Multinational study of allergic sensitization to ten fish species indicates patient-dependent tolerance of specific fish

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Abstract

Background: Recent studies indicated that fish-allergic patients may safely consume certain fish species. Multiplex IgE testing facilitates the identification of species tolerated by individual patients. **Methods:** Sera were collected from 263 fish-allergic patients from Austria, China, Denmark, Luxembourg, Norway and Spain. Specific (s) IgE to parvalbumins (PVs) from 10 fish species along with IgE to 7 raw and 6 heated fish extracts was quantified using a research version of the ALEX ^{2} assay. IgE-signatures of individual patients and patient groups were analyzed using SPSS and R. **Results:** sIgE to alpha-PV from ray, a cartilaginous fish, was not detected in 78% of the patients while up to 41% of the patients, depending on their country of origin, tested negative for at least one beta-PV. sIgE values were highest for mackerel and tuna PVs (>10 kUA/L) and significantly lower for cod (4.9 kUA/L) and sole PVs (2.55 kUA/L). 17% of the patients, although negative for PVs, tested positive for the respective fish extracts. Based on the absence of IgE to PVs and extracts, up to 21% of the patients were identified as potentially tolerating one or more bony fish. Up to 90% of the patients tested negative for ray. The probability of negativity to one fish based on negativity to others was calculated. Negativity to tuna and mackerel emerged as a good marker of negativity to additional bony fish. **Conclusion:** Measuring sIgE to PVs and extracts from evolutionary distant fish species indicates bony and cartilaginous fish species for tolerance-confirming food challenges.

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Abbreviations:

FC- food challenge

HE- heated extract

PCA- principal component analysis

PPT- prick-to-prick test

PV- parvalbumin

RE- raw extract

sIgE- specific IgE

SPT- skin prick test

Abstract

Background: Recent studies indicated that fish-allergic patients may safely consume certain fish species. Multiplex IgE testing facilitates the identification of species tolerated by individual patients.

Methods: Sera were collected from 263 fish-allergic patients from Austria, China, Denmark, Luxembourg, Norway and Spain. Specific (s) IgE to parvalbumins (PVs) from 10 fish species along with IgE to 7 raw and 6 heated fish extracts was quantified using a research version of the ALEX^2 assay. IgE-signatures of individual patients and patient groups were analyzed using SPSS and R.

Results: sIgE to alpha-PV from ray, a cartilaginous fish, was not detected in 78% of the patients while up to 41% of the patients, depending on their country of origin, tested negative for at least one beta-PV. sIgE values were highest for mackerel and tuna PVs (>10 kUA/L) and significantly lower for cod (4.9 kUA/L) and sole PVs (2.55 kUA/L). 17% of the patients, although negative for PVs, tested positive for the respective fish extracts. Based on the absence of IgE to PVs and extracts, up to 21% of the patients were identified as potentially tolerating one or more bony fish. Up to 90% of the patients tested negative for ray. The probability of negativity to one fish based on negativity to others was calculated. Negativity to tuna and mackerel emerged as a good marker of negativity to additional bony fish.

Conclusion: Measuring sIgE to PVs and extracts from evolutionary distant fish species indicates bony and cartilaginous fish species for tolerance-confirming food challenges.

Keywords: fish allergy, fish tolerance, multiplex allergy diagnosis, parvalbumin, fish extracts

COI statement:

All authors declare that they have no conflicts of interest.

Introduction

Fish is a highly valuable and healthy food; however, it is among the most common foods that elicit IgE-mediated allergy. Over 90% of fish-allergic patients are sensitized to the major allergen beta-parvalbumin (PV), and for the last decades the cross-reactivity among PVs from various fish species was considered a hallmark of fish allergy.[1,2] Recent studies indicated that the real-life situation is far more complex. The prevalence of fish-allergic patients with a broad or limited cross-reactivity to various species was recently estimated to be 70% and 30%, respectively.[3]We previously demonstrated low IgE-reactivity to the alpha-PV from thornback ray, a cartilaginous fish, and oral tolerance of this fish by the majority of patients sensitized to bony fish beta-PVs.[4] In that study, 18 patients from Luxembourg were analyzed. Future studies investigating the tolerance of ray in larger multinational cohorts remained to be performed. Certain bony fish species may also be tolerated by patients sensitized to only one fish species.[5]

Precise diagnosis of fish allergy and identification of tolerated species is challenging due to the large number of fish species consumed worldwide, their varying allergen content, differences in allergenicity of alpha- and beta-PVs, and different fish preparation and processing methods.[6] Besides the PV, additional allergens, heat stable or not, may also be implicated.[7] Clinicians face the challenge to precisely determine tolerated versus symptom-eliciting species for each individual, as performing food challenges (FC) with multiple species and differently processed fish is nearly impossible. Patients with confirmed fish sensitization are routinely advised to strictly avoid all fish, which is often unnecessary. Hence, there is an unmet need to improve *in vitro*diagnostics to reduce the number of required FCs.[8] For improved *in vitro* diagnostics, the complementary use of purified natural or recombinant allergens and whole extracts should be considered. Purified allergens overcome the problem of low abundance of some allergens in aqueous extracts.[9,10] Whole extracts contain multiple allergens and may be used in raw or heated versions, which may be of different diagnostic value due to changes in allergenic properties of some allergens upon thermal processing.[11,12] Multiplex technologies allow the simultaneous quantification of serum IgE specific for individual allergens and whole extracts from multiple species.[13]

This is the first study which investigates IgE sensitization to PVs from 10 fish species, along with heated and raw fish extracts, using a large multinational cohort (n=263) of fish-allergic patients from 6 countries (Austria, China, Denmark, Luxembourg, Norway and Spain). This study setup allowed us to observe differences in sensitization patterns among individuals with different exposures and eating patterns. The selected species covered various fish families important for human consumption. Up to 90% of the patients, depending on the country, showed no IgE to PV and extracts from ray, while up to 21% tested negative for some of the bony fish species. For certain patients and species, IgE to extracts was higher than to PVs, demonstrating the importance of individual allergens, but also of extracts for accurate diagnosis.

Methods

Study subjects

Patients with a documented clinical history of fish allergy (n=263) were recruited from six countries (Austria, China, Denmark, Luxembourg, Norway and Spain). Their demographic and clinical characteristics are summarized in **Table I** and detailed in **Table SI** . Sensitization to fish was confirmed by ImmunoCAP (ThermoFisher Scientific), skin prick tests (SPT) to fish extracts and/or prick-to-prick test (PPT) to fresh fish for 255 patients. Total IgE measured by ALEX^2 (MacroArray Disgnostics) and ImmunoCAP demonstrated a significant positive correlation (Spearman's Rho=0.88) (**Figure S1**). Fish allergy symptoms' severities were scored from 1 to 4 (**Table 1)** . Anaphylaxis scoring was performed according to Ring et al.[14] Distribution of age groups and sex among the participants from each country is shown in**Figure S2** .

Informed written consent was obtained from all participants or their legal representatives and the study was approved by the Ethics Committees of the participating institutions: Austria- Ethics Committee of the

city of Vienna (EK-12-126-0712); China- Joint CUHK-NTEC CREC (2017.542); Denmark- (S-20210170); Luxembourg- CNER approval (201307/04); Norway- REK (2013/ 757); Spain- Hospital Clínic de Barcelona (HCB/2021/1129) and Hospital Sant Joan de Déu, Esplugues de Llobregat (PIC-97-20).

Fish parvalbumins and extracts

The parvalbumins (PV), raw extracts (RE) and heated extracts (HE) used in the ALEX^2 assay are listed in **Table SII** . PV purity and protein composition of the extracts are visualized in**Figure S3** . The methods used for PV and extract preparation are provided in the supplementary material. The phylogenetic analysis of PV sequences was performed in Jalview 2.11.1.3.[15] The sequences were aligned using ClustalO and the phylogenetic tree computed using the neighbor-joining algorithm.

IgE quantification

Patients' sera were applied individually to the research version of the ALEX^{2}Allergy Explorer (MacroArray Diagnostics). Total and allergen-specific IgE were quantified as described previously.[16] Values above 0.3 kU/L were considered positive.

Statistical analysis

The statistical analyses were performed using GraphPad Prism 9 (San Diego, CA), IBM SPSS Statistics 26 (Armonk, NY), R programming language and RStudio (Boston, MA), as explained in supplementary material.

Results

The low allergenicity of ray parvalbumin is confirmed in a multinational study of fish allergy

sIgE to each PV for all 263 patients was first quantified (**Figure 1A**). The lowest median sIgE was observed for ray alpha-PV followed by sole and cod beta-PVs. The highest values were found for mackerel and tuna PVs (>10 kUA/L). A comparison of IgE levels to PVs from different species demonstrated statistically significant differences for most pairs (**Table SIII)** . In total, IgE of only 22% of the patients recognized ray PV, while for bony fish beta-PVs sensitization was observed for between 75% (sole) and 92% (tuna) of patients (**Figure 1B**).

The pairwise correlation of the PV-sIgE levels showed a weak correlation between ray and any other PV (Spearman correlation coefficient [?]0.42) (**Figure 1C**), while the correlations between values for beta-PV pairs were strong. The correlations were statistically significant for all PV pairs (data not shown). To further support these results, the principal component analysis (PCA) was applied to the PV-sIgE levels. PC1 and PC2 explained approximately 82.0% and 8.6% of the variance contained in the PV data, respectively (**Figure S4**). All PV-sIgE scores except for ray showed a strong correlation with PC1 while correlating weakly with PC2. Conversely, ray PV-sIgE correlated strongly with PC2. The probability of IgE test to be positive for one PV if positive for another was also determined (**Figure S5**). A positive test for any beta-PV indicated >80% probability of positivity to other beta PVs.

Next, the median IgE levels to each PV in each country were determined, and the highest were observed for the Norwegian cohort, while ray PV demonstrated lower IgE than any other in each country (**Figure 1D**). To analyze whether the evolutionary distance among the PVs may be related to their different IgE levels, their sequences were aligned (**Figure S6A**) and a neighbor-joining phylogenetic tree was computed (**Figure S6B**). Interestingly, not only ray alpha-PV, but also sole beta-PV clustered separately from the others.

Diversity of parvalbumin-specific IgE levels varies in demographic groups but not as a function of symptom severity, age or gender

To investigate whether the diversity of IgE responses to different PVs depends on clinical or demographic characteristics, Shannon's diversity index was calculated for each patient based on four categories of the IgE levels to 10 parvalbumins (<0.3 kUA/L, 0.3-3.5 kUA/L, 3.5-13 kUA/L, >13 kUA/L). The diversity of the

PV-sIgE levels in serum did not depend on symptom score (**Figure 2A**), age group (**Figure 2B**) or sex (**Figure 2C**). A significantly higher diversity of IgE levels to different PVs was observed for the Chinese cohort than for the Austrian, Spanish or Norwegian cohorts (**Figure 2D**).

Up to 41% of fish-allergic patients may tolerate at least one bony fish based on the absence of PV-sIgE

To investigate possibly tolerated fish species based on the absence of PV-sIgE, we first determined the percentage of the patients negative to each investigated PV in each analyzed country (**Figure 3A**). IgE of 78% of patients in total did not recognize ray PV, with the highest proportion of such patients in Luxembourg (97%) and the lowest in Spain (64%). 25% of patients were negative to sole PV (highest proportion in Luxembourg, 41%), while 19% in total and up to 38% (China) were negative to cod PV. To investigate how to best identify the absence of IgE to PV from specific species, the probability of an IgE test to be negative for a specific PV, when already negative for another specific PV, was calculated (**Figure 3B**). Among the beta-PVs, tuna, mackerel and carp appeared as the best predictors of highly probable negative IgE tests for other beta PVs. For example, if negative for tuna PV, a probability of [?]90% exists for an IgE test to be negative for any other tested PV, except for salmon (71%). These findings should be considered when designing a panel of species for tolerance-confirming FC.

The complementary use of fish extracts enhances the diagnostic performance of the molecular approach

Besides PVs, the presence of IgE to other fish allergens should be investigated before continuing with FC to confirm specific fish tolerance. Our analyses showed lower IgE levels to raw and heated fish extracts than to PVs for all bony fish (**Figure 4A**). To determine the correlation between the IgE levels for PV and RE, PV and HE, or RE and HE, Spearman's rank correlation coefficient was calculated. Data were interpreted (weak/moderate/strong/very strong correlation) according to Schober et al.[17] A strong significant positive correlation (Rho>0.7) of all three pairs was observed for herring and salmon (**Figure 4B**). In addition, a very strong significant positive correlation (Rho=0.98) between values for cod PV and RE was shown, indicating that for cod, a RE is sufficient to detect the PV sensitization. For other analyzed species, correlations of IgE levels to PVs and extracts were weak to moderate (**Figure 4B**). Data for REs and HEs correlated strongly for herring (Rho=0.85) and salmon (Rho=0.75), while moderate to weak correlations were observed for other species. All correlations were significant, except for tuna PV vs RE and tuna PV vs HE (**Figure S7**). Individual scatterplots for each species and calculated P values for the correlation coefficients are shown in **Figure S7** .

We next assembled data for patients with higher IgE values to extracts than to PV from one or more species (44 patients in total). The percentage of these patients varied across the countries (8% - 28%) (**Figure 4C**). Data for each patient was then analyzed in detail (**Figure 4D**). Up to 7 patients per fish species had no IgE for the respective PV, but were positive for RE and/or HE. These patients could be falsely diagnosed as negative to some species if only the PV-sIgE would be quantified.

A combinatory approach of negative IgE to PVs and extracts allows stratifying the patients for tolerance-confirming FC with bony or cartilaginous fish

At last, the proportion of the patients who may tolerate specific fish was investigated, based on negative *in vitro* IgE tests to PV, RE and HE. Between 60% (Spain) and 90% (Luxembourg) of the patients were negative to ray PV, RE and HE (**Figure 5A)** . The percentage of the patients negative to each tested bony fish varied across the countries and up to 21% of the patients (mackerel, Denmark) were negative to one or more bony fish. For the cohorts from Austria, Denmark, Luxembourg and Norway, all tested species were found to be potentially tolerated by some patients. Only ray and herring were shown to be the potentially tolerated in the Chinese cohort, while for Spain, except for tuna, IgE tests to all other species were negative for some patients (**Figure 5A**). All patients negative to PV, REs and HEs from specific species are listed in **Table SIV** . Based on these findings, 8 patients underwent prick-to-prick (PPT) with ray (*Dipturus innominatus* available in China), of which 6 showed skin reactivity neither to raw nor to cooked ray. These

patients are candidates for future FCs to confirm their clinical tolerance. The patients with absence of reactivity to ray by PPT are labelled with an asterisk (*) in **Table SIV** .

Figure 5B shows the probability that all 3 IgE tests (to PV, RE and HE) are negative for species X (x-axis) if negative for species Y (y-axis). These data can be used to predict possible tolerance of specific species if tolerance of others is known. Negativity to tuna and mackerel showed the highest likelihood of negativity not only to ray but also to other bony fish. Specifically, patients negative to tuna have 92% probability to be negative to ray and swordfish. If negative to mackerel, 93% chance exists to be negative to herring and ray, and 86% to swordfish. However, if negative to salmon or swordfish, the probability of IgE tests to be negative to other bony fish is [?]58%.

Discussion

This multinational study indicates that, based on fish PV- and extract-sIgE detection for 263 fish-allergic patients, up to 90% may tolerate cartilaginous fish. In addition, up to 21% of fish-allergic patients may tolerate some bony fish species. Multiplex *in vitro*IgE quantification, using purified allergens and extracts from various fish species, may be utilized to identify patient candidates and fish species for FCs, with the ultimate goal to confirm the clinical tolerance of some species.

Fish allergens from different species share limited similarities. While some IgE epitopes of parvalbumins are highly conserved, species-specific epitopes have also been identified.[18,19] Additionally, not all species have equal allergenic potential.[20] We previously showed the low reactivity to cartilaginous fish among bony-fish allergic patients due to the evolutionary distance between beta- and alpha-PVs from bony and cartilaginous fish, respectively.[4] In addition, a significant proportion of fish-allergic patients may tolerate certain bony fish.[5] Besides PVs, other fish allergens have been identified (e.g. tropomyosin, lactate dehydrogenase, glucose-6-phosphate isomerase, creatine kinase) but their cross-reactivity potential is unknown.[21,22]

In addition to species and allergen diversity, eating habits differ across the world. Fish is subject to different cooking methods, which may impact allergen stability and IgE reactivity.[23,24]Beta-PVs demonstrated higher heat stability than enolase and aldolase.[3] The variety of species, allergens and processing methods present a challenge for diagnosis. Certain region-specific important species may be absent from common diagnostic tests, and routine approaches often focus on limited number of species.[25] High-risk patients with fish allergy are recommended strict avoidance of all fish, which is often unnecessary.[25] Next-generation diagnostic approaches are hence necessary and multiplex molecular allergy diagnosis emerges as a promising tool.[26,27]

A research version of the ALEX^2 multiplex assay (Macro Array Diagnostics), with values of sIgE strongly correlated to those obtained by ImmunoCAP-ISAC (Thermo Fischer)[28] was used here to quantify total, fish extract- and PV-sIgE in fish-allergic patients' sera. The ALEX^2 platform was previously successfully utilized in quantifying serum IgE of patients with atopic dermatitis, dust mite allergy and nut allergy.[29-31] We first investigated the IgE levels to PVs from 10 fish species. The species were selected based on published phylogenetic analyses, covering species relevant for consumption, and those responsible for monosensitizations.[3,32] The median sIgE values were the highest for tuna and mackerel PVs (>10 kU/L), followed by herring, carp, salmon and swordfish (> 8kU/L), ocean perch (6 kU/L), cod (5 kU/L), sole (2.5 kU/L) and ray (<0.3 kU/L) (**Figure 1A**). High IgE levels to tuna and swordfish PVs were unexpected, considering previous reports on their lower allergenicity due to low PV abundance in their dark muscles.[33,34] Our data indicate the presence of shared IgE epitopes with other beta-PVs. However, the clinical relevance of these findings, and their clinical cross-reactivity require future investigations. Significantly lower IgE for cod PV than for several other beta PVs demonstrated that cod PV, a commonly used diagnostic marker, may not be sufficient for accurate fish allergy diagnosis. In this study, 22% of the patients were sensitized to ray PV (**Figure 1B**), showing that tolerance of cartilaginous fish may be a possibility for many but not all bony-fish sensitized individuals.

Fish consumption differs across geographic regions in terms of quantity and processing.[8] According to the Food and Agriculture Organization, China was the top fish consumer in 2017 worldwide based on total supply. Among the countries from our study, the highest per capita fish consumption was estimated for

Norway (51.4 kg) followed by Spain (42.5 kg) and China (38.8 kg).[35] IgE to PVs from different species may reflect exposure and indicate the species most relevant for diagnosis. Interestingly, we observed the highest IgE to all PVs for cohorts from Norway, China and Spain, and each tested country displayed weaker IgE to ray and sole PVs than to others (**Figure 1D**). However, IgE levels to most commonly used PVs in diagnostics differed depending on the country. Cod PV-sIgE was overall low and the highest for patients from Spain and Norway, while tuna PV-IgE was high (in comparison to other PVs) in all cohorts. Currently we cannot reach conclusions about the clinical relevance of IgE binding to tuna PV.[34] Nevertheless, tuna PV appears as highly cross-reactive protein to other beta PVs and its potential use in diagnostic assays should be investigated in the future. Although the country-specific distribution of IgE to different PVs may in part be a result not only from IgE specific to single PVs but also cross-reactive IgE, our data indicate important PVs for fish-allergy diagnosis for each country.

The diversity of PV-sIgE levels was analyzed next (**Figure 2**). Shannon's diversity did not depend on patient's symptom severity, age or gender, suggesting that IgE profiles to different PVs are largely independent of these factors, and each patient should be subjected to a wide-range diagnosis with multiple fish species.

In our study, up to 41% of patients were negative to some of the bony fish PVs and up to 97% to ray PV (**Figure 3A**). Previous studies indicated that up to 30% of patients may tolerate some fish species.[3] This variable reactivity to different PVs emphasized the importance of including allergens from diverse fish families into diagnostic approaches. Furthermore, based on probability calculations (**Figure 3B**), we demonstrated that tuna PV may be utilized to predict whether patients may be negative to other PVs when negative to this protein.

Additional allergens such as enolase, aldolase or collagen may be important for some patients. Our previous study on 101 fish-allergic patients showed sensitization to fish collagen by 21%.[36] Another study reported the varying prevalence of sensitization to enolase and aldolase depending on fish species, being as high as 70% for cod enolase.[5] Some allergens lost their IgE-binding capacity upon heat treatment.[37]In contrast, HEs may contain gelatin, still able to bind IgE.[38,39] In this study, lower IgE levels to extracts were observed compared to PVs (**Figure 4**), indicating that for patients with predominant sensitization to PV, the dilution factor of the parvalbumin in whole extracts coated on allergen-detection systems may yield a negative test result. In contrast, to determine the sensitization to other known and yet unknown allergens, whole extracts may be required. This was emphasized by negative result for PVs but positive results for extracts for some patients. Additionally, for most of the species, correlation between IgE levels to PVs and extracts was weak, indicating that all three components (PV, RE and HE) are required for diagnosis.

Finally, we investigated patients with no IgE to PV, RE and HE from the same fish, as potential candidates for the tolerance-confirming FCs. As many as 90% of the patients were detected as candidates for future FCs with ray and up to 21% with specific bony fish species (**Figure 5A**). Negativity to ray was confirmed for 6 of 8 tested Chinese patients by PPT. For the PPT, the *Dipturus innominatus* ray, available in China, was used. Although *Dipturus innominatus* and *Raja clavata* (used in the ALEX^2 assay) belong to the same fish family (Rajidae)[40] and high IgE cross-reactivity is expected, the possibility of unshared epitopes on some allergens cannot be excluded, possibly explaining the 2 positive PPT outcomes. Larger studies are required to confirm negative *in vitro* IgE data using functional assays or FC, and to calculate the predictive values of the multiplex *in vitro* diagnosis. When IgE quantification for many species cannot be performed, our calculations of probability to be negative to some fish if known to be negative to another (**Figure 5B**) may be used as a guideline for selecting the panel for *in vitro* testing. A recently published prospective clinical study showed development of fish tolerance with age.[41] Our data may therefore also be used in the context of determining the species for testing tolerance development over time. A limitation of our study is the absence of FCs for tolerance confirmations. Schulkes et al. indicated a frequent serological, but limited clinical cross-reactivity between fish species.[42] Although a further investigation of the clinical significance of our data is required, we provide a clear direction for future studies which should carefully choose the species for specific patients to explore tolerance.

Together, the presented data demonstrate the need for fish extracts in both raw and heated form, and

the PVs from several evolutionary distant species for next-generation fish allergy diagnosis, which will enable identification of potentially safe-to-consume species for individual patients. Ultimately, combining the knowledge about the important species and allergens with novel bioinformatic approaches will permit the design of region-specific diagnostic arrays, which will significantly improve safety and wellbeing of fish-allergic individuals.

Figure legends:

Figure 1. sIgE to fish PVs. A) The concentration of PV-sIgE in patients' sera (n=263). Dashed line-threshold for positive signal; full line-median; dotted line-quartiles. Statistical analysis demonstrating significant differences between the PVs is provided in Table SIII. B) Percentage of patients positive to each PV. C) Spearman correlation of PV-sIgE levels (numbers show the correlation coefficient). The order of the PVs on the x-axis is according to the degree of IgE levels' correlation to those for ray PV. D) Median levels of IgE to each PV in each country.

Figure 2. Diversity of PV-sIgE levels. Shannon's diversity index is shown for the patient groups with different symptom scores (A), gender (B), sex (C) and country of origin (D). Groups for diversity calculations were based on four categories of sIgE levels (kUA/L): 1) <0.3 2) 0.3-3.5 3) 3.5-13 4)>13. Statistical tests included the Kruskal-Wallis test with Dunn's multiple comparison test for comparison between more than two groups (A, B and D), and the Mann Whitney test for two groups (C). *P<0.05; **P<0.01.

Figure 3. Distribution of the patients negative to one or more PVs. A) Percentage of the patients with IgE<0.3 kUA/L for each PV in each investigated country. B) The probability of the IgE test to be negative for PV on x-axis, if negative for PV on y-axis. The order of the PVs is according to the strongest correlation to results obtained for ray PV.

Figure 4. The complementary use of fish extracts in molecular diagnosis. A) Concentration of IgE specific to PVs, raw extracts (RE) and heated extracts (HE) from seven fish species in sera of fish-allergic patients (n=263). Full line indicates median. Dashed line indicates threshold for positive signal. Each dot presents a signal for an individual patient. B) Spearman correlation coefficient demonstrating correlations between IgE values obtained for PVs and extracts from each fish PV for all 263 patients. Error bars indicate 95% confidence interval for the calculated correlation coefficient. C) Country-specific percentage of patients with higher IgE to raw and/or heated extracts than to PVs from one or more species. D) Comparison of IgE values to PVs and extracts for each patient with higher IgE to raw and/or heated extract than to PV from the same species. Dashed line is a threshold for positive signal.

Figure 5. Patients negative to *in vitro* IgE tests with parvalbumins, raw and heated extracts from specific fish. A) Percentage of patients (n=263) negative in ALEX^2 assay to PV&RE&HE from each tested fish. B) Probability (0.0-1.0) that a patient is negative for PV&RE&HE tests for fish on x-axis if negative for PV&RE&HE for fish on y-axis. Fish species are in alphabetical order.

Tables:

Table I. Demographic and clinical characteristics of recruited fish-allergic patients.

Number of patients	263
Austria China Spain Denmark Luxembourg Norway	81 5
Age (years)	**Age**
Median Range	12 1
Sex (male/female)	160/
Total IgE by ImmunoCAP (kU/L)*	**Tot**
Median Range	545
Total IgE by ALEX^2 (kU/L)	**Tot**
Median Range	729
Cod-sIgE by ImmunoCAP (kUA/L)**	**Cod**

Number of patients	263
Median Range	4.57
Symptoms of fish allergy	**Syn**
Mild local (Score 1) Anaphylaxis grade 1 (Score 2) Anaphylaxis grade 2 (Score 3) Anaphylaxis grade 3 (Score 4) NA	17 1
Fish SPT (positive/ negative/ not done)* **	184/
Fish PPT (positive/ negative/ not done)* **	33/1

*Determined for 209 patients. Due to the upper ImmunoCAP cutoff for total IgE, values >5000 kU/L were counted as 5000 for the calculation of median. **Determined for 209 patients. Due to the upper ImmunoCAP cutoff for specific IgE, values >100 kUA/L were counted as 100 for the calculation of median. ***Positive was considered any wheel diameter of or larger than 3 mm after subtraction of negative control.

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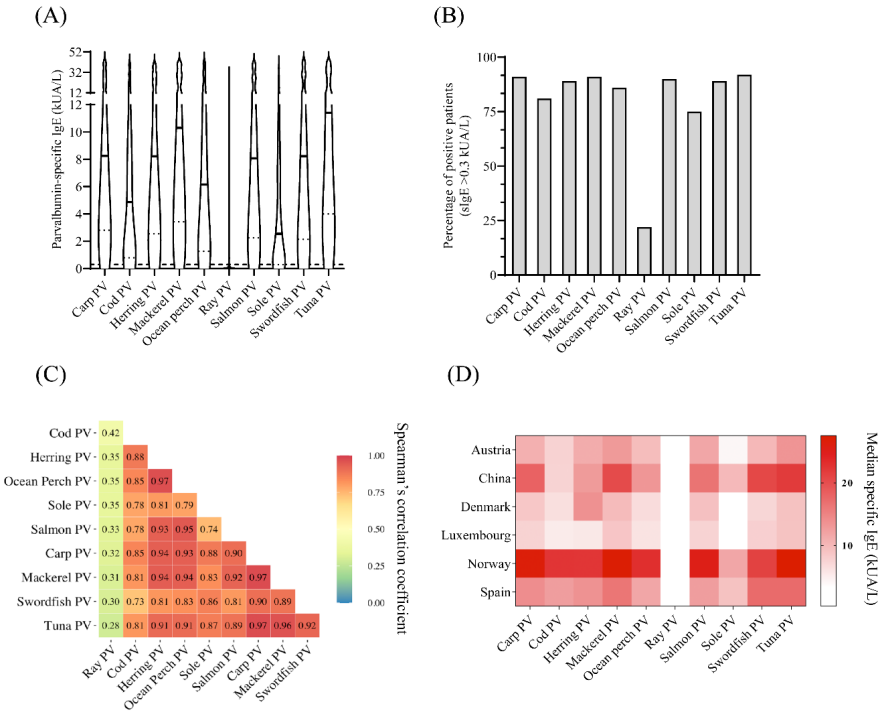

Figure 1_Kalic et al.

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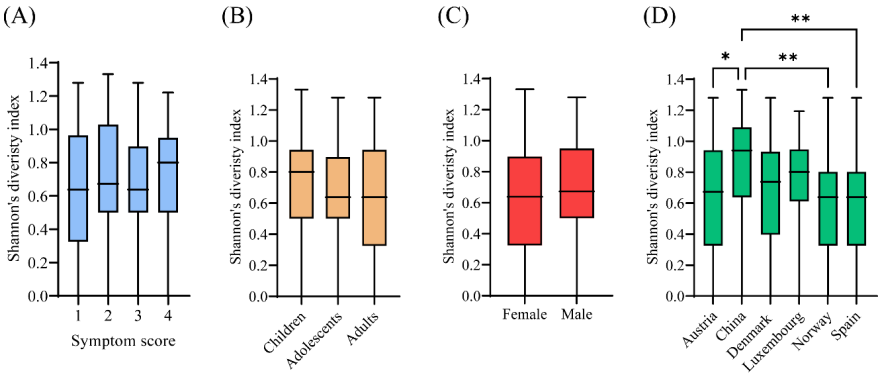

Figure 2_Kalic et al.

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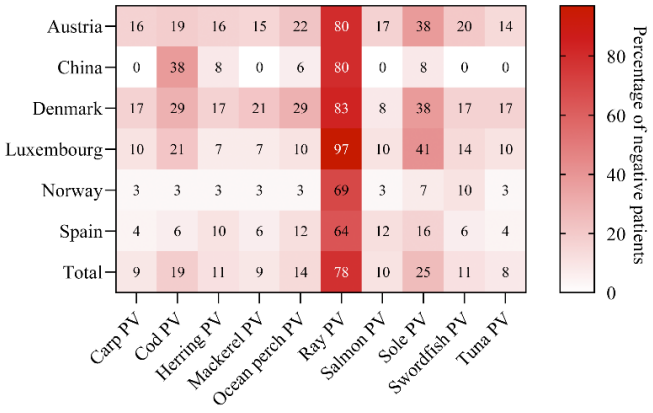

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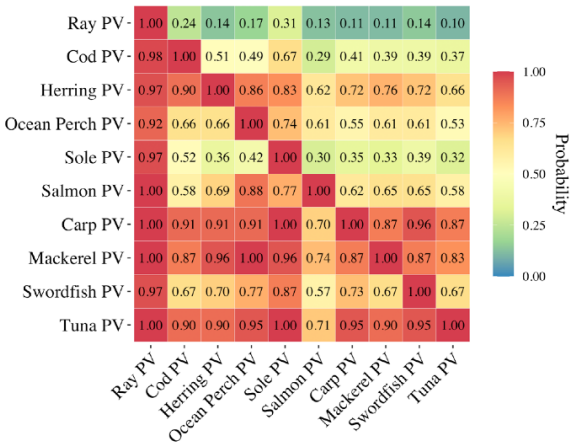

Figure 3_Kalic et al.

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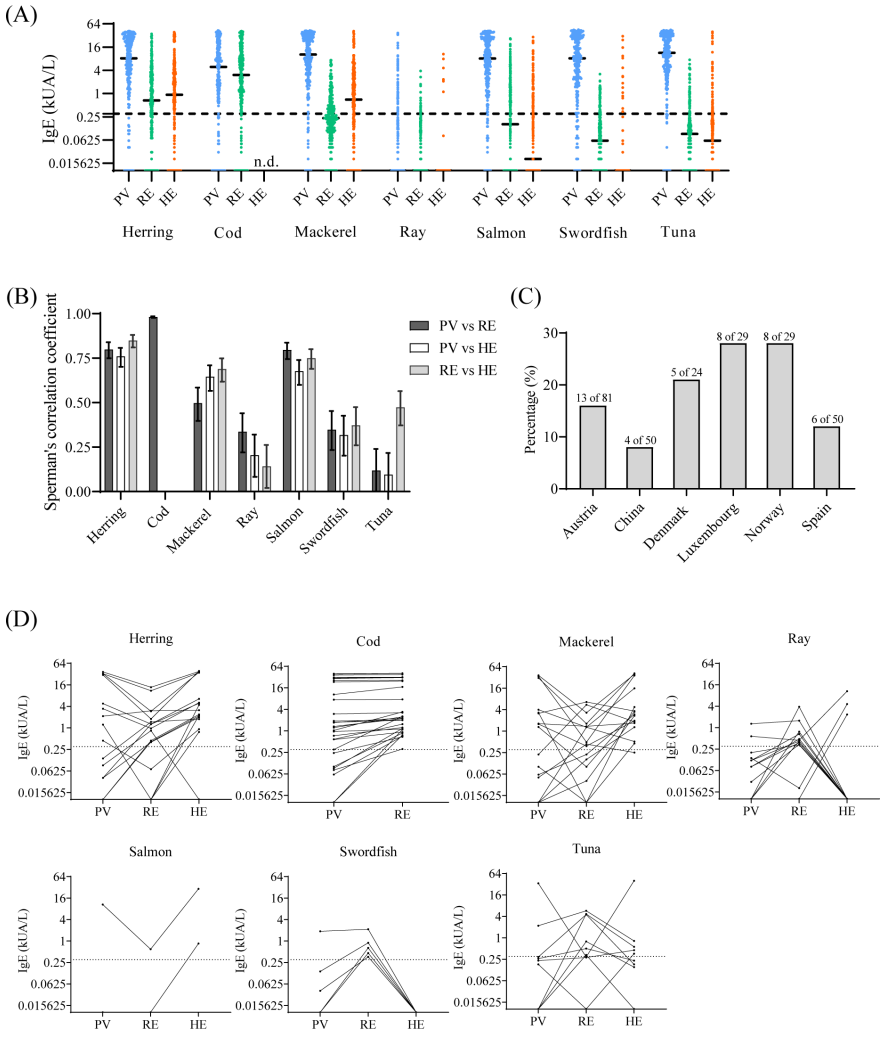

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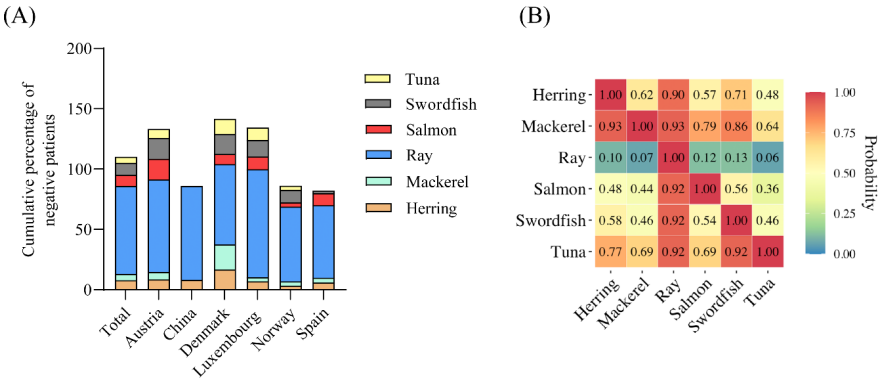

Figure 5_Kalic et al.

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