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Measurement of Ara h 6 can improve diagnosis in patients suspected of peanut allergy

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EE and CBJ contributed with conceptual idea and study design. EE was main contributor of manuscript, with adding and editing contribution from PSS, KB. EE and KB developed and performed calculations. All reviewed / edited the manuscript.

Key messages

- 4% of all peanut allergic are mono-sensitized to Ara h 6
- Peanut-tolerant patients are more often sensitized to Ara h 2 than Ara h6.
- Adding Ara h 6 in the diagnostic workup, increases the accuracy in patients suspected of peanut allergy.

Conflicts of interest:

PSS is acting as scientific advisor for RefLab ApS and EP Medical. KB is employed as consultant by RefLab ApS. EE and CBJ have received educational grants from Thermo Fisher Scientific.

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To the editor

The majority of peanut allergic patients have elevated IgE against the peanut protein Ara h 2, which is considered the superior serological marker for clinical peanut allergy [1]. However, not all patients' reactivity can be explained by Ara h 2 [2], indicating that IgE against other major peanut proteins might play a role. Ara h 6 belongs to the same protein family as Ara h 2 (2S Albumin) and they share 59% sequence homology [3], however, the clinical usage of Ara h 6 is much less widespread than Ara h 2. This originates partly from a common misunderstanding of an almost complete cross-reactive overlap between IgE to the two entities [4], and thereby unawareness of the clinical utility of IgE against Ara h 6, illustrated by only two published studies [5,6] applying the frequently used, commercially available, singleplex test (ImmunoCAP). A few other studies have assessed the importance of specific IgE against Ara h 6 in relation to IgE against Ara h 2 using an experimentally-coupled ImmunoCAP [2], semi-quantitative multiplex (ISAC) [3,7-9], or basophil activation test (BAT) [6], but neither of these are comparable with a true-quantitative and validated outcome, nor provide a clear conclusion between the comparative performance of the two markers and the added clinical value of including IgE against Ara h 6 in the routine diagnostic assessment of peanut allergic patients.

This retrospective chart review includes previously published data [1] from all patients suspected of peanut allergy, i.e. referred due to a positive case history to peanut and/or a positive skin prick test (SPT) and/or s-IgE against peanut extract. Patients were routinely challenged according to EAACI guidelines with up to 25 gram of peanut (6150 mg peanut protein) at Odense University Hospital (Odense, Denmark) and needed a stored serum-sample available for further analysis (n=157). Samples from 133 challenge-positive and 24 challenge-negative peanut patients (mean age 5.6Y; range 1-26Y), previously analyzed [1] for specific IgE against peanut extract, Ara h 1, Ara h 2, Ara h 3, Ara h 8, and Ara h 9, were reanalyzed for levels of Ara h 6-specific IgE. All previous IgE determinations and the newly performed Ara h 6-specific IgE measurements were performed by Thermo Fisher Scientific (Allerød, Denmark) using the ImmunoCAP platform. Spearman correlation between specific IgE levels were compared using t-test after bootstrapping. IgE levels were correlated to challenge-outcome using receiver operating characteristic (ROC) curve analysis, 95% confidence intervals of diagnostic values were estimated using Wilson score interval, and area under the curve (AUC) were compared using DeLong test. The study was approved by the regional ethics committee (no. s-201300086) and the Danish Data Protection Agency (no. *19/1055*) with informed consent from all participants. All analyses were performed in Stata 11.

We found a high correlation between IgE levels against Ara h 6 and Ara h 2 for the 133 patients in the challenge-positive group (ρ=0.92) (**Figure 1**), especially for values above 1 kU/L, as seen in other studies [5,6,8],

whereas the correlation for the challenge-negative group was significantly ($p<0.01$) lower ($\rho=0.71$). The latter was driven by significantly higher levels of Ara h 2 compared to Ara h 6 ($p = 0.003$), found in 13/24 of the peanut tolerant patients. These patients were referred to an oral peanut challenge based on serological suspicion, i.e. specific IgE against peanut extract and Ara h 2, which is measured routinely in our clinic.

Diagnostically, IgE against Ara h 2 and Ara h 6 are superior compared to IgE towards other peanut proteins, however, their internal hierarchy is unclear. Some studies report Ara h 2 to perform slightly better diagnostically than Ara h 6 [2,5,6], one study found similar diagnostic values [7], and yet others have shown Ara h 6 to be superior [8,9]. However, all these studies report relatively small differences in diagnostic performances between the two IgE measurements, and most studies lack statistical tests to confirm this. In our study, specific IgE against Ara h 6 was slightly, but still significantly ($p<0.05$), better in predicting challenge outcomes than IgE towards Ara h 2, as illustrated by the ROC-curve analysis AUC (0.95 vs. 0.90) (see **Figure 1**). Ara h 6 could successfully identify 91% of the suspected patients compared to 88% for Ara h 2-specific IgE. A combined cut-off of >0.1 kU/L for Ara h 6 and Ara h 2 increased the overall sensitivity to 98%, in line with previous findings by Kukkonen et al [8]. Additional results and figures describing patterns of sensitization are available in the **online repository https://osf.io/v2hj3.**

Although the clinical reactivity of most peanut allergic patients can be explained by IgE against Ara h 2, around 2-7% of patients are reported to be mono-sensitized towards Ara h 6 [2,3,5,6,9]. This variation can partly be explained by the different definitions of mono-sensitization; from clinically relevant (patients without Ara h 2 sensitization) to truly mono-sensitized (lacking IgE against any other peanut protein). We found that 16 of the 133 challenge-positive peanut allergic patients had levels of IgE against Ara h 2 <0.35 kU/L (**Table 1**) and 7 of them <0.10 kU/L (pt. 10-16). Five of these (4% of all challenge-positive) were mono-sensitized against Ara h 6 (≥ 0.10 kU/L including one patient (pt. 10) who had low, but detectable IgE level against Ara h 8). The remaining two patients (pt. 13-14) had either high IgE levels against Ara h 8 or low levels of Ara h 9, meaning that all challenge-positive patients could have an explicit serological peanut marker explaining their clinical peanut allergy.

Ten of the 133 patients developed tolerance within a year after challenge; these included one patient who was neither sensitized to Ara h 2 nor Ara h 6, four patients (of a total of 122) sensitized to both Ara h 2 and Ara h 6, four patients (of a total of five) mono-sensitized to Ara h 6, and one patient (of a total of 4) mono-sensitized to Ara h 2. There was no statistical difference between these groups, but it is interesting that 4/5 patients mono-sensitized to Ara h 6 developed tolerance and thus could indicate that Ara h 6 might be used as a marker for tolerance progression. A similar trend was found in the peanut-tolerant group, where sensitization with IgE to peanut extract and Ara h 2, but not to Ara h 6, equals a negative challenge in 7/11

patients. Overall, we have equally frequent mono-sensitization to either Ara h 2 or Ara h 6 (4%), whereas the majority (91%) are poly-sensitized. The consensus has been that most poly-sensitization is due to cross-reactive IgE, recognizing both 2S-albumins, however recently it has been shown, that only a minor fraction (17.1%) of IgE count for this response [4]. Instead, the mean fraction of IgE specifically recognize either Ara h2 (57.4%) or Ara h 6 (25.5%), where the higher level of IgE to Ara h 2 is driven by a repetitive hydroxyproline motif (DPYDP-OH-S), which cannot be found in Ara h 6 [4]. Ara h 2 for commercial IgE tests is recombinant and of prokaryotic origin, which lacks these repetitive hydroxyproline motifs. It is therefore unclear how this affects the conformational structure, and thereby IgE affinity, and thus the result and clinical interpretation.

In conclusion, our data show that measurements of IgE against Ara h 6 could add clinically relevant serological information, i.e. identifying Ara h 6 mono-sensitized peanut allergic patients as well as patients who are sensitized but tolerate peanut, or are in the process of developing clinical tolerance. This could suggest an improvement of the diagnostic algorithm and could thereby reduce the number of patients having an oral peanut challenges.

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Table 1: IgE sensitization pattern (in kU/L) in the 16 challenge-positive patients with the lowest s-IgE levels to Ara h 2, and one-year clinical development (challenge-verified persistent positive or tolerance development within one year).

ID	Ara h 2	Ara h 1	Ara h 3	Ara h 6	Ara h 8	Ara h 9	Peanut extract	Development
Pt.1	**0.32**	0.0	0.0	**1.88**	**0.13**	0.0	**0.9**	Pers. Pos.
Pt.2	**0.30**	**0.35**	0.02	0.00	**0.2**	**0.11**	**0.35**	Tolerance>1y
Pt.3	**0.27**	**0.28**	0.01	**0.14**	**2.69**	0.0	**0.6**	Pers. Pos.
Pt.4	**0.23**	0.0	0.0	**0.13**	0.0	0.0	**1.1**	Pers. Pos.
Pt.5	**0.21**	0.01	**0.2**	**5.06**	0.0	0.0	**3.1**	Pers. Pos.
Pt.6	**0.21**	0.07	0.05	**4.65**	**0.12**	**0.15**	**3.6**	Pers. Pos.
Pt.7	**0.17**	0.03	0.0	**0.16**	0.04	0.0	**0.35**	Pers. Pos.
Pt.8	**0.13**	0.05	0.01	0.09	0.01	0.0	**0.6**	Pers. Pos.
Pt.9	**0.10**	0.02	0.15	**4.12**	**1.29**	0.02	**13.8**	Tolerance>1y
Pt.10	0.09	0.02	0.03	**0.17**	**0.16**	0.0	**1.0**	Tolerance>1y
Pt.11	0.08	0.0	0.0	**0.15**	0.0	0.0	**8.4**	Tolerance>1y
Pt.12	0.05	0.04	0.04	**0.3**	0.01	0.0	**0.35**	Pers. Pos.
Pt.13	0.04	0.04	0.05	0.06	0.06	**0.15**	**4.2**	Pers. Pos.
Pt.14	0.01	0.0	0.03	0.02	**68.3**	0.08	**9.8**	Tolerance>1y
Pt.15	0.01	0.0	0.02	**0.18**	0.0	0.0	**3.3**	Tolerance>1y
Pt.16	0.0	0.0	0.01	**1.61**	0.0	0.0	**5.2**	Tolerance>1y

Figure Legend:

Figure 1: Levels of specific IgE against Ara h 2 and Ara h 6 with corresponding correlation coefficients (left), and receiver operating characteristic (ROC) curves with the area under the curve (AUC) of IgE towards peanut extract and six peanut components (right) in 157 patients challenged on suspicion of peanut allergy.