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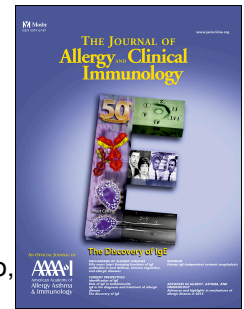
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Cross-reactivity in Fish Allergy: A Double-Blind Placebo-Controlled Food

Challenge Trial

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Clinical Implications: Measurement of specific IgE to fish allergen molecules reduce the number of oral fish challenges needed to identify allergic or tolerant patients.

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50 **Capsule summary:** One third of fish-allergic patients tolerate at least one fish species.

51 Specific IgE testing can identify most patients with fish allergy and reduce the need of oral

52 challenges in the diagnostic work-up.

53

54 **Key words:** Fish allergy, sIgE, sensitization, cross-reactivity, cod, salmon, mackerel,

55 parvalbumin, aldolase, enolase, threshold dose.

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To the Editor:

Fish is a healthy nutrient and a common food allergen. Cross-reactivity between fish species exist and some patients report tolerance to certain species¹ but data from food-challenge trials are scarce.^{2,3} Parvalbumin is the major fish-allergen⁴⁻⁶ while recently fish muscle enolases and aldolases were identified as new fish-allergens.⁷ We present the first double blind, placebo-controlled food challenge (DBPCFC) trial with different fish species, evaluating the correlation between clinical reactivity and IgE-reactivity to fish allergen molecules in fish-allergic patients.

In this trial, 35 subjects (5-19 years) with physician-diagnosed fish allergy and sensitization to fish-allergen underwent DBPCFC with cod, salmon and mackerel, followed by open food challenges (OFC) if DBPCFC's were negative. Study design (Figure E1), patients' background characteristics (Table E1), and methods are described in this article's Online Repository at www.jacionline.org.

Any allergic symptoms were observed/reported from 33 (cod), 28 (salmon) and 28 (mackerel) participants. Five participants reported mild, transient subjective symptoms on placebo challenge but had unequivocal findings during the DBPCFC-active arm and are thus included in the analysis (Table E 2, Online Repository).

We found tolerance to at least one of three fish species (partially tolerant) in 10 (29%) participants regarding any symptoms (Table 1a). Table 1b shows reactivity in partially tolerant participants. There was no difference between non-tolerant (reacting to all three fish species), and partially tolerant participants regarding age, sex, other allergies, parental allergy, asthma or atopic dermatitis.

Oral itching and swellings/blisters were the most frequent subjective and objective symptoms, respectively (Figure E 2a-b, Online Repository). Involvement of more than one organ was

seen in 14/35 participants. Upon challenge, two participants received adrenaline, but none had severe anaphylaxis requiring further observation or treatment.

Sensitization to all three fish-allergen extracts and parvalbumins was found in nearly all participants, whereas sensitization to enolases/aldolases was predominantly found in participants with objective symptoms (Figure 1a-c). However, many participants with objective symptoms were not sensitized to the corresponding enolase/aldolase.

Non-tolerant participants had higher sIgE levels to fish-allergen extracts and parvalbumins compared to partially tolerant participants, whereas no difference was seen for enolase and aldolase (Table E 3, Online Repository). Specific IgE to extracts from cod and salmon discriminated best between non-tolerant and partially tolerant groups. IgE to cod extract >8.2 kU_A/L or salmon extract >5.0 kU_A/L identified 18/24 and 19/24 non-tolerant participants, respectively, whereas below these cut-off values, 8/10 and 9/10 partially tolerant participants were identified (Figure E 3, Online Repository).

Finally, we estimated the eliciting dose predicted to provoke an allergic reaction in 10% of individuals (ED₁₀), based on dose distribution curves (Table E4 and Figure E 4, Online Repository). We found ED₁₀ to be 0.7 mg and 23.8 mg of cod protein for subjective and objective symptoms, respectively, in line with previously published data.⁸ Low number of participants with objective reactions to salmon and mackerel restricted us to produce reliable dose distribution curves but a comparison of lowest observed adverse effect levels (LOAEL) for objective symptoms to cod, salmon and mackerel (Figure E 5a-c, Online Repository) shows more participants with low LOAELs for cod compared to salmon and mackerel.

Table E 5, Online Repository, shows individual results from 35 participants with sIgE values and the results from DBPCFC/OFC.

This is the first DBPCFC-trial comparing clinical and sIgE cross-reactivity to fish allergen molecules with different fish species. More than half of all participants had objective tolerance, and around one third had subjective tolerance to at least one fish species. By combining sIgE against conventional fish-allergen extracts and fish-allergen molecules, we could predict the outcome of challenge in the majority of patients. Cod allergy was most prevalent, cod elicited more serious symptoms and had lower ED₁₀/LOAEL, compared to salmon and mackerel. This may be due to local dietary traditions with high consumption of cod and cod being the primary sensitizer. Although unproven, differences in parvalbumin stability between species may theoretically play a role. The poor ability of fish-allergen extracts and parvalbumins to identify tolerance to specific fish species may be due to sIgE-cross-reactive parvalbumins.^{4,9} More sensitization to cod enolase/aldolase, compared to salmon and mackerel enolase/aldolase may be due to less inter-species IgE cross-reactivity between enolases/aldolases and cod being the primary cause of sensitization.

Although allergen levels may vary in different allergen extract batches⁹, patients with an obvious history of self-reported fish allergy and sIgE to cod extract > 8.2 kU_A/L or salmon extract >5 kU_A/L may be advised to avoid all fish species. Mackerel extract is less reliable since median IgE to extract is lower than to parvalbumin (Table E3, online repository). Sensitization to enolase/aldolase most likely reflects true fish allergy, whereas non-sensitized patients may be allergic and must undergo a challenge. However, based on our results, an OFC protocol could be prepared for low-risk patients to test new fish species in small amounts without the need of a full DBPCFC. Our results indicate that sIgE to enolase/aldolase may have a role in diagnosing fish allergy when it comes to cod, salmon and mackerel.

A limitation of this study is low number of participants, attenuating the power to detect differences between groups. Secondly, children of lower age had higher dropout rates due to poor palatability of the test food, but none dropped out due to allergic reactions. We

believe the data represent good estimates of cross-reactivity and severity in the fish-allergic population but results may not be applicable to other regions. The main strength is the randomized, placebo-controlled design using standardized test food, followed by OFC. The basophil activation test will be included in a follow-up to further elaborate on the diagnostic differentiation between sensitization and true allergy.

In summary, more than half of participants had no objective symptoms, and around one third, no subjective symptoms to at least one fish species. Doses provoking allergic reactions are lower for cod compared to salmon and mackerel. Patients with partial tolerance should be identified to avoid unnecessary food restrictions. A combination of clinical history and sIgE to fish-allergen extracts and molecules can significantly reduce the number of food challenges needed for specific diagnosis of fish allergy.

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Author contributions

Martin Sørensen wrote the protocol, participated in data retrieval, analysed the data, wrote the first version of the manuscript and revised the manuscript. **Annette Kuehn** measured specific IgE to the fish-allergen molecules at the Department of Infection and Immunity, Luxembourg Institute of Health, and reviewed and revised the manuscript. **Clare Mills** supervised and helped conceptualize the protocol and study analysis, reviewed and revised the manuscript, and were responsible for the manufacture of test-food for DBPCFC. **Carol-Ann Costello**, The Molecular Allergology Group, Manchester Institute of Biotechnology, manufactured and quality checked the chocolate desert matrixes used in DBPCFC and revised the manuscript. **Markus Ollert** supervised on fish-allergen molecules, reviewed, and revised the manuscript. **Lars Småbrekke and Raoul Primicerio** performed statistical analyses and estimated threshold doses, using dose distribution curves, and reviewed and revised the manuscript. **Magnus Wickman** supervised and helped conceptualize the protocol and study analysis, reviewed and revised the manuscript. **Claus Klingenberg** conceptualized and designed the study, directed all phases of the study, and revised the final manuscript.

All nine authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work. Martin Sørensen and Claus Klingenberg had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Table 1a Status of tolerance to cod, salmon and/or mackerel, confirmed with DBPCFC/OFC

Status of Tolerance	Objective allergic symptoms n (%)	Any allergic symptoms n (%)
Non-tolerant	15 (43%)	24 (68%)
Partially tolerant	19 (54%)	10 (29%)
Tolerant*	1 (3%)	1 (3%)
Total	35 (100%)	35 (100%)

Non-tolerant: Symptoms to cod, salmon and mackerel. Partially tolerant: Symptoms to one or two of cod, salmon and mackerel. Tolerant: No symptoms to cod, salmon and mackerel. *One participant with a previous convincing history of fish allergy turned out to be clinically tolerant to all three species. He was sIgE-positive to cod, salmon and mackerel extracts (sIgE 0.87, 0.79 and 0.15 kU_A/L, respectively), but not to any of the fish-allergen molecules, and had most likely outgrown his fish allergy.

Table 1b. Phenotypes of fish allergy in partially tolerant participants

Symptoms to	Objective allergic symptoms n (%)	Any allergic symptoms n (%)
Cod only	7 (20%)	2 (6%)
Salmon only	2 (6%)	0
Mackerel only	0	0
Cod and Salmon	6 (17%)	3 (9%)
Cod and Mackerel	4 (11%)	4 (11%)
Salmon and Mackerel	0	1 (3%)
Total	19 (54%)	10 (29%)

Figure legends

Figure 1a. Sensitization to fish extracts and molecules in participants with objective (n=32), only subjective (n=1) and no symptoms (n=2) to cod during DBPCFC/OFC.

Legend:

* DBPCFC/OFC = Food challenge cod. Light blue bar, first group of bars: Number of participants with objective symptoms during cod challenge. Light blue bar, second group of bars: Number of participants with only subjective symptoms during cod challenge. Light blue bar, third group of bars: Number of participants with no symptoms during cod challenge. All other bars are number of participants sensitized (sIgE > 0.1 kU_A/L) to cod using four different fish-allergens assays.

Figure 1b. Sensitization to fish extracts and molecules in participants with objective (n=23), only subjective (n=5) and no symptoms (n=7) to salmon during DBPCFC/OFC.

Legend:

* DBPCFC/OFC = Food challenge salmon. Light blue bar, first group of bars: Number of participants with objective symptoms during salmon challenge. Light blue bar, second group of bars: Number of participants with only subjective symptoms during salmon challenge. Light blue bar, third group of bars: Number of participants with no symptoms during salmon challenge. All other bars are number of participants sensitized (sIgE > 0.1 kU_A/L) to salmon using four different fish-allergens assays.

Figure 1c. Sensitization to fish extracts and molecules in participants with objective (n=19), only subjective (n=9) and no symptoms (n=7) to mackerel during DBPCFC/OFC.

Legend:

* DBPCFC/OFC = Food challenge mackerel. Light blue bar, first group of bars: Number of participants with objective symptoms during mackerel challenge. Light blue bar, second group of bars: Number of participants with only subjective symptoms during mackerel challenge. Light blue bar, third group of bars: Number of participants with no symptoms during mackerel challenge. All other bars are number of participants sensitized (sIgE > 0.1 kU_A/L) to mackerel using four different fish-allergens assays.

Figure 1a. Sensitization to fish extracts and molecules in participants with objective (n=32), only subjective (n=1) and no symptoms (n=2) to cod during DBPCFC/OFC.

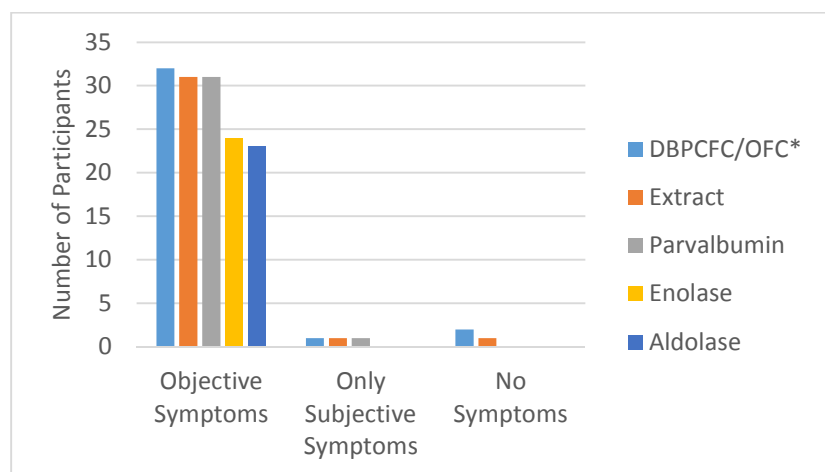


Figure 1b. Sensitization to fish extracts and molecules in participants with objective (n=23), only subjective (n=5) and no symptoms (n=7) to salmon during DBPCFC/OFC.

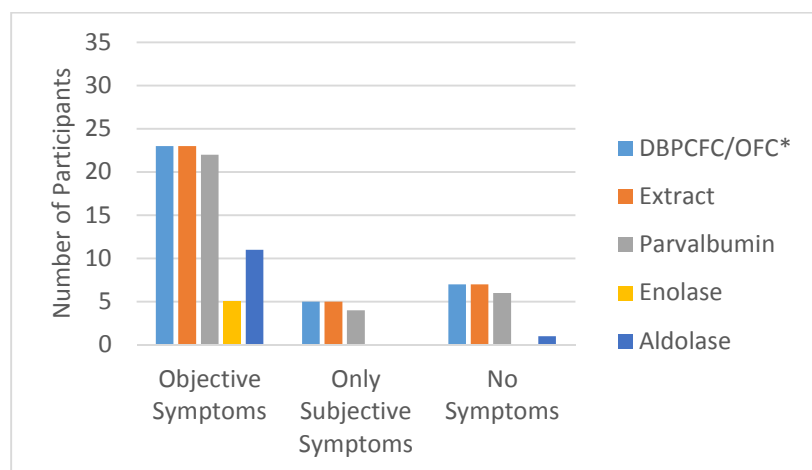
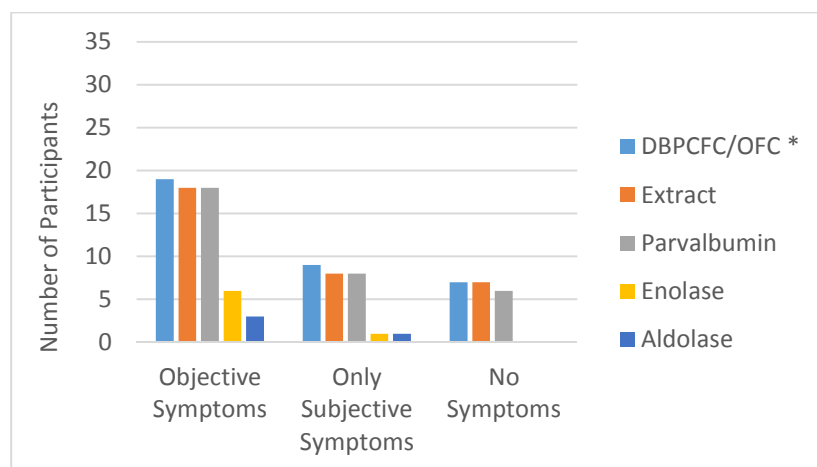


Figure 1c. Sensitization to fish extracts and molecules in participants with objective (n=19), only subjective (n=9) and no symptoms (n=7) to mackerel during DBPCFC/OFC.



Cross-reactivity in Fish Allergy: A Double-Blind Placebo-Controlled Food Challenge Trial

Online Repository Material:

Methods

Patient cohort

Seventy-three children and adolescents (age 5-19 years, median 11.0 years) with a convincing clinical history of fish allergy from medical records, and positive sIgE to fish-allergen extracts were invited to this study (Figure E 1, Online Repository). Eligible participants were identified from the pediatric outpatient clinic, University Hospital of North Norway (63 patients) and the 2009-2013 hospital laboratory database (additional 10 patients).

Double-blind, placebo-controlled food challenge (DBPCFC)

The National Food Research Institute in Norway produced dried powder from cooked, skinned and boned cod, salmon and mackerel fillets. Fish-powder protein content was calculated according to established food analysis procedures using the Kjeldahl method and nitrogen conversion factor of 6.25. The Molecular Allergology Group, Manchester Institute of Biotechnology, manufactured chocolate dessert matrices based on a EuroPrevall recipe¹, alone (placebo) and containing varying doses of the dried fish-powders. Blinding of fish content was assessed using an informal, blinded, sensory panel. The chocolate matrices were stored at -20°C and warmed to 4°C overnight prior to reconstituting with sweetened water and flavor. All reconstituted desserts were stored at 4°C and used within 24 h.

Low-dose DBPCFCs with cod, salmon, mackerel and placebo were performed in a randomized order (Microsoft Access, Random Numbers-RND Function) according to international guidelines.² Fish-containing desserts and placebo dessert were administered on

four separate challenge days, with six weeks intervals. Escalating fish protein doses of 3 µg, 600 µg, 12.5 mg, 120 mg and 1 g were served with 30 min intervals. Trained study nurses, blinded to the test food, recorded subjective and objective allergic symptoms based on previous published approaches³ (Table E VI, Online Repository). Subjective symptoms were assessed using a visual analogue scale (VAS). A pediatric allergist, blinded to the test food, classified all DBPCFCs as negative or positive, regarding subjective and objective symptoms. The DBPCFC was stopped if either the predefined stop criteria were achieved or all doses completed. Anaphylaxis was defined and treated according to international guidelines.⁴

Open food challenge (OFC)

Participants without objective symptoms during DBPCFC underwent OFC using oven-baked (80°C for 10 min) fish-burgers produced from minced fish fillet (cod, salmon or mackerel), potato flour, water, salt and pepper, vacuum packed and stored frozen at -20°C. The fish burgers were served after thawing over night at 4 °C and heating in vacuum pack for 3 min in water at 80°C. Fish-burger protein content was measured with the same method as for the fish powders. Escalating fish protein doses of 2 g, 6 g and 12 g were served, with 30 min intervals. A cumulative dose of 20 g fish protein corresponds to approximately 100 g of fish fillet. Both the participant and the pediatric allergist were blinded to the food being tested. As a test for blinding, all participants guessed which fish species they had eaten and they guessed correctly after 31% (mackerel), 46% (cod) and 56% (salmon) of each completed test meal.

Specific IgE to fish-allergen extracts and molecules

sIgE to cod, salmon and mackerel allergen extracts was determined by ImmunoCAP (Phadia-ThermoFisher, Uppsala, Sweden). Sensitization was defined by sIgE > 0.10 kU_A/L. sIgE to the fish-allergen molecules parvalbumin, enolase and aldolase from cod, salmon and mackerel were determined at the Department of Infection and Immunity, Luxembourg Institute of Health using enzyme linked immunosorbent assays, as previously reported.⁵ Parvalbumins,

enolases and aldolases from the three fish species were purified by column chromatography to homogeneity followed by purity and identity check⁵, and classified according to the International Union of Immunological Societies.⁶

Statistical methods

Statistical analyses were performed using IBM SPSS® statistics, version 23. Study participants and results from the fish challenges were characterized using summary statistics including Pearson's chi-square test in univariate comparisons of categorical variables and Mann-Whitney U test for univariate comparisons of non-normally distributed continuous data. Receiver operating characteristic (ROC) curves were used to analyze the performance of fish-allergen sIgE to discriminate between partially tolerant and non-tolerant participants. To estimate threshold doses, data were analyzed using an interval censoring survival analysis approach.⁷ Dose intervals were arranged from low to high doses by ordering lower and upper bounds of the intervals, and the step intervals were calculated according to the expectation-maximization algorithm. Dose distributions for objective and subjective reactions to each fish species were fitted by log-normal, log-logistic and Weibull distribution functions using Maximum Likelihood Estimation. As the three distributions were comparable in goodness of fit, the log-normal distribution was used to estimate the eliciting dose predicted to provoke an allergic reaction in 10% of individuals with fish allergy (ED₁₀). Confidence bands for the fitted distribution functions, and 95% confidence intervals of the ED₁₀ values, were calculated by bootstrapping (1,000 resamples). The analyses were conducted with the statistical software R using the “fitdistrplus” package (version 1.07) for interval censoring survival analysis (<https://cran.r-project.org/web/packages/fitdistrplus/index.html>).

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Table E 1, Online Repository. Background characteristics and allergy phenotype of participants, dropouts and eligible patients not included.

	Included			Not included	
	Completed	Dropouts			
	N=35	N=9	P†	N=29	P‡
Mean age (SD)	11.6 (3.0)	6.7 (1.4)	<0.001	11.6 (3.9)	0.256
Sex (Boys)	22 (63%)	7 (78%)	0.695	20 (69%)	0.619
Asthma*	21 (60%)	4 (44%)	0.467	15 (52%)	0.811
Atopic dermatitis*	24 (69%)	9 (100 %)	0.085	22 (76%)	1.000
Allergic rhinitis*	23 (66%)	6 (67%)	1.000	20 (69%)	1.00
Median (IQR) sIgE Cod	10.8 (5.5-25.9)	38.6 (10.3-115.1)	0.089	12.7 (2.4-53.3)	0.727
Median (IQR) sIgE Salmon	8.4 (2.6-19.8)	40.5 (5.9-55.0)	0.190	15.3 (5.5-74.1)	0.413
Parental allergic disease	28 (80%)	7 (78%)	1.000	18/22 (82%)	1.000
Food allergy, other than fish	31 (89%)	8 (89%)	1.000	27 (93%)	0.160

Included = Included in the study (n=44), Not included = Not consenting to participate (n=26) or did not meet inclusion criteria (n=3). Dropouts = dropouts from originally included participants. *Symptoms recorded at the Paediatric outpatient clinic, University Hospital of North Norway. SD = Standard Deviation. IQR= Interquartile range. P†; comparison between completed and dropouts. P‡ comparison between all included and not included (comparison of sIgE to cod and salmon extracts by Mann Whitney U Test).

Table E 2, Online Repository. Symptoms upon DBPCFC/OFC in participants with placebo reactions

		Fish challenge								Clinical reactivity	
		DBPCFC doses					OFC doses			Non-tolerant/Partially tolerant	
Pat Nr.	Fish Spec.	3 µg	600 µg	12.5mg	120 mg	1 g	2 g	6 g	12 g	Any symptoms	Objective symptoms
22	Cod		Itchy Mouth VAS 2	Itchy Mouth VAS 6	Itchy Mouth VAS 7 Lip Blisters					NT	NT
	Salmon			Erytema Face	Erytema Face Itchy Mouth VAS 3	Erytema Face Urticaria Itchy Mouth VAS 5					
	Mackerel			Erytema Face Urticaria Itchy Mouth VAS 1	Erytema Face Urticaria Itchy Mouth VAS 6	Erytema Face Urticaria Lip Blister Ichy Mouth VAS 2					
	Placebo			Itchy Mouth VAS 3	Itchy Mouth VAS 6	Itchy Mouth VAS 6					
23	Cod			Itchy Mouth VAS 6	Itchy Mouth VAS 8 Abd. Pain VAS 10	Skin Erytema Itchy Mouth VAS 3 Abd. Pain VAS 10				NT	PT Tolerant to mackerel
	Salmon		Abd. Pain VAS 7	Itchy Skin VAS 10 Urticaria Eye lid edema							
	Mackerel						Itchy Mouth VAS 6				
	Placebo					Abdominal Pain VAS 6 *					
31	Cod			Itcy Mouth VAS 7 Throat pain VAS 5	Itchy Mouth VAS 7 Nausea VAS 7 Throat pain VAS 8 Lip Blisters					NT	NT
	Salmon				Itchy Mouth VAS 7	Itchy Mouth VAS 8	Itchy Mouth VAS 9 Itchy Eye Sneeze x 6				
	Mackerel					Itchy Mouth VAS 6	Itchy Mouth VAS 9 Itchy Eyes				

							Red Eyes				
	Placebo		Itchy Mouth VAS 5	Itchy Mouth VAS 5							
32	Cod			Itchy Mouth VAS 4	Itchy Mouth VAS 8 Red spots on swollen tounge					PT Tolerant to salmon and mackerel	PT Tolerant to salmon and mackerel
	Salmon										
	Mackerel										
	Placebo	Itchy Mouth VAS 1	Itchy Mouth VAS 1	Itchy Mouth VAS 1							
33	Cod		Itchy Mouth VAS 2	Itchy Mouth VAS 1	Itchy Mouth VAS 2 Chest pain VAS 5 Itchy skin, erytema Tired/Fatigue					NT	NT
	Salmon						Itchy skin VAS 4 Throat tightness VAS 6 Red eyes, Urticaria				
	Mackerel				Itchy Mouth VAS 2	Itchy Mouth VAS 3 Itchy Skin VAS 6 Skin eryteme					
	Placebo			Itchy Mouth VAS 2	Itchy Mouth VAS 2	Itchy Mouth VAS 2					

NT = Non-Tolerant. PT = Partially tolerant*The participant thought he experienced abdominal pain because he was satisfied after the last dose of DBPCFC. The pain was relieved after 10 minutes. VAS = Visual Analog Scale (0-10)

Table E 3, Online Repository. Median sIgE in partially tolerant vs non-tolerant participants regarding objective and subjective symptoms.

	Objective symptoms	Subjective symptoms*
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Allergen (IUIS code)	Partially Tolerant N=19 Median (IQR)	Non- Tolerant N=15 Median (IQR)	p-value	Partially Tolerant N=10 Median (IQR)	Non- Tolerant N=24 Median (IQR)	p-value
Cod Extr. sIgE	8.8 (1.6-18.9)	19.5 (6.0-49.3)	0.064	4.0 (1.1-13.2)	15.0 (6.0-36.0)	0.017
Cod Parv. sIgE (Gad m 1)	8.0 (1.5-20.6)	13.4 (5.5-41.1)	0.103	5.1 (0.7-13.6)	12.5 (5.5-36.9)	0.034
Cod Enol. sIgE (Gad m2)	0.8 (0.3-1.4)	0.9 (0.1-1.6)	0.792	0.4 (0.3-1.2)	0.9 (0.1-1.6)	0.478
Cod Aldo. sIgE (Gad m 3)	0.4 (0.1-1.5)	1.3 (0.1-1.8)	0.217	0.4 (0.1-0.9)	1.2 (0.1-1.8)	0.144
Salmon Extr. IgE	4.7 (0.9-13.1)	13.5 (6.7-23.6)	0.052	1.8 (0.4-6.8)	12.2 (5.7-25.3)	0.003
Salmon Parv. IgE (Sal s 1)	4.7 (2.3-12.1)	12.4 (4.9-30.3)	0.052	4.5 (1.2-11.1)	11.2 (2.7-26.6)	0.085
Salmon Enol. sIgE (Sal s 2)	0.1 (0.1-0.1)	0.1 (0.1-0.4)	0.076	0.1 (0.1-0.1)	0.1 (0.1-0.1)	0.125
Salmon Aldo. sIgE (Sal s 3)	0.1 (0.1-0.1)	0.7 (0.1-2.1)	0.015	0.1 (0.1-0.4)	0.1 (0.1-1.4)	0.288
Mackerel Extr. sIgE	2.1 (0.4-4.7)	5.5 (1.7-9.7)	0.026	0.6 (0.1-2.4)	5.4 (1.8-9.6)	0.002
Mackerel Parv. sIgE (Sco s 1)	3.4 (1.9-13.4)	13.1 (5.6-49.2)	0.029	3.1 (1.1-9.3)	12.9 (2.7-26.3)	0.039
Mackerel Enol. sIgE (Sco s 2)	0.1 (0.1-0.1)	0.1 (0.1-0.1)	0.022	0.1 (0.1-0.1)	0.1 (0.1-0.3)	0.273
Mackerel Aldo. sIgE (Sco s 3)	0.1 (0.1-0.1)	0.1 (0.1-0.1)	0.853	0.1 (0.1-0.1)	0.1 (0.1-0.1)	0.813

Comparison of IgE (kU_A/L) between groups are performed with Mann Whitney U test. Partially tolerant: No symptoms to at least one fish species (cod, salmon or mackerel).

Non-tolerant: Symptoms to all three of cod, salmon and mackerel. *All participants with subjective symptoms, including participants with objective symptoms. Significant

differences in bold. One participant did not show any allergic reaction during fish challenge, and is excluded from the analysis. Extr. = extract, Parv. = parvalbumin, Enol. = enolase, Aldo. = aldolase, IUIS = International Union of Immunological Societies, IQR = Interquartile Range.

Table E 4, Online Repository. ED₁₀ in mg for subjective and objective symptoms to cod, salmon and mackerel compared to ED₁₀ values for cod in EuroPrevall.

Fish species (95% CI)	Tromsø Fish Allergy Study		EuroPrevall ¹	
	Objective Symptoms	Subjective Symptoms	Objective Symptoms	Subjective Symptoms
Cod ED ₁₀	23.8 (9.5-58.7)	0.7 (0.2-2.4)	27.3 (5.3-171.2)	0.2 (0.005-8.2)
Salmon ED ₁₀	*	3.5 (0.7–20.1)	Not reported	Not reported
Mackerel ED ₁₀	*	42.9 (11.2-138.9)	Not reported	Not reported

ED₁₀: doses at which an allergic reaction would be elicited in 10% of the population. CI: confidence interval. ED₁₀ were determined by using an interval-censoring approach with log-normal parametric curve fitting with subjective and objective symptoms. *Not enough participants to calculate reliable dose distribution curves and estimate ED₁₀.

Table E 5, Online Repository. Subjective, objective and anaphylactic symptoms at each dose during DBPCFC/OFC and IgE values for each participant.

.		Fish challenge								sIgE to fish-allergens				Clinical Reactivity	
		DBPCFC doses					OFC doses			Extr.	Parv.	Enol.	Aldo.	Non-tolerant /Partially tolerant	
Pat. Nr.	Fish Spec.	3µg	600 µg	12.5mg	120 mg	1 g	2 g	6 g	12 g					Any symptoms	Objective symptoms
1	Cod				S	S	S		X	10.70	4.90	.10	.10	PT	PT
	Sal				SO	SOX				14.50	11.70	.10	6.70	Tolerant to Mackerel	Tolerant to Mackerel and Cod
	Mac								X	4.70	19.00	.10	.10		
	Pla					X									
2	Cod					SOAX				117.00	121.70	61.50	63.10	NT	NT
	Sal					SO	S	SOA	SOAX E	144.00	30.30	9.50	18.30		
	Mac						S	S	SOAX	52.10	49.20	1.10	.50		
	Pla					X									
3	Cod				S	SOX				5.98	5.50	.10	1.00	NT	NT
	Sal				S	SO	SOX			6.69	6.60	.10	.80		
	Mac					SO	S	SOX		1.67	8.10	.10	.10		
	Pla					X									
4	Cod		S	S	SO	SOX				288.00	153.00	5.40	4.90	NT	NT
	Sal				S	S	SOX			254.00	205.00	7.10	46.10		
	Mac				S	S	S	SO	SOX	41.00	200.00	.10	.10		
	Pla					X									
5	Cod				SOX					28.30	20.60	3.50	4.10	PT	PT
	Sal							X		2.64	13.50	.10	.10	Tolerant to salmon	Tolerant to salmon
	Mac					S		SOX		2.34	11.20	.10	.10		
	Pla					X									
6	Cod				S	SOAX				25.90	16.80	.10	.10	NT	NT
	Sal				S	SOAX				20.10	14.10	.10	.10		
	Mac						S	S	SOAX	7.25	13.10	.10	.10		
	Pla					X									
7	Cod						S	S	SOAX	6.38	5.30	1.10	1.50	PT	PT
	Sal								X	4.65	4.70	.10	.10	Tolerant to salmon and mackerel	Tolerant to salmon and mackerel
	Mac								X	1.84	3.40	.10	.10		
	Pla					X									
8	Cod			S	SOX					3.38	2.70	.50	.80	NT	NT

	Sal					SO	SO	SOX		3.24	3.00	.10	.40		
	Mac							SOX		.89	2.50	.10	.10		
	Pla					X									
9	Cod			S	S	SOX				15.30	20.80	.10	.10	NT	PT
	Sal				SO	SO	SOX			27.30	30.10	.10	2.10		Tolerant to mackerel
	Mac				S	S	S	S	SX	5.53	16.20	.10	.10		
	Pla					X									
10	Cod					SO		SOX		.07	11.30	1.50	.10	PT	PT
	Sal							X		.92	10.90	.10	1.40	Tolerant to salmon	Tolerant to salmon
	Mac					SO		SOX		.04	6.80	.30	.40		
	Pla					X									
11	Cod					S	S	SO	SOX	6.40	5.60	1.30	1.80	NT	NT
	Sal				SO	SO	SOAX			7.75	5.40	.10	1.60		
	Mac						SO	SO	SOX	2.13	7.50	.10	.10		
	Pla					X									
12	Cod				S	SO	SO	SOAX		1.46	1.50	.50	.60	PT	PT
	Sal			S	S	S	S	S	SX	4.20	3.20	.10	.10	Tolerant to mackerel	Tolerant to salmon and mackerel
	Mac								X	.39	2.10	.10	.10		
	Pla					X									
13	Cod			SO	SOAX					70.00	108.0	2.70	3.90	NT	NT
	Sal			S	S	SOAX				45.90	69.70	.10	.10		
	Mac			S	SOAX		SOAX			9.69	67.70	.10	.10		
	Pla					X									
14	Cod							X		.87	.10	.10	.10	Tolerant to all three species	Tolerant to all three species
	Sal							X		.79	.10	.10	.10		
	Mac							X		.15	.10	.10	.10		
	Pla					X									
15	Cod				SO	SOX				3.20	4.80	.10	.30	NT	PT
	Sal				O	O	SO	SO	SOX	1.80	2.50	.10	.10		Tolerant to mackerel
	Mac							S	SX	.67	1.90	.10	.10		
	Pla					X									
16	Cod						SO	SOX		1.57	.50	.30	.30	PT	PT
	Sal							SO	SOX	.23	.70	.10	.10	Tolerant to mackerel	Tolerant to mackerel
	Mac							X		.12	.60	.10	.10		
	Pla					X									
17	Cod			SO	SOAX					14.00	10.80	.90	1.30	NT	NT
	Sal				S	S	SOX			10.80	12.40	.10	2.10		

	Mac				S	SO	SO	SOX		5.49	12.70	.10	.10		
	Pla					X									
18	Cod				S	SOAX				20.90	27.60	.30	.10	PT	PT
	Sal							X		13.10	10.70	.10	.10	Tolerant to salmon	Tolerant to salmon and mackerel
	Mac							SX		2.58	8.70	.10	.10		
	Pla					X									
19	Cod				S	S	SOAX			8.78	.10	.90	1.70	NT	PT
	Sal				S	S	S	S	X	8.37	.10	.10	.10		Tolerant to salmon
	Mac					S	SOAX			2.37	.10	.40	.10		
	Pla					X									
20	Cod				S	SO	SOX			46.30	44.60	70.00	83.00	NT	PT
	Sal				SO		SOX			88.60	17.00	.90	.10		Tolerant to mackerel
	Mac						SX			13.30	23.70	1.10	1.00		
	Pla					X									
21	Cod				SO		SO	SOX		1.47	.70	.30	.40	PT	PT
	Sal							X		.48	1.40	.10	.10	Tolerant to salmon and mackerel	Tolerant to salmon and mackerel
	Mac							X		.28	1.30	.10	.10		
	Pla					X									
22	Cod		S	S	SOX					5.47	5.30	.10	1.30	NT	NT
	Sal			O	SO	SO		SO	SOX	4.37	4.90	.10	.80		
	Mac			SO	SO	SOX				1.37	5.60	.10	.10		
	Pla			S	S	SX									
23	Cod			S	S	SOX				18.90	11.60	.90	1.10	NT	PT
	Sal		S	SOX						11.70	8.90	.10	.10		Tolerant to mackerel
	Mac						SX			5.70	9.20	.10	.10		
	Pla					SX									
24	Cod				S	SOAX				10.10	10.00	.80	1.40	NT	PT
	Sal						S	S	SX	5.36	2.60	.10	.10		Tolerant to salmon and mackerel
	Mac				S	S	S	S	SX	2.12	2.50	.10	.10		
	Pla					X									
25	Cod								X	.01	.10	.10	.10	PT	PT
	Sal					S			SOX	.10	.10	.10	.10	Tolerant to cod	Tolerant to cod and mackerel
	Mac						S	S	SX	.01	.10	.10	.10		
	Pla					X									
26	Cod						S	SOX		31.60	32.50	6.20	.10	NT	PT
	Sal						S	SOX		25.80	19.80	.10	.10		Tolerant to mackerel
	Mac						SX			12.20	15.90	.10	.10		

	Pla														
27	Cod					S	S	SOX		22.00	25.40	.10	.10	NT	NT
	Sal		S	S	S	S	S	SOX		13.50	27.90	.40	.70		
	Mac						SOX			3.88	27.10	.10	.10		
	Pla					X									
28	Cod				S	SO	SO	SOX		49.30	41.10	.10	.10	NT	NT
	Sal						S	SO	SOX	19.80	22.70	.10	.10		
	Mac						S	SO	SOX	5.33	27.80	.10	.10		
	Pla					X									
29	Cod			S	S	SOAX				14.70	8.20	1.20	1.60	NT	NT
	Sal						S	SOX		7.69	2.30	.10	.10		
	Mac						S	S	SOAX	7.02	2.30	.10	.10		
	Pla					X									
30	Cod			S	S	SOAX				5.89	6.50	1.30	1.70	NT	PT Tolerant to salmon
	Sal						SX			2.08	2.30	.10	.10		
	Mac					S	SOA	SOAX		.35	3.30	.50	.10		
	Pla					X									
31	Cod			S	SOX					19.50	13.40	1.60	1.80	NT	NT
	Sal				S	S	SOX			16.10	10.30	1.30	3.70		
	Mac					S	SOX			9.37	16.00	.30	.20		
	Pla		S	SX											
32	Cod			S	SOX					7.77	8.00	.60	.70	PT Tolerant to salmon and mackerel	PT Tolerant to salmon and mackerel
	Sal					X				.89	4.20	.10	.10		
	Mac					X				.71	2.70	.10	.10		
	Pla	S	S	S		X									
33	Cod		S	S	SOAX					0.92	.80	.10	.10	NT	NT
	Sal						SOAX			0.23	1.00	.10	.10		
	Mac				S	SOX				0.39	.80	.10	.10		
	Pla			S	S	SX									
34	Cod			S	SO	SOX				37.50	38.40	.90	.70	NT	NT
	Sal				S	S	S	SO	SOX	23.60	35.60	.10	.10		
	Mac				S	S	SOAXE			9.76	58.50	.40	.10		
	Pla					X									
35	Cod			S	S	SOX				13.60	15.40	1.40	.10	NT	
	Sal				S		S	S	SX	12.70	12.10	.10	.10		
	Mac				S		S	S	SX	2.94	13.40	.10	.10		
	Pla					X									

DBPCFC = Double Blind Placebo Controlled Food Challenge. OFC = Open Food Challenge. Sal = salmon, Mac = mackerel, Pla = placebo, Extr = extract, Parv. = parvalbumin, Enol. = enolase, Aldo. = aldolase, S = subjective allergic reaction, O = objective allergic reaction, A = anaphylactic reaction, X = challenge stopped during/after this dose. E = Epinephrine auto injector 0.3 mg intramuscular injection. Participants are numbered 1-35. IgE is measured in kU_A/L. NT = Non-tolerant.

Table E 6, Online Repository. Subjective and objective symptoms recorded during food challenges

Organ System	Symptoms	S/O	Grading	Comment
Mouth/throat	Itching	S	None or VAS 1-10	
	Edema	O	Presence/absence	
	Blisters	O	Presence/absence	
Skin	Erytema/flushing	O	% body surface	«Rule of nines» Body surface diagram
	Itching	S	None or VAS 1-10	
	Urticaria/hives	O	0-3	1=Mild: ≤ 3 wheals 2=Moderate: > 3 wheals 3=Severe: Generalized involvement
	Angioedema	O	Presence/absence	
Nasal/ocular	Sneezing/itchy nose	O	0-3	1=Mild: rare bursts 2=Moderate: <10 bursts, intermit. rubbing 3=Severe: continuous rubbing (nose, eyes, external ear canals)
	Runny nose	O	0-3	1=Mild: occasional sniffing 2=Moderate: frequent sniffing, use tissues 3=Severe: nose runs despite sniffing/tissues

	Nasal congestion	O	0-3	1=Mild: some hindrance to breathing 2=Moderate: nostrils feels blocked, 3=Severe: nostrils occluded
	Red itchy eyes	O	Presence/absence	
	Watery eyes	O	Presence/absence	
Larynx/Respiration	Feeling throat tightness	S	None or VAS 1-10	
	Inspiratory stridor	O	Presence/absence	
	Chest tightness	S	None or VAS 1-10	
	Wheezing	O	0-3	1=Mild: expiratory wheeze on auscultation 2=Moderate: dyspnea, insp and exp wheeze 3=Severe: dyspnea, acc. muscles, wheeze
	Coughing	O	Presence/absence	
	Dyspnea	S	No or VAS 1-10	
Gastrointestinal	Difficult to swallow	S	No or VAS 1-10	

	Nausea	S	No or VAS 1-10	
	Vomiting/diarrhea	O	0-3	1=Mild: 1 episode of vomiting or diarrhea 2=Moderate: 2-3 episodes of vomiting or diarrhea or 1 of each 3=Severe: 3 episodes of vomiting/diarrhea or 2 of each
	Abdominal pain	S	None or VAS 1-10	
Heart/circulation	Tachycardia	O	Presence/absence	
	Arrhythmia	O	Presence/absence	
	Chest pain	S	No or VAS 1-10	
	Hypotension	O	Presence/absence	Yes=Drop in BP > 20 mmHg from baseline
Other	Syncope	O	Presence/absence	
	Seizures	O	Presence/absence	
	Incontinence	O	Presence/absence	
	Panic/anxiety	S	No or VAS 1-10	

S= subjective, O= objective. VAS= Visual Analog Scale, BP= Blood Pressure

Figure E 1, Online Repository. Flow chart with patient inclusion and completed tests.

Legend:

* One participant did not undergo DBPCFC due to poor palatability of the test-food but performed OFC with all three fish species.

Figure E 2a, Online Repository. Number of participants (N=35) with objective symptoms to cod, salmon and mackerel during DBPCFC/OFC.

Legend:

Mouth: swellings/blisters in the mouth or on the lips. Skin: Erythema, urticaria or angioedema of the skin. Nose: Sneezes or itchy, runny, blocked nose. Eye:

Itchy, red or watery eyes. No participants had stridor, wheeze, tachycardia, arrhythmia, syncope, seizures or incontinence.

Figure E 2b, Online Repository. Number of participants (N=35) with subjective symptoms to cod, salmon and mackerel during DBPCFC/OFC.

No legend

Figure E 3, Online Repository. Receiver Operating Curves (ROC) for discrimination between non-tolerant and partially tolerant fish allergic patients.

Legend

Non-tolerant: Reaction with any symptom to cod, salmon and mackerel. Partially tolerant: tolerant regarding any symptoms to at least one of cod, salmon and mackerel. AUC: Area Under Curve.

Fig E 4, Online Repository. Dose distributions for subjective and objective symptoms to cod and subjective symptoms to salmon and Mackerel

No legend

Figure E 5a, Online Repository. Lowest Observed Adverse Effect Level (LOAEL) for objective symptoms to cod during DBPCFC/OFC, n=32.

No Legend

Figure E 5b, Online Repository. Lowest Observed Adverse Effect Level (LOAEL) for objective symptoms to salmon during DBPCFC/OFC, n=23.

No Legend

Figure E 5c, Online Repository. Lowest Observed Adverse Effect Level (LOAEL) for objective symptoms to mackerel during DBPCFC/OFC, n=19.

No Legend

Figure E 1, Online Repository. Flow chart with patient inclusion and completed tests.

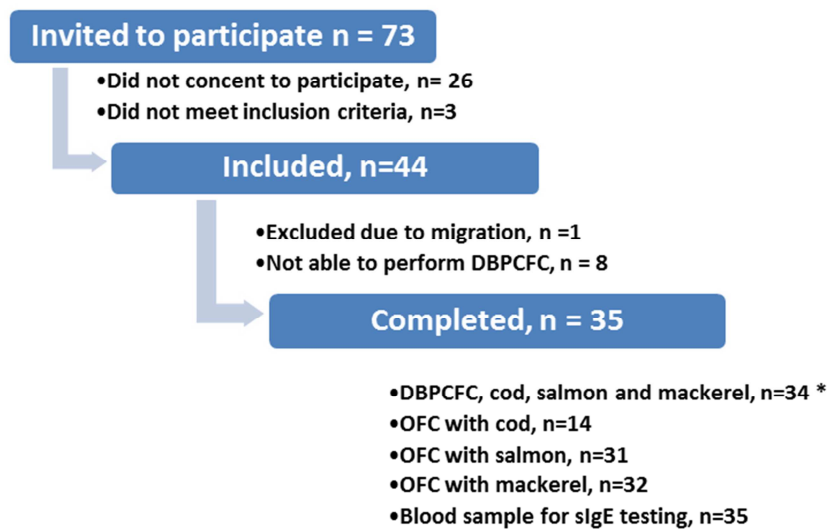


Figure E 2a, Online Repository. Number of participants (N=35) with objective symptoms to cod, salmon and mackerel during DBPCFC/OFC.

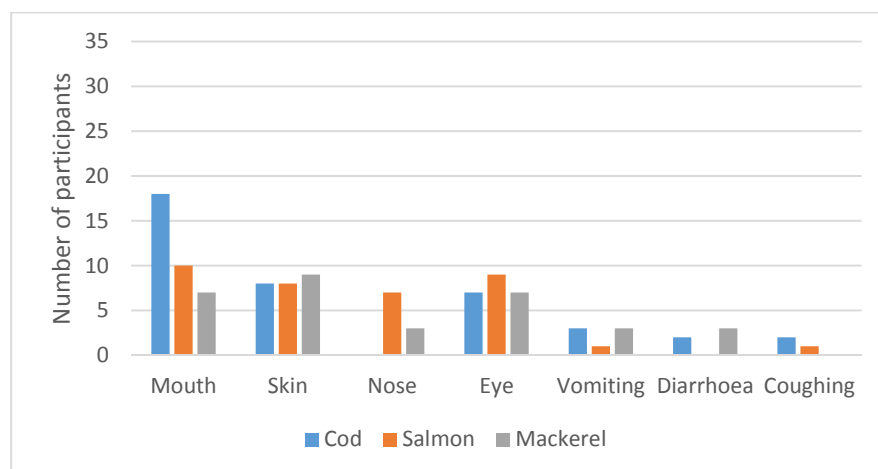
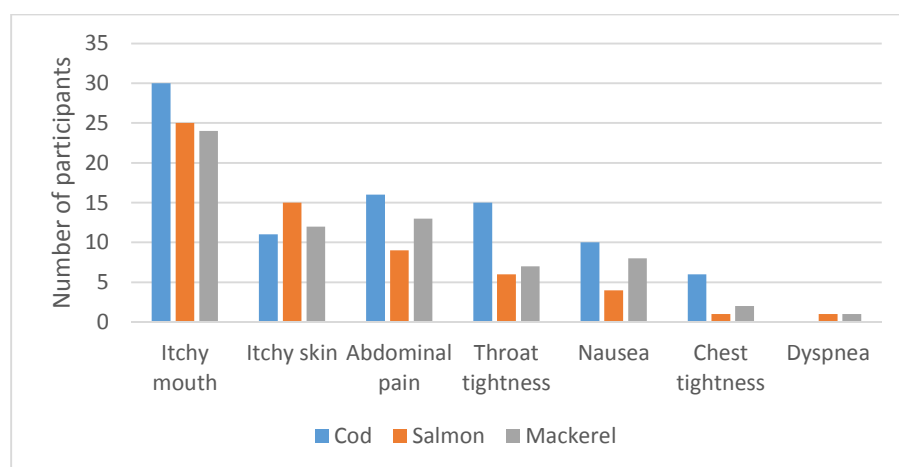
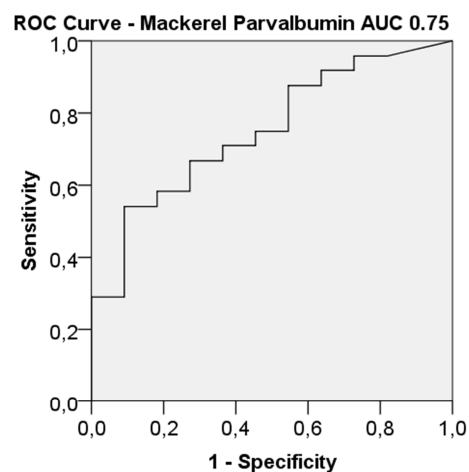
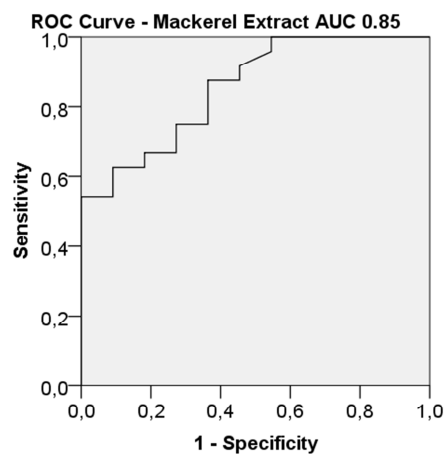
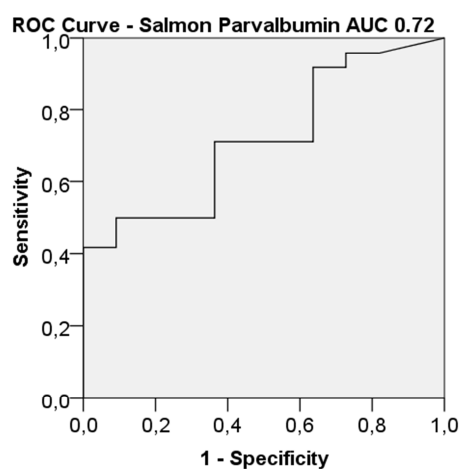
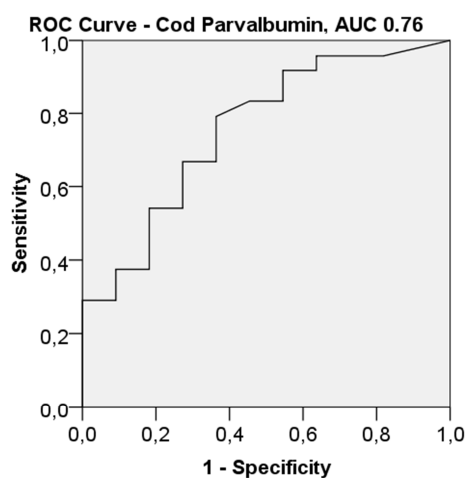
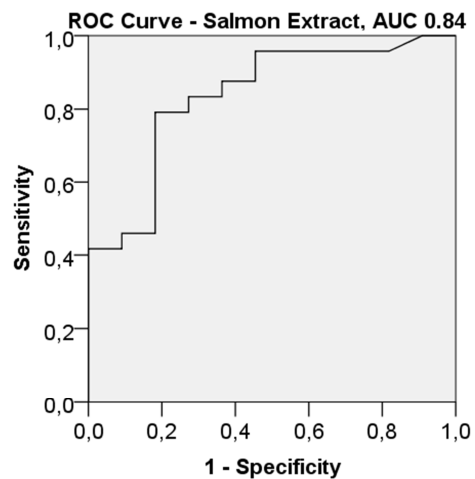
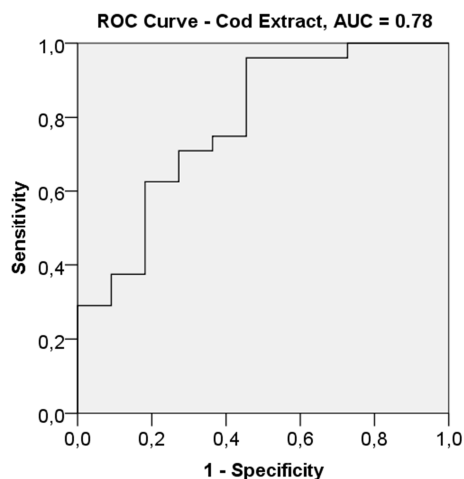
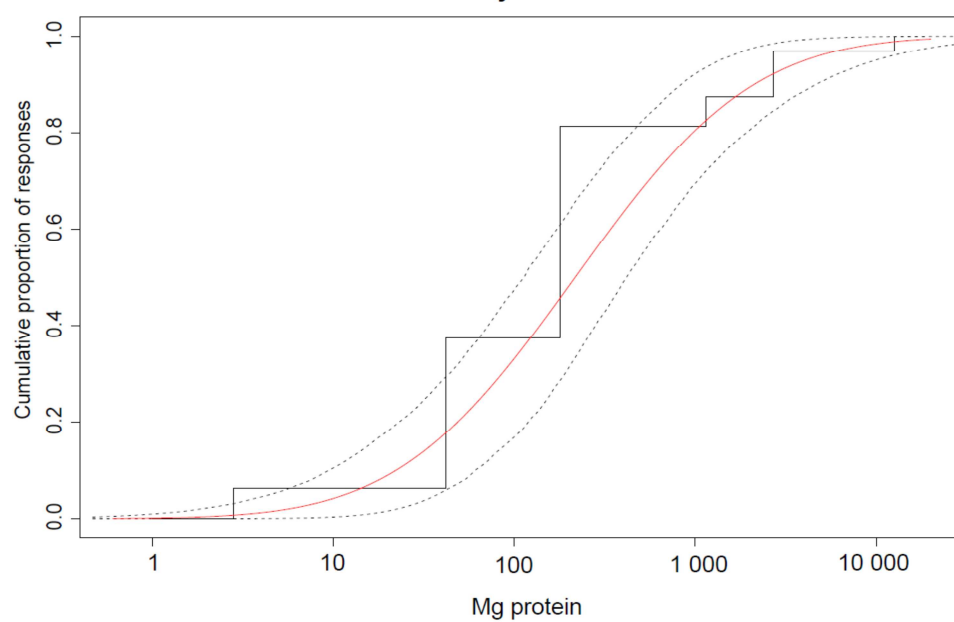
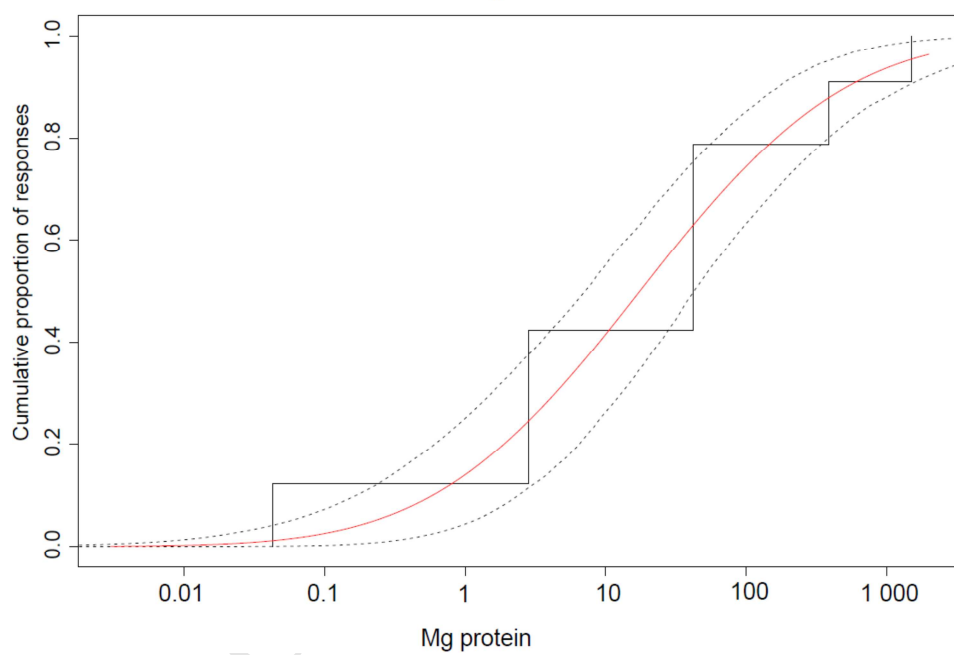
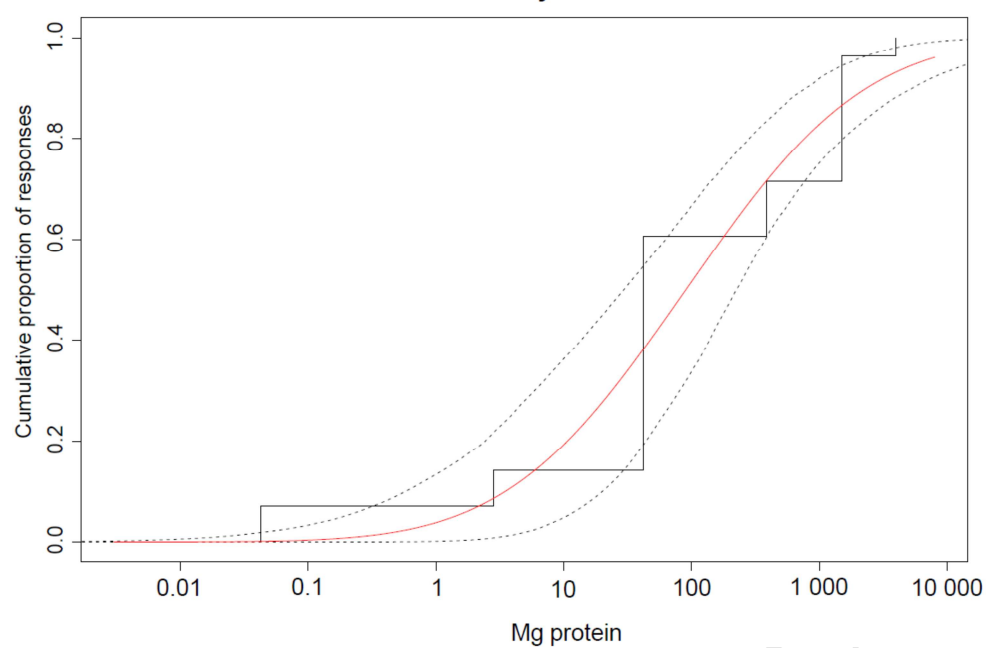


Figure E 2b, Online Repository. Number of participants (N=35) with subjective symptoms to cod, salmon and mackerel during DBPCFC/OFC.





Cod. Objective reactions**Cod. Subjective reactions**

Salmon. Subjective reactions**Mackerel. Subjective reactions**