Co-sensitization to the three non-homologous major cashew allergens Ana o 1, Ana o 2 and Ana o 3 is caused by IgE cross-reactivity

Short title: IgE cross-reactivity of cashew allergens

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ABSTRACT

Background: Cashew nuts often cause strong allergic reactions, even exceeding those of peanuts. Ana o 1 (vicilin), Ana o 2 (legumin) and Ana o 3 (2S albumin) are major cashew allergens. Co-sensitization to all three non-homologous cashew nut allergens has been observed. We hypothesize that this might be due to IgE cross-reactivity.

Methods: IgE cross-inhibitions were performed with Ana o 1-3 using sera from cashew nut allergic patients. Related hazelnut allergens Cor a 11, 9 and 14 were used as controls. For comparison, IgE cross-reactivity between the hazelnut allergens was investigated using sera from hazelnut allergic patients.

Results: Median percentages of cross-inhibitions between Ana o 1-3 were 84-99%. In comparison, medians of cross-inhibitions between hazelnut allergens were 33-62%. The IC₅₀ values revealed the highest IgE affinity to Ana o 3 and Cor a 14. Hazelnut legumin Cor a 9 inhibited IgE-binding to Ana o 1, 2, and 3 with median percentages of 75%, 56%, and 48%, respectively. No cross-reactivity was observed between allergenic vicilins or between 2S albumins from cashew and hazelnut. In silico identified potentially cross-reactive peptides of Ana o 3 overlapped with previously reported IgE epitopes of all three allergens.

Conclusion: IgE with high affinity to Ana o 3 that cross-reacts with the other two major non-homologous cashew nut allergens might be responsible for the high allergenic potency of cashew nut. These cross-reactive IgE comprises the major fraction of specific IgE in cashew allergic patients, and might be responsible for cross-reactivity between unrelated tree nuts.

Key words: Cashew nut allergy. IgE cross-reactivity. Food allergens. Food allergy. Hazelnut allergy. Hazelnut allergens. Tree nut allergy.

RESUMEN

Antecedentes: Los anacardos suelen causar fuertes reacciones alérgicas, incluso mayores a las del cacahuete. Los principales alérgenos del anacardo son: Ana o 1 (vicilina), Ana o 2 (legumina) y Ana o 3 (albúmina 2S). Se ha observado cosensibilización a los tres alérgenos no homólogos de anacardos. Nuestra hipótesis es que esto podría deberse a la reactividad cruzada de IgE.

Métodos: Se realizaron inhibiciones cruzadas de IgE con Ana o 1-3 utilizando sueros de pacientes alérgicos al anacardo. Los alérgenos de avellana relacionados Cor a 11, 9 y 14 se usaron como controles. A modo de comparación, se investigó la reactividad cruzada de IgE entre los alérgenos de las avellanas utilizando sueros de pacientes alérgicos a las avellanas.
**Resultados:** Las medianas del porcentaje de inhibición cruzada entre Ana o 1-3 fueron del 84-99%. En comparación, las medianas de las inhibiciones cruzadas entre alérgenos de avellana fueron del 33 al 62%. Los valores de IC50 revelaron mayor afinidad de la IgE por Ana o 3 y Cor a 14. La legumina de avellana (Cor a 9) inhibió la unión de IgE a Ana o 1, 2 y 3 con medianas de 75 %, 56 % y 48 %, respectivamente. No se observó reactividad cruzada entre las vicilinas o entre las albúminas 2S de anacardo y avellana. *In silico* se identificaron péptidos que potencialmente eran responsables de la reactividad cruzada de Ana o 3 superpuestos con epitopos IgE previamente identificados de los tres alérgenos.

**Conclusión:** La IgE con alta afinidad por Ana o 3 que reacciona de forma cruzada con los otros dos alérgenos principales no homólogos del anacardo, podría ser responsable de la alta potencia alergénica del anacardo. Estas IgE de reactividad cruzada comprenden la fracción principal de IgE específica en pacientes alérgicos al anacardo y podrían ser responsables de la reactividad cruzada entre frutos secos no relacionados.

**Palabras clave:** Alergia al anacardo. Reactividad cruzada de IgE. Alérgenos alimentarios. Alergia a alimentos. Alergia a las avellanas. Alérgenos de las avellanas. Alergia a los frutos secos.
INTRODUCTION
Peanut and tree nuts such as cashew nut, hazelnut or walnut are sources of potent allergenic proteins. Especially, reactions induced by cashew nut and peanut are associated with potential severity[1-4] and are recognized as leading causes of food allergy-induced anaphylaxis, mainly among children and young adults [5-7]. Also, a higher incidence of severe clinical reactions to cashew nut compared to peanut was reported [3, 4, 8, 9]. It seems that the increased reports on cashew nut allergy have paralleled the increasing consumption of this nut over the last three decades [5]. Studies on threshold dose distributions have shown that ingestion of an amount as small as 0.9 mg of cashew nut protein [3, 10, 11], or even only skin and mucosal contact [12, 13], may cause severe clinical reactions. This suggests a high potency of cashew nut allergens, equivalent to or even higher than that of peanut [14]. Notably, the allergic reactions were triggered in more than three-quarters of cases at the first known exposure [10, 12, 15, 16]. Similar clinical observation in peanut allergy led to the hypothesis that early environmental exposure to peanut (through the impaired skin or the airway) may account for early sensitization [17].

Cashew nut contains about 44% lipids and 19% proteins [18], and most of the protein content is made up by seed storage proteins comprising major cashew allergens Ana o 1-3 [19]. Ana o 1 [20] (vicilin) and Ana o 2 [21] (legumin) belong to the cupin superfamily, and Ana o 3 belongs to the 2S albumin protein family [22]. Studies in populations from different geographical regions, performed with the only commercially available cashew nut allergen Ana o 3, showed that sensitization to this allergen is highly predictive for clinical reactivity in cashew nut sensitized patients [23-25]. However, outcomes of a large study including 173 patients with suspected cashew nut allergy showed that all three components, namely Ana o 1-3, were each individually predictive for oral food challenge failure [26]. Interestingly, higher median values of specific IgE to cashew nut components Ana o 2 and 3 were measured than to whole cashew nut extract, a phenomenon also observed in peanut [27].

In our previous study, we found unexpected IgE cross-reactivity between the three non-homologous peanut allergens Ara h 1-3 [28]. A later study, analyzing antibodies produced by B-cells from peanut allergic patients confirmed the presence of IgEs with high affinity and cross-reactivity to the unrelated peanut allergens [29]. Meanwhile, it became evident that highly similar Ara h 2-specific IgE sequences are shared between different peanut allergic patients [30, 31] indicating that common immunoglobulin rearrangements may contribute to pathogenesis and that peanut allergens are recognized in a similar manner.

Given the high clinical relevance of cashew nut allergy and the above described similarities to peanut allergy, we hypothesized that the high allergenic potency of cashew nut is due to IgE cross-reactivity between the three individual cashew nut allergens. To test our hypothesis, we performed IgE cross-inhibitions with cashew nut allergens Ana o 1-3 using well characterized sera from
cashew allergic patients. For comparison, IgE cross-reactivity between the three hazelnut allergens was assessed using sera from hazelnut allergic patients.

METHODS

Patients’ sera
In this study, sera from 10 cashew nut and 10 hazelnut allergic patients were used (see Table I). Sera were collected from patients with a convincing history of cashew nut or hazelnut allergy or a positive double-blind, placebo-controlled food challenge (DBPCFC) to cashew nut or hazelnut plus sIgE ≥ 0.35 kU/L. DBPCFCs were performed at the Sean N. Parker Center for Allergy and Asthma Research at Stanford University using a standardized methodology according to validated guidelines [32, 33] and as previously described [34]. As a negative control group, 4 sera from atopic patients without a history of food allergy and negative sIgE were used. Total IgE levels were measured by ALEX (Macro Array Diagnostics GmbH, Austria) and allergen-specific IgE levels by quantitative IgE ELISA [35]. The use of clinical data and serum samples for this study was approved by the local ethics committees (Stanford Institutional Review Board, the Ethics Committee of the Medical University of Vienna and the Ethics Committee of the Medical University of Graz) and signed informed consent was obtained from all patients.

Purification and identification of cashew nut and hazelnut allergens
Purification and identification of cashew nut (Ana o 1-3) and hazelnut allergens (Cor a 9, 11, and 14) as well as recombinant Ana o 3 and Cora 14 is described in Methods section and Fig. S1A in the Supplement.

Dose-dependent IgE inhibition ELISA assay
Dose-dependent IgE inhibition ELISA assays with the three cashew nut allergens as well as with hazelnut allergens were performed as described in the Supplement. To evaluate the nature of the epitopes involved in the cross-reactivity reduction and alkylation of Ana o 2, Ana o 3 and Cor a 9 were performed as described in the Supplement.

IgE immunoblots and inhibitions
The IgE binding as well as cross-reactivity were analyzed by immunoblotting and inhibitions using a human-derived monoclonal anti-Ana o 3 IgE antibody (Indoor Biotechnologies Ltd, Cardiff, UK) as well as a pool of sera from cashew allergic patients as described in Supplementary material.

Identification of potentially cross-reactive peptides
For identification of potentially cross-reactive peptides, the mature protein sequence of Ana o 3 was compared with the mature sequences of Ana o 1, 2 and Cor a 9. The mature sequence of Cor a 14 was compared with Cor a 9, 11, and Ana o 3. The sequence comparisons were performed as previously described for peanut allergens Ara h 1-3[28] with some modifications which are provided in Supplementary material.
RESULTS

Cashew nut allergic patients show high IgE co-reactivity to Ana o 1-3
Using 10 sera from cashew nut allergic patients (Table 1), IgE co-reactivity to Ana o 1, 2, and 3 was observed with statistically significant Pearson’s correlation coefficients between 0.87 and 0.98 (p < 0.001 and p < 0.0001) (Fig. 1A). As a control group, 10 sera from hazelnut allergic patients were tested for IgE reactivity to hazelnut extract and the three hazelnut allergens (Table 2 and Fig. 1B). All patients were sensitized to Cor a 9 and nine had sIgE to Cor a 14. Five patients were co-sensitized to Cor a 9, 11, and 14. A statistically significant Pearson’s correlation coefficient of 0.91 (p = 0.004) was only found for the correlation between sIgE to Cor a 9 and 11. The correlation between IgE to Cor a 11 and 14 was 0.24, and between Cor a 14 and 9 0.05.

The three non-homologous cashew nut allergens are highly cross-reactive among each other
To test the cross-reactivity between the three cashew nut allergens, dose-dependent IgE inhibitions were performed with 10 individual sera from cashew nut allergic patients (Fig. 2, 3, and Fig. S2 in Supplement). Pre-incubation of the sera with Ana o 3 at a concentration of 10 µg/ml reduced IgE binding to Ana o 1 by a median of 99% (range: 87-100%) and to Ana o 2 by 90% (range: 51-100%). Similarly, IgE binding to Ana o 1 and 3 was inhibited by Ana o 2 by 96-100% (median: 99%) and 82-100% (median: 94%) (Fig. 2A). Ana o 1 inhibited IgE binding to Ana o 2 by 54-96% (median: 84%) and to Ana o 3 by 64-100% (median: 86%).

Among the three allergens, Ana o 3 was the most potent in reducing IgE binding to Ana o 1, 2 and itself, with median half maximal inhibitory concentrations (IC 50 ) of ≤ 0.01 µg/ml (Fig. 2C). Ana o 2 was the second most potent cashew nut allergen in inhibiting IgE binding to Ana o 1 and 3 with median IC 50 values of 0.01 and 0.025 µg/ml, respectively. In contrast, Ana o 1 inhibited IgE binding to Ana o 2 with a median IC 50 value of 0.13 µg/ml, and to Ana o 3 with 0.16 µg/ml.

As control inhibitors the homologous hazelnut allergens, Cor a 9, 11, and 14 were selected due to their high amino acid sequence identities (Table S2) to the three cashew allergens. However no inhibition was observed between hazelnut vicilin, Cor a 11, and its homologue from cashew nut, Ana o 1, as well as between the 2S albumins Cor a 14 and Ana o 3 (Fig. 2 and 3). Interestingly, Cor a 9 reduced IgE binding to Ana o 1-3 at a concentration of 10 µg/ml by 75%, 56%, and 48% (Fig. 2 and 3). However, those maximal inhibitions were reached only with median IC 50 values of 1.3 µg/ml for Ana o 1 and 2 as well as 1.7 µg/ml for Ana o 3.

The cross-reactivity of the three cashew nut allergens was also confirmed by IgE-immunoblot inhibitions. As visualized in Figure S1B, pre-incubation of the serum pool with Ana o 1, 2, or 3 completely abolished IgE binding to all three allergens.

To test the contribution of conformational epitopes in the cross-reactivity Ana o 3, Ana o 2 as well as Cor a 9 were reduced and alkylated (RA) to destroy disulfide bridges and consequently the
intact conformation of the proteins. Pre-incubation of the five representative sera with RA Ana o 3 at a concentration of 10 µg/ml reduced IgE binding to Ana o 1 by a median of 73% and to Ana o 2 by 38% (range: 0-21%). RA Ana o 2 inhibited 21% (range: 9-41%) of IgE binding to Ana o 1, and 4% (range: 0-21%) to Ana o 2. Similarly, compared to native Cor a 9, RA Cor a 9 showed very low inhibitions of IgE binding to Ana o 1-3 (Figure S3).

The three non-homologous hazelnut allergens show moderate cross-reactivity among each other

In contrast to cashew nut allergens, not only co-reactivity but also the extent of cross-reactivity between the three hazelnut allergens varied greatly (Fig. 4, 5 and Fig. S4). Three sera (HA5, HA8, and HA9) showed high content of cross-reactive IgE specific to all three allergens (Fig. 5). For the other seven sera the extent of cross-reactivity was lower, especially at an inhibitor concentration of 1 µg/ml (Fig. 4B and Fig. 5). Overall, the most potent inhibitor was Cor a 14 with median of inhibition of IgE binding to Cor a 11 of 62%, and to Cor a 9 of 43% (Fig. 4A). Median IC50 values were in both cases 0.01 µg/ml for self-inhibition with a median inhibition of 98% (Fig. 4C). Although similar medians of inhibition values to Cor a 11 (61%) and to Cor a 14 (62%) were reached using Cor a 9 as inhibitor, the median IC50 concentrations of 0.58 µg/ml and 0.68 µg/ml were more than 50-fold higher compared to the inhibitions with Cor a 14.

In general, low percentages of IgE inhibitions to homologous and to non-homologous hazelnut allergens were observed after preincubation of sera with cashew nut allergens Ana o 1-3 (Fig. 5).

Conformation of the cross-reactivity using recombinant allergens and a monoclonal anti-Ana o 3 antibody

To exclude that cross-reactivity was result of impurities in the allergen preparations, IgE-binding inhibition values of Ana o 3 and Cor a 14 to the three cashew and hazelnut allergens were compared with their recombinant counterparts. No significant differences (Wilcoxon matched-pairs test; p=0.1094) of inhibition values to Ana o 1 and 3 was observed when rAna o 3 was used as inhibitor compared to Ana o 3 (Fig. 2A and 3). A small but statistically significant difference (p=0.0273) in the IgE inhibition to Ana o 2 was observed. However, the inhibitions of IgE binding by rAna o 3 to Ana o 2 was still high with a median percentage of inhibition of 82% compared with inhibition of nAna o 3 by 94%. Likewise, no significant differences between recombinant and natural Cor a 14 in their abilities to inhibit IgE-binding to the three hazelnut allergens was found (Fig. 4A and 5).

Furthermore, the high purity of the isolated natural allergens was confirmed by immunoblotting using an Ana o 3-specific monoclonal IgE antibody derived from a cashew allergic patient (Fig. S1C). The purified Ana o 3 migrating near the 10 kDa marker was strongly recognized by the antibody. No Ana o 3 either under reducing or non-reducing conditions could be detected in the
Ana o 1 and Ana o 2 samples. However, similar to IgE from sera from cashew allergic patients, the Ana o 3-specific antibody showed, cross-reactivity to Ana o 1 and 2. Strong signals to both, Ana o 1 at about 50 kDa and Ana o 2 at about 55 kDa, were obtained by analysis under non-reducing conditions and under reducing conditions where binding to the 30 kDa acidic and 20 kDa basic Ana o 2 subunits was visible.

Interestingly, the Ana o 3-specific antibody showed cross-reactivity to hazelnut legumin Cor a 9, but not to the hazelnut 2S albumin Cor a 14 and vicilin Cor a 11. The binding of the antibody to all 4 allergens could be inhibited by rAna o 3 confirming the specificity of the antibody.

**Identification of potentially cross-reactive peptides**

Sequence alignments using EMBOSS Needle revealed significant identities only between homologous allergens from cashew nut and hazelnut (Table S1).

Using a search for similar short peptides, a comparison of Ana o 1 and 2 with Ana o 3 yielded two regions on the Ana o 3 sequence, that matched similar peptides on Ana o 1 or 2 (Fig. S5). Ana o 3 amino acid residues 40-49 located on the surface exposed N-terminal helices 1 and 2 matched four Ana o 1 peptides, whereas the loop region (residues 101-108) connecting helices 5 and 6 was similar to one peptide of Ana o 2. All matching peptides from Ana o 3 were located on the surface and have been previously identified as important IgE binding regions (Fig. S5) [22]. No peptides sharing significant sequence similarity were identified by comparison of Ana o 3 and its hazelnut homologue Cor a 9. Using the same method, only one peptide of Cor a 14 was found that matched the sequence of Cor a 11 and no peptide that matched the sequence of Cor a 9.
Discussion

Cashew nut has been reported to cause strong allergic reactions, even exceeding those observed for peanut, suggesting a high potency of its allergens. However, there is little evidence to explain the nature of cashew nut allergenicity that could inform studies to identify targets to treat cashew nut allergy.

Our findings suggest that strong IgE co-reactivity to the three unrelated major cashew nut allergens Ana o 1-3 observed in our and in a previous study[26] is due to high cross-reactivity among them. Similar to peanut, the high percentages of IgE cross-inhibitions between the three allergens indicate that cross-reactive IgE antibodies comprise the major portion of IgE specific to these allergens and might contribute to the high specific IgE values to each of the three allergens. The affinity of these IgE antibodies was highest for the 2S albumin, Ana o 3. This result could explain findings of several studies reporting Ana o 3 as a predictive marker of clinical reactivity to cashew nut [23-25]. Similarly, previous studies showing cross-reactivity of the three non-homologous peanut allergens [28] and evidence of cross-reactive IgE antibodies [29] observed the highest affinity of cross-reactive IgE to the 2S albumin Ara h 2, which was confirmed as a predictor of clinical reactivity to peanut [36, 37]. Interestingly, in vivo [38, 39] and in vitro [40, 41] studies on peanut allergens showed that treatment with a single allergen or a single allergen-specific antibody induce protection against the peanut extract consisting of multiple allergens. Based on the data achieved with peanut allergens and our present results, we could hypothesize that one cashew allergen might be sufficient for successful immunotherapy in cashew allergic patients.

Furthermore, we showed that IgE cross-reactivity among unrelated allergens from the same nut occurs also in hazelnut but to a lesser extent compared to cashew nut and peanut. The cross-reactivity was only high in patients co-sensitized to all three hazelnut allergens and appears not to be associated with sensitization to cashew allergens. Co-sensitization and cross-reactivity was strongest between Cor a 9 and 14, however similar to peanut and cashew nut the highest IgE affinity was seen for the 2S albumin Cor a 14. For hazelnut, it was shown that sIgE to Cor a 9 had a diagnostic value comparable to sIgE to Cor a 14, and that sensitization to both allergens is associated with a more severe hazelnut allergic phenotype [42, 43], however Cor a 14 was shown to be the most potent hazelnut allergen in basophil activation assays [43].

It is a commonly held view that cross-reactivity relies on highly similar primary and tertiary structures between allergens and is hence generally observed between members of the same protein family. Consequently, frequent co-sensitization of peanut and/or tree nut allergic individuals to multiple nuts and seeds and their extensive in vitro IgE cross-reactivity have been interpreted by cross-reactive epitopes present in homologous allergens from the vicilin, legumin, or 2S albumin protein families [44-46]. Among cashew nut allergic patients, co-sensitization was observed to pistachio, peanut, hazelnut, and almond [3, 8, 16, 47]. Yet, in the majority of cases, the identity of the cross-reactive allergens was not investigated. Strong clinically relevant co-sensitization was
found only between cashew nut and the botanically related pistachio [25, 48], which could be explained by the high sequence identities (≥70%) between their homologous allergens.

As a control, in our study we used the three homologous allergens from hazelnut for inhibition of IgE binding to cashew nut allergens in the cashew nut allergic group and the three cashew nut allergens in the hazelnut allergic group. We did not find cross-reactivity between cashew and hazelnut 2S albumins and vicilins. Interestingly, at the highest inhibitor concentration, the hazelnut legumin, Cor a 9, could moderately inhibit not only IgE-binding to the cashew nut legumin, Ana o 2, which is not unexpected considering a sequence identity of 54%, but also to the non-homologues cashew nut vicilin, Ana o 1, and the 2S albumin, Ana o 3. This indicates that binding of cross-reactive IgE to Cor a 9 might be responsible for the previously observed cross-reactivity between cashew nut and hazelnut, where hazelnut extract was a strong inhibitor of cashew nut sIgE, while cashew nut extract was less able to inhibit IgE binding to hazelnut extract [47, 49].

This is also in line with our results showing that low or no inhibition of IgE binding to hazelnut allergens was achieved by pre-incubation of sera from hazelnut allergic patients with individual cashew nut allergens. The differences between cashew and hazelnut allergic patients may be explained by different extents of cross-reactivity of IgE, depending on the sensitizing allergen. Cashew-allergic patients are primarily sensitized by Ana o 3, which induces highly cross-reactive IgE that also recognize Cor a 9. In contrast, hazelnut allergic patients, primarily sensitized to Cor a 9, develop IgE with low cross-reactivity. Thus, our study highlights the importance of using purified individual allergens instead of total protein extracts to correctly assess cross-reactivity and commonly observed co-sensitization to diverse tree nuts.

Using recombinant Ana o 3 and Cor a 14 allergens and an Ana o 3-specific monoclonal antibody we showed that the observed high cross-reactivity among the unrelated cashew nut and hazelnut allergens is not due to contamination of each allergen preparation by the other allergens. Recombinant Ana o 3 and rCor a 14 and therefore not contaminated with other nut proteins, showed no significant differences in inhibiting the IgE compared to their natural counterparts. Furthermore, neither sera of patients with cashew allergy nor the Ana o 3-specific monoclonal antibody detected other allergens in the lanes containing each purified allergen. Besides, the Ana o 3-specific antibody isolated from a cashew nut allergic patient showed the same pattern of cross-reactivity as IgE in sera of cashew nut allergic subjects used in our study.

Our present and previous findings[28] showing that cross-reactivity occurs between unrelated seed storage proteins further emphasize that protein sequence identity alone cannot accurately predict cross-reactivity. Using an approach searching for short matching peptides we previously identified cross-reactive IgE epitopes of the unrelated peanut allergens Ara h 1-3 [28]. By a similar approach, we identified potential epitopes on Ana o 3 with sequence similarities to peptides from Ana o 1 and 2 that could be recognized by cross-reactive antibodies. This assumption is supported by the fact that the identified peptides overlap peptides which have previously been reported to bind IgE
from the majority of cashew nut allergic subjects [20-22]. The adjacent location of the surface-exposed patches presenting the two herein identified Ana o 3 peptides indicates that they could be part of a conformational epitope. Indeed, our experimental data showed that the native structure of Ana o 3, but especially of Ana o 2 and Cor a 9 is substantial for binding of the cross-reactive IgE, since their disruption strongly reduced or eliminated the inhibitory potency of the three allergens. Various studies on the three cashew nut allergens have shown that their epitopes are conformational and depend on the three-dimensional structure of the protein [50].

In summary, our results indicate the presence of IgE with high affinity to Ana o 3 that cross-reacts with the two other unrelated major cashew nut allergens. Although this needs to be demonstrated, it is highly possible that such cross-reactive antibodies with high affinity are particularly potent at inducing allergen aggregation and IgE-mediated mast cell activation and might therefore be responsible for the high allergenic potency of cashew nut.

This substantial new information on the cross-reactivity between unrelated cashew nut and hazelnut allergens may have important consequences for diagnosis and immunotherapy not only of cashew nut but also of other tree nut allergies.
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Conflicts of interest statement:
Sharon Chinthrajah reports grants from NIAID, CoFAR, Aimmune, DBV Technologies, Astellas, Regeneron, FARE, MCHRI and is an advisory board member for Alladapt Therapeutics, Novartis, Genetech, Sanofi, Allergenis, and Nutricia. Sayantani B. Sindher reports grants from NIH, grants from Regeneron, grants from DBV Technologies, grants from AIMMUNE, grants from Novartis, grants from CoFAR, grants and personal fees from FARE, other from Astra Zeneca, other from DBV, outside the submitted work; Kari Nadeau reports grants from National Institute of Allergy and Infectious Diseases (NIAID), National Heart, Lung, and Blood Institute (NHLBI), National Institute of Environmental Health Sciences (NIEHS), and Food Allergy Research & Education (FARE); stock options from IgGenix, Seed Health, ClostraBio, and ImmuneID; is Director of World Allergy Organization (WAO), Advisor at Cour Pharma, Consultant for Excellergy, Red tree ventures, Eli Lilly, and Phylaxis, Co-founder of Before Brands, Alladapt, Latitude, and IgGenix; and National Scientific Committee member at Immune Tolerance Network (ITN), and National Institutes of Health (NIH) clinical research centers, outside the submitted work; patents include, “Mixed allergen composition and methods for using the same”, “Granulocyte-based methods for detecting and monitoring immune system disorders”, “Methods and Assays for Detecting and Quantifying Pure Subpopulations of White Blood Cells in Immune System Disorders” and “Methods of isolating allergen-specific antibodies from humans and uses thereof”. The rest of the authors declare that they have no relevant conflict of interest related to this study.
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Table 1. Clinical characteristics and sIgE levels to cashew nut extract and allergens (Ana o 1, 2, 3) of 10 cashew nut allergic patients used for the study.

| Patient no. | Age (y) | Sex | Allergy confirmed by | Cashew nut-related symptoms | Other nut allergies | Total IgE (kU/L) | qELISA sIgE (kU/L) |
|-------------|---------|-----|----------------------|-----------------------------|--------------------|----------------|------------------|
|             |         |     |                      | Cashew nut                  | Ana o 1            | Ana o 2         | Ana o 3          | Hazelnut         | Cor a 11 | Cor a 9 | Cor a 14 |
| CA1         | 4       | M   | Positive history     | Anaphylaxis                 | None               | 360            | >100            | >100            | >100     | 2.34    | <0.35   | 1.54   | 6.41   |
| CA2         | 6       | F   | Positive history     | Urticaria, vomiting         | Hazelnut           | 1324           | >100            | >100            | >100     | >100    | 13.91   | 93.54  | >100    |
| CA3         | 11      | F   | DBPCFC               | Wheezing                    | Peanut, almond, hazelnut, walnut | 956            | >100            | >100            | >100     | 83.42   | 18.83   | 66.14  | 2.83   |
| CA4         | 6       | M   | DBPCFC               | Wheezing                    | Peanut, almond, hazelnut, walnut | 652            | >100            | >100            | >100     | 24.68   | <0.35   | 16.73  | 15.77  |
| CA5         | 8       | M   | DBPCFC               | Cough                       | Peanut, almond, hazelnut, walnut | 1355           | >100            | >100            | >100     | 99.23   | 4.40    | <0.35  | <0.35  |
| CA6         | 5       | M   | Positive history     | Urticaria                   | Peanut, hazelnut   | 317            | 49.15           | 56.30           | 65.74    | 51.74   | 2.05    | <0.35  | <0.35  | 1.66   |
| CA7         | 4       | M   | Positive history     | Anaphylaxis                 | Peanut             | 87             | 36.63           | 29.43           | 48.77    | 27.04   | 1.61    | <0.35  | 1.45   | <0.35  |
| CA8         | 5       | F   | Positive history     | Urticaria, angioedema, eczema| Hazelnut, walnut, pistachio, macadamia | 374            | >100            | >100            | >100     | >100    | 17.32   | 27.17  | >100    |
| CA9         | 4       | M   | Positive history     | Rash, vomiting, angioedema  | None               | 227            | >100            | 52.07           | >100     | 37.42   | 2.93    | 11.92  | 0.83   | 3.92   |
| CA10        | 3       | M   | Positive history     | Rash, urticaria, cough      | None               | ND             | 62.93           | >100            | 91.73    | <0.35   | <0.35   | <0.35  | 1.83   |

Median: 374, 128.54, 150.94, 170.38, 154.82, 4.40, 15.62, 9.80, 5.17
Range: 87-1355, 37-850, 29-837, 49-1199, 27-624, 2-155, 12-19, 1-94, 2-141
Table 2. Clinical characteristics and sIgE levels to hazelnut extract and allergens (Cor a 9, 11, 14) of 10 hazelnut allergic patients used for the study.

| Patient no. | Age (y) | Sex | Allergy confirmed by | Hazelnut-related symptoms | Other nut allergies | Total IgE (kU/L) | qELISA sIgE (kU/L) |
|-------------|---------|-----|----------------------|--------------------------|---------------------|----------------|-------------------|
|             |         |     |                      |                          |                     | Hazelnut | Cor a 11 | Cor a 9 | Cor a 14 | Ana o 3 |
| HA1         | 33      | F   | Positive history     | Anaphylaxis              | All tree nuts (not almond) | 1982     | 38.62   | 7.53    | 20.59   | 24.9    | 16.63   |
| HA2         | 27      | F   | DBPCFC               | Anaphylaxis              | Peanut, walnut         | 1470     | 24.00   | <0.35   | 7.90    | 9.56    | 24.10   |
| HA3         | 7       | M   | Positive history     | Dyspnoe                  | Peanut, walnut         | 2119     | 75.37   | 6.63    | 59.42   | 14.18   | 59.92   |
| HA4         | 2       | M   | Positive history     | Angioedema               | None                  | 68       | 28.85   | <0.35   | 3.00    | 32.11   | <0.35   |
| HA5         | 34      | F   | Positive history     | Diarrhea, vomiting, angioedema, rash | Peanut              | 864      | 20.96   | 2.95    | 4.88    | 15.18   | <0.35   |
| HA6         | 1       | M   | Positive history     | Angioedema, dyspnoe, stridor | Peanut, walnut, almond | ND       | 56.58   | <0.35   | 3.50    | 92.91   | ND      |
| HA7         | 27      | F   | Positive history     | Angioedema, dyspnoe      | Almond, walnut        | ND       | 8.93    | <0.35   | 2.7     | 4.93    | <0.35   |
| HA8         | 10      | F   | DBPCFC               | Vomiting                 | Almond, cashew nut, walnut | 1249     | 87.67   | 13.05   | 23.83   | 84.26   | 31.42   |
| HA9         | 11      | F   | DBPCFC               | Vomiting                 | Peanut, almond, cashew nut, walnut | 956      | >100    | 32.29   | >100    | 38.58   | 29.26   |
| HA10        | 26      | F   | Positive history     | Angioedema               | Peanut, walnut, almond, pecan | 729      | 32.85   | <0.35   | 5.41    | <0.35   | <0.35   |

Median: 1103 35.74 7.53 5.41 24.90 29.26
Range: 68-2119 9-173 3-32 3-130 5-93 17-60

ND: Not determined.
**Figure 1.** Correlation of specific IgE to cashew nut and hazelnut allergens using sera from (A) cashew nut and (B) hazelnut allergic patients. Negative values were set to 0.01. Pearson’s correlation coefficients (r values) and p values were calculated using GraphPad Prism.
Figure 2. IgE cross-reactivity between cashew nut allergens Ana o 1, Ana o 2, and Ana o 3 tested using an IgE ELISA inhibition assay. Cross-inhibitions were tested in a dose-dependent manner with sera from patients with cashew nut allergy (n=10). (A) Values are expressed as maximum inhibition at 10 µg/ml (B) Values represent inhibition at 1 µg/ml of inhibitor. Recombinant Ana o 3 and Bet v 1 were used for inhibitions as additional control allergens only at a concentration of 10 µg/ml. (C) Inhibitor concentrations for half-maximal inhibition (IC₅₀) with interquartile ranges. Inhib.: Inhibitor. Median values (solid lines) with interquartile ranges are shown.
**Figure 3.** Summary heat map of percent IgE binding inhibition to cashew nut allergens at inhibitor concentrations of 1 µg/ml and 10 µg/ml.

**Inhibition of IgE binding to cashew allergens**

| Inhibitor (1 µg/ml) | Ana o 1 | Ana o 2 | Ana o 3 | Cor a 11 | Cor a 9 | Cor a 14 | Betv 1 |
|---------------------|---------|---------|---------|----------|---------|---------|--------|
| CA1                 | 92      | 100     | 100     | 4        | 11      | 2       |        |
| CA2                 | 69      | 96      | 98      | 6        | 26      | 33      |        |
| CA3                 | 90      | 92      | 81      | 0        | 18      | 1       |        |
| CA4                 | 86      | 91      | 89      | 1        | 47      | 0       |        |
| CA5                 | 90      | 99      | 98      | 2        | 33      | 0       |        |
| CA6                 | 100     | 100     | 100     | 3        | 33      | 1       |        |
| CA7                 | 70      | 100     | 97      | 4        | 43      | 0       |        |
| CA8                 | 97      | 97      | 97      | 37       | 37      | 100     |        |
| CA9                 | 94      | 99      | 99      | 0        | 29      | 2       |        |
| CA10                | 95      | 99      | 99      | 0        | 8       | 0       |        |

| Inhibitor (10 µg/ml) | Ana o 1 | Ana o 2 | Ana o 3 | Cor a 11 | Cor a 9 | Cor a 14 | Betv 1 |
|----------------------|---------|---------|---------|----------|---------|---------|--------|
| CA1                  | 100     | 100     | 95      | 10       | 27      | 11      | 3      |
| CA2                  | 99      | 100     | 98      | 8        | 19      | 51      | 49     |
| CA3                  | 96      | 96      | 87      | nd       | 10      | 60      | 2      |
| CA4                  | 100     | 100     | 100     | 95       | 9       | 55      | 5      |
| CA5                  | 98      | 99      | 99      | 100      | 13      | 74      | 3      |
| CA6                  | 99      | 100     | 99      | 98       | 10      | 70      | 11     |
| CA7                  | 95      | 99      | 98      | 74       | 9       | 100     | 5      |
| CA8                  | 98      | 99      | 99      | 98       | 100     | 91      | 100    |
| CA9                  | 100     | 100     | 100     | 100      | 3       | 86      | 0      |
| CA10                 | 95      | 99      | 99      | 100      | 1       | 8       | 0      |

% IgE inhibition to Ana o 1

| Inhibitor (1 µg/ml) | Ana o 1 | Ana o 2 | Ana o 3 | Cor a 11 | Cor a 9 | Cor a 14 | Betv 1 |
|---------------------|---------|---------|---------|----------|---------|---------|--------|
| CA1                 | 70      | 91      | 86      | 2        | 9       | 1       |        |
| CA2                 | 68      | 95      | 82      | 1        | 26      | 19      |        |
| CA3                 | 39      | 90      | 46      | 0        | 21      | 0       |        |
| CA4                 | 58      | 96      | 80      | 0        | 37      | 0       |        |
| CA5                 | 78      | 93      | 80      | 3        | 26      | 0       |        |
| CA6                 | 85      | 100     | 93      | 3        | 26      | 4       |        |
| CA7                 | 60      | 96      | 69      | 5        | 27      | 0       |        |
| CA8                 | 42      | 93      | 46      | 37       | 29      | 90      |        |
| CA9                 | 83      | 95      | 91      | 7        | 23      | 5       |        |
| CA10                | 86      | 97      | 95      | 6        | 7       | 5       |        |

% IgE inhibition to Ana o 2

| Inhibitor (1 µg/ml) | Ana o 1 | Ana o 2 | Ana o 3 | Cor a 11 | Cor a 9 | Cor a 14 | Betv 1 |
|---------------------|---------|---------|---------|----------|---------|---------|--------|
| CA1                 | 84      | 97      | 97      | 80       | 9       | 25      | 3      |
| CA2                 | 78      | 98      | 78      | 60       | 10      | 44      | 31     |
| CA3                 | 54      | 98      | 51      | nd       | 7       | 44      | 0      |
| CA4                 | 84      | 99      | 79      | 57       | 2       | 53      | 0      |
| CA5                 | 90      | 98      | 94      | 91       | 10      | 61      | 2      |
| CA6                 | 95      | 100     | 94      | 82       | 6       | 60      | 5      |
| CA7                 | 79      | 99      | 75      | 59       | 6       | 50      | 0      |
| CA8                 | 82      | 99      | 87      | 100      | 96      | 100     | 98     |
| CA9                 | 91      | 98      | 94      | 85       | 6       | 83      | 1      |
| CA10                | 96      | 100     | 96      | 90       | 7       | 77      | 10     |

% IgE inhibition to Ana o 3

| Inhibitor (1 µg/ml) | Ana o 1 | Ana o 2 | Ana o 3 | Cor a 11 | Cor a 9 | Cor a 14 | Betv 1 |
|---------------------|---------|---------|---------|----------|---------|---------|--------|
| CA1                 | 64      | 82      | 82      | 81       | 13      | 9       | 3      |
| CA2                 | 80      | 90      | 88      | 65       | 11      | 24      | 24     |
| CA3                 | 83      | 91      | 93      | nd       | 9       | 47      | 0      |
| CA4                 | 100     | 100     | 100     | 93       | 4       | 71      | 0      |
| CA5                 | 90      | 100     | 99      | 92       | 8       | 50      | 5      |
| CA6                 | 74      | 86      | 79      | 73       | 4       | 46      | 1      |
| CA7                 | 89      | 98      | 94      | 65       | 8       | 45      | 4      |
| CA8                 | 89      | 100     | 94      | 100      | 99      | 100     | 20     |
| CA9                 | 81      | 95      | 95      | 100      | 8       | 68      | 0      |
| CA10                | 92      | 98      | 99      | 94       | 11      | 64      | 6      |
Figure 4. IgE cross-reactivity between hazelnut allergens Cor a 11, Cor a 9, and Cor a 14 tested using an IgE ELISA inhibition assay. Cross-inhibitions were tested in a dose-dependent manner with sera from patients (n=10) with hazelnut allergy. (A) Values are expressed as maximum inhibition at 10 µg/ml and (B) values represent inhibitions at 1 µg/ml of inhibitor. Recombinant Cor a 14 and Bet v 1 were used for inhibitions as additional control allergens only in a concentration of 10 µg/ml. (C) IC_{50} with interquartile ranges. Due to negligible inhibition of hazelnut allergens by Ana o 3, IC_{50} values were excluded from the graph. Inhib.: Inhibitor. Median values (solid lines) with interquartile ranges are shown.
**Figure 5.** Summary heat map of percent IgE binding inhibition to hazelnut allergens at inhibitor concentrations of 1 µg/ml and 10 µg/ml.