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Allergenicity of bony and cartilaginous fish – molecular and immunological properties

Abstract:

Allergy to bony fish is common and probably increasing worldwide. 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% to 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.

1. Introduction:

Allergy to seafood is common, potentially life threatening, often lifelong and accounts for up to 5% of food allergies in children and 2% of food allergy in adults worldwide [1, 2]. There are major regional differences, with apparent much higher rates of sensitization to fish in Asia. The move towards healthy lifestyles and eating habits has seen a steady increase in the consumption of fish, which suggests that seafood allergy will continue to be a major health

issue into the foreseeable future. Recent research has focused on the molecular characterization of seafood proteins for identification of major allergens and for improved diagnostics.

The major allergen identified in fish, parvalbumin (PV), binds calcium, is heat stable and is involved in the muscle relaxation and contraction cycle [3] as well as signal transduction [4]. PV is reported to account for up to 90% of cross-reactive allergic immunological reactions to fish [1, 2]. There are two distinct lineages of PV, α & β [5]. While most fish contain both lineages of PV, β-PV is predominantly reported as major allergen in bony fish. Reported allergic sensitization to α-PV are limited. In this review, the prevalence of fish allergy and clinical implication of allergenicity of various fish species are explored. The two lineages of PV are extensively detailed at the molecular level to emphasize the immunological and clinical impacts of this two diverse allergen lineages.

2. Fish allergy epidemiology: How common is it?

Allergic reactions to bony fish are well documented. However, the true prevalence of fish allergy is unknown, with a lack of high quality population based data. Reported prevalence ranges from self-reported rates of 5% to challenge proven rates of 0.1%. Most estimates are based upon small samples, patient-based questionnaires or allergic cohorts from specialist centers. From current available data is appears that there are significant regional differences in rates of bony fish allergy (see Table 1). It is likely that prevalence of fish allergies is diet (and exposure) related, with higher reported rates of bony fish allergy in regions with high consumption [6].

Asia has one of the largest populations of fish consumers with reported yearly consumption rates significantly above the world average rate of 16 kg per capita with up to 54 kg per capita in Japan (2011) [7]. A review of seafood allergy in South East Asia reported a higher rate of fish allergy in the Philippines (n=11,434; 2.29%) compared to Singapore (n=6,498; 0.26%) and

Thailand (n=2,034; 0.29%) [8]. Fish, shrimp and crab were amongst the leading 5 causes of food allergy in Thai preschool children, and fish allergy was estimated to account for 13.2% of food allergy in a cohort of food allergic children in Singapore [9]. As a cause of food-related anaphylaxis fish and shellfish accounted 3.3% and 66.7%, respectively in a small cohort in Singapore [10]. Similarly, fish allergy was reported to account for 4.1% of IgE-mediated food allergy in adults attending for care of their food allergy in Singapore [11].

While prevalent across Asia, fish allergy also appears to be relatively common in Europe (Table 1), again with significant sub-regional variation. Self-reported prevalence of fish allergy is as high as 3% [12], while the incidence of challenge proven fish allergy challenge is reported to be only 0.1% [13]. Across European cities, approximately 0.2% of the population is reported to be allergic to some type of fish [14].The populations of Portugal, Spain and Scandinavian are likely to be the highest consumers of fish in Europe [15] and the highest rates of fish allergy are also generally reported in Northern Europe [16]. In a Norwegian prospective observational cohort of 3,623 children [12]- 3% were reported to have fish allergy by 2 years of age . Early onset of fish allergy was also described in a cohort of Spanish food allergic children who had allergy to cod, tuna, hake and whiff predominantly before the age of 2 years [17].

In Australia, 5.6% of a large cohort of food allergic children were identified as having fish allergy, with the most commonly implicated fish being tuna, salmon, barramundi and flathead [18]. Of those with a fish allergy, 20% had a history of fish-related anaphylaxis. Clinical and allergy skin test (SPT) cross-sensitivity between the fish species was also common within this fish allergic cohort, with 93% being atopic to at least one other fish type on SPT and 29% with a previous clinical allergic reaction to another species of fish. In a separate Australian cohort study (n=94) of children with proven seafood allergy, 64% of the cohort were allergic to fish [19], with 'white fish', salmon and tuna being most commonly implicated species and allergic reactions to basa [catfish], barramundi, bream, and cod all reported.

In the USA, 0.4% of the population was estimated to be fish allergic and 2% shellfish allergic based upon a large national random telephone survey [20]. The self-reported major allergenic fish species included salmon, tuna and cod. A similar random telephone survey was conducted across Canada in 2002 and estimated the population prevalence of fish allergy to be 0.1% [21]

with the most common fish allergens being cod and salmon. Similarly, a US self-report survey found a prevalence of 0.7% and 0.6% for self-reported and self-reported doctor-diagnosed fish allergy, respectively [22].

3. Classification of cartilaginous and bony fish

In order to understand the clinical, diagnostic and allergen characterization implications of differences between various fish, it is useful to review taxonomy and evolution of fish. Biologically, fish are divided into two classes, Chondrichthyes (cartilaginous fish) and Osteichthyes (bony fish). Bony fish are the largest group of all vertebrates consisting of 45 orders and over 435 families. Bony fish possess stable cranial bones, swim bladders and, in certain species, even primitive lungs. Bony fish can be further divided into ray finned fish (Actinopterygii) and lobe-finned fish (Sacropterygii). Actinopterygii is the biggest class and possess fins made of webs of skin supported by bony spines and includes all edible fish. Sacropterygii consists of fish with fleshy, lobed, paired fins, which are joined to the body by a single bone. The West Indian Ocean coelacanth discussed in this review is the oldest, extant lineage of Sacropterygii with evidence of continuous presence for 100 million years.

The Chondrichthyes (cartilaginous fish) in contrast are jawed fish with skeletons made of cartilage. They can be further divided into two subclasses – Elasmobranchii and Holocephali. Elasmobranchii consists of sharks (Selachii) and rays and skates (Batoidea). Lack of swim bladders, rigid dorsal fins, small placoid scales and five to seven pairs of gill cleft openings to the exterior are characteristic of the Elasmobranchii. In contrast, the subclass Holocephali consists of only one surviving Chimaeriformes order, which constitutes rat fish, rabbit fish and elephant fish. They are bottom dwellers, having simplified guts where the stomach is merged with the intestine. An interesting species of study is the chimaeric elephant or Australian ghost shark [*Callorhinchus milli*], as it is closely related to sharks but also have distinct features similar to bony fish, such as gill covers.

4. Fish Allergens

4.1 Parvalbumin

Isoforms: The major pan-fish allergen, parvalbumin (PV), belongs to the EF-hand helix-loop-helix domain family of calcium binding proteins and constitutes the biggest group of animal derived food allergens [23]. It has been characterized in detail from 12 fish species (registered with the IUIS) across various continents including Europe and the USA [1, 9, 14, 20] and is reported to account for up to 90% of cross-reactive fish allergy [2, 24]. PV is abundant in muscle and nerve tissue of lower vertebrates and is capable of binding Ca^{2+} and Mg^{2+}. Two distinct phylogenetic lineages of PV, α and β, have been identified, with the β isoform seems to be predominant in muscle tissue of bony fish [25, 26]. The characteristics of PV from bony and cartilaginous fish [α isoform] along with their isoforms, molecular weights and isoelectric points are listed in ascending order of discovery in Table 2.

Chemistry: PV is an acidic, sarcoplasmic, 10 - 15 kDa protein that is extremely resistant to heat [27], proteolytic and chemical degradation [28, 29]. It was first identified as an allergen in Baltic cod (*Gadus morhua)* in 1969 and named Gad c 1 or Allergen M [29]. The isoelectric points range from 3.9 – 4.5 for the β-PVs, whereas it is generally above 5.0 for different α-PVs [30].

PV's are globular in shape and contain six α-helices (A, B, C, D, E, and F). These helices form three helix-loop-helix EF hand motifs (AB, CD and EF). The heat stability and resistance to protease degradation is attributed to chelated calcium which changes the conformation of PV [31]. As a consequence of conformational changes, the allergenicity of PV is also higher in the presence of calcium due to better exposure of IgE binding epitopes [2, 32]. The calcium-binding epitopes in PV are well conserved across bony and cartilaginous fish (Figure 1A and 1B), demonstrating the similar functionality of this protein.

Characterization: PV has been extensively studied in bony fish including in Atlantic cod (*Gadus morhua*) [28, 29, 33-37], Alaska Pollack (*Theragra chalcogramma*) [38, 39], common carp (*Cyprinus carpio*) [24, 30, 37, 40, 41], silver carp (*Hypophthalmichthy molitrix*) [42], Atlantic salmon (*Salmo salar*) [39, 43-47] and recently Asian seabass (*Lates calcarifer*) [48, 49]. In comparison, studies on PV from cartilaginous fish are limited to leopard shark [25], red stingray [26], Atlantic stingray [50] and thornback ray [51]. β-parvalbumin is more abundant in bony fish

and appears to be responsible for the majority of allergic reactions, while the α isoform seems to be predominant in cartilaginous fish [25, 26]. Some fish species seem also to express multiple isoforms of β-PV such as β1 and β2 seen in barramundi and isoforms 1 to 9 in zebrafish [48, 52].

Differences between α- and β-PV: There appears to be low clinical cross-reactivity between α- and β-PV of the two fish classes (bony and cartilaginous) as evident from the lack of detailed published reports of individuals being allergic to both [53-55]; however the reasons for this low cross reactivity are poorly understood. One of the key differences between the lineages is the presence of more acidic amino acid residues with pI 4.5 or lower in β-PV, while α-PV consists of less acidic amino acid residues with pI 5.0 or higher [26]. The α and β lineages also differ slightly in length, with α-PV usually consisting of 109 or more amino acids while β-PV generally have 108 or fewer amino acids [31]. This extensive molecular variation may be the key to understanding why the α-PV predominant fish are less allergenic and the limited clinical cross-reactivity observed between different fish species and groups.

Leopard shark α-PV was the first shark PV to have its crystal structure studied [25]. The amino acid sequence homology of PV from one shark and several bony fish [carp, cod, salmon] is less than 50% (Table 3). Interestingly, bony fish PV's demonstrate relatively high sequence identity with muscle from amphibians (63-76%), reptiles (56-69%) and birds (54-71%), whereas human muscles are devoid of β-PV with only 56% sequence homology between human α-PV and fish β-PV. The homological distance is an indicator of the differential allergenicity of the two isoform lineages [56], with evolutionary divergence of α and β-PV lineages in vertebrates (Figure 2). Interestingly, PV amino acid sequences (α and β) for the West Indian Ocean coelacanth (UniProtKB ID: P02629.1 and UniProtKB ID: P02623.1) have high amino acid identity (62% and 70%) to leopard shark α-PV but also carp β-PV, respectively (Table 3). Here, the degree of sequence identity is a good indicator of the extent of molecular conservation of PV across a vast time period.

4.2 IgE binding epitopes and clinical cross-reactivity

Elucidation of the PV IgE binding epitopes is necessary to understand allergen-antibody interactions which result in clinical reactivity. IgE binding epitopes of PV from five fish, Atlantic cod (Gad m 1), Baltic cod (Gad c 1), common carp (Cyp c 1), chub mackerel (Sco j 1) and Atlantic salmon (Sal s 1), have been elucidated in detail (Figure 3A). Epitopes for Atlantic cod, Atlantic salmon and chub mackerel have been individually mapped using overlapping peptides of PV and analyzed using allergic patient's serum IgE [44, 57, 58]. The epitopes for Baltic cod were elucidated via mutated derivatives of PV and immunological reactivity of overlapping tryptic peptides [34]. Carp PV epitopes have been identified using patient sera by screening against a decamer phage library, computational matching and alignment techniques [32]. While PV from Atlantic cod, Baltic cod, common carp, chub mackerel and Atlantic salmon differs in amino acid sequences, they seem to share some of the linear antibody binding epitopes. Currently there is very little information available about conformational IgE binding epitopes, but it is very likely that they are very different from each other. A comparative study by Kobayashi et al [59] demonstrated the importance of stereoscopic conformation for IgE binding in a range of different PVs, with a structural dependence on Ca2+ binding.

Fish allergic patients generate a variety of epitope specific IgE which have been used in epitope mapping for five fish species including cod, salmon, carp and mackerel. In Gad c1 (cod) there are five regions reported to include IgE-binding epitopes; residues 13-32 in the AB domain, 33-44 on the region joining the AB and CD domains, 49-64 in the calcium-binding region in the CD domain, 65-74 in the region joining CD and EF domains and the 88-96 calcium binding region in the EF domain [34]. Three additional epitopes were later identified by mapping mimotopes onto the molecular surface of natural carp PV via a surface-matching algorithm [32]. While two epitopes were found within regions joining the domains AB and CD, and CD and EF, the third epitope was found in the calcium-binding loop of the EF domain. The carp β-PV model has been used to specify the known IgE binding epitopes (Figure 3B). Epitope alignment has shown that region IV is highly antigenic, while region I seems to be more species-specific (which is implicated especially in the case of clinical mono-sensitivity to salmon) and perhaps several species in the group of salmonidae [1].

4.3 Other fish allergens

Collagen, vitellogenin, fructose bisphosphate-aldolase and beta-enolase [45, 60-65] have been reported as potentially important allergens in fish. The first instance of collagen as a putative fish allergen was reported in bigeye tuna [66] where collagen was shown to elicit IgE-reactivity in five of eight tuna-allergic patients. Here, an inhibition ELISA [enzyme linked immunosorbent assay] strongly implicated the allergenicity of collagen being independent of fish species. Additionally, the importance of collagen as a pan-allergen has been demonstrated recently [67, 68]. In a cohort of 36 Japanese fish (raw and cooked) allergic patients, 50% were found to have IgE to mackerel collagen. Allergenicity of fish collagen from cartilaginous fish has recently been demonstrated to be lower than that of bony fish collagen [67]. In a separate study, α and β-chain collagen (120 and 240 kDa, respectively) were identified as allergens in two species of tuna [albacore and yellowfin] [69]. The hormone vitellogenin, from Beluga caviar and salmon roe, has been shown to cause IgE-reactivity [45, 65]. Fish allergic patients have also been reported to produce IgE to fish gelatin, a type I collagen [62], which has been reported to cause anaphylaxis [64].

Fish contaminated with the parasitic nematode, *Anisakis*, may cause allergic reactions, where IgE is directed not at fish allergen, but at parasite proteins. These reactions can be misdiagnosed as fish allergy [70-74]. A number of allergens have been characterized from *Anisakis* including tropomyosin and protease inhibitors. Occupational allergy due to inhalation of heat stable parasite allergens has also been shown to cause allergic reactions in fish processing workers [75, 76].

5. Changes in allergenicity related to exposure

Oral ingestion of fish allergens is the primary route of exposure and in sensitized individuals may result in IgE-mediated immune responses due to the allergen being absorbed via the mucosa of the gastrointestinal tract [77]. Absorption of allergen across mucosa can be rapid and codfish proteins have been identified in the sera of healthy individual within 10 minutes of consumption. Here the biological activity of the allergens was lost at pH 2.0, but at pH 3.0 the

protein patterns and histamine releasing capacity of the proteins were maintained up to 2 hours post digestion. The increased pH was a result of antacid medication leading to incomplete digestion and rapid uptake of the allergens.

5.1 Patterns of clinical cross reactivity and tolerance across fish species in fish allergic individuals

Determining true clinical cross sensitivity between fish species in fish allergic individuals is problematic, particularly when using current standard IgE testing. *In vitro or* SPT cross reactivity is not always predictive of *in vivo* cross sensitization and actual clinical allergy. In South Africa, a study of 10 fish allergic patients using five commonly consumed fish, pilchard, anchovy, hake, snoek, and yellowtail, reported pilchard PV as being the major cross-reactive allergen using different *in vitro* tests [78]. Serum from 10 fish allergic patients in Norway demonstrated cross-reactivity amongst 9 different commonly consumed fish species [39]. Those individuals sensitive to cod were also sensitized to salmon, whereas Halibut, flounder, tuna and mackerel were found to be the least cross-reactive. A Japanese study of 43 fish species, consisting of both bony and cartilaginous fish, used sera from 38 fish allergic patients and, utilizing radioallergosorbent test (RAST), reported low IgE reactivity to smoothhounds, spotted smoothhounds, blue sharks and salmon sharks [46]. Halibut was also shown to have low IgE reactivity, whereas, whiting, mackerel, saurel, Japanese sardine, silver salmon and bigeye tuna, all commonly consumed fish in Japan, were reported to have high IgE-reactivity within this cohort.

In a single case of anaphylaxis to dogfish, (a type of shark), the patient was tolerant to horse mackerel, hake, rays and mollusks [79]. A 14 kDa protein was detected although the biochemical, immunological properties and lineage of the allergen was not confirmed. This may be explained by the fact that Chondrichthyes are likely to have low levels of expression of allergenic fast muscle switching β-PV and more non-allergenic α-PV above [27, 80].

Allergies to cartilaginous fish are rarely reported, possible due to their low incidence, despite the fact that they are commonly consumed. Although not from fish, in a single case of near fatal anaphylaxis to frog's legs [81], the causative allergen was identified as α-PV. In a separate

study, 15 fish allergic patients also cross-reacted, *in vitro*, to PV from frog [82]. While cross-reactions to both α and β-PV were observed, β-PV demonstrated significantly more IgE-binding than α-PV in serum from 11 fish allergic patients. With 60-70% amino acid homology between bony fish β-PV and frog β-PV, this potentially explains the high degree of cross-reactivity between the two proteins [82]. In comparison, only 45-54% amino acid identity was seen between α-PV from frog and fish PV.

In a Japanese cohort (with fish-allergy based upon questionnaire) [68], PV and collagen were identified as highly cross-reactive fish pan-allergens and causative factors of fish allergy [83]. Sera from 16 patients with fish parvalbumin or collagen allergy were used to analyze IgE reactivities and cross-reactivities using ELISA and inhibition ELISA in 26 fish species. IgE reactivities of extracts from 22 species were extensively variable as determined by ELISA using sera obtained from patients with fish PV specific allergies. These patients showed little to no reactivity to mackerel and salmon extracts whereas all patients with fish collagen-specific allergies indicated IgE cross-reactivity to 22 types of fish. Collagen specific allergic patients strongly reacted to rainbow trout, Atlantic salmon, skipjack, and bigeye tuna extracts. In contrast, patients with fish parvalbumin-specific allergies reacted weakly to the same fish species. Fish collagen was indicative of a panallergen, in addition PV, when all 26 species of fish were found to be cross-reactive with Pacific mackerel collagen.

5.2 Occupational exposure

Workplace related fish allergies are of major concern. Inhalation of aerosolized allergens is the common exposure route, as reported previously in large occupational and industrial processing venues [60, 84-87]. Anaphylaxis is rare, but dermatitis and respiratory conditions have been commonly observed upon inhalation of aerosolized allergens [73]. Although occupational allergy has also been extensively studied in several countries [6, 85, 86, 88-94], there are very few reports of the specific proteins, which drive this occupational fish sensitization.

Two separate case reports of fatal occupational asthma in adult males have been attributed to "dust" inhalation from powdered shark cartilage [54, 55]. Shark cartilage dust has also been

implicated as an asthma inducing agent in the USA [55] [54]. In these reports, the specific causative allergens were not identified, but shark cartilage has been shown to contain several proteins, including collagen.

As little as 30ng/m^3 of aerosolized fish antigen is reported to cause allergic asthmatic reactions [91]. In a study of workplace exposure to saltwater bony fish (pilchard and anchovy), 7% of workers reported allergic symptoms - including 2.6% with occupational allergic rhino-conjunctivitis and 1.8% with asthma [86]. In a salmon processing plant where workers were exposed to up to 75ng/m^3 of aerosolized allergen Sal s 1 [85], 50% of workers developed respiratory symptoms after commencement of employment.

7. Diagnosis of fish allergy

The exact identity of the type/species of fish consumed by individuals experiencing fish allergy is often unknown and as a result, it is often difficult to determine which specific fish allergen/s are responsible for the allergy in any individual fish allergic patient. *In vivo* skin prick testing (SPT) is an inexpensive, rapid and relatively safe means of screening patients with IgE-mediated fish allergy. One limitation of this technique is the possibility of clinically irrelevant *in vivo* cross-sensitization to other fish allergens, resulting in false-positive results- especially in patients with poor clinical history [95]. Additionally for many fish species, particularly outside Europe, there are no commercial SPT preparations available, so clinicians are reliant on fresh prick-to-prick testing using the candidate raw fresh fish.

Likewise, serum specific IgE (ssIgE) and other qualitative *in vitro* assays are also good indicators of IgE-sensitization, but their presence does not necessarily correlate with clinical reactivity. For a few fish species, positive predictive values (PPV) for clinical reactivity are available, such that individuals with a very high ssIgE above the 95% PPV can be relatively confidently diagnosed as being allergic to this specific food. In general however, the negative predictive value of fish ssIgE, and the ability to distinguish clinical reactivity and sensitization with levels below the 95% PPV is, poor. For cod fish allergy, IgE levels of 20 kU/l [>95% confidence] have been used to predict clinical reactivity.

The amount of fish ingested may be important for some individuals with fish allergy. Moreover important fish pan-allergens may vary in concentration between fish. However, information on threshold doses and lowest observed adverse effect level (LOAEL) values for triggering of fish related allergic reactions is limited. The fish ED10 based upon data from the EuroPrevall cohort [eliciting dose to which 10% of an allergic population would be expected to first clinically react] is estimated to be 27 mg [96]. A LOAEL of 5 mg for herring or cod was estimated as the lowest dose of fish required to elicit a clinical response, using results of DBPCFC from 14 fish allergic patients [97]. Other factors may influence thresholds, such as impaired gastric digestion. Codfish proteins digested in hypo-acidic conditions (pH 3.0) were demonstrated to reduce the tolerance levels between 10 to 30 times as compared to digestion at pH 2.0. Lower dosage levels of less than 3 mg were demonstrated to trigger allergic reactions [77].

A study from South Africa highlighted the importance of food challenges in diagnosis of fish allergy by using DBPCFC to confirm or refute suspected species-specific fish allergy [98]. In a cohort of 105 patients with perceived or a clinical history of fish allergy, reported allergy was most common to hake (24%), yellowtail (21.9%), salmon (15.2%) and mackerel (15.2%). The patients were subject to SPTs and DBPCFCs; ssIgE and Western blots were performed. Species-specific IgE-mediated fish allergy was confirmed in one patient and refuted in six patients by food challenge.

Avoidance of the causative food is the primary means of management of food allergy. Accurate and extensive labeling of food and conscientious reading of labels is important to prevent ingestion of allergens. Accidental ingestion is still relatively common due to factors such as misleading labels, cross-contamination during processing and preparation equipment processing different foods or unknown allergens hidden in foods. A decision tree has been suggested to manage fish allergy in a stringent manner that also takes into account adverse parasitic and toxin reactions [1].

A recent review highlights the progression of detecting and quantifying allergens in food products, including fish, using mass spectroscopy (MS) [99]. Antibody based methods, such as ELISA, currently used for the detection and quantification have their limitations, including under- and overestimation of allergenic proteins as well as detection of allergens in very small

quantities. More than 600 different food allergens are currently known. This extensive variety has led to the development of more robust, reliable and comparable methods such as mass spectrometry, which has a better resolution with respect to the limits of detection and quantification. A challenge that currently persists is the absence of commercial methods to test the presence of specific fish species in food which consequently makes the management of fish allergy, with respect to specific fish species, very challenging.

8. Novel therapeutics

Current management of fish allergy relies upon allergen avoidance; management plans for accidental exposure, and in many cases, provision of adrenaline auto injectors. However, advances in the understanding of the molecular characteristics of fish allergens [24, 41, 73, 100, 101] are leading to novel approaches. Current approaches include targeting the calcium binding, muscle protein PV [102]. Recombinant hypoallergenic carp-PV is currently under investigation for potential subcutaneous (and potentially other routes) immunotherapy [103]. Sera from 26 proven fish allergic patients (by DBPCFC) were used to show 10-5,000 fold reduction in IgE binding to this hypoallergenic PV, while retaining immunogenicity [as confirmed in a murine model]. Currently, clinical trials are in progress and the efficacy of this hypoallergenic protein has yet to be established [104] (ClinicalTrials.gov identifier: NCT02017626).

There are currently no published reports of oral or sublingual fish sensitization trials. However, as oral and sublingual immunotherapy regimes for other food allergies such as cow's milk, egg and peanut are refined, with improved efficacy and long term tolerance; it is likely that oral fish immunotherapy, with modified allergen or with adjuvant will eventually be available.

9. Conclusion

The consequences of fish allergy for adults and children are significant, and immunotherapeutic approaches to induce desensitization or sustained tolerance to fish allergens are currently only

in early phase development. PV has been demonstrated as the major allergenic protein in fish. The high degree of PV amino acid sequence variability between different fish species and lineages seems to be central to clinical sensitization. The molecular phylogenetic similarity among beta-PVs of bony fish best explains the high allergenicity across species and groups and low clinical cross-reactivity to the alpha-lineage of PV, present in most cartilaginous and ancient fish species. Consequently the distance of amino acid sequence homology between different PVs could be the ultimate indicator for clinical-cross-reactivity. A better understanding of the molecular, immunological and biochemical characteristics of fish allergens is required to aid the development of better clinical diagnostics and patient management, food processing technologies, allergen detection methods as well as novel immunotherapeutics.

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Figure Legends:

Figure 1: A) Parvalbumin amino acid sequence alignment of bony fish species (Baltic Cod, Common carp, Atlantic salmon α and β, Atlantic mackerel), Coelacanth (isoforms α and β) and

672 cartilaginous fish species (Leopard shark, Thornback ray, Australian ghost shark) generated in
673 Clustal Omega. Highly conserved amino acids are shaded dark blue. Note: Ca^{2+} binding sites are
674 boxed in red. (B) Conservation analysis shows degree of conservation of amino acids among the
675 various different fish. The scores range from 1-10, 1 (dark brown) being least conserved amino
676 acid to 10 (yellow) being 100% conservation across all the fish species analyzed.
677
678 Figure 2: Unrooted neighbor joining dendrogram showing evolutionary relationships of
679 parvalbumin from vertebrates constructed in MEGA5. (A) α-parvalbumins. (B) β-parvalbumins.
680 The tree is drawn to scale. The evolutionary distances were computed using the Poisson
681 correction method. Amino acid sequences were obtained from UniProt.
682
683 Figure 3: A) Amino acid sequence alignment of Atlantic cod (UniprotKB Accession number:
684 A5I874), Baltic cod (UniprotKB Accession number: P02622), common carp (UniprotKB Accession
685 number: E0WD92), Atlantic salmon (UniprotKB Accession number: B5DH15) and chub mackerel
686 (UniprotKB Accession number: P59747) showing known IgE-binding regions coloured in yellow,
687 orange, blue, purple and green. (B) Space filling parvalbumin models of common carp, Chub
688 mackerel, Atlantic salmon, Atlantic cod and Baltic cod. The corresponding coloured IgE binding
689 epitopes from (A) are depicted on the space filling models.
690
691

692 **Table 1: Epidemiological studies on fish allergy across different continents. Report: based on symptoms. Information provided by**
693 **parents or self-reported. Patient history: symptoms occurring in less than 2 hours. SPT: skin prick test. OFC: oral food challenge.**
694 **DBPCFC: double blind placebo controlled food challenge. FE: Food elimination.**

Country	Age of individuals	Number of individuals	Sensitization to fish (%)	Methods of confirmation	References
ASIA					
China	0-2	1,604	0.21	Report, SPT, FE, DBPCFC	[105]
Hong Kong	2-7	3,677	0.25	Report, Physician-diagnosis	[106]
Korea	<1	1,177	-	Telephone interview, patient history	[107]
Philippines	14-16	13,989	2.29	Patient history	[8]
Singapore	14-16	9,570	0.26	Patient history	[9]
Taiwan	<3 4-18 Adults	813 15,169 14,036	0.49 0.49 1.17	Patient history, SPT, Serum IgE levels, FC	[108]
Thailand	3-7 14-16	656 2,536	0.22 0.29	Report, SPT, OFC, Serum IgE levels, patient history	[9] [8]
EUROPE					

	3	486	-	Report, SPT, Serum IgE levels, Histamine release, OFC	[109]
Denmark	Adults	936	0.2		[109]
	22	1,282	0.1		[13]
Norway	0-2	3623	3.0	Report	[12]
Sweden	0-4	2,614	0.69	Report, Serum IgE levels	[110]
	0-3	891	-		[111]
UK	11	775	1.16	Report, SPT, OFC, DBPCFC	[112]
	15	757	1.19		[113]
AFRICA					
South Africa	Adults	594	7.0	Report, SPT, Serum IgE levels, determination of omega-3 fatty acids, Spirometry, MCT	[86]
NORTH AMERICA					
Canada	Adults	9,667	0.10	Telephone interview, Report, Patient history, Physician-diagnosis, SPT, IgE levels, OFC	[21]
	0-2	5,429	0.30		[114]
	6-10	9,911	0.50		[114]
USA	14-18	10,514	0.60	Report, Physician-diagnosis,	[114]
	0-17	2,707	0.22	SPT, Serum IgE levels, OFC	[20]
	18-61	9,816	0.48		[20]

OCEANIA					
Australia	2-5	154	62	Report, SPT, OFC	[18]
	3-8	94	-		[19]

695

696

697 **Table 2: Selection of allergenic parvalbumins in muscle-tissue of bony and cartilaginous fish, listed in ascending order of**
698 **discovery. Common and scientific names of species, their molecular weight in kDa and the isoform lineage have been listed. For**
699 **cartilaginous fish (A) or (B) identifies the species belonging to the Elasmobranchii (A) or Holocephali (B), respectively.**

Common Name	Scientific Name	Isoform Lineage	Isoelectric Point	Molecular Weight (kDa)	References
OSTEICHTHYES (BONY FISH)					
West Indian Ocean coelacanth	*Latimeria chalumnae*	α or β	4.93 & 4.68	12.2 & 11.732	[115]
Baltic cod	*Gadus callarias*	β	4.37	12.1	[116]
Atlantic salmon	*Salmo salar*	β	4.95	11.9	[117]
Carp	*Cyprinus carpio*	β	4.25	11.5	[113]
Atlantic cod	*Gadus morhua*	β	4.56	11.5	[118]
Chub mackerel	*Scomber japonicus*	β	4.64	11.5	[119]
Alaska pollock	*Theragra chalcogramma*	β	4.60	11.5	[120]
Barramundi	*Lates calcarifer*	β1, β2	4.48	11.6	[49]

Pacific pilchard	*Sardinops sagax*	β	6.07	11.9	[78]
Whiff	*Lepidorhombus whiffiagonis*	β	4.50	11.7	[80]
Swordfish	*Xiphias gladius*	β	4.43	11.5	[80]
CHONDRICHTHYES (CARTILAGINOUS FISH)					
Thornback ray (A)	*Raja clavata*	α or β	4.45	11.8	[121]
Leopard shark (A)	*Triakis semifasciata*	α	5.14	12	[25]
Common dogfish (A)	*Scyliothinus canicula*	-	-	-	[122]
Atlantic stingray (A)	*Dasyatis sabina*	α or β	4.95 & 5.02	12.2 & 12	[50]
Red stingray (A)	*Dasyatis akajei*	α or β	~5	12.3 & 12	[26]
Australian ghost shark (B)	*Callorhinchus milii*	α	5.04	12.1	[123]

Table 3: Comparison of amino acid sequence identities of fish alpha- and some beta-parvalbumin calculated using ClustalW. The fish species have been grouped into Chondrichthyes (red) and Osteichthyes (blue) (Values are shown in percentage). Sequences obtained from UniProt.

	Leopard shark α	Thornback ray α	Australian ghost shark α	Baltic cod β	Common carp β	Atlantic salmon α	Atlantic salmon β	Chub mackerel β	West Indian Ocean coelacanth α	West Indian Ocean coelacanth β	Human α
Leopard shark α	**100**										
Thornback ray α	49	**100**									
Australian ghost shark α	46	48	**100**								
Baltic cod β	46	43	47	**100**							
Common carp β	50	51	56	66	**100**						
Atlantic salmon α	55	49	62	54	61	**100**					
Atlantic salmon β	48	46	49	51	70	60	**100**				
Chubb mackerel β	52	56	52	61	79	57	64	**100**			
West Indian Ocean coelacanth α	62	50	58	50	57	59	48	58	**100**		
West Indian Ocean coelacanth β	47	55	53	54	70	49	58	63	53	**100**	
Human α	58	47	67	49	58	67	50	51	57	51	**100**

Figures: Allergenicity of bony and cartilaginous fish – molecular and immunological properties

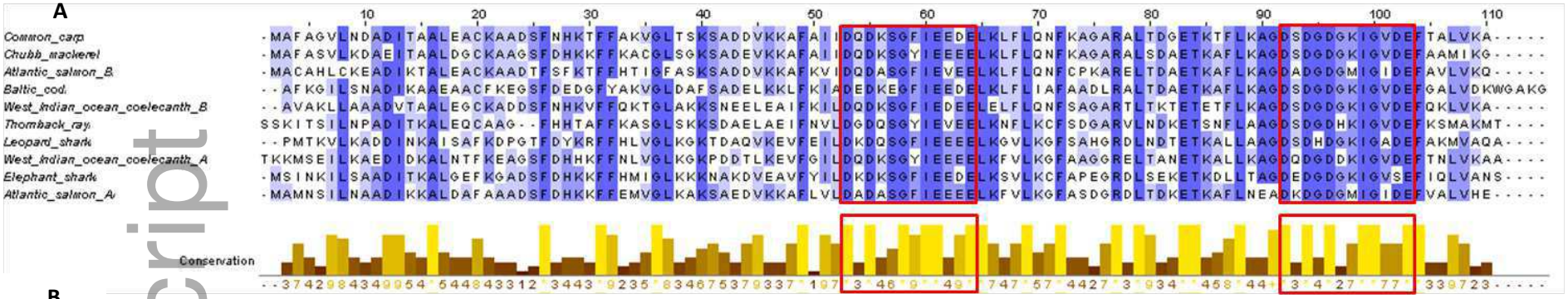

Figure 1

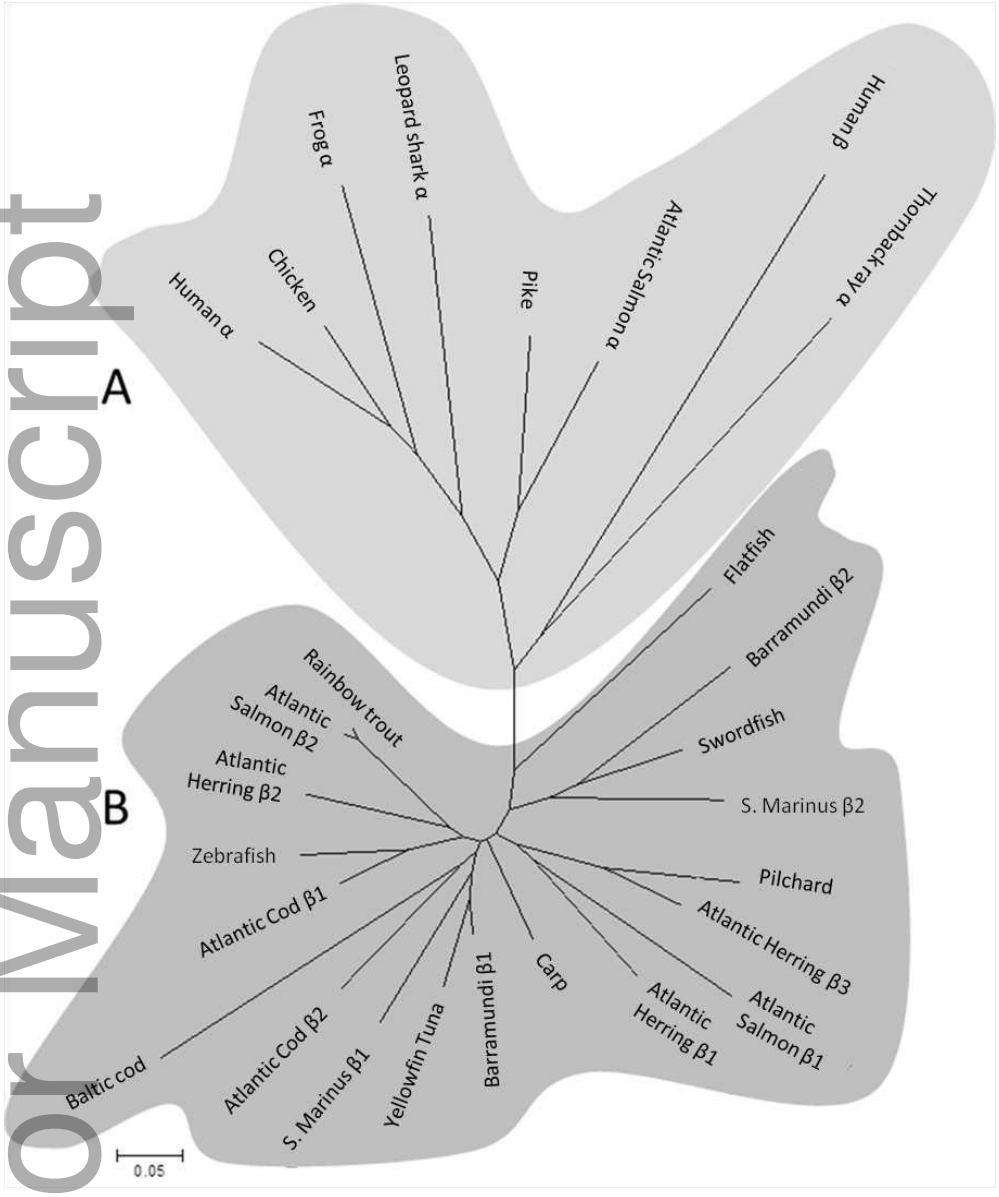

Figure 2

A

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Chub_mackerel     MAFASVLKDAEVTAALDGCKAAGSFDHKKFFKACGLSGKSTDEVKKAFAIIDQDKSGFIE
Atlantic_salmon   MACAHLCKEADIKTALEACKAADTFSFKTFFHTIGFASKSADDVKKAFKVIDQDASGFIE
Carp              MAFAGVLNDADITAALEACKAADSFNHKTFFAKVGLTSKSADDVKKAFAIIDQDKSGFIE
Atlantic_cod      MAFAGILNDADITAALAACKAEGSFDHKAFFTKVGLAAKSPADIKKVFEIIDQDKSDFVE
Baltic_cod        -AFKGILSNADIKAAEAACFKEGSFDEDGFYAKVGLDAFSADELKKLFKIADEDKEGFIE
                                                                  <-- Ca2+

Chub_mackerel     EEELKLFLQNFKAGARALSDAETKAFLKAGDSDGDGKIGIDEFAAMIKG
Atlantic_salmon   VEELKLFLQNFCPKARELTDAETKAFLKAGDADGDGMIGIDEFAVLVKQ
Carp              EDELKLFLQNFKAGARALTDGETKTFLKAGDSDGDGKIGVDEFTALVKA
Atlantic_cod      EDELKLFLQNFSAGARALSDAETKVFLKAGDSDGDGKIGVDEFGAMIKA
Baltic_cod        EDELKLFLIAFAADLRALTDAETKAFLKAGDSDGDGKIGVDEFGALVDKWGAKG
                  -->                  <-- Ca2+
```

B

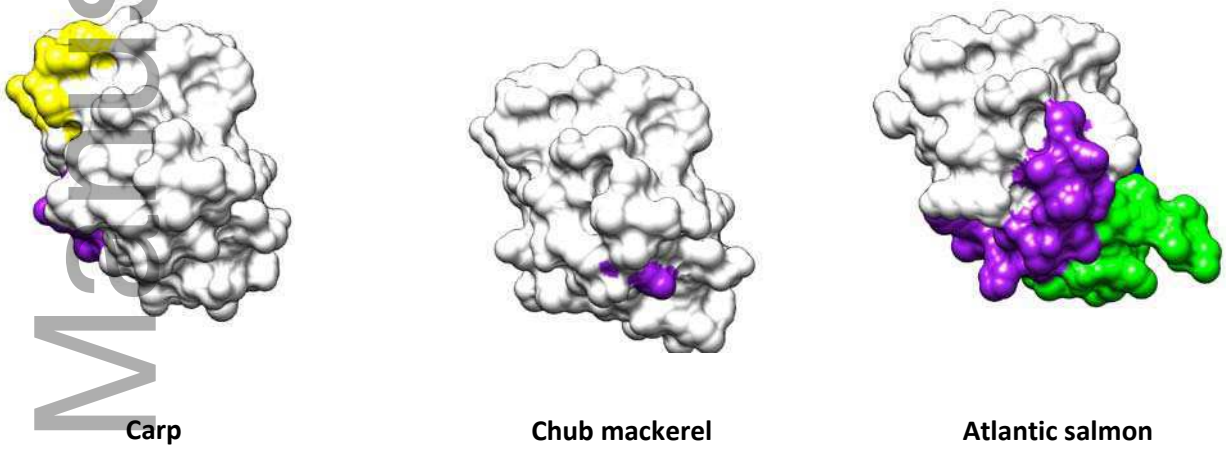

Figure 3