

# Dissecting the Sensitization Profiles to Parvalbumins From 12 Freshwater Fish Species to Improve Diagnosis of Fish Allergy

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J Investig Allergol Clin Immunol 2025; Vol. 35(6)

doi: 10.18176/jiaci.1069

## ■ Abstract

**Background:** Many fish-allergic patients only react to certain fish species and may tolerate others, mostly because of IgE-mediated recognition of specific epitopes on the major allergen parvalbumin. However, the considerable number of fish species consumed makes it challenging to identify which species are allergenic and which are tolerated by individual patients.

**Objective:** In order to improve the diagnosis of fish allergy, we investigated IgE-mediated reactivity to parvalbumins from 12 freshwater fish species that are largely underrepresented in diagnostic tests.

**Methods:** Parvalbumins were purified from 12 freshwater fish species belonging to 8 families, and their isoform composition was analyzed using mass spectrometry. IgE specific for each parvalbumin was quantified in serum samples from 66 fish-allergic individuals, and basophil activation tests were performed for 5 patients. Crosswise inhibition assays were carried out for all parvalbumins for 7 patients to investigate cross-reactivity between the parvalbumins from the different species.

**Results:** IgE binding and cross-linking potency of the parvalbumins differed, with the strongest reactivities observed for 4 parvalbumins from the salmonid family (results positive for 89%-95% of patients) and the weakest for parvalbumins from Wels catfish, European eel, and tench (results negative for  $\geq 50\%$  of patients). Ninety percent of the patients with negative results for Wels catfish parvalbumin also had negative results for additional parvalbumins from multiple species. Inhibition assays revealed variable recognition of epitopes by several patients, with the primary sensitizers most frequently being parvalbumins from salmonids and percids.

**Conclusion:** Including freshwater salmonids in the diagnostic work-up for fish allergy may help to identify most fish-allergic patients. IgE to Wels catfish could help distinguish between polysensitized and oligosensitized patients.

**Key words:** Parvalbumin. Fish allergy. Freshwater fish. Salmonids. IgE. Wels catfish. Fish tolerance. Oligosensitization.

## ■ Resumen

**Antecedentes:** Pacientes alérgicos al pescado sólo reaccionan a determinadas especies de pescado y pueden tolerar otras, debido principalmente al reconocimiento por IgE de epítomos específicos del alérgeno mayor, la parvalbúmina. Sin embargo, el considerable número de especies de pescado consumidas dificulta la identificación de las especies que provocan reacciones alérgicas y las que tolera cada paciente.

**Objetivo:** Para mejorar el diagnóstico de la alergia al pescado, investigamos la reactividad IgE a las parvalbúminas de 12 especies de peces de agua dulce que están muy poco representadas en las pruebas diagnósticas.

**Métodos:** Se purificaron parvalbúminas de 12 especies de peces de agua dulce de 8 familias diferentes y se analizó la composición de sus isoformas por espectrometría de masas. Se cuantificó la IgE específica para cada parvalbúmina en sueros de 66 individuos alérgicos al pescado y se realizaron test de activación de basófilos en 5 pacientes. Se llevaron a cabo ensayos de inhibición cruzada de todas las parvalbúminas en 7 pacientes para investigar la reactividad cruzada entre las parvalbúminas de diferentes especies.

**Resultados:** La unión IgE y la reactividad cruzada de las parvalbúminas difirieron, observándose las reactividades más fuertes para cuatro parvalbúminas de la familia de los salmónidos (89-95% de pacientes positivos) y las más débiles para las parvalbúminas de siluro, anguila europea y tenca, con  $\geq 50\%$  de pacientes negativos. El 90% de los pacientes negativos a la parvalbúmina del siluro fueron negativos a otras

parvalbúminas de múltiples especies. Los ensayos de inhibición indicaron un reconocimiento epitópico variable, siendo los sensibilizantes primarios más frecuentes las parvalbúminas de salmónidos y pércidos.

**Conclusión:** La inclusión de los salmónidos de agua dulce en el diagnóstico de la alergia al pescado puede ayudar a identificar a la mayoría de los pacientes alérgicos al pescado, mientras que la IgE frente al siluro puede ser indicadora de los pacientes polisensibilizados frente a los oligosensibilizados.

**Palabras clave:** Parvalbúmina. Alergia al pescado. Peces de agua dulce. Salmónidos. IgE. Siluro. Tolerancia al pescado. Oligosensibilización.

## Summary box

- **What do we know about this topic?**

It is now generally recognized that up to 40% of patients with fish allergies can tolerate certain types of bony fish. Most studies focus on tolerance of saltwater fish species, while freshwater fish are less studied.

- **How does this study impact our current understanding and/or clinical management of this topic?**

We demonstrated strong reactivity to freshwater salmonids and significantly weaker and less frequent reactivity to other species, especially Wels catfish. Inclusion of these species in the diagnostic work-up may help to identify oligosensitized patients and potentially tolerated fish species.

## Introduction

Consumption and aquaculture of fish are increasing worldwide because of this food's recognized nutritional value. In addition to its health benefits, consumption of fish is associated with a decrease in allergic rhinitis in children [1]. For patients with fish allergy, it has been increasingly observed that strict avoidance of all fish species is unnecessary. Based on double-blind placebo-controlled food challenge with cod, salmon, and mackerel, tolerance to at least one of these species was demonstrated for 29% of fish-allergic patients [2]. Also using food challenges, we showed that 10 of 11 patients sensitized to different bony fish species can tolerate thornback ray, a cartilaginous fish [3]. Leung et al [4] demonstrated tolerance to some species, especially those with low levels of the major allergen  $\beta$ -parvalbumin, in 40% of patients.

Identifying tolerated species remains a challenge, as the ultimate test to confirm allergy or tolerance, the double-blind placebo-controlled food challenge, cannot be performed with many species. Multiplex IgE tests are increasingly used in allergy diagnostics, and the quantification of serum IgE specific for different fish species may be used as a prerequisite to reduce the number of species required for food challenges [5,6]. Eventually, a comprehensive understanding of cross-reactive IgE epitopes on the major fish allergen parvalbumin from various species and detailed diagnostic algorithms will enable us to predict reactivity based on a known sensitization profile. A necessary step towards achieving this goal is the creation of a meaningful panel of fish species and allergens for in vitro IgE-based diagnosis.

To this end, we have recently been investigating IgE-mediated reactivity to parvalbumin from multiple species

in different patient cohorts. We showed the lower allergenicity of ray, a cartilaginous fish, and its  $\alpha$ -parvalbumin in patients allergic to bony fish [3]. We also demonstrated the absence of reactivity to the parvalbumins from specific bony fish for up to 38% of patients and recorded different sensitization patterns in different parts of the world [3,6]. Further research is needed to thoroughly assess the allergenicity of locally available fish species across different geographical regions. In Europe, many freshwater species from families such as salmonids, cyprinids, and percids are farmed and consumed. Based on the European Market Observatory for Fisheries and Aquaculture Products report on freshwater aquaculture in the EU released in April 2021, the 4 main European aquaculture producers are France, Greece, Spain, and Italy, which, together, supply 67% of Europe's aquaculture products. However, the freshwater fish aquaculture sector is present in almost all countries. Trout and carp are the most farmed freshwater species, especially in France, Italy, and Denmark, followed by Spain and Poland [7].

Except for rainbow trout and common carp, the allergenicity of other freshwater species has not been well characterized to date. In this study, we investigated sensitization patterns to parvalbumins from 12 freshwater fish species belonging to 8 families in 66 patients with clinically confirmed fish allergy. Our data showed that IgE-mediated reactivity was strongest to salmonid parvalbumins and remarkably low to those from Wels catfish, European eel, and tench. The findings of the present study will improve diagnosis of fish allergy by contributing to future designs of diagnostic panels. These should facilitate the selection of species for food challenges aiming to confirm allergy or tolerance, ultimately leading to a reduction in dietary restrictions and an improvement in patient safety and quality of life.

## Methods

### Protein Purification

Twelve freshwater fish species belonging to 8 families (Table S1) were obtained from fish farming facilities in Austria. Parvalbumins were purified from fish muscle using a 3-step protocol consisting of the following: (a) heating of the fish extracts to 90°C to precipitate some of the other proteins from the extract, based on known stability and solubility of parvalbumins under these conditions; (b) precipitation with ammonium sulfate according to established protocols [8]; and (c) phosphate-buffered saline-based size-exclusion chromatography using a 26/60 Sephacryl S-200 High Resolution column (GE HealthCare) to remove any remaining contaminating proteins.

### Identification of Parvalbumins by Mass Spectrometry

Tryptic digestion of the samples and further preparation for liquid chromatography-tandem mass spectrometry (LC-MS/MS) was carried out according to the method described by Shevchenko et al [9], and the resulting peptides were used for LC-MS/MS according to the methodology described by Abdelhameed et al [10]. Detailed data on sample preparation and the mass spectrometry approach used for the identification of parvalbumin isoforms are provided in the Supplementary material. The fish protein database was created for each species using entries under the specific taxonomy IDs (Table S2) from the UniProtKB and NCBI GenBank databases (accessed in December 2023 for Wels catfish, tench, and eel parvalbumins and in October 2021 for the other species).

### Study Participants

Patients with clinically confirmed fish allergy were recruited in allergy clinics in Austria. The inclusion criteria comprised a clinical history of immediate allergic reaction after fish consumption and the confirmation of sensitization to fish by ImmunoCAP (for cod, Gad c 1, salmon, or fish mix) and/or skin prick test. Based on the clinical history, the causative fish species were known for 22 patients, 10 of whom reported reactivity to some of the freshwater species included in our study.

A multiplex assay was used to determine sensitization profiles to parvalbumins from 12 freshwater fish species, as described below. Initially, 72 patients were recruited; of these, 6 were not sensitized to any of the parvalbumins tested and are likely sensitized to other fish allergens. Considering that the present study focused on parvalbumins, these 6 patients were excluded from further analyses, leaving the total number of patients assessed at 66. The demographic and clinical characteristics of these patients are summarized in the Table and detailed in Table S3. In addition, 4 adults with no clinical history of food allergy and 3 adults with other allergies were recruited as negative controls for the IgE quantification assays. The study was approved by the Ethics Committee of Lower Austria (GS1-EK-4/503-2017). Written informed consent was obtained from all participants or their legal representatives.

**Table.** Overview of Demographic and Clinical Characteristics of the Fish-Allergic Individuals Studied.

No. of patients	66
Sex	
Male	41
Female	25
Age, y	
≤10	28
11-19	11
≥20	27
Fish allergy symptoms	
Asthma	2
Angioedema	22
Conjunctivitis	2
Difficulty breathing	9
Eczema	10
Gastrointestinal symptoms	10
Heat sensations	3
Oral allergy syndrome	17
Urticaria	20
Rhinitis	2
Other additional symptoms	6
Total IgE by ImmunoCAP*, kU/L	
Median	472
Range	19-4202
Total IgE by multiplex assay, kU/L	
Median	322
Range	8-3142
Cod ImmunoCAP**, kU <sub>A</sub> /L	
Median	2.9
Range	0.5-22.7
Skin prick test with cod (≥3 mm/1-3 mm/not done)	54/2/10

\*Determined for 60 patients. \*\*Determined for 41 patients.

### Multiplex IgE Quantification

Patients' sera were applied individually to a multiplex research chip that was custom-designed for this study. The chip contained the freshwater fish parvalbumins and was based on the same principle as the ALEX<sup>2</sup> Allergy Xplorer (MacroArray Diagnostics). It was designed following the protocols used for the parvalbumins that are present on the commercial ALEX<sup>2</sup> chip, and its performance was tested prior to the study using an internal set of serum samples with known reactivity to the different fish species available at MacroArray Diagnostics. Total and parvalbumin-specific IgE were quantified as described elsewhere [11]. IgE values above 0.3 kU/L were considered positive.

### **Multiplex Inhibition Assay**

Chip-based multiplex inhibition assays were used to study IgE-mediated cross-reactivity to parvalbumins from different species. Individual serum samples from 7 patients for whom sufficient amounts of serum were available were preincubated with 1 µg/mL of each of the 12 parvalbumins for 2 hours at room temperature or used without the inhibitors. Sera were added to the chips, and the assay was performed as usual.

### **Basophil Activation Test**

After stimulation with increasing concentrations of the parvalbumins (0.1 ng/mL to 100 ng/mL), basophil activation was assessed for 5 patients, as described previously [3], using the Flow-CAST Basophil Activation Test kit (Bühlmann Laboratories AG) according to the manufacturer's protocol.

### **Statistical Analysis**

Significant differences in IgE levels specific to the parvalbumins were determined using the Friedman test with the post hoc Dunn test. *P* values below .05 were considered significant. The statistical analyses were performed using GraphPad Prism 10 (GraphPad Software).

## **Results**

### **Parvalbumin Sequences**

Parvalbumins from 12 freshwater fish species (Table S1) were purified and visualized using Coomassie Brilliant Blue staining (Figure S1). The identities of the proteins and the protein sequences of the isoforms present in each parvalbumin preparation were detected using LC/MS-MS (Table S1). Sequence coverages were 82%-99% for the parvalbumins from 10 of the 12 species, with the numbers of identified peptides between 13 and 44 and unique peptides between 1 and 44, depending on the sequence. In the case of tench and Wels catfish, the absence of parvalbumin sequences of these species from the databases meant that sequences belonging to other species from the same fish families were identified with coverages of between 46% and 77% (Table S1). Detailed mass spectrometry data for each protein with all identified hits are shown in Table S4.

### **Demographic and Clinical Characteristics of Fish-Allergic Patients**

The study population comprised patients with a documented clinical history of fish allergy and confirmed sensitization to fish (28 children, 11 adolescents, and 27 adults) (overview in the Table and details in Table S3). Table S3 shows specific data about the symptoms experienced upon exposure, positive allergy test results for fish, and information about other food allergies for each patient. The clinical manifestations of fish allergy ranged from mild to severe, with angioedema, urticaria, and oral allergy syndrome being the most frequent (Table and Table S3). Skin prick testing for cod was performed for 56 patients, while ImmunoCAP for fish mix, cod, Gad c 1, and/or salmon was performed for 65 patients (Table and

Table S3). Specifically, the cod ImmunoCAP (f3) test was performed for 41 patients, the Gad c 1 ImmunoCAP (f426) for 37 patients, the fish mix ImmunoCAP (fx74) for 8 patients, and the salmon ImmunoCAP (f41) for 4 patients.

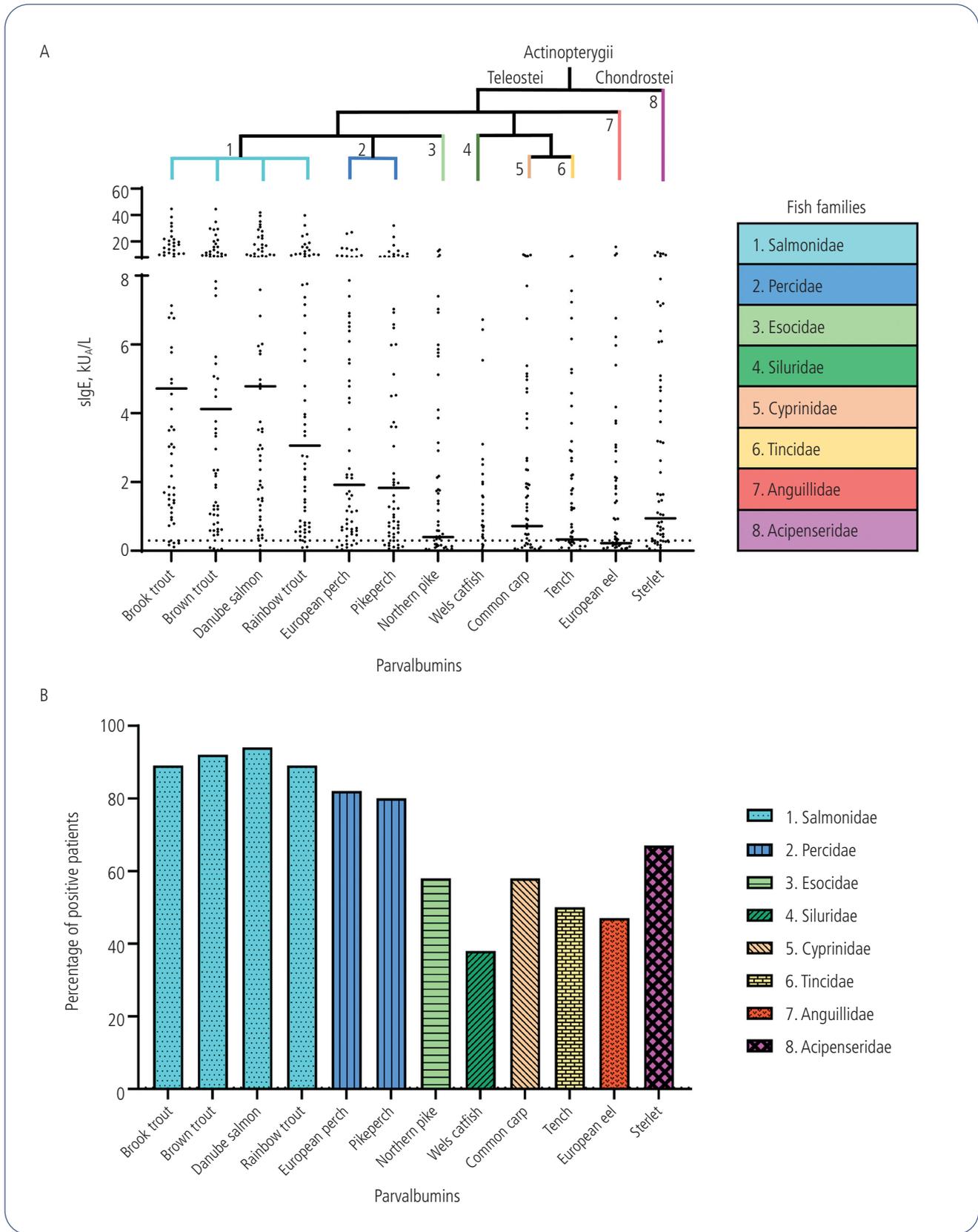
### **Specific IgE Highest for Freshwater Salmonid Parvalbumins and Lowest for Wels Catfish Parvalbumin**

Based on the multiplex IgE quantifications, the strongest reactivity was found for parvalbumins from brook trout (median IgE, 4.7 kU<sub>A</sub>/L; maximum, 44.68 kU<sub>A</sub>/L), brown trout (median, 4.1 kU<sub>A</sub>/L; maximum, 44.50 kU<sub>A</sub>/L), Danube salmon (median, 4.8 kU<sub>A</sub>/L; maximum, 41.87 kU<sub>A</sub>/L) and rainbow trout (median, 3.1 kU<sub>A</sub>/L; maximum, 39.83 kU<sub>A</sub>/L), all belonging to the salmonids (Salmonidae fish family) (Figure 1A). The next most recognized parvalbumins were from European perch and pikeperch, which are members of the percids (Percidae family). The weakest reactivity was observed for parvalbumins from Wels catfish and European eel (median IgE, ≤0.3 kU<sub>A</sub>/L; maximum, 6.72 kU<sub>A</sub>/L for Wels catfish and 15.93 kU<sub>A</sub>/L for eel parvalbumin), followed by tench (median, 0.33 kU<sub>A</sub>/L; maximum, 8.88 kU<sub>A</sub>/L). Statistically significant differences between IgE levels for various parvalbumins are presented in Table S5. The percentage of patients with positive results for specific species followed a similar trend: ≥89% of the patients were sensitized to salmonid parvalbumins, while ≤50% had IgE specific to Wels catfish, eel, and tench parvalbumins (Figure 1B). IgE binding to Wels catfish parvalbumin was not detected in 40 patients (62%), indicating possible tolerance of this species. Moreover, of the 40 patients with negative results for Wels catfish parvalbumin, 36 were negative for parvalbumins from additional fish species, most commonly Northern pike, carp, tench, and eel (Table S6).

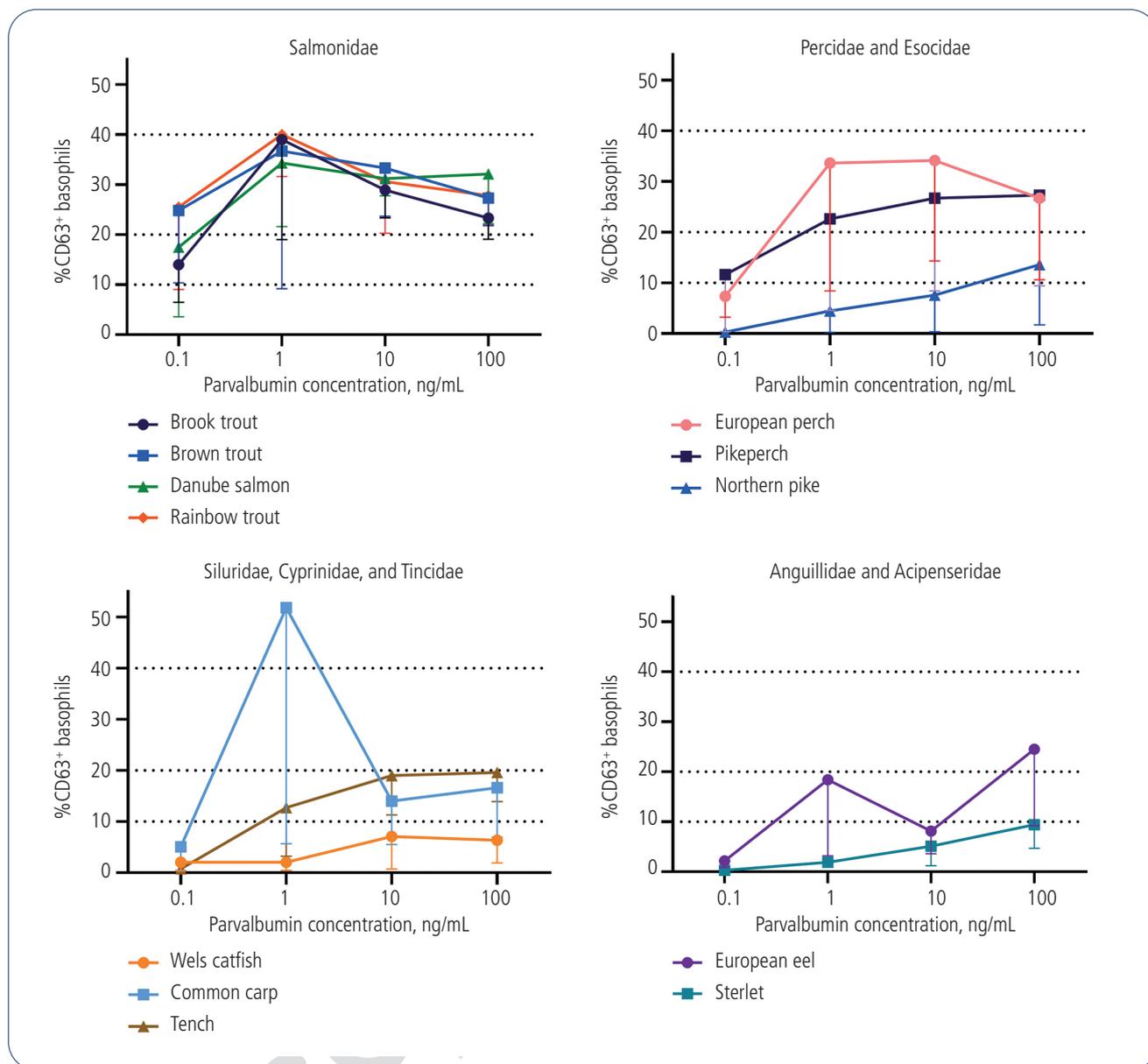
Results were positive for all 12 parvalbumins in 23 patients (35%) (polysensitized patients), for 7-11 parvalbumins in 22 patients (33%), for 2-6 parvalbumins in 19 patients (29%), and to 1 parvalbumin (Danube salmon) in 2 patients (3%) (Figure S2 and Table S6).

### **Basophil Activation Strongest With Salmonid Parvalbumins and Weakest With Wels Catfish Parvalbumin**

The ability of all 12 parvalbumins to induce basophil activation was investigated by a direct basophil activation test (BAT) for 5 patients. Each parvalbumin induced basophil activation above the recommended threshold of 10% for between 2 and 5 patients, thus demonstrating their allergenicity in a functional assay (Figure 2 and Figure S3). The parvalbumins with the strongest potency to activate basophils were from the salmonids and percids (Figure 2), while the weakest were from Northern pike, Wels catfish, and sterlet. For all 4 salmonid parvalbumins at all concentrations, the median percentage of activated (CD63<sup>+</sup>) basophils was 14%-40%, while it was below 7% for Wels catfish parvalbumin. All 5 patients tested reacted to all 4 salmonid parvalbumins in the BAT (Figure S3), 4 reacted to European perch and/or pikeperch, while only 2 patients reacted to the Wels catfish parvalbumin.



**Figure 1.** IgE specific to fish parvalbumins. A, Concentration of parvalbumin-specific IgE in patients' sera (n=66). The dotted line represents the threshold (0.3 kU<sub>A</sub>/L) for a positive signal; solid lines represent the median. See Table S5 for the statistical analysis demonstrating significant differences between levels of IgE to different parvalbumins. B, Percentage of patients with IgE>0.3 kU<sub>A</sub>/L for each parvalbumin tested.



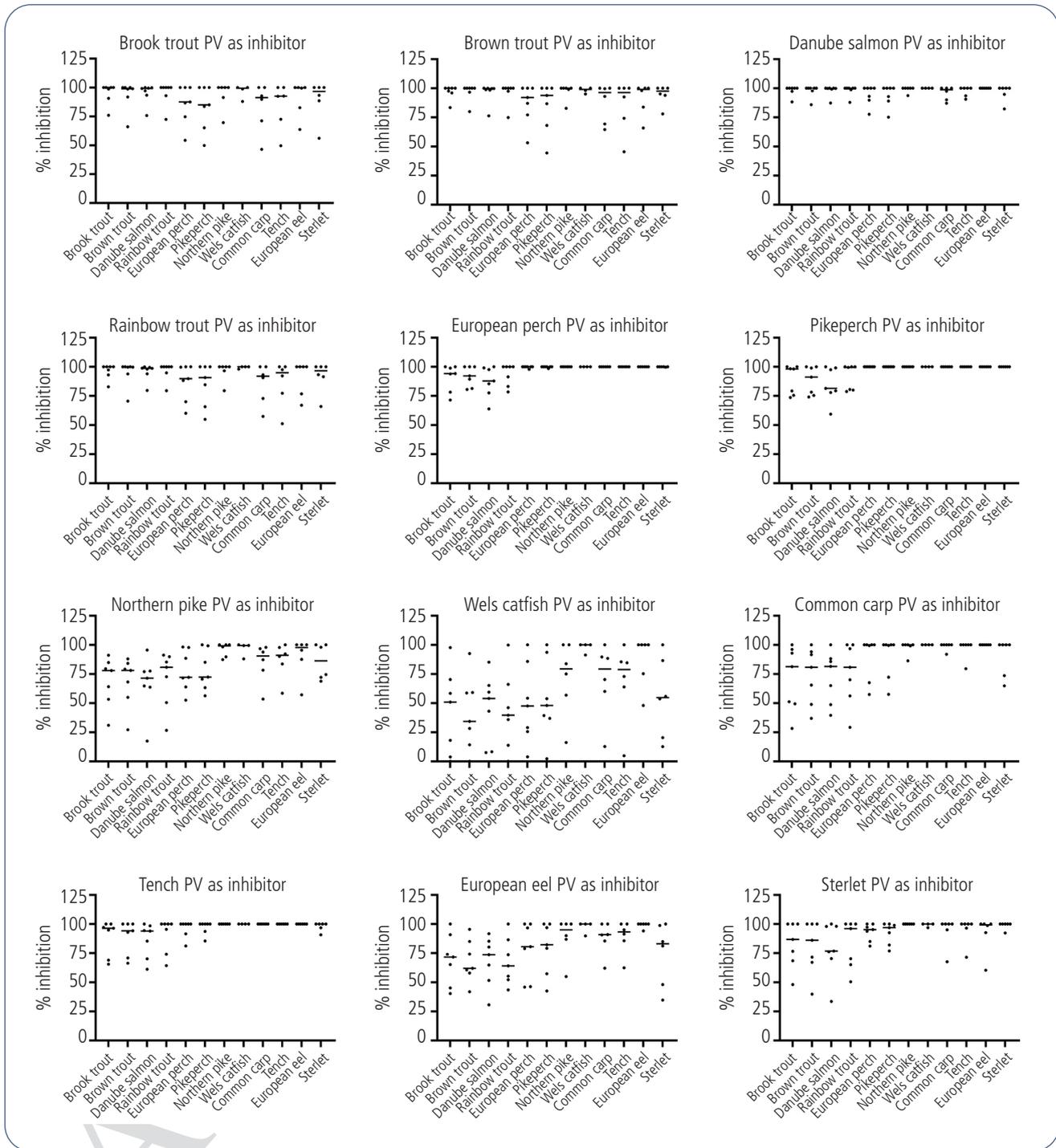
**Figure 2.** Basophil responses to parvalbumins from the fish species studied. Data indicate basophil activation (expressed as the percentage of CD63<sup>+</sup> basophils) upon stimulation with varying doses of fish parvalbumins in fish-allergic patients (n=5). Data are shown as median (IQR).

### Cross-Reactivity Analysis Indicates Recognition of Salmonid and Percid Parvalbumin Epitopes by Most Patients' IgE

To investigate the IgE cross-reactivity to parvalbumins from the different species, inhibition assays were performed for 7 patients. First, we analyzed the efficacy of the individual parvalbumins in inhibiting IgE binding to parvalbumins of all other fish species used in the study, considering all patients together. Among the salmonid fish parvalbumins tested, that from Danube salmon showed the strongest potency to inhibit IgE binding to all other investigated parvalbumins (>75% inhibition of binding to all parvalbumins for all patients tested), indicating that the IgE of these patients

predominantly recognizes epitopes shared between Danube salmon parvalbumin and the others (Figure 3).

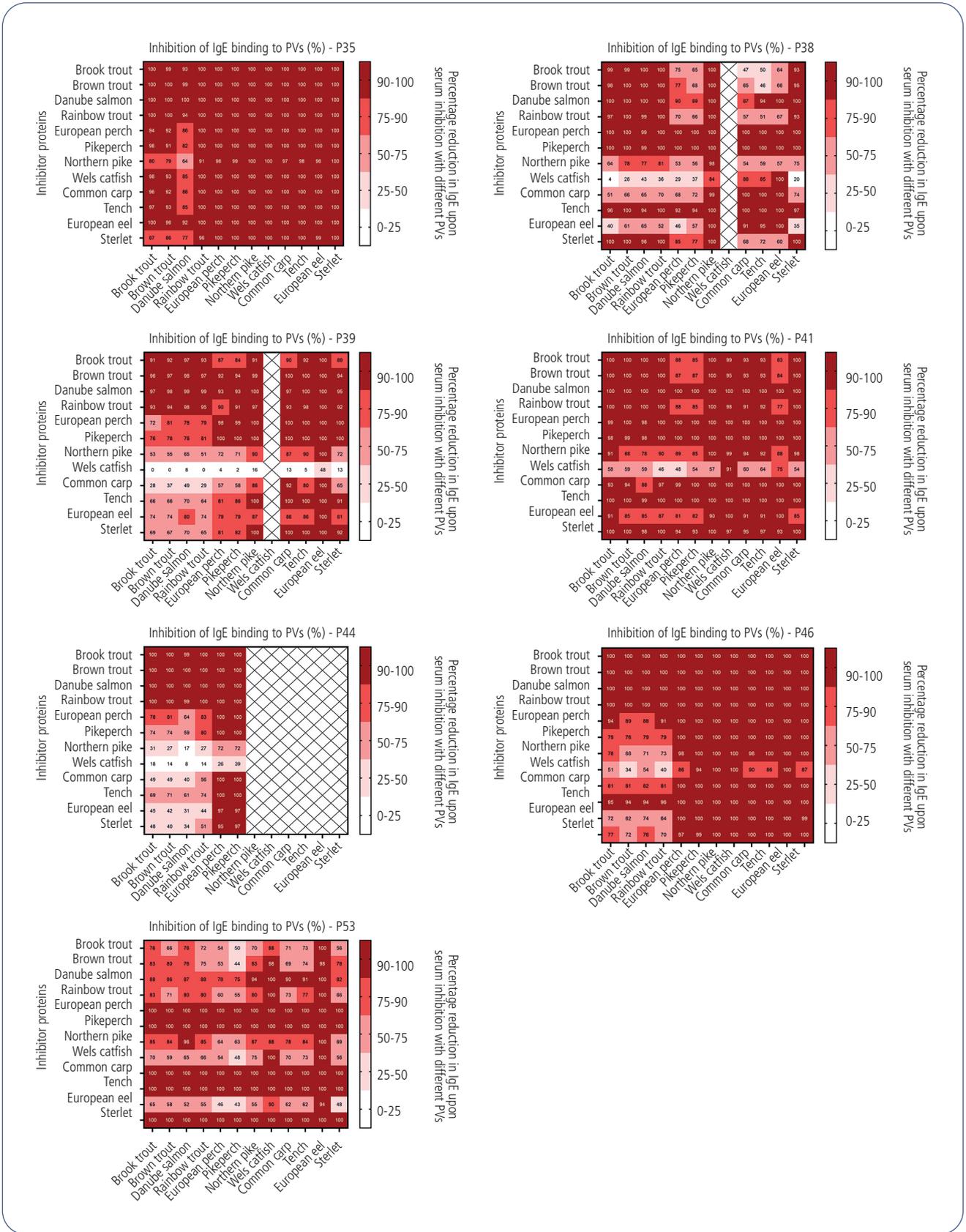
European perch and pikeperch parvalbumins inhibited 100% of IgE binding to all other tested parvalbumins except those from salmonids, indicating highly cross-reactive IgE epitopes between percids and all nonsalmonid parvalbumins investigated. Tench parvalbumin also strongly inhibited IgE binding, especially to parvalbumins of species that are not members of the salmonid or percid families. The weakest inhibitors were parvalbumins from Northern pike, Wels catfish, and European eel (Figure 3). However, this low cross-reactivity is not reflected by the low sequence identities between these and other parvalbumins (Figure S4), suggesting that some of the cross-reactive IgE epitopes are likely absent from these 3 parvalbumins.



**Figure 3.** Scatter dot plots demonstrating the ability of each parvalbumin to inhibit IgE binding to any of the other parvalbumins investigated. A solid line indicates the median based on data from the serum samples of 7 patients. The y-axis displays the percentage of the inhibition of IgE binding to each parvalbumin shown on the x-axis. PV, parvalbumin.

Crosswise inhibition results for all parvalbumins for each patient tested are shown in Figure 4. Here, we also observed that the strongest inhibitory potential was generally that of parvalbumins from salmonids and percids and from tench (Tincidae, order Cypriniformes) and that the weakest was from Northern pike, Wels catfish, and European eel.

However, each patient had a different sensitization profile. Specifically, for patients P35, P39, P44, and P46 (Figure 4), salmonid parvalbumins appeared to be the primary sensitizers (84%-100% inhibition of IgE binding to any other investigated parvalbumin after preincubation of serum with any of these proteins). For patient P38, parvalbumins from European perch,



**Figure 4.** Multiplex-based IgE-inhibition assay for each patient tested. The data show the percentage of inhibition of IgE binding to various parvalbumins after preincubation of serum samples with parvalbumins at a concentration of 1 µg/mL. PV, parvalbumin.

pikeperch, and tench inhibited  $\geq 97\%$  of IgE binding to other proteins. For patients P41 and P53, the strongest inhibitors were parvalbumins from the percids, sterlet (*Acipenseridae*), tench, and carp (both belonging to *Cypriniformes*) (Figure 4).

The specific-IgE quantification data without inhibitions for these patients is presented in Figure S5, which shows that the 7 patients tested in the inhibition assays reflect different reactivity profiles: P35, P41, P46, and P53 reacted to all parvalbumins tested; P38 and P39 were negative only to parvalbumin from Wels catfish; and P44 was positive to parvalbumins from salmonids and percids and negative to all others.

## Discussion

After decades of assuming that fish-allergic patients must avoid all fish, it has recently become evident that 30%-40% of patients with confirmed fish allergy are oligosensitized to some fish species and can tolerate others [12]. Correct diagnosis of allergy or tolerance to specific species could provide patients with safe dietary alternatives [13]. In addition, up to 63% of patients develop tolerance to fish over time [14,15]. Although an oral food challenge is required to confirm tolerance or allergy [16], multiplex quantification of IgE for the major allergen parvalbumin is a useful first step towards recognizing potentially tolerated fish [6]. The identification of highly allergenic and well-tolerated fish is a component of tailored precision medicine that will become an important strategy for patient management [17].

In order to determine the correct fish species to be used in diagnosis, locally relevant fish species and their allergens should be investigated. We previously showed differences in IgE sensitization patterns to 9 saltwater and 1 freshwater fish species in patients from different parts of the world [6]. Freshwater fish species have generally been less frequently investigated than saltwater fish. However, freshwater fish, especially salmonids, percids, and cyprinids, are farmed and consumed on a large scale [18]. Many landlocked European countries do not receive sufficient seafood supplies to meet their health needs [19], and locally farmed freshwater fish are an important source of valuable nutrients. In addition, aquaculture contributes indirectly to the conservation of some fish species by satisfying demand without exploiting natural resources.

This study investigated the IgE reactivity of fish-allergic patients to parvalbumins from 12 farmed freshwater fish species, with the aim of understanding which are the most reactive and whether some may be hypoallergenic and tolerated. As commercial extracts for the diagnosis of fish allergy are not well standardized [20], molecular allergy diagnosis is receiving increased attention. To better understand patients' response patterns, we included less commonly farmed and consumed species from distant fish families, in addition to the more commonly consumed freshwater species such as trout and carp.

We first used a custom-designed multiplex-based IgE quantification assay to identify the individual reactivity patterns. IgE reactivity to parvalbumins from the 4 freshwater salmonids investigated (brook trout, brown trout, Danube

salmon, and rainbow trout) was stronger than for other fish families, possibly reflecting frequent exposure of patients to salmonids. For example, according to the 2020 FAO Fishery and Aquaculture Country Profiles ([www.fao.org/fishery/en/facp](http://www.fao.org/fishery/en/facp)), 4527 tons of freshwater fish was produced in Austria from aquaculture and used solely for national consumption. The most abundantly cultivated species were rainbow trout, brook trout, and carp. Atlantic salmon is commonly consumed in Central Europe, in addition to freshwater salmonids. Menozzi et al [21] investigated the preferred consumer choice for various fish species in 5 European countries and found that salmon and cod have the largest market share. In our study, percent sequence identities between the allergenic parvalbumin from Atlantic salmon (Sal s 1.0101, GenBank ID CAA66403) and the sequences of parvalbumins identified in freshwater salmonids using mass spectrometry are between 71% and 98%. For each freshwater salmonid, we identified 1 parvalbumin sequence with an identity of  $\geq 96\%$  to Sal s 1.0101. Therefore, high IgE levels to salmonids may be due to sensitization to either freshwater salmonids or to Atlantic salmon. However, as the culprit fish species in our patient cohort is known for only 22 patients, 9 of whom reported reactivity to salmon or freshwater salmonids (Table S3), it cannot be concluded with certainty which species was the primary sensitizer for each patient. Salmonid parvalbumins were also the most frequently recognized allergens in our study cohort (95% of the patients tested positive to parvalbumin from at least 1 salmonid species). This is in contrast to, for example, Atlantic cod, to which 19% of Austrian patients reacted negatively in an earlier study [6]. Salmonid parvalbumins should therefore be considered crucial allergens in the diagnosis of fish allergy in this part of the world. The next highest IgE levels were for parvalbumins from European perch and pikeperch, with positive results in 82% and 80% of patients, respectively. Surprisingly low specific IgE levels (median  $< 0.3$  kU<sub>A</sub>/L) and a low percentage of positive results (38% of patients) were observed for the parvalbumin from Wels catfish. Similarly, tench and eel parvalbumins were not strongly reactive. These 3 fish species are distant in evolutionary terms from salmonids and may be tolerated by some patients. However, this possibility requires confirmation by skin prick tests and subsequent food challenges.

Our data represent good estimates of the reactivity patterns for a Central European cohort of fish-allergic patients exposed mainly to the species investigated; however, different results may be obtained for other geographical regions. For example, while Wels catfish may be tolerated by many European fish-allergic patients, Leung et al [4] showed that carp, tilapia, and catfish were the least tolerated species in China, while salmon, tuna, and halibut were tolerated by 8%-28% of patients. We had the opportunity to test serum samples from 12 fish-allergic patients from Korea in our multiplex IgE assay (approved by the Institutional Review Board of the Yonsei University Health System, approval number 4-2013-0397). Four samples were negative to all the parvalbumins included. Among the other 8 sera, the strongest reactivity was observed to salmonid and percid parvalbumins and the weakest to Wels catfish (data not shown), similar to the European cohort. Nevertheless, reactivity to various freshwater fish species in other geographical regions may differ and should be investigated in future studies.

We also tested all 12 parvalbumins in a direct BAT for 5 patients who agreed to additional blood sampling. The BAT is considered more specific than IgE sensitization tests [22] and, despite the small number of patients tested in our study, served as a confirmation of the allergenic properties of various parvalbumins. The strongest basophil activation was observed for salmonid parvalbumins and the weakest for Wels catfish parvalbumin. Quantification of specific IgE followed by the BAT was shown to be indicative when diagnosing peanut allergy, and this process was suggested as a prerequisite for food challenges [23]. Comparable results were observed in our previous study, where patients tolerating ray were negative to ray parvalbumin in IgE ELISA and BAT [3]. In the current study, 2 of 3 patients with negative results to Wels catfish parvalbumin in BAT were invited to undergo a skin prick test with this protein, and negative results were confirmed (data not shown). While our data indicate possible tolerance of Wels catfish by many patients, food challenges in a clinical setting are required before any clinical recommendation about possible consumption of this fish can be made. Moreover, allergens other than parvalbumin may be implicated in some patients [24].

IgE reactivity to parvalbumins from different species may result from primary sensitization or from cross-reactivity based on shared IgE epitopes [25]. It is well-known that the conserved calcium-binding region of parvalbumins contains important IgE epitopes responsible for cross-reactivity to various fish species [26]. However, additional IgE epitopes are present on parvalbumins from specific species, leading to monosensitization, as shown for salmonids and recently for Wedge sole [27,28]. While the existence of both linear and conformational IgE-binding epitopes on parvalbumins has been reported [29-31], further, detailed investigation of cross-reactivity will be required for the prediction of patients' reactivity to different species. In this study, multiplex inhibition assays enabled the simultaneous investigation of cross-reactivity between 12 distinct proteins using a minimal serum volume. All salmonid parvalbumins tested were strong inhibitors, with that of Danube salmon demonstrating the strongest efficacy in inhibiting IgE binding to all the other parvalbumins tested (>75% inhibition of binding to all proteins in all patients tested), suggesting the presence of shared IgE epitopes. Other strong inhibitors were the parvalbumins from European perch, pikeperch, and tench. Interestingly, parvalbumins from percids inhibited 100% of IgE binding to all other parvalbumins except those from salmonids for all 7 patients. This finding indicates that most parvalbumin epitopes from percids are shared with other fish families. The parvalbumins from Northern pike, Wels catfish, and European eel demonstrated low cross-reactivity of their IgE epitopes with the other parvalbumins. When examining the inhibition assay data for each patient separately, we observed that while some patients had IgE against highly cross-reactive epitopes, others were more likely sensitized to specific parvalbumins, such as those from salmonids and percids.

The IgE inhibition data obtained during this study serve as a starting point for further investigations of the parvalbumin epitopes from different species. However, clinical relevance of the cross-reactivity observed remains to be elucidated [32].

Taken together, the data presented in this study suggest that freshwater salmonids, especially Danube salmon, should be included in the diagnosis of fish allergy to identify up to 95% of allergic patients. Moreover, in order to enhance the likelihood of identifying oligosensitized patients and potentially tolerated freshwater species, Wels catfish should be included in routine diagnostic procedures.

## Acknowledgments

Support with mass spectrometry and related analyses was provided by the VetCore Facility (Proteomics) of the University of Veterinary Medicine, Vienna. The authors would like to acknowledge Mr. Gregor Gravogl from the Fishing Association of Lower Austria (NÖ Landesfischereiverband) for his help obtaining the fish species required for the study. Fish were kindly provided by Dr. Thomas Friedrich (The University of Natural Resources and Life Sciences, Project LIFE Sterlet, Danube Island, Vienna), Mr. Günther Gratzl (Dobersberg), Mr. Johann Haimel (Traismauer), and Mr. Ferdinand Trauttmansdorff (Gut Dornau Vertrieb und Verarbeitung Gesellschaft m.b.H, Leobersdorf). Furthermore, the authors wish to acknowledge the NÖ Landesgesundheitsagentur, the legal entity of University Hospitals in Lower Austria, for providing the organizational framework for this research.

## Funding

This study was supported by the Danube Allergy Research Cluster 1.0-P06 and 2.0-P10, funded by the Federal State of Lower Austria, Austrian Science Fund (FWF, grant number M 3295), the Medical University of Vienna, and the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education (RS-2023-00241714).

## Conflicts of Interest

Sandip D. Kamath, Peter Forstenlechner, and Martina Aumayr are employees of MacroArray Diagnostics. The remaining authors declare that they have no conflicts of interest.

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- *Manuscript received July 16, 2024; accepted for publication January 21, 2025.*
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