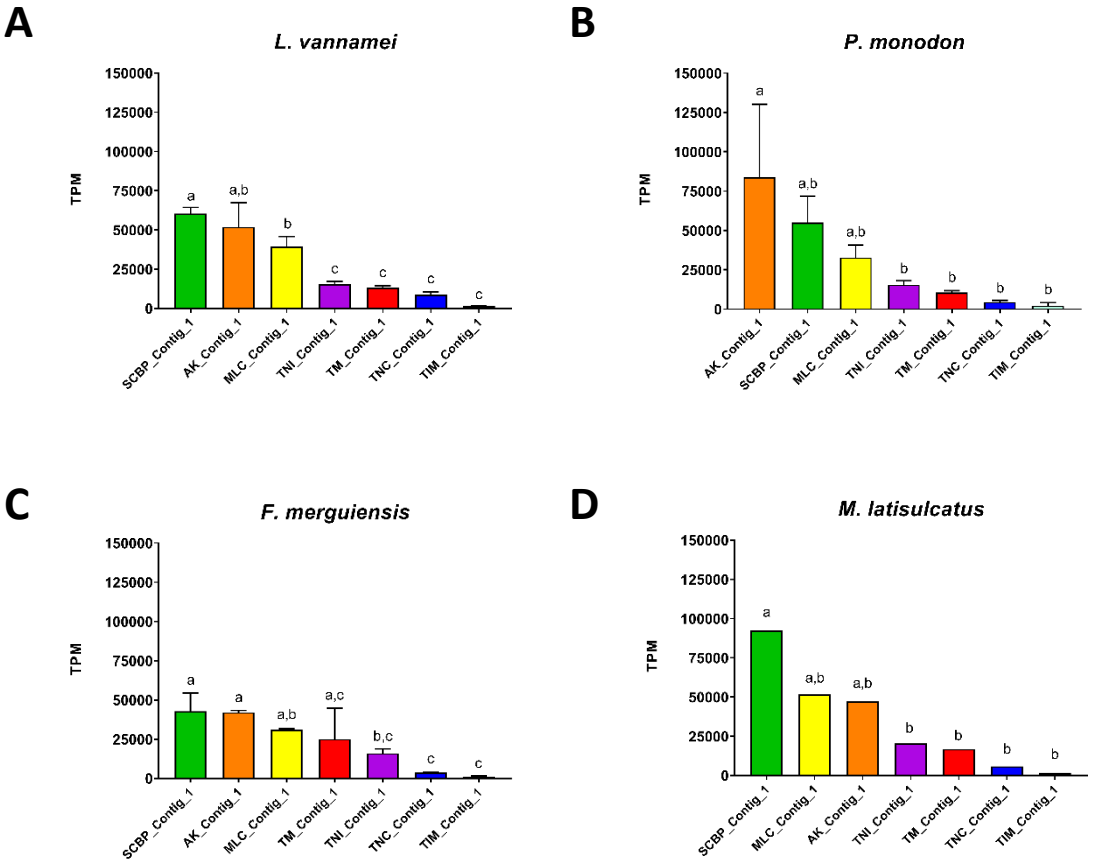

Figure 8: Abundance estimation values in transcript-per-million (TPM) for contigs that matched with shrimp allergens in the 5 analysed shrimp species. **A**: *L. vannamei*, **B**: *P. monodon*, **C**: *F. merguiensis*, **D**: *M. latisulcatus*, **E**: *M. endeavouri*. ANOVA test was employed to measure the significance of difference between the seven shrimp allergens. Only one contig with the highest Pairwise Identity with known shrimp allergens value was included where there was more than one contig for one allergen in each species. The contigs are arranged in descending order of on their abundance. Allergen abundance values with the same letter are not significantly different to each other.

Tables

Table 1: Results of Trinity transcriptome assembly, TransRate, and BUSCO. Shrimp species name (common name) and their 1-3 biological replicates are shown here with their transcriptomes' number of contigs and assembly size after assembly by Trinity. TransRate score and BUSCO scores (C: complete, F: fragmented, M: missing) of each transcriptome are also shown here.

Shrimp species		Replicates	RNA-Seq	Transcriptome assembly metrics			Transrate quality assessment		BUSCO scores		
			Normalized read count	No. of contigs	Assembly size	GC content (%)	Proportion of read pairs mapped (%)	Assembly score	Complete (%)	Fragmented (%)	Missing (%)
	L. vannamei (Whiteleg shrimp)	1	1,412,587	32,302	28.6Mb	43.4	93.2	0.413	56	21	23
		2	1,412,010	33,574	29.4Mb	43.0	92.6	0.401	56	23	21
		3	1,070,376	28,101	22.7Mb	44.8	92.8	0.419	48	25	27
	P. monodon (Black Tiger shrimp)	1	1,609,374	41,971	37.9Mb	44.3	91.9	0.387	66	20	14
		2	1,443,066	40,927	36.5Mb	45.1	91.0	0.364	66	19	14
		3	1,643,259	42,510	38.1Mb	43.7	92.3	0.390	64	21	14
	F. merguiensis (Banana shrimp)	1	1,486,264	37,572	31.4Mb	43.0	91.8	0.385	64	17	19
		2	1,657,940	41,336	34.8Mb	42.6	91.7	0.385	67	16	17
		3	1,602,775	38,638	33.5Mb	42.5	92.6	0.389	65	19	16
	M. latisulactus (King shrimp)	1	1,130,898	37,128	25.6Mb	42.9	90.7	0.410	46	26	27
		2	1,052,237	28,125	21.7Mb	42.8	92.2	0.411	43	25	32
	M. endeavouri (Endeavour shrimp)	1	1,142,169	35,407	25.9Mb	42.5	90.6	0.374	48	25	27
		2	1,035,324	30,879	23.2Mb	42.3	91.2	0.399	48	24	27
		3	1,081,301	38,204	25.5Mb	43.3	87.9	0.355	49	26	25

Table 2: List of unreported allergens identified that have a minimum of 70% pairwise identity value in at least one species. List includes protein name, the common and scientific name of the allergen source, along with the allergen sequence's IUIS nomenclature. % Pairwise identity and E-values. Proteins with a % Pairwise identity of 70% or higher (highly likely to be allergenic) are highlighted in red.

Allergens				LV Whiteleg shrimp (E Value)	PM Black tiger shrimp (E Value)	FM Banana shrimp (E Value)	ML King shrimp (E Value)	ME Endeavour shrimp (E Value)
Protein name	Source name		IUIS nomen-clature					
	Common	Scientific						
Heat shock-like protein	Storage mite	*Tyrophagus putrescentiae*	Tyr p 28	**85.1%** (0)	**82.7%** (0)	**83.3%** (0)	**82.7%** (0)	**84.3%** (0)
Alpha-tubulin	American house dust mite	*Dermatophagoides farinae*	Der f 33	**81.8%** (0)	**81.7%** (0)	**81.6%** (0)	**81.6%** (0)	**81.6%** (0)
Chymotrypsin	American house dust mite	*Dermatophagoides farinae*	Der f 6	**78.7%** (4.3E-94)	**78.7%** (2.13E-94)	**79.3%** (1.45E-94)	**79.9%** (3.71E-97)	**80.5%** (3.93E-95)
Enolase 3-2	Atlantic salmon	*Salmo salar*	Sal s 2	**74.8%** (0)	**74.6%** (0)	**74.1%** (0)	**74.6%** (0)	**74.5%** (0)
Glyceral-dehyde-3-phosphate de-hydrogenase	Wheat	*Triticum aestivum*	Tri a 34	**72.3%** (1.25E-168)	**72.0%** (2.87E-172)	**71.7%** (1.86E-170)	**72.0%** (1.21E-174)	**72.3%** (4.91E-175)
Cyclophilin	Common mould	*Aspergillus fumigatus*	Asp f 27	**61.9%** (8.45E-65)	**62.5%** (1.87E-67)	**70.3%** (2.63E-75)	**70.7%** (4.38E-75)	**69.3%** (5.53E-76)
Aldolase A	Yellowfin tuna	*Thunnus albacares*	Thu a 3	**66.0%** (2.29E-164)	**64.9%** (2.25E-164)	**70.1%** (4.09E-169)	**69.6%** (1.47E-166)	**70.1%** (3.65E-167)

Conservation Analysis of B-Cell Allergen Epitopes to Predict Clinical Cross-Reactivity Between Shellfish and Inhalant Invertebrate Allergens

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Understanding and predicting an individual's clinical cross-reactivity to related allergens is a key to better management, treatment and progression of novel therapeutics for food allergy. In food allergy, clinical cross-reactivity is observed in patients reacting to unexpected allergen sources containing the same allergenic protein or antibody binding patches (epitopes), often resulting in severe allergic reactions. Shellfish allergy affects up to 2% of the world population and persists for life in most patients. The diagnosis of shellfish allergy is however often challenging due to reported clinical cross-reactivity to other invertebrates including mites and cockroaches. Prediction of cross-reactivity can be achieved utilizing an in-depth analysis of a few selected IgE-antibody binding epitopes. We combined available experimentally proven IgE-binding epitopes with informatics-based cross-reactivity prediction modeling to assist in the identification of clinical cross-reactive biomarkers on shellfish allergens. This knowledge can be translated into prevention and treatment of allergic diseases. To overcome the problem of predicting IgE cross-reactivity of shellfish allergens we developed an epitope conservation model using IgE binding epitopes available in the Immune Epitope Database and Analysis Resource (http://www.iedb.org/). We applied this method to a set of four different shrimp allergens, and successfully identified several non-cross-reactive as well as cross-reactive epitopes, which have been experimentally established to cross-react. Based on these findings we suggest that this method can be used for advanced component-resolved-diagnosis to identify patients sensitized to a specific shellfish group and distinguish from patients with extensive cross-reactivity to ingested and inhaled allergens from invertebrate sources.

Keywords: shellfish allergens, IgE epitopes, B-cell epitopes, shellfish cross-reactivity, tropomyosin

INTRODUCTION

The prevalence of food allergy is steadily increasing over the past decade, with ∼4% of adults and up to 10% of children having some type of food allergy. The shellfish group is included among the "Big Eight" food groups that are responsible for more than 90% of all food allergy cases (1). It is estimated that 2% of the general population are affected by food allergy to shellfish (2). Shellfish allergy has, similar to peanut allergy, one of the highest rates of food-induced anaphylaxis with nearly 42% among affected adults and 12% in children (3). Shellfish allergy is typically persistent, with only 13% of patients likely to outgrow their shellfish allergy (4).

In the sensitized individual, subsequent exposure to the shellfish allergen, via ingestion or inhalation, triggers the generation of specific IgE antibodies by activated B-cells of the immune system. These antibodies subsequently bind to immune effector cells, including mast cells and basophils, resulting in degranulation and clinical manifestation of allergic symptoms (5). Currently, eight proteins from different shellfish species are known as the main provocateur of shellfish allergy and have been registered in the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (2).

Two allergens of major importance in the development of shellfish allergy are the muscle protein tropomyosin (TM) and the enzyme arginine kinase (AK). Tropomyosin is the major allergen of shellfish allergy with specific IgE antibody responses in 60–80% of shellfish allergic patients recognizing this protein, and it is suggested to be a good biomarker of severe clinical-reactivity to shellfish (6, 7). TM has been identified as a major allergen in over 14 crustacean and 5 mollusc species (www.allergen.org). Meanwhile, AK has been considered as the second invertebrate pan-allergen, implicating allergy cross-reactivity to shellfish, mite and insect. IgE-sensitization toward AK has been found in 10–51% of shrimp allergic patients (2).

The primary structure of TM's alpha-helical coiled-coil dimeric protein is highly conserved across various invertebrates and has been identified in over 150 species, including insects and mites (8). This structural similarity seems to be one of the main reason for the high degree of immunological cross-reactivity and as a result, is responsible for inducing allergic immune responses in over 30% of the world-population (9, 10). Furthermore, the immunogenic properties of TM have made it a prominent vaccine candidate for several pathogens in livestock, including nematodes, filaria, scabies and ticks (5–7).

Edible crustacean and mollusc are commonly discussed as "shellfish." However, the group of "shellfish" comprises the two invertebrate phyla arthropods and molluscs. Although all shellfish are invertebrate animals, these two groups are very distinct in evolutionary terms and subsequently contain different molecular repertoires of food allergens. In fact, crustacean are placed closer to insects and arachnids (spiders), and this seems to be the major factor for molecular sensitization and clinical reactivity between crustacean, dust-mite, insects and parasites (11). Severe acute allergic reactions upon accidental ingestion of different shellfish species and insects have been observed for a long time and studies attempted to understand the underlying immunological cross-reactivity have been conducted (12, 13). Immunological cross-reactivity was also demonstrated in unexpected populations, never exposed to shrimp TM (14). Tropomyosin from shrimp and mite seem to cross-react considerably, as recently demonstrated in patients clinically reacting to TM in ingested insects (15, 16). Furthermore, IgE responses to TM in mite and the parasite Ascaris are significant risk factors for asthma (17). While the clinical relevance of cross-reactivity to TM is known for as long time, lack of specific diagnostic tools are the main problem for correct diagnosis of crustacean and/or mollusc allergy and extensive immunological cross-reactivity with other invertebrate allergen sources containing similar proteins.

Cross-reactivity occurs when the IgE antibodies recognize identical or very similar protein patches (epitopes) from different proteins as compared to the primary sensitizing protein (18). IgE cross-reactivity to unrelated peanut allergens has recently been demonstrated (19), resulting from amino acid similarities of short peptides. Furthermore, it is known that phylogenetically related species often have similar proteins, and this similarity can implicate IgE cross-reactivity (20). Allergens which share highly conserved protein sequences, but also structure and function, can be termed pan-allergens if they are responsible for antibody binding cross-reactivity and subsequent clinical cross-reactivity (21).

Cross-reactivity of related proteins could be predicted computationally by comparing the identity of the amino acid sequence to the known allergen. Aalberse (22) reviewed potentially cross-reactive structures of known allergens and noted that proteins with >50% identity throughout the length of the protein compared to an allergen are likely cross-reactive. However, some recent studies (19, 23, 24) demonstrated that this predictive value is only useful to predict the allergenicity of new protein of unknown structure but is not accurate to predict cross-reactivity. This is of particular importance for invertebrate tropomyosin, which shares over 50% of amino acid identify with human tropomyosin. Moreover, in many cases the demonstrated cross-reactivity is patient-specific and seem only to occur when the patient IgE antibodies bind to conserved epitopes. In the context of food allergy, sequential IgE binding epitopes seem to be much more relevant as conformational epitopes are easily degraded due to the digestion in the gastrointestinal tract (25).

Based on these observations, we consider that a large-scale analysis of sequential IgE epitope conservation is of great importance for predicting clinical cross-reactivity between crustacean and mollusc, as well as mite and cockroach, in allergic patients. While there are eight allergenic proteins known among different shellfish (26), conclusive epitope data are only available for shrimp allergens including TM, AK, SCP, and MLC. We developed a database of positive IgE-binding epitopes of these shrimp allergens collected from the Immune Epitope Database (IEDB). Subsequently, we determined the conserved epitope sequences responsible for cross-reactivity using the Epitope Conservancy Analysis program (27). Shrimp allergen epitopes were considered to be conserved if the sequence of the other invertebrate homologous peptide had less than two

amino acid mismatches (28–31). These epitopes could be used to design better predictive diagnostic tools for shellfish allergic patients as well as enabling the early detection of risk factors for developing food allergy to shellfish in dust mite allergic patients, thereby leading to the improved management of this severe clinical condition.

METHODS

Collection of Shrimp Allergens IgE-Binding Epitopes

The dataset for the subsequent analysis was built from available IgE binding epitopes from four shrimp allergens: tropomyosin (TM), arginine kinase (AK), myosin light chain (MLC), and sarcoplasmic calcium-binding protein (SCBP). The data were assembled from the Immune Epitope Database and Analysis Resource (IEDB) (32). The collected epitopes were restricted to peptides with positive serum IgE antibody binding from patients with confirmed shrimp allergy.

Sequence Retrieval and Phylogenetic Analysis

Protein sequences were obtained from the UniProt database and aligned using MUSCLE v3.8.31 (33). Different numbers of protein sequences could be retrieved for TM, AK, MLC, and SCBP, with, respectively 54, 30, 20, and 10 protein sequences. The selected sequences represent 18 different crustacean and 30 mollusc species within the shellfish group. The subsequent phylogenetic analyses of relatedness of proteins were performed using MrBayes v3.2.6 (34) (50,000 generations) and RAxML v8.2.9 (35) (1,000 replicates) via the CIPRES Science Gateway (36).

Conservation Analysis of Shrimp Allergens in Invertebrate Species

The conservation of amino acid residues for each allergen among the different invertebrate species was estimated using the Rate4Site algorithm in Consurf (37) server by calculating position-specific evolutionary rates under an empirical Bayesian methodology. The rates were normalized and grouped into 9 grades, where high conserved residues receive a score of 9 and very variable residues receive a score of 1. The conservation rate of the amino acids was then mapped using Chimera (38) to the structure model of the allergens generated by Swiss-Model (39).

Conservation Analysis of IgE-Binding Epitopes of Shrimp Allergens

The degree of conservation of the epitopes within the sequences of the respective allergens was calculated using conservancy analysis tool (27) on the IEDB website. The degree of conservation of an epitope is calculated as the fraction of the protein sequence that matched the aligned epitope above a chosen identity level. An epitope was considered to be conserved if the homologous peptide had less than two amino acid mismatches.

Data Analysis

Data analysis was performed using GraphPad Prism version 7. One-way ANOVA was applied to determine the statistically significant difference of the conserved epitopes between groups of invertebrate species.

RESULTS

Amino Acid Sequence Analysis of Shrimp Allergens

Phylogenetic analysis was performed to determine the relationships between shrimp allergen sequences in different invertebrate groups and to infer the evolutionary trends among the wide representation of the allergens. In this study, four shrimp allergens, TM, AK, SCP, and MLC were selected based on the availability of the IgE-binding epitopes. A dataset of TM, AK, SCP, and MLC protein sequences from crustaceans and molluscs species in UniProt database was assembled to construct a tree using the Maximum Likelihood and Bayesian approach. A consensus tree generated for all protein groups showed similar topologies with good branch support (>70%) for major branches for TM and AK (**Figure 1A**). These trees, particularly for TM, are in good agreement with previously published trees (40, 41), demonstrating the expected distant phylogenetic relationship between crustacean and mollusc. Crustacean clustered closer with other allergy-causing arthropods, including mite and cockroach, while the mollusc form a distinct cluster.

The degree of evolutionary conservation at individual amino acid sites of TM and AK were determined using Consurf server by applying the Rate4Site algorithm. In ConSurf, the evolutionary rate is estimated based on the evolutionary relatedness between the protein and its homologs and considers the similarity between amino acids as reflected in the substitutions matrix. The conservation grades identified using ConSurf are mapped to the query sequence and/or structure using the ConSurf color-code, with cyan-through-purple corresponding to a variable (grade 1)-through-conserved (grade 9) positions (**Figure 1B**). As the analysis can only be conducted if there are at least five homologous proteins, the conservation analysis could only be conducted for the two major allergens, TM and AK, but not for SCP and MLC. In general, TM had more conserved amino acids than AK. Most of the conserved amino acids in the TM were located at the N- and C-terminal regions. A total of 175 out of 284 TM residues had conservation grades >5, while 86 residues (30%) had the full grade 9. For AK, 200 out of 356 residues had conservation grades >5, of which 96 residues (27%) had grade 9.

Conservation of IgE Binding Epitopes of Shrimp Allergens

To determine the likelihood of cross-reactivity between shrimp and other invertebrates, the conservation analysis of epitopes in those groups was conducted. A database of linear 176 epitopes was generated, containing 96 epitopes from TM, 39 epitopes from AK, 27 epitopes from MLC, and 12 epitopes

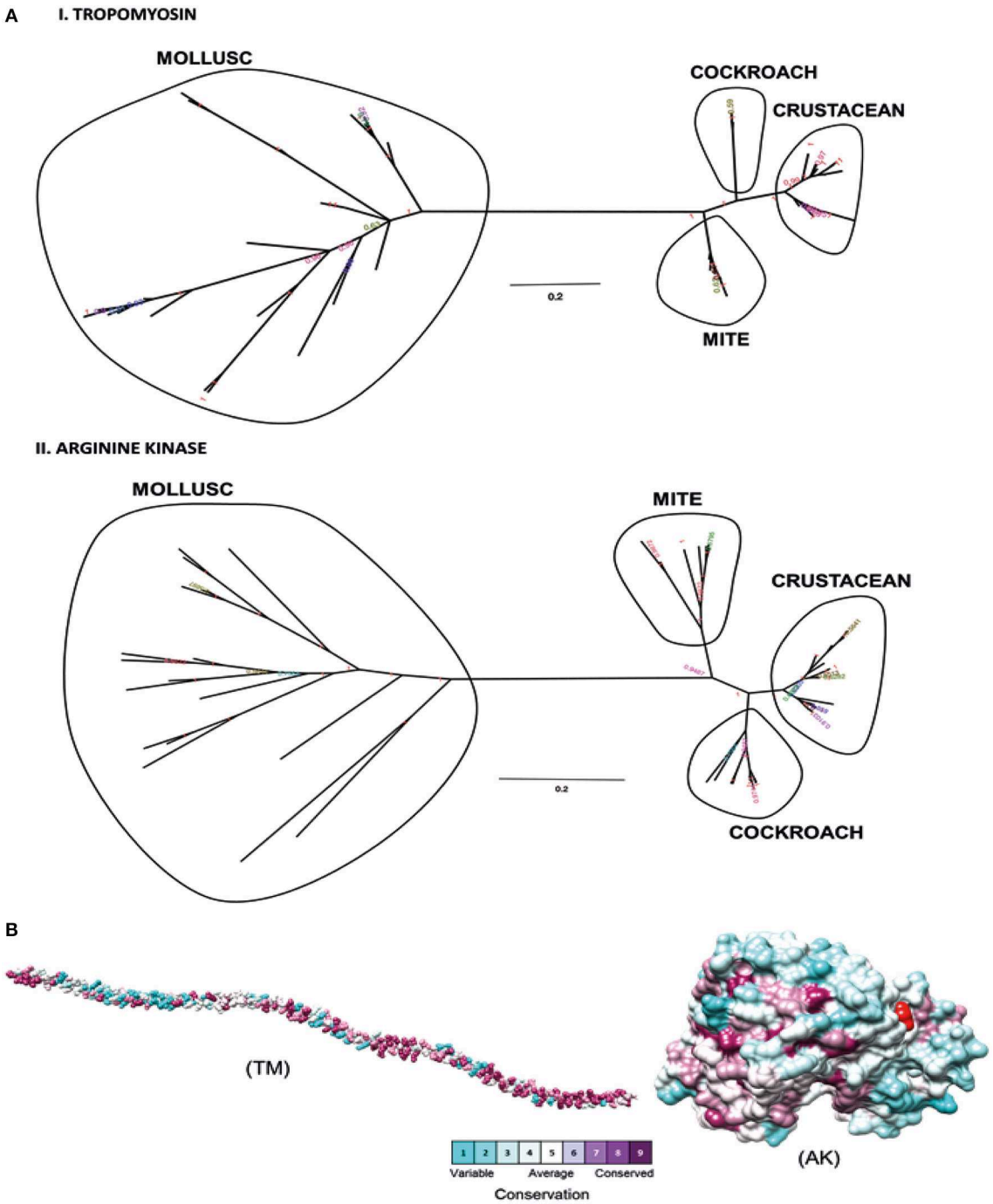

FIGURE 1 | Molecular evolutionary analysis of shellfish allergens. **(A)** The phylogenetic tree was drawn using Bayesian and Maximum Likelihood approach for (I) Tropomyosin and (II) Arginine kinase. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. **(B)** Conservation analysis of individual amino acids in tropomyosin (TM) and arginine kinase (AK) was determined using the Consurf server. The conservation grades were mapped onto the query sequence and structure using the ConSurf color-code, with cyan-through-purple corresponding to a variable (grade 1)-through-conserved (grade 9) positions.

from SCBP. These epitopes were identified to be recognized by IgE antibodies from over 100 patients with shellfish allergy as determined in previous studies (6, 29, 42–45). The epitope conservation was determined using epitope conservancy analysis tool within the IEDB webpage. Epitopes were conserved when they share less than two amino acid mismatches within the

aligned sequence. The conservation analysis showed a similar trend for TM and AK (**Figure 2**). The number of species containing peptide regions similar with the epitopes was found to be the highest within the crustacean followed by cockroach, mite, and mollusc.

Approximately, 91% of the IgE epitopes were found to be conserved in crustacean, 56% in cockroach and 48% in mite species. The number of conserved epitopes in crustacean, cockroach, and mites epitopes were significantly higher than in the three mollusc groups where only <20% of TM epitopes were conserved (**Figure 3A**) and even less for AK with 9% of the epitopes (**Figure 3B**). Nevertheless, these conservations were very high with the majority having only one or two amino acid mismatches. Within the molluscs, the cephalopods had the highest number of conserved epitopes, followed by gastropods and bivalves. The analysis of 20 MLC and 10 SCBP proteins resulted in no conserved epitopes to be identified within the mollusc group.

Invertebrate Allergen Pan-Epitopes

Species-specific conservation analysis of IgE binding epitopes was carried out to identify the allergen epitope sequences that could be termed "pan-epitope." Of the 97 shrimp TM IgE-binding epitopes, 22 invertebrate pan-epitope were

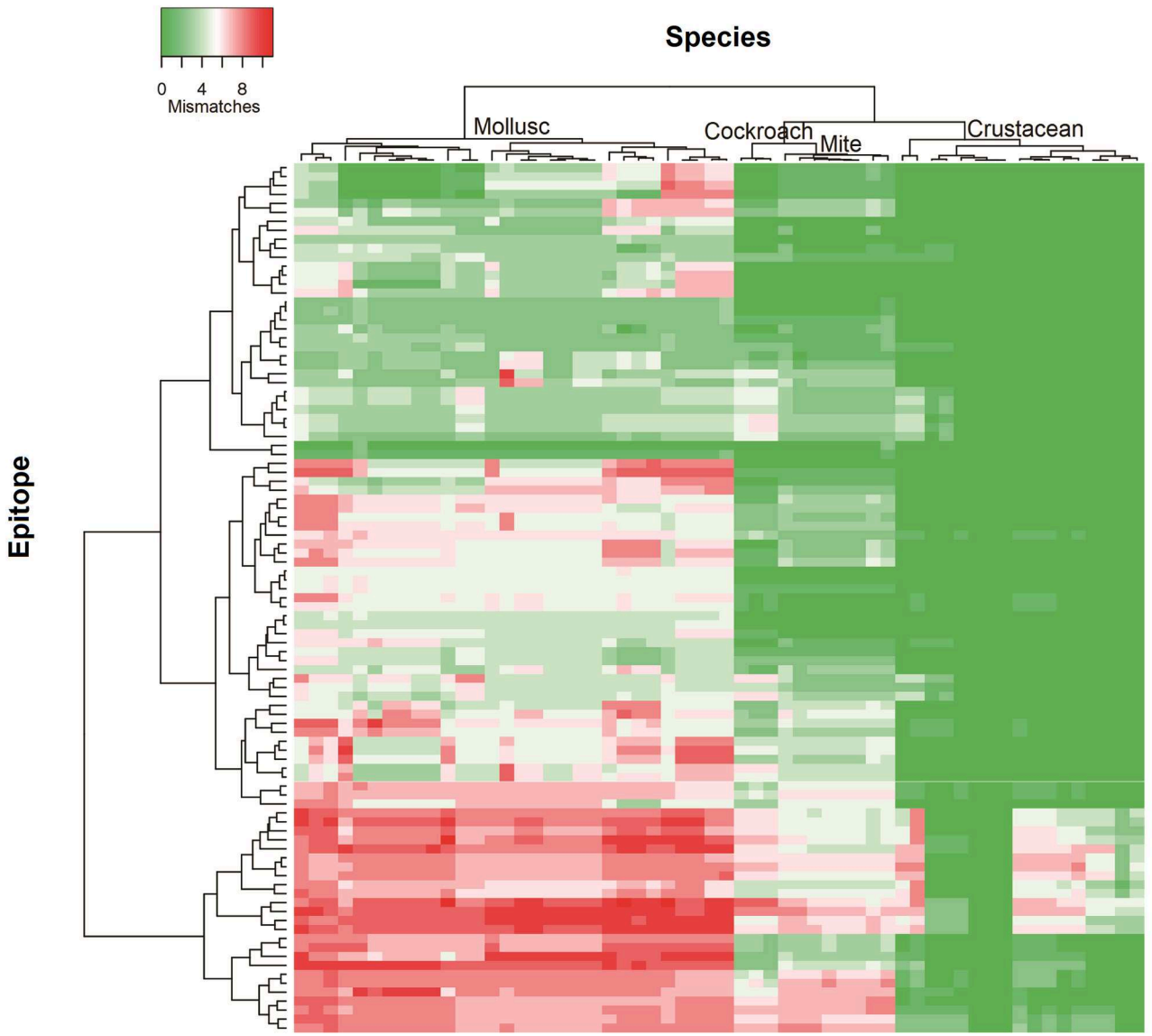

FIGURE 2 | Heatmap representing number of mismatches in homologous peptide of shrimp TM IgE-binding epitopes in different invertebrate species. The heatmap was generated using Heatmapper and clustered using Manhattan distance metric approach. The colors grading as indicated in the top left represents the number of amino acid mismatches found in the homologous peptides of epitopes. Green indicates no mismatches and red indicates maximum mismatches.

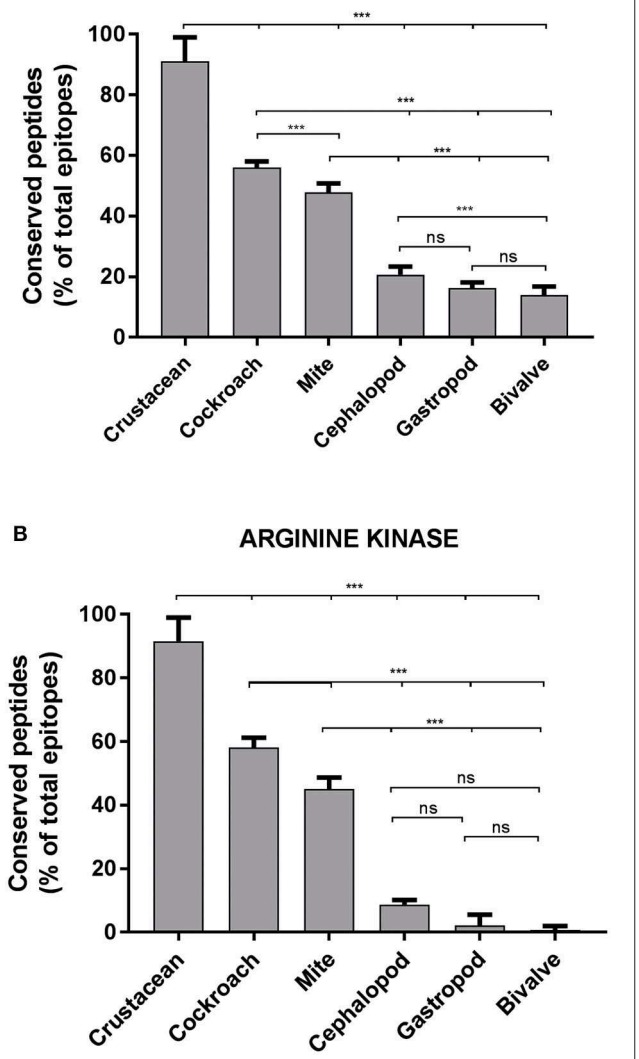

FIGURE 3 | Percentage of conserved shrimp IgE-binding epitopes between invertebrate species. Ninety-Eight B-cell epitopes from tropomyosin **(A)** and 39 B-cell epitopes from arginine kinase **(B)** were examined for their conservation within shellfish and between other allergenic invertebrate species. An epitope was considered conserved if the sequence matched to a homolog or a peptide variant with not more than 2 amino acid substitutions in another species. Significance differences ($p < 0.05$) were calculated using One-way ANOVA. ns, not significant, ***$p < 0.05$.

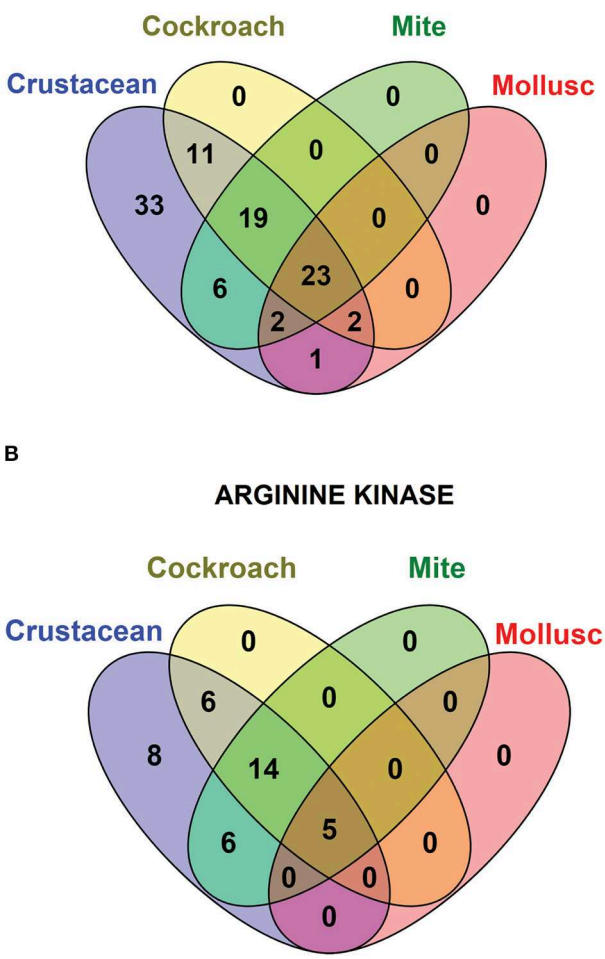

FIGURE 4 | Venn diagram showing the similarities among the conserved shrimp IgE-binding epitopes across invertebrate species. In total 96 epitopes on TM **(A)** and 39 on AK **(B)** were analyzed.

identified (**Figure 4A**). These epitopes were conserved and shared by crustacean, cockroach, mite as well as the mollusc. The epitope sequences are summarized in **Table 1**. The species belonging to the Arthropoda—crustacean, cockroach, and mite, shared 19 IgE-binding epitopes, while crustacean-cockroach and crustacean-mite shared 11 and 6 epitopes, respectively. Thirty-three IgE-binding epitopes were specific to crustaceans and may be used to diagnose crustacean-specific IgE sensitization.

In contrast, only 5 of the 39 shrimp AK IgE-binding epitopes were conserved across crustacean, cockroach, mite, and mollusc (**Figure 4B** and **Table 2**) and unlike TM IgE-binding epitopes, only few AK IgE-binding epitopes were specific to crustacean. **Table 1** shows that most of the invertebrate TM pan-epitopes are located at the N- and C-terminals of the TM sequence. The epitopes at the amino acid position 243–264 were of importance for the cross-reactivity across the tested sequences due to high conservation in over 90% of the invertebrate species analyzed.

Shellfish Allergen Pan-Epitopes

Avoidance of other shellfish species including molluscs is one of the management strategies for shrimp-allergic patients. However, **Figure 1** shows that the major allergenic proteins from shrimps and mollusc are distinctly different, supported by previous studies showing that cross-reactivity

TABLE 1 | Sequences of TM IgE-binding epitopes responsible for cross-reactivity between crustacean, cockroach, mite, and mollusc in prawn allergic patients and their presentation in each invertebrate group.

Epitope sequence	Position	Presentation (%)			
		Crustacean (%)	Cockroach (%)	Mite (%)	Mollusc (%)
MDAIKKKMQAMKLEK	1–15	100	100	100	77
IKKKMQAMKLEKDNA	4–18	100	100	100	10
VAALNRRIQLLEEDL	85–99	100	100	100	3
LNRRIQLLEEDLERS	88–102	100	100	100	33
NRRIQLLEEDLERSEER	89–105	100	100	100	33
RIQLLEEDLER	91–101	100	100	100	43
RIQLLEEDLERSEER	91–105	100	100	100	33
EASQAADESERMRK	115–128	100	100	100	50
EASQAADESERMRKV	115–129	100	100	78	50
LENQLKEA	144–151	100	100	100	37
LAEEADRKYDEVARK	154–168	100	100	100	10
EADRKYDEVARKLAM	157–171	100	100	100	10
ESKIVELEEELRVVG	187–201	100	100	100	17
IVELEEELRVVGNNL	190–204	100	100	100	20
LEEELRVVGNNLKSL	193–207	100	100	100	50
KEVDRLEDELVNEKEKYKSI	241–260	100	100	100	60
ERSVQKLQKEVDRLEDE	243–259	100	100	100	90
QKLQKEVDRLEDELV	247–261	100	100	100	93
LQKEVDRLEDELV	249–261	100	100	100	100
QKEVDRLEDELVNEK	250–264	100	100	100	93
KEVDRLEDE	251–259	100	100	100	100
VDRLEDELVNEKEKY	253–267	100	100	100	63

TABLE 2 | Sequences of AK IgE-binding epitopes responsible for cross-reactivity between crustacean, cockroach, mite, and mollusc in prawn allergic patients and their presentation in each invertebrate group.

Epitope sequence	Position	Present in (%)			
		Crustacean (%)	Cockroach (%)	Mite (%)	Mollusc (%)
SLLKKYLTKEVFDKL	25–39	57	100	33	9
EGGIYDISNKRRMGL	319–333	100	100	67	36
IYDISNKRRMGLTEF	322–336	100	100	67	55
ISNKRRMGLTEFQAV	325–339	100	100	100	45
KRRMGLTEFQAVKEM	328–342	100	50	100	27

between shrimp and mollusc is species-specific (46). Based on this rationale, further detailed analysis of shrimp IgE-binding epitope conservation across three edible mollusc classes, including bivalves, cephalopods and gastropods were carried out. In total, 28 shrimp TM and 5 AK IgE-binding epitope sequences were aligned with less than two amino acid mismatches with at least one mollusc species (**Figure 4**).

Of the 28 conserved TM epitopes, only 22 epitopes were present in over 50% of each mollusc classes, with three epitopes were conserved in all of the mollusc species (**Figure 5**). Detailed analysis of the conserved epitopes revealed that half of the amino acid residues in shrimp TM were responsible for cross-reactivity to at least one species of mollusc and these amino acids were distributed across the entire protein sequence. Some of the epitopes showed group-specific conservation, such as Epitope 3, 14, 15, 17, and 18 in **Figure 5**. Unlike TM, where the conserved epitopes were aligned with various species of mollusc, the conserved epitopes of AK were mostly aligned with peptides of the cephalopod. Of the five conserved AK epitopes, only four epitopes were present in over 50% of mollusc species, with two of those were aligned to all species of cephalopods (**Figure 5**). While the complete amino acid sequence of the 4 analyzed shrimp TMs are identical, the length and composition of the identified IgE epitopes differs. Nevertheless, the overall trend of high conservation to cephalopod peptides is similar, as well as the low conservation to bivalves.

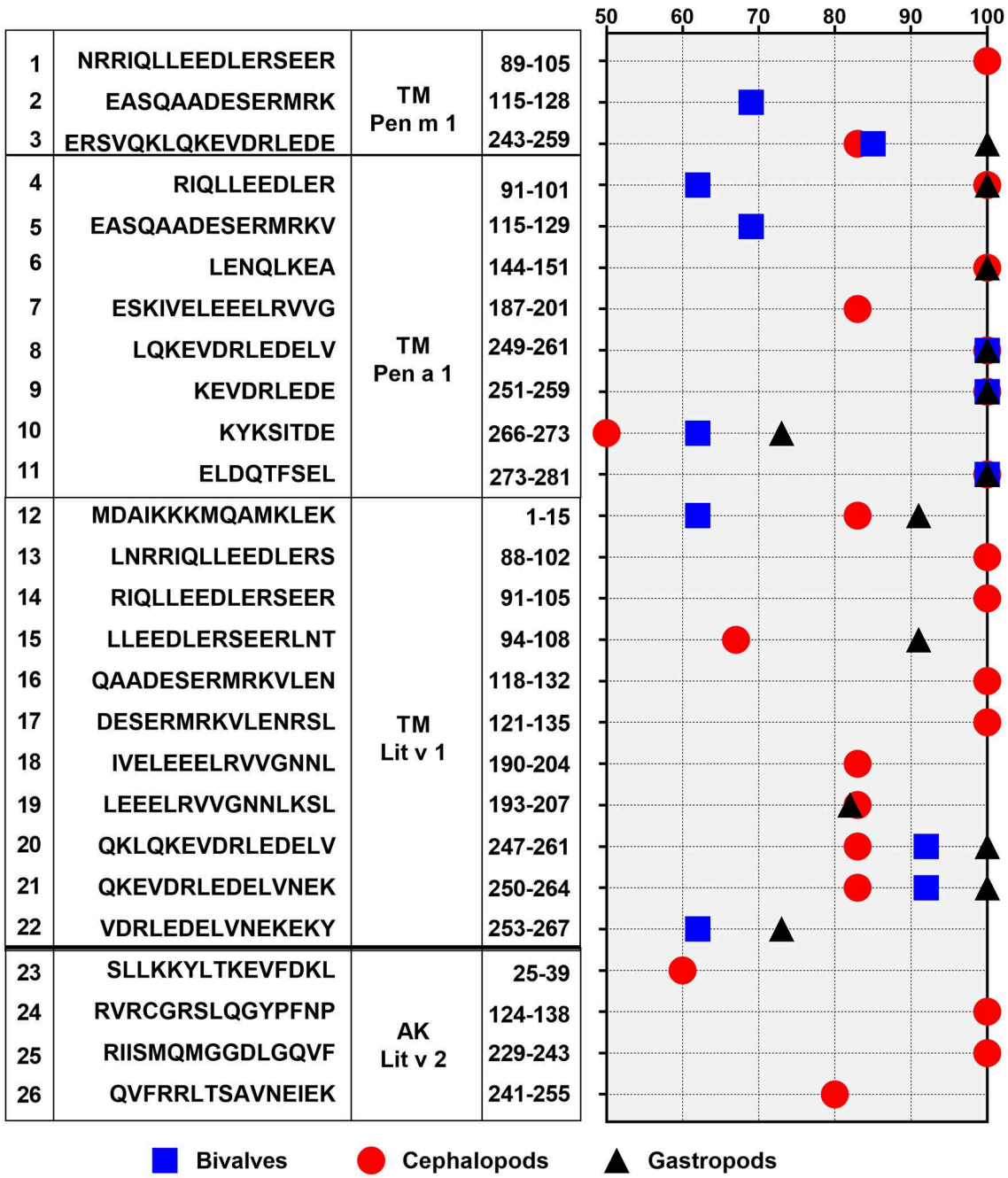

FIGURE 5 | Percentage representation of conserved epitopes in the three mollusc classes: bivalves (square), cephalopod (circle), and gastropod (triangle). Only epitopes which are present in over 50% of each mollusc classes are shown. The epitope allergen sources, their amino acid sequences and positions in the protein are indicated on the left.

Combining the conserved epitopes divided the shrimp TM into three possible cross-reactivity scenarios, located on distinct areas on the TM allergen. Three regions with a total of 35 amino acids residues, or 12%, of the total 284 amino acids were conserved across all classes of the mollusc phyla (**Figure 6**, yellow boxes) and responsible for shellfish pan-allergy. Three regions (**Figure 6**, green boxes) were conserved within the cephalopods and gastropods, and one region (**Figure 6**, gray box) was conserved within the bivalves and cephalopods.

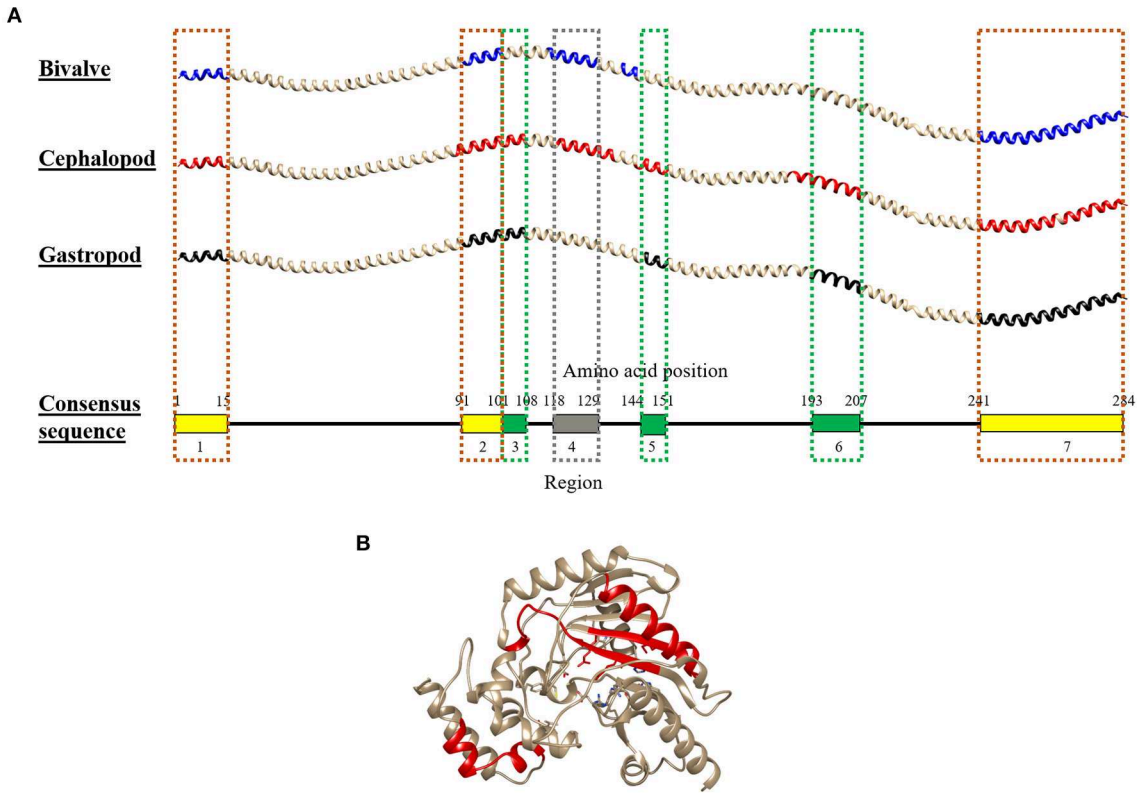

FIGURE 6 | Molecular modeling of the conserved IgE-binding epitopes of **(A)** tropomyosin (TM) and **(B)** arginine kinase (AK) that are presented in over 50% of each mollusc class. For tropomyosin, the epitopes were remapped to their consensus tropomyosin sequence and color-coded based on mollusc classes in which the conserved epitopes are found: yellow (all mollusc classes), green (cephalopod and gastropod), and gray (bivalve and cephalopod). The red color in AK protein model highlights the conserved sequence. The protein structure of TM and AK were modeled using SWISS-MODEL based on reference proteins 1C1G and 4BG4, respectively.

Decision Tree to Identify Potential Crustacean-Mollusc Cross-Reactivity in Shrimp-Allergic Patients

A decision tree was developed to identify potential shrimp-mollusc cross-reactivity in shrimp-allergic patients based on the conservation patterns of shrimp TM and AK IgE-binding epitopes in mollusc species (**Figure 7**). In the diagnosis of shrimp allergy, where clinical history does not give a clear conclusion, sensitization tests against a whole shrimp extract and specific allergens are needed prior to oral food challenge. If TM-specific IgE results are positive, with quantitative IgE-levels to TM being similar than to the whole protein extract, immune-dominant sensitization to shellfish TM is likely, and broad (serological) cross-reactivity to other shellfish species is to be expected. Analysing IgE-binding to shrimp TM epitopes can further improve the diagnosis of cross-reactivity. Four reactivity patterns are suggested including; crustacean mono-reactivity, crustacean-mollusc cross-reactivity, crustacean-cephalopod-gastropod cross-reactivity, and crustacean-cephalopod-bivalve cross-reactivity. However, if TM-specific IgE are not present (negative), then cross-reactivity due to sensitization to AK is still possible, however only Region 1 would be responsible for crustacean-cephalopod cross-reactivity.

DISCUSSION

The development of safe and reliable diagnostic tools is crucial to accurately diagnose allergic sensitization in patients and determine the primary allergen sources. Diagnosis of shellfish allergy, in particular, is a major challenge for the management of the allergic patients due to highly cross-reactive nature of some shellfish allergens. This problem is clearly evident in a recent study by Pascal et al. (6) where 44% of their negative controls positively reacted with tropomyosin, resulting in an overall false-negative rate of 17% in their diagnosis. A preceding study demonstrated that IgE recognition of allergic patients toward identical and/or similar homologous peptides to the allergen epitopes are the basis of the molecular cross-reactivity (31). Thus, the present study was conducted to determine shrimp IgE-binding epitopes that could be used to predict cross-reactivity toward other invertebrate species in shrimp allergic individuals, through which better predictive diagnostic tools for shellfish allergic patients could be developed.

The present study demonstrates that within a large directory of shrimp IgE-binding epitopes, there are a substantial fraction of epitopes that are highly conserved across invertebrate species. These conserved epitopes might play a key role in cross-reactivity between shrimp and other invertebrate species. Shrimp TM and

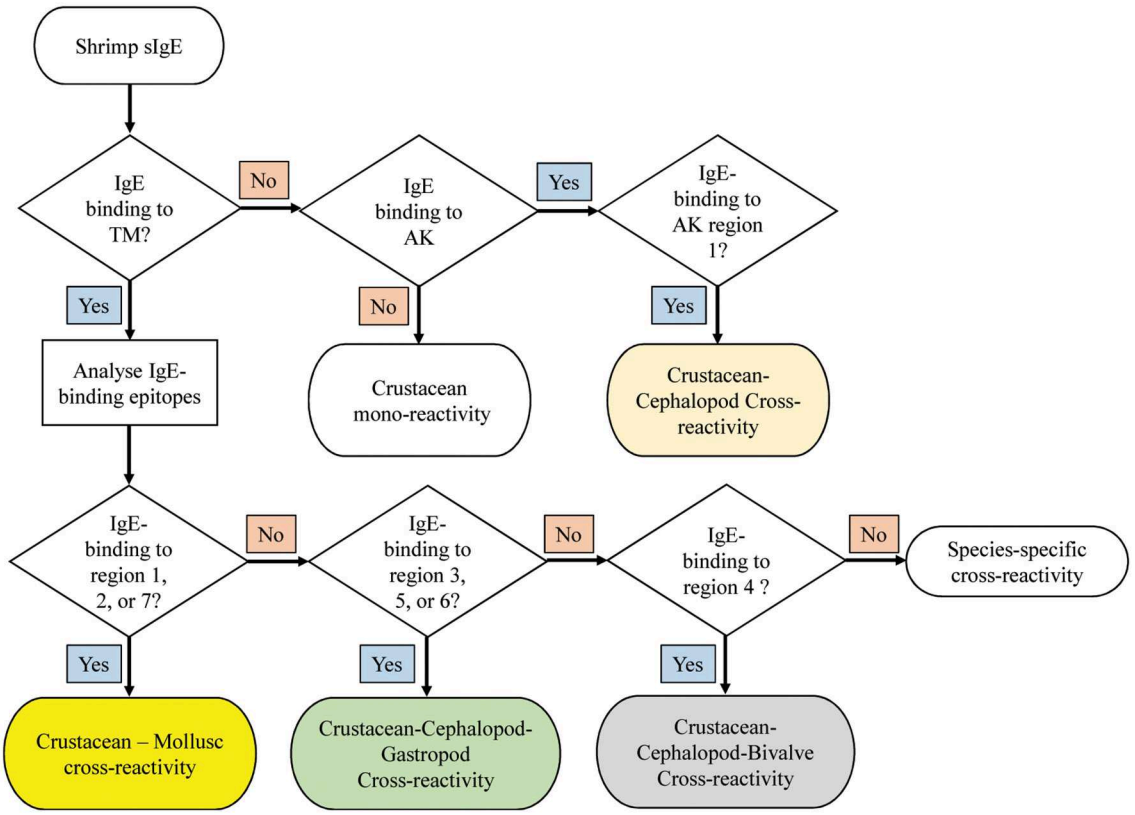

FIGURE 7 | Decision tree to diagnose potential molecular cross-reactivity to the invertebrate allergens TM and AK in shrimp allergic patients based on the reactivity to specific IgE epitopes. The region numbers refer to the epitope mapping in **Figure 5**.

AK shared a higher number of conserved epitopes compared to shrimp SCP and MLC; in fact, no conserved epitope could be found for SCP, while the MLC only shared two epitopes in one region with cockroach MLC. A recent study demonstrated the absence of IgE recognition to shrimp SCP and MLC in house dust mite allergic infants (7). These results suggest that TM and AK are the major contributing proteins in immunological and clinical cross-reactivity between crustacean and other invertebrate groups. Furthermore, comparative evaluation of the number of conserved epitopes in TM and AK revealed a clear cross-reactive hierarchy where cockroach is on the top followed by mite, while molluscs are on the bottom of this hierarchy.

House dust mite and cockroach are the most common sources of indoor allergens worldwide with up to 85 and 60% of asthmatic patients eliciting sensitization to at least one of the mite or cockroach allergens, respectively. In a retrospective study of 95 shrimp-allergic patients in Canada, 90.5% of those patients had a positive test to HDM (47). Similarly, a study on an Asian population found a majority of patients with shrimp allergy have positive skin tests to HDM and cockroach (48). Vivid evidence of this cross-reactivity was demonstrated by a study on Orthodox Jews who positively tested against shrimp yet had no prior exposure to seafood due to strict religious laws prohibiting shellfish consumption (14). TM and AK seem to be the allergens responsible for this cross-reactivity as supported by the current study. Shrimp TM shares about 82 and 81% amino acid identity with cockroach and house dust mite tropomyosin, respectively. Likewise, shrimp AK shares about 82 and 78% amino acid identity with cockroach and house dust mite AK, with 66% of shrimp TM IgE-binding epitopes and 80% of shrimp AK IgE-binding epitopes are also existing within TM and AK of cockroach and house dust mite. These identified IgE-binding epitopes could be used to diagnose shrimp sensitization in patients sensitized to cockroach or house dust mite, without a previous history of allergic reactions to shrimp. Reciprocally, we can use the non-conserved IgE-binding epitopes to diagnose shrimp mono-sensitization. These findings are of significant impact on the diagnosis of shrimp allergy as current diagnostic tools using tropomyosin are not specific. Although tropomyosin is a good predictor of allergy reaction to crustacean (49), the rate of false positive reaction is still high due to IgE binding of antibodies developed in patients against tropomyosin from other invertebrate sources, in particular house dust mite and cockroach (11). Furthermore, the identification of specific IgE binding epitopes allows the prediction of allergic reactions to ingested crustacean, in patients allergic to cockroach and house dust mite. As over 30% of the global population is sensitized to dust mite allergens,

the developed predictive model in this study could be of major importance.

Crustaceans and molluscs are generally referred to as "shellfish" in the context of seafood consumption and avoidance to both groups are often advised for shellfish allergic patients (50). Patients with allergy to shellfish may fail to identify the offending seafood species, often as a result of confusion regarding the different common names used to describe diverse seafood. Crustaceans are classified as arthropods together with spiders and insects, while the group of molluscs is a large and diverse group, subdivided into different classes such as bivalves, gastropods, and cephalopods. Precise diagnosis of allergy to crustacean or mollusc species is difficult as no species-specific allergens have been identified so far. Moreover, true sensitization to shellfish-specific allergens can be hampered due to the highly cross-reactive nature of some allergenic proteins, including TM and AK (51, 52). While crustacean TMs and AKs show very high amino acid sequence identity (up to 98 and 97%, respectively) with demonstrated IgE cross-reactivity (53, 54), the reported sequence identity between crustacean and mollusc TMs and AKs is much lower with only up to 68 and 58%, respectively. The gold standard to determine food allergy is an oral food challenge. However, due to the risk of severe reactions to shellfish allergens, this test is not frequently performed. Our present findings suggest that specific shrimp allergen IgE-binding epitopes could be used as a robust, alternative way to diagnose cross-reactivity between crustacean and mollusc species among shellfish-allergic patients. Among the known 97 shrimp TM IgE-binding epitopes, 71 epitopes are only existing within crustacean TMs, while 26 epitopes are shared with mollusc TMs. Meanwhile, of the 39 shrimp AK IgE-binding epitopes, only five epitopes are shared with cephalopod AK. In contrast, no shrimp SCBP and MLC IgE-binding epitopes are present in mollusc SCBP or MLC. These findings indicate that only shellfish-allergic patients sensitized to TM have a risk of reacting to both crustacean and mollusc species. This supports the conclusion of the previous study where crustacean-allergic patients with concurrent mollusc allergy reacted more frequently to tropomyosin than without mollusc allergy (93 vs. 35%, respectively, $P = 0.004$), while recognition of the other allergens were not different in both patient cohorts (55).

Our findings also demonstrate different patterns of conserved IgE-binding epitopes among the three mollusc classes, suggesting that some crustacean-allergic patients will cross-react to one but tolerate another class of molluscs. The cephalopods have a higher probability to cross-react with crustacean than the other mollusc classes. The cephalopod TM amino acid sequences have a higher identity with crustaceans than those of gastropod and bivalve (68 vs. 63 vs. 62.3%, respectively), and therefore contained more homologous peptides of shrimp TM and AK IgE-binding epitopes. From the study of Vidal et al. (55), of the 14 crustacean-allergic patients with mollusc allergy that were examined by skin prick tests against different mollusc species, 11 patients were positive to cephalopods, and 6 patients were positive to bivalves. While no study identified IgE-cross-reactivity due to AK between crustacean and gastropods or bivalves, cross-reactivity between cephalopods and crustacean has been reported (56). Nevertheless, immunological cross-reactivity between shrimp and other mollusc classes, the gastropods and bivalves, has been demonstrated in several studies (57–59).

Gastropod and bivalve TMs share only 60% sequence identities with crustacean TMs, however, unlike cephalopod where the identity of TM amino acid sequence is very high among the group, the identity of TM amino acid sequence in the species among those two classes is very variable, particularly among bivalve species. The variability of bivalve TM is very apparent where out of 23 conserved IgE-binding epitopes, only five epitopes were shared across all species of bivalve providing a molecular basis of selective cross-reactivity (46). It is to be noted that most experimental IgE epitope mapping studies published in the database use short overlapping peptide libraries for each allergen. This inevitably leads to elucidation of only linear IgE epitopes. However, since tropomyosin has an alpha-helical structure, all its IgE binding epitopes are known to be linear epitope (29). In case of arginine kinase, there may be a possibility of conformational IgE epitopes but this hasn't yet been proven experimentally, and is a caveat of this study.

Based on the abovementioned findings on the different pattern of IgE-binding epitope conservations in the three mollusc classes, we developed a decision tree to predict immunological cross-reactivity between shrimp and mollusc classes based on TM and AK. This decision tree could contribute significantly toward patient management, particularly on the aspect of food avoidance and diet. It has been well-known that shrimp allergic patients are advised to avoid all shellfish species including mollusc due to the risk of cross-reactivity. Our decision tree suggests that this advice should not be generalized as only patients sensitized to TM which account for 70–80% of total shrimp-allergic patients have a risk of cross-reacting with mollusc allergens. Moreover, the cohort of shrimp-allergic patients could be further divided into five groups based on their cross-reactivity patterns to specific IgE binding epitopes.

CONCLUSION

Prediction of immunological cross-reactivity between an allergen and close related proteins based on similarity of the IgE-binding epitopes has been confirmed to be more accurate than the prediction based on similarity of the complete amino acid sequence of the allergenic proteins. Food allergens including shellfish allergens tend to have sequential IgE-binding epitopes due to digestion in the gastrointestinal tract. Thus, epitope sequence comparison is more relevant and conceivable for assessing the potential cross-reactivity of allergenic proteins, then comparing the whole protein sequence. The shrimp allergen IgE-binding epitope conservation results outlined in this study illustrate that a clear hierarchy of cross-reactivity is discovered, with TM being the most cross-reactive allergen among allergenic invertebrate species. This is most likely the main reasons that TM is one, if not the major pan-allergen in inhalant and ingestion animal allergy. The IgE binding epitopes located at the N- and C-terminal regions of TM are highly

conserved and could be used as biomarkers to predict allergic cross-reactivity of shrimp-allergic patients. Unexpectedly, more than half of the TM as well as the AK IgE epitopes were found to be conserved in cockroach and mite TM and AK, respectively. In contrast, only few shrimp IgE-binding epitopes were conserved across the molluscs. This suggests a low risk of cross-reactivity of shrimp allergic patients to molluscs, while a high risk of cross-reactivity to cockroach or mite is predicted. We developed for the first time a decision tree to predict cross-reactivity between shrimp and molluscs based on the major allergens TM and AK. These fundamental findings could simplify the diagnosis of cross-reactivity among shellfish-allergic patients, thereby avoiding potential life-threatening food challenges.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.iedb.org/ - list of epitopes can be found in the **Supplementary Material**.

AUTHOR CONTRIBUTIONS

RN, SDK, and AL conceived the study and wrote the manuscript. RN, EJ, TR, and SK performed and analyzed the experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02676/full#supplementary-material

Data Sheet 1 | List of epitopes.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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IgE and IgG$_4$ epitopes revealed on the major fish allergen Lat c 1

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Abstract

Background: The IgE- and IgG4-binding patterns of the major fish allergen parvalbumins are not clearly understood. IgE antibody-binding to parvalbumin from Asian seabass, Lat c 1.01, is implicated in up to 90% of allergic reactions, although the region of IgE or IgG4 epitopes are unknown. In the present study, we characterized the specific IgE- and IgG4-binding regions of Lat c 1.01 using serum from pediatric and adult patients with clinically-confirmed fish allergy.

Methods: A comparative investigation of patient IgE- and IgG4-binding to recombinant Lat c 1.01 was performed by immunoblotting and indirect ELISA using serum from 15 children and eight adults with clinically confirmed IgE-mediated reactions to fish. The IgE- and IgG4-binding regions of Lat c 1.01 were determined by inhibition ELISA using seven overlapping peptides spanning the entire 102 amino acid sequence. Eluded IgE-binding regions were modelled and compared to known antibody-binding regions of parvalbumins from five other fish species.

Results: Twenty-two out of 23 sensitized patients demonstrated IgE-binding to rLat c 1.01, while fewer patients (10/15 children and 7/8 adults) demonstrated robust IgG4-binding when determined by immunoblots. IgE-binding for both cohorts was significantly higher compared to IgG4-binding by ELISA. All patients in this study presented individual IgE and IgG4 epitope-recognition profiles. In addition to these patient-specific antibody binding sites, general IgE epitopes were also identified at the C- and N-terminal regions of this major fish allergen.

Conclusions and Clinical relevance: Our findings demonstrate two specific IgE epitopes on parvalbumin from Asian seabass, while IgG4-binding is much lower and patient-specific. This study highlights the importance of advancement in epitopes analysis regardless of the age group for diagnostics and immunotherapies for fish allergy.

Keywords: Epitope mapping, Fish allergy, IgE epitope, IgG4 epitope, Parvalbumin

1. Introduction

IgE-mediated fish allergy is an adverse immunological response to fish which persists through adulthood (Davis et al., 2020), with sensitization rates of up to 3% in the general population (Moonesinghe et al., 2016). Fish is the second most common trigger of food allergy after crustacean in countries with high seafood consumptions (Le et al., 2019). A wide range of clinical symptoms, including life-threatening anaphylaxis, can be caused by exposure to fish allergens (Matricardi et al., 2016). There are no effective therapeutics and strict avoidance of the implicated fish is the only measure currently available to affected patients (Lopata and Kamath, 2012). The major fish allergen is parvalbumin (Kuehn et al., 2014), a calcium-binding protein expressed in muscle tissues, and detailed characterization is essential to ensure a correct diagnosis (Kuehn et al., 2013) and the development of therapeutic approaches (Zuidmeer-Jongejan et al., 2015). The main challenges for the development of reliable diagnostics are the considerable molecular differences in amino acid sequences of parvalbumins between fish species and the presence of multiple isoforms (Ruethers et al., 2018).

Linear IgE-binding epitopes of different parvalbumin allergens have been characterized (Stephen et al., 2017) and might contribute to species-specific clinical reactivity often seen in fish-allergic patients. There are five studies mapping the specific immunoglobulin (Ig)E-binding epitopes of parvalbumin from fish species, including Baltic cod (allergen nomenclature: Gad c 1; Elsayed and Apold, 1983), common carp (Cyp c 1; Untersmayr et al., 2006), Pacific mackerel (Sco j 1; Yoshida et al., 2008), Atlantic salmon (Sal s 1; Perez-Gordo et al., 2012) and Atlantic cod (Gad m 1; Perez-Gordo et al., 2013). For the identification of IgE-binding epitopes, various techniques including phage display library, overlapping synthetic peptides and tryptic digests of parvalbumin were utilized. The allergens analyzed display both linear and conformational epitopes; however, no epitopes share identical amino acid sequences between these five fish species. While the IgE-binding epitopes of these parvalbumins commonly recognized by fish-allergic patients have been established, patient-specific epitopes are also considered to exist for other fish species based on the findings on Gad m 1 from Atlantic cod (Perez-Gordo et al., 2013).

Identifying IgE epitopes on food allergens may help to develop and predict successful specific immunotherapies (sIT) for allergy sufferers (Vickery et al., 2013; Savilahti et al., 2014). An increase in specific IgG_4 response could be an indicator of successful sIT due to this antibody being able to out-compete IgE-binding for the allergen (Santos et al., 2020). In addition, elevated IgG_4 levels have been associated with protective effects (Du Toit et al., 2015). However, a comparative study of the IgE- and IgG_4-binding regions on parvalbumin has never been performed despite being the major allergen for fish.

Worldwide consumption of Asian seabass (*Lates calcarifer*), also known as barramundi, is rapidly growing due to increased availability from aquaculture industries and its culinary popularity (Loughnan et al., 2013). We have recently identified and characterized parvalbumin from this species and two related allergen isoforms, Lat c 1.01 and Lat c 1.02 (Sharp et al., 2014) (www.allergen.org). Asian seabass can cause allergic reactions in up to 90% of fish-allergic patients, with IgE reactivity to at least one of the two parvalbumin isoallergens identified in this species (Turner et al., 2011; Sharp et al., 2014). The parvalbumin isoform, Lat c 1.01 appears to have the greatest IgE reactivity (Sharp et al., 2014). With an aim to elucidate the IgE- and IgG_4-binding patterns of fish parvalbumins, we sought to characterize the most IgE-reactive Asian seabass parvalbumin, Lat c 1.01, using 23 pediatric and adult patients with clinically confirmed IgE-mediated fish allergy, and ultimately, to define patient-specific IgE- and IgG_4-binding regions on fish parvalbumins.

2. Materials and Methods

2.1 Patients

Fish-allergic patients were recruited based on a convincing recent history of an IgE-mediated reaction (including urticaria, angioedema, stridor, cough, wheeze, hypotension and/ or gastrointestinal symptoms of vomiting and/or diarrhea) to any fish, occurring within 2 h of ingestion. Sensitization to fish was confirmed *in vivo* by skin prick testing using commercial reagents and/or *in vitro* by determining the levels of serum IgEs (ImmunoCAP, Phadia-Thermo Fisher Scientific; Uppsala, Sweden) to cod (*Gadus morhua*), tuna (*Thunnus albacares*), and/or salmon (*Salmo salar*). The 23 patients, consisting of eight adults and 15 children (details are listed in Table 1), were selected based on positive IgE reactivity to recombinant Asian Seabass parvalbumin isoform (rLat c 1.0101) (Sharp et al., 2014). Sera from five non-atopic patients were used as negative controls. Ethics approval for this study was granted by James Cook University's Ethics committee (JCU-H4313) in collaboration with The Westmead Children's Hospital Network (LNR-14/SCHN/185) and The Alfred Hospital (Project number 192/07) and Monash University's Ethics Committees (MUHREC CF08/0225).

2.2 Recombinant Asian Seabass parvalbumin expression

Recombinant (r)Lat c 1.01, Asian seabass parvalbumin β1, was obtained as previously described (Sharp et al., 2014). In brief, the β1 parvalbumin gene (GenBank accession number: KF021278.1) was cloned into the expression vector pPROEX HTb (Invitrogen; Waltham, USA) and used to transform NM522 *Escherichia coli* cells. The expression of hexa-histidine (his 6)-tagged rLat c 1.01 was induced with 1 mM IPTG over 3 h at 37 °C, and purified from clarified cell lysate using HisTrap HP column (GE Healthcare; Little Chalfont, UK). Purified rLat c 1.01 was applied onto a 15% polyacrylamide gel and separated under reducing condition according to its molecular weight using a Mini-PROTEAN® SDS-PAGE system (BioRad laboratories; Hercules, USA) as described by Laemmli (Laemmli, 1970). rLat c 1.01 was visualized by Coomassie Brilliant Blue R-250 staining and confirmed by subsequent immunoblotting with two allergen-specific antibodies; monoclonal anti-parvalbumin PARV-19 antibody (Sigma-Aldrich; St. Louis, USA), previously evaluated for the detection of fish parvalbumin (Saptarshi et al., 2014), and in-house generated polyclonal antibodies against parvalbumin from Asian seabass raised in rabbits (Sharp et al., 2015).

2.3 IgE and IgG$_4$ immunoblot analysis

Patient IgE- and IgG$_4$-binding to denatured rLat c 1.01 was analyzed by immunoblotting. Purified rLat c 1.01 was separated on a 15% polyacrylamide gel under reducing condition, then transferred onto a nitrocellulose membrane (BioRad laboratories). The membrane was blocked with tris-buffered saline (TBS)/0.05% Tween-20 (TBS-T) for 2 h at room temperature. Both sera and detection antibodies were diluted in 0.1% BSA in TBS-T. Membranes were placed into the surf-blot manifold (Idea Scientific, Minneapolis, USA), then applied with diluted patient serum (1:20) and incubated for 16 h at 4 °C, with subsequent washing with TBS-T at room temperature. For the detection of IgE, the membranes were further incubated with horseradish peroxidase (HRP)-labelled goat anti-human IgE antibody (1:10,000, Genetex; Irvine, USA). The IgG$_4$ were detected with monoclonal anti-human IgG$_4$ MAB1313 antibody (1:2,000, Merck; Kenilworth, USA), followed by subsequent detection with anti-mouse IgG HRP-labelled antibody (1:20,000, Sigma-Aldrich). Both IgE and IgG$_4$ binding complexes were visualized by ECL technique.

2.4 IgE and IgG$_4$ indirect enzyme-linked immunosorbent assay (ELISA)

Patient IgE- and IgG$_4$-binding to native (non-denatured) rLat c 1.01 was analyzed by ELISA. The 96-well EIA/RIA plates (Costar; St. Louis, USA) were coated with 20 µL of rLat c 1.01 (1 µg/mL in sodium carbonate/bicarbonate buffer, pH 9.6), and incubated overnight at 4 °C. Plates were blocked with 5% skim milk/PBS-T for 1 h, then serum (1:10) in 1% skim milk/PBS-T was added and incubated for 3 h at room temperature while shaking (45 rpm). The wells were washed and then incubated with either anti-human IgE HRP-labelled antibody (1:10,000, Genetex) or monoclonal anti-human IgG$_4$ MAB1313 antibody (1:2,000, Merck) in 1% skim milk/PBS-T. Serum IgG$_4$ plates were further incubated with anti-mouse IgG HRP-labelled antibody (1:20,000, Sigma-Aldrich) with gentle shaking. Plates were washed extensively, and the antibody-binding was detected using TMB (3,3',5,5'-tetramethylbenzidine) substrate (BD Biosciences; Franklin Lakes, USA). The reaction was terminated using 1 M hydrochloric acid and the absorbance (O.D.) measured at 450 nm by spectrophotometry (Molecular Devices; Sunnyvale, USA).

2.5 Overlapping peptides of parvalbumin

Seven overlapping peptides spanning Lat c 1.01 sequence, each 25 amino acids in length, were synthesized and purified to 95% homogeneity (Mimotopes; Clayton, Australia). Six peptides were designed with nine amino acids overlapping on both termini. The seventh peptide, covering the C-terminal end of parvalbumin, was designed to provide complete coverage of one of the two calcium-binding sites. Peptides positions are depicted in Figure 5.

2.6 Peptide binding analysis for IgE and IgG_4

Patient IgE- and IgG_4-binding to each peptide were evaluated by inhibition ELISA using 96-well EIA/RIA plates (Costar), coated with 20 µL of rLat c 1.01 (1 µg/mL in sodium carbonate/bicarbonate buffer, pH 9.6) overnight at 4 °C. The wells were blocked with 5% skim milk/PBS-T prior to the assay. Patient serum (1:10) was pre-adsorbed to 12.5 µg/mL synthesized peptides for 2 h, then added to the wells in 20 µL and incubated for 1 h at room temperature. The assays were completed using the same protocol as for the indirect ELISA. All washes were performed in TBS-T and all detection antibodies were diluted in TBS-T with 0.1% BSA. Antibody binding was detected by colorimetric assay using TMB substrate and O.D. measured at 450 nm by spectrophotometry. The percentage of inhibition was calculated as follows: 100 – [(O.D. 450 nm of antibody with inhibitor x 100) / O.D. 450 nm of antibody without inhibitor].

2.7 Comparative modelling of parvalbumins

The homology search was conducted and homology models generated using the carp parvalbumin crystal structure (PDB ID: 4cpv) with SWISS-MODEL (Basel, Switzerland) (Arnold et al., 2006; Biasini et al., 2014). The amino acid sequences of allergens, Gad c 1.01, Cyp c 1.01, Sal s 1.01, Sco j 1.01, Gad m 1.01 and Lat c 1.01, were aligned with Clustal Omega (EMBL-EBI). Theoretical models of parvalbumin were built using UCSF Chimera (version 1.7; San Francisco, USA) (Pettersen et al., 2004).

2.8 Data analysis

Cut-off values for the indirect ELISA were determined using the mean of three non-fish-allergic patients plus two times the standard deviation. For the peptide binding assay, multiple T-tests were used to compare overall serum IgE and IgG_4 reactivity to each peptide between the negative and fish-allergic patients. Analyses were performed using GraphPad Prism (version 8; GraphPad, San Diego, CA).

3. Results

3.1 Patient IgE and IgG$_4$ bind to rLat c 1.01

The analysis of IgE- and IgG$_4$-binding to denatured rLat c 1.01 was first performed by immunoblotting using sera of 23 fish-allergic patients (Figure 1). IgE from all patients but one child (C5) reacted to monomeric rLat c 1.01 (Figure 1A). Five children (C4, C9 and C13-15) also showed reactivity to dimeric forms of rLat c 1.01 at 30 kDa (Figure 1B), while none of the adults demonstrated IgE-binding to the dimers. IgG$_4$ from fewer patients demonstrated binding to rLat c 1.01 (10/15 pediatrics; C1-6, C9, C12-14, 6/8 adults; A1-5 and A8) (Figure 1A). In contrast to IgE-binding, more IgG$_4$-reactive patients detected the dimeric form of parvalbumin (7/15 children; C1-2, C6, C9, C12-14, 2/8 adults; A1 and A4). Two children (C1 and C9) also showed reactivity to a rLat c 1.01 oligomer with a molecular weight above 50 kDa. All five negative control patients (N1-5) demonstrated no IgE or IgG$_4$ binding to rLat c 1.01.

3.2 Patient IgE and IgG$_4$ bind to the native form of rLat c 1.01

Further analysis of IgE- and IgG$_4$-binding to the native form of rLat c 1.01 was performed by indirect ELISA. Binding was considered positive if O.D. values were greater than the cut-off determined based on the non-atopic controls (dotted line) (Figure 2A). rLat c 1.01-specific IgE was detected in 22 out of 23 patients, except for one child (C5) who also demonstrated low reactivity on the immunoblot. Similar to the immunoblot analysis, IgG$_4$ from fewer patients bound to rLat c 1.01 when analyzed by ELISA, with 14 out of 23 as compared to 16 out of 23 patients showing reactivity on the immunoblot. IgG$_4$-binding observed by ELISA presented however a different antibody-binding profile for some patients compared to the immunoblots. rLat c 1.01-specific IgG$_4$ was detected in 10 out of 15 children (C1-3, C7, C9 and C11-15), while five out of the eight adults were reactive (A1-2, A4, A6 and A8). Discrepancies in the reactivity detected on immunoblots and ELISA were found in six children (C4-6 for reactivity on immunoblot only, C7, C11, C15 for reactivity by ELISA only) and three adults (A3 and A5 for reactivity on immunoblot only, A6 for reactivity by ELISA only) for IgG$_4$. Uniformly, three patients (C8, C10, A7) did not show IgG$_4$ binding in either of the experiments.

The mean IgE- and IgG$_4$-binding, as compared to the negative controls, within each cohort revealed that IgE binding was significantly higher than that of IgG$_4$ for both children and adults (Figure 2B). The adult cohort demonstrated markedly higher IgE-binding (8.2-fold

increase) as compared to IgG4-binding (1.6-fold increase; P = 0.0004), while the children cohort observed lower differences between IgE (5.2-fold increase) and IgG4-binding (1.5-fold increase; P = 0.02).

3.3 Patient-specific IgE and IgG4 epitopes on Lat c 1.01

The IgE- and IgG4-binding regions on Lat c 1.01 were further investigated using seven overlapping peptides. Inhibition ELISAs were performed using these peptides to inhibit IgE- and IgG4-binding to rLat c 1.01 and the IgE and IgG4 inhibition profiles for each of the 15 children and eight adults compared (Figure 3). The maximum peptide inhibition reached for IgE 95.5% (C12) and for IgG4 up to 72.9% (C3). In general, the binding profiles differed considerably between the patients, but no single peptide demonstrated outstanding inhibition of either IgE or IgG4 antibodies, suggesting the presence of patient-specific epitopes. Two children demonstrated only IgE-binding (C1 and C7), or with marginal IgG4-binding (C4, C6 and C9), while all adults demonstrated both IgE- and IgG4-binding. Two children with only IgG4 binding were also identified. Some patients demonstrated distinctly different IgE- and IgG4-bindings patterns to each peptide, as seen for example for patient C2. All seven peptides exhibited relatively uniform IgE inhibition in six patients (C8, C11, C15, A2-3 and A5).

3.4 Unanimous IgE epitopes identified on Lat c 1.01

The inhibition of IgE-binding to rLat c 1.01 by seven overlapping peptides for all 23 patients was combined and analyzed to determine the unanimous epitopes (Figure 4A). Five peptides demonstrated inhibition of IgE-binding significantly higher than that of non-atopic controls. Two peptides (1 and 7) showed the greatest inhibitions with mean percentages of inhibitions of 31.5% (P = 0.005; peptide 1) and 34.4% (P = 0.009; peptide 7), while three peptides (2, 3 and 4) followed with high inhibitions of 45.5% (P = 0.02; peptide 2), 45.4% (P = 0.03; peptide 3) and 48.2% (P = 0.04; peptide 4). Peptide 5 and 6 achieved the highest inhibition percentage of 48.2% and 48.0%; however, not significantly different to non-atopic controls due to large distributions of negative controls. Furthermore, no difference in the IgE inhibition was observed between adult and children, with both cohorts following a similar trend (Figure 4B).

For IgG_4, no peptide demonstrated significantly higher inhibitions than the non-atopic controls (Figure 4C), with peptide 6 demonstrating the highest inhibition with only an average of 19.5%. None of the seven peptides significantly inhibited IgG_4 more than the other, and no difference in the IgG_4 inhibition between adults and children was observed (Figure 4D).

3.5 Two epitope regions predicted on Lat c 1.01

To evaluate the proximity of potential liner IgE epitopes on Lat c 1.01, the amino acid sequences of fish parvalbumins with known IgE epitopes and Lat c 1.01 were aligned (Figure 5A), as well as 3D models generated for each protein (Figure 5B). Peptides 1, 2 and 7 inhibited significantly more serum IgE-binding to rLat c 1.01 than other peptides. The matching regions to these peptides on Lat c 1.01 were found on both the N- and C-terminal ends of the allergen (underlined in Figure 5A and shaded in Figure 5B in a *navy-blue* gradient). The most probable IgE-binding region on the N-terminus of Lat c 1.01 is where peptide 1 and 2 overlap by nine amino acids (AACQAADSF). As both peptides were able to considerably inhibit serum IgE-binding, it is very likely that one epitope is located within these nine-amino acids. Peptides 6 and 7 share 21 amino acids; however, only peptide 7 could significantly inhibit IgE-binding to rLat c 1.01. Therefore, C-terminal region of Lat c 1.01, including the four amino acids distinct for peptide 7, represent the second most likely IgE epitope of this parvalbumin. Both possible IgE-binding regions are very similar to the identified regions on parvalbumin from cod (Gad m 1) and carp (Cyp c 1). The location of possible IgG_4 epitopes could not be predicted due to the peptides demonstrating no significant inhibition in these experiments.

4. Discussion

This is the first published study to characterize and compare IgE and IgG4 antibody-binding epitopes on the major fish allergen parvalbumin. This study focused in particular on parvalbumin from one of the most consumed fish species from the South East Asia. Lat c 1.01 is the major allergen from Asian seabass (Sharp et al., 2014), and in this study, the reactivity of 23 fish-allergic patient was analyzed.

Serum IgE-binding detected by immunoblotting under reducing conditions for a high number of patients (22/23 patients) suggests the presence of linear IgE epitopes on Lat c 1.01. However, IgE-binding of the same patients (22/23 patients) to the native form of Lat c 1.01 indicates the possible presence of conformational epitopes. In general, much fewer patients demonstrated IgG_4-binding by ELISA compared to immunoblotting, suggesting IgG_4 may recognize epitopes that are not easily accessible in the tertiary structure of rLat c 1.01. No notable differences were found between two cohorts of different age groups in binding patterns expect that a higher level of serum IgE-binding to rLat c 1.01 was observed for adults (individual and as a cohort). The lack of difference in IgE binding between these two cohorts is possibly due to the fact that fish allergy, similar to shellfish and peanut allergy, is rarely outgrown and continues into adulthood (Sicherer et al., 2004; Ruethers et al., 2018).

Antibody isotype switching in B cells usually occur from IgG_4 to IgE as a consequence of the sequence order of the antibody isotypes situated on their chromosome (Aalberse, 2011; Aalberse et al., 2009), suggesting that IgE is more likely to be present than IgG_4. However, it was also demonstrated that non-sequential class switch to IgE can occur independently (Niederberger et al., 2002). In this study, we found more fish-allergic patients with a robust IgE-binding (22 out of 23) than IgG_4-binding (17 out of 23) to rLat c 1.01, demonstrating a dissociation between IgG_4 and IgE in fish-allergic patients as reported previously (Swoboda et al., 2002; Swoboda et al., 2007). This was consistent regardless of the age of patients as significantly higher levels of IgE-binding were observed compared to IgG_4-binding in both cohorts. Despite IgG_4 being associated with tolerance and desensitization in allergic disease, our findings further support that specific IgE is a reliable antibody marker that directly correlates with allergic symptoms (Aalberse et al., 2009; Aalberse, 2011; Eckl-Dorna et al., 2019).

Using overlapping peptides, we demonstrated that IgG_4 epitopes of Lat c 1.01 are most likely patient-specific, while common IgE epitopes are eluded more at terminal regions of the allergen. The probable IgE epitope in the C-terminal epitope was previously identified in Gad m 1 (Perez-Gordo et al., 2013), while the probable epitopes on the N-terminal end, combining peptide 1 and 2, cover two previously identified epitope regions for four different fish parvalbumins. This most likely indicates that this protein region is of immunological importance (amino acid position 1 to 56). Peptide 1, situated in the least conserved region of fish parvalbumin (Sharp and Lopata, 2014), has previously only been identified in an epitope region parvalbumin Sal s 1 from Atlantic salmon (Perez-Gordo et al., 2012). The absence of epitope discovery in this region for other fish parvalbumins may explain the clinically unique mono-sensitization to salmon within fish-allergic patients (Perez-Gordo et al., 2012; Vàzquez-Cortés S, 2012; Sharp et al., 2015). Patients whose IgE specifically identify this N-terminal epitope may have low cross-reactivity to other fish due to the low sequence identity in this region. No patient from this study however presented IgE that solely binds to this region. In contrast, peptide 2 aligns with a region that contains the most frequent epitopes mapped on parvalbumins from fish including Baltic cod, Atlantic salmon, Pacific mackerel and carp (Sharp and Lopata, 2014). This epitope seems to be in a highly cross-reactive region of parvalbumin and patients who recognize peptide 2 would most likely react to many other fish parvalbumins. It is not surprising that this cross-reactive epitope has been identified in Lat c 1.01, as Asian seabass parvalbumin has previously been characterized as a highly cross-reactive allergen among sensitized individuals (Sharp et al., 2015). Further analysis using shorter overlapping peptides for more precise coverage as well as peptide alanine screening could increase the resolution of the mapped Lat c 1.01 epitopes (Zhao et al., 2017; Liu and Sathe, 2018).

This study highlights the importance of advancement in epitopes analysis regardless of the age group of patients. Our findings indicate the greater attribution of IgE over IgG_4 on the reactivity to parvalbumin in all fish-allergic patients of two age groups. Patient profile of both age groups revealed common IgE epitopes, while many patient-specific IgG_4 epitopes were demonstrated. Understanding IgE epitopes on allergenic proteins is crucial for the development of immunotherapies. The application of hypoallergenic proteins in sIT must demonstrate a lower probability of anaphylaxis after administration (Swoboda et al., 2002; Swoboda et al., 2007), as well as alleviating allergic Th2 responses (van der Ventel et al., 2011). Based on our data, the employment of modified (mutated) common IgE epitopes, that are highly reactive

amongst the majority of fish-allergic patients of all ages, should be considered during designing sIT against fish allergy to Asian seabass. The use of mutated hypoallergenic parvalbumin may suit treating patients with independent IgE and IgG_4 epitopes – as the disruption or loss of linear and/or conformational epitopes of allergenic proteins can lead to reduction in allergenicity through loss of IgE-binding ,while the interruption of IgG_4-binding is prevented (Swoboda et al., 2007; Freidl et al., 2017). One of the speculated IgE epitopes spans over the calcium-binding region of at least four parvalbumins (Gad c 1, Cyp c 1, Gad m 1 and Lat c 1), including the N-terminal epitope in Lat c 1.01 established in the present study. A modification in this epitope could be useful in treating patients who are sensitized to fish species from European and Asia Pacific regions. Indeed, it was recently demonstrated that antibodies raised against a mutant carp parvalbumin protected against allergic reactions in a murine model (Freidl et al., 2017). Furthermore, elevated allergen-specific IgG_4 is thought to play a key role in successful immunotherapy, yet to be demonstrated for fish allergy (van de Veen and Akdis, 2016; Eckl-Dorna et al., 2019). It is possible that patients with shared IgE and IgG_4 epitopes would positively respond to a sIT using the native (no modification) allergens due to the increase of serum IgG_4 which acts as a competitive blocking antibody to IgE, as has been demonstrated for milk allergy (Savilahti et al., 2014). However, there is a greater risk of IgE-specific antibody upregulation associated in using unmodified allergens.

In conclusion, our investigation of antibody-binding epitopes of parvalbumin from Asian seabass contributes greatly to the development of improved allergy diagnostics and future immunotherapies for fish allergy. The consumption and international trade of Asian seabass is greater than some of the previously studied fish species in the Asia-Pacific region, and is growing rapidly worldwide. This is the first study to characterize IgE- and IgG_4-binding epitopes of the major fish allergen parvalbumin and identify the IgE-binding regions of Lat c 1.01 from Asian seabass. This knowledge is an important discovery to pave the way for developing new and targeted therapies for the Asia-Pacific region.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Author contributions

M.F.S.: Data curation; Formal analysis; Investigation; Methodology; Resources; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. A.C.T.: Formal analysis; Visualization; Roles/Writing - original draft; Writing - review & editing. T.R.: Visualization; Writing - review & editing. J.N.S.: Resources; Writing - review & editing. N.D.: Conceptualization; Writing - review & editing. S.D.K.: Writing - review & editing. A.L.L.: Conceptualization; Funding acquisition; Investigation; Project administration; Resources; Supervision; Validation; Roles/Writing - original draft; Writing - review & editing.

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Table 1. Clinical characteristics of 23 fish-allergic patients recruited for the study.

ID	Age (years)	Sex	ImmunoCAP (kU/L)			Skin Prick Test (mm)			Symptoms upon ingestion	Implicated fish species
			Cod (f3)	Tuna (f40)	Salmon (f41)	Cod	Tuna	Salmon		
A1	29	M	6.45	0.41	0.43	10	7	8	R, OAS	blue grenadier, salmon, trevally
A2	26	F	1.40	-	1.35	-	-	-	A, GIS, U	Sea perch, unknown
A3	26	F	0.91	4.46	5.75	-	6	10	A, OAS	Trout, unknown
A4	32	F	7.62	5.67	13.6	-	-	-	A, R, OAS, AE, U	Unknown
A5	19	M	4.23	7.75	7.14	-	-	-	A, R, AE, U	Unknown
A6	46	M	2.74	3.78	-	-	-	-	R, OAS	Snapper, tuna
A7	32	F	3.36	2.17	3.20	-	-	-	A, R, OAS, U	Unknown
A8	33	F	-	13.9	25.8	-	-	-	A, AN, U, R, OAS	Trout
C1	7	M	-	-	-	6	3	4	OAS, GIS	White fish
C2	5	F	-	-	-	5	6	3	U	Salmon
C3	4	M	-	-	-	5	3	4	AN, OAS	Trout, tuna, white fish
C4	10	M	-	-	-	8	3	3	AN	Catfish
C5	7	M	-	-	-	6	0	2	AE, U	Smoked salmon, white fish
C6	5	M	-	-	-	0	4	0	AE, U	Bream
C7	9	F	-	-	-	11	5	6	AN, R, U	Milkfish, tilapia
C8	12	F	-	-	-	8.5	5.5	0	AE, U	-
C9	15	F	-	-	-	5	5	6	R, OAS, AE	Red mowong, whiting
C10	14	M	-	-	-	11.5	8	8	AE, OAS, GIS	Salmon, silver perch, shark

C11	12	F	-	-	-	11	3.5	5	U	Asian seabass, ling, whiting,
C12	16	M	-	-	-	8	4.5	3.5	OAS	White fish
C13	2	F	-	-	-	4	5	5	U	Salmon
C14	7	F	-	-	-	10	5.5	3	AN	Catfish
C15	14	M	-	-	-	0	0	0	AN, GIS, OAS	Catfish, white fish
N1	21	M	<0.01	-	-	-	-	-	-	None
N2	53	M	<0.01	-	-	-	-	-	-	None
N3	29	F	<0.01	-	-	-	-	-	-	None
N4	41	F	<0.01	-	-	-	-	-	-	None
N5	29	F	<0.01	-	-	-	-	-	-	None

Patient ID: A, C and N denote for adult, children and negative control, respectively. Symptoms: A, asthma; AN, anaphylaxis; AE, angioedema; E, eczema; GIS, gastrointestinal syndrome; OAS, oral allergy syndrome; R, rhinitis; U, urticaria. '-'=Not performed.

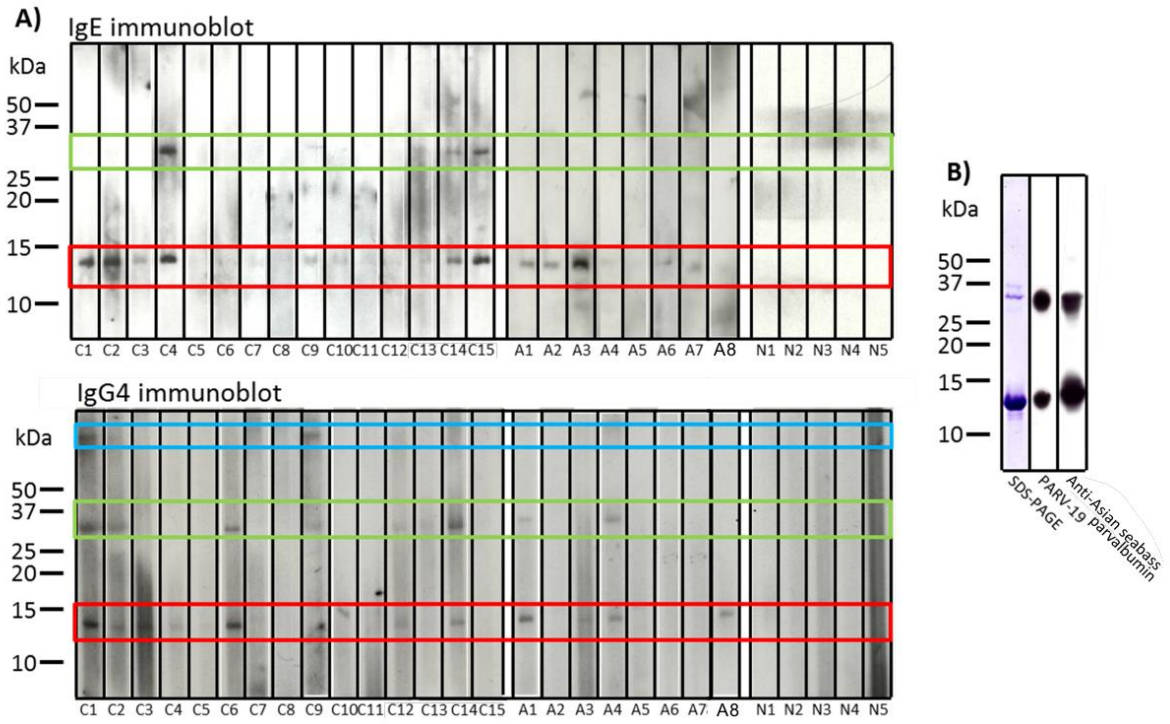

Figure 1. Serum IgE and IgG4 analyses of 23 fish-allergic patients against Asian seabass major allergen rLat c 1.01, by immunoblotting in reduced condition. A) Serum from 15 pediatric (C1-15) and eight adult (A1-8) fish-allergic patients were subjected to purified recombinant rLat c 1.01 and analyzed for IgE (top) and IgG_4 (bottom) binding. Five non-fish allergic patients (N1-5) were included as negative controls. Parvalbumin monomers, dimers and oligomers are boxed in red, green and blue, respectively. **B)** Purified rLat c 1.01 was detected and the protein purity and integrity were confirmed on a Coomassie Brilliant Blue stained 15% polyacrylamide gel (left lane), immunoblots with monoclonal anti-parvalbumin PARV-19 antibody (middle lane) and polyclonal rabbit anti-Asian seabass parvalbumin antibody (right lane).

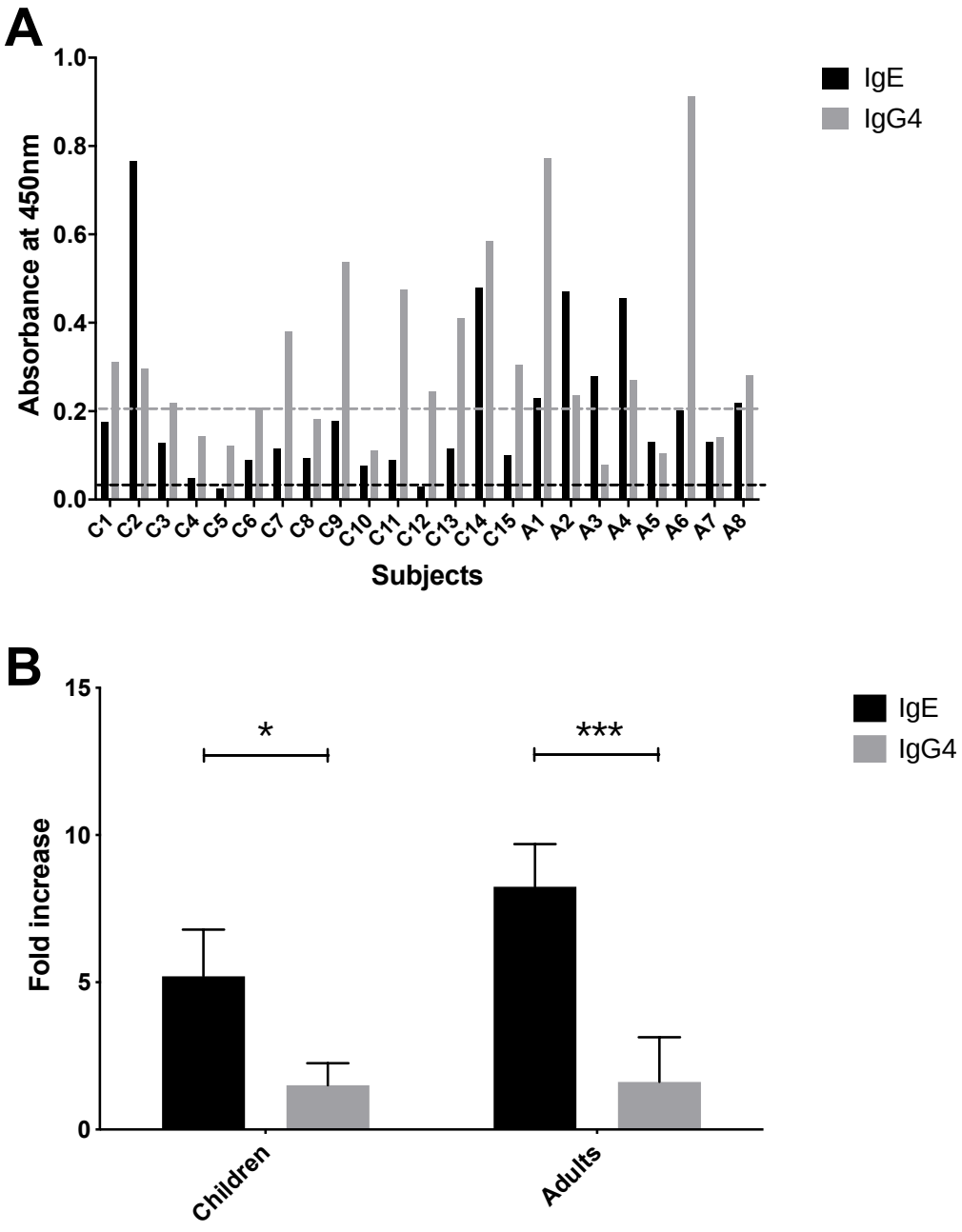

Figure 2. Evaluation of serum IgE and IgG$_4$ binding to native form of Asian seabass parvalbumin, rLat c 1.01 by indirect ELISA. A) The serum IgE (black) and IgG$_4$ (grey) reactivities to rLat c 1.01 (1 µg/mL) were evaluated for 15 fish-allergic children (C1-15) and eight adults (A1-8) by measuring the absorbance at 450 nm. The reactivity for individual patient serum was confirmed using a cut-off derived from two standard deviations above the mean reactivity of five non-allergic subjects to each antibody isotype [indicated by the dotted line (IgE; black line=0.03, IgG$_4$; grey line=0.20)]. **B)** Mean fold increase values of IgE and IgG$_4$ against rLat c 1.01 from the cut-off values were compared for each of children and adult cohorts. Error bars represent standard error of the mean. *$P < 0.05$, ***$P < 0.0005$.

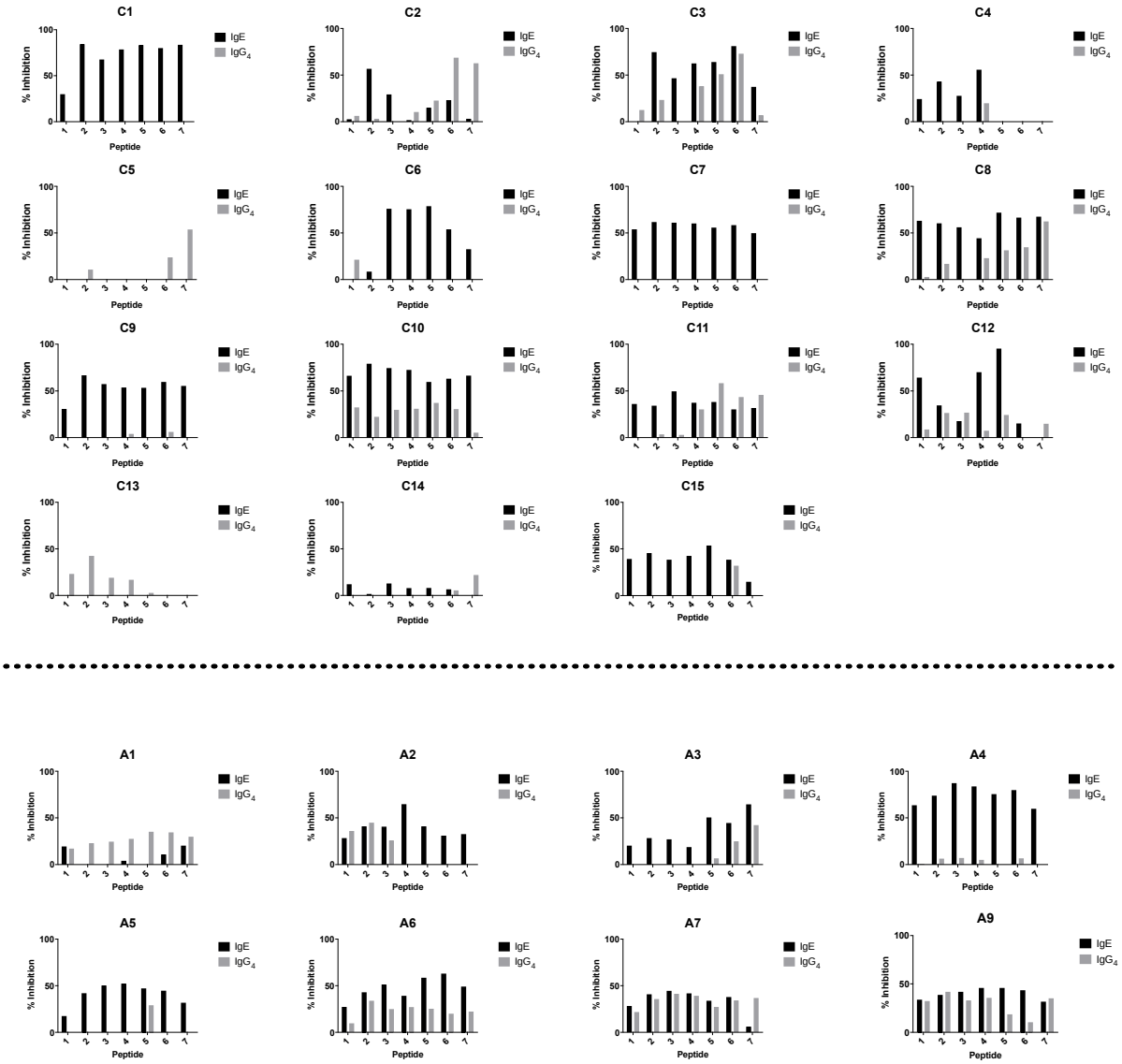

Figure 3. Analysis of peptide binding regions using serum IgE and IgG4 by inhibition ELISA. Binding regions were analyzed using the synthetic overlapping peptides for individual fish-allergic patient serum IgE (black) and IgG4 (grey). Binding profiles of individual 15 children (C1-15) and eight adults (A1-8), were determined for each of IgE and IgG4. Each cohort is grouped and shown separately by dotted line. Y-axis represents the percentage of inhibition of serum IgE and IgG4 binding to rLat c 1.01 (20 ng) induced with the seven generated peptides (250 ng; x-axis).

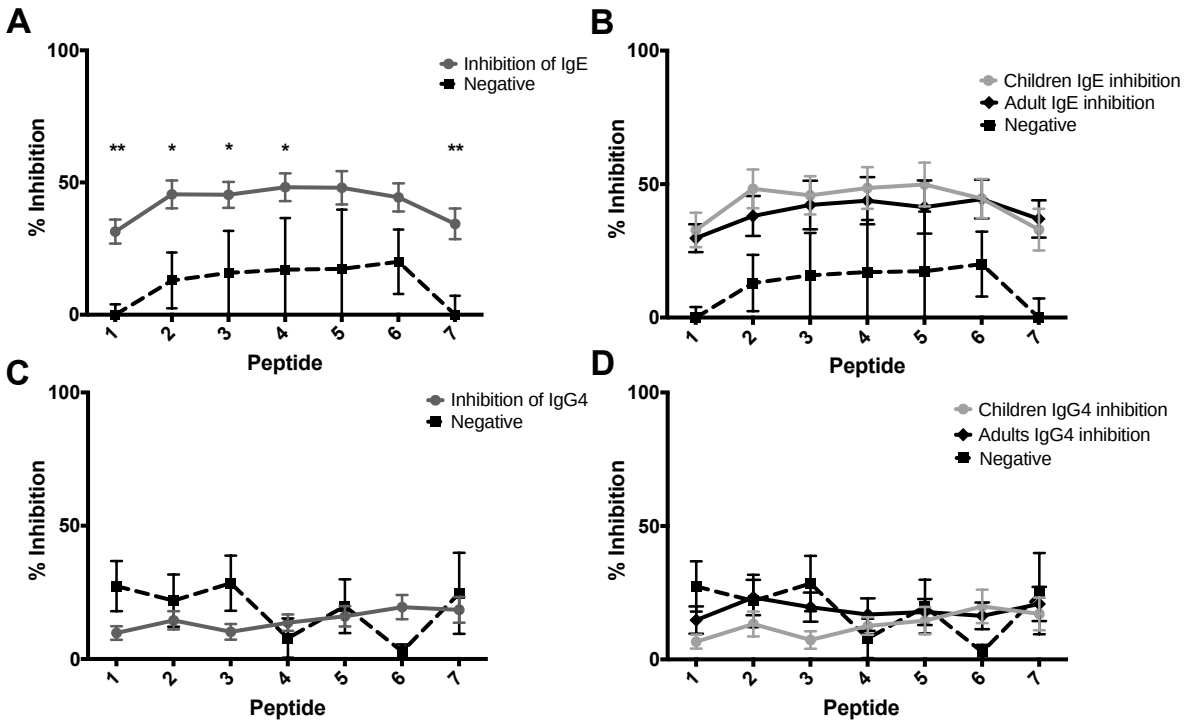

Figure 4. Determination of IgE and IgG4 binding regions in rLat c 1.01 for pediatric and adult fish-allergic patients using seven overlapping peptides. Percentages of IgE inhibition against seven peptide regions were calculated for **A)** all 24 patients (dark grey; 15 children and eight adults) and five non-atopic controls (dotted black), and **B)** each cohort of children (light grey), adults (black) and non-atopic controls (dotted black). Percentages of IgG_4 inhibition against seven peptide regions were calculated for **C)** all 24 patients (dark grey; 15 children and eight adults) and five non-atopic controls (dotted black), and **D)** each cohort of children (light grey), adults (black) and non-atopic controls (dotted black). Y-axis represents the percentage of inhibition of respective serum IgE and IgG_4 (1:10) binding to 20 ng rLat c 1.01 with 250 ng of the seven generated peptides (x-axis). Error bars represent standard error of the mean. $*P < 0.05$, $**P < 0.01$.

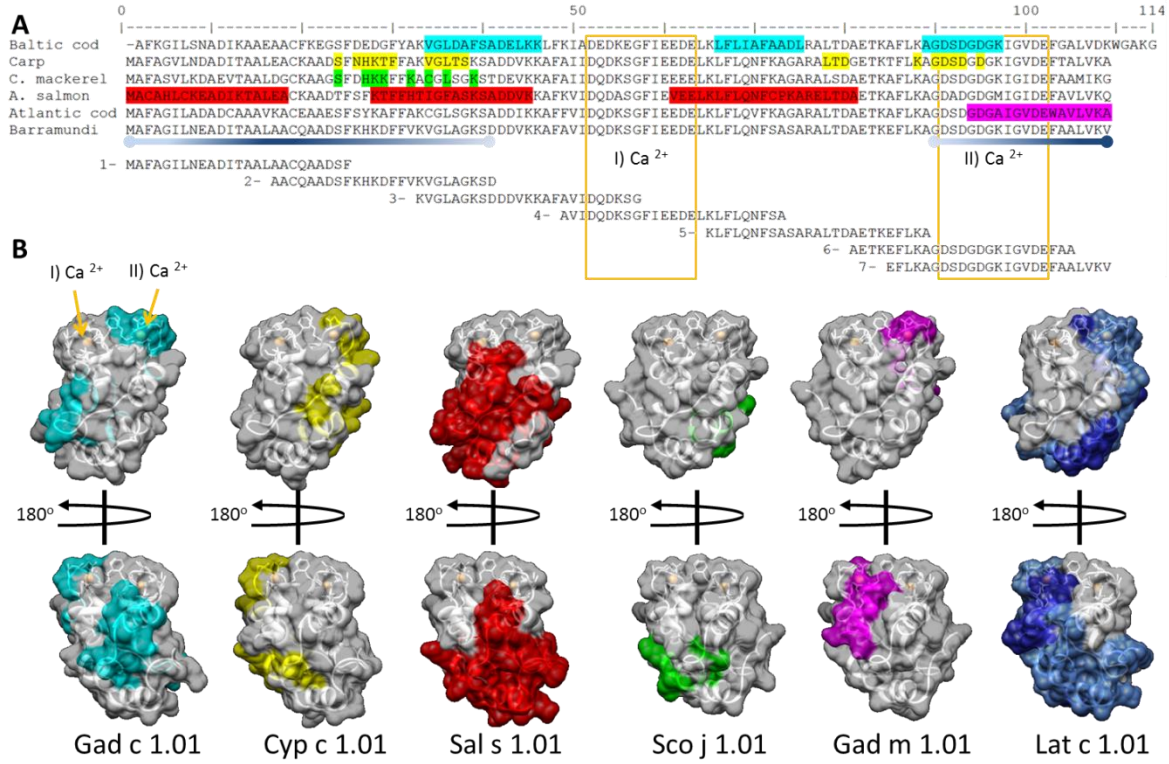

Figure 5. Comparative modelling of fish parvalbumin and elucidation of IgE epitopes. A) Amino acid sequence alignment of five fish parvalbumins. IgE epitopes previously elucidated are indicated in color shading as follows; Baltic cod (Gad c 1.01; UniProtKB Accession number, P02622.1) *cyan*, common carp (Cyp c 1.01; Q8UUS2) *yellow*, Atlantic salmon (Sal s 1.01; B5DH15) *red*, Pacific mackerel (Sco j 1.01; P59747) *green* and Atlantic cod (Gad m 1.01; Q90YL0) *pink*. The suspected IgE epitope regions is underlined in two *navy-blue* gradient lines under Lat c 1.01 sequence. Sequences of the overlapping peptides from this study are aligned under the parvalbumin sequences with assigned peptide numbers. The two calcium-binding sites are boxed in orange. **B)** Protein models of the five fish parvalbumins with their elucidated IgE epitopes highlighted in their respective colors. The model of Lat c 1.01 is shown with matching *navy-blue* gradient regions for the speculated IgE epitopes.

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Exposure to bioaerosols during fish processing on board Norwegian fishing trawlers

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Abstract

Introduction: There is a lack of knowledge regarding exposure to bioaerosols during seafood processing on board fishing trawlers.

Methods: Exposure sampling was carried out during the work shifts when processing fish in on five deep-sea fishing trawlers. Three of these trawlers were old ships, not built for all the machines currently present in the processing area. Two trawlers were newer and built for these machines. On four of the trawlers they produced round fish; they remove the head and entrails of the fish before they are frozen in blocks. On one trawler they processed the fish further and produced fish fillets. Samples were analyzed for total protein, trypsin activity, parvalbumin and endotoxin. T-test were used to compare levels of exposure on the different trawlers.

Results: Personal exposure levels to total protein were higher on the three oldest trawlers compared to the two new trawlers. The highest activity of trypsin was detected on the four trawlers producing round fish. Parvalbumin was detected in 58% of the samples from the fillet-trawler compared to 13% of the samples from the four trawlers producing round fish. The highest level of endotoxin was detected when using high pressure water during cleaning machines and floors in the processing area.

Conclusion: Fishers in the processing area on board Norwegian trawlers are exposed to airborne proteins, trypsin, parvalbumin and endotoxin. Levels varied between trawlers and type of production.

Keywords

exposure to bioaerosols, fishing trawlers, fish processing, proteins, trypsin, parvalbumin, endotoxin

Introduction

Fishers are exposed to potentially harmful working conditions. Due to a high number of occupational deaths and reported accidents in fisheries, research emphasis has been on safety, accidents, and documenting the number of fatalities and injuries (Windle, 2008; Aasjord, 2012; Frantzeskou et al., 2014; Jensen, 2014). Exposure to bioaerosols during seafood processing may increase the risk of developing upper and lower respiratory symptoms, asthma and allergy (Jeebhay et al., 2010; Bang et al., 2005; Douglas et al., 1995; Shiryaeva et al., 2014; Dahlman-Höglund et al., 2012; Thomassen et al., 2016). There is, however, a lack of knowledge on workers exposures to bioaerosols during seafood processing on board fishing trawlers. Bioaerosols are airborne particles with biological origin. Several studies in onshore seafood industry plants have shown that production workers are exposed to bioaerosols generated by eg mechanical processing of the fish or cleaning by water beams directed towards biological materials (Bang et al., 2005; Jebhay et al., 2004; Jeebhay et al., 2005; Dahlman-Høglund et al., 2013; Shiryaeva et al., 2014; Thomassen et al., 2016). Many processes involve extensive use of water, generating droplets as well as particulate matter that may be inhaled by the workers. These aerosols may contain bioactive agents, including enzymes, allergens, and endotoxin. Confined spaces are common on vessels, and the processing areas on board are characterized by non-shielded machines and processing lines. Previous research has revealed higher levels of crab allergens during processing the crab on board crab fishing vessels compared to a land-based crab plant (Baudet, 2002; Weytjens, 1999). It has also been shown that Russian trawler fishers were more likely to have asthma, respiratory symptoms and impaired lung function compared to Russian merchant seafarers, suggesting that the work environment on board trawlers contains risk factors for respiratory symptoms (Shiryaeva at al., 2011).

Total protein levels give an indication of the total load of proteins workers are exposed to and may be used as a crude measure of bioaerosol exposure. Trypsin is a digestive enzyme, and exposure to trypsin is linked to innate inflammatory responses in cell models and suspected to play a role in allergy (Sun and Lopata, 2010; Baur 2005; Larsen et al., 2008). Endotoxin, a lipopolysaccharide component of the cell-wall of Gram-negative bacteria, is a well-known pro-inflammatory mediator, and inhalation induces an inflammatory response in the respiratory system (Eduard et al., 2009). Parvalbumin is the major allergen in bony fish, a muscle protein, and has been demonstrated to be one of the airborne allergens responsible for allergic respiratory disease in seafood-processing workers (Lopata and Jeebhay, 2013; Beale at al., 2009)

Exposure to bioaerosols in the work environment in fishing vessels is sparsely described. Based on previous investigations in fish processing industry on shore, and the sparse existing data from fish vessels, we hypothesize that fishers working in the processing area on board trawlers are exposed to aerosols containing biological active compounds as enzymes, allergens and endotoxin.

Objectives

The main objective was to gain more knowledge on exposure to bioaerosols in the processing area on trawlers through quantifying total protein, trypsin, endotoxin and parvalbumin levels, collected through personal and stationary aerosol sampling, and to compare the exposure levels on different trawlers.

Materials and Methods

Study population

Fishing trawlers were recruited in collaboration with ship owners. Fishers working in the processing area on board voluntarily carried bioaerosol sampling equipment during their work shifts. In total, 64 fishers from the five trawlers participated in the study.

During sampling on board the fishers in the processing area were asked to report work tasks they performed during the shift, breaks and also whether they cleaned machines, equipment and floors during the shift, and for how long time they performed the different tasks.

Written informed consent was obtained from all participants. The study was approved by the Regional Committee for Medical Research Ethics in Northern Norway.

Participant trawlers

Data were collected on five Norwegian trawlers in 2014 and 2015 during normal activity/fishing in the Norwegian Sea and in the Barents Sea. Included was four freeze-trawlers (numbered 1-4), and one fillet-trawler (number 5). At the time of data-collection the age of the trawlers 2, 3, and 4 were 15-21 years old, and trawler 1 and 5 was 3 years old. Each trip at sea lasted from 9 to-18 days.

Fishers on freeze-trawlers operates machinery that removes head and entrails of each fish. The fishes are stored in tanks to bleed out, before they gets sorted and cleaned. The fishes are distributed into vertical plate freezers and are frozen into blocks of 25 or 50 kg. The frozen blocks are removed from the plate freezer, and stored at the cold storage on a lower deck until they are offloaded onshore. The fillet-trawler process the fish further into fillets through filleting machinery. The fillets are manually checked for bones and trimmed, sorted after

weight and packed, frozen, and stored in the freezer hold. Trawler 1 and the trawler 5 produced fish-meal in a separate room.

Scedule

Seafood processing on board is continuous work, divided into four shifts, each lasting for six hours. Fishers follow a rotation of six hours on and six hours off. A work period lasts for 4-6 weeks, included fishing, fish processing, steaming to new fishing areas, and delivering the catch on shore when the freezer hold is fully loaded, sometimes several times in the work period. During the shift work was performed on both the trawler deck and in the processing area or only one of these.

Exposure sampling

This study was performed with stationary and personal samplers from fishers breathing zone on five Norwegian trawlers in 2014 and 2015 during normal activity in the Norwegian and Barents Sea. Exposure sampling were performed during processing fish in the processing area using SKC Sidekick (SKC Ltd., Dorset, UK) sampling pumps connected to samplers containing filters specific for each exposure agent. The flow rate for each pump was controlled before and after collection, using Bios Defender 520 (SKC Ltd., Dorset, UK). If the flow deviated with more than 10% from the start of the sampling, the samples were discarded.

Due to confined spaces in the processing area, fishers carried only one sampling pump connected to samplers due to safety considerations. At break times, the pumps were stored away from the processing area to prevent overestimation of the exposure, but kept running to represent the mean exposure during a shift, including time away from the processing area.

Samples for proteins, trypsin and allergen were collected using SurealSeal Air monitoring cassettes (37 mm, 3-pc, styrene SKC Ltd. UK) on polytetrafluoroethylene (PTFE/Teflon) filters with polypropylene support (37 mm, 1.0 mm SKC Ltd. UK). The cassettes were stored at -20°C in the field, and further -70°C and until extraction and analysis. Samples for endotoxin were collected using preloaded cassettes with glass fiber filters, type AE, 37 mm, 3 piece, pre-banded (SKC 225-706). The cassettes were stored at -20°C until extraction.

262 personal samples from 64 fishers and 108 stationary samples on the five trawlers were sampled and analysed for total protein. Trypsin was analysed in 252 of 262 of the personal protein samples. In addition, 72 stationary samples for parvalbumin analysis and 67 stationary samples for endotoxin analysis were sampled.

Total protein analysis

The protein filters was extracted in 1000 µl PBS with 0.5% Tween20. Manual QuantiPro BCA Assay Kit (Sigma-Aldrich, St. Louis, USA) was used to determine the total amount of proteins in filter extracts in the samples by colorimetric reading in a spectrophotometer at 560nm.

Trypsin analysis

Trypsin activity in filter extracts was analyzed by zymography, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 0.1% gelatin as substrate added in the polyacrylamide gel (Bang et al 2018). Due to low precision in the early assay set up, the results are presented using a semi-quantitative scale based on the 25 and 75 percentile of positive results: Low ≤ 25 percentile; 25 percentile > medium percentile ≤ 75 percentile; high

> 75 percentile. Low level ≤25th percentile (~0.0014 mU/m^3) of positive samples. High level ≥ 75th percentile (~0.0098 mU/m^3) of positive samples. One Unit is defined as dA/dt x 1/(ε x optical path length) x 10^6 x V_{final} , where dA/dt is the rate of absorbance change, and ε is the extinction coefficient 8800 $M^{-1}cm^{-1}$. Analysis of protein and trypsin were performed at the Department of Occupational and Environmental Medicine, University Hospital North Norway, Tromsø.

Allergen analysis

The bioorganic matter from the air-filters were extracted using 1 mL of extraction buffer (100 mM ammonium Bicarbonate, 0.05% Tween10, and 2 nM sodium azide) under agitation for 3h at 28ºC using a previously established protocol (Kamath et al 2014). For quantification of the major fish allergen, parvalbumin 3x20 ul of the filter extract were loaded on a nitrocellulose membrane (Biorad, USA) using the PR 600 Slot Blot Filtration Manifolds (Amersham Biosciences), followed by air drying for 10 min. Membranes were then blocked in 5% skim milk (in PBS) for one hour, followed by incubation with an anti-parvalbumin antibody cocktail (antibody against parvalbumin from barrmundi (*Lates calcarifer*), basa (*Pangasius hypophthalmus*), Atlantic salmon (*Salmo salar*), and pilchard (*Sardinops sagax*)); diluted 1:1,000 in PBS with 1% skim milk and 0.1% Tween20 (PBS-T) for 20 minutes (Sharp et al 2015). After washing with PBS-T the membranes were incubated with the secondary (anti-rabbit IgG) antibody labeled with horseradish peroxidase (HRP) for 20 minutes and washed as before. Detection of parvalbumin was visualized using the enhanced chemiluminescent (ECL) assay as described previously (Kamath et al 2013). For the quantification of parvalbumin in the filter extracts, a standard curve was generated by serial dilution of purified parvalbumin from Atlantic cod (*Gadus morhua*) dotted on the membrane as described above. Analyses were performed by co-operators at James Cook University, Australia

Endotoxin analysis

The glass fibre filters were extracted in 4.0 ml pyrogen-free water with 0.05% Tween 20 by orbital shaking (300 rpm) at room temperature for 60 min and centrifuging (1000 g) for 15 min. The filters were analysed for endotoxin content by a quantitative kinetic chromogenic Limulus Amoebocyte Lysate assay (Kinetic-QCL endotoxin kit, Lonza, Walkersville, MA, USA), and results are expressed in EU m^{-3} (EU = endotoxin units, 10 EU = 1 ng) (Madsen et al 2014). Analyses were performed at the National Research Centre for Working Environment, Copenhagen, Denmark.

Statistical analysis

Descriptive analysis are presented as arithmetic mean and median with standard deviations. Single variable analysis were performed with t-test. The statistical analyses were done using the IBM SPSS software package, version 25 where $p< 0.05$ were considered statistical significant.

Results

Four freeze-trawlers producing round frozen fish, and one fillet-trawler producing fish fillets, were included in the study. The catch consisted mainly of Atlantic cod, Haddock, Pollack and Deepwater redfish. Exposure samples were collected in the processing area on board trawlers during fish processing, and samples were analysed for total protein, trypsin, endotoxin and parvalbumin.

Personal exposure levels to total protein were significantly higher on the three oldest trawlers (2, 3 and 4): n=129, range 0-83.2 µg/m^3, compared to the two new trawlers (1 and 5): n= 133, range 0-34.1 µg/m^3, table 1. Environmental levels of total protein measured at stationary stations were significantly lower on trawler 1 compared to trawler 2, 3, 4 and 5, table 2.

Based on a semi-quantitative scale, high level (> 75 percentile) of trypsin was detected in 13% (mean), (range 11.8-52%) of samples from the four freeze-trawlers compared to 2.6% of samples from the fillet trawler, table 3.

The highest level of endotoxin was detected in the processing area on the fillet-trawler (n=9, range 0.5-483.6 EU/m^3) compared to the freeze-trawlers (n=58, range 1.5-111.6 EU/m^3), table 4. Endotoxin levels from fish-meal production on trawler 1 and 5, indicated exposure levels < 16 EU/m^3 (n=6).

The mean and range values shows no difference in parvalbumin exposure levels between the 4 freeze-trawlers and the fillet-trawler, table 5. Parvalbumin was detected in 58% of the samples on the fillet trawler in contrast to 13% of the samples on the 4 freeze-trawlers. Parvalbumin was not detected in samples from fish-meal production.

Discussion

To our knowledge this is the first study reporting exposure to bioaerosols in the fish processing area aboard fishing trawlers. In this study we measured total proteins, trypsin, endotoxin and parvalbumin in bioaerosols collected in fishers breathing zone and at stationary stations.

In common with land based seafood industry there has been a technological development in the processing area on board fishing trawlers which has led to less manual work and more mechanical processes. Older trawlers were originally designed and built for manual handling and manual processing of the fish. In the processing area of the three oldest trawlers (2-4), space was maximally utilized with machines, and was characterized by lack of space for movement and low ceiling in contrast to the two newest trawlers (1 and 5) with higher ceiling and more space. The total protein levels in the processing area on the trawlers (LOD-83 ug/m^3) were higher compared to studies performed in land based seafood industries; Norwegian salmon industry: 1-13 µg/m^3 (Shiryaeva et al., 2014), Norwegian crab industry: 2-48 µg/m^3 (Thomassen et al., 2016), African pilchard and anchovy industry: LOD-11.5 µg/m^3 (Jeebhay et al., 2005). Even if new trawlers are designed and built for machineries, with higher ceiling and more space, the processing area was generally smaller compared to land based seafood industries.

Fish-processing operations aboard the four freeze-trawlers were in general similar. Personal exposure samples showed significantly higher levels of total protein on the three oldest trawlers (2, 3 and 4) compared to the two newest (1 and 5). This was not reflected in the stationary samples, where significant higher levels was detected on trawler, 2, 3, 4 and 5 compared to trawler 1. Several factors may contribute for the higher personal exposure levels on the oldest trawlers, as more confined spaces in the processing area, lower ceiling, and

placement and efficacy of the ventilation systems. In addition, levels may be influenced by catch type, different processing techniques in freeze-trawlers and fillet-trawlers, and lay-out of the processing area, season, as well as work load during sampling. Even if the freeze-trawlers had almost identical processing lines, the observed differences might be attributable to the difference in physical layout and ventilation.

Trypsin is a serine protease located mainly in pancreatic tissue and intestines. Proteases may have an immunological or non-immunological effect on the lungs when inhaled (Sun and Lopata, 2010; Baur, 2005; Florsheim et al., 2015; Madsen et al., 2015) In a previous study in the crab industry protease activity in bioaerosol samples varied by industrial processes as well as factory-specific factors (Thomassen et al., 2016). An important work task on freeze-trawlers is to remove the entrails of the fish. Even if this work is partly automated, fishers are in close contact with entrails, and this may explain that the highest trypsin levels were found on board freeze-trawlers.

Parvalbumin is an allergen located mostly in the fish muscle, and may explain why parvalbumin in was detected more often in the fillet-trawler compared to the freeze-trawlers. Parvalbumin was detected in 58% of the samples on the processing area on the fillet-trawler (7 of 12 samples) in contrast to 13% of the samples on the 4 freeze-trawlers (8 of 60 samples). This finding is in accordance with previous studies in in the processing area in herring and salmon industries. A study in the herring industry showed that workers were highly exposed to parvalbumin during handling and controlling of the herring fillets; and the lowest exposure levels in the loading and packing areas (Dahlman-Høglund et al., 2013). In the salmon industry exposure levels of parvalbumin were higher in the fillet department compared to the slaughter department (Shiryaeva at al., 2014).

Endotoxin exposure levels were highest in samples from the fillet-trawler (table 4). Several factors may contribute, and a known risk factor for increased endotoxin concentration is use of high pressure water in cleaning procedures (Visser et al., 2006; Madsen et al., 2013; Shiryaeva et al., 2014). In land-based seafood plants, the production area is normally cleaned after work shifts, during the night, and often performed by external workers. On board trawlers, the cleaning of machines, floors and other equipment is done by the fishers themselves. They normally use high pressure water during cleaning, and main cleaning is performed when the trawler steams to another fishing location, or is on its way to offload frozen fish on shore.

Normal use of high pressure cleaning in the processing area is often several times during a shift, 5-15 min each time. In contrast, on the shift with the highest measured endotoxin levels, 520 and 548 EU/m^3, the fishers reported use of high pressure cleaning in total for several hours. The high endotoxin levels may be due to accumulation and growth of microorganisms in the machines and other equipment in the processing area. Endotoxin exposure samples were taken as stationary samples, however, this indicates that fishers may be exposed to high levels of endotoxin during cleaning with extensive use of high pressure water in the processing area over a longer period of time. We studied protein exposure levels the specific day with high endotoxin exposure levels, however, protein exposure levels did not seem to be affected by this work process.

Conclusions

Fish processing workers on board deep-sea fishing vessels are exposed to proteins, trypsin, endotoxin and the major fish allergen parvalbumin. The bioaerosol levels varied between vessels, and may be influenced by different processing techniques and cleaning procedures. Retrofitting of processing machines trawlers built for manual processing should be avoided due to confined space and risk for higher aerosol levels and then in turn increased risk for adverse respiratory health effects. Older trawlers are replaced with new-built trawlers from time to time, however, there are still many of the old vessels in operation.

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Fish-derived low molecular weight components modify bronchial epithelial barrier properties and release of pro-inflammatory cytokines

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ABSTRACT

The prevalence of fish allergy among fish-processing workers is higher than in the general population, possibly due to sensitization via inhalation and higher exposure. However, the response of the bronchial epithelium to fish allergens has never been explored. Parvalbumins (PVs) from bony fish are major sensitizers in fish allergy, while cartilaginous fish and their PVs are considered less allergenic. Increasing evidence demonstrates that components other than proteins from the allergen source, such as low molecular weight components smaller than 3 kDa (LMC) from pollen, may act as adjuvants during allergic sensitization.

We investigated the response of bronchial epithelial cells to PVs and to LMC from Atlantic cod, a bony fish, and gummy shark, a cartilaginous fish. Polarized monolayers of the bronchial epithelial cell line 16HBE14o- were stimulated apically with fish PVs and/-or the corresponding fish LMC. Barrier integrity, transport of PVs across the monolayers and release of mediators were monitored.

Intact PVs from both the bony and the cartilaginous fish were rapidly internalized by the cells and transported to the basolateral side of the monolayers. The PVs did not disrupt the epithelial barrier integrity nor did they modify the release of proinflammatory cytokines. In contrast, LMC from both fish species modified the physical and immunological properties of the epithelial barrier and the responses differed between bony and cartilaginous fish. While the barrier integrity was lowered by cod LMC 24 h after cell stimulation, it was increased by up to 2.3-fold by shark LMC. Furthermore, LMC from both fish species increased basolateral and apical release of IL-6 and IL-8, while CCL2 release was increased by cod but not by shark LMC.

In summary, our study demonstrated the rapid transport of PVs across the epithelium which may result in their availability to antigen presenting cells required for allergic sensitization. Moreover, different cell responses to LMC derived from bony versus cartilaginous fish were observed, which may play a role in different allergenic potentials of these two fish classes.

1. Introduction

Fish allergy is a potentially life-threatening disease. Its prevalence has increased worldwide over the past decade, most likely due to increased fish processing, trade and consumption (Sharp and Lopata, 2014). The current prevalence of fish allergy worldwide ranges from

Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; CBB, Coomassie brilliant blue; DPBS, Dulbecco's phosphate-buffered saline; FBS, fetal bovine serum; FD10, FITC-Dextran 10; LAMP2, lysosome-associated membrane protein 2; LMC, low molecular weight components; PV, parvalbumin; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline with Tween 20; TEER, trans-epithelial electrical resistance

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0.2% to 3% in the general population, whereas in fish-processing workers it is much higher and reaches up to 8% (Sharp and Lopata, 2014; Tjalvin et al., 2018). The major fish allergens are parvalbumins (PVs) which are proteins of 11–13 kDa and belong to the EF-hand protein superfamily (Arif, 2009). Several PV isoforms can be present in the muscle tissue of a fish (Saptarshi et al., 2014). Fish PVs belong to one of two evolutionary lineages, alpha or beta. Beta PVs are abundant in bony fish and are the primary sensitizers in 75–95% of patients with fish allergy, whereas alpha PVs are abundant in cartilaginous fish and higher vertebrates, and are considered less allergenic (Kuehn et al., 2014; Ruethers et al., 2018b).

The mechanisms underlying allergic sensitization to fish are not well understood. Allergic sensitization and reactions may occur due to exposure to fish allergens via ingestion. However, in an occupational setting, inhalation of or skin contact with fish allergens may contribute to the higher prevalence of fish allergy (James and Crespo, 2007; Jeebhay and Lopata, 2012). In allergic sensitization via inhalation, airway epithelia are exposed to air-borne fish allergens released from fish during food processing (Jeebhay et al., 2005). It has been estimated that the amount of fish allergens present in aerosols ranges from 0.001 to 5 μg/m^3 (Jeebhay et al., 2001). Currently, it is not known why there is such a high prevalence of allergic sensitization to fish among fish-processing workers (exposed to fish allergens mainly by inhalation and skin contact) as compared to ingestion-induced fish allergy. Food processing and different routes of exposure may impact the sensitizing capacity, as well as the number of retained IgE epitopes of the allergenic food proteins (Bogh and Madsen, 2016; Kuehn and Hilger, 2015).

To be able to induce allergic sensitization, allergens need to cross epithelial barriers in an immunologically active state before being presented to the immune system. Airway epithelial cells do not just represent a passive barrier stopping allergens from penetration into the body, but they initiate an innate response by secretion of mediators needed for sensitization (Lambrecht and Hammad, 2014). However, the mechanisms of interaction between fish PVs and airway epithelial cells have not been explored. Increasing evidence points towards a role of the food matrix, which contains lipids and other low molecular weight components (LMC), as potential adjuvants during allergic sensitization (Blume et al., 2015; Palladino et al., 2018). LMC (< 3 kDa) from pollen increased the barrier integrity of bronchial epithelial cells and induced production of proinflammatory cytokines by these cells (Blume et al., 2015). In addition, LMC enhanced the allergen-specific immune response *in vivo* (Gilles-Stein et al., 2016).

We sought to understand the mechanisms of the allergic sensitization to fish via inhalation. We thus explored the interaction of two fish PVs with 16HBE14o- cells, a widely used model for investigating the barrier function of bronchial epithelium (Georas and Rezaee, 2014). The PVs used in this study were isolated from Atlantic cod (*Gadus morhua*), a highly allergenic bony fish, and from gummy shark (*Mustelus antarcticus*), a cartilaginous fish. We hypothesized that the LMC present in the fish-derived organic matter might play a role in the immune response leading to allergic sensitization via inhalation. Thus, we investigated the epithelial barrier integrity, the transport of the PVs across the cell monolayers and the release of mediators upon exposure to PVs as well as LMC from both bony and cartilaginous fish. For the first time, we demonstrated that fish PVs are internalized by bronchial epithelial cells and rapidly transported across the tight epithelial monolayers in an intact and immunologically active form. Furthermore, fish-derived LMC modified the physical barrier properties and the release of pro-inflammatory cytokines in a fish-source dependent pattern. Our data suggest a role of fish LMC as possible adjuvants in allergic sensitization to fish via inhalation.

2. Methods

2.1. Parvalbumins and low molecular weight components (LMC) from fish

Natural beta-PV from Atlantic cod (*Gadus morhua*) and alpha-PV from gummy shark (*Mustelus antarcticus*) were purified from extracts of muscle tissue of the respective fish species according to the previously described protocols (Griesmeier et al., 2010; Ruethers et al., 2018a). Purified PVs were visualized on 15% SDS-PAGE gels by Coomassie brilliant blue (CBB) staining (Supplementary Fig. S1A). Protein identity was confirmed by Western blotting using anti-beta PV antibody (Swant, Switzerland, cat. number 235) and anti-alpha PV antibody (Abcam, UK, cat. number ab11427) (Supplementary Fig. S1B). Presence of the alpha helical secondary structure in the purified PVs was determined by circular dichroism (CD) spectroscopy using a Jasco J-810 spectropolarimeter (Jasco International Co., Hachioji, Tokyo). The spectra were measured from 190 to 250 nm at 25 °C using a 1 mm path length quartz cell and represent the average of 5 accumulations (Supplementary Fig. S1C). The concentration of the PVs was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). MALDI-TOF mass spectrometry was used for mass determination of intact parvalbumins. Proteins were mixed with matrix (α-cyano-4-hydroxycinnamic acid) in 1:1 ratio and spotted onto a ground steel target plate. The mass was measured using a MALDI-TOF mass spectrometer (Microflex, Bruker Daltonics, Bremen, Germany). For each parvalbumin, two isoforms were detected (data not shown). Endotoxins were removed from the purified protein preparations using MACS endotoxin removal beads (Miltenyi Biotec, Germany). The absence of measurable endotoxins was demonstrated using the EndoZyme recombinant Factor C endotoxin detection assay (Hyglos GmbH, Germany) according to the protocols recommended by the manufacturer. The endotoxin levels in the PV preparations were below the detection limit of the assay (0.005 EU/mL).

For the preparation of fish LMC, muscle tissues of Atlantic cod or gummy shark were cut into pieces of 1 cm^3, frozen in liquid nitrogen and ground to a fine powder. Samples were resuspended in 3 volumes of minimum essential medium (MEM) without supplements (Gibco, cat. number 11095080), extracted by gentle shaking for 3 h at 4 °C and centrifuged (17,000 × *g*, 4 °C, 45 min). The supernatant was collected and sterile filtered. To obtain the allergen-free LMC, extracts were ultrafiltrated using 3 kDa cut-off filters (Merck Millipore, Germany). Absence of low molecular weight peptides in LMC which might bind IgE was demonstrated by CBB staining of a 16.5% Tris-Tricine gel (Bio-Rad, cat. number 456–3064) (Supplementary Fig. S2A). Furthermore, a dot blot using serum of a patient with sensitization to cod PV was performed with cod LMC. Cod PV (positive control), cod LMC or bovine serum albumin (BSA, negative control) were spotted onto a nitrocellulose membrane. The membrane was blocked using 3% BSA (Carl Roth GmbH, Germany) in Tris-buffered saline with Tween 20 (TBST) and incubated with the serum of the patient 1 diluted 1:10 in TBST with 1% BSA. Detection of the IgE bound to the membrane was performed using an alkaline phosphatase-conjugated mouse anti-human IgE monoclonal antibody (BD Biosciences, cat. number 555859) and the BCIP/NBT substrate system (BCIP, 5-Brom-4-chlor-3-indoxylphosphat, Biomol; NBT, Nitroblue Tetrazolium chloride, Biomol, Austria). No IgE-binding to the LMC was detected, confirming the absence of allergen degradation products in LMC preparations (Supplementary Fig. S2B).

2.2. Bronchial epithelial cell culture

The human bronchial epithelial cell line 16HBE14o- was used as a model for bronchial epithelium. Cells were cultured as previously described (Waltl et al., 2018). Briefly, cells were cultured in collagen-fibronectin coated tissue culture flasks in MEM supplemented with 10% fetal bovine serum (FBS) (Gibco, 10270-106), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich) at 37 °C in a humidified

atmosphere containing 5% CO_2. Cells were passaged when they reached 80–90% confluence. All experiments were performed with cells within four passages.

If not indicated otherwise, cells were seeded on permeable filter supports (0.4 μm, Corning, 3460) at a density of 4×10^5 cells per mL in MEM supplemented with FBS, penicillin and streptomycin as described above. Formation of polarized monolayers was monitored by measurement of the transepithelial electrical resistance (TEER) as well as the permeability for the tracer FITC-dextran (FD10; Sigma-Aldrich). TEER was determined using an EVOM2 Epithelial Voltohmmeter (World Precision Instruments, Sarasota, Florida). Permeability of monolayers for FD10 was measured 24 h, 36 h and 48 h after seeding. FD10 solution (3 mg/mL) in MEM supplemented with 1.5% FBS was added apically to the cells for 4 h at 37 °C. Basolateral medium was collected, and the fluorescence measured using the Spark multimode microplate reader (Tecan, Switzerland). The excitation and emission wavelengths were 490 nm and 520 nm, respectively. The amount of transported FD10 was calculated from a standard curve.

All subsequent experiments were performed only after the formation of polarized monolayers with a TEER exceeding 1500 $\Omega \cdot cm^2$ and a permeability for FD10 of less than 1% compared to the empty transwell filters.

2.3. Patients' sera

Serum samples from three patients with clinical histories of IgE-mediated type 1 fish allergy were used. Sensitization to fish was verified by determination of fish-specific IgE using ImmunoCAP (Thermo-Fisher Diagnostics, Vienna, Austria). Additionally, levels of PV-specific IgE were determined for each patient. For patients 1 and 2, levels of IgE specific to Cyp c 1 (parvalbumin from European carp) were determined by radioallergosorbent testing and were 15.8 kU/L and 1.4 kU/L, respectively. For patient 3, the level of IgE to Gad c 1 (parvalbumin from Baltic cod) was measured by ImmunoCAP ISAC and was 19.7 ISAC standardized units.

The study was approved by the Ethics Committee of Lower Austria (GS4-EK-4/242–2013).

2.4. Detection and quantification of transepithelial parvalbumin transport

Cod and shark PVs (1 mg/mL) were labelled with Alexa Fluor 488 5-TFP (Alexa Fluor 488 Carboxylic Acid, 2,3,5,6-Tetrafluorophenyl Ester), 5-isomer (Thermo Fisher Scientific) using the protocol recommended by the manufacturer. Labelled proteins were extensively dialyzed against Dulbecco's Phosphate-Buffered Saline (DPBS; Gibco, Thermo Fisher Scientific) and sterile filtered. Labelled proteins were visualized by CBB staining of 15% SDS-PAGE gels, and by fluorescence detection after blotting to a nitrocellulose membrane using a VersaDoc Imaging System (Bio-Rad Laboratories, Hercules, CA, USA).

Polarized cell monolayers were treated apically with fluorescence-labelled PVs (80 μg/mL or 160 μg/mL, 350 μl per well) diluted in MEM supplemented with 1.5% FBS at 37 °C. The volume of basolateral medium was 1.5 mL per well. Samples of basolateral medium were collected after 1 h and 24 h and subjected to SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, and fluorescence was detected using a VersaDoc Imaging System. To quantify the amounts of PVs transported to the basolateral side, aliquots of the supernatants were taken, and the fluorescence intensity was measured by a Spark multimode microplate reader (Tecan, Switzerland). The concentration of the PVs was calculated from a standard curve using a third-degree polynomial curve fitting. The experiments were performed in triplicates.

To explore whether the PVs transported across the epithelial monolayers were able to bind patients' IgE, monolayers were treated apically with cod PV (160 μg/mL) for 24 h, the basolateral cell culture medium was collected, separated by 15% SDS-PAGE and proteins were transferred to nitrocellulose membrane. The membrane was blocked using 5% BSA in TBST and incubated with sera of three fish-allergic patients diluted 1:10 in TBST with 1% BSA. Detection of bound IgE was performed using an alkaline phosphatase-conjugated mouse anti-human IgE monoclonal antibody (BD Biosciences, cat. number 555859) and the BCIP/NBT substrate system.

2.5. Evaluation of the barrier integrity upon stimulation with PVs and LMC

Confluent monolayers were stimulated apically with the purified fish PVs (80 μg/mL or 160 μg/mL) or with fish LMC diluted in MEM supplemented with 1.5% FBS. MEM to LMC ratios were as follows: Dose I – 1:6, Dose II – 1:3, Dose III – 1:2. In control wells, only MEM containing 1.5% FBS was used (untreated cells). The calcium chelator EGTA (5 mM) was used as a control to increase paracellular permeability and decrease TEER (Tomita et al., 1996). TEER was measured at several time points up to 24 h after stimulation. Data represent results of three independent experiments, each performed in duplicate. Furthermore, the permeability of the monolayers for FD10 and the PVs after 24 h of stimulation with fish LMC was measured as indicated above.

2.6. Cell viability analysis

Cell viability after apical exposure to different stimuli was assessed by an MTT assay. Cells were cultured for 55 h (average time needed to form tight, polarized monolayers as determined by TEER measurements in separate experiments) in 96-well plates coated with collagen and fibronectin as described before (Gangl et al., 2015). Confluent cell monolayers were stimulated for 24 h with PVs, fish LMC, or 1% SDS (sodium dodecyl sulfate) as a control for cell toxicity. The supernatant was removed and replaced by thiazolyl blue tetrazolium bromide (MTT) dissolved at 5 mg/ml in Tyrode's salt solution with 0.1% BSA. After incubation for 45 min at 37 °C, the supernatant was removed, and the MTT formazan was solubilized by adding a 1:6 solution of 10 mM glycine in DMSO (dimethyl sulfoxide). Absorbance at 565 nm was measured and the percentage of viable cells was calculated using untreated cells as a reference.

2.7. Confocal microscopy

Cells were seeded (3.4×10^5 cells/mL) on sterilized round 12 mm coverslips coated with collagen and fibronectin and grown for 55 h. Monolayers were washed with DPBS and incubated in serum-free MEM for 30 min at 37 °C to remove serum components from cellular receptors. Alexa Fluor 488-labelled PVs (100 μg/mL) were added and cells were incubated for 1 h at 37 °C. Cells were washed twice with cold DPBS and fixed using 4% formaldehyde solution in DPBS. After washing, the cells were blocked and permeabilized (0.05% saponin, 1% BSA in DPBS) for 1 h at room temperature. Monolayers were incubated with a primary antibody against the late endosome/lysosome marker lysosomal-associated membrane protein-2 (LAMP 2; BD555803, BD Biosciences) diluted 1:50 in blocking buffer, followed by washing with DPBS and incubation with Alexa Fluor 568-conjugated secondary antibody (A-11004, Thermo Fisher Scientific), diluted 1:2000 in blocking buffer). Both antibody incubations were performed for 1 h at room temperature. Nuclei were stained with DRAQ5 (Abcam, UK) and coverslips were mounted onto microscope glass slides using Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Intracellular localization of PVs and cellular markers was explored by confocal microscopy, using an UltraVIEW ERS Rapid Confocal Imager (PerkinElmer, Waltham, MA, USA) connected to a Zeiss Axiovert 200 microscope fitted with a 63x/1.4 oil objective lens (Plan-Apochromat, Zeiss). Pictures were digitalized and processed by the Volocity software (Version 6.1 PerkinElmer).

2.8. Quantification of cytokine release

Concentrations of cytokines released to apical and basolateral cell culture supernatants upon stimulation with fish PVs or LMC were measured 24 h after apical cell stimulation. Quantification of the cytokines was performed using the xMAP Technology reagents and the Luminex 100 System supported by the xPONENT software, according to the protocols recommended by the manufacturer (Merck Millipore, Massachusetts, USA). IL-6, IL-8 and CCL2 were measured using the HCYTOMAG-60K kit. IL-25 and IL-33 were measured by the HTH17MAG-14K kit, and TSLP by the HCYP2MAG-62K kit (all three kits from Merck Millipore). Stimulation of the cells with TNFα (50 ng/mL) and IL-1β (20 ng/mL) was used as a positive control for release of proinflammatory cytokines (Ozaki et al., 1996; Tanaka et al., 2014; Tobe et al., 2002). Cytokines were measured in supernatants derived from 3 independent experiments performed in duplicates. The selection of the cytokines was made based on the published literature on the importance of these cytokines in epithelial cell damage response and allergic sensitization (Chow et al., 2010; Kosaka et al., 2011; Lee et al., 2015; Yi et al., 2017).

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Significant differences in TEER and cytokine release upon treatments with PVs and LMC compared to untreated cells were determined by the Friedman test with Dunn's post-test. Significant differences in permeability for FITC-Dextran of monolayers stimulated with cod LMC, shark LMC or without stimulation, as well as differences between permeability for PVs in presence or absence of LMC were determined by One-way ANOVA, followed Tukey's and Sidak's test, respectively. P values below 0.05 were regarded as significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

3. Results

3.1. Fish PVs cross the bronchial epithelial monolayers and remain intact

PVs from Atlantic cod, a bony fish, and gummy shark, a cartilaginous fish, were fluorescence-labelled using Alexa Fluor 488 (Fig. 1A). To explore whether the PVs have the capability to cross the epithelial barrier, 16HBE14o- bronchial epithelial cells were cultured on permeable filter supports. PVs were added to the apical compartment of polarized cell monolayers, and the basolateral cell culture medium was collected after 24 h. Labelled PVs of the expected molecular weight (11–13 kDa) were detected in the medium (Fig. 1B). Furthermore, the PVs transported across the epithelial monolayers into the basolateral medium were quantified after 1 h and 24 h of stimulation (Fig. 1C). Already after 1 h of apical exposure to the PVs, we were able to detect PVs transported to basolateral cell culture medium (Fig. 1C). After 24 h of apical stimulation with 80 µg/mL of cod or shark PV, the basolateral PV concentration was ˜200 ng/mL, while the stimulation with 160 µg/mL resulted in basolateral PV concentration of around 400 ng/mL (Fig. 1C). Thus, when the volumes of apical and basolateral media were taken into account, the calculated amount of transported PVs was approximately 1%. The amount of the transported PVs was concentration-dependent and no significant difference in the amount of cod or shark PV transported to basolateral medium was observed. Importantly, the PVs which crossed the epithelial barrier remained intact and were able to bind patients' IgE, as demonstrated for cod PV by IgE immunoblots using sera of three fish-allergic individuals sensitized to cod (Fig. 1D).

3.2. PVs do not interfere with physical barrier integrity and viability of bronchial epithelial cells

Transepithelial transport is the result of transcellular as well as paracellular pathways. Paracellular transport of molecules is controlled by tight junctions. To test whether the PVs interfered with the barrier properties of 16HBE14o- cells, the TEER of the monolayers exposed apically to two different concentrations of the PVs was monitored. Although a higher concentration (160 µg/mL) of both PVs induced an initial moderate reduction of TEER (5–10%), the barrier fully recovered after 16 h of treatment and this effect was not statistically significant (Fig. 2A and B). Interestingly, no difference was observed between the influence of cod PV (highly allergenic) or shark PV (considered to be less allergenic) on the physical barrier integrity (Fig. 2A). The calcium chelator EGTA, used as a control for increase of paracellular permeability, drastically reduced TEER (Fig. 2C). Furthermore, the PVs did not affect cell viability (Fig. 2D). Thus, fish PVs do not change the paracellular permeability of 16HBE14o- cell monolayers.

3.3. PVs are rapidly taken up by bronchial epithelial cells

Transcellular transport of proteins occurs by transcytosis, which is a unique endocytic route of polarized cells (Garcia-Castillo et al., 2017). To explore whether the PVs were internalized by the cells, cell monolayers were exposed apically to fluorescently labelled PVs and incubated at 37 °C. After one hour of exposure, the cells were fixed and analyzed by confocal microscopy. Both cod and shark PV were internalized by the cells and localized in a perinuclear area (Fig. 3). The PVs did not colocalize with LAMP2, a marker of late endosomes/lysosomes, indicating that the majority of PVs did not enter the degradative pathway (Fig. 3). There was no apparent difference in internalization patterns between cod and shark PVs (Fig. 3).

3.4. PVs do not influence the release of proinflammatory cytokines

To explore the possible influence of PVs on the release of mediators from the cells, aliquots of basolateral and apical media were taken after 24 h of apical stimulation of the cells with two different concentrations of PVs. None of the PVs induced significant changes in release of IL-6, IL-8 and CCL2, neither from the basolateral nor the apical side (Fig. 4A and B). TSLP, IL-25, IL-33, and IL-10 were below the detection limit of the assay in all samples (data not shown). Stimulation with TNFα and IL-1β as a positive control increased basolateral and apical release of IL-6, IL-8 and CCL-2 to 180–250% compared to untreated cells (100%) (Supplementary Fig. S3).

3.5. LMC from fish modify the physical barrier properties

As we did not observe an effect of PVs on the physical and immunological properties of the epithelial barrier, we next explored the influence of LMCs from bony and cartilaginous fish on 16HBE14o- cell monolayers. Apical stimulation with the LMC from both bony (cod) and cartilaginous fish (shark) resulted in an initial significant increase in TEER which was dose-dependent and persisted for both fish species for up to 8 h (Fig. 5A). Thereafter, monolayers treated with cod LMC demonstrated a significant drop in TEER to values below those of untreated monolayers (Fig. 5A). Interestingly, shark LMC showed the opposite effect from cod after a prolonged exposure, by continuously increasing the resistance of the monolayers for up to 24 h (Fig. 5A).

To investigate whether these changes in TEER reflected alterations of the physical barrier integrity, the permeability of the monolayers for FD10 was determined. The permeability for FD10 was significantly higher ($p < 0.05$) for monolayers treated with cod LMC (Fig. 5B), which was in line with the decreased resistance of these monolayers after 24 h. Also in line with the results from the TEER measurements, shark LMC significantly reduced ($p < 0.01$) the permeability for FD10 in 16HBE14o- cell monolayers (Fig. 5B). As expected, the calcium chelator EGTA drastically increased permeability for FD10 which was 10-fold higher compared to untreated monolayers (data not shown).

To elucidate whether the exposure to LMC interferes with the

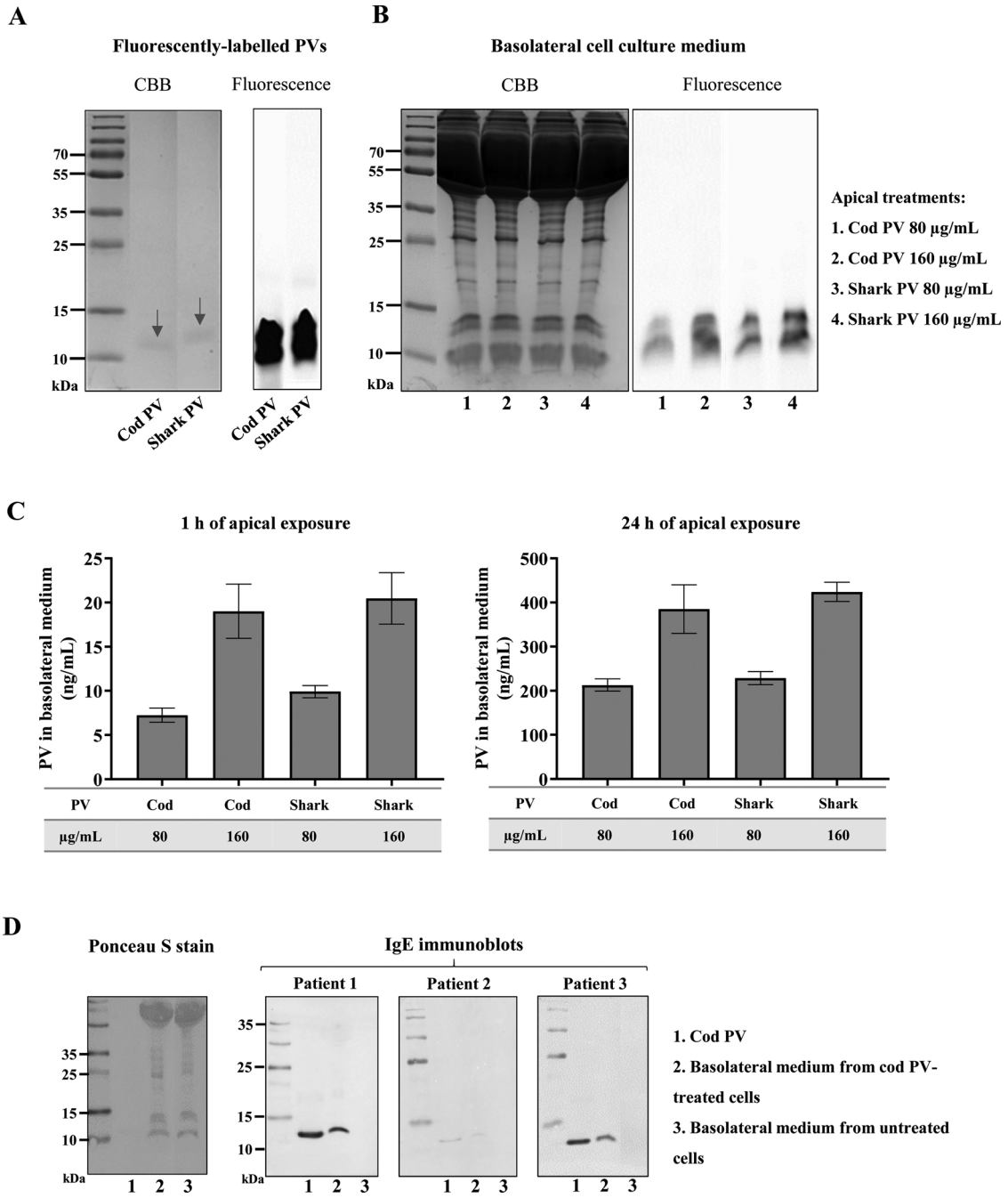

Fig. 1. PV transport across epithelial monolayers. A. Detection of 0.1 µg of PVs conjugated to Alexa Fluor 488 by CBB staining of an SDS-PAGE gel and fluorescence detection on a nitrocellulose membrane. B. CBB-stained gel and fluorescence detection of proteins in the basolateral medium from monolayers treated apically with 80 µg/mL or 160 µg/mL of labelled PVs for 24 h. C. Quantification of PVs in the basolateral medium after 1 h and 24 h of apical stimulation (n = 3, data shown as mean ± SD). D. Fish allergic patients' IgE binding to cod PV in basolateral medium. Purified cod PV (0.2 µg) was used as a positive control.

transport of PVs across the epithelium, cells were co-incubated with PVs and corresponding LMC for 24 h and the amount of PVs transported to the basolateral medium was quantified (Fig. 5C). When cells were treated with the combination of PV and LMC from the corresponding fish, the PVs were still transported to basolateral medium. However, their concentrations were significantly lower compared to the concentrations measured after exposure to PVs in absence of fish LMC (Fig. 5C). No difference between the amounts of cod versus shark PV was observed.

Athough they influenced TEER of the monolayers (Fig. 5A), the fish LMC did not reduce viability of the cells, as determined by MTT assay 24 h after exposure of the cells to different concentrations of LMC (Fig. 5D).

3.6. Fish LMC increase release of proinflammatory cytokines

In contrast to the PVs which did not influence the release of IL-6, IL-8 and CCL2 from the cells, notable differences in release of these cytokines were observed upon stimulation of the cells with fish LMC. Basolateral and apical release of IL-6 and IL-8 was increased by exposure to both cod and shark LMC (Fig. 6A and B). This effect was clearly dose-dependent for cod LMC, reaching an increase of up to 2.2-fold in IL-6 release and up to 50% in IL-8 release after exposure to Dose III (Fig. 6B). The influence of shark LMC on cytokine release was not

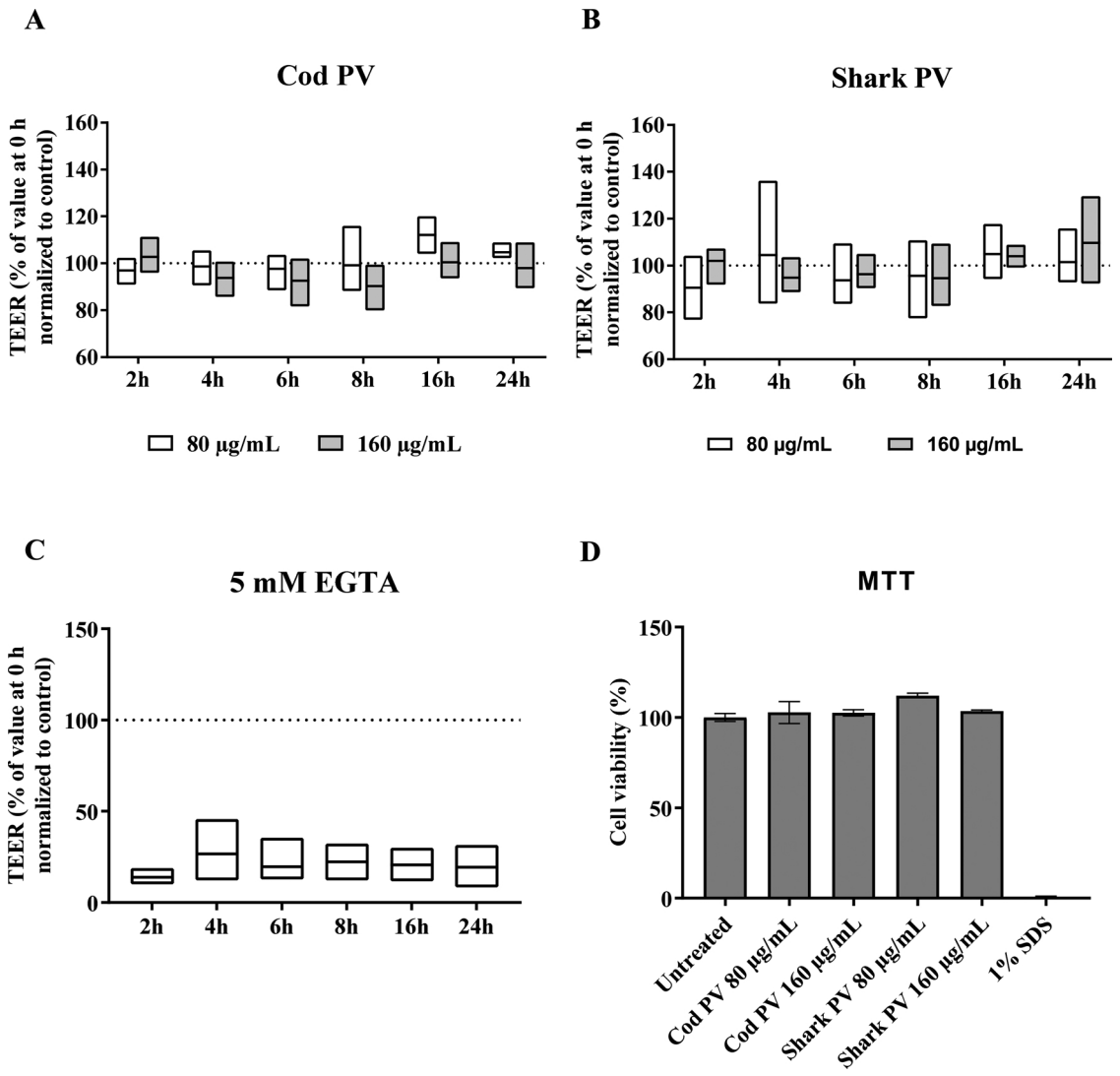

Fig. 2. PVs do not significantly influence epithelial barrier integrity and the viability of bronchial epithelial cells. Polarized 16HBE14o- cell monolayers were exposed apically to (A) cod PV, (B) shark PV (80 or 160 µg/mL) or to (C) 5 mM EGTA and TEER was monitored for 24 h. Untreated monolayers were used as controls. Data are obtained from three independent experiments performed in duplicates and show means with ranges (min to max), normalized to control monolayers. D. Cell viability analyzed by an MTT assay 24 h after apical exposure to PVs. 1% SDS was used as a control for toxicity. Data show mean ± SD (n = 3).

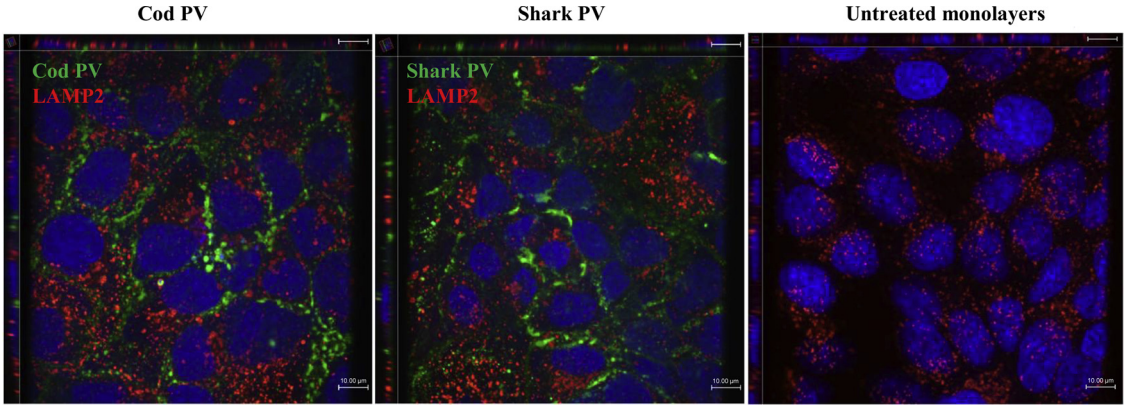

Fig. 3. PVs are internalized by 16HBE14o- cells and do not co-localize with LAMP-2-positive late endosomes/lysosomes. The figure shows results of confocal microscopy of 16HBE14o- cell monolayers after apical exposure to cod or shark PV (100 µg/mL) for 1 h at 37 °C, or monolayers without treatment with PVs. Alexa Fluor 488-labelled PVs (green), late endosome/lysosome marker LAMP2 detected by a specific primary and Alexa Fluor 568-labelled secondary antibody (red), DRAQ5-labelled nuclei (blue). Bar = 10 µm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

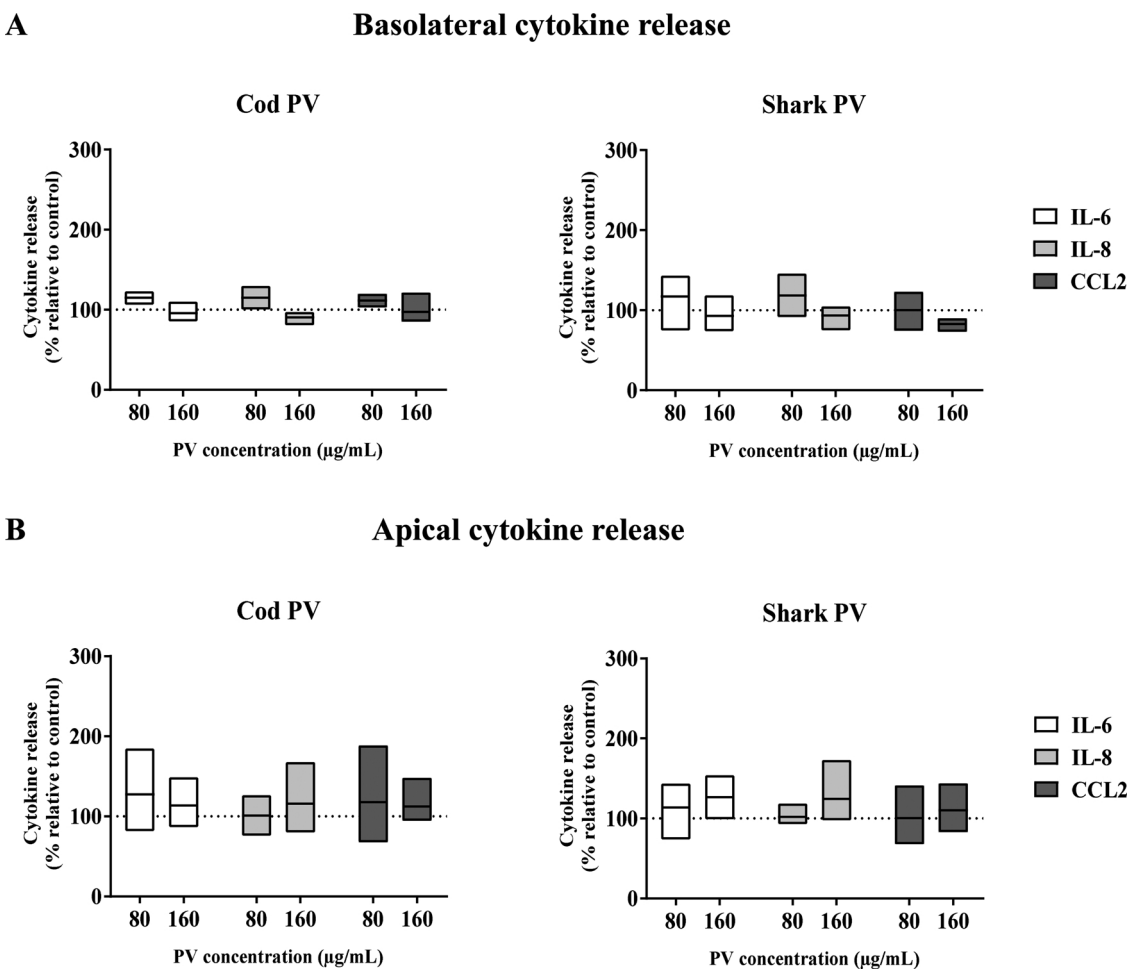

Fig. 4. PVs do not modify the release of IL-6, IL-8 and CCL2 by 16HBE14o- cells. Epithelial cell monolayers were stimulated apically with cod or shark PV (80 or 160 µg/mL) and the concentrations of the cytokines released to the basolateral (A) or apical (B) media were measured after 24 h of stimulation. Cytokine concentrations were normalized to the concentrations measured for untreated monolayers. Data are representative of three independent experiments performed in duplicates and depict means with ranges (min to max).

dose-dependent and was statistically significant only for basolateral release of IL-6 (Fig. 6A). Furthermore, basolateral and apical release of CCL2 was increased by exposure to cod (up to 50%), but not to shark LMC (Fig. 6A and B).

IL-10, IL-25, IL-33 and TSLP were below the detection limit for all treatments (data not shown).

4. Discussion

The prevalence of fish allergy has been increasing worldwide and is especially high in fish processing workers who are mainly exposed to fish allergens via inhalation (Jeebhay and Lopata, 2012; Moonesinghe et al., 2016). However, the mechanisms of the interaction between the major fish allergen PV and the bronchial epithelial cells, as well as the possible role of the food matrix in allergic sensitization to fish have not been explored before. Our study is the first to demonstrate that PVs from both a highly allergenic bony and a less allergenic cartilaginous fish are internalized by bronchial epithelial cells and rapidly cross the epithelial barrier in an intact form. Furthermore, LMC from fish exerted an effect on the barrier integrity of the cell monolayers and on mediator release. This activity differed between bony and cartilaginous fish. Therefore, LMC possibly play a role as adjuvants in allergic sensitization to fish via inhalation.

The respiratory epithelium is a physical, chemical and immunological barrier which plays an active role in detecting local changes of the environment to protect the body from possibly harmful molecules such as allergens (McClain et al., 2014; Vroling et al., 2008). Besides the need for the allergens to pass the epithelium and be presented to the immune system to induce allergic sensitization, the role of the food matrix as an adjuvant has emerged in recent years (McClain et al., 2014). Components other than allergens found in allergen sources may increase the stability of the allergens, act as danger signals or drive the immune system towards Th2 response (Aglas et al., 2018; Palladino et al., 2018; van Wijk et al., 2005; Wavrin et al., 2015).

The major fish allergens belong to the beta lineage of PVs and are abundant in bony fish. Cartilaginous fish contain mainly PVs of the alpha lineage and their allergenic potential is not well understood (Kuehn et al., 2014). Our recent study demonstrated the low reactivity to Thornback ray, a cartilaginous fish, in a cohort of patients sensitized to bony fish (Kalic et al., 2019). Possible explanations for the low allergenicity of cartilaginous fish may include the low sensitizing capacity of fish alpha PVs or different properties of their fish matrices, which could differently interact with epithelial barrier cells and the immune system resulting in different responses.

Epithelial cells are able to endocytose antigens, however most of the endocytosed material is sorted into lysosomal compartments and degraded thus preventing transcytosis of potentially antigenic proteins to the luminal side (Vroling et al., 2008). Moreover, the tight junction complexes between epithelial cells exclude the paracellular transport of most of the proteins (Georas and Rezaee, 2014). It is therefore being extensively investigated which mechanisms allergens use to cross the epithelial barrier. While several allergenic proteins use a paracellular

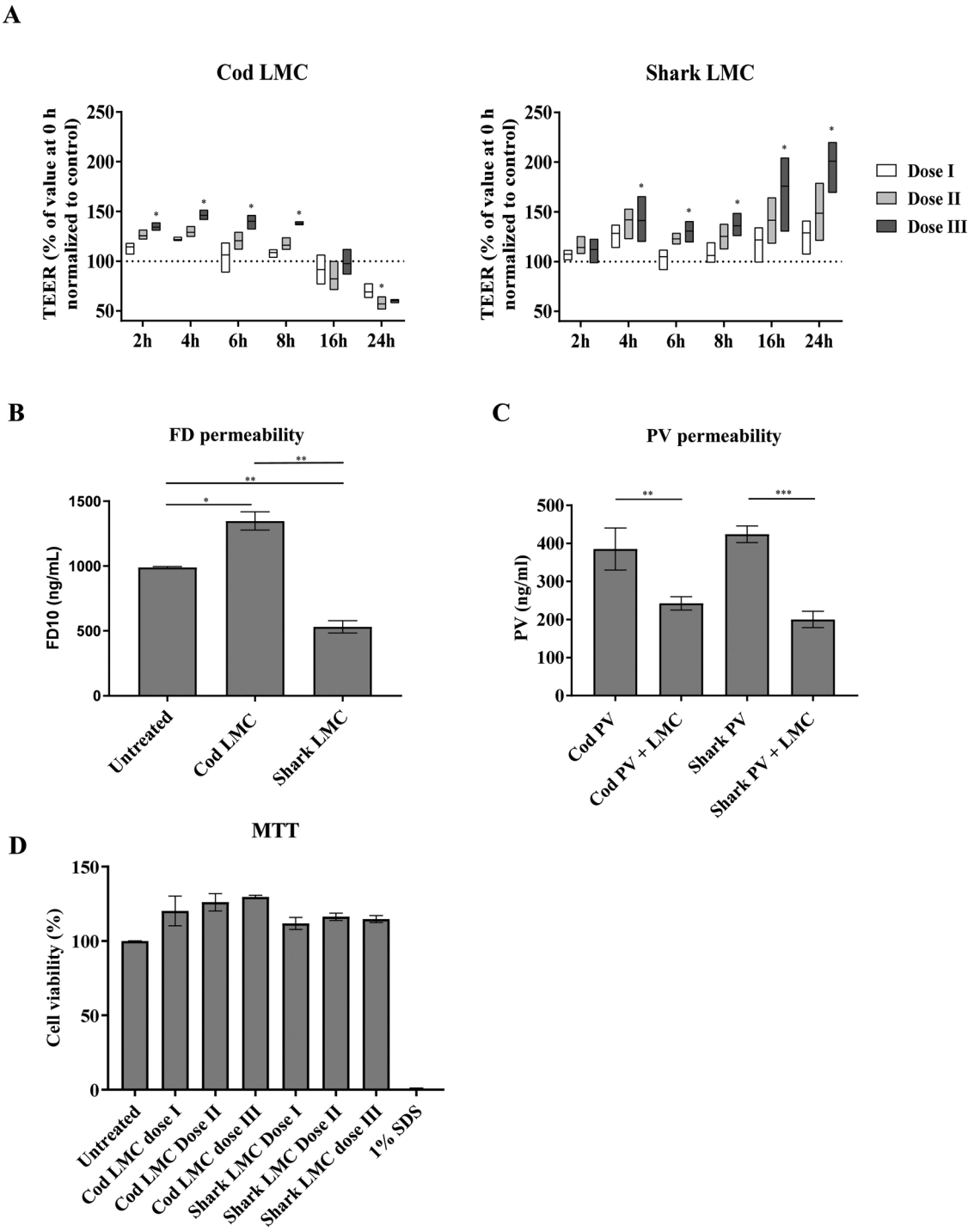

Fig. 5. Fish LMC modify the epithelial barrier properties. A. 16HBE14o- cell monolayers were exposed apically to three doses of cod or shark LMC and TEER measured over time. TEER was normalized to untreated monolayers for each time point. Data show means with ranges (min to max) from three independent experiments performed in duplicates. B. Polarized monolayers were treated for 24 h with fish LMC (Dose III) and the permeability for FD10 was explored. Untreated cells were used as reference. n = 2, data show mean ± SD. C. Quantification of the PVs in basolateral cell culture medium after 24 h of apical stimulation in presence or absence of LMC. n = 3, data show mean ± SD. D. Cell viability analyzed by MTT assay 24 h after apical exposure to different doses of LMC. n = 3, data show mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001.

epithelial transport by interfering with the epithelial barrier integrity (by direct protease activity or by inducing an inflammatory epithelial response) (Georas and Rezaee, 2014), we demonstrated the rapid internalization of PVs and their subsequent transport to the basolateral side without their degradation in endo-lysosomal compartments, suggesting a transcellular transport route. On the basolateral side, these allergens are accessible to antigen presenting cells possibly leading to allergic sensitization. Furthermore, the rapid transport of PVs across the epithelium may be an explanation for the rapid onset of allergic reactions to fish in already sensitized individuals, when exposed to minute amounts of fish allergens found in aerosols or due to ingestion of trace amounts of fish (Crespo et al., 1995; Untersmayr et al., 2007).

In our study, PVs did not change the viability of the cells, nor did they significantly influence the release of the proinflammatory

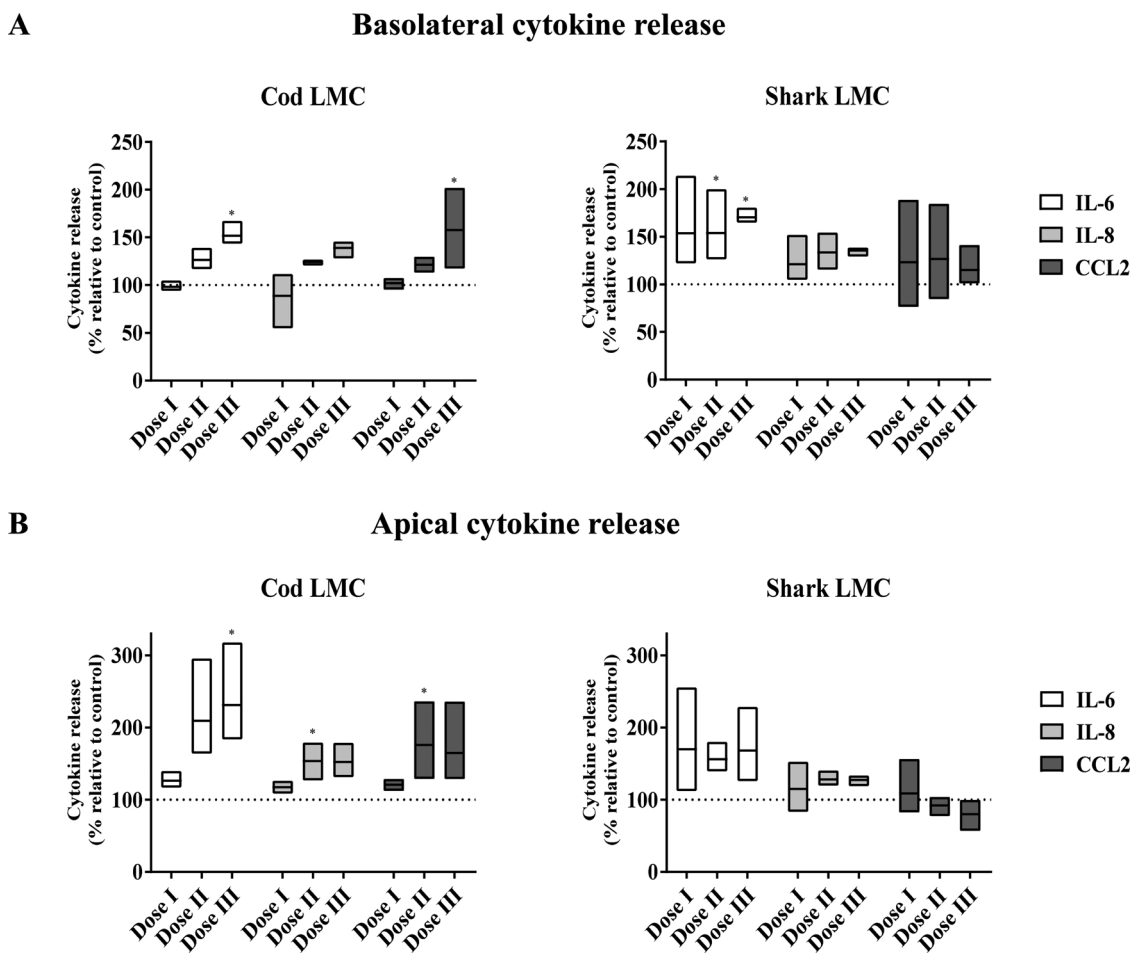

Fig. 6. Fish LMC modify proinflammatory cytokine release. Protein levels of IL-6, IL-8 and CCL2 in basolateral (A) and apical (B) media were quantified 24 h after apical stimulation of the cells. Concentrations are normalized to concentrations of cytokines released from untreated cells. Data are representative of three independent experiments performed in duplicates and depict means with ranges (min to max). *$p < 0.05$.

cytokines IL-6, IL-8 and CCL2. Interestingly, no difference between the interaction of cod versus shark PV with the cells was observed. Therefore, although being an important step in allergic sensitization, the ability of PVs to cross a tight epithelial barrier did not explain the different sensitizing capacities of bony versus cartilaginous fish. It is, however, important to note that our study used a cell line and that future studies on primary cells will be required to understand the mechanisms of allergic sensitization via inhalation in more detail.

Besides the allergens themselves, multiple external and internal factors contribute to polarization of the immune system towards allergic sensitization or tolerance when exposed to allergenic proteins (Curotto de Lafaille et al., 2010).

To further explore the differences in the response of epithelial cells to bony versus cartilaginous fish, we next investigated the possible influence of food matrix components as potential adjuvants in allergic sensitization. The role of LMC from the allergen source as an adjuvant had already been demonstrated for pollen (Blume et al., 2015). Birch pollen extract but not the major birch pollen allergen Bet v 1 was able to efficiently activate dendritic cells and induce a Th2 polarization (Aglas et al., 2018). We used fish LMC instead of whole fish extract to study the role of components other than proteins which are small enough to be aerosolized and to act as potential adjuvants in allergic sensitization via inhalation. Interestingly, the response of epithelial cells to cod or shark LMC was different, these differences possibly being the reason for their different allergenic potential. After 24 h of exposure to fish LMC, the TEER decreased below that of the control monolayers in case of cod, while it became higher in case of shark LMC, showing a potential protective effect of shark LMC on the epithelial barrier. Quantities of transported cod and shark PV after 24 h of cell stimulation were lower in presence than in absence of LMC from the corresponding fish. However, the amounts of transported cod versus shark PV were similar, although the TEER was different upon treatment with cod versus shark LMC. A possible explanation is that transcellular transport of PVs may be altered in the presence of LMC but this effect did not depend on the fish species. The mechanism of PV transport across the epithelium in the presence or absence of LMC will be the subject of further studies.

Furthermore, fish LMC modified the proinflammatory cytokine release from the cells which differed between cod and shark and may play a role in differential recruitment of other immune cells or further release of mediators, subsequently contributing to the different allergenicity of these two fish classes.

IL-6 and IL-8 are classic proinflammatory cytokines which influence the function of the bronchial epithelium and their polarized secretion by 16HBE14o- cells in response to the potential cell damage was demonstrated by the study of Chow et al. (2010). In our study, basolateral and apical release of IL-6 and IL-8 was increased by exposure to LMC from both species, while CCL2 release was increased only by cod LMC. The induction of CCL2 release by airway epithelial cells had been demonstrated for several allergens such as house dust mite proteins and chitin (Plantinga et al., 2013; Roy et al., 2012). It was demonstrated that CCL2 can induce differentiation of monocytes into inflammatory $CD11b^{+}$ dendritic cells, which are one of the crucial players in induction of allergic sensitization, biasing the differentiation of naïve T cells

towards a Th2 phenotype (Lee et al., 2015; van Ree et al., 2014). Furthermore, the role of neutrophil recruitment by IL-8 was implicated in allergic sensitization in several studies (Hosoki et al., 2016; Lambrecht and Hammad, 2014). In our study, while LMC from both cod and shark increased the release of IL-8 from the cells, the increased release of CCL2 was observed only upon exposure to LMC from cod, possibly playing a role in the higher allergenic potential of this bony fish.

The role of IL-6 in allergic sensitization to fish is not fully understood as it can elicit both Th1 and Th2 responses based on the signaling pathways activated in the cells, as well as the microenvironment which may include different cell types and the presence or absence of other cytokines (Lambrecht and Hammad, 2014; Tanaka et al., 2014). In our study, IL-6 release was increased after exposure to both cod and shark LMC. Possible differences in signaling pathways activated in the cells upon exposure to LMC from different fish species, as well as the composition of the LMC and the molecules responsible for the observed effects need to be elucidated in future studies. Furthermore, a larger study including multiple species of bony and cartilaginous fish classes is required to further explore the possibility of fish LMC as a contributing factor to the lower sensitizing capacity of cartilaginous compared to bony fish.

The importance of our study is the demonstration of the rapid transport of PVs in an immunologically active form across the epithelium, resulting in their availability to antigen presenting cells which is required for allergic sensitization. Furthermore, LMC from fish modified the barrier properties and the cytokine response which differed between bony and cartilaginous fish, possibly contributing to the different allergenic potential of these two fish classes.

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Conflicts of interest

All authors declare no conflicts of interest regarding the work presented in this manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.04.029.

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Supplementary figures *Kalic et al, 2019*

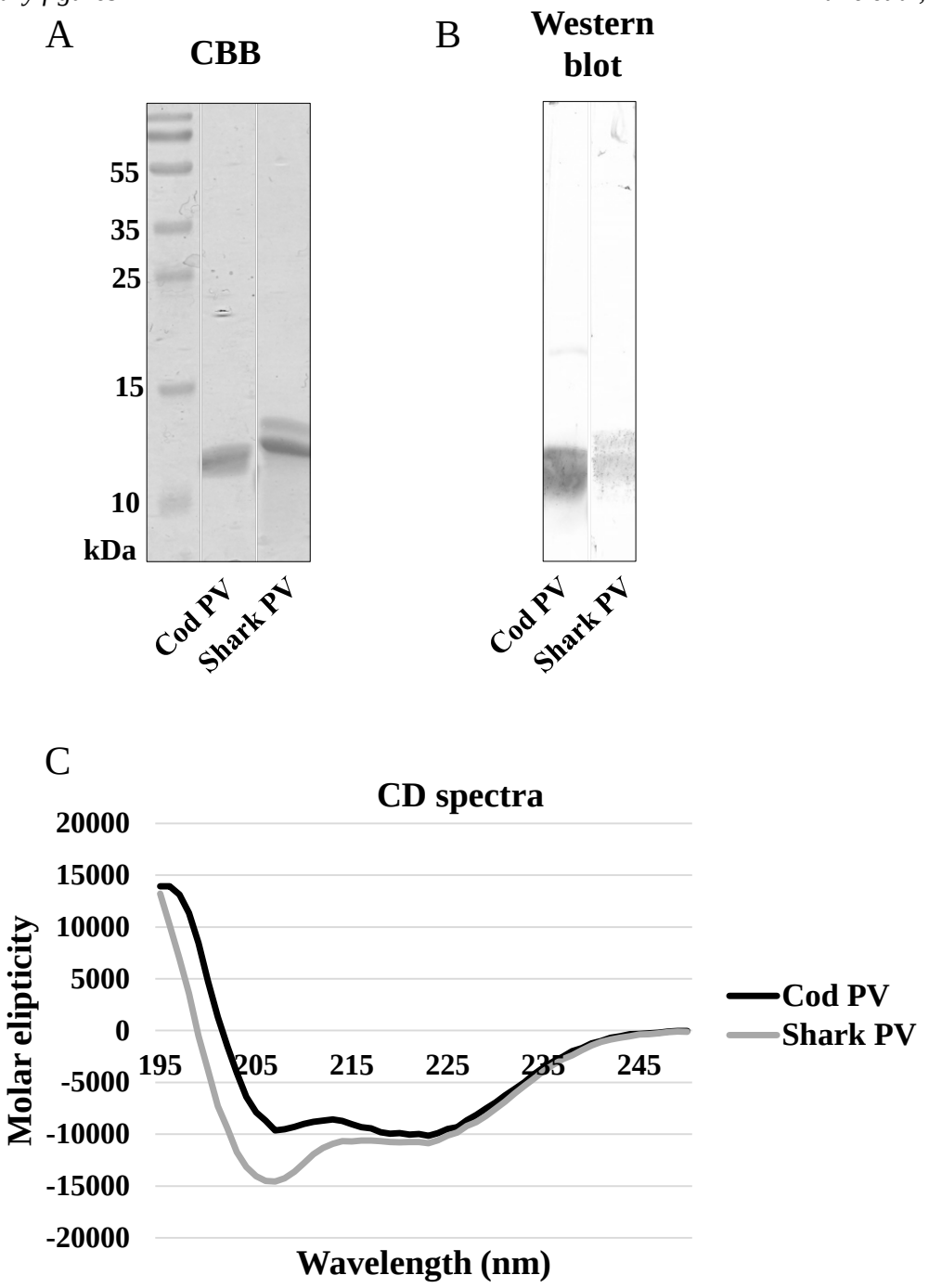

Figure S1. Characterization of the study parvalbumins. A: Coomassie brilliant blue staining of an SDS-PAGE gel demonstrating purity of the proteins. B: Western blot using anti-parvalbumin antibodies, confirming the protein identity. C: Circular dichroism (CD) spectroscopy, showing the expected alpha helical secondary structure of the proteins.

Supplementary figures *Kalic et al, 2019*

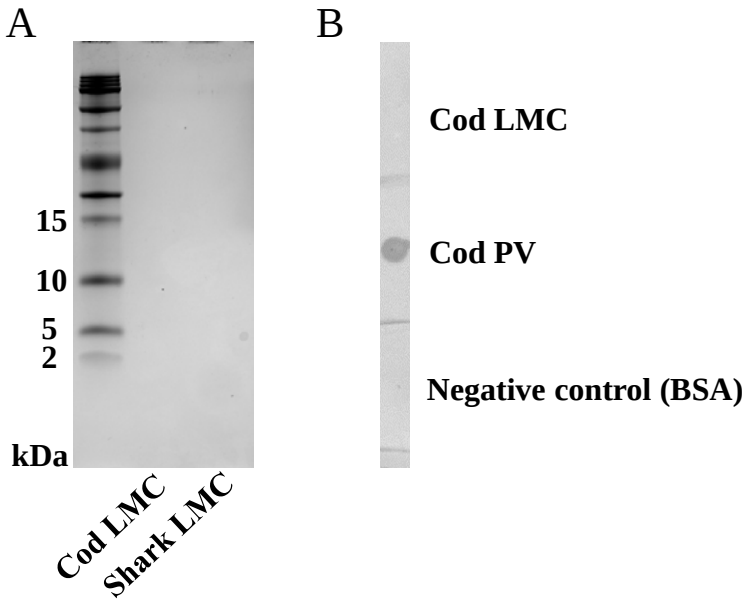

Figure S2. A: Absence of low molecular weight peptides in cod and shark LMC demonstrated by Coomassie Brilliant Blue staining of 16.5 % Tris-Tricine gel. B: Absence of IgE-binding to cod LMC demonstrated by dot blot using serum of Patient 1.

Supplementary figures *Kalic et al, 2019*

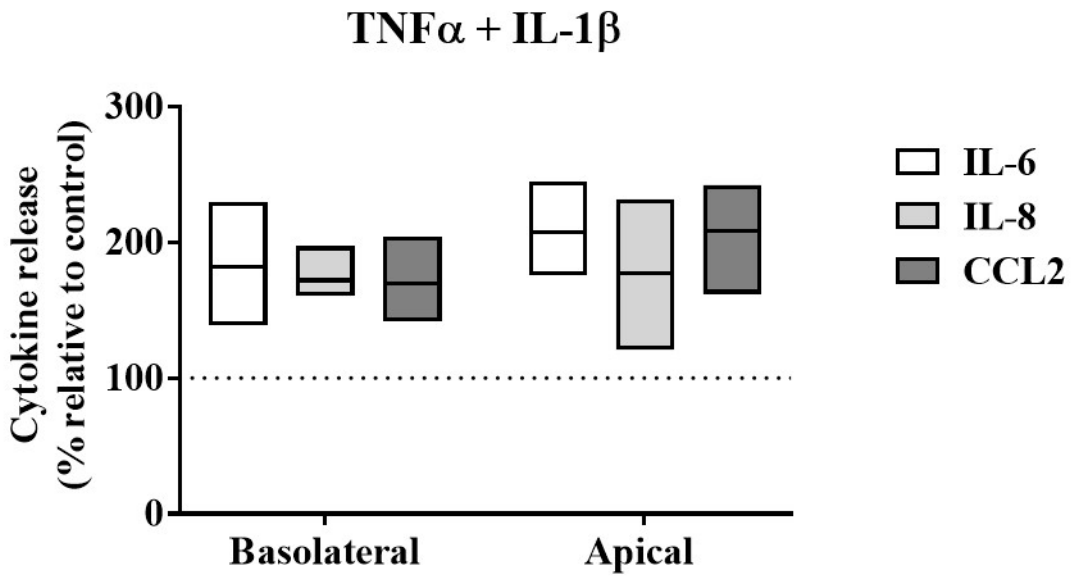

Figure S3. Basolateral and apical cytokine release after apical stimulation of 16HBE14o- cells with a mixture of TNFa and IL-1b as a positive control for induction of inflammation. Data show means with ranges obtained from three independent experiments performed in duplicates and represent cytokine release relative to release from untreated cells.

The end.