

# 1 Allergenicity of bony and cartilaginous fish – molecular and 2 immunological properties

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4

## 5 **Abstract:**

6 Allergy to bony fish is common and probably increasing worldwide. The major heat stable pan-  
7 fish allergen, parvalbumin (PV), has been identified and characterized for numerous fish  
8 species. In contrast, there are very few reports of allergic reactions to cartilaginous fish despite  
9 widespread consumption. The molecular basis for this seemingly low clinical cross-reactivity  
10 between these two fish groups has not been elucidated. PV consists of two distinct protein  
11 lineages,  $\alpha$  and  $\beta$ . The  $\alpha$ -lineage of this protein is predominant in muscle tissue of cartilaginous  
12 fish (Chondrichthyes), while  $\beta$ -PV is abundant in muscle tissue of bony fish (Osteichthyes). The  
13 low incidence of allergic reactions to ingested rays and sharks is likely due to the lack of  
14 molecular similarity, resulting in reduced immunological cross-reactivity between the two PV  
15 lineages. Structurally and physiologically both protein lineages are very similar, however the  
16 amino acid homology is very low with 47% to 54%. Furthermore, PV from ancient fish species  
17 such as the coelacanth demonstrates 62% sequence homology to leopard shark  $\alpha$ -PV and 70%  
18 to carp  $\beta$ -PV. This indicates the extent of conservation of the PV isoforms lineages across  
19 millennia. This review highlights prevalence data on fish allergy and sensitization to fish, and  
20 details the molecular diversity of the two protein lineages of the major fish allergen PV among  
21 different fish groups, emphasizing the immunological and clinical differences in allergenicity.

## 22 **1. Introduction:**

23 Allergy to seafood is common, potentially life threatening, often lifelong and accounts for up to  
24 5% of food allergies in children and 2% of food allergy in adults worldwide [1, 2]. There are  
25 major regional differences, with apparent much higher rates of sensitization to fish in Asia. The  
26 move towards healthy lifestyles and eating habits has seen a steady increase in the  
27 consumption of fish, which suggests that seafood allergy will continue to be a major health

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

31 The major allergen identified in fish, parvalbumin (PV), binds calcium, is heat stable and is  
32 involved in the muscle relaxation and contraction cycle [3] as well as signal transduction [4]. PV  
33 is reported to account for up to 90% of cross-reactive allergic immunological reactions to fish  
34 [1, 2]. There are two distinct lineages of PV,  $\alpha$  &  $\beta$  [5]. While most fish contain both lineages of  
35 PV,  $\beta$ -PV is predominantly reported as major allergen in bony fish. Reported allergic  
36 sensitization to  $\alpha$ -PV are limited. In this review, the prevalence of fish allergy and clinical  
37 implication of allergenicity of various fish species are explored. The two lineages of PV are  
38 extensively detailed at the molecular level to emphasize the immunological and clinical impacts  
39 of this two diverse allergen lineages.

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## 43 **2. Fish allergy epidemiology: How common is it?**

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

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

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

62 While prevalent across Asia, fish allergy also appears to be relatively common in Europe (Table  
63 1), again with significant sub-regional variation. Self-reported prevalence of fish allergy is as  
64 high as 3% [12], while the incidence of challenge proven fish allergy challenge is reported to be  
65 only 0.1% [13]. Across European cities, approximately 0.2% of the population is reported to be  
66 allergic to some type of fish [14]. The populations of Portugal, Spain and Scandinavian are likely  
67 to be the highest consumers of fish in Europe [15] and the highest rates of fish allergy are also  
68 generally reported in Northern Europe [16]. In a Norwegian prospective observational cohort of  
69 3,623 children [12]- 3% were reported to have fish allergy by 2 years of age . Early onset of fish  
70 allergy was also described in a cohort of Spanish food allergic children who had allergy to cod,  
71 tuna, hake and whiff predominantly before the age of 2 years [17].

72 In Australia, 5.6% of a large cohort of food allergic children were identified as having fish  
73 allergy, with the most commonly implicated fish being tuna, salmon, barramundi and flathead  
74 [18]. Of those with a fish allergy, 20% had a history of fish-related anaphylaxis. Clinical and  
75 allergy skin test (SPT) cross-sensitivity between the fish species was also common within this  
76 fish allergic cohort, with 93% being atopic to at least one other fish type on SPT and 29% with a  
77 previous clinical allergic reaction to another species of fish. In a separate Australian cohort  
78 study (n=94) of children with proven seafood allergy, 64% of the cohort were allergic to fish  
79 [19], with 'white fish', salmon and tuna being most commonly implicated species and allergic  
80 reactions to basa [catfish], barramundi, bream, and cod all reported.

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

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

### 88 **3. Classification of cartilaginous and bony fish**

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

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

110

### 111 **4. Fish Allergens**

#### 112 **4.1 Parvalbumin**

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

123 Chemistry: PV is an acidic, sarcoplasmic, 10 - 15 kDa protein that is extremely resistant to heat  
124 [27], proteolytic and chemical degradation [28, 29]. It was first identified as an allergen in Baltic  
125 cod (*Gadus morhua*) in 1969 and named Gad c 1 or Allergen M [29]. The isoelectric points range  
126 from 3.9 – 4.5 for the  $\beta$ -PVs, whereas it is generally above 5.0 for different  $\alpha$ -PVs [30].

127 PV's are globular in shape and contain six  $\alpha$ -helices (A, B, C, D, E, and F). These helices form  
128 three helix-loop-helix EF hand motifs (AB, CD and EF). The heat stability and resistance to  
129 protease degradation is attributed to chelated calcium which changes the conformation of PV  
130 [31]. As a consequence of conformational changes, the allergenicity of PV is also higher in the  
131 presence of calcium due to better exposure of IgE binding epitopes [2, 32]. The calcium-binding  
132 epitopes in PV are well conserved across bony and cartilaginous fish (Figure 1A and 1B),  
133 demonstrating the similar functionality of this protein.

134 Characterization: PV has been extensively studied in bony fish including in Atlantic cod (*Gadus*  
135 *morhua*) [28, 29, 33-37], Alaska Pollack (*Theragra chalcogramma*) [38, 39], common carp  
136 (*Cyprinus carpio*) [24, 30, 37, 40, 41], silver carp (*Hypophthalmichthys molitrix*) [42], Atlantic  
137 salmon (*Salmo salar*) [39, 43-47] and recently Asian seabass (*Lates calcarifer*) [48, 49]. In  
138 comparison, studies on PV from cartilaginous fish are limited to leopard shark [25], red stingray  
139 [26], Atlantic stingray [50] and thornback ray [51].  $\beta$ -parvalbumin is more abundant in bony fish

140 and appears to be responsible for the majority of allergic reactions, while the  $\alpha$  isoform seems  
141 to be predominant in cartilaginous fish [25, 26]. Some fish species seem also to express multiple  
142 isoforms of  $\beta$ -PV such as  $\beta$ 1 and  $\beta$ 2 seen in barramundi and isoforms 1 to 9 in zebrafish [48, 52].

143 Differences between  $\alpha$ - and  $\beta$ -PV: There appears to be low clinical cross-reactivity between  $\alpha$ -  
144 and  $\beta$ -PV of the two fish classes (bony and cartilaginous) as evident from the lack of detailed  
145 published reports of individuals being allergic to both [53-55]; however the reasons for this low  
146 cross reactivity are poorly understood. One of the key differences between the lineages is the  
147 presence of more acidic amino acid residues with pI 4.5 or lower in  $\beta$ -PV, while  $\alpha$ -PV consists of  
148 less acidic amino acid residues with pI 5.0 or higher [26]. The  $\alpha$  and  $\beta$  lineages also differ slightly  
149 in length, with  $\alpha$ -PV usually consisting of 109 or more amino acids while  $\beta$ -PV generally have  
150 108 or fewer amino acids [31]. This extensive molecular variation may be the key to  
151 understanding why the  $\alpha$ -PV predominant fish are less allergenic and the limited clinical cross-  
152 reactivity observed between different fish species and groups.

153 Leopard shark  $\alpha$ -PV was the first shark PV to have its crystal structure studied [25]. The amino  
154 acid sequence homology of PV from one shark and several bony fish [carp, cod, salmon] is less  
155 than 50% (Table 3). Interestingly, bony fish PV's demonstrate relatively high sequence identity  
156 with muscle from amphibians (63-76%), reptiles (56-69%) and birds (54-71%), whereas human  
157 muscles are devoid of  $\beta$ -PV with only 56% sequence homology between human  $\alpha$ -PV and fish  $\beta$ -  
158 PV. The homological distance is an indicator of the differential allergenicity of the two isoform  
159 lineages [56], with evolutionary divergence of  $\alpha$  and  $\beta$ -PV lineages in vertebrates (Figure 2).  
160 Interestingly, PV amino acid sequences ( $\alpha$  and  $\beta$ ) for the West Indian Ocean coelacanth  
161 (UniProtKB ID: P02629.1 and UniProtKB ID: P02623.1) have high amino acid identity (62% and  
162 70%) to leopard shark  $\alpha$ -PV but also carp  $\beta$ -PV, respectively (Table 3). Here, the degree of  
163 sequence identity is a good indicator of the extent of molecular conservation of PV across a vast  
164 time period.

165

166 4.2 IgE binding epitopes and clinical cross-reactivity

167 Elucidation of the PV IgE binding epitopes is necessary to understand allergen-antibody  
168 interactions which result in clinical reactivity. IgE binding epitopes of PV from five fish, Atlantic  
169 cod (Gad m 1), Baltic cod (Gad c 1), common carp (Cyp c 1), chub mackerel (Sco j 1) and Atlantic  
170 salmon (Sal s 1), have been elucidated in detail (Figure 3A). Epitopes for Atlantic cod, Atlantic  
171 salmon and chub mackerel have been individually mapped using overlapping peptides of PV  
172 and analyzed using allergic patient's serum IgE [44, 57, 58]. The epitopes for Baltic cod were  
173 elucidated via mutated derivatives of PV and immunological reactivity of overlapping tryptic  
174 peptides [34]. Carp PV epitopes have been identified using patient sera by screening against a  
175 decamer phage library, computational matching and alignment techniques [32]. While PV from  
176 Atlantic cod, Baltic cod, common carp, chub mackerel and Atlantic salmon differs in amino acid  
177 sequences, they seem to share some of the linear antibody binding epitopes. Currently there is  
178 very little information available about conformational IgE binding epitopes, but it is very likely  
179 that they are very different from each other. A comparative study by Kobayashi et al [59]  
180 demonstrated the importance of stereoscopic conformation for IgE binding in a range of  
181 different PVs, with a structural dependence on Ca<sup>2+</sup> binding.

182 Fish allergic patients generate a variety of epitope specific IgE which have been used in epitope  
183 mapping for five fish species including cod, salmon, carp and mackerel. In Gad c1 (cod) there  
184 are five regions reported to include IgE-binding epitopes; residues 13-32 in the AB domain, 33-  
185 44 on the region joining the AB and CD domains, 49-64 in the calcium-binding region in the CD  
186 domain, 65-74 in the region joining CD and EF domains and the 88-96 calcium binding region in  
187 the EF domain [34]. Three additional epitopes were later identified by mapping mimotopes  
188 onto the molecular surface of natural carp PV via a surface-matching algorithm [32]. While two  
189 epitopes were found within regions joining the domains AB and CD, and CD and EF, the third  
190 epitope was found in the calcium-binding loop of the EF domain. The carp  $\beta$ -PV model has been  
191 used to specify the known IgE binding epitopes (Figure 3B). Epitope alignment has shown that  
192 region IV is highly antigenic, while region I seems to be more species-specific (which is  
193 implicated especially in the case of clinical mono-sensitivity to salmon) and perhaps several  
194 species in the group of salmonidae [1].

195

196 4.3 Other fish allergens

197 Collagen, vitellogenin, fructose biphosphate-aldolase and beta-enolase [45, 60-65] have been  
198 reported as potentially important allergens in fish. The first instance of collagen as a putative  
199 fish allergen was reported in bigeye tuna [66] where collagen was shown to elicit IgE-reactivity  
200 in five of eight tuna-allergic patients. Here, an inhibition ELISA [enzyme linked immunosorbent  
201 assay] strongly implicated the allergenicity of collagen being independent of fish species.  
202 Additionally, the importance of collagen as a pan-allergen has been demonstrated recently [67,  
203 68]. In a cohort of 36 Japanese fish (raw and cooked) allergic patients, 50% were found to have  
204 IgE to mackerel collagen. Allergenicity of fish collagen from cartilaginous fish has recently been  
205 demonstrated to be lower than that of bony fish collagen [67]. In a separate study,  $\alpha$  and  $\beta$ -  
206 chain collagen (120 and 240 kDa, respectively) were identified as allergens in two species of  
207 tuna [albacore and yellowfin] [69]. The hormone vitellogenin, from Beluga caviar and salmon  
208 roe, has been shown to cause IgE-reactivity [45, 65]. Fish allergic patients have also been  
209 reported to produce IgE to fish gelatin, a type I collagen [62], which has been reported to cause  
210 anaphylaxis [64].

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

217

218 5. Changes in allergenicity related to exposure

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



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

227

### 228 5.1 Patterns of clinical cross reactivity and tolerance across fish species in fish allergic individuals

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

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

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

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

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

## 269 5.2 Occupational exposure

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

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

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

281 As little as 30ng/m<sup>3</sup> of aerosolized fish antigen is reported to cause allergic asthmatic reactions  
282 [91]. In a study of workplace exposure to saltwater bony fish (pilchard and anchovy), 7% of  
283 workers reported allergic symptoms - including 2.6% with occupational allergic rhino-  
284 conjunctivitis and 1.8% with asthma [86]. In a salmon processing plant where workers were  
285 exposed to up to 75ng/m<sup>3</sup> of aerosolized allergen Sal s 1 [85], 50% of workers developed  
286 respiratory symptoms after commencement of employment.

287

## 288 **7. Diagnosis of fish allergy**

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

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

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

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

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

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

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

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343

#### 344 **8. Novel therapeutics**

345 Current management of fish allergy relies upon allergen avoidance; management plans for  
346 accidental exposure, and in many cases, provision of adrenaline auto injectors. However,  
347 advances in the understanding of the molecular characteristics of fish allergens [24, 41, 73, 100,  
348 101] are leading to novel approaches. Current approaches include targeting the calcium  
349 binding, muscle protein PV [102]. Recombinant hypoallergenic carp-PV is currently under  
350 investigation for potential subcutaneous (and potentially other routes) immunotherapy [103].  
351 Sera from 26 proven fish allergic patients (by DBPCFC) were used to show 10-5,000 fold  
352 reduction in IgE binding to this hypoallergenic PV, while retaining immunogenicity [as  
353 confirmed in a murine model]. Currently, clinical trials are in progress and the efficacy of this  
354 hypoallergenic protein has yet to be established [104] (ClinicalTrials.gov identifier:  
355 NCT02017626).

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

360

#### 361 **9. Conclusion**

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

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

374

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377

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669 **Figure Legends:**

670 Figure 1: A) Parvalbumin amino acid sequence alignment of bony fish species (Baltic Cod,  
671 Common carp, Atlantic salmon  $\alpha$  and  $\beta$ , Atlantic mackerel), Coelacanth (isoforms  $\alpha$  and  $\beta$ ) 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<sup>2+</sup> 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.

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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.

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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
<b><u>ASIA</u></b>					
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	813	0.49	Patient history, SPT, Serum IgE levels, FC	[108]
	4-18	15,169	0.49		
Thailand	Adults	14,036	1.17	Report, SPT, OFC, Serum IgE levels, patient history	[9]
	3-7	656	0.22		
	14-16	2,536	0.29		[8]
<b><u>EUROPE</u></b>					

	3	486	-		[109]
Denmark	Adults	936	0.2	Report, SPT, Serum IgE levels,	[109]
	22	1,282	0.1	Histamine release, OFC	[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]
<b><u>AFRICA</u></b>					
South Africa	Adults	594	7.0	Report, SPT, Serum IgE levels, determination of omega-3 fatty acids, Spirometry, MCT	[86]
<b><u>NORTH AMERICA</u></b>					
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]

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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
<b><u>OSTEICHTHYES (BONY FISH)</u></b>					
West Indian Ocean coelacanth	<i>Latimeria chalumnae</i>	$\alpha$ or $\beta$	4.93 & 4.68	12.2 & 11.732	[115]
Baltic cod	<i>Gadus callarias</i>	$\beta$	4.37	12.1	[116]
Atlantic salmon	<i>Salmo salar</i>	$\beta$	4.95	11.9	[117]
Carp	<i>Cyprinus carpio</i>	$\beta$	4.25	11.5	[113]
Atlantic cod	<i>Gadus morhua</i>	$\beta$	4.56	11.5	[118]
Chub mackerel	<i>Scomber japonicus</i>	$\beta$	4.64	11.5	[119]
Alaska pollock	<i>Theragra chalcogramma</i>	$\beta$	4.60	11.5	[120]
Barramundi	<i>Lates calcarifer</i>	$\beta$ 1, $\beta$ 2	4.48	11.6	[49]

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

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

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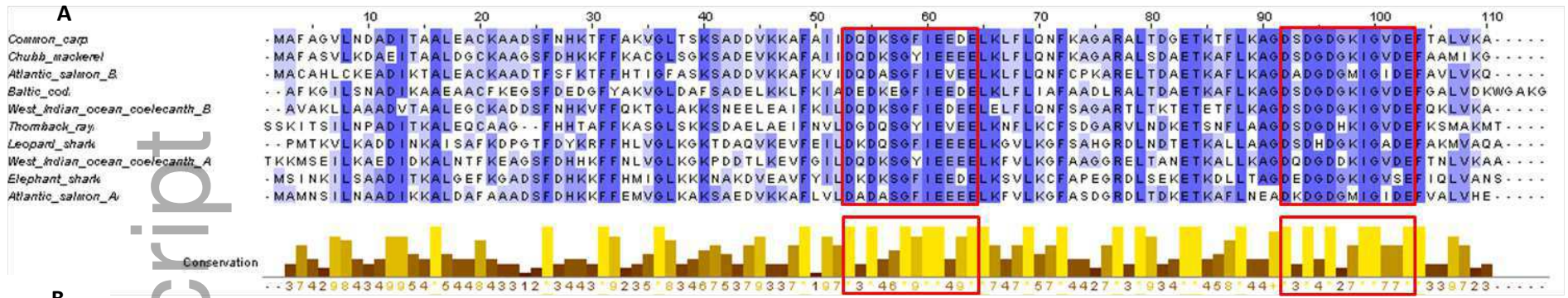
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	Leopard shark $\alpha$	Thornback ray $\alpha$	Australian ghost shark $\alpha$	Baltic cod $\beta$	Common carp $\beta$	Atlantic salmon $\alpha$	Atlantic salmon $\beta$	Chubb mackerel $\beta$	West Indian Ocean coelacanth $\alpha$	West Indian Ocean coelacanth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

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# **Figures: Allergenicity of bony and cartilaginous fish - molecular and immunological properties**

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**Figure 1**

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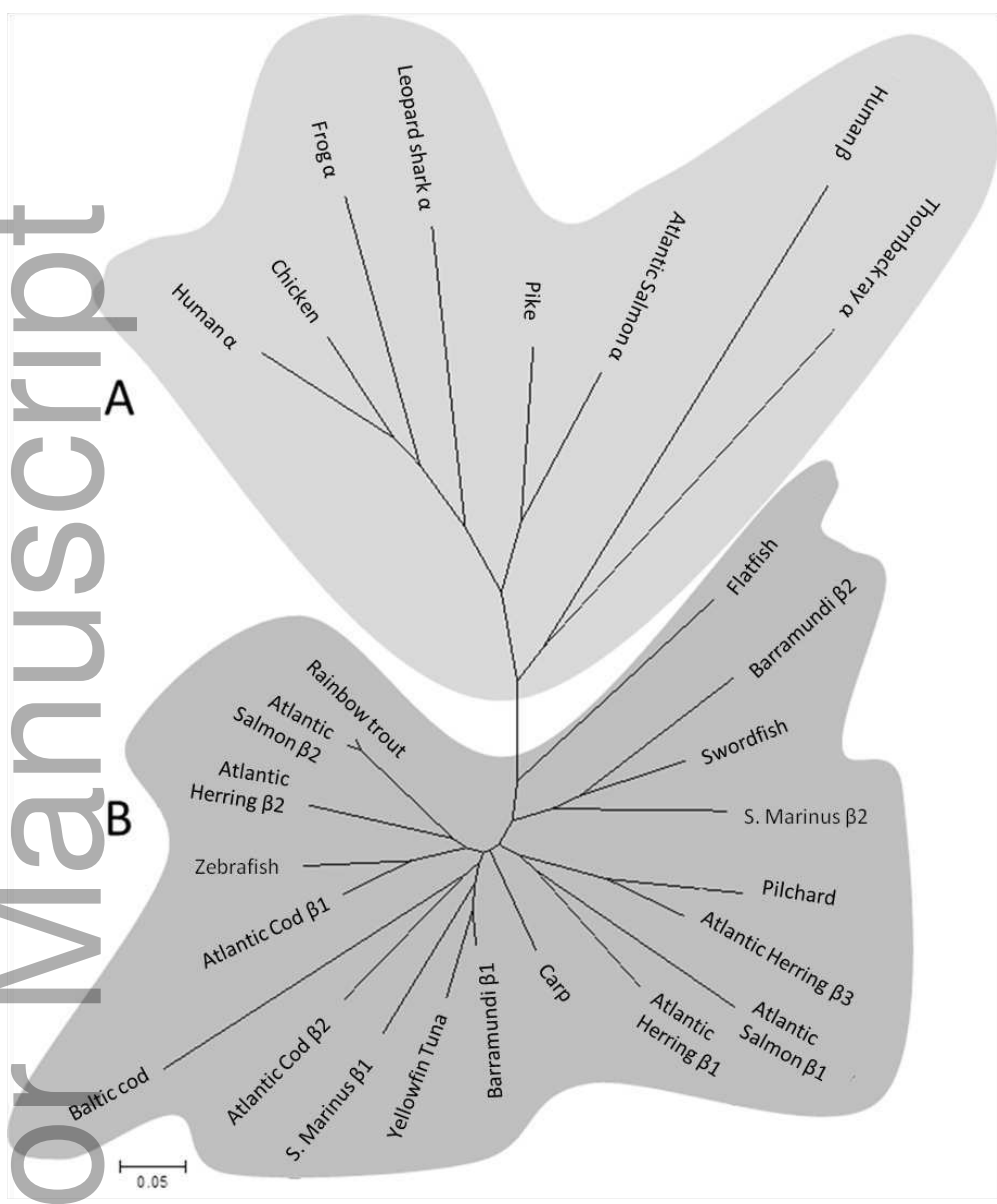


Figure 2

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**A**

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Chub_mackerel	MAFASVLKDAEVTAALDGCKAAGSFDHKKFFKACGLSGKSTDEVKKAFIIDDQDKSGFIE
Atlantic_salmon	MACAHLCKEADIKTALEACKAADTFSFKTFFHTIGFASKSADDVKKAFKVIDQDASGFIE
Carp	MAFAGVLNDADITAAL EACKAADSFNHKTFFAKVGLTSKSADDVKKAFIIDDQDKSGFIE
Atlantic_cod	MAFAGILNDADITAALAACKAEGSFDHKAFFTKVGLAAKSPADIKKVF EIDDQDKSDFVE
Baltic_cod	-AFKGILSNADIKAAEAACFKEGSFDEDFGYAKVGLDAFSADELKCLFKIADEDKEGFIE

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← Ca<sup>2+</sup>

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Chub_mackerel	EEELKFLQNFKAGARALSDAETKAFKAGDSDGDGKIGIDEFAAMIKG
Atlantic_salmon	VEELKFLQNFPCPKARELTD AETKAFKAGDADGDMIGIDEFAVLVKQ
Carp	EDELKFLQNFKAGARALTDGETKTFLKAGDSDGDGKIGVDEFTALVKA
Atlantic_cod	EDELKFLQNF SAGARALSDAETKVFLKAGDSDGDGKIGVDEFGAMIKA
Baltic_cod	EDELKFL IAFADLRAL TDAETKAFKAGDSDGDGKIGVDEFGALVDKKGAKG

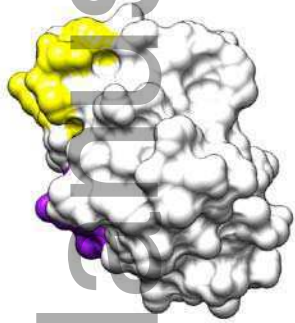
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→ Ca<sup>2+</sup>

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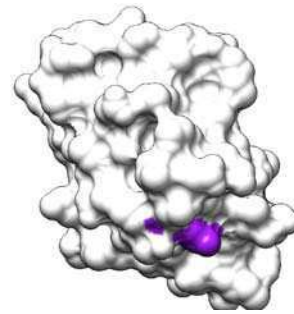
**B**

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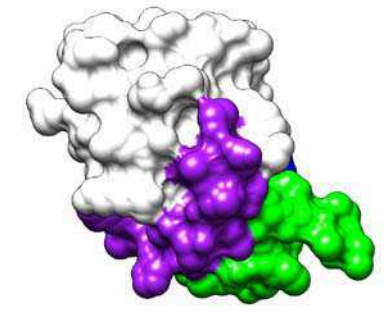
Carp

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Chub mackerel

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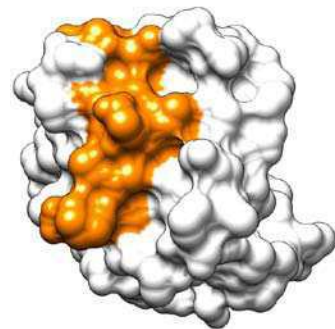


Atlantic salmon

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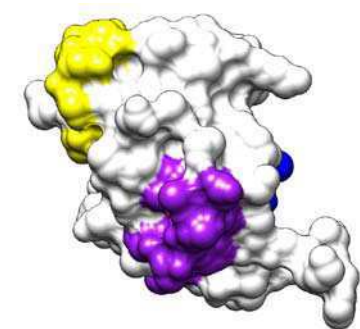
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Atlantic cod

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Baltic cod

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**Figure 3**



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