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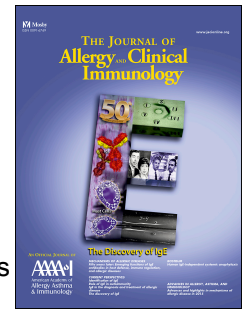
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Peanut oleosins associated with severe peanut allergy - Importance of lipophilic allergens for comprehensive allergy diagnostics

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Abstract**Background**

Peanut allergy is one of the most common and most severe food allergies in Western countries and its accurate diagnosis to prevent potential life-threatening allergic reactions is crucial. However, aqueous extracts used for routine diagnostic measurements are devoid of lipophilic allergens such as oleosins. We have recently succeeded in the isolation and purification of these unique proteins, and the current study evaluates their allergenic potential and clinical relevance.

Objective

We sought to assess allergenicity and sensitization prevalence of oleosins obtained from both raw and in-shell roasted peanuts. Additionally, we tested the utilization of natural and recombinant oleosins for allergy diagnostic purposes.

Methods

Oleosin sensitization, prevalence, and impact of thermal processing were analyzed by immunoblot with sera from 52 peanut-allergic individuals displaying different clinical phenotypes. The application of natural and recombinant oleosins for allergy diagnostics was investigated by basophil activation test (BAT). IgE-binding epitopes were identified by oligopeptide microarray.

Results

Sensitization to oleosins was observed exclusively in peanut-allergic subjects suffering from severe systemic reactions. IgE-binding capacity of oleosins derived from in-shell roasted peanuts was increased as shown by immunoblot analysis and BAT. Both natural and recombinant molecules can be used to identify oleosin-sensitized patients by BAT. A linear epitope of Ara h 15 was determined which displays high similarity to other seed-derived oleosins.

Conclusion

Oleosins are clinically relevant peanut allergens and most likely associated with severe allergic symptoms. In-shell roasting increases their allergenicity, which is consistent with the observation that most allergic reactions are in connection with roasted peanuts.

Key Messages

Peanut oleosins are major allergens most probably related to severe peanut allergy. In-shell roasting further increases the IgE-binding potency of these allergens.

Capsule Summary

Peanut oleosins are novel lipophilic allergens associated with severe allergic reactions. Their IgE-binding capacity is increased in roasted peanuts. Application of either purified natural or recombinant oleosins now closes a diagnostic gap.

Key words

peanut allergy, lipophilic allergens, oleosins, Ara h 10, Ara h 11, Ara h 14, Ara h 15, BAT, roasting, epitope mapping

Abbreviations used

Ara h: *Arachis hypogaea*

BAT: Basophil activation test

CI: Confidence interval

CRD: Component-resolved diagnostics

fMLP: Formyl-methionyl-leucyl-phenylalanine

HCD: Hydrophobic core domain

- 96 NA: Non-peanut-sensitized nonallergic peanut consumers
- 97 PA: Peanut-allergic patients
- 98 PS: Peanut-sensitized but tolerant peanut consumers
- 99 PVDF: Polyvinylidene fluoride
- 100 ROC: Receiver-operating characteristic
- 101 TBST: Tris-buffered saline with Tween 20

INTRODUCTION

Peanut allergy is one of the most frequent and most severe food allergies worldwide with an increasing prevalence over the past decades.¹⁻³ It affects up to 3% of children and adolescents in Western countries and is usually persistent.⁴⁻⁶ As accidental peanut exposure can trigger potentially life-threatening allergic reactions, an accurate diagnosis is essential.⁷⁻⁹ However, a diagnosis by use of routine diagnostic procedures can be difficult, particularly in patients with a medical history of peanut allergy but negative results in skin prick tests and specific IgE measurements.¹⁰ As a consequence, an increased need exists to perform costly and potentially dangerous provocation tests to verify inconclusive clinical examination findings.^{11, 12} Advancement of existing diagnostic tests to reduce the necessity of food challenges in case of ambiguous clinical pictures would therefore be highly desirable. Thus, the availability of allergens for diagnostic procedures is a prerequisite to improve risk assessment and management of peanut allergy.¹³ While diagnostic extracts already include a certain number of water-soluble allergens, others, in particular lipophilic allergens are still missing.¹⁴ Hidden in the lipid matrix of oil-rich crops, lipophilic allergens tend to elude standard protein purification procedures. Consequently, only a few allergens of this type have been identified so far, but those discovered have been linked to rather severe systemic reactions.¹³⁻¹⁵ Peanuts are important oil seeds composed of approximately 50% lipids.¹⁶ In these seeds, triacylglycerols are harbored in organelles called oil bodies.¹⁷ These oil bodies are stabilized by oleosins which represent roughly 80 to 90% of the oil body proteins.^{18, 19} Oleosins consist of a conserved hydrophobic core domain which is flanked by two less conserved amphipathic domains.^{20, 21} Their tight association with lipids and the membrane protein structure renders them poorly soluble in aqueous buffers and difficult to handle.²² So far, they elude canonical extraction, separation and purification procedures and are only soluble in the presence of detergents, which restricts their application in immunological test systems.²³ Yet, we recently

purified four distinct peanut oleosin prototypes and demonstrated their IgE-binding capability with sera from three peanut-allergic subjects.²² In addition to the oleosins Ara h 10 and Ara h 11, we were able to register a new variant of Ara h 11 as well as oleosins with molecular masses of 17 and 17.5 kDa as new allergens according to the WHO/IUIS Allergen Nomenclature Subcommittee criteria. The latter two were termed Ara h 14 and Ara h 15. Since nothing is known about the prevalence of sensitization to the various peanut oleosins and their association with the grade of disease severity, we screened a study population of 52 peanut-allergic patients for oleosin-specific IgE. Moreover, we investigated potential differences in the IgE-binding potency of oleosins from both raw and commercially available in-shell roasted peanuts since roasting has been shown to significantly alter the IgE-binding capacity of peanut storage proteins.²⁴⁻²⁶ As oleosin isolation and purification is a challenging task, the use of a modified water-soluble recombinant oleosin was assessed as a promising concept for routine diagnostic measurements. Finally, an oleosin oligopeptide microarray was created to provide further insight into IgE-binding epitopes.

METHODS

Study population

Peanut-allergic patients (PA) with a convincing medical history were recruited during clinical work in the allergy outpatient clinics of Borstel and Luebeck on an ongoing basis. The German guideline for standardization of oral food challenges²⁷ is strict regarding the indication for challenge tests in patients who have a convincing history of anaphylactic reactions. In addition, patients generally refused to have provocation tests with foods to which they know themselves to be allergic. The patients were categorized according to the severity of allergic symptoms. Sera of peanut-sensitized but tolerant peanut consumers (PS) and non-peanut-sensitized nonallergic peanut consumers (NA) were used as controls. Concentrations of IgE specific to peanut extract, Ara h 2 and Ara h 8 were determined by ImmunoCAP. For an overview of the study population see Table I, for individual sensitization profiles and allergic symptoms see Table E1 in the Online Repository. The study was approved by the local ethics committee of the University of Luebeck (approval numbers 10-126 and 16-268). All patients gave written informed consent.

Recombinant expression of a water-soluble Ara h 15

The gene sequence of Ara h 15 (GenBank no. AY722696) with a modified sequence encoding the hydrophobic domain was optimized for expression in *E.coli* and chemically synthesized by Geneart (Regensburg, Germany). Instead of the hydrophobic domain, a His-tag flanked by a Glycin-Alanin-Glycin-spacer on each side was inserted between the N- and C-terminal domains (Fig 1) as proposed by Riecken.²⁸ The water-soluble Ara h 15 was produced in *E. coli* BL21 (DE3) cells and purified using cobalt chelate chromatography. Purified rAra h 15 was used for BAT and for production of an anti-Ara h 15 antibody (the procedure is described in the Methods section in this article's Online Repository).

Isolation and purification of peanut oleosins

Peeled kernels of raw and in-shell roasted peanuts (Seeberger, Ulm, Germany) were ground and oleosin isolation and purification was performed as described by Schwager and colleagues.²² As the resolution power of the preparative electrophoresis cell is limited, separation of oleosins with a molecular mass difference of less than 1 kDa was not successful. Therefore, fractions containing oleosin mixtures (Ara h 10/11 or Ara h 14/15) were pooled and subjected to immunoblot analysis. For BAT, fractions of oleosins (Ara h 10/11 and Ara h 14/15) were combined in equal parts to achieve a defined mixture, and then dialyzed intensively against PBS. Although the majority of oleosins precipitated after dialysis against PBS, a quantity sufficient for BAT remained soluble. Non-solubilized oleosins were removed by centrifugation and the protein concentration of the supernatant was determined using the Bradford assay with BSA as standard (Thermo Fisher Scientific, Waltham, Mass).

SDS-PAGE and immunoblot analysis

Tricine-SDS-PAGE was conducted according to the protocol of Haider et al.²⁹ and immunoblot analysis was performed as stated elsewhere²² with the following modifications: application of membrane blocking solution (Thermo Fisher Scientific) instead of nonfat dry milk dissolved in buffer and an oleosin concentration of 25 µg/cm. The anti-Ara h 10/Ara h 14 antibody²² was diluted 1:10,000 in TBST, the anti-Ara h 15 antibody 1:100,000 in TBST.

Basophil activation test (BAT)

BAT was performed with heparinized whole blood which was stimulated for 30 minutes at 37°C with serial 10-fold dilutions of the defined oleosin mixtures or rAra h 15, starting at 10,000 ng/mL. Stimulation with formyl-methionyl-leucyl phenylalanine (fMLP, 1 µM,

Sigma-Aldrich, Steinheim, Germany), polyclonal goat anti-human IgE (1 µg/mL, Sigma-Aldrich) or dilution media (PBS buffer) served as controls. Cells were stained using labeled mouse (IgG1, IgG2a or IgG2b, κ) anti-human antibodies; FITC lineage cocktail (CD3, CD14, CD16, CD19, CD20, CD56), PerCP/Cy5.5 HLA-DR, PE/Cy7 FcεRIα, BV421 CD123, BV510 CD45, PE CD203c, APC CD63 or included matched isotype controls as appropriate (Biolegend, San Diego, Calif). Following lysis of erythrocytes (1-Step Fix/Lyse Solution, eBioscience, San Diego, Calif) and two washing steps, samples were analyzed using flow cytometry on an LSR II instrument (BD Biosciences, San Jose, Calif). Analysis was performed with the FCS Express 5 program (De Novo Software, Thornton, Canada). Established markers (CD45^{pos}, LIN^{neg}, CD123^{pos}, FcεRIα^{pos}, HLA-DR^{neg}) were used to characterize basophils³⁰⁻³² (see Fig E1 in the Online Repository). The CD63^{pos} percentage of CD203c^{pos} cells was calculated. Data were analyzed using GraphPad Prism software package (version 6, GraphPad Software, San Diego, Calif), compiled as box plots and subjected to Mann-Whitney *U* tests where applicable. The performance of BAT with oleosins was examined by receiver-operating characteristic (ROC) analysis.

IgE epitope mapping of Ara h 15

Overlapping 15-mer peptides derived from the amino acid sequence of Ara h 15 (Uniprot accession no. Q647G3) were offered as linear target epitopes to the serum samples investigated. Ara h 15 peptides with a GRAVY-score above 0 (the majority of the hydrophobic core domain) were omitted from the analysis because it was found that they cause non-specific binding of secondary antibodies (data not shown). Peptides were synthesized with an offset of 2 amino acids each by Fmoc solid phase synthesis on amine-derivatized cellulose disks of 2.7 mm diameter (Intavis Bioanalytical Instruments AG, Cologne, Germany) using an automated multiple peptide synthesizer (MultiPep RS, Intavis

Bioanalytical Instruments AG) as described by Homann and colleagues.³³ After completion of synthesis, the cellulose disks were disintegrated and the 15-mer peptides were spotted in quadruplicate onto glass slides in four arrays with 96 positions each. Slides were air-dried and stored at -20°C . Thawed microarrays were rehydrated with 100% ethanol for 10 min and subsequently washed 3 times thereafter, 10 min each, with TBST and TBS. Slides were blocked with Synblock ELISA blocking buffer (ImmunoChemistry Technologies, Bloomington, Minn) for 1 hour and washed with TBST. A liquid blocking pen (Pap Pen, Daido Sangyo, Japan) was used to encircle the spotted grid. Two hundred μl serum either of a PA suffering from severe peanut allergy (P7) or control sera (PA having mild allergic symptoms (P76, P77), PS (P51 – P55) and NA (P36 – P40)) were each diluted 1:2 in TBST, applied to the microarrays and left overnight at 4°C on a rocking shaker. After thorough washing with TBST, a monoclonal mouse anti-human IgG₂ antibody (Pharmingen, San Diego, Calif; diluted 1:100 in TBST) was added for 3 hours to block nonspecific binding of the secondary antibody. After 3 washings with TBST, a HRP-conjugated polyclonal swine anti-human IgE antibody (Nordic-MUBio, Susteren, the Netherlands; diluted 1:300 in TBST) was applied. Next, microarrays were washed with TBST, dried, and bound IgE was visualized using ECL detection reagents (Clarify Western ECL substrate, Bio-Rad Laboratories). Emitted light was detected with a ChemiDoc MP System (Bio-Rad Laboratories), intensity was documented spatially resolved in a tif-file and quantified with Image Studio Version 4 (Li-Cor Biosciences, Lincoln, Neb). Data analysis and statistics were performed using Microsoft Excel (MS Office 2013). True positive signals were identified using the procedure of Frey and colleagues.³² Signals are means of single measurements of quadruplicate spots of each peptide in a given array that was either exposed to serum from an allergic patient or to control serum. The positivity cut-off was set to values that represent a confidence interval of 99.5% for negativity in the control group ($n = 12$), i.e. any signal higher than the positivity

241 cut-off for a given peptide has a probability of $\geq 99.5\%$ of being a true positive IgE-reaction
242 with the respective sequence motif.

RESULTS

Study population

82 subjects were included in this study (52 PA, 15 PS and 15 NA, see Table I and Table E1 in the Online Repository). Categorization of PA into subgroups according to symptom severity resulted in a group of 16 individuals displaying mild allergic symptoms (e.g. oral allergy syndrome) and a group of 36 subjects suffering from severe allergic symptoms (objective symptoms for example gastrointestinal symptoms, urticaria, angioedema, dyspnoe, cardiac symptoms).

IgE reactivity of patients' sera to oleosins isolated from raw and in-shell roasted peanuts

In order to find out whether oleosin recognition is associated with the severity of symptoms in peanut allergy, balanced oleosin mixtures of Ara h 10/11 or Ara h 14/15 were analyzed with respect to their IgE-binding ability. Serum from all PA with severe allergic symptoms (except P5) showed IgE reactivity to oleosins isolated from in-shell roasted peanuts (Fig 2), both with Ara h 14/15 (Fig 2, A) and Ara h 10/11 (Fig 2, B). Moreover, IgE binding was also observed to oleosin dimers. PS, NA and PA with mild allergic symptoms showed no IgE reactivity to oleosins obtained from roasted peanuts (see Fig E2 in the Online Repository). In contrast to oleosins extracted from roasted peanuts, IgE binding to oleosins obtained from raw peanuts is notably decreased or almost undetectable in individuals suffering from severe allergic symptoms (see Fig E3 in the Online Repository). Thus, oleosin reactivity among peanut sensitized individuals appears to be predominantly directed against oleosins that underwent a roasting procedure and seems to be restricted to PA presenting with severe allergic symptoms.

Identification of oleosin-sensitized patients by BAT

After identification of PA with specific IgE to peanut oleosins, we further assessed whether this sensitization is of clinical relevance, i.e. whether the apparent correlation is based on a causative relationship. The basophil activation test (BAT) is an acknowledged *in vitro* assay mimicking the *in vivo* type I hypersensitivity reaction in a physiological environment,³⁴ and thus should be able to reveal such a cellular/molecular link. Reactivity of basophils from voluntary donors was analyzed by BAT (PA with severe allergic symptoms (P1 – P10, P20 – P24), NA (P36 – P50) and PS (P51 – P65), see Fig 3). In PA with severe systemic reactions the percentage of CD63^{pos} basophils was significantly higher for the starting concentration (10,000 ng/mL) of oleosins obtained from in-shell roasted and raw peanuts ($P < 0.0001$) as well as for recombinant Ara h 15 ($P < 0.001$) compared to the respective PS controls, and decreased in a dose-dependent manner. Median percentages of CD63^{pos} basophils for the 10,000 ng/mL stimulus were 43% for oleosins obtained from in-shell roasted peanuts, 35% for its raw counterpart and 17% for rAra h 15 in the PA group (Fig 3, A). Basophils from NA did not respond to natural and recombinant oleosins (Fig 3, C). ROC analysis of BAT with oleosins between PA and controls showed a high discriminative power of the test with area under the curve (AUC) values of 1.0 (95% CI, 1.0 – 1.0, in-shell roasted), 0.99 (95% CI, 0.97 – 1.0, raw) and 0.88 (95% CI, 0.74 – 1.0, modified rAra h 15) for the highest concentrations used (see Fig E4 in the Online Repository).

Identification of an IgE-binding epitope of Ara h 15

For the identification of IgE-binding epitopes, an oligopeptide microarray was created and probed with the serum of P7 or controls. IgE binding to five sequential peptides representing the overall amino acid sequence IADKARDVKDRAKDYAGAGRAQE located in the C-terminal domain of Ara h 15 was observed with a confidence interval of > 99.5%. The

293 consensus motif present in all five peptides is KDRAKDY whereas the peptide
294 DKARDVKDRAKDYAG displayed the highest IgE-binding affinity. Alignment of the C-
295 terminal domain of Ara h 15 and further homologous oleosins from other plant species
296 revealed a conservation of this IgE epitope (Fig 4).

297

DISCUSSION

Within the scope of the immuno-allergological characterization of oleosins, the major oil body proteins of plant seeds, we recently published an isolation and purification procedure.²² In this way, we were able to identify and characterize 8 peanut oleosins using mass spectrometry and N-terminal sequencing.²² In the present study, we show that oleosins are clinically relevant peanut allergens that are most likely associated with severe allergic symptoms. Moreover, our results indicate that thermal treatment used in industrial processing of peanuts increases the IgE-binding properties of oleosins as has already been shown for other peanut allergens.^{25, 26} Oleosins of commercially available peanuts which were dried and roasted in-shell to prevent molding (roasted peanuts) are capable of more IgE binding than those of peanuts that have only been dried (raw peanuts). In addition, we were able to produce a water-soluble recombinant oleosin which can be applied in physiological test systems (e.g. BAT), alongside natural molecules, to identify oleosin-sensitized patients. Finally, an IgE-binding epitope in the Ara h 15 sequence was identified which might be a cause of cross-reactivity between oleosins of different plants.

The starting point of this investigation was the assessment of the oleosin sensitization rate among peanut-allergic patients (PA) and the effect of roasting on the IgE-binding potency of oleosins. This was initially analyzed by immunoblot and BAT using purified mixtures of Ara h 10/11 and Ara h 14/15 obtained by preparative electrophoresis.²² Our results indicate that more than 65% (35/52) of the recruited PA are sensitized to peanut oleosins. Surprisingly, all of the tested individuals suffering from severe peanut allergy, except one (P82) (see Fig E2 in the Online Repository), showed a reaction to the distinct oleosins in immunoblot and BAT (P5 only in BAT). The single outlier (P82) is most probably due to an Ara h 8 monosensitization (30.4 kU/L), as such indicative for a birch pollen-associated peanut allergy

that in general is mild or moderate but has been shown to cause serious symptoms in some patients.^{35, 36}

Although earlier reports provided first hints on the potential allergenicity of peanut oleosins,³⁷ this study is the first comprehensive allergological investigation on peanut oleosins known to date with a panel of well-characterized patients. Moreover, this investigation is the first to reveal the allergenicity of 4 peanut oleosins, designated Ara h 10, Ara h 11, Ara h 14 and Ara h 15 on at least 5 individuals (criteria for allergen submission according to the WHO/IUIS Allergen Nomenclature Subcommittee). Our findings are in line with published data for sesame and hazelnut that highlight the relationship between oleosin sensitization and severe food allergy.^{14, 15} Besides that, our data may also explain an observation of Ballmer-Weber and colleagues who speculated about a peanut oleosin sensitization in 6 peanut-allergic patients without specific IgE to storage proteins but a history of anaphylaxis.¹⁰ In this context, it should be noted that all of the identified oleosin-sensitized patients are sensitized to Ara h 2 (range 1 – >100 kU/L) as well. This concomitance may not be coincidental but rather point to a new indicator for allergy severity. Despite the fact that several studies consider IgE against Ara h 2 to be the best predictor for a clinically relevant peanut allergy, it seems not to be discriminative for the degree of severity nor could its absence exclude a peanut allergy.^{10, 38, 39} A multiple sensitization to peanut allergens seems to be more predictive of symptom severity so far,⁴⁰⁻⁴² and our data show that severity closely correlates with the occurrence of anti-oleosin IgE. Thus, addition of oleosins to the established routine diagnostics might further improve risk assessment and management of peanut allergy. This could be important for two reasons. First, sensitization to oleosins seems to be highly prevalent as up to 25% of hazelnut-sensitized patients across Europe possess IgE against oleosins.⁴³ Second, children have been found to be more frequently sensitized to oleosins than adults.⁴³ Thus, inclusion of oleosins in routine testing shall increase assay sensitivity and may resolve inconclusive clinical findings.

Yet, the physical status of the oleosins appears to be important, too, as the strong correlation of severity and occurrence of anti-oleosin IgE was most prominent for the roasted molecules. This phenomenon has also been observed for other peanut allergens such as Ara h 1, Ara h 2 and Ara h 8.^{25, 44, 45} It is most likely a result of the Maillard reaction which takes place during food processing.^{46, 47} In addition to thermal processing, association with lipids may be another key factor for the allergenicity of some allergens, simply because a shell consisting of allergen-associated lipids may exert protection against gastrointestinal degradation and may foster the uptake of allergens via fat-carrier mediated transport.^{45, 48} Binding and incorporation of diverse molecules to oil bodies has been demonstrated to elevate blood concentrations of these substances following oral administration.^{49, 50} Furthermore, an adjuvant effect of lipids has also been demonstrated.⁴⁸ It thus seems highly probable that the close relationship between oleosins and lipids contributes to oleosin sensitization.

The striking differences observed in the IgE binding of PA to oleosins derived from raw and roasted peanuts in immunoblot experiments can similarly be found in BAT, particularly at low antigen concentrations (100 ng/mL). Again, the higher IgE affinity to and/or IgE cross-linking properties of Maillard-modified oleosins might explain this effect. In contrast to immunoblot, the advantage of BAT experiments is a more physiological setting. Our results show for the first time the ability of peanut oleosins to trigger hypersensitivity reactions, and thus their clinical relevance. With regard to published data on oleosin allergenicity, we assume that the main methodological achievement of our study lies in the use of purified and solubilized lipophilic molecules in our experiments.

Owing to the hydrophobic core domain (HCD), oleosins possess a very restricted solubility which impedes their isolation, purification and use in routine diagnostics.^{21, 51, 52} More importantly, the HCD is thought to be non- or barely antigenic⁵³ as it functions as a membrane anchor (see Fig E5 in the Online Repository). These facts prompted us to produce a water-soluble recombinant oleosin by joining the N- and C-terminal domains via a His-tag-linker

bridge. From our point of view, such a molecule is beneficial for immuno-allergological research and possibly a first step towards component-resolved diagnostics (CRD). Although the percentage of basophil activation was lower than that for natural oleosins and not every PA responded, the recombinant Ara h 15 was able to act as a trigger in BAT for the majority of patients with severe peanut allergy. One possible explanation for the two dropouts observed with rAra h 15 as compared to the natural oleosins might be that the mixture of natural oleosins provides more epitopes than a single allergen, another one that an individual sensitization to different oleosins exist, as can be expected. In addition, interaction of the HCD of natural oleosins can lead to the formation of dimers or multimers in a detergent-free environment.^{51, 54-56} The resulting aggregates display multiple epitopes that cross-link FcεRI-bound IgE more efficiently and thus may increase basophil activation.^{57, 58} From the current basis of data, BAT with a mixture of oleosins from roasted peanuts seems to be superior to BAT with oleosins from raw peanuts and recombinant rAra h 15 in identifying PA suffering from severe allergic symptoms (see Fig E4 in the Online Repository). As a consequence, future work will focus on structure-function properties associated with IgE binding and IgE-dependent basophil activation by recombinant oleosins and assessment of the utilization of recombinant oleosin mixtures prior to their application in CRD.

Finally, to further support our findings on the activity of rAra h 15 in BAT, we analyzed the presence of IgE epitopes in the amphipathic domains of Ara h 15 by oligopeptide microarray. In our case, careful assay adjustment and optimization was required to gain the necessary sensitivity; most likely due to low sIgE titers⁴³ along with the absence of possible roasting-induced modifications or alterations which have been shown to increase IgE binding in natural oleosins. With fine-tuned assay conditions finally at hand, we were able to reveal an IgE-binding sequence of 23 amino acids in the C-terminal domain of Ara h 15 and identified DKARDVKDRAKDYAG as the peptide with the highest IgE affinity for patient P7 (Fig 4). Elements of this sequence are conserved among oleosins of diverse food sources such as

hazelnut (Cor a 13),⁵⁹ sesame (Ses i 5),¹⁵ almond⁶⁰, soybean⁶¹ and rapeseed⁶². Oleosins might therefore be a cause of IgE cross-reactivity (Fig 4) as patient P7 reported an allergy to soybean and rapeseed. Interestingly, the presence of IgE to the oleosin Cor a 12 has been shown to correlate with sensitization to oil-rich tree nuts, seeds and legumes.⁴³ However, the IgE-binding sequence of P7 might be different from other patients and other patients were not studied yet.

As dimerization and oligomerization of hydrophilic domains of rAra h 15 is conceivable,⁵⁵ such a single epitope might be sufficient for the IgE cross-linking on basophils in BAT. Moreover, the observed IgE binding to five sequential peptides might be the result of a polyclonal IgE response to adjacent epitopes. Additionally, it cannot be excluded that other non-linear conformational IgE epitopes on Ara h 15 which could not be detected by the applied peptide array system are present and may contribute to IgE binding and cross-linking. Generally, IgE epitope mapping can add a molecular basis for the identification and optimization of BAT triggers and for the prediction of individual cross-reactions.

In conclusion, we have demonstrated that peanut oleosins in total are major allergens that appear to be linked with severe peanut allergy. Hence, our study underscores the importance of lipophilic allergens, such as oleosins, for a comprehensive allergy diagnosis. Future multi-center studies will more precisely investigate the utilization of oleosins in different test systems and compare these results with the outcome of food challenges and the performance of other peanut allergens to evaluate their predictive value with regard to clinical severity of peanut allergy.

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TABLE LEGENDS

Table I. Characteristics of the study population according to peanut-allergic (n = 52) and peanut-tolerant (n = 30) individuals.

Characteristic	Peanut-allergic individuals		Peanut-tolerant individuals	
	severe allergic	mild allergic	PS	NA
	symptoms (n = 36)	symptoms (n = 16)	(n = 15)	(n = 15)
Age (y)	23 (6 – 55)	36 (13 – 58)	36 (21 – 61)	38 (25 – 72)
Males	18 (50%)	2 (12.5%)	2 (13%)	7 (47%)
Total IgE (IU/mL)	602 (93 – >2500)	261 (42 – 1517)	162 (46 – 762)	30 (2 – 251)
Specific IgE to				
Peanut (kU _A /L)	77 (5 – >100)	0 (<0.35 – 5)	0 (<0.35 – 2)	0 (<0.35)
Ara h 2 (kU _A /L)	35 (<0.35 – >100)	0 (<0.35)	0 (<0.35)	0 (<0.35)
Ara h 8 (kU _A /L)	1 (<0.35 – 66)	4 (1 – 29)	2 (<0.35 – 20)	0 (<0.35)
Specific IgE/total IgE [%]				
Peanut	8 (1 – 42)	0 (0 – 1)	0 (0 – 1)	0 (0 – 0)
Ara h 2	5 (0 – 24)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
Ara h 8	0 (0 – 5)	1 (0 – 21)	2 (0 – 8)	0 (0 – 0)

Values are expressed as numbers or medians (range).

For calculation, specific IgE values below 0.35 kU_A/L were considered as 0, values above 100 kU_A/L as 100 and for total IgE values above 2500 kU_A/L were considered as 2500.

FIGURE LEGENDS

FIG 1. Illustration of the modified recombinant Ara h 15 without hydrophobic domain (A) and the proposed oleosin structure (B) with respect to the three distinct domains (N-terminus, hydrophobic core domain (HCD) and C-terminus). The modified domain (MD) comprises of a His-tag linked to a Glycin-Alanin-Glycin-spacer on each side.

FIG 2. Western blot of mixed oleosin fractions (A) Ara h 14/15 and (B) Ara h 10/11 obtained from in-shell roasted peanuts. M, molecular mass marker; S, protein staining; A1, anti-Ara h 10/14 antibody; A2, anti-Ara h 15 antibody; C1, buffer control anti-human IgE antibody; C2, buffer control anti-rabbit IgG antibody; P1 – P35, PA with severe allergic symptoms; # patients included in BAT.

FIG 3. BAT of PA (n = 15, A), PS (n = 15, B) and NA (n = 15, C) using defined oleosin mixtures and the modified rAra h 15. The *P* value refers to comparisons of median percentages of CD63^{pos} basophils at indicated doses of allergens between PA and PS: **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$.

FIG 4. Location of the IgE epitope identified in the Ara h 15 sequence. Alignment of the C-terminal domains of selected oleosins registered as allergens of peanut, hazelnut, sesame and three additional homologous oleosins of almond, soybean and rapeseed. Dashes represent gaps introduced for best alignment. The boxed sequence indicates the determined IgE epitope of Ara h 15.

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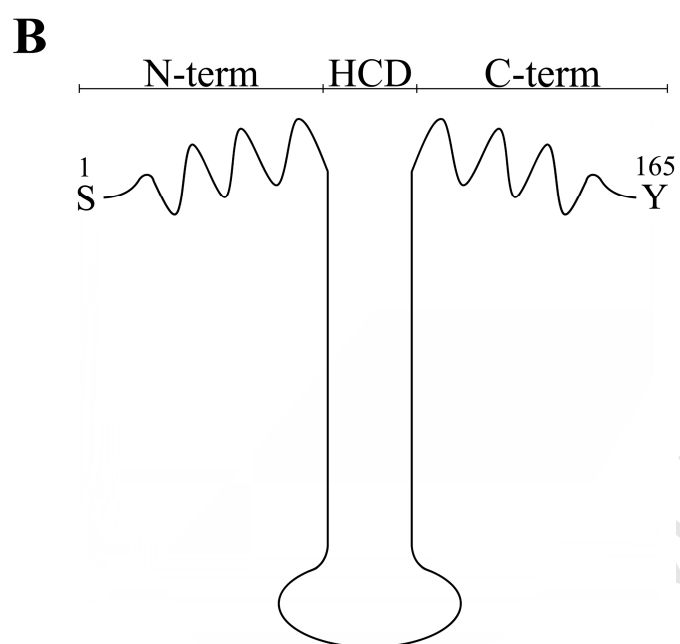
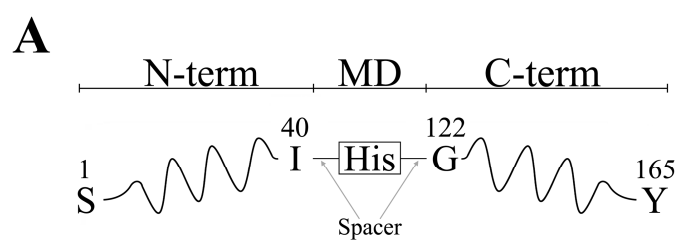
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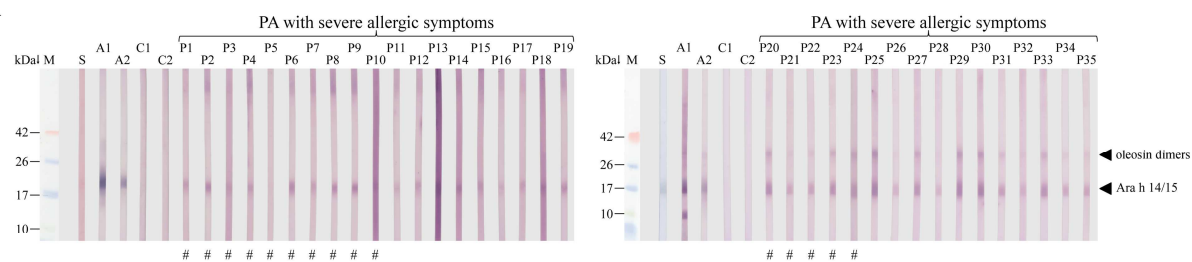
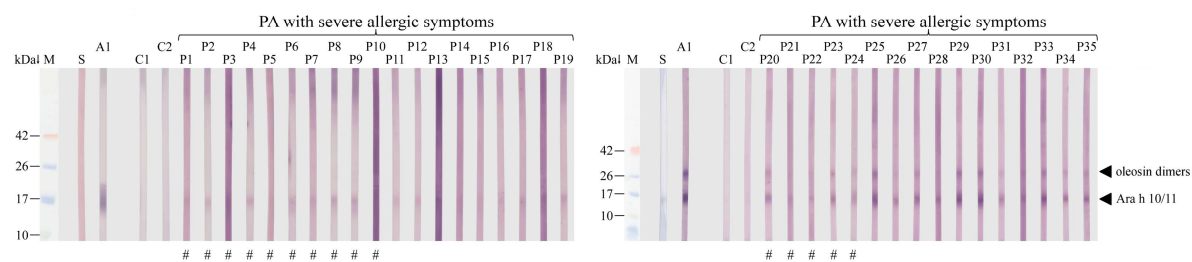
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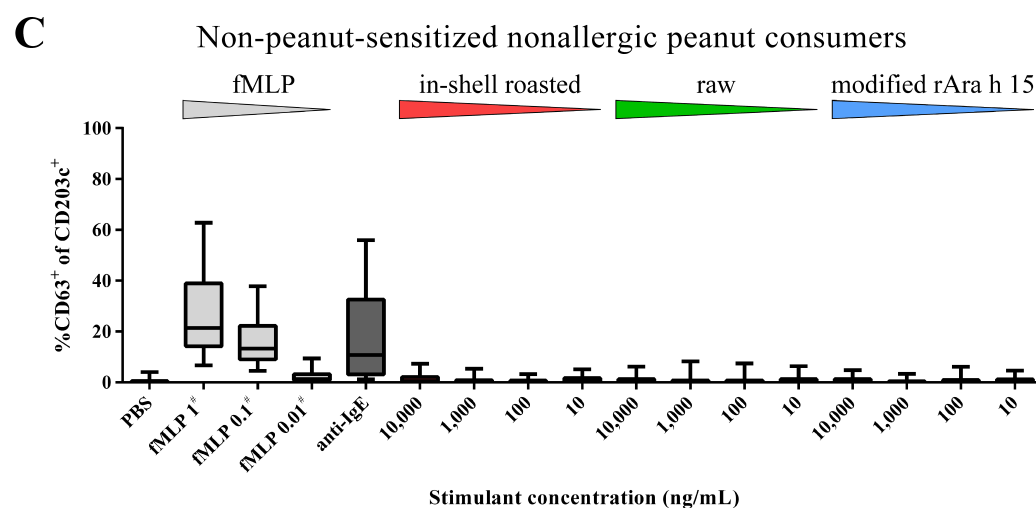
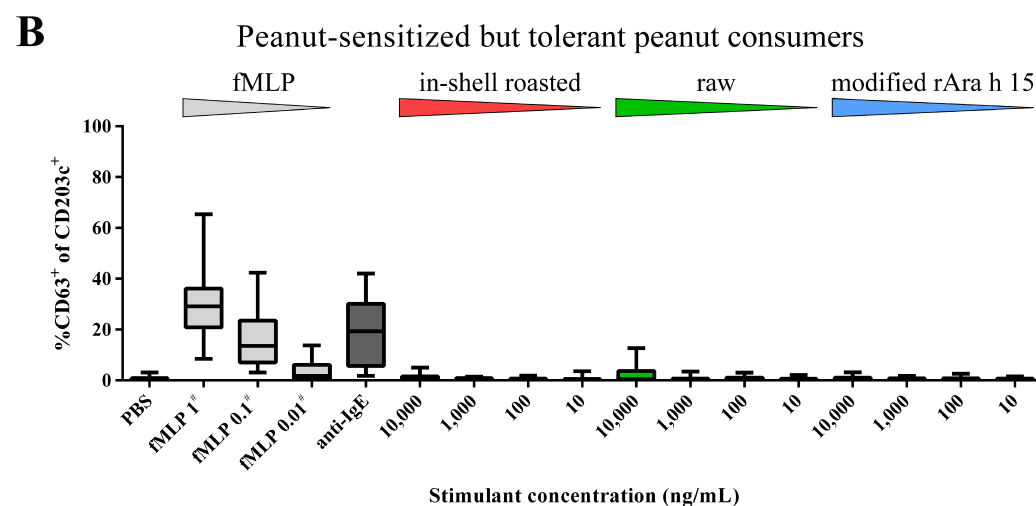
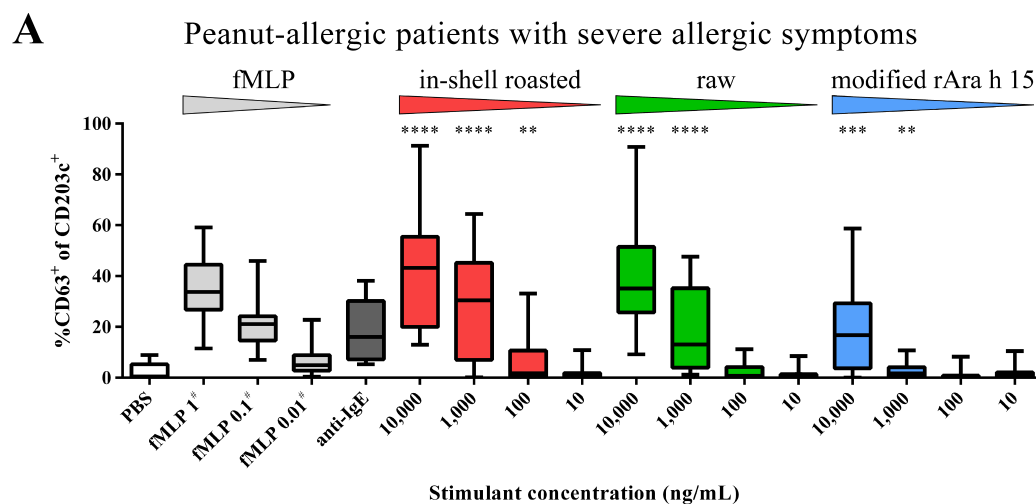
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- 626



A**B**



Rapeseed oleosin'	119	YKYATGEHPQGS	DKLDSARMKLGK	VQDMKDR	QYY-G---QQQTGGEDDRDR	TRGTQHTT	175
Almond oleosin"	100	YKYVTGKQPPG	ADQLDQARHKL	AGKARDIK	DRAEQF-G---QQHVPSGQQSS	-----	148
Ses I 5.0101	99	YRYLTGRHPPG	ADQLESATKL	ASKAREMK	DRAEQF-S---QQPVAGSQTS	-----	145
Cor a 13.0101	94	YRYVTGRHPPG	ADQLDHARMKL	ASKAREMK	DRAEQF-G---QQHVTGSQGS	-----	140
Soybean oleosin"	114	YNYVSGNQFAG	YDTLDYAKGYL	ADKARDVKE	RAQY-GSTAQGRINEATQ	GGAY-----	165
Ara h 15.0101	118	YSYVTGKHFGS	DRLDYAKGVIA	<u>DKARDVKDRAKD</u>	<u>YAG</u> ---AGRAQEGT	PG-Y-----	166

* UniProt accession number P29111
 ** UniProt accession number Q43W4
 *** UniProt accession number C3VHQ8
 □ IgE epitope

ONLINE REPOSITORY MATERIAL

Peanut oleosins associated with severe peanut allergy - Importance of lipophilic allergens for comprehensive allergy diagnostics

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E-Methods:

Production of a polyclonal anti-Ara h 15 antibody

Purified rAra h 15 was extensively dialyzed against water and lyophilized thereafter. Two mg rAra h 15 were sent to BioGenes (Berlin, Germany) which carried out the antibody production on custom basis. Two rabbits were immunized for the production of antibodies against Ara h 15. Pre-immune serum was collected before immunization. Animals were bled 14 days after the second boost and the obtained sera were tested in different dilutions to determine the best concentration for immunoblot experiments. The animal experiments were approved by the local authorities (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern) and complied with the German Animal Protection law. Immunization of the animals was performed in strict accordance with recommendations in the National Institutes of Health guide for care and use of laboratory animals.

Legends of figures in the online repository:

FIG E1. Flow cytometric gating strategy for BAT displaying the basophil analysis of whole blood samples from PA (n = 15), PS (n = 15) and NA (n = 15). A representative flow cytometric gating strategy (P54) is shown using anti-IgE as stimulant. Cells were stained using labeled anti-human antibodies; FITC-lineage cocktail (CD3, CD14, CD16, CD19, CD20, CD56), PerCP/Cy5.5 HLA-DR, PE/Cy7 FcεRIα, BV421 CD123, BV510 CD45, PE CD203c and APC CD63. (A) Forward scatter area (FSC-A) versus sideward scatter area (SSC-A) was used for gating leucocytes. Doublets were excluded by using FSC-area versus FSC-height. (B) After gating CD45^{pos} cells, lineage negative cells were further assayed for CD123 and FcεRIα. CD123^{pos}FcεRIα^{pos} cells were gated and further analyzed to exclude HLA-DR^{pos} dendritic cells. (C) Finally, HLA-DR^{neg}CD45^{pos} cells were used and assessed for their CD63 and CD203c expression. The percentage of CD63^{pos} basophils (CD45^{pos}, LIN^{neg}, CD123^{pos}, FcεRIα^{pos}, HLA-DR^{neg}, CD203c^{pos}) were used as result for the box blot diagram.

FIG E2. Western blot of mixed oleosin fractions (A) Ara h 14/15 and (B) Ara h 10/11 from in-shell roasted peanuts probed with sera of PS, NA and PA with mild allergic symptoms. M, molecular mass marker; S, protein staining; A1, anti-Ara h 10/14 antibody; A2, anti-Ara h 15 antibody; P2, peanut-allergic individual with severe allergic symptoms (serving as positive control for IgE-reactivity to oleosins); C1, buffer control anti-human IgE antibody; C2, buffer control anti-rabbit IgG antibody; P36 – P50, NA; P51 – P65, PS; P66 – P81, PA with mild allergic symptoms; P82, patient with Ara h 8-IgE-positive pollen-associated peanut allergy with severe allergic symptoms but without oleosin-sensitization.

FIG E3. Western blot of mixed oleosin fractions (A) Ara h 14/15 and (B) Ara h 10/11 from raw peanuts probed with sera of PA with severe allergic symptoms. M, molecular mass marker; S, protein staining; A1, anti-Ara h 10/14 antibody; A2, anti-Ara h 15 antibody; C1, buffer control anti-human IgE antibody; C2, buffer control anti-rabbit IgG antibody; P1 – P35, PA with severe allergic symptoms.

FIG E4. (A) ROC curves and (B) characteristics of the basophil activation test performed with oleosins from raw and in-shell roasted peanuts as well as the modified rAra h 15 (n = 45). Values in parentheses represent the 95% confidence interval.

FIG E5. Prediction of the Ara h 15 protein sequence with regard to (A) hydrophobicity (Kyte-Doolittle), (B) antigenic index (Jameson-Wolf) and (C) surface probability (Emini) of the three structural domains (N-terminal domain, hydrophobic core domain (HCD), and C-terminal domain).

Table E1. Overview of demographic data, individual sensitization profiles and peanut allergy symptoms of PA, PS and NA.

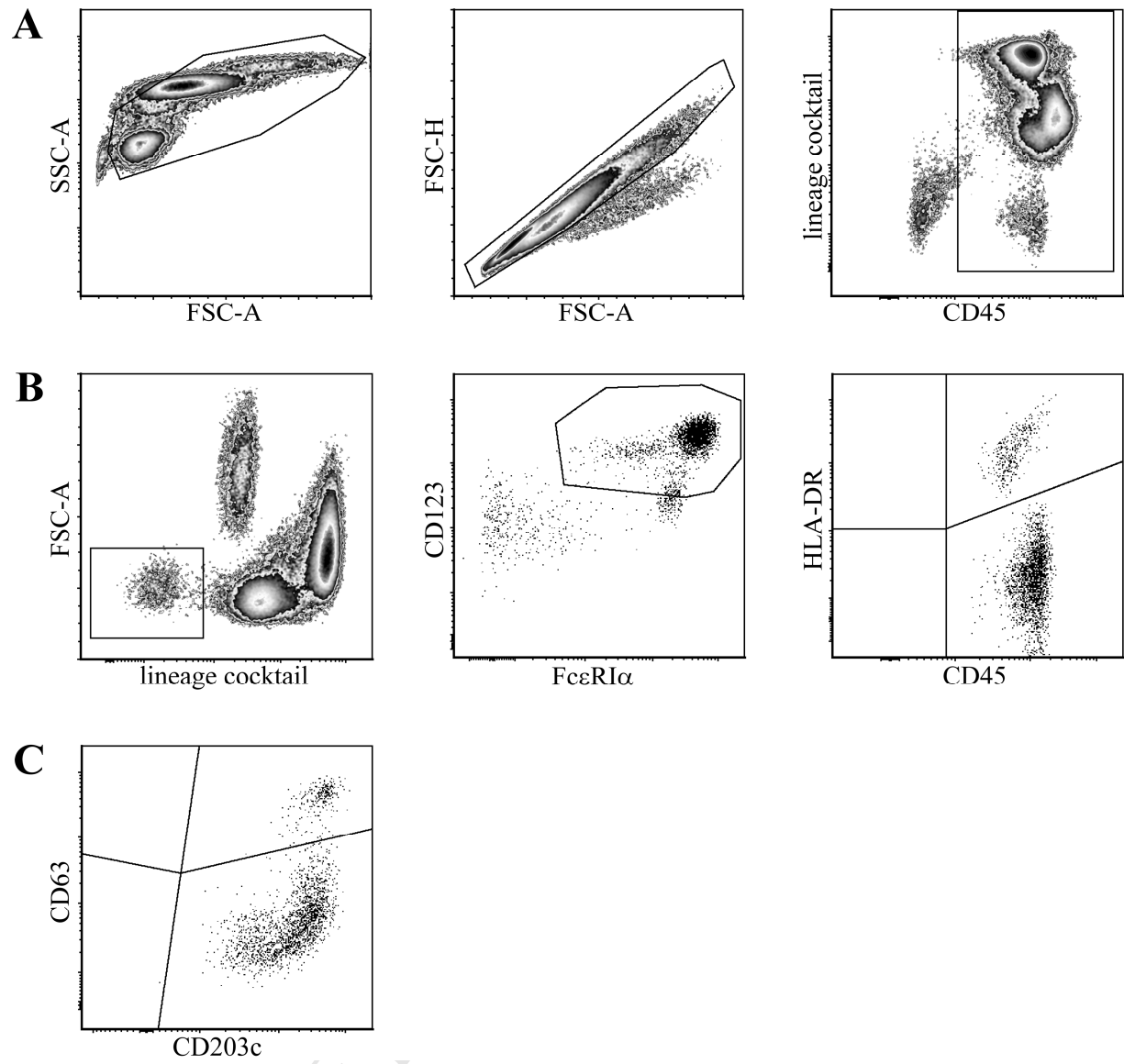
	Patient	Sex	Age	IgE ImmunoCAP [kU/L]				Western blot	Peanut allergy symptoms
				Total IgE	Peanut extract	Ara h 2	Ara h 8	Anti-oleosin IgE	
Peanut-allergic patients with severe allergic symptoms	P1	M	49	508	>100	60.50	17.30	+	SH
	P2	M	29	536	75.90	51.00	6.13	+	A, AE, DS, F, U
	P3	F	26	93	18.00	14.00	4.08	+	C, P, U
	P4	M	18	1267	>100	75.80	5.79	+	A, AE, F, G, GI
	P5	F	40	793	5.75	1.17	9.16	-*	A, G, GI, P
	P6	M	7	431	>100	>100	<0.35	+	A, AE, GI, U
	P7	M	21	260	66.30	22.00	1.79	+	A, AE, C, F
	P8	M	16	732	>100	96.50	<0.35	+	A, F, GI, R, U
	P9	F	7	2096	>100	96.00	<0.35	+	AD
	P10	F	23	1828	47.50	28.90	11.40	+	A, DS, G, OAS
	P11	M	44	175	32.90	18.90	<0.35	+	A, DS, G, OAS, U
	P12	M	42	555	19.50	1.15	<0.35	+	A, AE, U
	P13	M	20	238	>100	57.70	7.39	+	A
	P14	F	9	614	33.70	35.10	18.30	+	A, DS, GI
	P15	F	15	343	87.10	14.80	<0.35	+	A, GI
	P16	M	6	531	52.10	18.20	<0.35	+	A, AD, GI
	P17	M	14	1272	29.60	6.61	66.30	+	A, AE, C
	P18	M	15	>2500	>100	36.0	0.36	+	A, OAS
	P19	F	22	1206	13.70	8.56	4.70	+	A, AD, DS, G, OAS
	P 20	F	23	1616	>100	>100	<0.35	+	A, AE, DS, F, G, GI
	P 21	M	11	706	21.30	3.21	<0.35	+	AD, AE
	P 22	F	23	271	5.09	4.92	0.42	+	A, AE, DS, F, G, GI, OAS, U
	P 23	F	17	1773	>100	>100	<0.35	+	A, AE, DS, F, G, GI, U
	P24	F	6	>2500	>100	>100	<0.35	+	AE, DS, G, GI, R
	P25	F	15	1489	>100	>100	5.16	+	A, AE, DS, U
	P 26	F	40	533	47.50	11.60	12.20	+	A, AD, AE, DS, OAS, U
	P 27	F	42	590	>100	91.80	0.95	+	A, AD, AE, DS, GI, OAS, R, U
	P 28	M	29	194	77.40	25.40	<0.35	+	A, OAS, R, U
	P 29	F	43	2062	>100	67.50	0.54	+	A, AD, DS, GI, OAS, R, U
	P 30	F	20	>2500	>100	>100	3.79	+	A, GI, R, U
	P 31	M	43	479	95.60	38.20	12.20	+	A, C, P
	P 32	M	53	321	36.10	5.66	0.79	+	A, AD, AE, DS, GI, OAS, U
	P 33	M	40	2355	>100	23.70	7.86	+	A, AD, AE, DS, GI, OAS, R
	P 34	F	55	308	23.10	22.60	<0.35	+	A, AD, AE, DS, GI, OAS, R, SH, U
	P 35	M	20	208	39.80	36.40	0.67	+	A, AE, DS, G, GI, P, SH
	P82	F	50	894	5.52	<0.35	30.40	-	A, AE, C, DS, GI

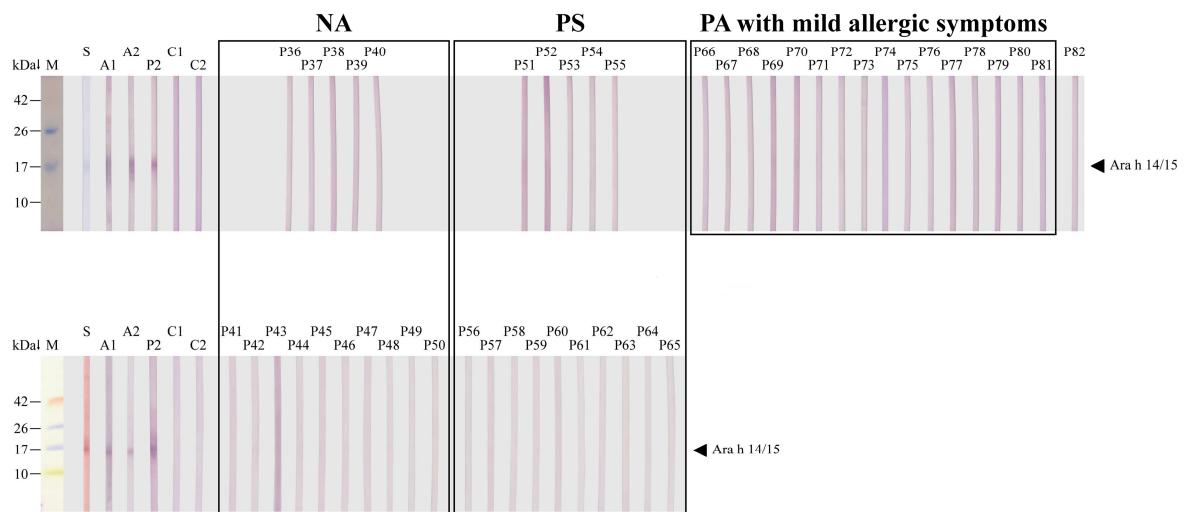
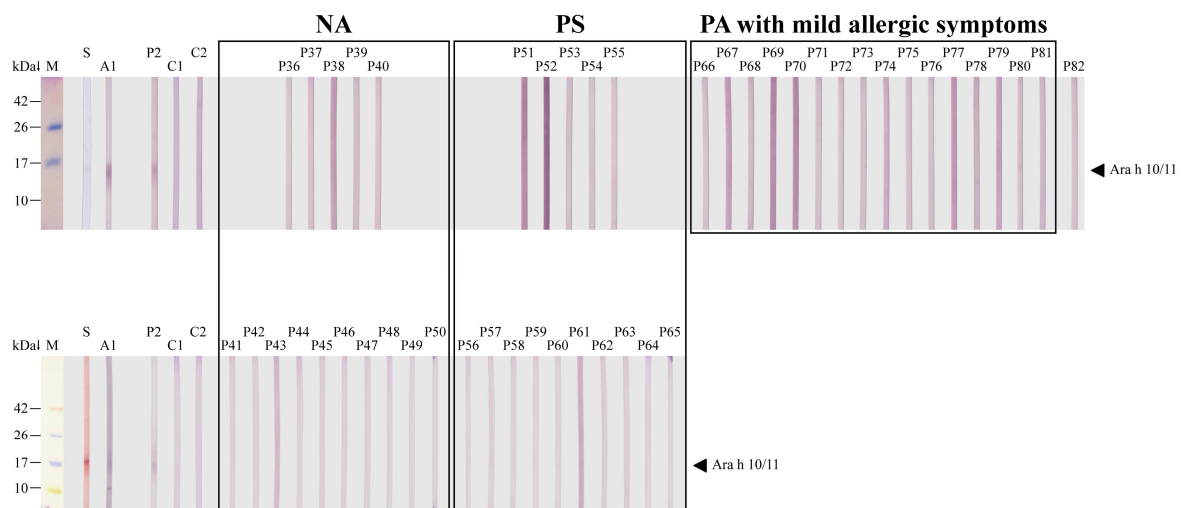
	Patient	Sex	Age	IgE ImmunoCAP [kU/L]				Western blot	Peanut allergy symptoms
				Total IgE	Peanut extract	Ara h 2	Ara h 8	Anti-oleosin IgE	
NA	P36	M	72	12	<0.35	<0.35	<0.35	-	no symptoms
	P37	M	49	5	<0.35	<0.35	<0.35	-	no symptoms
	P38	M	38	30	<0.35	<0.35	<0.35	-	no symptoms
	P39	F	25	39	<0.35	<0.35	<0.35	-	no symptoms
	P40	F	53	2	<0.35	<0.35	<0.35	-	no symptoms
	P41	F	38	34	<0.35	<0.35	<0.35	-	no symptoms
	P42	M	41	4	<0.35	<0.35	<0.35	-	no symptoms
	P43	F	38	8	<0.35	<0.35	<0.35	-	no symptoms
	P44	M	44	61	<0.35	<0.35	<0.35	-	no symptoms
	P45	M	33	22	<0.35	<0.35	<0.35	-	no symptoms
	P46	F	36	15	<0.35	<0.35	<0.35	-	no symptoms
	P47	M	31	251	<0.35	<0.35	<0.35	-	no symptoms
	P48	F	31	54	<0.35	<0.35	<0.35	-	no symptoms
	P49	F	29	64	<0.35	<0.35	<0.35	-	no symptoms
	P50	F	26	54	<0.35	<0.35	<0.35	-	no symptoms
PS	P51	F	26	227	<0.35	<0.35	0.56	-	no symptoms
	P52	F	32	147	<0.35	<0.35	11.00	-	no symptoms
	P53	F	35	762	0.41	<0.35	20.30	-	no symptoms
	P54	F	22	241	<0.35	<0.35	0.59	-	no symptoms
	P55	F	48	46	<0.35	<0.35	1.40	-	no symptoms
	P56	F	37	97	<0.35	<0.35	1.34	-	no symptoms
	P57	F	50	85	<0.35	<0.35	2.46	-	no symptoms
	P58	F	47	63	<0.35	<0.35	0.62	-	no symptoms
	P59	F	21	385	<0.35	<0.35	7.88	-	no symptoms
	P60	F	39	222	0.90	<0.35	3.15	-	no symptoms
	P61	F	32	112	<0.35	<0.35	0.43	-	no symptoms
	P62	F	51	142	0.40	<0.35	3.08	-	no symptoms
	P63	F	61	162	<0.35	<0.35	4.84	-	no symptoms
	P64	M	36	178	1.20	<0.35	5.42	-	no symptoms
	P65	M	26	546	1.99	<0.35	<0.35	-	no symptoms

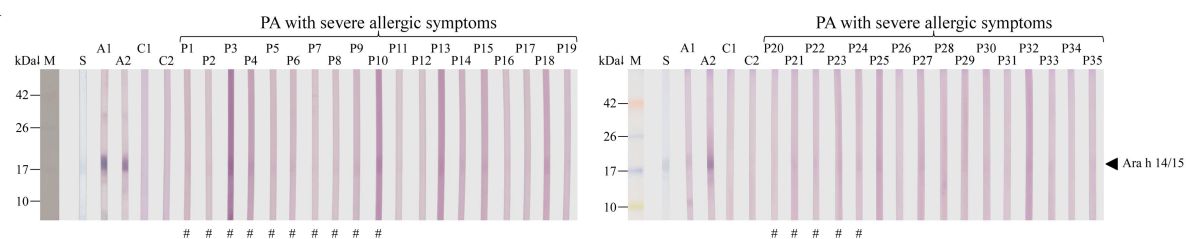
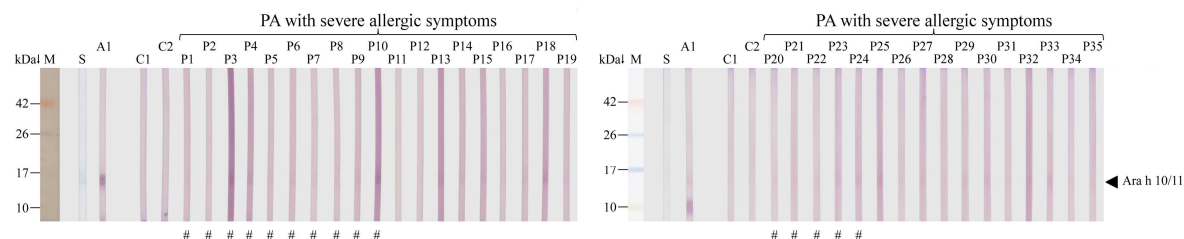
	Patient	Sex	Age	IgE ImmunoCAP [kU/L]				Western blot	Peanut allergy symptoms
				Total IgE	Peanut extract	Ara h 2	Ara h 8	Anti-oleosin IgE	
Peanut-allergic patients with mild allergic symptoms	P66	F	35	42	<0.35	<0.35	3.41	-	OAS
	P67	F	29	214	<0.35	<0.35	1.65	-	OAS
	P68	F	27	224	<0.35	<0.35	0.73	-	OAS, prickling of the hands
	P69	M	44	357	0.38	<0.35	0.84	-	AE, OAS
	P70	F	51	1517	1.35	<0.35	12.80	-	OAS
	P71	F	29	70	<0.35	<0.35	2.32	-	OAS
	P72	F	57	459	0.97	<0.35	28.70	-	OAS
	P73	F	35	291	<0.35	<0.35	4.69	-	OAS, R
	P74	F	13	898	5.38	<0.35	10.80	-	C, OAS
	P75	F	53	357	4.20	<0.35	6.20	-	OAS
	P76	F	28	129	0.84	<0.35	1.51	-	OAS
	P77	F	58	107	<0.35	<0.35	3.35	-	DS, OAS
	P78	M	40	887	0.51	<0.35	7.46	-	DS, OAS
	P79	F	45	338	<0.35	<0.35	4.10	-	OAS
	P80	M	36	231	0.47	<0.35	3.92	-	OAS
	P81	F	33	49	<0.35	<0.35	10.43	-	AE, OAS

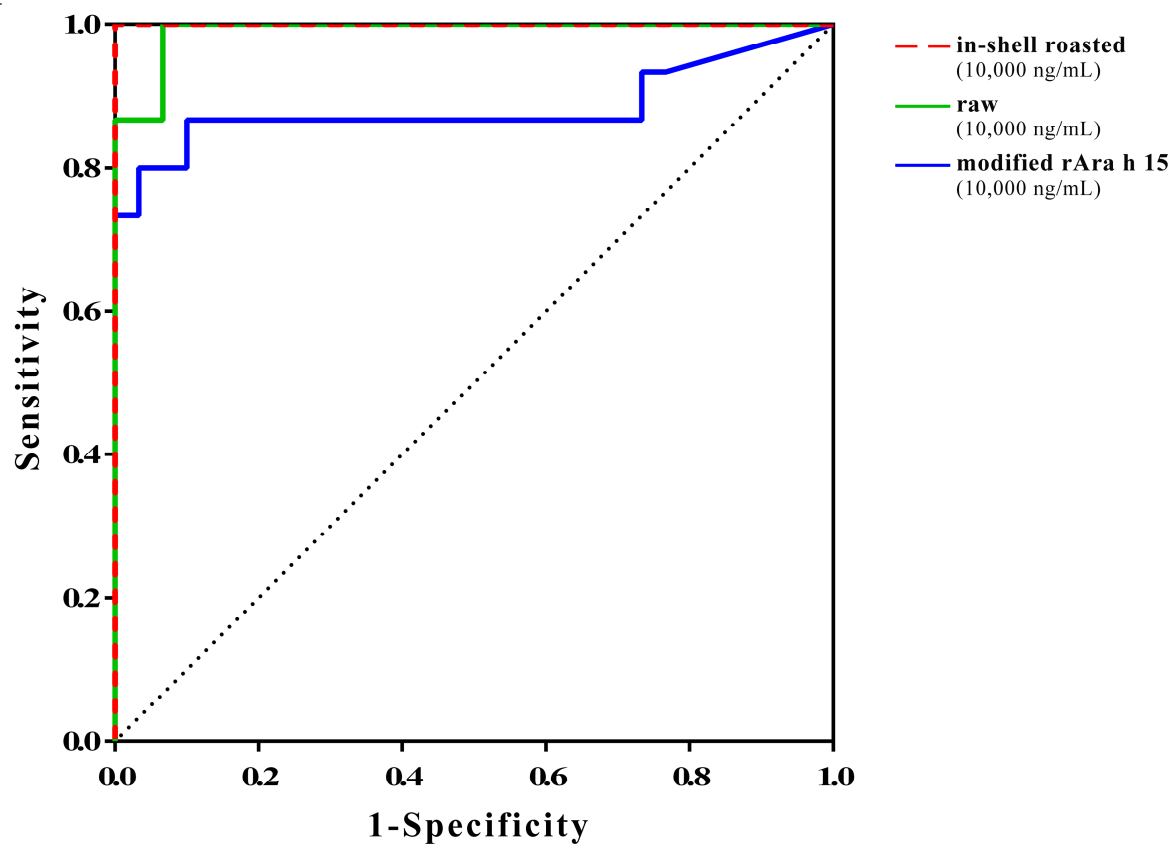
Symbol	Explanation
+	detected (positive)
-	not detected (negative)
-*	no IgE detection in Western blot but showed activation of basophils by oleosins in BAT
A	asthma, dyspnoe
AD	atopic eczema
AE	angioedema
C	conjunctivitis
DS	difficulties swallowing
F	flush
G	maximal fatigue, general malaise, hypotonia, tremor
GI	gastrointestinal
OAS	oral allergy syndrome
P	generalized pruritus
R	rhinitis
SH	shock

ACCEPTED MANUSCRIPT



A**B**

A**B**

A**B**

BAT parameters	AUC ROC (95% CI)	Optimal cut-off (% CD63 ⁺)	Sensitivity (95% CI)	Specificity (95% CI)
In-shell roasted oleosins (10,000 ng/mL)	1.0 (1.0 – 1.0)	10.16	1.0 (0.78 – 1.0)	1.0 (0.88 – 1.0)
Raw oleosins (10,000 ng/mL)	0.99 (0.97 – 1.0)	7.72	1.0 (0.78 – 1.0)	0.93 (0.78 – 0.99)
Modified recombinant Ara h 15 (10,000 ng/mL)	0.88 (0.74 – 1.0)	2.05	0.87 (0.60 – 0.98)	0.90 (0.73 – 0.98)

