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DOI:

[10.1111/all.14301](https://doi.org/10.1111/all.14301)

Document Version

Peer reviewed version

[Link to publication record in King's Research Portal](#)

Citation for published version (APA):

Santos, A., Barbosa-Morais, N. L., Hurlburt, B. K., Ramaswamy, S., Hemmings, O., Kwok, M., O'Rourke, C., Bahnson, H. T., Cheng, H., James, L., Gould, H., Sutton, B. J., Maleki, S. J., & Lack, G. (2020). IgE to epitopes of Ara h 2 enhance the diagnostic accuracy of Ara h 2-specific IgE. *Allergy*, 75(9), 2309-2318. <https://doi.org/10.1111/all.14301>

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5
6
7 Article type : Original Article: Food Allergy and Gastrointestinal Disease

8
9
10 **Title:** IgE to epitopes of Ara h 2 enhance the diagnostic accuracy of Ara h 2-specific IgE

11
12 **Short title:** Ara h 2 peptides improve peanut allergy diagnosis

13
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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/ALL.14301](https://doi.org/10.1111/ALL.14301)

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42 **Acknowledgments:**

43 N.L.B.-M. was supported by a Marie Curie International Outgoing Fellowship (European

44 Commission) and by an FCT Investigator Starting Grant (Portugal). B.J.S and H.J.G. acknowledge

45 support from the Medical Research Council (G1100090). The authors are grateful to Dr Åsa Marknell

46 DeWitt and her team at the Special Allergen Service (SAS) at Thermofisher in Uppsala, Sweden for

47 coupling the JPT peptides to the ImmunoCAP technology through a collaboration agreement with

48 King's College London.

49

50 **Source of funding:** This work was supported by the Medical Research Council (MRC Clinical

51 Research Training Fellowship G090218, MRC Centenary Early Career Award and MRC Clinician

52 Scientist Fellowship MR/M008517/1, all awarded to A. F. Santos), the Department of Health via the

53 National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to

54 Guy's & St Thomas' NHS Foundation Trust in partnership with King's College London and King's

55 College Hospital NHS Foundation Trust; the US Department of Agriculture, Agricultural Research

56 Service (USDA-ARS6054-43440-046-00D; USDA-NIFA) and the National Peanut Board
57 (GRANT12229460).

58

59 **Competing interests:** Dr. Santos reports grants from Medical Research Council, non-financial
60 support from Thermo Scientific, during the conduct of the study; grants from Immune Tolerance
61 Network/NIAID, grants from Asthma UK, personal fees from Thermo Scientific, Buhlmann, Nutricia,
62 Infomed, other from Allergy Therapeutics, outside the submitted work; Mr. O'Rourke reports grants
63 from Benaroya Research Institute/Immune Tolerance Network, other from Kings College, during the
64 conduct of the study; Henry Bahnson reports grants from Benaroya Research Institute / Immune
65 Tolerance Network, personal fees from King's College London, during the conduct of the study; Dr.
66 Lack reports other from UK Dept of Health through NIHR, during the conduct of the study; personal
67 fees and other from DBV Technologies, other from Mission Mighty Me, personal fees from Aravax,
68 personal fees from Novartis, personal fees from ALK Abello , outside the submitted work.

69

70 **Authors contributions:** AFS designed the study, recruited the patients, led the experimental work,
71 data analyses and interpretation of results and wrote the manuscript; BKH, HC and SJM designed and
72 performed the microarray experiments; NLBM analysed the microarray data; OH and MK performed
73 the ImmunoCAP experiments; HTB performed statistical analyses of ImmunoCAP data; SR, LKJ,
74 HJG and BJS performed the structural analyses and advised on the experimental approach; GL
75 contributed to study design, patient recruitment and interpretation of results; all authors contributed to
76 the manuscript and approved the final version.

77

78

79 **Abstract**

80 **Background:** Understanding the discrepancy between IgE sensitization and allergic reactions to
81 peanut could facilitate diagnosis and lead to novel means of treating peanut allergy.

82 **Objective:** To identify differences in IgE and IgG4 binding to peanut peptides between peanut
83 allergic (PA) and peanut sensitized but tolerant (PS) children.

84 **Methods:** PA (n=56), PS (n=42) and non-sensitized non-allergic (NA, n=10) patients were studied.
85 Synthetic overlapping 15-mer peptides of peanut allergens (Ara h 1-11) were spotted onto microarray
86 slides and patients' samples were tested for IgE and IgG4 binding using immunofluorescence. IgE
87 and IgG4 levels to selected peptides were quantified using ImmunoCAP. Diagnostic model
88 comparisons were performed using likelihood ratio tests between each specified nominal logistic
89 regression models.

90 **Results:** Seven peptides on Ara h 1, Ara h 2 and Ara h 3 were bound more by IgE of PA compared to
91 PS patients on the microarray. IgE binding to one peptide on Ara h 5 and IgG4 binding to one Ara h 9
92 peptide were greater in PS than in PA patients. Using ImmunoCAP, IgE to the Ara h 2 peptides
93 enhanced the diagnostic accuracy of Ara h 2-specific IgE. Ratios of IgG4/IgE to 4 out of the 7
94 peptides were higher in PS than in PA subjects.

95 **Conclusions:** Ara h 2 peptide-specific IgE added diagnostic value to Ara h 2-specific IgE. Ability of
96 peptide-specific IgG4 to surmount their IgE counterpart seems to be important in established peanut
97 tolerance.

98

99 **Keywords:**

100 Ara h 2, diagnosis, epitopes, food allergy peanut allergy

101

102 **Figure count:** 5

103 **Table count:** 1

104 **Online Repository Material:** 4 tables and 13 figures

105 **Word count:** 3924

106

107 **Abbreviations:**

108 DBPCFC, double-blind placebo-controlled food-challenge

109 FBR, foreground to background ratio

110 MBP, maltose-binding protein

111 MAT, mast cell activation test

112 OFC, oral food challenge
113 P-sIgE, peanut-specific IgE
114 PA, peanut allergic
115 PS, peanut-sensitized tolerant
116 PPV, positive predictive value
117 RBL, rat basophilic leukemia
118 SPT, skin prick test
119
120

121 **Introduction:**

122 Allergen-specific IgE is necessary but not sufficient for the development of allergic reactions to a
123 food allergen. Thus, IgE sensitization to foods can often be identified without proven clinical
124 relevance. For instance, in the case of peanut, about 11.8% of school-age children in the United
125 Kingdom have detectable specific IgE to peanut and only 2.6% are actually peanut allergic as
126 confirmed by double-blind placebo-controlled food-challenge (DBPCFC)¹. This discrepancy between
127 allergic sensitization and clinical reactivity poses diagnostic difficulties and raises fundamental
128 questions about the mechanisms of food allergy and oral tolerance. If IgE binds to the allergen in
129 immunoassays to peanut, why is it not able to cause effector cell activation in the majority of patients?
130 We have been addressing two non-mutually exclusive hypotheses to explain the discrepancy between
131 the presence of peanut-specific IgE (P-sIgE) and peanut allergy. The first hypothesis is that there may
132 be differences in the characteristics of allergen-specific IgE between peanut allergic (PA) and peanut-
133 sensitized but tolerant (PS) patients. We have previously shown that, at the population level, PA
134 patients tend to have higher levels of P-sIgE and to have IgE directed to the major peanut allergens,
135 Ara h 1, Ara h 2 and Ara h 3². However, there is a large overlap in the distribution of specific IgE
136 levels in PA and PS patients. At the individual level, many cases can be found of PS patients who eat
137 peanut without developing any symptoms and have relatively higher levels of P-sIgE compared to PA
138 patients who develop allergic reactions, often severe, when exposed to peanut. This is also the case
139 when considering specific IgE to Ara h 2, which has proved to be particularly discriminative between
140 allergic and tolerant individuals³. Refining IgE specificity at the epitope level may clarify this further;
141 with existing experimental approaches using microarray and other platforms allowing to primarily
142 identify linear epitopes⁴⁻⁷. Various groups have studied IgE epitopes on peanut allergens; however,
143 most studies focused on searching for peanut epitopes bound by IgE of peanut allergic patients and
144 were limited to Ara h 2 or at most Ara h 1, Ara h 2 and Ara h 3. Ours was the first study to test IgE
145 and IgG4 binding to all peanut allergens and to analyze the differential binding between PA and PS
146 who were mostly sensitized to peanut major allergens, some able to cause allergic symptoms (as in
147 the case of PA patients) and some not (as in the case of PS subjects).

148 The second hypothesis to explain the discrepancy between sensitization and allergy is that PS patients
149 may have a peanut-specific antibody, such as IgG4, that are able to interfere with the allergen-IgE

150 interaction. We previously showed that the levels of IgG4 to peanut were higher in PS compared to
151 PA patients but it was the relative amount of IgG4 compared to IgE in individual patients, i.e. the
152 IgG4/IgE ratio, that enabled a clearer distinction between PA and PS patients with PS patients having
153 higher IgG4/IgE ratios for peanut, Ara h 1, Ara h 2 and Ara h 3². Depletion of IgG4 antibodies from
154 plasma samples with detectable IgE to the major peanut allergens that would otherwise be predictive
155 of peanut allergy partially restored mast cell activation, which supported a role of IgG4 in the absence
156 of an effector cell response characteristic of peanut tolerance².

157 A complete understanding of the mechanisms by which IgE and allergen may or may not be able to
158 elicit effector cell activation that is responsible for the clinical manifestations of allergic disease
159 requires a molecular approach. In this study, we aimed to identify the epitope specificities of IgE and
160 IgG4 in PA and PS children to improve our understanding of the interplay between IgE and IgG4 in
161 modulating peanut allergen-induced effector cell responses and consequent allergic reactions.

162

163 **Methods:**

164 **Study procedures**

165 Patients undergoing diagnostic evaluation for suspected peanut allergy were studied. The study was
166 approved by the South East London 2 Research Ethics Committee. Written informed consent was
167 obtained from the parents of all participants. Study participants underwent detailed clinical
168 assessment, skin prick testing, specific IgE and IgG4 testing and oral peanut challenges, as previously
169 described⁸. Skin prick testing was performed using a commercially available peanut extract (ALK-
170 Abelló). Serum specific IgE and IgG4 to peanut were measured using an immunoenzymatic assay
171 (ImmunoCAP, ThermoFisher). Specific IgE to 112 allergens was determined using the ISAC
172 microarray (ThermoFisher).

173 Study participants were grouped as PA, PS and non-sensitized non-allergic (NA). Peanut allergy was
174 confirmed by a positive oral food challenge (OFC) or by the combination of reported immediate-type
175 allergic reactions to peanut and skin prick test (SPT) and/or P-sIgE greater or equal than the validated
176 95% positive predictive value (PPV) cut-offs of 8 mm and 15 kU/L, respectively. Peanut allergy was

177 excluded by a negative OFC or the ability to eat ≥ 4 g of peanut protein twice a week, as assessed by a
178 validated peanut consumption questionnaire. Peanut-sensitization was defined by a wheal size of
179 peanut SPT ≥ 1 mm and/or specific IgE ≥ 0.10 KU_A/L. Out of the 108 patients studied, 78 (72%) had
180 OFC, 68 had DBPCFC and 10 had open OFC for logistical reasons, as previously reported. 12 (15%)
181 of OFC were positive and 66 (85%) were negative.

182

183 **Peanut peptide microarray**

184 Synthetic overlapping 15-mer peptides representing the entire amino acid sequence of ten peanut
185 allergens (Table E1), offset by 5 amino acids, were synthesized and printed in triplicate onto
186 microarray slides (JPT Peptide Technologies GmbH, Berlin, Germany) together with peptides from
187 other nuts and plant foods (see Table E2 for a full list of the peptides tested). Slides were placed in
188 individual chambers of a HS400 ProTM (Tecan, San Jose, CA) and blocked in filtered Superblock
189 (Thermo, Rockford, IL) for 30 minutes at room temperature (RT). Following a wash with Tris-
190 buffered saline containing Tween-20, patients' plasma were injected and incubated at 4°C overnight.
191 Slides were sequentially washed and incubated with mouse anti-human IgE and Cy3-conjugated goat
192 anti-mouse IgG (both Life technologies, Grand Island, NY). Slides were scanned using GenePix-
193 4000B and the software GenePix-Pro7. The same slide was then re-blocked with Superblock and
194 sequentially washed as above and incubated with rabbit-anti-human IgG4 (Abcam, Cambridge, MA)
195 and anti-rabbit IgG Alexa Fluor (Life Technologies, Grand Island, NY) at RT for 30 minutes. The
196 slides were washed and dried before scanning as above. IgE binding was measured by the Cy3, green
197 fluorescence at 532 nm, and IgG4 binding by Alexa Fluor red fluorescence at 635 nm wavelength.

198

199 **Microarray data analysis**

200 Scanning slides with GenePix Pro 7 (GP7) software generated multi-layer TIFF files which were
201 analyzed by GP7 to generate GPR data files. These were read into the statistical software environment
202 *R*, where all statistical analyses were done⁹. Quality assessment, pre-processing and differential
203 binding analysis of the microarray data were performed using tools included in the *limma* package¹⁰
204 available through the Bioconductor project¹¹. Pre-processing of data comprised log-subtraction of

205 mean background for each probe, mean-summarization of replicate probes followed by single-channel
206 quantile-normalization between arrays for contrasts involving only one antibody, and probe-level two-
207 channel loess-normalization within arrays before computing IgG4/IgE ratios. IgE and IgG4 binding
208 was expressed as the base 2 logarithm of the foreground to background ratio (FBR). See methods'
209 section of the online repository for more details.

210

211 **Identification of the epitopes in the 3D structure of the allergens**

212 The locations of the peptides in the 3D structures of the allergens, as deposited in the RCSB Protein
213 Data Bank, were identified and visualized using PyMOL¹².

214

215 **Quantification of specific IgE and IgG4 to peptides**

216 Unblocked peptides (JPT Peptide Technologies GmbH, Berlin, Germany) were conjugated to the
217 solid phase of ImmunoCAP by Thermofisher (Uppsala, Sweden). IgE and IgG4 binding was
218 quantified using the Phadia 100 following the manufacturer's instructions. IgG4/IgE ratios were
219 calculated as previously described by converting IgG4 levels from milligrams per liter to nanograms
220 per milliliter and the peanut-specific IgE levels from kilo unit per liter to nanograms per milliliter with
221 the use of the formula $\log_{10}((\text{IgG4} \times 1000) \div (\text{IgE} \times 2.4))^{2,13}$. Diagnostic model comparisons were
222 performed using likelihood ratio tests between each specified nominal logistic regression models
223 using SAS version 9.4 and JMP Pro 14.

224

225 **Results:**

226 **Study population**

227 Plasma samples of patients consecutively and prospectively recruited were tested on a peanut peptide
228 allergen microarray. Demographic and clinical data can be found in Table E3. Most patients were
229 sensitized to the three major peanut allergens Ara h 1-3 (Table E4). Data of the 89 patients for whom
230 there were results for both IgE and IgG4 binding following appropriate quality control (53 PA, 27 PS
231 and 9 NA) were analyzed and compared between PA and PS patients. Total IgE ($p=0.402$) and

232 peanut-specific IgG4 ($p=0.122$) was not significantly different between the 2 groups. PA patients had
233 higher sIgE to peanut, ($p<0.001$), Ara h 1 ($p=0.007$), Ara h 2 ($p<0.001$) and Ara h 3 ($p=0.017$) than
234 PS patients. Forty-six per cent of patients assessed on ISAC were sensitized to Ara h 6 (84% of PA
235 and 10% of PS) and 22% were sensitized to profilins (18% of PA and 33% of PS) with 13% being
236 sensitized to Phl p 12 (6% of PA and 25% of PS) – data not shown.

237

238 **Ara h 1, Ara h 2 and Ara h 3 peptides were differentially bound by IgE of peanut allergic and** 239 **sensitized but tolerant patients**

240 Various peanut peptides were able to bind IgE of peanut sensitized patients, including both PA and PS
241 patients (Figure E1). When analyzing the differences between PA and PS patients, 7 peptides
242 associated with the major peanut allergens Ara h 1, Ara h 2 and Ara h 3 emerged in the differential
243 binding analyses as having a higher degree of IgE binding in PA compared to PS patients (Figure 1,
244 Table I and Figure E2). Peptide 10, on Ara h 5 (AA51-65) was bound preferentially by IgE of PS than
245 by IgE of PA patients (Table I). There was a positive association between IgE to the Ara h 5 peptide
246 and IgE to peptides from Ara h 8 (Table E5). IgE binding to peptides from other peanut allergens was
247 not significantly different between PA and PS patients.

248

249 **All peptides identified on Ara h 1, Ara h 2 and Ara h 3 were located on the surface of the** 250 **allergens and thus were susceptible to antibody binding**

251 The identified Ara h 1, Ara h 2 and Ara h 3 peptides were located on the surface of the allergens in
252 structurally disordered or partially disordered loop regions (Figure 2). The Ara h 1 peptides (peptides
253 7 and 8) overlapped by 10 amino acids, ranging between amino acid 85 and 105 of the allergen, and
254 were located in a part of the protein that is absent from the crystal structure and was predicted as
255 disordered. Two of the Ara h 2 peptides, peptide 1(AA61-75) and peptide 3 (AA81-95) were located
256 on a flexible loop in a partially disordered region. The other two overlapping Ara h 2 peptides,
257 peptide 2 (AA26-40) and peptide 4 (AA31-45) consisted of parts of two helices linked by a loop,
258 located in the N-terminal region close to the link to maltose-binding protein (MBP) with which Ara h
259 2 was expressed and crystallized as a fusion protein¹⁴. The peptide identified in Ara h 3, peptide 9

260 (AA324-338) was located on a loop of a partially disordered region in an exposed part of the protein
261 crystal structure.

262

263 **Quantification and diagnostic utility of IgE to the 7 peptides using ImmunoCAP**

264 In order to validate our findings, we quantified IgE levels using the ImmunoCAP technology to the 7
265 peanut peptides that were bound more by IgE of PA than by IgE of PS on the microarray (Figures 3
266 and E3). The differences in IgE binding to the peanut peptides between PA and PS patients were
267 independent of their peanut-specific IgE levels (Figure E4). Specific IgE to the individual allergen
268 components was detectable both in PA and in PS subjects (Table E3) and was generally related to
269 specific IgE to the peptides from the respective allergen in both PA and PS subjects (Figures E5 and
270 E6).

271 Considering the utility of ImmunoCAP to peptides to discriminate between peanut allergic and non-
272 allergic subjects among sensitized individuals, specific IgE to the four Ara h 2 peptides showed good
273 diagnostic discrimination and enhanced the accuracy of Ara h 2-specific IgE (Figure 4). Specific IgE
274 to peptides from Ara h 1 or Ara h 3 did not offer advantage over the respective allergen-specific IgE
275 (Figures E7 and E8). Specific IgE to peanut was inferior to specific IgE to Ara h 2 (Figure E9).

276

277 **Similarity of IgE and IgG4 binding to peanut peptides**

278 On the microarray, IgG4 binding to one Ara h 9 peptide, to one peptide from another lipid-transfer
279 protein from peach Pru p 3 and from Gly m 5 from soya was greater in PS than in PA patients (Figure
280 1B, Table I). For the remaining peanut allergen peptides, there were no statistically significant
281 differences in IgG4 binding between the two groups of patients. Overall, there was a strong
282 association between peanut peptides bound by IgG4 and IgE both in PA and PS patients (Figure
283 E10A). The number of peanut peptides bound by IgG4 and IgE was similar between the two groups of
284 patients (Figure E10B); however, overall the ratio of IgG4/IgE to peanut peptides was higher in PS
285 than in PA patients (Figure E11).

286 Using ImmunoCAP, we were able to quantify the levels of antibodies directed to specific peptides and
287 to calculate with precision the relative amounts of IgE and IgG4. IgG4 levels to any of the 7 peptides

288 were not significantly different between PA and PS. IgG4/IgE ratios were higher in PS than in PA
289 patients for antibodies directed to peptides 1, 3 and 4 of Ara h 2, as well as to peptide 9 of Ara h 3
290 (Figure 5). Interestingly, we observed correlation of peptide-specific IgE and IgG4 with age (Figure
291 E12).

292

293 **Discussion:**

294 The discrepancy between the presence of P-sIgE and IgE-mediated allergic reactions to peanut is
295 intriguing in that patients with similar levels of P-sIgE and even Ara h 2-specific IgE can have
296 different clinical outcomes, some being PA and some being able to eat peanut without developing any
297 symptoms. To explore the underlying reasons for this discrepancy, we have tested PA and PS patients
298 for IgE and IgG4 binding to 15-mer peptides covering the sequence of all peanut allergens known at
299 the time when the microarray was generated. We identified four Ara h 2 peptides, two Ara h 1
300 peptides and one Ara h 3 peptide that were bound preferentially by IgE of PA than by IgE of PS. One
301 peptide of the profilin Ara h 5 was bound preferentially by IgE of PS. Quantification of IgE and IgG4
302 to selected peptides using ImmunoCAP technology revealed that specific IgE to the Ara h 2 peptides
303 showed very good diagnostic utility and taken together with IgE to Ara h 2 were the best serologic
304 marker for peanut allergy and better than Ara h 2-specific IgE alone. Additionally, ImmunoCAP
305 allowed precise calculations of IgG4/IgE ratios to individual peptides, which were higher in PS than
306 in PA patients, suggesting that the balance of IgG4 and IgE is important in established peanut
307 tolerance.

308 For the first time, we have quantified IgE to peanut peptides that were discriminative between allergy
309 and tolerance using ImmunoCAP technology and determined the diagnostic value of this approach.
310 Importantly, we were able to show that the differences in IgE binding to the peptides between PA and
311 PS were independent of the level of peanut-specific IgE. The peptides we have identified as most
312 discriminative between PA and PS status overlap with some of the IgE binding epitopes previously
313 reported in pioneering studies in which IgE binding was assessed using samples from PA patients¹⁴⁻²⁰.
314 However, few studies have looked at the comparison of epitope specificity between PA and PS
315 patients like ours^{21,22}. The peptides we identified align totally or partially with epitopes reported by

316 Lin et al²². Four peptides were particularly important to differentiate PA from PS using a machine
317 learning method, decision tree and support vector machine in the latter study²²: two peptides on Ara h
318 2 which coincide with two of the peptides we identified and one peptide on Ara h 1 and one peptide
319 on Ara h 3, which in turn are different from those that we have identified. There are some differences
320 in the methodology used that could explain the different findings; for instance, Lin et al²² used 15-mer
321 peptides with an offset of 3 amino acids and only 9% of PS and 13% of PA had IgE to Ara h 1 or Ara
322 h 3 and 4% of PS and 74% of PA had IgE to Ara h 2; whereas in our study, we used 15-mer peptides
323 with an offset of 5 amino acids and the majority of patients both from PA and PS groups were
324 sensitized to all three major peanut allergens (see Table E4).

325 We tested for all peanut allergens known at the time of generation of the microarray. Going beyond
326 testing for the well-studied major allergens Ara h 1, Ara h 2 and Ara h 3 enabled us to explore the
327 importance of allergens that are not commonly tested for. The fact that the peptides that are bound
328 preferentially by IgE of PA than by IgE of PS are located on Ara h 1, Ara h 2 and Ara h 3 is a
329 confirmation that these major allergens are indeed the most important in peanut allergy. More
330 recently, oleosins²³ and defensins²⁴ have been described in peanut and may also be important;
331 however, IgE to these lipophilic allergens is probably not as dominant as IgE to Ara h 1, Ara h 2 and
332 Ara h 3, given that it is uncommon to find PA patients with negative SPT or specific IgE, which are
333 tests that use extracts that are defatted and thus lack lipophilic proteins.

334 The crystal structures of Ara h 1^{25,26}, Ara h 2¹⁴ and Ara h 3²⁷ have been totally or partially solved;
335 thus, we were able to establish the location of the relevant peptides in these 3D structures. The
336 identified peptides were all located on the surface of peanut allergens, in structurally disordered or
337 partially disordered loop regions, accessible to antibody binding. Two Ara h 2 peptides adopt a partly
338 alpha-helical conformation in the fusion protein; however, in Ara h 2 alone they might be more
339 flexible. A crystal structure of Ara h 2 on its own, without MBP, would be necessary to clarify this.
340 Epitopes located on the surface of the allergens are indeed particularly susceptible to antibody
341 binding, including receptor-bound IgE on the membrane of mast cells and basophils, and thus are
342 more likely to be able to elicit effector cell activation and allergic symptoms. Finding mainly epitopes
343 located on the surface of the allergens could indicate that the linear epitopes were part of epitopes
344 formed by parts of the protein that are close in the 3D structure but distant in the protein sequence (i.e.

345 conformational epitopes). Because we used linear short peptides to test for IgE and IgG4 binding we
346 will not have been able to detect conformational epitopes. Conformational epitopes are likely to be
347 important in IgE binding, particularly to allergens that are labile to heating and digestion, such as
348 pollen-cross-reactive allergens like Ara h 8 and Ara h 5. In our study, a peptide from the peanut
349 profilin Ara h 5 was identified as being bound preferentially by IgE of PS than by IgE of PA. Profilins
350 are pan-allergens with unclear clinical relevance in peanut allergy²⁸. Profilin is likely to be an
351 important cause of false-positives in P-sIgE testing and its importance is probably underestimated as
352 specific IgE to peanut profilin is not commercially available and thus is not usually tested in isolation.
353 Additional characteristics of IgE, apart from epitope specificity, may contribute to the discrepancy
354 between sensitization and clinical allergy, notably differences in diversity and affinity of IgE
355 antibodies for the peanut epitopes and the spatial distribution of these epitopes²⁹.

356 We moved from a semi-quantitative microarray to the quantitative method ImmunoCAP to show the
357 statistical, biological and clinical impact of the epitopes contained in the peptides we identified. The
358 ImmunoCAP technology allowed us to quantify the levels of IgE to the peptides and showed that IgE
359 to the Ara h 2 peptides improved the diagnostic utility of IgE to Ara h 2, which could have direct
360 practical clinical implications. It was impressive that IgE to Ara h 2 peptides alone had good
361 diagnostic performance and could enhance the accuracy of Ara h 2-specific IgE, a diagnostic test that
362 is already able to discriminate very well peanut allergic from non-allergic individuals^{3,8}. Even if the
363 number of subjects with equivocal levels of Ara h 2-specific IgE is a small proportion of the
364 population tested, it is clinically relevant for those individuals and could enable us to reduce the
365 number of patients we need to subject to an oral peanut challenge. The combination of IgE to the four
366 Ara h 2 peptides can improve the diagnostic utility of Ara h 2-specific IgE in equivocal cases and, to
367 make this approach more practical, could be provided as a single test in the ImmunoCAP platform in
368 the future, following additional validation to confirm that such approach would not lead to a loss in
369 sensitivity.

370 The alternative hypothesis we explored related to IgG4 interfering with the interaction between IgE
371 and the allergen. No differences in IgG4 binding to peanut peptides could be identified on the
372 microarray between PA and PS patients, except for one Ara h 9 peptide. As IgE to Ara h 9 was very
373 low in the studied patients and not significantly different between PA and PS patients, the clinical

374 relevance of this finding is unclear. This peptide is predicted to adopt an exposed helix and loop
375 structure, based upon a model of Ara h 9 generated from the highly homologous structure of the pea
376 lipid transfer protein (PDB ID: 2N81). Both PA and PS patients tended to produce IgE and IgG4 to
377 the same peptides, as reported in a previous study of Ara h 2⁷; however, the relative amounts of
378 specific IgE and IgG4 present were different with IgG4/IgE ratios prevailing in PS patients, as we
379 previously showed at the level of the whole allergen². As the microarray is semi-quantitative, and thus
380 not an accurate method to precisely quantify the amount of IgE and IgG4 antibodies that bound each
381 peptide, we quantified the levels of IgG4 to the peanut peptides that were distinct between PA and PS
382 in the microarray using the ImmunoCAP technology. IgG4/IgE ratios were calculated as in previous
383 studies and were higher in PS than in PA patients for peptides 1, 3 and 4 of Ara h 2 and peptide 9 of
384 Ara h 3. These findings support the role of the IgG4/IgE balance in established peanut tolerance.

385 Competition for binding to the peptides could potentially have interfered with the results of the
386 microarray given that IgE and IgG4 binding were measured using the same slide for each patient,
387 particularly in the PS group, similar to what occurs in other assays using microarrays³⁰. However, the
388 peptides were printed onto the slide in large amounts and in excess of what was expected to be bound
389 by IgE, therefore there should have been enough peptide to prevent saturation of the system and to
390 allow enough antibody binding. As there was serial exposure to the antibodies used for detection of
391 the IgE and the IgG4, controls were included in which the detection antibodies were reversed with
392 anti-IgG4 being added first followed by anti-IgE; no significant differences were observed. Sera from
393 non-peanut allergic individuals with undetectable peanut-specific IgE and elevated total IgE were
394 additionally used as negative controls. Human sera from non-allergic controls and chicken sera were
395 also used to check for non-specific binding.

396 In the future, we would like to integrate information about the exact location of epitopes, the distance
397 between them, and their repetition and combination in the allergen structure, with the affinity of
398 binding. Understanding the interplay between all these factors could clarify what determines the
399 ability of IgE and allergen to cause effector cell activation. Clarifying the mechanism by which PS
400 patients do not react to peanut despite the presence of IgE could help to identify targets for novel
401 curative treatments for peanut and other food allergies.

402

Tables

Table 1. Peanut peptides differentially bound by IgE or IgG4 of peanut allergic and peanut sensitized but tolerant patients. Probes are ranked inversely by the log-odds of being differentially bound between PS and PA (positive log₂-fold changes (logFC) express greater binding by IgE of PA patients; negative logFC expresses greater binding by IgE or IgG4 of PS patients).

Antibody isotype	Allergen	Peptide name	Peptide name based on position in the protein sequence	Peptide sequence	logFC	B	% recognition PS (max NA)	% recognition PA (max NA)
IgE	Ara h 1	Peptide 7	Peptide 18	SPPGERTRGRQPGDY	1.22	3.96	3.70	54.76
	Ara h 2	Peptide 1	Peptide 17	RDPYSPSPYDRRGAG	0.96	2.53	18.52	54.76
	Ara h 3	Peptide 9	Peptide 61	EDEYEYDEEDRRRGR	1.15	2.03	33.33	64.29
	Ara h 2	Peptide 2	Peptide 7	RRCQSQLERANLRPC	0.93	1.79	3.70	40.48
	Ara h 1	Peptide 8	Peptide 19	RTRGRQPGDYDDDRR	0.66	1.41	11.11	50.00
	Ara h 2	Peptide 3	Peptide 13	GRDPYSPSQDPYSPS	1.03	1.40	25.93	61.90
	Ara h 2	Peptide 4	Peptide 6	ELQGDRRCQSQLERA	1.01	0.23	59.26	76.19
	Ara h 5	Peptide 10	Peptide 11	MNDFAEPGSLAPTGL	-0.29	0.07	44.44	11.90
IgG4	Ara h 9	Peptide 11	Peptide 12	GSLHGLNQGNAAALP	-0.52	2.09	18.52	0.00
	Gly m 5	Peptide 12	Peptide 6	QHGEKEEDEGEQPRP	-0.42119	1.43	29.63	4.76
	Pru p 3	Peptide 13	Peptide 12	GAVKGINPGYAAALP	-0.55018	1.35	11.11	2.38

Footnote: B , empirical Bayes statistic which gives the logarithm (natural base) of the ratio between the odds of a peptide being differentially bound between PA and PS and the odds of not being differentially bound. $B > 0$ (i.e. odds of differential binding higher than those of no effect, with false discovery rate (FDR) < 0.05) was considered statistically significant.

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Figure legends:

Figure 1. Volcano plots for (A) IgE and (B) IgG4 binding to peanut peptides between peanut allergic (PA) and peanut-sensitized tolerant (PS) patients. The x-axis represents the log₂-fold-changes of average foreground-to-background ratio between PA and PS patients. The y-axis represents the empirical Bayes log-odds (B) of differential binding between PS and PA. Peanut peptides (blue dots) that are differentially bound between the groups (B>0, i.e. odds of differential binding higher than those of no effect, with FDR < 0.05) are named in the figure.

C. Heatmap of IgE binding to peptides significantly more bound in PA (in red) than PS (in green) patients (NA patients are shown in grey for comparison) expressed as the binary logarithm of foreground to background ratio (log₂(FBR)).

Figure 2. Peptides indicated on the X-ray structures for (A) Ara h 1 (B) Ara h 2 and (C) Ara h 3. The two overlapping peptides (7 & 8) on Ara h 1 are not shown as they are present in a disordered N-terminal region that has been truncated in the crystal structure. The four peptides for Ara h 2 are shown as sticks in red (peptides 1 & 3) and blue (overlapping peptides 2 & 4). Only the ordered residues are shown; most of these peptide residues are in flexible/disordered loop regions. The peptide for Ara h 3 (peptide 9) is present in the disordered loop region of the structure that is indicated by an arrow. The PDB codes for the structures used to generate the figures were: 3S7I (for Ara h 1), 3OB4 (for Ara h 2) and 3C3V (for Ara h 3).

Figure 3. Box and violin plots of specific IgE to individual peanut peptides and the respective peanut allergen. Peptides and grouped and displayed sequentially from left to right overtop each of their respective peanut allergen by peanut allergic (PA), peanut-sensitized tolerant (PS) and non-sensitized non-allergic (NA) measured by ImmunoCAP (ThermoFisher). Statistically significant comparisons (p<0.01) between PA and PS are marked with an * above each boxplot. The exact p-values are as follows for each IgE to the respective peptides and to Ara h1-h3: 1 (<0.001), 2 (0.5427), 3 (<0.001), 4 (0.001), 7 (0.1001), 8 (0.2804) and 9 (0.004), Ara h 1 (<0.001), Ara h 2 (<0.001), Ara h 3 (0.002).

Figure 4. ROC curves for identifying peanut allergy using: 1. the combination of IgE to Ara h 2 and IgE to each of its peptides (1,2,3,4), labeled “All”; 2. Ara h 2-specific IgE alone and 3. combination of specific IgE to the 4 Ara h 2 peptides (labeled Peptides). The hypothesis test that the AUC from all models are equal was

rejected ($p < 0.0001$) and we made the following pairwise comparisons of the difference between the AUCs of each model with (95% CI), p-values using likelihood ratio tests. All vs Peptides: 0.095 (.032, .158), $p = 0.003$. All vs Ara h2: 0.028 (0.013, 0.043), $p = 0.0002$. Peptides vs Ara h2: -.067 (-0.138, 0.004), $p = 0.0656$. Note: All data was used for the model comparisons and no imputation of missing data was performed. However, because each predictor had different amounts of missing data, the model comparisons used slightly different cohorts of participants.

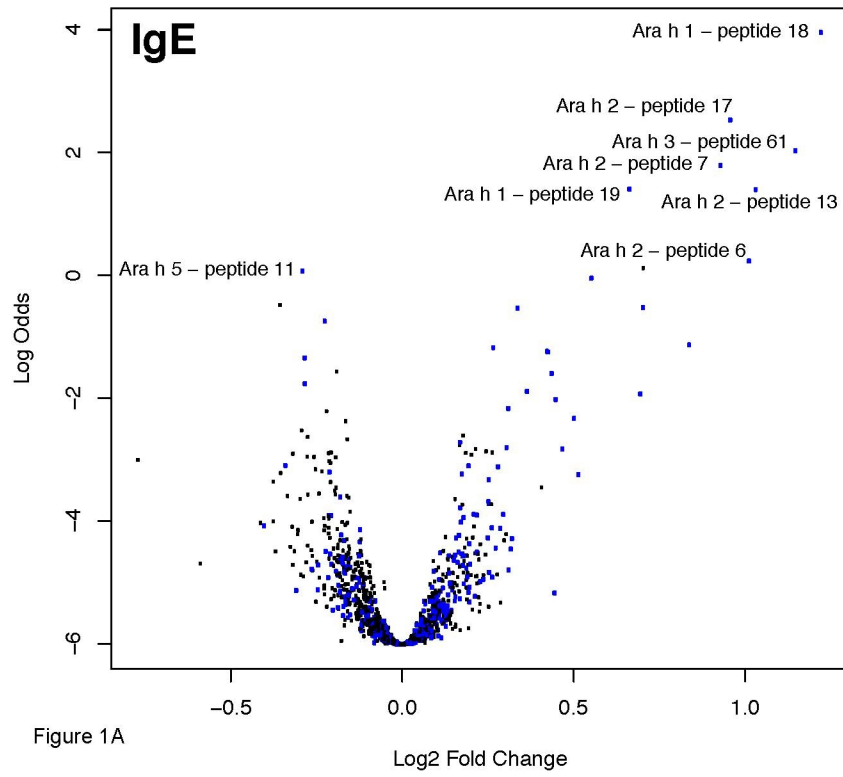
Figure 5. IgG4/IgE ratios to individual peptides from (A) Ara h 2 and (B) Ara h 1 and Ara h 3 in peanut allergic (PA), peanut-sensitized tolerant (PS) and non-sensitized non-allergic (NA). IgE and IgG4 levels to the peptides were measured by ImmunoCAP and IgG4/IgE ratios were calculated following conversion of IgG4 levels from milligrams per liter to nanograms per milliliter and of IgE levels from kilo unit per liter to nanograms per milliliter using the formula $\log_{10}((\text{IgG4} \times 1000)/(\text{IgE} \times 2.4))$. p-values are represented for the comparison across the three groups. For the comparison between PA and PS, the p-values are given in brackets for the respective peptides: 1 (< 0.001), 2 (0.9627), 3 (< 0.001), 4 (< 0.001), 7 (0.0564), 8 (0.1651) and 9 (< 0.001).

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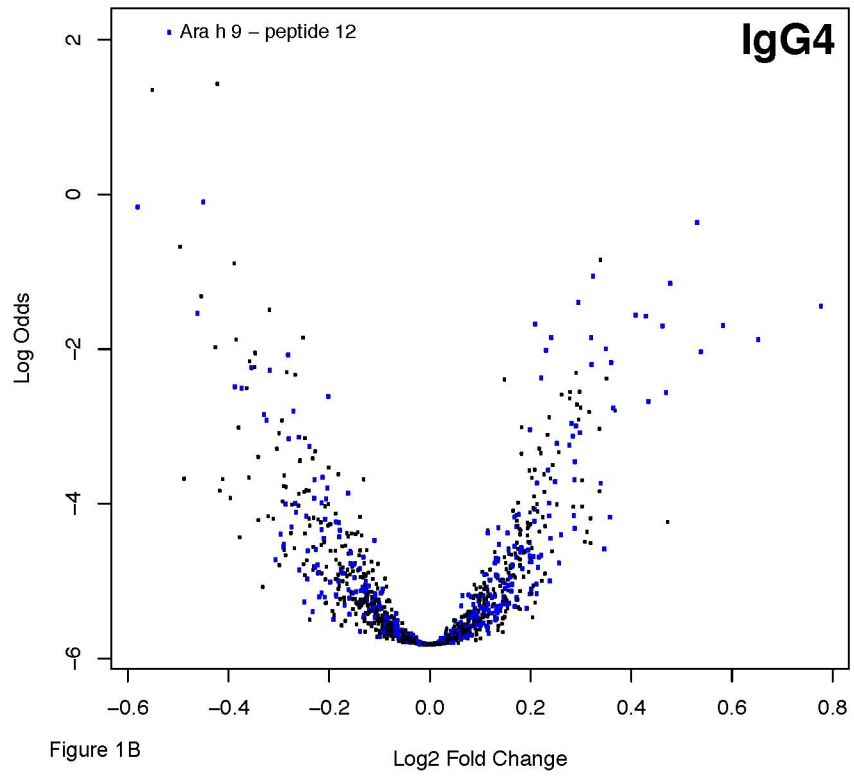


Figure 1B

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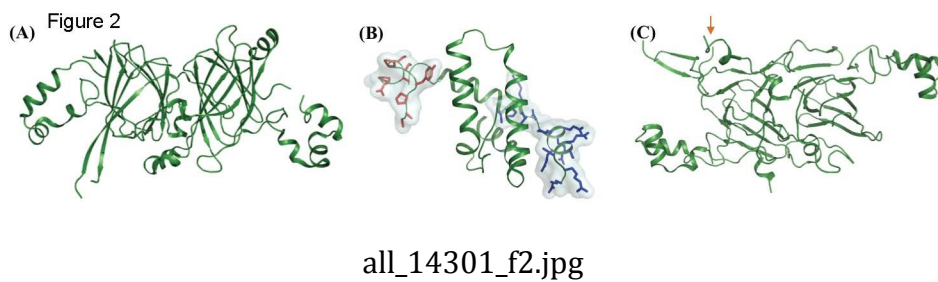
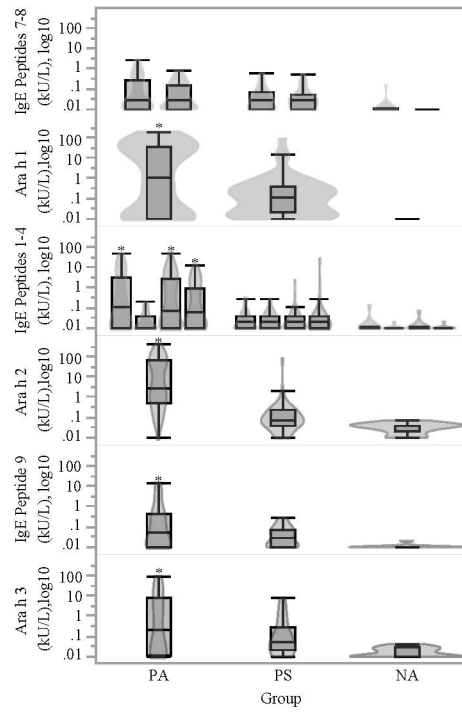
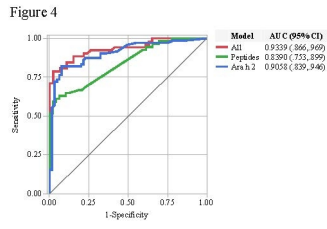


Figure 3

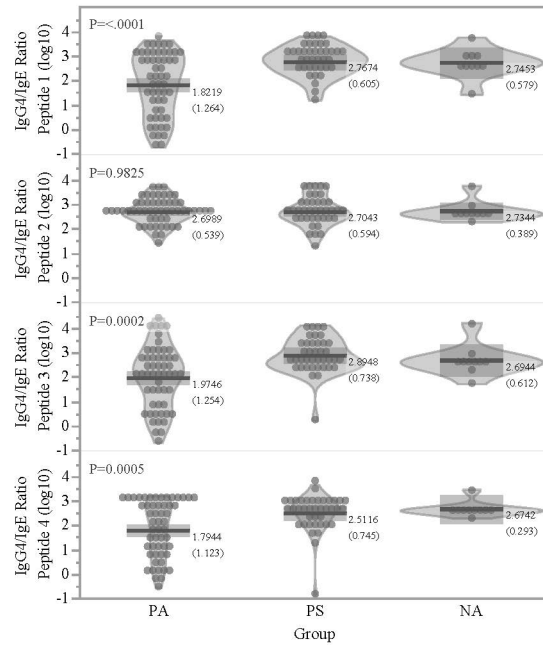


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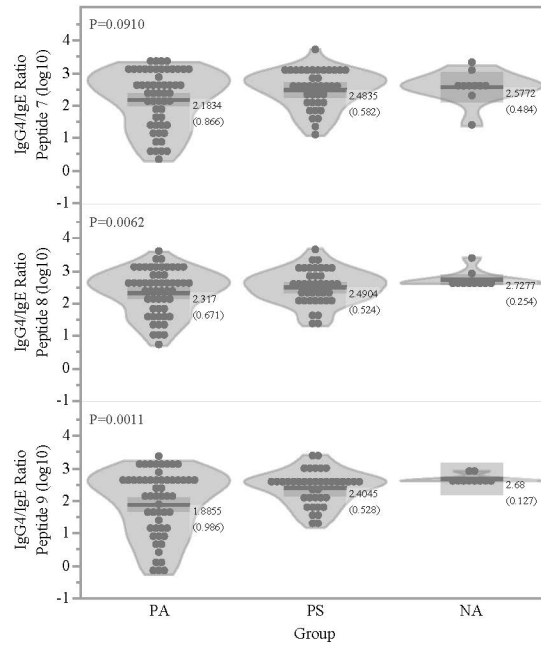
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Figure 5A



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Figure 5B



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