

Original Article

Association of Clinical Reactivity with Sensitization to Allergen Components in Multifood-Allergic Children

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What is already known about this topic? It is estimated that 6% to 8% of children suffer from food allergy, and of these, 30% are multifood allergic. Multifood-allergic children frequently suffer from nut allergies and can exhibit varied reactions to their food allergens.

What does this article add to our knowledge? This study of multifood-allergic children is the largest and most comprehensive of its kind, providing a systematic analysis of associations among different food allergies based on clinical double-blind, placebo-controlled food challenge as well as serological (IgE levels) data.

How does this study impact current management guidelines? Cashew/pistachio and walnut/pecan/hazelnut allergies show significant clinical associations, suggesting that this be considered when giving avoidance advice.

BACKGROUND: Thirty percent of children with food allergies have multiple simultaneous allergies; however, the features of these multiple allergies are not well characterized serologically or clinically.

OBJECTIVE: We comprehensively evaluated 60 multifood-allergic patients by measuring serum IgE to key allergen components, evaluating clinical histories and medication use, performing skin tests, and conducting double-blind, placebo-controlled food challenges (DBPCFCs).

METHODS: Sixty participants with multiple food allergies were characterized by clinical history, DBPCFCs, total IgE, specific IgE, and component-resolved diagnostics (IgE and IgG4) data. The food allergens tested were almond, egg, milk, sesame, peanut, pecan, walnut, hazelnut, cashew, pistachio, soy, and wheat.

RESULTS: Our data demonstrate that of the reactions observed during a graded DBPCFC, gastrointestinal reactions occurred more often in boys than in girls, as well as in individuals with high levels of IgE to 2S albumins from cashew, walnut, and hazelnut. Certain food allergies often occurred concomitantly in individuals (ie, cashew/pistachio and walnut/pecan/hazelnut).

IgE testing to components further corroborated serological relationships between and among these clustered food allergies.

CONCLUSIONS: Associations of certain food allergies were shown by DBPCFC outcomes as well as by correlations in IgE reactivity to structurally related food allergen components. Each of these criteria independently demonstrated a significant association between allergies to cashew and pistachio, as well as among allergies to walnut, pecan, and hazelnut. © 2017 American Academy of Allergy, Asthma & Immunology (J Allergy Clin Immunol Pract 2017;■:■-■)

Key words: Multifood allergy; Component-resolved testing; IgE; Double-blind, placebo-controlled food challenge

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It has been estimated that approximately 8% of children suffer from food allergy, and of those, 30% are allergic to multiple

Abbreviations used

au-Approximately unbiased
 CRD-Component-resolved diagnostics
 DBPCFC-Double-blind, placebo-controlled food challenge
 FDR-False discovery rate
 GI-Gastrointestinal
 LTP-Lipid transfer protein
 sIgE-Specific IgE
 SPT-Skin prick test

foods.^{1,2} However, little information is available regarding the clinical and serological characteristics associated with multiple food allergies, and such information could advance our understanding of phenotypes and endotypes, respectively, of food allergy.³ Clinically, it has been well recognized that certain food allergies occur concurrently. For example, studies have indicated that approximately 92% of patients with cow's milk allergy are also allergic to goat's milk,⁴ and individuals with shrimp allergy are often allergic to other shellfish. Among tree nuts, allergies to pistachios have been found more commonly in individuals with cashew nut allergy⁵ and individuals with walnut allergy are often allergic to pecan nuts.⁶ Studies by Goetz et al⁷ and Masthoff et al⁸ found that there was little cross-reactivity between proteins found in peanuts and tree nuts suggesting that concurrent sensitization between these foods is likely due to independent sensitization to both peanuts and tree nuts. Clinical associations between allergens are more commonly seen among allergens that are closely related botanically like tree nuts⁷; however, links between allergens from very different taxonomical groups have also been observed.⁹ Many foods that are closely related share homologous proteins, and sensitization to one food is therefore likely to result in positive tests or clinical reactivity to related foods. In addition, sensitization to proteins that are particularly highly conserved can result in associated allergies to foods that are not closely related. Therefore, we set out to test if individuals with multifood allergies have clinically relevant food allergies to proteins belonging to similar food categories and/or to similar protein classes.

Understanding the patterns of cross-reactivity between and among foods may aid the clinician in evaluating the risk of food reactions as well as reactions to multiple foods. Moreover, the application of food allergy diagnostics, using knowledge of relevant protein relationships, along with clinical history, could enable more precise allergen avoidance strategies to avoid unnecessarily eliminating entire food groups from the diet. Also, recent data indicate that multifood-allergic patients carry a higher risk for developing asthma and other comorbid conditions compared with those with single-food allergies, which further underscores the need to test relationships of cross-reactivity in multifood-allergic patients.¹⁰

There has been great interest in developing novel *in vitro* tests that can more accurately predict food allergies. One of these approaches is based on component-resolved diagnostics (CRD) in which native or recombinant allergens are used to test IgE sensitivity to individual allergen proteins.¹¹ At the present time, CRD tests are mainly performed in research settings. However, Food and Drug Administration-approved milk, egg, peanut, and tree nut CRD tests are available for clinical use in the USA.

In this study, our goal was to comprehensively evaluate the characteristics of patients with multiple food allergies. We focused

TABLE I. Demographics

Number participants	60
Female, n (%)	30 (50%)
Age, y (median, range)	8 (4-15)
With atopic dermatitis, n (%)	46 (77%)
With allergic rhinitis, n (%)	44 (73%)
With asthma, n (%)	30 (50%)
DBPCFCs performed	311
Positive DBPCFCs, n (%)	273 (88%)
No. of DBPCFCs performed for participant (one per food) (median, range)	5 (2-8)

DBPCFC, Double-blind, placebo-controlled food challenge.

especially on the association of multifood allergies with the phenotype (for our study, clinical symptoms during a double-blind, placebo-controlled food challenge [DBPCFC] and comorbidities) and endotype (for our study, the component IgE levels) of the participants. We analyzed the baseline data of a study in which the participants were screened and eligible only if they had a high likelihood of reactions to more than one food allergen in separate DBPCFCs. Accordingly, the study was not aimed at evaluating the ability of new diagnostic tools to predict negative versus positive reactions to DBPCFCs. Instead, we focused on testing whether there were associations between the extent and type of food challenge reactions and relatedness of allergen proteins. To our knowledge, this is the first study that systematically investigates multiallergic participants with the aim of identifying associations among food challenge outcomes, component testing, and levels of whole allergen-specific IgE (sIgE).

METHODS

The protocol for this study was reviewed and approved by the Institutional Review Board of Stanford University.

Study population

Sixty pediatric participants allergic to multiple foods were included in this study. Their demographic characteristics are summarized in Table I. Information about participant selection (screening criteria, DBPCFC, skin prick test [SPT], food flours) can be found in this article's Online Repository at www.jaci-inpractice.org.

Antibody measurements

Total IgE and allergen-sIgE and IgG4 antibody concentrations were determined using the ImmunoCAP 250 assay (Thermo Fisher Scientific, Portage, Mich). Antibodies to the following foods and allergen components were measured: peanut (Ara h 1, Ara h 2, Ara h 3, Ara h 8, Ara h 9), hazelnut (Cor a 1, Cor a 8, Cor a 9, Cor a 14), walnut (Jug r 1, Jug r 3), cashew (Ana o 3), egg white (Gal d 1, Gal d 2, Gal d 3), cow's milk (Bos d 4, Bos d 5, Bos d 8), soy (Gly m 4, Gly m 5), wheat (Tri a 14, Tri a 19), and birch (Bet v 1).

Statistical analysis

Differences between nonparametric unpaired variables were assessed using a 2-sided Mann-Whitney *U* test. *P* values were adjusted for multiple comparisons using the approach by Benjamini and Hochberg¹² to control the false discovery rate (FDR) and the corrected values were noted as *Q* values.

To characterize the multiallergic character of the participants, for each allergen combination, the number of participants who were allergic against both allergens in DBPCFC tests was determined. To

take the different counts of allergic participants for the various allergens into account, the number of co-allergic participants was examined using the Jaccard similarity coefficient (Jaccard index).¹³ This coefficient measures the similarity between sample sets; in our case each set was the list of participants who were allergic against one of the allergens. The Jaccard similarity coefficient is calculated by dividing the size of the intersection of the sample sets by the size of the union between the sample sets. The result is a value between 0 and 1, with 1 being a perfect overlap between the sample sets and 0 meaning that the sample sets do not overlap at all. To determine the pairs of allergens against which participants were more often concurrently allergic than by random chance, a resampling permutation approach was applied. Keeping the count of DBPCFC-positive participants per allergens the same as present in the study, we permuted the DBPCFC-positive cases so that they get randomly assigned to the 60 participants. Each time we calculated for each allergen combination the Jaccard similarity coefficient and recorded the results. This way, for each allergen combination we estimated the null distribution for the case if participants would be randomly co-allergic against them. Afterward, for each allergen combination, the count of permutations that resulted in a Jaccard similarity coefficient greater than or equal to our original study data was determined and divided by 1000 (the number of permutations). These results were the *P* values that we adjusted for multiple comparisons.¹²

To evaluate the association between the IgE responses against different components, hierarchical clustering of IgE values against the components across all 60 participants was done using 1 minus the absolute Spearman's rank correlation as distance measure and complete linkage as the agglomeration method. To assess the uncertainty of the clusters in the hierarchical clustering procedure, the *pvcust* R package¹⁴ was used. This function uses a multiscale bootstrap resampling approach to assign a *P* value to each cluster. Approximately unbiased (au) *P* values, which are computed by multiscale bootstrap resampling, and bootstrap probability values were calculated and presented as percentages. The authors described that the au *P* value is a better approximation to the unbiased *P* value. Clusters with *Q* values (FDR adjusted *P* values) < .05 are highlighted by a red rectangle in the cluster dendrogram.

RESULTS

Multifood allergen testing by DBPCFC

The study examined a total of 12 different food allergens, and each participant underwent a DBPCFC for between 2 and 8 foods (Figure 1, A). Overall 311 DBPCFCs were performed, and the details of the food allergens, DBPCFC outcomes, as well as the sIgE and SPT results for positive DBPCFCs are presented in Table II. The number of positive DBPCFCs per allergen ranged from 46 (cashew) to 2 (soy and wheat) (Table II, diagonal in Figure 2, A). Participants were allergic (positive DBPCFC) to between 2 and 7 of the tested foods (Figure 1, A). Combinations of foods to which participants were allergic varied widely (Figure 1, B). Six participants were allergic to exactly the same food allergen combination of cashew, peanut, and pistachio; however, most combinations were unique to participants. Twenty-eight participants (47%) were allergic to a unique combination of food allergens. Figure 2(A) presents the number of participants being co-allergic to defined pairs of allergens. The number of participants who were allergic to a pair of food allergens does depend on the number of occurrences of the single allergies in this data set (eg, more than 4 times the number of

participants were allergic to pistachio than to almond). To adjust for this, Figure 2(B) presents a pairwise plot between the allergens, showing the Jaccard similarity coefficient. The greater this coefficient, the greater is the similarity between the lists of participants who were shown to be allergic to either of the 2 allergens. We used a resampling permutation test to identify the allergen pairs that occurred with a greater Jaccard similarity coefficient than by chance in our data set. These significant allergen pairs ($Q < .05$) are represented by red stars in Figure 2(B).

Participants were significantly frequently co-allergic to pecan and walnut, as reported before, based on a strong correlation of their sIgE and SPT values.^{6,15} In our set of multiallergic participants, all participants who were allergic to pecan (29 participants) were also allergic to walnut and only 3 of 32 walnut-allergic participants (9%) tolerated pecan. Goetz et al⁷ showed a strong serological cross-reactivity not only between walnut and pecan but also with hazelnut. Our data reflect this observation, as we identified significant ($Q < .05$) co-occurrences of allergies to hazelnut and pecan or walnut (Figure 2, B). Even though they were significant in our analysis, the Jaccard similarity coefficients were much lower for the hazelnut-pecan pair (0.55) and hazelnut-walnut pair (0.59) than for a co-occurrence of pecan and walnut allergies (Jaccard similarity coefficient: 0.91). From the 39 participants who were allergic to walnut, pecan, and/or hazelnut, 21 (54%) were allergic to all 3 allergens. Eight had a positive DBPCFC against walnut and pecan, 2 against walnut and hazelnut, while only 1 participant for walnut and 7 participants for hazelnut were solely allergic to these allergens, respectively.

All of our 42 pistachio-allergic participants were DBPCFC positive against cashew. This co-occurrence of pistachio and cashew allergies is in line with previous findings.^{5,6,15-17}

The reason for the small proportion of DBPCFCs being negative was that a challenge was only performed if an allergy to a specific food was suspected based on SPT and IgE data screening (see the DBPCFC methods section in this article's Online Repository at www.jaci-inpractice.org for details). Even though there were few negative DBPCFCs in this data set, we analyzed the overlap between all available DBPCFC outcomes (positive or negative) of each allergen pair for the participants. Figure E1 in this article's Online Repository at www.jaci-inpractice.org shows the percentage of pairwise DBPCFC outcomes in which the participants tested positive for both allergens, negative for both allergens, or positive for one allergen and negative for the other.

Organ system involvement with reaction and comorbid conditions

For each positive DBPCFC, the reacting organ system was recorded as per Bock's criteria.¹⁸ The organ involved most frequently was the skin, followed by gastrointestinal (GI) and respiratory (93%, 36%, and 6% of positive DBPCFCs, respectively). The respective frequencies of a skin ($P = .94$) and respiratory reaction ($P = .74$) were similar across food allergens (Table II), whereas the probability of a GI reaction significantly varied among allergens ($P < .01$). After adjusting for age and allergen, the sex of the participant was associated with the probability of having a GI reaction ($P < .01$); specifically a higher rate of GI reactions occurred in boys (42% of all positive DBPCFCs in boys; 24 of 30 boys [80%] had at least 1 GI reaction) than in girls (27% of all positive DBPCFCs in girls; 16 of 30 girls [53%] had at least 1 GI reaction). Sex was also found

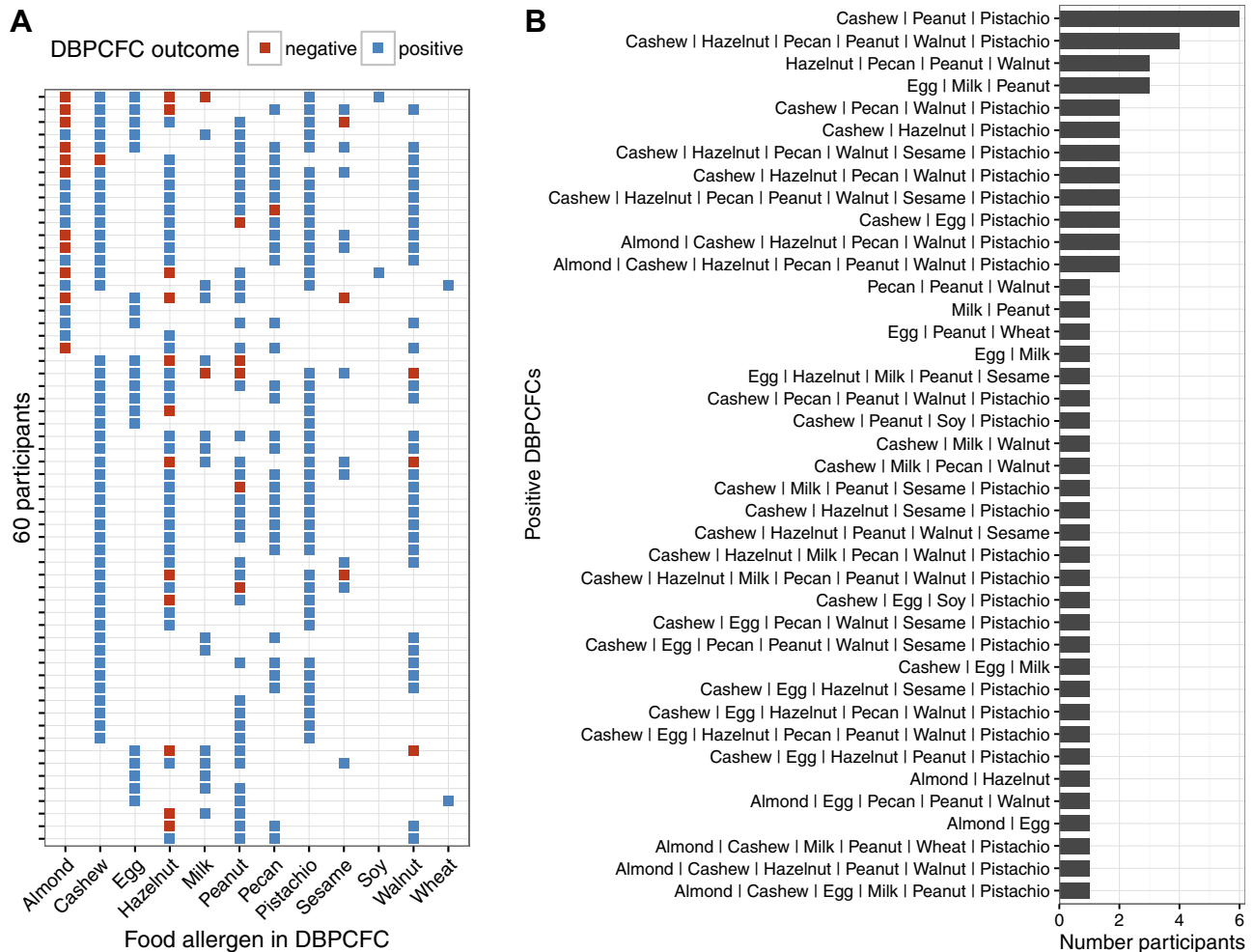


FIGURE 1. Food allergen and DBPCFC outcome combinations. **A**, Overview of DBPCFC outcomes in the study population. **B**, Number of participants who had positive DBPCFCs against different foods. *DBPCFC*, Double-blind, placebo-controlled food challenge.

to be significant for the frequency of having a skin reaction ($P < .01$), but with a lower rate of skin reaction in boys (87% of all positive DBPCFCs in boys; every boy had at least 1 skin reaction) than girls (99% of all positive DBPCFCs in girls; every girl had at least 1 skin reaction). The occurrence of a respiratory reaction did not vary significantly between male and female participants ($P = .1$). More than half of the participants had at least one atopic comorbid condition (asthma, atopic dermatitis, or allergic rhinitis) (Table 1). None of these conditions was significantly associated with a specific organ system showing an allergic reaction during the DBPCFCs.

IgE levels for whole allergens and components

We measured serum IgE levels for all 12 whole allergens and the components of 9 allergens (overview in Table E1, available in this article's Online Repository at www.jaci-inpractice.org). Components for 8 of the studied food allergens and birch were included. Figure 3 shows the IgE levels for whole food allergens and components for participants who were either DBPCFC positive or negative against the allergen or the source allergen of the respective component. For peanut (Figure 3, C), Ara h 1, Ara h 2, and the whole allergen showed significantly ($Q < .05$) different IgE levels between the 2 groups. The hazelnut

DBPCFC-positive group showed significantly greater IgE values against Cor a 14 ($Q < .05$) than the negative group. The other hazelnut components as well as the whole allergen were not significantly different between the groups (Figure 2, D). Notably, for all the components, some participants with negative DBPCFCs showed greater IgE levels than some participants with positive DBPCFCs.

Walnut and its components, as well as almond, showed no significant difference in IgE levels between the positive and negative DBPCFC groups. For milk, egg, pistachio, cashew, pecan, sesame, soy, and wheat, no (or a too small a number of) DBPCFC negative participants prevented a statistical analysis.

In addition to IgE responses, available IgG4 levels are shown in Figure E2 in this article's Online Repository at www.jaci-inpractice.org. None of the IgG4 levels were significantly different between the participants who were DBPCFC positive or negative.

IgE correlation analyses

To evaluate the association between the IgE responses against different components across our multiallergic participants, Spearman's correlation matrix was calculated. By using a multi-scale bootstrap resampling approach on the hierarchical clustering of these correlation data (R package *pvcust*¹⁴), we identified

TABLE II. Participant's food allergy and outcome overview

Food allergen	Cashew	Pistachio	Hazelnut	Peanut	Walnut	Pecan	Almond	Egg	Milk	Sesame	Soy	Wheat
No. of participants undergoing DBPCFC	47	42	42	41	35	30	21	19	16	14	2	2
Positive DBPCFCs												
n	46	42	30	36	32	29	10	19	14	11	2	2
%	98%	100%	71%	88%	91%	97%	48%	100%	88%	79%	100%	100%
Of positive DBPCFCs:												
Cumulative tolerated dose in DBPCFC (mg)												
Median	2.5	0	5	5	0	5	5	0	2.5	5	75	187.5
1st-3rd quartile	0-25	0-5	0-75	0-25	0-5	0-5	0-58	0-25	0-25	2.5-15	75	94-281
Range	0-275	0-175	0-375	0-375	0-75	0-375	0-75	0-75	0-175	0-375	75	0-375
Skin reaction in DBPCFC												
n	42	40	28	32	30	27	10	16	13	11	2	2
%	91%	95%	93%	89%	94%	93%	100%	84%	93%	100%	100%	100%
GI reaction in DBPCFC												
n	28	16	6	9	11	12	0	7	2	5	0	0
%	61%	38%	20%	25%	34%	41%		37%	14%	45%		
Respiratory reaction in DBPCFC												
n	5	1	2	2	1	1	1	1	2	0	0	0
%	11%	2%	7%	6%	3%	3%	10%	5%	14%			
Specific IgE (kU/L)												
Median	10.5	13	19.7	57.6	36	13.4	5.2	6.1	3.1	16.3	36.5	67.2
Range	0.4-100	0.4-100 [1 NA]	0.1-91.6	0.3-100	1.1-100	0.4-91.8	0.4-100	1.8-84	0.4-100	0.1-100	1-71.9	37.3-97.1
Skin prick test wheal diameter (mm)												
Median	15	12.5	9.5	16	12.5	8.5	9.5	11.5	15.5	16.5	7.3	13.3
Range	6.5-32 [1 NA]	3.5-31 [8 NA]	3.5-27	7-40	5-24.5	4.5-20.5 [4 NA]	6-25	7-36.5	4.5-40	6-27.5	7-7.5	10.5-16
Epis used												
n	1	0	0	3	0	0	0	0	1	0	0	0

DBPCFC, Double-blind, placebo-controlled food challenge; GI, gastrointestinal; NA, not applicable (not measured).

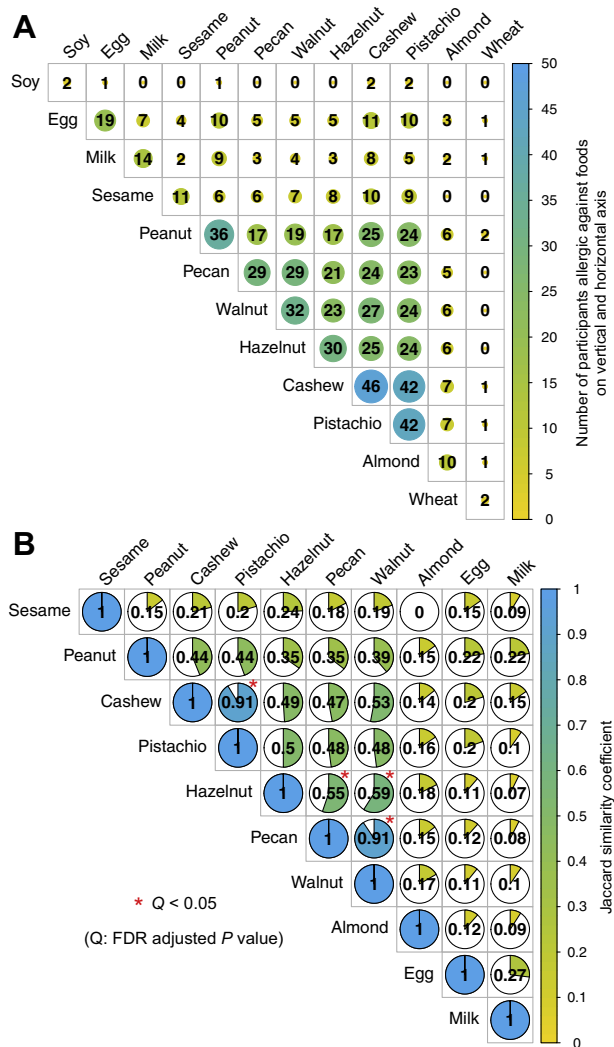


FIGURE 2. Concurrent occurrences of food allergies. **A**, Number of participants who were allergic against a specific food (diagonal) and any pairwise combination of 2 food allergens (intersection of column and row foods). **B**, Pie charts and numbers showing the Jaccard similarity coefficient. This similarity measure represents the overlap of participants who were allergic against the 2 food allergens adjusted for the number of participants being allergic against each of the food allergens separately. A coefficient of 1 means perfect overlap of participants, and 0 means no participant is allergic against the 2 food allergens. Soy and wheat were excluded because only 2 participants were allergic against each of them. *FDR*, False discovery rate.

significant clusters (Figure 4). The IgE levels against the components built 6 significant clusters that can be characterized by the function/family of the proteins. One cluster consisted of all pathogenesis-related class-10 (PR-10) proteins in this analysis (Cor a 1, Bet v 1, Ara h 8) that share significant structural similarity.^{19,20} Another cluster was formed by 3 of the lipid transfer protein (LTP) components (Cor a 8, Ara h 9, and Jug r 3). Jug r 1, Cor a 14, and Ana o 3, all being 2S albumin storage proteins, formed another significant cluster with Cor a 9 (an 11S globulin), despite a lack of structural homology among members of these

protein families. The 3 peanut storage proteins Ara h 1, Ara h 2, and Ara h 3 clustered significantly together. The final 2 significant clusters were built out of the 3 egg white proteins (Gal d 1, Gal d 2, Gal d 3) and the cow's milk proteins (Bos d 4, Bos d 5, Bos d 8), respectively.

To study the relationship between the IgE levels to the components and to the whole allergen extracts, Spearman's rank correlation coefficients are shown in Figure E3 in this article's Online Repository at www.jaci-inpractice.org. All measured IgE levels were included, even when no DBPCFC was performed for the respective allergen. Significant correlations ($Q < .05$) that show an absolute Spearman's rank correlation of greater than 0.5 were marked with a colored background. Looking at the pairs of components and their sources (red-bordered boxes in Figure E3 and listed in Table E1 in this article's Online Repository at www.jaci-inpractice.org), the correlations reflect the trends of components that were different between the groups of DBPCFC-positive and -negative participants (Figure 3). Ara h 1, Ara h 2, and Ara h 3 were significantly positively correlated with peanut sIgE levels. Also Jug r 1 IgE levels were significantly positively correlated with walnut sIgE levels, as well as Ana o 3 with cashew, and Cor a 9 and Cor a 14 with hazelnut sIgE levels. Focusing on the pairs between components and food allergens that are not their source allergen (outside of the red boxes in Figure E3), several strong significant correlations were detected. IgE levels to Ana o 3, a cashew component, correlated not only to cashew sIgE levels (Spearman's $\rho = 0.86$) but also as strongly positively with pistachio sIgE levels (Spearman's $\rho = 0.85$). It has previously been shown that cashew and pistachio sIgE levels are correlated,^{5,6,15-17} and we can see from Figure 2(B) that in our cohort, a significant percent of patients were allergic to both pistachio and cashew. Jug r 1 from walnut is another example of strong correlation (Spearman's $\rho = 0.89$) with sIgE levels to another species, in this case pecan. Pecan and walnut allergies are known to occur together,^{6,7,15} and our study shows this connection by IgE levels as well as DBPCFC outcomes (Figure 2, B), thereby enhancing our understanding of the possible molecular underpinnings of the coexistence of these allergies (ie, protein sequence homology). Figure 2(B) also shows a significant co-occurrence of pecan or walnut allergy with hazelnut allergy. This association can also be seen by significant correlations of pecan as well as walnut sIgE levels with Cor a 14 and Cor a 9 from hazelnut. Furthermore, hazelnut sIgE levels were significantly correlated to Jug r 1, even though less strongly (Spearman's $\rho = 0.59$) than to pecan (0.84) or walnut (0.89) sIgE.

Allergen component IgE levels and DBPCFC reactions

After adjusting the data for sex and age, we performed a logistic regression to analyze the contribution of the IgE level for each component to the chance of having a GI reaction in any positive DBPCFC. High IgE levels against Ana o 3 (cashew), Jug r 1 (walnut), or Cor a 14 (hazelnut) were significantly ($Q < .05$) associated with the chance of participants exhibiting a GI reaction in a DBPCFC (Figure 5). All 3 components are 2S albumin storage proteins. In models analyzing sIgE values against whole cashew, walnut, or hazelnut, respectively, rather than their 2S albumins, these sIgE values were not significantly associated with having a GI reaction or not. Because almost every positive DBPCFC resulted in a skin reaction and nearly none in a respiratory reaction, a similar analysis for these reactions was not possible.

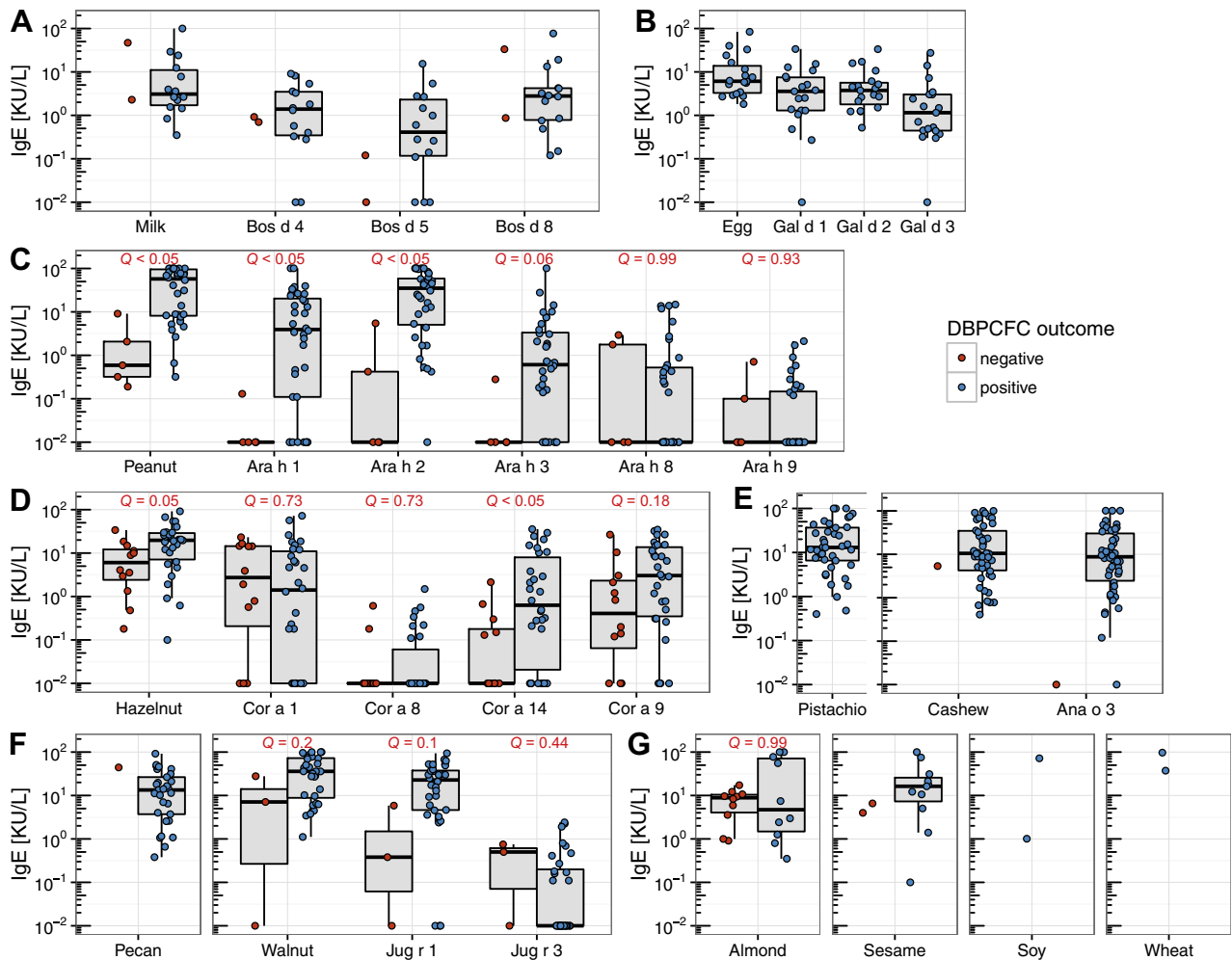


FIGURE 3. Whole food allergen and component IgE levels stratified by DBPCFC outcomes. **A**, Milk and its components; **B**, egg and its components; **C**, peanut and its components; **D**, hazelnut and its components; **E**, pistachio, cashew, and its component; **F**, pecan, walnut, and its components; **G**, almond, sesame, soy, wheat. Soy and wheat components were not plotted because only 2 values were obtained. (Q value is the FDR adjusted *P* value). *DBPCFC*, Double-blind, placebo-controlled food challenge; *FDR*, false discovery rate.

DISCUSSION

To our knowledge, the study of multifood-allergic children presented here is the largest and most comprehensive of its kind to date. Cashew, pistachio, peanut, walnut, and hazelnut caused the majority of reactions, but extensive variation was seen in individual challenge patterns. Strong associations, both serologically and clinically, were seen for cashew and pistachio as well as for walnut, pecan, and to some extent hazelnut. GI reactions were more frequently seen in boys than in girls, as well as in individuals with high IgE levels to 2S albumins from cashew, walnut, and/or hazelnut.

Our study population represents a selected group of multifood-allergic children, who were at high risk of reacting to a variety of food allergens. A strength of our study is that all participants were DBPCFC challenged to all possible offending foods (determined by skin and blood testing) to verify their food hypersensitivity clinically. They were, on average, challenged with 5 food items.

We observed serological associations between cashew and pistachio as well as among walnut, pecan, and hazelnut,

suggestive of cross-reactivity within these groups of nuts. Consistent with these observations, our data demonstrate, to our knowledge for the first time, the association of positive DBPCFC outcomes for these groups of nuts (Figure 2). Significant associations of positive DBPCFC outcomes, reflected in high, significant Jaccard similarity coefficients, were seen between cashew and pistachio as well as among hazelnut, pecan, and/or walnut (Figure 2, B). This extensive clinical association was not seen, for example, between peanut and pecan. Savvatanos et al¹⁷ have earlier described that extensive cross-reactivity occurs at the clinical level between the 2 Anacardiaceae nuts (cashew and pistachio). This was based on open nut challenges, and we can now verify that this also can be observed through DPBCFCs. Our data confirm that individuals diagnosed with cashew allergies should also avoid pistachio and vice versa. The same likely applies to walnut, pecan, and possibly hazelnut, for which we could show serological relationships as well as an increased probability of coexisting allergies by DBPCFC.

This association analysis was based on proven allergy by positive DBPCFCs. A DBPCFC was only performed if the

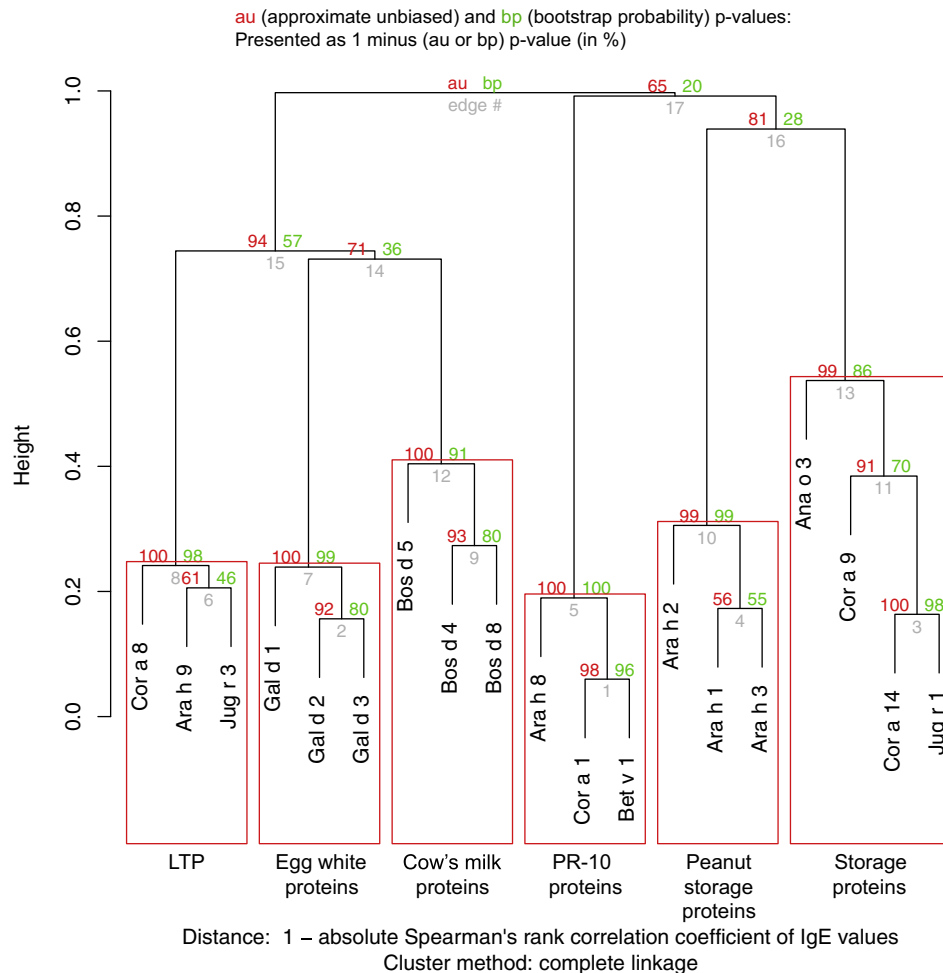


FIGURE 4. Hierarchical clustering of the component IgE indicates a clustering of components by protein family or origin. Significant clusters (au $P < .05$) are shown by red rectangles. After FDR adjusting the P value, all rectangles are also significant at $Q < .05$ (numbers not in plot). Soy and wheat components were excluded in this analysis because of too few positive observations. au, Approximately unbiased; FDR, false discovery rate; PR-10, pathogenesis-related class-10.

screening by SPT and IgE level suggested a possible allergy. So, for the foods for which no DBPCFC was performed, we assume that it would be a negative DBPCFC. With this assumption, we might miss a few positive DBPCFCs, but we do not think that this weakens our findings because these possible additional positive DBPCFCs would not reduce the number of co-occurrences between allergies that we found.

The male/female ratio in our study population was 1:1. Boys showed a higher chance than girls of having a GI reaction during DBPCFCs, whereas girls showed a greater probability than boys of having a skin reaction. Sex differences in food allergy during childhood were previously reported with boys appearing more likely than girls to develop food allergies.^{21–26} To our knowledge, the association between sex and the type of reaction has not before been explicitly reported. However, eosinophilic esophagitis, an allergic/immune condition affecting the esophagus, has been shown to be 3 to 4 times more common among males than females.²⁷ The results in our study population, which was comparatively small for this kind of analysis, point toward a trend in sex differences in reactions to food challenges that should be studied in larger cohorts in the future.

In line with previous findings,^{28–32} the comparison of IgE levels among peanut allergic and nonallergic participants showed greater IgE responses to Ara h 1, Ara h 2, and whole peanut extract in peanut DBPCFC-positive than DBPCFC-negative children (Figure 3, C). The IgE levels against hazelnut component Cor a 14 were also significantly different between hazelnut DBPCFC-positive and -negative participants (Figure 3, D). Cor a 14 levels were previously shown to be useful in predicting the outcome of a DBPCFC against hazelnut.^{31,33}

A cluster analysis of the IgE levels to the components over all 60 participants revealed several significant groupings (Figure 4) reflecting, as expected, both phylogenetically and/or structurally defined protein families and the food from which the proteins originate. Two clusters of LTP and PR-10 proteins, respectively, reflect structural homology, whereas 2 other clusters (egg white and cow's milk proteins) reflect the component source origin. In the last 2 clusters, 4 storage proteins from different sources cluster separately from the 3 peanut storage proteins.

To study the quantitative relationship between IgE responses to individual components and whole food extracts, we analyzed the significant Spearman's correlations across all

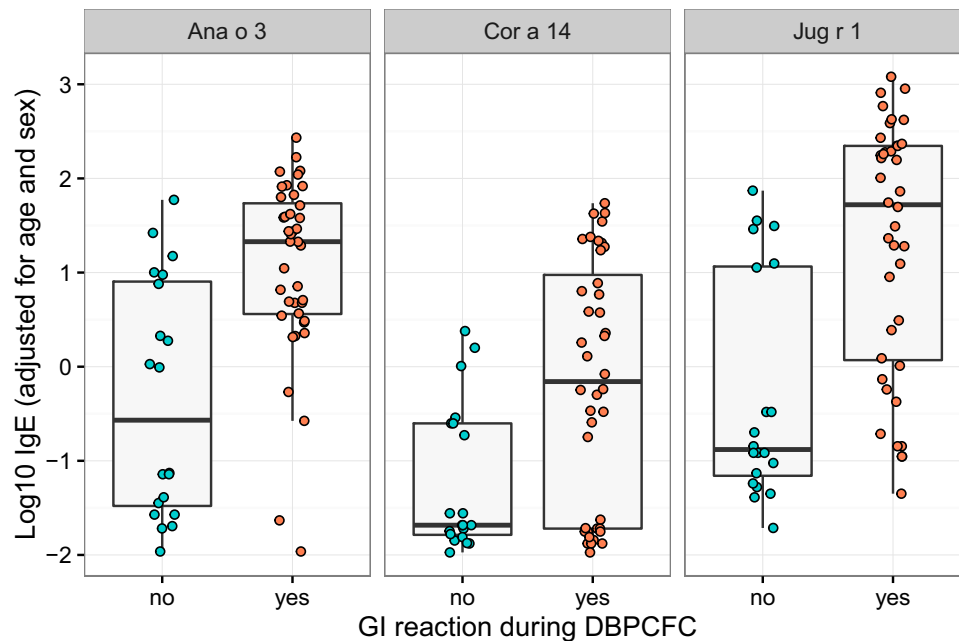


FIGURE 5. After adjusting the component IgE levels for age and sex, Ana o 3, Cor a 14, and Jug r 1 levels were significantly different between participants showing a GI reaction during any DBPCFC and those who did not. Q value (FDR adjusted P value) $< .05$ for all 3 components. DBPCFC, Double-blind, placebo-controlled food challenge; FDR, false discovery rate; GI, gastrointestinal.

60 participants (Figure E3, available in this article's Online Repository at www.jaci-inpractice.org). The levels of IgE to Ara h 1, Ara h 2, and Ara h 3 were significantly positively correlated with the sIgE level against peanut, as well as Jug r 1 with walnut, Ana o 3 with cashew, and Cor a 9 and Cor a 14 with hazelnut. Similarly, levels of IgE to Ana o 3 and pistachio were strongly correlated. It has previously been shown that cashew and pistachio sIgE levels are correlated.^{5,6,15-17} Also, Jug r 1 was strongly significantly correlated with pecan sIgE values. Pecan and walnut allergies are known to occur together.^{6,7,15} Hazelnut, pecan, and walnut sIgE levels were significantly correlated with Cor a 14, Cor a 9 as well as Jug r 1 IgE levels. Cross-reactivity between walnut, pecan, and hazelnut by IgE enzyme-linked immunosorbent assay inhibition was previously shown.⁷ These IgE correlations are consistent with the clinical associations among these 3 allergens reported in this paper.

Spearman's correlation matrix between IgE levels against components and the whole allergen extracts (Figure E3, available in this article's Online Repository at www.jaci-inpractice.org) is not free of bias because the allergy status was not known for every participant and every food. Even though we found known patterns of co-occurring allergies, we cannot assume that this is a perfect reflection of the overall population of multiallergic individuals. Furthermore, allergies that cannot be attributed as IgE-mediated will most likely not be revealed in this correlation analysis.

After adjusting for age and sex, Ana o 3, Jug r 1 and Cor a 14 IgE levels were significantly higher in participants who experienced GI reactions during any DBPCFC than in those who did not. These 3 components are all 2S albumin storage proteins. Members of this protein family are recognized as major food allergens of nuts and seeds.

Little is known about how sensitization occurs both for 2S albumins and for other allergens; however, it was reported previously that 2S albumins are thought to sensitize directly via the GI tract.³⁴ Several 2S albumins have been shown to be highly resistant to heat and GI digestion.³⁴⁻³⁶ Both stability and abundance of a food component are important factors that determine the amount of the protein that can reach the "effector system" and cause a reaction. Although all 3 classes of seed storage proteins (2S albumins, 7S, and 11S globulins) are highly abundant, 2S albumins are small and compact proteins containing intramolecular disulfide bonds acting to maintain a tight conformation, which may contribute to their demonstrated stability under conditions in the stomach that would rapidly denature or degrade many other proteins.³⁷

Although the data set is very complex and too small to allow a firm conclusion, the results indicate a trend that sensitization against 2S albumin proteins might serve to predict a higher probability of having a GI reaction.

This study provides evidence for the significant associations of certain food allergies, namely cashew and pistachio and walnut, pecan and hazelnut, as shown both by DBPCFC outcomes and by correlations in IgE reactivity to structurally related food allergen components. These findings thus help to elucidate the clinical and serological features of multiallergic children. However, many open questions remain that in part reflect the design of our study, which attempted to minimize the risk associated with exposure of multiallergic individuals to allergenic food items and to reduce stress and discomfort to the study participants and their families. For example, because DBPCFCs were only performed with foods for which there was strong clinical evidence for a suspected allergy, the low number of DBPCFCs that were negative prevented certain analyses, such as

a systematic study of the sensitivity and specificity of whole food extract or component IgE levels for predicting allergies. In summary, our study enhances understanding of the clinical relevance of the molecular connections among various allergen protein groups, and that this may aid in diagnosing and managing patients with food allergies.

Acknowledgments

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**ONLINE REPOSITORY
SUPPLEMENTARY METHODS****Participant selection**

Screening data from 165 participants in an IRB-approved clinical trial (Clinical trial number: NCT02643862) were included. Participants in the study were eligible for inclusion if they were between 4 and 55 years of age with moderate-to-severe allergy to at least 2 studied food allergens (milk, egg, peanut, almond, wheat, cashew, pistachio, sesame seed, soy, pecan, walnut, and/or hazelnut). They must have shown a positive skin prick test (SPT) greater than or equal to 6 mm wheal diameter to each allergen and/or a specific IgE greater than 4 kU_A/L (ImmunoCAP) for each allergen; a double-blind, placebo-controlled food challenge (DBPCFC) to the respective food allergen was performed only in those meeting these criteria. To be included in the subsequent clinical trial, participants needed to have had a clinical reaction during a DBPCFC^{E1} to small doses of food ($\leq$ dose of 500 mg food protein) and no clinical reaction during the placebo (oat) challenge. In the analysis of the screening data in this paper, participants were included regardless of their DBPCFC outcome.

Exclusion criteria included a history of cardiovascular or other chronic disease (other than asthma, atopic dermatitis, or rhinitis) requiring therapy, a history of severe anaphylaxis (requiring intubation or admission to an ICU) to food allergens used in the study, or a total IgE >1500 kU/L. We also excluded women who were pregnant or breastfeeding.

DBPCFC

A DBPCFC was performed to any of the food proteins tested to which patients had either an IgE > 4 kU_A/L and/or an SPT reactivity ≥ 6 mm wheal diameter during screening. These screening criteria suggested a possible allergy to the food.^{E2} Additionally, 3 DBPCFCs were performed for 3 different participants, each of whom exhibited wheal diameters of 5.5 and IgE < 4 kU_A/L for the allergens tested in the DBPCFCs. Before the food challenge, participants were required to restrict the use of oral antihistamines (5 half-lives), beta-agonists (12 hours), theophylline (12 hours), and cromolyn (12 hours).

Seven doses of food allergens were given every 15 to 30 minutes in increasing amounts (5 mg, 20 mg, 50 mg, 100 mg, 100 mg, 100 mg, and 125 mg) up to a cumulative total of 500 mg of protein. Before the challenges, the participants were given a physical assessment administered by a trained physician's assistant, registered nurse, nurse practitioner, and/or physician of the study team who was blinded to the testing material. The supervising investigator was also blinded to the testing material.

Reactions were scored using a Food Challenge Symptom Score sheet.^{E3} After the development of objective symptoms, the food challenge was terminated and the participant was given appropriate treatment. The participants were observed for a minimum of 2 hours after the final administered dose and discharged only when deemed clinically stable by a study physician/clinician. All food challenges were performed under physician supervision.

Skin prick tests

Skin prick tests were performed as per published methods^{E4} with the following extracts: peanut (1:20 W/V), almond (1:20 W/V), pecan (1:20 W/V), milk (1:20 W/V), sesame seed (1:20 W/V), egg white (1:20 W/V), cashew (1:20 W/V), walnut (1:20 W/V), whole wheat (1:20 W/V), hazelnut (1:20 W/V), soybean (1:40 W/V) (Greer Laboratories, Lenoir, NC), and pistachio (1:10 W/V, ALKPort Washington, NY). Histamine (10 mg/mL, Hollister-Stier, Spokane, WA) was used as a positive control and physiological saline as a negative control. Wheal size was calculated as the average of the largest diameter and the perpendicular midpoint diameter.

Food powders

This study used food flours/powders dispensed through a food flour/powder good manufacturing practice facility at Stanford per FDA guidelines (Guidance for Industry, CGMP for Phase 1 Investigational Drugs, July 2007) and as previously described.^{E4} Each dose was weighed out by a trained dietician on a professional-grade balance. Flour/powder protein content was calculated according to nutritional information provided by manufacturers and confirmed through protein assays run at Stanford laboratories.

		Almond	Cashew	Egg	Hazelnut	Milk	Peanut	Pecan	Pistachio	Sesame	Soy	Walnut	Wheat	
DBPCFC outcome for food allergen on vertical		n = 21	n = 16	n = 8	n = 16	n = 4	n = 14	n = 13	n = 15	n = 7	n = 2	n = 13	n = 1	Almond
	negative	52%	6% 50%	62%	25% 38%	25% 25%	50%	54%	53%	29% 71%	100%	54%		
	positive	48%	44%	38%	38%	50%	7% 43%	8% 38%	47%			46%	100%	
		n = 16	n = 47	n = 11	n = 34	n = 10	n = 31	n = 26	n = 42	n = 12	n = 2	n = 30	n = 1	Cashew
	negative	6%	2%		3%		3%	4%				3%		
	positive	50% 44%	98%	100%	24% 74%	20% 80%	16% 81%	4% 92%	100%	17% 83%	100%	7% 90%	100%	
		n = 8	n = 11	n = 19	n = 11	n = 9	n = 12	n = 5	n = 10	n = 6	n = 1	n = 7	n = 1	Egg
	negative													
	positive	62% 38%	100%	100%	55% 45%	22% 78%	17% 83%	100%	100%	33% 67%	100%	29% 71%	100%	
		n = 16	n = 34	n = 11	n = 42	n = 10	n = 30	n = 24	n = 31	n = 13	n = 2	n = 28	n = 0	Hazelnut
	negative	25%	24%	55%	29%	10% 50%	3% 27%	8%	23%	15% 15%	100%	7% 7%		
	positive	38% 38%	3% 74%	45%	71%	10% 30%	13% 57%	4% 88%	77%	8% 62%		4% 82%		
		n = 4	n = 10	n = 9	n = 10	n = 16	n = 11	n = 3	n = 7	n = 4	n = 1	n = 7	n = 1	Milk
	negative	25%	20%	22%	10% 10%	12%	9%		29%	25%	100%	14%		
	positive	25% 50%	80%	78%	50% 30%	88%	9% 82%	100%	71%	25% 50%		29% 57%	100%	
		n = 14	n = 31	n = 12	n = 30	n = 11	n = 41	n = 20	n = 28	n = 11	n = 1	n = 24	n = 2	Peanut
	negative	7%	16%	17%	3% 13%	9% 9%	12%	10%	14%	18%		4% 8%		
	positive	50% 43%	3% 81%	83%	27% 57%	82%	88%	5% 85%	86%	27% 55%	100%	8% 79%	100%	
		n = 13	n = 26	n = 5	n = 24	n = 3	n = 20	n = 30	n = 24	n = 6	n = 0	n = 30	n = 0	Pecan
	negative	8%	4%		4%		5%	3%	4%			3%		
	positive	54% 38%	4% 92%	100%	8% 88%	100%	10% 85%	97%	96%	100%		97%		
		n = 15	n = 42	n = 10	n = 31	n = 7	n = 28	n = 24	n = 42	n = 11	n = 2	n = 26	n = 1	Pistachio
	negative													
	positive	53% 47%	100%	100%	23% 77%	29% 71%	14% 86%	4% 96%	100%	18% 82%	100%	8% 92%	100%	
		n = 7	n = 12	n = 6	n = 13	n = 4	n = 11	n = 6	n = 11	n = 14	n = 0	n = 9	n = 0	Sesame
	negative	29%	17%	33%	15% 8%	25%	27%		18%	21%				
	positive	71%	83%	67%	15% 62%	25% 50%	18% 55%	100%	82%	79%		22% 78%		
		n = 2	n = 2	n = 1	n = 2	n = 1	n = 1	n = 0	n = 2	n = 0	n = 2	n = 0	n = 0	Soy
	negative													
	positive	100%	100%	100%	100%	100%	100%		100%		100%			
		n = 13	n = 30	n = 7	n = 28	n = 7	n = 24	n = 30	n = 26	n = 9	n = 0	n = 35	n = 0	Walnut
	negative		7%	29%	7% 4%	14% 29%	4% 8%		8%	22%		9%		
	positive	54% 46%	3% 90%	71%	7% 82%	57%	8% 79%	3% 97%	92%	78%		91%		
		n = 1	n = 1	n = 1	n = 0	n = 1	n = 2	n = 0	n = 1	n = 0	n = 0	n = 0	n = 2	Wheat
	negative													
	positive	100%	100%	100%		100%	100%		100%				100%	
		negative	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative	
		positive	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive	DBPCFC outcome for food allergen on horizontal

FIGURE E1. DBPCFC outcomes for each allergen or allergen pairs. For each single allergen (squares on the diagonal with only blue percentages) or pair of allergens, the number of participants having a DBPCFC performed against the single allergen or both allergens (tested separately) is shown in black (n). The percentages show the portion of these participants who had a positive or negative DBPCFC against the single allergen (on the diagonal) or both allergens (blue % numbers) as well as how many have discordant DBPCFC outcomes for the pair of allergens (pink % numbers). *DBPCFC*, Double-blind, placebo-controlled food challenge.

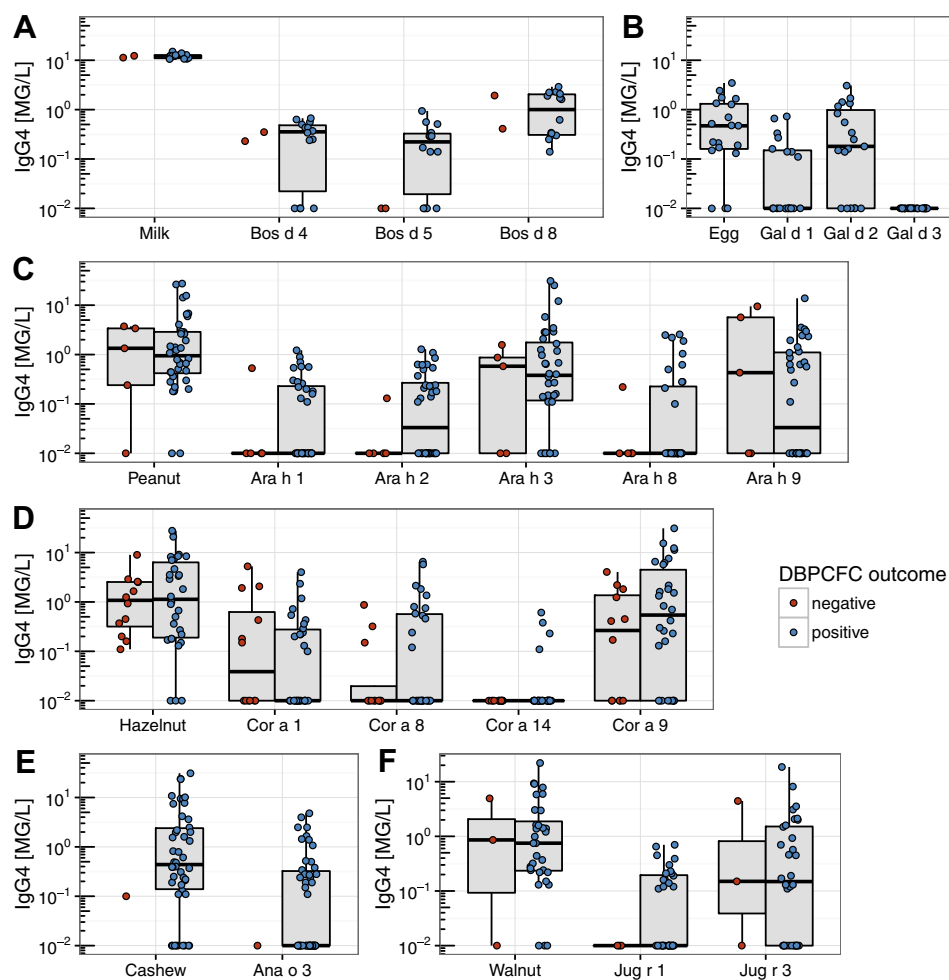


FIGURE E2. Whole food allergen and component IgG4 stratified by DBPCFC outcomes. **A**, Milk and its components; **B**, egg and its components; **C**, peanut and its components; **D**, hazelnut and its components; **E**, cashew and its component; **F**, walnut and its components. Turkey boxplots: shown are the medians; the “hinges” represent the 1st and 3rd quartile. The whiskers are the smaller and largest values after outliers are excluded. Outliers are defined as values greater than the 75th percentile plus 1.5 times the interquartile range (IQR), or less than 25th percentile minus 1.5 times the IQR. *DBPCFC*, Double-blind, placebo-controlled food challenge.

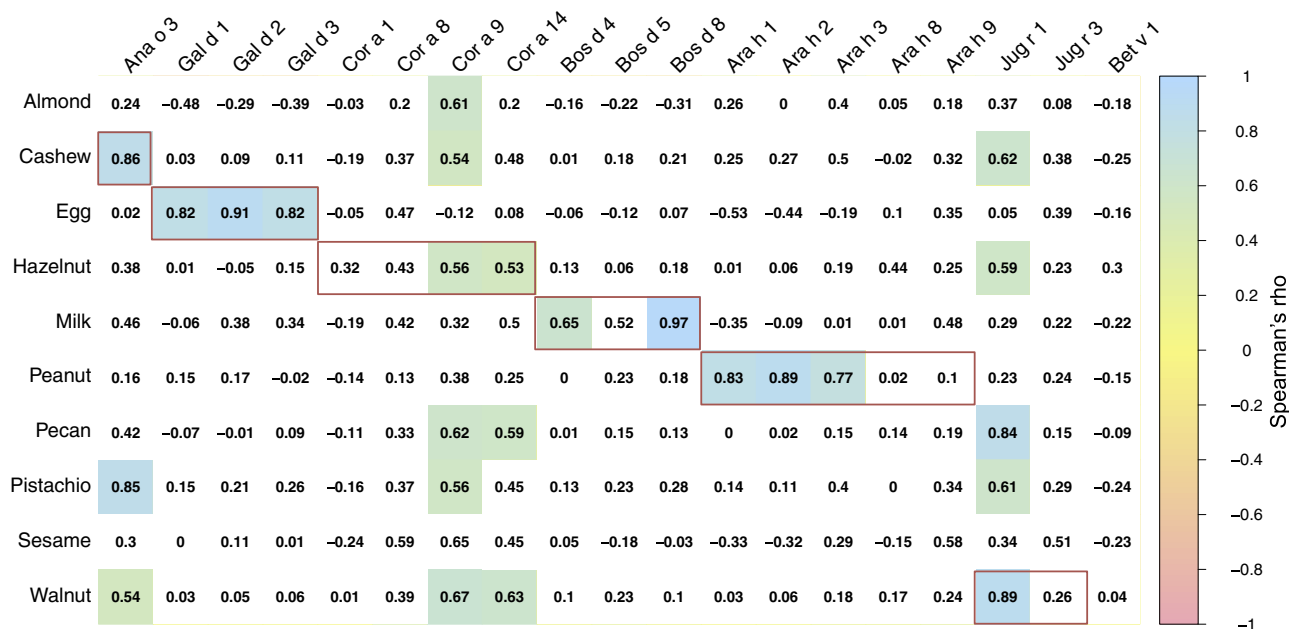


FIGURE E3. Spearman's rank correlation between component IgE and whole allergen extract IgE values. Significant correlations ($P < .05$), which show an absolute Spearman's rank correlation of greater than 0.5, are marked with a colored background. Red rectangles mark the components and their respective source food allergens.

TABLE E1. Protein families and functional designations of food components in this study

Source	Component	Protein family/function
Birch	Bet v 1	PR-10
Cashew	Ana o 3	Storage protein (2S albumin)
Egg (white)	Gal d 1	Ovomucoid
	Gal d 2	Ovalbumin
	Gal d 3	Conalbumin/ovotransferrin
Hazelnut	Cor a 1	PR-10
	Cor a 8	LTP
	Cor a 9	Storage protein (11S globulin)
	Cor a 14	Storage protein (2S albumin)
Cow's milk	Bos d 4	Alpha-lactalbumin
	Bos d 5	Beta-lactoglobulin
	Bos d 8	Casein
Peanut	Ara h 1	Storage protein (7S globulin)
	Ara h 2	Storage protein (2S albumin)
	Ara h 3	Storage protein (11S globulin)
	Ara h 8	PR-10
	Ara h 9	LTP
Soy	Gly m 4	PR-10
	Gly m 5	Storage protein (beta-conglycinin)
Walnut	Jug r 1	Storage protein (2S albumin)
	Jug r 3	LTP
Wheat	Tri a 14	LTP
	Tri a 19	Omega-5 gliadin

LTP, Nonspecific lipid transfer proteins; PR-10, pathogenesis-related class-10.

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