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1 # IgE and IgG_4 epitopes revealed on the major fish allergen Lat c 1

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25 **Abstract**

26 **Background:** The IgE- and IgG_4-binding patterns of the major fish allergen parvalbumins are
27 not clearly understood. IgE antibody-binding to parvalbumin from Asian seabass, Lat c 1.01,
28 is implicated in up to 90% of allergic reactions, although the region of IgE or IgG4 epitopes
29 are unknown. In the present study, we characterized the specific IgE- and IgG_4-binding regions
30 of Lat c 1.01 using serum from pediatric and adult patients with clinically-confirmed fish
31 allergy.

32 **Methods:** A comparative investigation of patient IgE- and IgG_4-binding to recombinant Lat c
33 1.01 was performed by immunoblotting and indirect ELISA using serum from 15 children and
34 eight adults with clinically confirmed IgE-mediated reactions to fish. The IgE- and IgG_4-
35 binding regions of Lat c 1.01 were determined by inhibition ELISA using seven overlapping
36 peptides spanning the entire 102 amino acid sequence. Elucidated IgE-binding regions were
37 modelled and compared to known antibody-binding regions of parvalbumins from five other
38 fish species.

39 **Results:** Ninety five percent (22/23) patients demonstrated IgE-binding to rLat c 1.01, while
40 fewer patients (10/15 children and 7/8 adults) demonstrated robust IgG_4 binding when
41 determined by immunoblots. IgE-binding for both cohorts was significantly higher compared
42 to IgG_4-binding by ELISA. All patients in this study presented individual IgE and IgG_4 epitope-
43 recognition profiles. In addition to these patient-specific antibody binding sites, general IgE
44 epitopes were also identified at the C- and N-terminal regions of this major fish allergen.

45 **Conclusions and Clinical relevance:** Our findings demonstrate two specific IgE epitopes on
46 parvalbumin from Asian seabass, while IgG_4 binding is much lower and patient specific. This
47 study highlights the importance of advancement in epitope analysis regardless of the age group
48 for diagnostics and immunotherapies for fish allergy.

49

50 ***Keywords:*** Epitope mapping; Fish allergy; IgE epitope; IgG_4 epitope; Parvalbumin

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52

53

54 **1. Introduction**

55 IgE-mediated fish allergy is an adverse immunological response to fish which persists through
56 adulthood (Davis et al., 2020), with sensitization rates up to 3% in the general population
57 (Moonesinghe et al., 2016). Fish is the second most common trigger of food allergy after
58 crustacean in countries with high seafood consumptions (Le et al., 2019). A wide range of
59 clinical symptoms, including life-threatening anaphylaxis, can be caused by exposure to fish
60 allergens (Matricardi et al., 2016). There are no effective therapeutics and strict avoidance of
61 the implicated fish is the only measure currently available to affected patients (Lopata and
62 Kamath, 2012). The major fish allergen is parvalbumin (Kuehn et al., 2014), a calcium-binding
63 protein expressed in muscle tissues. Characterization of parvalbumin has the potential to aid in
64 the diagnosis (Kuehn et al., 2013) and the development of therapeutic approaches (Zuidmeer-
65 Jongejan et al., 2015) for fish allergy. However, the main challenges for the development of
66 reliable diagnostics are the considerable molecular differences in amino acid sequences of
67 parvalbumins between fish species and the presence of multiple isoforms (Ruethers et al.,
68 2018).

69 Linear IgE-binding epitopes of different parvalbumin allergens have been characterized
70 (Stephen et al., 2017) and might contribute to species-specific clinical reactivity often seen in
71 fish-allergic patients. There are five studies mapping the specific immunoglobulin (Ig)E-
72 binding epitopes of parvalbumin from fish species, including Baltic cod (allergen
73 nomenclature: Gad c 1; Elsayed and Apold, 1983), common carp (Cyp c 1; Untersmayr et al.,
74 2006), Pacific mackerel (Sco j 1; Yoshida et al., 2008), Atlantic salmon (Sal s 1; Perez-Gordo
75 et al., 2012) and Atlantic cod (Gad m 1; Perez-Gordo et al., 2013). Various techniques were
76 used in the identification of these IgE-binding epitopes, including phage display library,
77 overlapping synthetic peptides and tryptic digests of parvalbumin. The allergens analyzed
78 display both linear and conformational epitopes; however, no epitopes share identical amino
79 acid sequences between these five fish species. While the IgE-binding epitopes of these
80 parvalbumins commonly recognized by fish-allergic patients have been established, patient-
81 specific epitopes are also considered to exist for other fish species based on the findings on
82 Gad m 1 from Atlantic cod (Perez-Gordo et al., 2013).

83 Identifying IgE epitopes on food allergens may help to develop and predict successful
84 specific immunotherapies (sIT) for allergy sufferers (Vickery et al., 2013; Savilahti et al.,
85 2014). An increase in specific IgG_4 response could be an indicator of successful sIT due to this

86 antibody being able to out-compete IgE binding for the allergen (Santos et al., 2020). In
87 addition, elevated IgG_4 levels have been associated with protective effects (Du Toit et al.,
88 2015). However, a comparative study of the IgE- and IgG_4-binding regions on parvalbumin
89 has never been performed despite being the major allergen for fish.

90 Worldwide consumption of Asian seabass (*Lates calcarifer*), also known as
91 barramundi, is rapidly growing due to increased availability from aquaculture industries and
92 its culinary popularity (Loughnan et al., 2013). We have identified and characterized
93 parvalbumin from this species and two related allergen isoforms, Lat c 1.01 and Lat c 1.02
94 (Sharp et al., 2014) (www.allergen.org). Asian seabass can cause allergic reactions in up to
95 90% of fish-allergic patients, with IgE reactivity to at least one of the two parvalbumin
96 isoallergens identified in this species (Turner et al., 2011; Sharp et al., 2014). The parvalbumin
97 isoform, Lat c 1.01 appears to have the greatest IgE reactivity (Sharp et al., 2014). With an aim
98 to elucidate the IgE- and IgG_4-binding patterns of fish parvalbumins, we sought to characterize
99 the most IgE-reactive Asian seabass parvalbumin, Lat c 1.01, using 23 pediatric and adult
100 patients with clinically confirmed IgE-mediated fish allergy, and ultimately, to define patient-
101 specific IgE- and IgG_4-binding regions on fish parvalbumins.

102

103 **2. Materials and Methods**

104 ***2.1 Patients***

105 Fish-allergic patients were recruited based on a convincing recent history of an IgE-mediated
106 reaction (including urticaria, angioedema, stridor, cough, wheeze, hypotension and/ or
107 gastrointestinal symptoms of vomiting and/or diarrhea) to any fish, occurring within 2 h of
108 ingestion. Sensitization to fish was confirmed *in vivo* by skin prick testing using commercial
109 reagents and/or *in vitro* by determining the levels of serum IgEs (ImmunoCAP, Phadia-Thermo
110 Fisher Scientific; Uppsala, Sweden) to cod (*Gadus morhua*), tuna (*Thunnus albacares*), and/or
111 salmon (*Salmo salar*). The 23 patients, consisting of eight adults and 15 children (details are
112 listed in Table 1), were selected based on positive IgE reactivity to recombinant Asian Seabass
113 parvalbumin isoform (rLat c 1.0101) (Sharp et al., 2014). Sera from five non-atopic patients
114 were used as negative controls. Ethics approval for this study was granted by James Cook
115 University's Ethics committee (JCU-H4313) in collaboration with The Westmead Children's

116 Hospital Network (LNR-14/SCHN/185) and The Alfred Hospital (Project number 192/07) and
117 Monash University's Ethics Committees (MUHREC CF08/0225).

118

119 ***2.2 Recombinant Asian Seabass parvalbumin expression***

120 Recombinant (r)Lat c 1.01, Asian seabass parvalbumin β1, was obtained as previously
121 described (Sharp et al., 2014). In brief, the β1 parvalbumin gene (GenBank accession number:
122 KF021278.1) was cloned into the expression vector pPROEX HTb (Invitrogen; Waltham,
123 USA) and used to transform NM522 *Escherichia coli* cells. The expression of hexa-histidine
124 (his 6)-tagged rLat c 1.01 was induced with 1 mM IPTG over 3 h at 37 °C, and purified from
125 clarified cell lysate using HisTrap HP column (GE Healthcare; Little Chalfont, UK). Purified
126 rLat c 1.01 was applied onto a 15% polyacrylamide gel and separated under reducing condition
127 according to its molecular weight using a Mini-PROTEAN® SDS-PAGE system (BioRad
128 laboratories; Hercules, USA) as described by Laemmli (Laemmli, 1970). rLat c 1.01 was
129 visualized by Coomassie Brilliant Blue R-250 staining and confirmed by subsequent
130 immunoblotting with two allergen-specific antibodies; monoclonal anti-parvalbumin PARV-
131 19 antibody (Sigma-Aldrich; St. Louis, USA), previously evaluated for the detection of fish
132 parvalbumin (Saptarshi et al., 2014), and in-house generated polyclonal antibodies against
133 parvalbumin from Asian seabass raised in rabbits (Sharp et al., 2015).

134

135 ***2.3 IgE and IgG_4 immunoblot analysis***

136 Patient IgE and IgG_4 binding to denatured rLat c 1.01 was analyzed by immunoblotting.
137 Purified rLat c 1.01 (15 µg or 1.2 nmol) was separated on a 15% polyacrylamide gel under
138 reducing condition, then transferred to a nitrocellulose membrane (BioRad laboratories, CA,
139 USA). The membrane was blocked with tris-buffered saline (TBS)/0.05% Tween-20 (TBS-T)
140 for 2 h at room temperature. Both sera and detection antibodies were diluted in 0.1% BSA in
141 TBS-T. Membranes were placed into the surf-blot manifold (Idea Scientific, Minneapolis,
142 USA), then applied with diluted patient serum (1:20) and incubated for 16 h at 4 °C, with
143 subsequent washing with TBS-T at room temperature. For the detection of IgE, the membranes
144 were further incubated with horseradish peroxidase (HRP)-labelled goat anti-human IgE
145 antibody (1:10,000, Genetex; Irvine, USA). The IgG_4 were detected with monoclonal anti-
146 human IgG_4 MAB1313 antibody (1:2,000, Merck; Kenilworth, USA), followed by subsequent

147 detection with anti-mouse IgG HRP-labelled antibody (1:20,000, Sigma-Aldrich). Both IgE
148 and IgG_4 binding complexes were visualized by enhanced chemiluminescence (ECL)
149 technique.

150

151 ***2.4 IgE and IgG_4 indirect enzyme-linked immunosorbent assay (ELISA)***

152 Patient IgE and IgG_4 binding to native (non-denatured) rLat c 1.01 was analyzed by ELISA.
153 The 96-well EIA/RIA plates (Costar; St. Louis, USA) were coated with 20 μL of rLat c 1.01
154 (1 μg/mL in sodium carbonate/bicarbonate buffer, pH 9.6), and incubated overnight at 4 °C.
155 Plates were blocked with 5% skim milk/PBS-T for 1 h, then serum (1:10) in 1% skim
156 milk/PBS-T was added and incubated for 3 h at room temperature while shaking (45 rpm). The
157 wells were washed and then incubated with either anti-human IgE HRP-labelled antibody
158 (1:10,000, Genetex) or monoclonal anti-human IgG_4 MAB1313 antibody (1:2,000, Merck) in
159 1% skim milk/PBS-T. Serum IgG_4 plates were further incubated with anti-mouse IgG HRP-
160 labelled antibody (1:20,000, Sigma-Aldrich) with gentle shaking. Plates were washed
161 extensively, and the antibody binding was detected using TMB (3,3',5,5'-
162 tetramethylbenzidine) substrate (BD Biosciences; Franklin Lakes, USA). The reaction was
163 terminated after 30 min using 1 M hydrochloric acid and the absorbance (O.D.) measured at
164 450 nm by spectrophotometry (Molecular Devices; Sunnyvale, USA).

165

166 ***2.5 Overlapping peptides of parvalbumin***

167 Seven overlapping peptides spanning Lat c 1.01 sequence, each 25 amino acids in length, were
168 synthesized and purified to 95% homogeneity (Mimotopes; Clayton, Australia). Six peptides
169 were designed with nine amino acids overlapping on both termini. The seventh peptide,
170 covering the C-terminal end of parvalbumin, was designed to provide complete coverage of
171 one of the two calcium-binding sites. The peptide sequences and locations within the full-
172 length protein are given in Figure 5.

173

174 ***2.6 Peptide binding analysis for IgE and IgG_4***

175 Patient IgE and IgG_4 binding to each peptide were evaluated by inhibition ELISA using 96-
176 well EIA/RIA plates (Costar), coated with 8.67 nM of rLat c 1.01 (20 µL sodium
177 carbonate/bicarbonate buffer, pH 9.6) overnight at 4 °C. The wells were blocked with 5% skim
178 milk/PBS-T prior to the assay. Patient serum (1:10) was pre-adsorbed to 4.38-5.02 µM,
179 equating to 12.5 µg/mL, of synthesized peptides for 2 h, then added to the wells in 20 µL and
180 incubated for 1 h at room temperature. The assays were completed using the same protocol as
181 for the indirect ELISA. All washes were performed in TBS-T and all detection antibodies were
182 diluted in TBS-T with 0.1% BSA. Antibody binding was detected by colorimetric assay using
183 100 µL TMB substrate and O.D. measured at 450 nm by spectrophotometry. The percentage
184 of inhibition was calculated as follows: 100 – [(O.D. 450 nm of antibody with inhibitor x 100)
185 / O.D. 450 nm of antibody without inhibitor].

186

187 ***2.7 Comparative modelling of parvalbumins***

188 The homology search was conducted and homology models generated using the carp
189 parvalbumin crystal structure (PDB ID: 4cpv) with SWISS-MODEL (Basel, Switzerland)
190 (Arnold et al., 2006; Biasini et al., 2014). The amino acid sequences of allergens, Gad c 1.01,
191 Cyp c 1.01, Sal s 1.01, Sco j 1.01, Gad m 1.01 and Lat c 1.01, were aligned with Clustal Omega
192 (EMBL-EBI). Theoretical models of parvalbumin were built using UCSF Chimera (version
193 1.7; San Francisco, USA) (Pettersen et al., 2004).

194

195 ***2.8 Data analysis***

196 Cut-off values for the indirect ELISA were determined using the mean of three non-fish-
197 allergic patients plus two times the standard deviation. For the peptide binding assay, multiple
198 T-tests were used to compare overall serum IgE and IgG_4 reactivity to each peptide between
199 the negative and fish-allergic patients. Analyses were performed using GraphPad Prism
200 (version 8; GraphPad, San Diego, CA).

201

202 **3. Results**

203 ***3.1 Patient IgE and IgG_4 bind to rLat c 1.01***

204 The analysis of IgE- and IgG_4-binding to denatured rLat c 1.01 was first performed by
205 immunoblotting using sera of 23 fish-allergic patients (Figure 1). IgE from all patients but one
206 child (C5) reacted to monomeric rLat c 1.01 (Figure 1A). Five children (C4, C9 and C13-15)
207 also showed reactivity to dimeric forms of rLat c 1.01 at 30 kDa (Figure 1B), while none of the
208 adults demonstrated IgE-binding to the dimers. IgG_4 from fewer patients demonstrated binding
209 to rLat c 1.01 (10/15 pediatrics; C1-6, C9, C12-14, 6/8 adults; A1-5 and A8) (Figure 1A). In
210 contrast to IgE-binding, more IgG_4-reactive patients detected the dimeric form of parvalbumin
211 (7/15 children; C1-2, C6, C9, C12-14, 2/8 adults; A1 and A4). Two children (C1 and C9) also
212 showed reactivity to a rLat c 1.01 oligomer with a molecular weight above 50 kDa. All five
213 negative control patients (N1-5) demonstrated no IgE or IgG_4 binding to rLat c 1.01. Two
214 patients were not positive for IgE binding, but C12 demonstrated very weak IgG4 binding to
215 the monomer and dimeric form of PV. Furthermore, A2 and A6 did not demonstrate IgG4
216 binding but IgE binding to the monomeric form of PV.

217

218 ***3.2 Patient IgE and IgG_4 bind to the native form of rLat c 1.01***

219 Further analysis of IgE and IgG_4 binding to the native form of rLat c 1.01 was performed by
220 indirect ELISA. Binding was considered positive if O.D. values were greater than the cut-off
221 determined based on the non-atopic controls (dotted line) (Figure 2A). rLat c 1.01-specific IgE
222 was detected in 22 out of 23 patients, except for one child (C5) who also demonstrated low
223 reactivity on the immunoblot. Similar to the immunoblot analysis, IgG_4 from fewer patients
224 bound to rLat c 1.01 when analyzed by ELISA, with 14 out of 23 as compared to 16 out of 23
225 patients showing reactivity on the immunoblot. IgG_4 binding observed by ELISA presented
226 however a different antibody-binding profile for some patients compared to the immunoblots.
227 rLat c 1.01-specific IgG_4 was detected in 10 out of 15 children (C1-3, C7, C9 and C11-15),
228 while five out of the eight adults were reactive (A1-2, A4, A6 and A8). Discrepancies in the
229 reactivity detected on immunoblots and ELISA were found in six children (C4-6 for reactivity
230 on immunoblot only, C7, C11, C15 for reactivity by ELISA only) and three adults (A3 and A5
231 for reactivity on immunoblot only, A6 for reactivity by ELISA only) for IgG_4. Uniformly, three
232 patients (C8, C10, A7) did not show IgG_4 binding in either of the experiments.

233 The mean IgE and IgG_4 binding, as compared to the negative controls, within each
234 cohort revealed that IgE binding was significantly higher than that of IgG_4 for both children
235 and adults (Figure 2B). The adult cohort demonstrated markedly higher IgE binding (8.2-fold

236 increase) as compared to IgG_4 binding (1.6-fold increase; $P = 0.0004$), while the children cohort
237 observed lower differences between IgE (5.2-fold increase) and IgG4 binding (1.5-fold
238 increase; $P = 0.02$).

239

240 ***3.3 Patient-specific IgE and IgG_4 epitopes on Lat c 1.01***

241 The IgE- and IgG_4-binding regions on Lat c 1.01 were further investigated using seven
242 overlapping peptides. Inhibition ELISAs were performed using these peptides to inhibit IgE
243 and IgG_4 binding to rLat c 1.01 and the IgE and IgG_4 inhibition profiles for each of the 15
244 children and eight adults compared (Figure 3). The maximum peptide inhibition reached for
245 IgE 95.5% (C12) and for IgG4 up to 72.9% (C3). In general, the binding profiles differed
246 considerably between the patients, but no single peptide demonstrated outstanding inhibition
247 of either IgE or IgG_4 antibodies, suggesting the presence of patient-specific epitopes. Two
248 children demonstrated only IgE binding (C1 and C7), or with marginal IgG_4 binding (C4, C6
249 and C9), while all adults demonstrated both IgE and IgG_4 bindings. Two children with only
250 IgG_4 binding were also identified. Some patients demonstrated distinctly different IgE and IgG_4
251 bindings patterns to each peptide, as seen for example for patient C2. All seven peptides
252 exhibited relatively uniform IgE inhibition in six patients (C8, C11, C15, A2-3 and A5).

253

254 ***3.4 Unanimous IgE epitopes identified on Lat c 1.01***

255 The inhibition of IgE binding to rLat c 1.01 by seven overlapping peptides for all 23 patients
256 was combined and analyzed to determine the unanimous epitopes (Figure 4A). Five peptides
257 demonstrated inhibition of IgE binding significantly higher than that of non-atopic controls.
258 Two peptides (1 and 7) were the most statistically significant, with mean inhibition percentages
259 of 31.5% ($P = 0.005$; peptide 1) and 34.4% ($P = 0.009$; peptide 7), while three peptides (2, 3
260 and 4) followed with inhibition percentages of 45.5% ($P = 0.02$; peptide 2), 45.4% ($P = 0.03$;
261 peptide 3) and 48.2% ($P = 0.04$; peptide 4). Peptides 5 and 6 achieved the highest inhibition
262 percentages of 48.2% and 48.0%, respectively; however, not significantly different to non-
263 atopic controls due to large distributions of negative controls. Furthermore, no difference in
264 the IgE inhibition was observed between adult and children, with both cohorts following a
265 similar trend (Figure 4B).

266 For IgG_4, no peptide demonstrated significantly higher inhibitions than the non-atopic
267 controls (Figure 4C), with peptide 6 demonstrating the highest inhibition with only an average
268 of 19.5%. None of the seven peptides significantly inhibited IgG_4 more than the other, and no
269 difference in the IgG_4 inhibition between adults and children was observed (Figure 4D).

270

271 ***3.5 Two epitope regions predicted on Lat c 1.01***

272 To evaluate the proximity of potential linear IgE epitopes on Lat c 1.01, the amino acid
273 sequences of fish parvalbumins with known IgE epitopes and Lat c 1.01 were aligned (Figure
274 5A), as well as 3D models generated for each protein (Figure 5B). Peptides 1, 2 and 7 inhibited
275 significantly more serum IgE-binding to rLat c 1.01 than other peptides. The matching regions
276 to these peptides on Lat c 1.01 were found on both the N- and C-terminal ends of the allergen
277 (underlined in Figure 5A and shaded in Figure 5B in a *navy-blue* gradient). The most probable
278 IgE-binding region on the N-terminus of Lat c 1.01 is where peptide 1 and 2 overlap by nine
279 amino acids (AACQAADSF). As both peptides were able to considerably inhibit serum IgE-
280 binding, it is very likely that one epitope is located within these nine-amino acids. Peptides 6
281 and 7 share 21 amino acids; however, only peptide 7 could significantly inhibit IgE-binding to
282 rLat c 1.01. Therefore, the C-terminal region of Lat c 1.01 including the four amino acids
283 distinct for peptide 7, represent the second most likely IgE epitope of this parvalbumin. Both
284 possible IgE binding regions are very similar to the identified regions on parvalbumin from
285 cod (Gad m 1) and carp (Cyp c 1). The location of possible IgG_4 epitopes could not be predicted
286 due to the peptides demonstrating no significant inhibition in these experiments.

287

288 **4. Discussion**

289 This is the first published study to characterize and compare IgE and IgG4 antibody-binding
290 epitopes on the major fish allergen parvalbumin. This study focused in particular on
291 parvalbumin from one of the most consumed fish species from South East Asia and Australia.
292 Lat c 1.01 is the major allergen from Asian seabass (Sharp et al., 2014), and in this study, the
293 reactivity of 23 fish-allergic patient was analyzed.

294 Serum IgE-binding detected by immunoblotting under reducing conditions for a high
295 number of patients (22/23 patients) suggests the presence of linear IgE epitopes on Lat c 1.01.

296 However, IgE binding of the same patients (22/23 patients) to the native form of Lat c 1.01
297 indicates the possible presence of conformational epitopes. In general, much fewer patients
298 demonstrated IgG_4-binding by ELISA compared to immunoblotting which was done under
299 reducing conditions, suggesting IgG_4 may recognize epitopes that are not easily accessible in
300 the tertiary structure of rLat c 1.01. No notable differences were found between the two cohorts
301 of different age groups in binding patterns, except that a higher level of serum IgE-binding to
302 rLat c 1.01 was observed for adults (individual and as a cohort). The lack of difference in IgE
303 binding between these two cohorts is possibly due to the fact that fish allergy, similar to
304 shellfish and peanut allergy, is rarely outgrown and continues into adulthood (Sicherer et al.,
305 2004; Ruethers et al., 2018).

306 Antibody isotype switching in B cells usually occur from IgG_4 to IgE as a consequence
307 of the sequence order of the antibody isotypes situated on their chromosome (Aalberse, 2011;
308 Aalberse et al., 2009), suggesting that IgE is more likely to be present than IgG_4. However, it
309 was also demonstrated that non-sequential class switch to IgE can occur independently
310 (Niederberger et al., 2002). In this study, we found more fish-allergic patients with a robust
311 IgE-binding than IgG_4-binding to rLat c 1.01, demonstrating a dissociation between IgG_4 and
312 IgE in fish-allergic patients as reported previously (Swoboda et al., 2002; Swoboda et al.,
313 2007). This was consistent regardless of the age of patients as significantly higher levels of
314 IgE-binding were observed compared to IgG_4-binding in both cohorts. Despite IgG_4 being
315 associated with tolerance and desensitization in allergic disease, our findings further support
316 that specific IgE is a reliable antibody marker that directly correlates with allergic symptoms
317 (Aalberse et al., 2009; Aalberse, 2011; Eckl-Dorna et al., 2019).

318 Beta parvalbumin represents an EF-hand superfamily with three highly conserved
319 helix-loop-helix motifs. Among these three regions only two are functional in binding Ca^{2+}.
320 Based on the extensive sequence alignment of 98 beta-parvalbumins by Carrera et al., the most
321 conserved region seems to be between position 46 to 77 (Carrera et al., 2019)**.** Subsequent
322 generation of peptides and IgE binding studies confirmed the results of previous studies that
323 major IgE binding for most parvalbumins are in the early N-terminus (Salmon), adjacent to the
324 first Ca^{2+} binding side (Salmon and Baltic cod) and in the C-terminal region (Atlantic and
325 Baltic cod) (Sharp eta l., 2014). Using overlapping peptides, we demonstrated that common
326 IgE epitopes are observed more at terminal regions of allergens, while IgG_4 epitopes of Lat c
327 1.01 are most likely patient-specific. Our current study demonstrates that the major IgE binding
328 for Asian seabass (Lat c 1.01) is also located in the N-terminal region (residues 10-30) as well

329 as C-terminal region (residues 90 to 110), which are very similar to Salmon (Sal s 1.01), Baltic
330 cod (Gad c 1.01) and Atlantic cod (Gad m 1.01) (Perez-Gordo et al., 2013; Elsayed and Apold,
331 1983; Untersmayr et al., 2006; Yoshida et al., 2008; Perez-Gordo et al., 2012). These findings
332 are of clinical importance as patients from the Asia-Pacific region, sensitised to Asian seabass,
333 would probably also react to ingested cod.

334 Peptide 1 (AAs 1 – 25), situated in the least conserved region of fish parvalbumin
335 (Sharp and Lopata, 2014), has previously only been identified in an epitope region of
336 parvalbumin Sal s 1 from Atlantic salmon (Perez-Gordo et al., 2012). The absence of epitope
337 discovery in this region for other fish parvalbumins may explain the clinically unique mono-
338 sensitization to salmon within fish-allergic patients (Perez-Gordo et al., 2012; Vàzquez-Cortés
339 S, 2012; Sharp et al., 2015). Patients whose IgE specifically identify this N-terminal epitope
340 may have low cross-reactivity to other fish due to the low sequence identity in this region. No
341 patient from this study however presented IgE that solely binds to this region. In contrast,
342 peptide 2 (aa 12-34) aligns with a region that contains the most frequent epitopes mapped on
343 parvalbumins from fish including Baltic cod, Atlantic salmon, Pacific mackerel and carp
344 (Sharp and Lopata, 2014). This epitope seems to be in a highly cross-reactive region of
345 parvalbumin and patients who recognize peptide 2 would most likely react to many other fish
346 parvalbumins. It is not surprising that this cross-reactive epitope has been identified in Lat c
347 1.01, as Asian seabass parvalbumin has previously been characterized as a highly cross-
348 reactive allergen among sensitized individuals (Sharp et al., 2015). Further analysis using
349 shorter overlapping peptides for more precise coverage as well as peptide alanine screening or
350 shotgun proteomics by mass spectrometry could increase the resolution of the mapped Lat c
351 1.01 epitopes (Zhao et al., 2017; Liu and Sathe, 2018; Carrera et al., 2019).

352 This study demonstrates age independent-IgE sensitization frequencies to parvalbumin.
353 Our findings indicate the greater attribution of IgE over IgG_4 on the reactivity to parvalbumin
354 in all fish-allergic patients of two age groups. Patient profile of both age groups revealed
355 common IgE epitopes, while many patient-specific IgG_4 epitopes were demonstrated.
356 Understanding IgE epitopes on allergenic proteins is crucial for the development of peptide-
357 based immunotherapies. The application of hypoallergenic proteins in sIT must demonstrate a
358 lower probability of IgE-mediated anaphylaxis after administration (Swoboda et al., 2002;
359 Swoboda et al., 2007), as well as alleviating allergic Th2 responses (van der Ventel et al.,
360 2011). Based on our data, the employment of modified (mutated) common IgE epitopes, that
361 are highly reactive amongst the majority of fish-allergic patients of all ages, should be

considered during the design of sIT against fish allergy to Asian seabass. The use of mutated hypoallergenic parvalbumin may suit treating patients with independent IgE and IgG_4 epitopes – as the disruption or loss of linear and/or conformational epitopes of allergenic proteins can lead to reduction in allergenicity through loss of IgE-binding ,while the interruption of IgG_4-binding is prevented (Swoboda et al., 2007; Freidl et al., 2017). One of the speculated IgE epitopes spans over the calcium-binding region of at least four parvalbumins (Gad c 1, Cyp c 1, Gad m 1 and Lat c 1), including the N-terminal epitope in Lat c 1.01 established in the present study. A modification in this epitope could be useful in treating patients who are sensitized to fish species from European and Asia Pacific regions. Indeed, it was recently demonstrated that antibodies raised against a mutant carp parvalbumin protected against allergic reactions in a murine model (Freidl et al., 2017). Furthermore, elevated allergen-specific IgG_4 is thought to play a key role in successful immunotherapy, yet to be demonstrated for fish allergy (van de Veen and Akdis, 2016; Eckl-Dorna et al., 2019). It is possible that patients with shared IgE and IgG_4 epitopes would positively respond to a sIT using the native (no modification) allergens due to the increase of serum IgG_4 which acts as a competitive blocking antibody to IgE, as has been demonstrated for milk allergy (Savilahti et al., 2014). However, there is a greater risk of IgE-specific antibody upregulation associated in using unmodified allergens.

In conclusion, our investigation of antibody-binding epitopes of parvalbumin from Asian seabass contributes greatly to our knowledge on allergic sensitization patterns among fish allergic adults and children. The consumption and international trade of Asian seabass is greater than some of the previously studied fish species in the Asia Pacific regions, and is growing rapidly worldwide. This is the first study to characterize IgE and IgG_4 binding epitopes of the major fish allergen parvalbumin and identify the IgE binding regions of Lat c 1.01 from Asian seabass. This knowledge is an important discovery to pave the way for developing new and targeted therapies for the Asia-Pacific region.

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401

402 **Conflicts of interest**

403 The authors declare that they have no conflicts of interest.

404

405 **Author contributions**

406 M.F.S.: Data curation; Formal analysis; Investigation; Methodology; Resources; Validation;
407 Visualization; Roles/Writing - original draft; Writing - review & editing. A.C.T.: Formal
408 analysis; Visualization; Roles/Writing - original draft; Writing - review & editing. T.R.:
409 Visualization; Writing - review & editing. J.N.S.: Resources; Writing - review & editing. N.D.:
410 Conceptualization; Writing - review & editing. S.D.K.: Writing - review & editing. A.L.L.:
411 Conceptualization; Funding acquisition; Investigation; Project administration; Resources;
412 Supervision; Validation; Roles/Writing - original draft; Writing - review & editing.

413

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568 **Table 1.** Clinical characteristics of 23 fish-allergic patients recruited for the study.

ID	Age (years)	Sex	ImmunoCAP (kU/L)			Skin Prick Test (mm)			Symptoms upon ingestion	Implicated fish species
			Cod (f3)	Tuna (f40)	Salmon (f41)	Cod	Tuna	Salmon		
A1	29	M	6.45	0.41	0.43	10	7	8	R, OAS	Salmon trevally, blue grenadier,
A2	26	F	1.40	-	1.35	-	-	-	A, GIS, U	Sea perch, unknown
A3	26	F	0.91	4.46	5.75	-	6	10	A, OAS	Unknown, trout
A4	32	F	7.62	5.67	13.6	-	-	-	A, R, OAS, AE, U	Unknown
A5	19	M	4.23	7.75	7.14	-	-	-	A, R, AE, U	Unknown
A6	46	M	2.74	3.78	-	-	-	-	R, OAS	Snapper, tuna
A7	32	F	3.36	2.17	3.20	-	-	-	A, R, OAS, U	Unknown
A8	33	F	-	13.9	25.8	-	-	-	A, AN, U, R, OAS	Trout
C1	7	M	-	-	-	6	3	4	OAS, GIS	White fish
C2	5	F	-	-	-	5	6	3	U	Salmon
C3	4	M	-	-	-	5	3	4	AN, OAS	Trout, tuna, whitefish
C4	10	M	-	-	-	8	3	3	AN	Catfish
C5	7	M	-	-	-	6	0	2	AE, U	Smoked salmon, fish finger (white fish)
C6	5	M	-	-	-	0	4	0	AE, U	Bream
C7	9	F	-	-	-	11	5	6	AN, R, U	Milkfish, tilapia
C8	12	F	-	-	-	8.5	5.5	0	AE, U	-
C9	15	F	-	-	-	5	5	6	R, OAS, AE	Red mowong, whiting

C10	14	M	-	-	-	11.5	8	8	AE, OAS, GIS	Salmon, silver Perch, flake (shark)
C11	12	F	-	-	-	11	3.5	5	U	Ling, whiting, Asian seabass
C12	16	M	-	-	-	8	4.5	3.5	OAS	White fish
C13	2	F	-	-	-	4	5	5	U	Salmon
C14	7	F	-	-	-	10	5.5	3	AN	Catfish
C15	14	M	-	-	-	0	0	0	AN, GIS, OAS	Catfish, white fish
N1	21	M	<0.01	-	-	-	-	-	-	None
N2	53	M	<0.01	-	-	-	-	-	-	None
N3	29	F	<0.01	-	-	-	-	-	-	None
N4	41	F	<0.01	-	-	-	-	-	-	None
N5	29	F	<0.01	-	-	-	-	-	-	None

569

570 Patient ID: A, C and N denote for adult, children and negative control, respectively. Symptoms: A, asthma; AN,
571 anaphylaxis; AE, angioedema; E, eczema; GIS, gastrointestinal syndrome; OAS, oral allergy syndrome; R,
572 rhinitis; U, urticaria. '-'=Not performed.

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577 Figure 1. Serum IgE and IgG4 analyses of 23 fish-allergic patients against Asian seabass
578 major allergen rLat c 1.01, by immunoblotting in reduced condition. A) Serum from
579 15 pediatric (C1-15) and eight adult (A1-8) fish-allergic patients were subjected to purified
580 recombinant rLat c 1.01 and analyzed for IgE (top) and IgG4 (bottom) binding. Five non-fish
581 allergic patients (N1-5) were included as negative controls. Parvalbumin monomers, dimers
582 and oligomers are boxed in red, green and blue, respectively. B) Purified rLat c 1.01 was
583 detected and the protein purity and integrity were confirmed on a Coomassie Brilliant Blue
584 stained 15% polyacrylamide gel (left lane), immunoblots with monoclonal anti-parvalbumin
585 PARV-19 antibody (middle lane) and polyclonal rabbit anti-Asian seabass parvalbumin
586 antibody (right lane).
587
588 Figure 2. Evaluation of serum IgE and IgG4 binding to native form of Asian seabass
589 parvalbumin, rLat c 1.01 by indirect ELISA. A) The serum IgE (black) and IgG4 (grey)
590 reactivities to rLat c 1.01 (1 µg/mL) were evaluated for 15 fish-allergic children (C1-15) and
591 eight adults (A1-8) by measuring the absorbance at 450 nm. The reactivity for individual
592 patient serum was confirmed using a cut-off derived from two standard deviations above the
593 mean reactivity of five non-allergic subjects to each antibody isotype [indicated by the dotted
594 line (IgE; black line=0.03, IgG4; grey line=0.20)]. B) Mean fold increase of IgE and IgG4
595 antibody bindings against rLat c 1.01 over non-atopic cpontrols were compared for each of
596 children and adult cohorts. Error bars represent standard error of the mean. *$P < 0.05$, ***$P <$
597 0.0005.
598
599 Figure 3. Analysis of peptide binding regions using serum IgE and IgG4 by inhibition
600 ELISA. Binding regions were analyzed using the synthetic overlapping peptides for individual
601 fish-allergic patient serum IgE (black) and IgG4 (grey). Binding profiles of individual 15
602 children (C1-15) and eight adults (A1-8), were determined for each of IgE and IgG4. Each
603 cohort is grouped and shown separately by dotted line. Y-axis represents the percentage of
604 inhibition of serum IgE and IgG4 binding to rLat c 1.01 (20 ng) induced with the seven
605 generated peptides (250 ng; x-axis).
606 Figure captions
607
608 Figure 4. Determination of IgE and IgG4 binding regions in rLat c 1.01 for pediatric and
609 adult fish-allergic patients using seven overlapping peptides. Percentages of IgE inhibition
610 against seven peptide regions were calculated for A) all 24 patients (dark grey; 15 children and
611 eight adults) and five non-atopic controls (dotted black), and B) each cohort of children (light
612 grey), adults (black) and non-atopic controls (dotted black). Percentages of IgG4 inhibition
613 against seven peptide regions were calculated for C) all 24 patients (dark grey; 15 children and
614 eight adults) and five non-atopic controls (dotted black), and D) each cohort of children (light
615 grey), adults (black) and non-atopic controls (dotted black). Y-axis represents the percentage
616 of inhibition of respective serum IgE and IgG4 (1:10) binding to 20 ng rLat c 1.01 with 250 ng
617 of the seven generated peptides (x-axis). Error bars represent standard error of the mean. *$P <$
618 0.05, **$P < 0.01$.
619
620 Figure 5. Comparative modelling of fish parvalbumin and elucidation of IgE epitopes. A)
621 Amino acid sequence alignment of five fish parvalbumins. IgE epitopes previously elucidated
622 are indicated in color shading as follows; Baltic cod (Gad c 1.01; UniProtKB Accession
623 number, P02622.1) cyan, common carp (Cyp c 1.01; Q8UUS2) yellow, Atlantic salmon (Sal s
624 1.01; B5DH15) red, Pacific mackerel (Sco j 1.01; P59747) green and Atlantic cod (Gad m

625 1.01; Q90YL0) pink. The suspected IgE epitope regions is underlined in two navy-blue
626 gradient lines under Lat c 1.01 sequence. Sequences of the overlapping peptides from
627 this study are aligned under the parvalbumin sequences with assigned peptide numbers.
628 The two calciumbinding sites are boxed in orange. B) Protein models of the five fish
629 parvalbumins with their elucidated IgE epitopes highlighted in their respective colors.
630 The model of Lat c 1.01 is shown with matching navy-blue gradient regions for the
631 speculated IgE epitopes.
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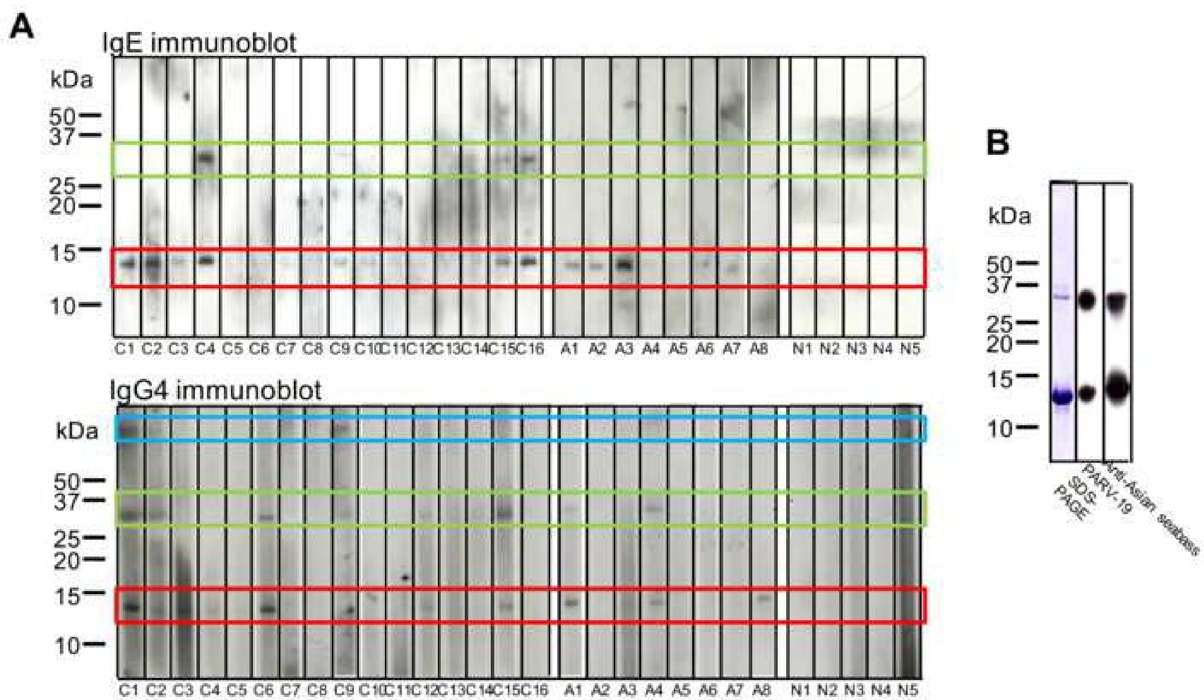

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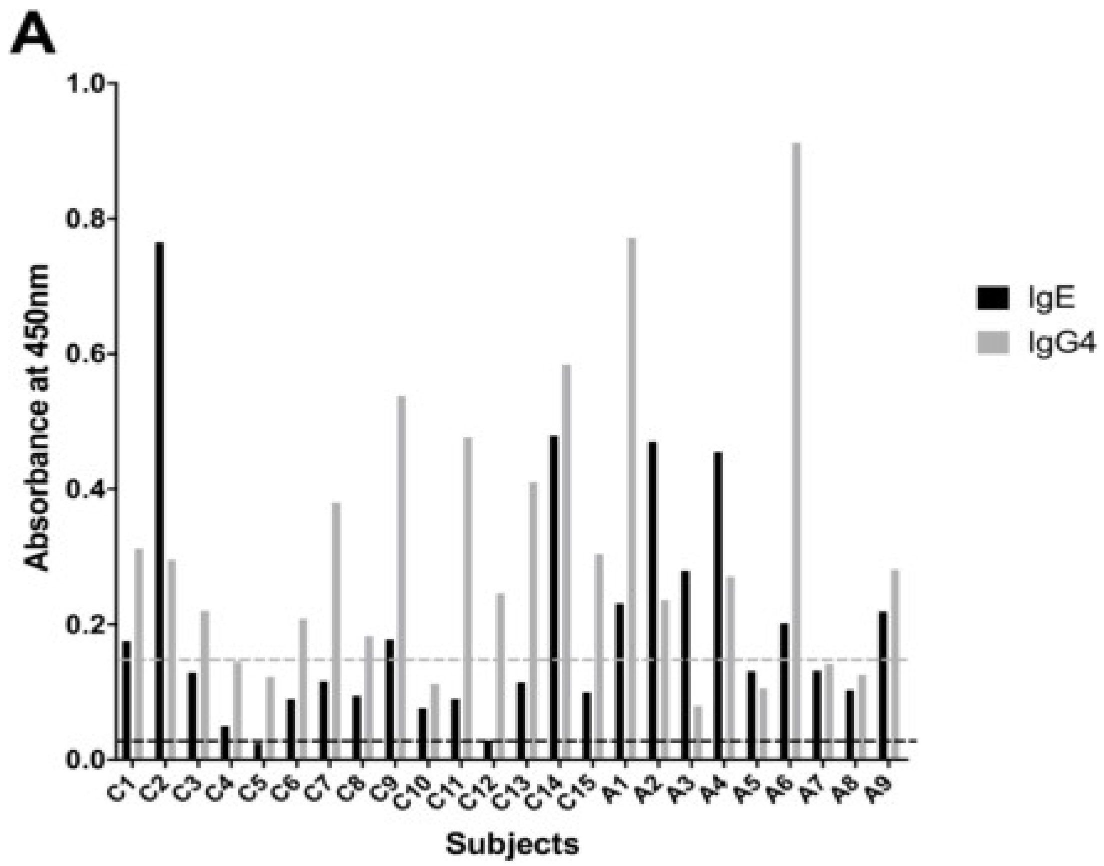

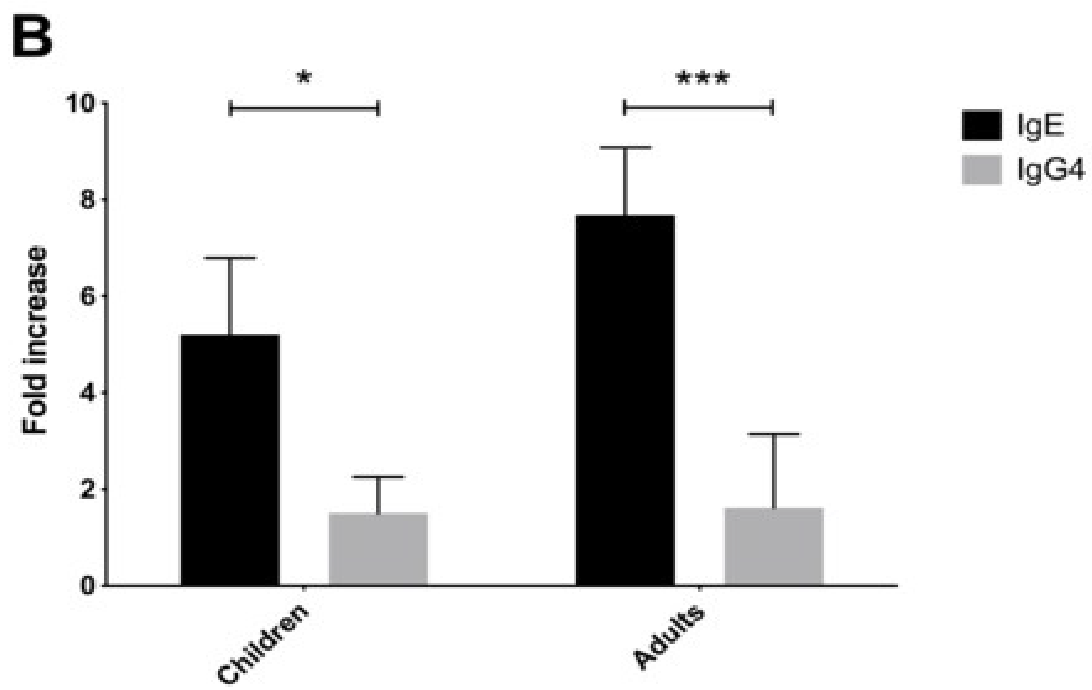

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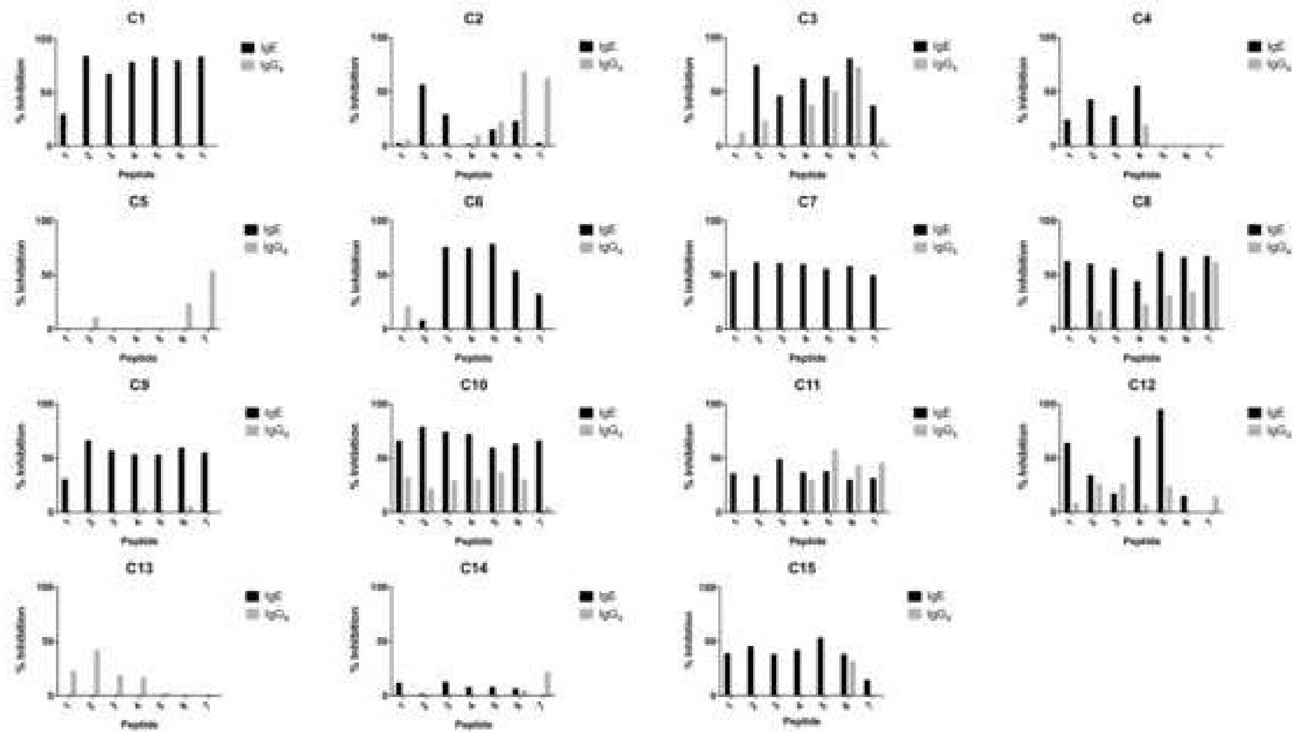

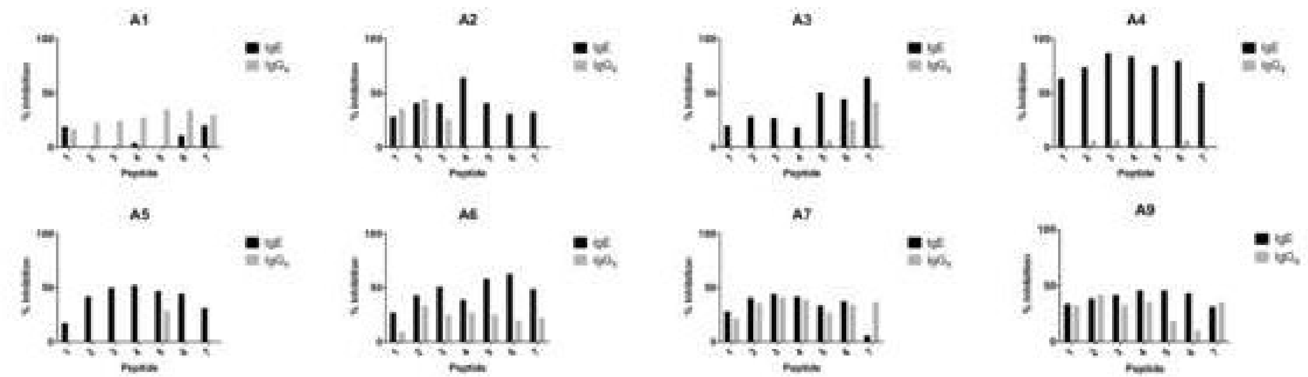

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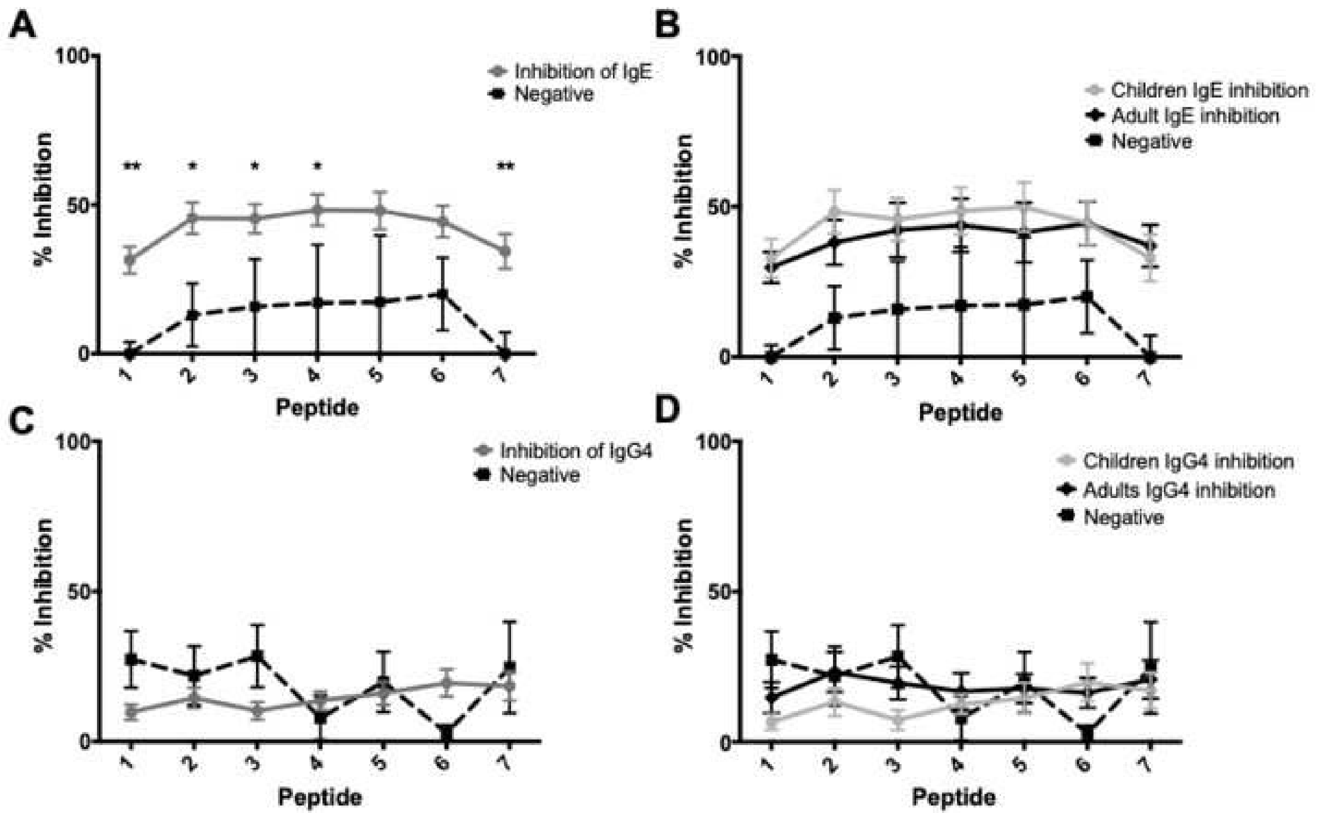

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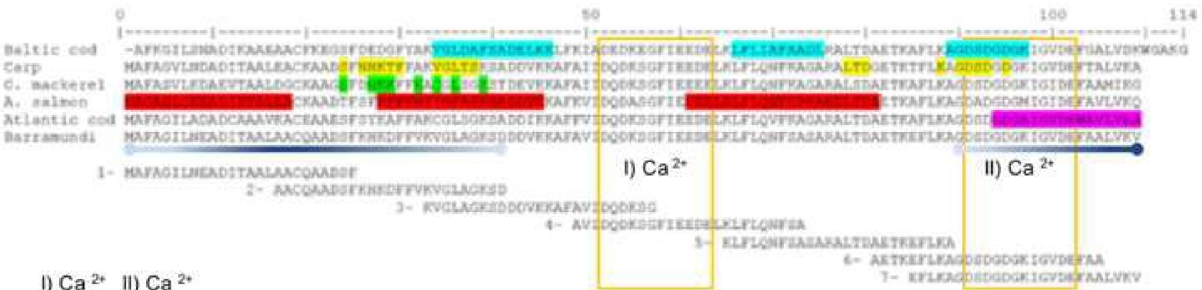

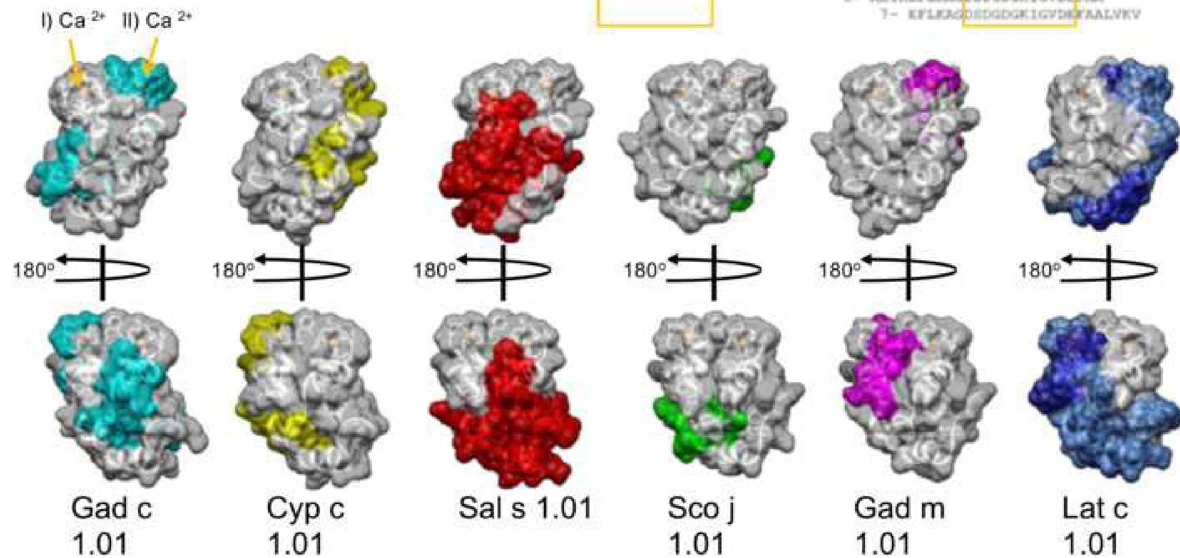

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