

Allergen Recognition Patterns in Walnut Allergy Are Age Dependent and Correlate with the Severity of Allergic Reactions



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What is already known about this topic? Walnut allergy is an important and prevalent tree nut allergy associated with severe allergic symptoms.

What does this article add to our knowledge? The study provides information on the relative importance of all known walnut allergens across Europe and evidence suggesting that vicilins may be as important as 2S albumin in walnut allergy.

How does this study impact current management guidelines? Patients with early onset walnut allergy have an elevated risk of severe reactions and should be given particular attention in the clinic.

BACKGROUND: Walnut is an important elicitor of food allergy in children and adults with a high rate of severe reactions. Multicenter studies using a common clinical protocol and a comprehensive allergen are lacking.

OBJECTIVE: To investigate potential correlations between molecular sensitization patterns and clinical characteristics of walnut-allergic patients.

METHODS: A total of 91 walnut-allergic subjects and 24 tolerant controls from Switzerland, Germany, and Spain were included. Walnut allergy was established by food challenge in all but anaphylactic subjects. Specific IgE (sIgE) to walnut extract, rJug r 1 (2S albumin), rJug r 3 (nonspecific lipid transfer protein 1), nJug r 4 (11S globulin), rJug r 5 (PR-10 protein), 2 vicilin fractions, profiling, and cross-reactive carbohydrate determinant was determined by ImmunoCAP. A threshold of 0.10 kU_A/L was used for positivity.

RESULTS: Sensitivity of sIgE to walnut extract was 87% and increased to 96% for the sum of all walnut components. sIgE to walnut extract and all walnut components, except rJug r 5, was

significantly higher in patients younger than 14 years at inclusion. Stratification by age at onset of walnut allergy led to similar results. All patients younger than 14 years had severe reactions, whereas 38% of patients 14 years or older were mild reactors. Severe reactors (n = 70) had higher sIgE levels than did mild reactors (n = 21) to walnut extract ($P < .0001$), rJug r 1 ($P < .0001$), nJug r 4 ($P = .0003$), and both vicilin fractions ($P < .0001$), but not to Jug r 3 and Jug r 5.

CONCLUSIONS: Sensitization to walnut storage proteins is acquired in childhood and correlates with severe reactions. sIgE levels to storage proteins Jug r 1 and Jug r 4 and vicilin fractions, but not to nonspecific lipid transfer protein and PR-10 proteins, correlate with systemic reactions to walnut. © 2019 American Academy of Allergy, Asthma & Immunology (J Allergy Clin Immunol Pract 2019;7:1560-7)

Key words: Walnut; Food allergy; Component-resolved diagnosis; Food challenge; Age; Severity; Vicilin

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Abbreviations used

DBPCFC- Double-blind placebo-controlled food challenge

nsLTP- Nonspecific lipid transfer protein

OAS- Oral allergy syndrome

OR- Odds ratio

sIgE- Specific IgE

SPT- Skin prick test

INTRODUCTION

Walnuts are seeds from the trees of *Juglans* genus, which consists of 24 different species. The *Juglans regia* species, the common or Persian walnut, is the most frequently consumed in Europe.

According to data from the EuroPrevall project, 3% of the adult European population is predicted to be sensitized to walnut, ranging from 0.1% in Iceland to 6% and 8% in Switzerland and Spain, respectively.¹ Among children and adolescents with anaphylaxis to tree nuts, walnut belonged to the most important elicitors, accounting for 16% of the cases.² According to telephone interviews with almost 5000 random households in the United States, walnut was the most common offending tree nut, reported as a trigger in 60% of individuals allergic to tree nut.³ In Chile, a cross-sectional questionnaire-based study indicated that allergy to walnut was the most prevalent food allergy in school-age children.⁴

Threshold dose distribution data revealed that 3.1 to 4.1 mg walnut protein, corresponding to 1/180 of a walnut kernel, elicited an allergic reaction in 5% of a walnut-allergic population.⁵ Despite the emerging importance of walnut allergy, there is a scarcity of data on the importance of individual walnut allergens.

Allergens identified in walnut to date are the storage proteins Jug r 1 (2S albumin), Jug r 4 (11S globulin), and Jug r 2 and Jug r 6 (vicilins) and the nonspecific lipid transfer protein (nsLTP) Jug r 3. Moreover, walnut allergens with cross-reactivity to pollen are known, mainly a profilin, Jug r 7, and the recently described Bet v 1 homologous protein Jug r 5.⁶⁻¹² Here, we present data of the most comprehensive study to date on molecular sensitization patterns to all known and officially accepted walnut allergens (www.allergen.org) in patients with confirmed walnut allergy, including children and adults, and patients from Mediterranean and from birch pollen-exposed regions, with a particular focus on severity and age of development of food allergy to walnut.

METHODS

Study design and subjects

Four allergy clinics in Switzerland (Zurich and Lucerne), Germany (Bonn), and Spain (Barcelona) participated in this prospective multicenter study. The clinical part of the study took place between 2013 and 2017. The study was approved by each center's local ethical committee, and all patients gave written informed consent before entering the study (KEK-ZH 2012-0519, EKNZ-2015-368; CEIC-2012/7085; AEKNO-2013310).

In total, 115 individuals were included. All patients underwent the same clinical evaluation, including an extensive interview using a uniform questionnaire and blood sampling.

Final inclusion criterion for the group of walnut-allergic patients ($n = 91$) was a positive food challenge (double-blind

placebo-controlled food challenge [DBPCFC] or an open titrated food challenge) or a convincing case history of anaphylaxis following ingestion of walnut. Sixty-one patients with a suggestive case history of a walnut allergy were included after agreeing to a food challenge. The remaining 30 patients were included on the basis of a case history of an anaphylactic reaction, defined as severe life-threatening reaction such as drop in blood pressure, severe bronchospasm, or laryngeal edema within 2 hours of ingestion of walnut without performing a food challenge. Atopic controls were recruited from CH ($n = 5$), DE ($n = 3$), and ES ($n = 6$) and 10 nonatopic controls from CH ($n = 5$) and ES ($n = 5$). The atopic controls had a history of pollen allergy with a positive skin prick test (SPT) result and/or positive specific IgE (sIgE) test result to relevant pollen allergens and were tolerant to walnut. The nonatopic controls had no history of pollen or food allergy and negative SPT results and/or sIgE test results to a panel of food and inhalant allergens.

Food challenges

Open titrated food challenges and DBPCFCs were performed according to a common protocol and consisted of the patient ingesting increasing doses of walnuts (5 mg, 10 mg, 50 mg, 100 mg, 1.0 g, 5.0 g, 8.0 g). A detailed description of the procedure is provided in this article's Online Repository at www.jaci-inpractice.org. For the purposes of the study, each patient was attributed to 1 of the following 4 clinical reaction groups: group 1: patients with isolated subjective oral symptoms under provocation (oral allergy syndrome [OAS]); group 2: patients with an "extended OAS" under provocation (OAS with either blisters of the oral mucosa, mild rhinitis or conjunctivitis, slight swelling of the lips, mild tightness of the throat, or mild dysphagia); group 3: objective systemic reactions under provocation; and group 4: history of anaphylaxis.

sIgE antibody measurements

For all patients and controls, serum sIgE to walnut, rJug r 1, rJug r 3, nJug r 4, rJug r 5, high- and low-molecular-weight walnut vicilin fractions, rPru p 3, rBet v 1, rBet v 2, and cross-reactive carbohydrate determinant was determined by ImmunoCAP. In addition, sIgE to rPla a 3 was determined in the Spanish patients and controls. sIgE values of greater than or equal to 0.1 kU_A/L were considered positive. Purification of nJug r 4 and high- and low-molecular-weight vicilin fractions from walnut, generation of rJug r 5, and generation of experimental ImmunoCAP tests are described in this article's Online Repository at www.jaci-inpractice.org.

Skin testing

Prick-to-prick tests with native walnuts were performed in all walnut-allergic patients and SPTs with walnut extract (ALK-Abelló, Madrid, Spain) in Swiss and Spanish patients.

Statistics

Differences between clinical reaction groups (mild vs severe reactors) and between age groups (<14 years vs ≥14 years) with respect to mean sIgE concentration were evaluated with 2-sample Wilcoxon test. For frequencies, Fisher exact test was used.

A logistic regression model was applied with clinical severity (occurrence of systemic reactions) as dependent variable and sex, age at inclusion into study (younger/older than 14 years), age at onset of walnut allergy (younger/older than 14 years), geographical region (Switzerland and Germany vs Spain), and sensitization to walnut components (below/equal or above threshold 0.10 kU_A/L) as independent variables. In addition, data were evaluated with univariate logistic regression models. The statistical analysis was performed

with SAS/STAT software, version 9.4, SAS System for Windows (SAS Institute, Heidelberg, Germany).

RESULTS

Patients' characteristics and food challenges

Overall, 91 walnut-allergic patients were enrolled, 31 adolescents and adults from CH (22 females, 9 males; age, 34 ± 12 years; range, 15-68 years), 31 from DE (15 females, 9 males; age, 6 ± 3 years; range, 2-15 years), and 29 from ES (15 females, 14 males; age, 32 ± 16 years; range, 3-53 years). Fifteen patients were included on the basis of a positive DBPCFC, 46 patients on a positive titrated oral food challenge, and 30 patients on a positive case history of walnut-induced anaphylaxis. Furthermore, 14 atopic controls with pollen allergy and a history of walnut tolerance and 10 nonatopic controls were analyzed. In 60 patients (68%), the onset of walnut allergy was before their 14th birthday.

Sixteen challenge-positive patients (18%) reported an isolated OAS under provocation and 5 an extended OAS with mild additional symptoms of the lips or throat, or rhinoconjunctivitis, and 40 patients developed a systemic reaction, 75% with skin symptoms such as flush, urticaria, and angioedema, 33% with respiratory symptoms, 23% with gastrointestinal symptoms, and 8% with laryngeal involvement. [Tables E1 to E3](#) in this article's Online Repository at www.jaci-inpractice.org summarize the clinical reactivity of all included walnut-allergic patients. Allergic reactions to the following tree nuts were reported by the 91 walnut-allergic subjects: pecan nut (9%), hazelnut (44%), almond (18%), brazil nut (13%), cashew (11%), pistachio (13%), and macadamia (8%).

Sensitization to walnut extract and individual allergens

Prick-to-prick test result was positive in all patients allergic to walnut from Spain and Germany ($n = 51$) and in 13 of 27 Swiss patients (overall sensitivity, 82%), whereas SPT result with walnut extract was positive in 25 of 29 Spanish patients and in 17 of 30 Swiss patients (overall sensitivity, 71%). For serum sIgE testing, the threshold for positivity was set to 0.10 kU_A/L. This cutoff was selected on the basis of a receiver operating characteristic curve analysis for sIgE to walnut extract, providing a sensitivity of 98.6% (95% CI, 92.3%-100%) and a specificity of 52.4% (95% CI, 29.8%-74.3%) for diagnosing patients with systemic reactions to walnut (groups 3 and 4).

Considering the entire walnut-allergic population, the sensitivity of sIgE to walnut extract was 87%, whereas 96% were sensitized to at least 1 walnut component. In patients with mild symptoms (groups 1 and 2), sensitivity of sIgE measurement to walnut extract was particularly compromised and was as low as 48%. Eight of 11 patients with mild symptoms but no sIgE to walnut extract were sensitized to the Bet v 1 homologous protein rJug r 5. Thus, only rJug r 5 accounted for the increased sensitivity of component testing compared with the whole walnut extract. Four patients, 3 with mild symptoms and 1 with anaphylaxis, tested negative to walnut extract and did not recognize any of the included components. In total, 53% of walnut-allergic subjects were sensitized to rJug r 1, 53% to rJug r 3, 48% to nJug r 4, 52% to rJug r 5, 57% to nJug r vicilin-H, 73% to nJug r vicilin-L, 17% to profilin (rBet v 2), and 19% to cross-reactive carbohydrate determinant. The concentration of IgE to the single walnut allergens is summarized in [Figure 1](#).

Sensitization profile in atopic and nonatopic controls

All nonatopic controls tested completely negative to walnut extract and allergens. Five Swiss and 1 German atopic controls were sensitized to rJug r 5 and all presented relatively high levels of sIgE to rBet v 1. Three Spanish and 2 German atopic controls were sensitized to rJug r 3, 4 of them to rPru p 3 and 1 to rPla a 3 at higher concentrations. Sensitization to rJug r 1 (3 of 3 patients) and nJug r 4 (1 of 3 patients) was observed only in the 3 German pediatric atopic controls. Sensitization rates to nJug r vicilin-H and nJug r vicilin-L among the atopic controls were 43% and 36%, respectively. Because the number of controls was limited, specificity in relation to the controls was not calculated.

Geographic differences in sensitization pattern

[Figure 2](#) summarizes the sensitization pattern of patients from the 3 geographic regions to walnut extract and the single allergen components. Particularly prominent differences were observed for sensitization to rJug r 3, which was higher in ES than in CH and DE, and for rJug r 5, which was highest in CH and DE. In DE, much higher sensitization rates to rJug r 1 and nJug r 4 were observed than in CH and ES, but this finding was confounded by the fact that in DE, a purely pediatric population was recruited.

Age dependency of the sensitization pattern to walnut allergens

Mean concentrations of sIgE to walnut extract and all walnut components except rJug r 5 were significantly higher in walnut-allergic patients younger than 14 years than in those above that age at inclusion into the study. Also, if stratified by the age of walnut allergy onset, patients who acquired walnut allergy before the age of 14 years had significantly higher sIgE concentrations to walnut extract and all walnut allergens except for rJug r 3 ($P < .0001$ for rJug r 1, nJug r 4, nJug r vicilin-H, and nJug r vicilin-L; $P < .01$ for walnut extract; $P < .05$ for rJug r 5) than did those with later onset ([Table 1](#)). Among the late-onset reactors, only 3 subjects were sensitized to rJug r 1 (11%), 2 to nJug r 4 (7%), and 6 to nJug r vicilin-H (21%), whereas sensitization rates to these storage proteins were significantly higher in the patients with early onset allergies (75%, 70%, and 77%, respectively; [Figure 3](#)), supporting the view that sensitization to these storage proteins is primarily acquired during childhood.

Of the patients with early onset allergies, 90% belonged to the severe reactors groups (groups 3/4), whereas 46% of the patients with late-onset allergies belonged to the mild reactors groups (groups 1/2). Thus, age at onset of allergy to walnut (<14 years vs ≥ 14 years) significantly correlated with the severity of the clinical reaction ($P = .0002$; Fisher exact test). If stratified by age at inclusion into the study, similar results were observed ($P < .0001$; Fisher exact test).

Are there any predictive markers for the severity of the clinical reaction?

Patients of clinical reaction groups 3/4 (severe reactors: $n = 70$; systemic reaction under challenge or anaphylaxis) had significantly higher concentrations of sIgE to walnut extract ($P < .0001$), rJug r 1 ($P < .0001$), nJug r 4 ($P < .0001$), nJug r vicilin-L ($P = .0001$), and nJug r vicilin-H ($P < .0001$) than did patients of clinical reaction groups 1/2 (mild reactors: $n = 21$; symptoms restricted to the oral cavity and mild symptoms of lip,

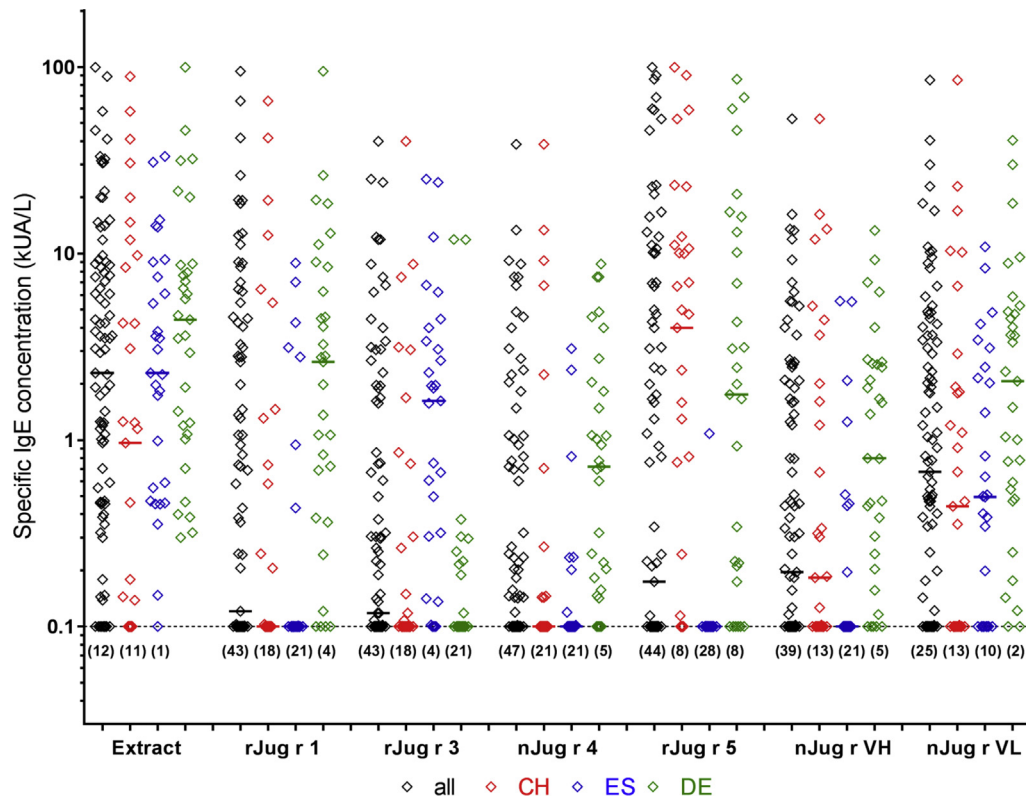


FIGURE 1. IgE concentration to walnut extract and to the different walnut allergen components in all walnut-allergic patients and in patients from CH, ES, and DE. IgE to profilin and cross-reactive carbohydrate determinant is not included, but described in the text. Numbers in parentheses indicate the number of observations below the 0.1 kUA/L cutoff. Horizontal bars, median; dotted line, cutoff.

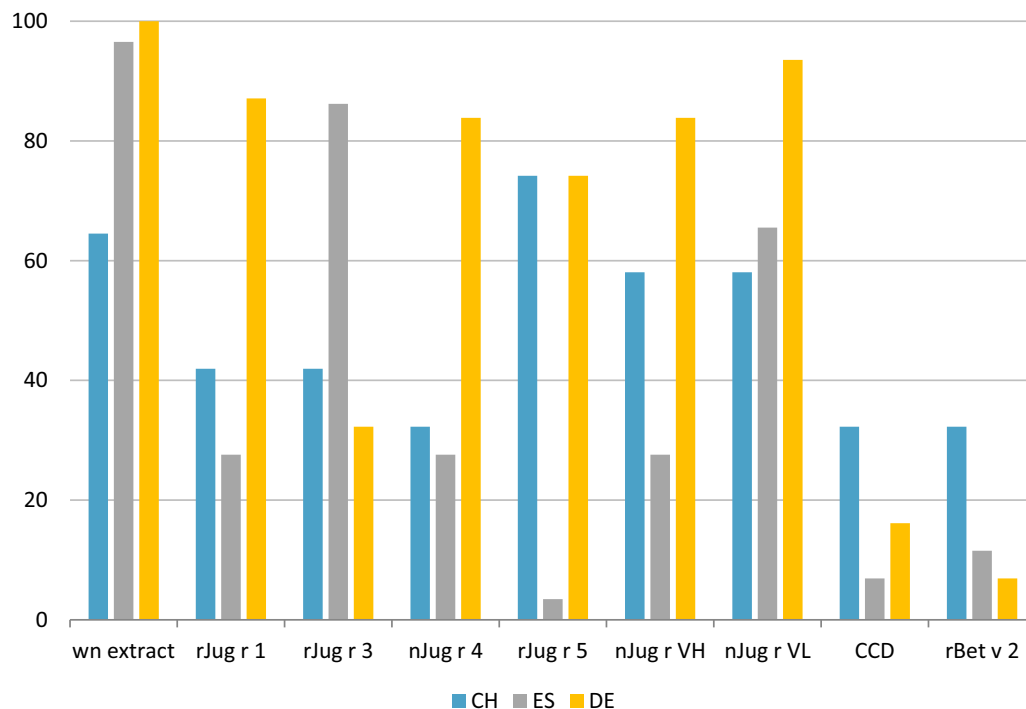
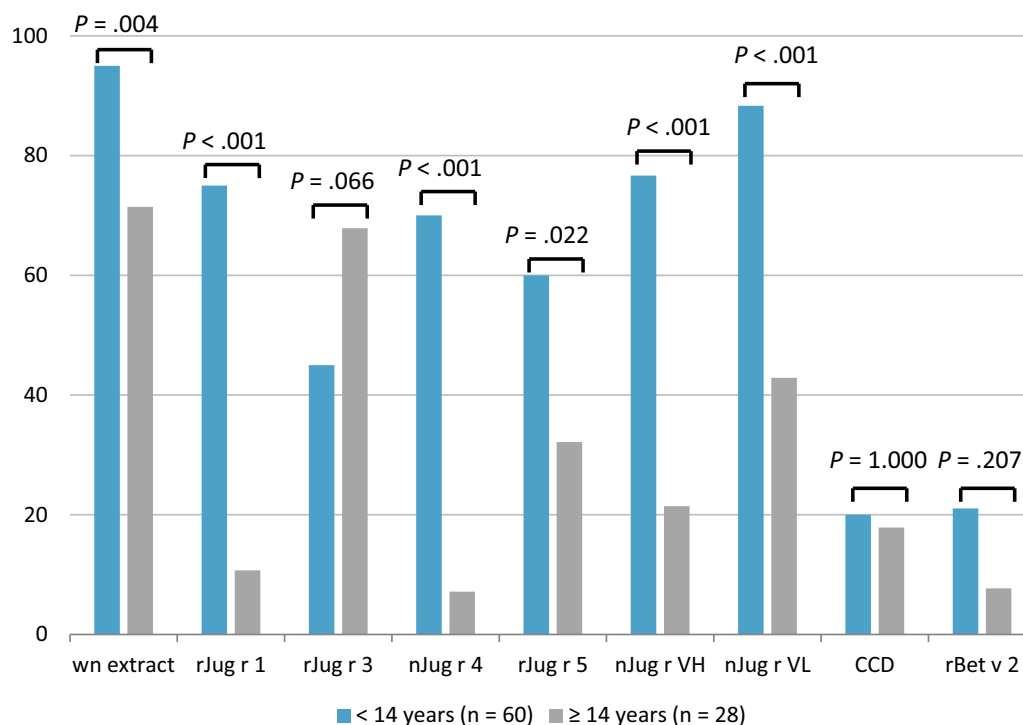


FIGURE 2. Percentage of patients with sensitization to walnut extract and to the different components in walnut-allergic patients from CH, ES, and DE. CCD, Cross-reactive carbohydrate determinant; *wn*, walnut.

TABLE I. Comparison of mean IgE concentrations to walnut allergens in patients with age of walnut allergy onset <14 y vs walnut allergy onset ≥14 y

Antigen	Allergy onset (y)	N	Mean ± SD	Minimum	Median	Maximum	95% CI		P value
Total IgE	<14	60	582 ± 720	23	324	3073	396	768	.0003
	≥14	28	219 ± 373	15	88	1598	75	364	
f256 walnut	<14	60	11.54 ± 19.74	0.10	4.32	100.00	6.44	16.64	.0025
	≥14	28	4.33 ± 8.56	0.10	0.47	33.16	1.01	7.65	
f441 rJug r 1	<14	60	7.04 ± 15.90	0.10	1.19	95.19	2.93	11.15	<.0001
	≥14	28	0.20 ± 0.51	0.10	0.10	2.79	0.01	0.40	
f442 rJug r 3	<14	60	1.86 ± 5.60	0.10	0.10	39.98	0.41	3.31	.0985
	≥14	28	3.26 ± 6.69	0.10	0.21	25.01	0.67	5.86	
nJug r 4	<14	60	2.23 ± 5.51	0.10	0.24	38.51	0.81	3.65	<.0001
	≥14	28	0.11 ± 0.03	0.10	0.10	0.27	0.09	0.12	
rJug r 5	<14	60	9.48 ± 19.09	0.10	0.29	86.24	4.55	14.41	.0368
	≥14	28	7.98 ± 24.83	0.10	0.10	100.00	-1.64	17.61	
nJug r vicilin-H	<14	60	3.13 ± 7.48	0.10	0.47	52.80	1.19	5.06	<.0001
	≥14	28	0.45 ± 1.16	0.10	0.10	5.23	0.00	0.90	
nJug r vicilin-L	<14	60	5.90 ± 12.85	0.10	1.45	85.22	2.58	9.21	<.0001
	≥14	28	0.75 ± 1.12	0.10	0.10	4.19	0.32	1.19	

Two-sample Wilcoxon test.

**FIGURE 3.** Percentage of patients with sensitization to walnut extract and to the different components in subjects with onset of walnut allergy before their 14th birthday and beyond the age of 14 years. CCD, Cross-reactive carbohydrate determinant; *wn*, walnut.

throat, or rhinoconjunctival mucosa under challenge). No significant differences between these groups were found for sIgE to rJug r 3 and rJug r 5 (Table II). Nevertheless, when stratified for geographic regions (CH/DE vs ES), significant differences between mild and severe reactors for sIgE concentrations to walnut extract, rJug r 1, nJug r 4, nJug r vicilin-L, and nJug r vicilin-H were found among the Swiss and German walnut-allergic patients but not among the Spanish patients. Thus, the

level of sensitization to all analyzed walnut allergens did not differ between mild and severe reactors from Spain.

Sensitization to rJug r 1 and nJug r 4 was observed only among the severe reactors (Figure 4). Furthermore, the sensitization rate to nJug r vicilin-H and vicilin-L was much higher in the severe reactors than in the mild reactors (71% vs 10% and 84% vs 33%, respectively). Symptom severity (group 3/4, severe reactors vs group 1/2, mild reactors) was highly significantly

TABLE II. Comparison of mean IgE concentration to walnut allergens in patients of clinical reaction group 1 and 2 (mild reactors) with patients of clinical reaction groups 3 and 4 (severe reactors)

Antigen	Symptom class	N	Mean $\pm$ SD	Minimum	Median	Maximum	95% CI	P value
Total IgE	1 + 2	21	168 $\pm$ 261	20	99	1240	49 to 287	.0036
	3 + 4	70	540 $\pm$ 699	13	274	3073	373 to 706	
f256 walnut	1 + 2	21	2.97 $\pm$ 7.32	0.10	0.10	30.92	−0.36 to 6.30	<.0001
	3 + 4	70	10.74 $\pm$ 18.70	0.10	3.73	100.00	6.28 to 15.20	
f441 rJug r 1	1 + 2	21	0.10 $\pm$ 0.00	0.10	0.10	0.10		<.0001
	3 + 4	70	6.09 $\pm$ 14.89	0.10	0.79	95.19	2.54 to 9.64	
f442 rJug r 3	1 + 2	21	2.39 $\pm$ 5.96	0.10	0.10	25.01	−0.32 to 5.11	.2917
	3 + 4	70	2.19 $\pm$ 5.90	0.10	0.15	39.98	0.78 to 3.60	
nJug r 4	1 + 2	21	0.10 $\pm$ 0.00	0.10	0.10	0.10		<.0001
	3 + 4	70	1.93 $\pm$ 5.15	0.10	0.19	38.51	0.70 to 3.16	
rJug r 5	1 + 2	21	3.82 $\pm$ 5.82	0.10	0.24	22.87	1.17 to 6.47	.9563
	3 + 4	70	10.38 $\pm$ 23.13	0.10	0.14	100.00	4.87 to 15.90	
nJug r vicilin-H	1 + 2	21	0.10 $\pm$ 0.02	0.10	0.10	0.19	0.10 to 0.11	<.0001
	3 + 4	70	2.83 $\pm$ 6.99	0.10	0.46	52.80	1.17 to 4.50	
nJug r vicilin-L	1 + 2	21	0.59 $\pm$ 1.06	0.10	0.10	4.19	0.10 to 1.07	.0001
	3 + 4	70	5.19 $\pm$ 12.02	0.10	1.15	85.22	2.32 to 8.05	

Two-sample Wilcoxon test.

($P < .0001$; Fisher exact test) associated with sensitization to walnut extract, rJug r 1, nJug r 4, nJug r vicilin-L, and nJug r vicilin-H but not with sensitization to rJug r 3 and rJug r 5. Results were consistent when applying a cutoff level for positivity of 0.35 rather than 0.10 kU_A/L.

Logistic regression revealed a significant influence of sensitization to walnut extract and to both vicilin fractions, as well as the age of onset of walnut allergy, on the severity of the clinical reaction. However, because all patients were included into the study at an age less than 14 years and all patients sensitized to rJug r 1 belonged to the severe reactors, the odds ratio (OR) for developing a severe reaction could not be calculated (not determinable). In the logistic regression analysis, sex, geographic region, and sensitization to rJug r 3, nJug r 4, and rJug r 5 all showed no influence on clinical severity ($P > .05$).

The risk of developing a systemic reaction was significantly higher for walnut extract-sensitized patients (OR, 76.9; 95% CI, 8.7-500; $P < .0001$), for nJug r vicilin-L- and vicilin-H-sensitized patients (OR, 10.5 and 23.3; 95% CI, 3.5-32.3 and 5.0-111, respectively; $P < .0001$), and for patients with an onset of walnut allergy before their 14th birthday (OR, 7.655; 95% CI, 2.487-23.563; $P = .0004$) than for patients not sensitized to walnut extract, neither of the vicilin fractions, or acquisition of walnut allergy after their 14th birthday.

DISCUSSION

To our knowledge, this study represents the most comprehensive component-resolved diagnostics analysis done in walnut-allergic children and adults so far. We quantified sIgE concentrations to walnut allergens in 91 European walnut-allergic pediatric and adult individuals recruited in 3 countries, Germany, Switzerland, and Spain, as well as in 24 walnut-tolerant controls.

For serological analysis, a cutoff of 0.10 kU_A/L was used for sIgE positivity, but most results, in particular on age dependency and severity, remained stable when the analyses were repeated with a cutoff of 0.35 kU_A/L.

sIgE to walnut extract showed a sensitivity of 87%, which increased to 96% when sIgE to the Bet v 1 homolog Jug r 5 was taken into consideration. However, at the same time, this led to a reduction in diagnostic specificity because 6 of the 14 atopic controls (43%) also showed positive test results to Jug r 5. Because the size of our atopic and nonatopic control groups was limited, an extended statistical analysis of our patient group data in comparison to the controls was not performed. However, the data appear to be in accordance with other component-resolved diagnostics studies in plant food allergy,¹³⁻¹⁵ showing very high specificity in comparison to nonallergic controls and negative effects on specificity caused by cross-reactivity of sIgE to Bet v 1 with PR-10 allergens from foods in pollen-allergic patients without food allergy. Notably, we found little effect on diagnostic performance by sensitization to profilin and cross-reactive carbohydrate determinant, as the sensitization rates among the walnut-allergic patients were below 20% and only 2 and 3 atopic controls were positive to these allergen components, respectively.

Sensitivity of prick-to-prick test and SPT with native walnuts and the commercial walnut extract, respectively, was particularly affected in the CH population (48% and 57%), which can be most likely explained by a low content of Bet v 1 homologous proteins in tree nuts and a consequently low representation in the SPT extract.

Our data analysis revealed statistically significant differences between patients with mild symptoms (groups 1 and 2) and more severe/systemic symptoms (groups 3 and 4) on the one hand, and between patients younger than versus older than 14 years on the other hand.

Geographic differences observed in our study need to be considered in view of the fact that the patient populations recruited in the different study centers were not fully comparable in terms of age. In Spain, adults/adolescents as well as children were included; in Switzerland, the youngest patient was 15 years old at inclusion, whereas in Germany only children up to age 15 years were included, most being younger than 10 years. Keeping in mind these differences, we found the highest sensitization rate

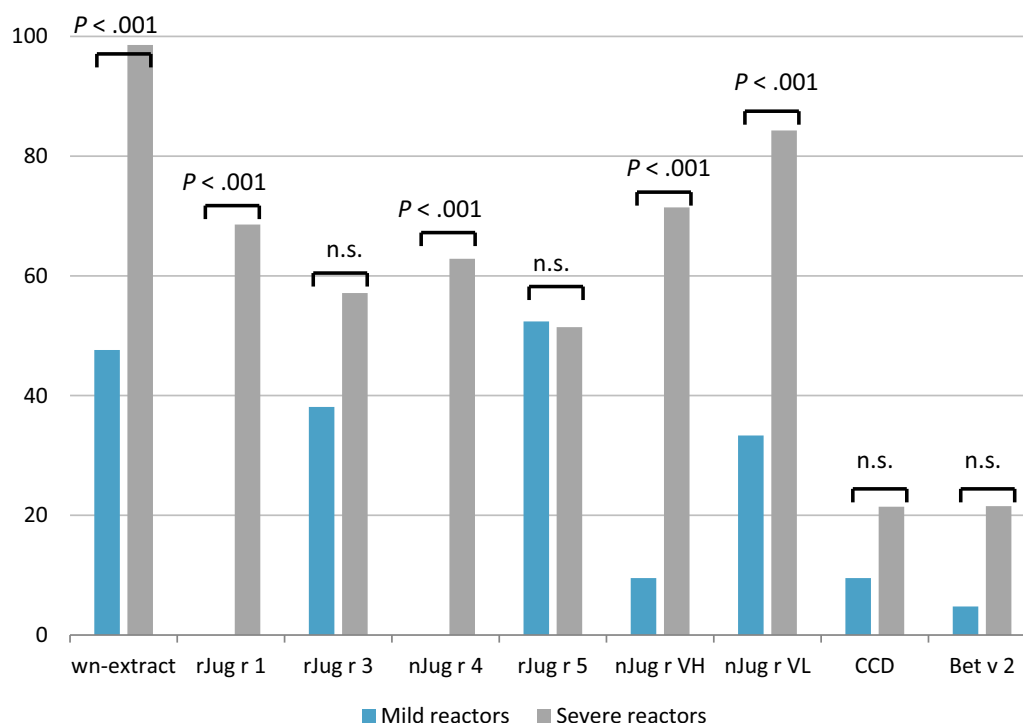


FIGURE 4. Percentage of patients with sensitization to walnut extract and to the different components in walnut-allergic patients with severe reactions (group 3/4, systemic reaction under provocation or anaphylaxis by history) and mild reactors (group 1/2, with mild symptoms under provocation). CCD, Cross-reactive carbohydrate determinant; n.s., not significant; wn, walnut.

to the nsLTP Jug r 3 in Spain, whereas sensitization to the Bet v 1 homolog Jug r 5 was much more common in Switzerland and Germany. This observation is in full accordance with other studies describing a predominance of nsLTP sensitization in plant food allergy in southern Europe, as well as a dominating role of Bet v 1-related food allergens in birch-endemic regions, such as Switzerland, Germany, and Scandinavia (reviewed in Egger et al¹⁶ and Werfel et al¹⁷). Moreover, higher sensitization rates to the storage proteins Jug r 1 (2S albumin) and Jug r 4 (11S globulin) were found in the German patient population. This finding reflects a more prominent role of the storage proteins in pediatric subjects with primary sensitization to walnuts.

Concerning age dependency, all patients younger than 14 years had severe reactions (group 3/4), as compared with 62% of patients 14 years or older. In regard to serology, we found significantly higher mean levels of sIgE to walnut extract and Jug r 1, Jug r 3, Jug r 4, and the 2 vicilin preparations in patients younger than 14 years at inclusion versus patients older than 14 years. When stratified for age of allergy onset, similar results were obtained, except that the difference was not significant for Jug r 3 ($P = .0985$), but turned significant for Jug r 5 ($P = .0368$) (Table I). The frequency of detectable sIgE (regardless of level) was also different between late- and early onset reactors, with sensitization to Jug r 1, Jug r 4, Jug r 5, and the vicilin preparations being significantly more frequent in patients with early onset allergies (Figure 3).

Taken together, our data on age dependency of sensitization patterns are comparable to findings obtained in peanut-allergic patients recruited within the EuroPrevall study.¹⁸

We also analyzed a potential correlation of sIgE levels with severity of clinical reactions to walnut. Mean sIgE concentrations to walnut extract and all walnut allergens except Jug r 3 and Jug r 5 were significantly higher in clinical reaction groups 3/4 than in groups 1/2. Similar to reports in peanut allergy¹⁸⁻²⁰ and hazelnut allergy,²¹ this result supports the view that severe reactions to legume seeds and nuts are predominantly caused by sensitization to storage proteins rather than by pollen-related allergens such as Bet v 1 homologs, or nsLTP. A plausible explanation for this phenomenon could be the relatively low content of Bet v 1-related proteins and nsLTP in such high-protein-containing foods, leading to a lower allergen intake in comparison to storage proteins, which are present in much higher amounts.

Interestingly, no significant differences in sIgE concentrations between symptom groups 3/4 versus groups 1/2 were detected in the Spanish patients. Although it is clear that this phenomenon cannot be explained by clinically insignificant sIgE binding to cross-reactive components such as PR-10 allergens, profilin, or nsLTP, it is difficult to establish a plausible hypothesis. We speculate that the more complex allergen environment in Spain in comparison to Switzerland and Germany may play a role, but a separate study will be required to confirm this finding and address potential reasons.

The 2 vicilin preparations used in this study showed a high frequency of sIgE binding (56% and 71% for vicilin-H and vicilin-L, respectively). These rates are the highest of all purified allergen preparations used in this study, suggesting that Jug r 2 and/or Jug r 6 may become important additions to the panel of allergens used in the diagnosis of walnut allergy.

In summary, our study showed that the sensitivity of sIgE testing in walnut allergy can be increased by component-resolved diagnostics and that this increase is mainly related to the Bet v 1-related walnut allergen Jug r 5. Our results support the view that sensitization to walnut storage proteins is usually acquired in childhood and is associated with severe allergic reactions. IgE to storage proteins Jug r 1 and Jug r 4 and vicilin fractions, but not to nsLTP and PR-10 proteins, is significantly associated with systemic reactions to walnut. Provided that these findings are confirmed by further studies in well-characterized patients with food allergy to walnut, sIgE to these storage proteins may come to serve as a marker for an increased risk of severe food allergy to walnut.

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ONLINE REPOSITORY

METHODS

Food challenges

Food challenges were performed according to a common protocol and consisted of the patient ingesting increasing doses of walnuts (5 mg, 10 mg, 50 mg, 100 mg, 1.0 g, 5.0 g, 8.0 g) mixed into soy- or milk pudding, yogurt, or apple sauce according to the patients' preference for open titrated food challenges or into a matrix consisting of Sinlac powder (Nestle, Vevey, Switzerland), chocolate powder, ground coconuts, and peppermint oil for DBPCFC. Symptoms were monitored and classified as either objective (blisters of the oral mucosa, rhinitis, conjunctivitis, flush, urticaria, angioedema, emesis, diarrhea, laryngeal edema, cough, bronchospasm, drop in blood pressure, shock) or subjective (OAS, defined as itch restricted to the oral cavity, localized pruritus of skin, generalized pruritus, dysphagia, nausea, gastric pain and/or burning, and dyspnea). The challenge procedure was stopped if the patient developed either objective (observable) symptoms or reported severe and/or persistent (lasting >45 minutes) subjective symptoms. As long as no objective or severe subjective symptoms occurred, the challenge continued until all doses were consumed.

Preparation of natural walnut allergens and experimental sIgE assays

Defatted walnut powder was extracted with 50 mM Tris, pH 8.0, containing 7 mM citric acid, 6 mM cysteine, 6 mM

ascorbic acid, 1.5 mol NaCl, and 2% (wt/vol) insoluble polyvinylpyrrolidone. Jug r 4 was purified by sequential steps of concanavalin A affinity chromatography (flow-through mode), mixed-mode anion exchange, and gel filtration, followed by bioaffinity adsorption to eliminate any residual amount of Jug r 1 and Jug r 3. High-molecular-weight (HMW) vicilins were purified by mixed-mode anion exchange and concanavalin A affinity chromatography. A low-molecular-weight (LMW) vicilin fraction was isolated by tangential flow ultrafiltration on 50-kDa and 5-kDa membranes, followed by mixed-mode anion exchange (flow-through mode). Both vicilin preparations were subjected to bioaffinity adsorption with mAbs to eliminate any residual amount of Jug r 1, Jug r 3, and Jug r 4. The HMW and LMW vicilin fractions both comprised the naturally occurring processing products of Jug r 2 and Jug r 6: the C-terminal cupin domain (HMW) and peptides derived from the N-terminal region (LMW).^{E1} Jug r 6-derived products dominated over Jug r 2-derived products in the HMW fraction, whereas the opposite was true in the LMW fraction. Purity, homogeneity, and identity of the allergen preparations were verified by SDS-PAGE and mass spectrometry and their immunologic activity assessed using the ImmunoCAP assay platform (Thermo Fisher Scientific, Uppsala, Sweden). All chromatography media and equipment were obtained from GE Healthcare Life Sciences (Uppsala, Sweden). Recombinant Jug r 5 and experimental ImmunoCAP tests were generated as described.^{E2,E3}

TABLE E1. Walnut-allergic patients (n = 21) with OAS (group 1) or OAS plus mild associated symptoms (group 2) under provocation: age at inclusion, reaction under provocation, and IgE sensitization pattern as determined by ImmunoCAP (kU_A/L) testing

Patient ID	Age at inclusion (y)	Symptoms under provocation	Extract	rJug r 1	rJug r 3	nJug r 4	rJug r 5	nJug r vicilin-H	nJug r vicilin-L	CCD	Profilin
CH-11002	32	OAS, R	0.46	<0.1	<0.1	<0.1	0.81	0.10	0.68	0.77	<0.1
CH-11008	50	OAS, R, mild Tght	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
CH-11017	41	OAS	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
CH-11020	15	OAS, R	<0.1	<0.1	<0.1	<0.1	22.9	<0.1	<0.1	<0.1	<0.1
CH-11021	25	OAS	<0.1	<0.1	<0.1	<0.1	11.1	<0.1	<0.1	<0.1	<0.1
CH-11032	48	OAS	0.14	<0.1	<0.1	<0.1	6.68	0.19	0.1	0.33	<0.1
CH-11036	35	OAS	<0.1	<0.1	<0.1	<0.1	0.24	<0.1	<0.1	<0.1	<0.1
CH-11044	40	OAS, itching face	<0.1	<0.1	<0.1	<0.1	4.73	<0.1	<0.1	<0.1	<0.1
CH-11045	34	OAS	<0.1	<0.1	<0.1	<0.1	7.0	<0.1	<0.1	<0.1	<0.1
CH-11046	32	OAS, itching face	0.14	<0.1	0.3	<0.1	10.0	<0.1	<0.1	<0.1	0.28
CH-11047	36	OAS, blisters, mild Dph	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
CH-11055	35	OAS	<0.1	<0.1	<0.1	<0.1	1.59	<0.1	<0.1	<0.1	<0.1
CH-11056	42	OAS, C, mild AE lip	<0.1	<0.1	<0.1	<0.1	3.99	<0.1	<0.1	<0.1	<0.1
CH-11059	46	OAS	<0.1	<0.1	<0.1	<0.1	10.1	<0.1	<0.1	<0.1	<0.1
ES-22003	48	OAS	9.29	<0.1	9.29	<0.1	<0.1	<0.1	2.15	<0.1	<0.1
ES-22005	54	OAS	0.35	<0.1	0.35	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
ES-22007	27	OAS	2.24	<0.1	2.24	<0.1	<0.1	<0.1	0.51	<0.1	<0.1
ES-22013	27	OAS	30.9	<0.1	30.9	<0.1	<0.1	<0.1	4.19	<0.1	<0.1
ES-22015	33	OAS	3.06	<0.1	3.06	<0.1	<0.1	<0.1	0.82	<0.1	<0.1
ES-22016	26	OAS	14.1	<0.1	14.1	<0.1	<0.1	<0.1	2.46	<0.1	<0.1
ES-22022	29	OAS	0.59	<0.1	0.59	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
>0.1 kU/L*			48% (n = 10)	0	38% (n = 8)	0	52% (n = 11)	5% (n = 1)	29% (n = 6)	10% (n = 2)	5% (n = 1)

AE, Angioedema; C, conjunctivitis; CCD, cross-reactive carbohydrate determinant; Dph, mild dysphagia; R, rhinitis; Tght, tightness of throat.

*%positive.

TABLE E2. Walnut-allergic patients with systemic reactions under provocation (group 3): age at inclusion, reaction under provocation, and IgE sensitization pattern as determined by ImmunoCAP (kU_A/L) testing

Patient ID	Age at inclusion (y)	Symptoms under provocation	Extract	rJug r 1	rJug r 3	nJug r 4	rJug r 5	nJug r vicilin-H	nJug r vicilin-L	CCD	Profilin
CH-11003	33	OAS, R, L, F, Dph	0.18	0.25	0.15	<0.1	2.37	<0.1	<0.1	<0.1	<0.1
CH-11015	20	OAS, R, Co	4.22	<0.1	3.15	<0.1	10.7	0.18	0.91	<0.1	1.38
CH-11016	25	OAS, F, D, N, V	1.15	1.31	<0.1	<0.1	<0.1	0.13	0.35	<0.1	0.68
CH-11023	16	OAS, D, Dph	19.9	19.3	<0.1	2.24	<0.1	4.41	6.69	<0.1	<0.1
CH-11038	27	OAS, D	3.09	0.21	0.26	0.27	>100	3.65	1.20	3.66	9.30
CH-11040	28	U	8.43	0.74	7.48	0.15	52.8	1.20	2.90	1.54	8.84
CH-11048	18	OAS, D, Dph, Whz	41.15	<0.1	40.0	0.71	0.11	2.01	10.2	0.79	0.18
CH-11054	24	Dph, Thgt, N	1.26	0.10	0.86	<0.1	59.0	1.61	1.78	1.01	0.12
CH-11058	68	OAS, R, C, Tght, AE, F	4.24	<0.1	0.11	<0.1	1.30	5.23	1.93	12.8	<0.1
ES-22002	47	U	3.51	<0.1	3.39	<0.1	<0.1	<0.1	0.20	<0.1	<0.1
ES-22006	37	U	3.83	<0.1	3.06	<0.1	<0.1	<0.1	1.41	<0.1	<0.1
ES-22009	29	U	1.73	<0.1	1.58	<0.1	<0.1	<0.1	<0.1	<0.1	ND
ES-22012	40	U, AE	1.84	<0.1	1.90	<0.1	<0.1	<0.1	0.40	<0.1	<0.1
ES-22030	52	U, AE	0.15	<0.1	0.10	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
ES-22032	45	U	0.45	<0.1	0.67	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
ES-22033	50	U	0.46	<0.1	0.14	<0.1	<0.1	<0.1	<0.1	<0.1	ND
ES-22035	6	R, E	2.29	0.94	0.61	0.24	<0.1	1.25	0.64	1.10	4.03
DE-33001	10	OAS, U	0.32	0.24	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
DE-33002	2	U	1.08	<0.1	<0.1	0.60	15.7	0.79	1.50	<0.1	<0.1
DE-33005	7	R, C, D, N, E	32.2	18.5	11.9	1.48	86.2	1.59	3.63	0.11	<0.1
DE-33006	6	U, F, N, E	3.63	4.57	<0.1	0.25	<0.1	0.30	0.78	<0.1	<0.1
DE-33008	4	OAS, U	3.51	0.84	0.30	1.06	1.66	2.10	2.33	<0.1	ND
DE-33009	6	U, R, C	0.47	0.38	0.25	<0.1	4.30	<0.1	0.18	0.10	<0.1
DE-33010	6	OAS, U, L, D	1.20	1.06	<0.1	0.32	0.17	0.12	0.25	<0.1	<0.1
DE-33011	6	U, R, C	6.09	4.49	<0.1	1.06	13.1	2.54	5.25	<0.1	<0.1
DE-33013	1	U, Co	8.81	1.98	<0.1	7.49	0.22	2.57	5.89	<0.1	<0.1
DE-33014	2	U	1.24	1.36	<0.1	<0.1	<0.1	<0.1	0.14	<0.1	<0.1
DE-33019	10	OAS, U	5.72	4.05	0.12	1.82	0.34	2.70	4.89	<0.1	<0.1
DE-33021	7	OAS, N, E	4.42	3.26	0.22	0.15	59.8	0.47	0.47	<0.1	<0.1
DE-33022	3	OAS, U, F, Co	0.39	<0.1	<0.1	0.77	0.93	0.46	0.54	<0.1	<0.1
DE-33023	4	U, AE, F	7.10	2.82	<0.1	4.58	<0.1	2.62	3.66	<0.1	<0.1
DE-33025	11	Co, N, E	20.0	12.9	<0.1	4.00	0.22	6.23	9.56	<0.1	<0.1
DE-33026	3	U, L, C, F, Dph	0.40	0.12	<0.1	0.18	0.21	0.25	0.48	<0.1	<0.1
DE-33028	2	OAS, U, Co, Dph	0.30	0.36	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
DE-33030	5	U, AE, R, C	1.42	0.72	<0.1	0.22	3.14	0.44	0.77	<0.1	<0.1
DE-33032	4	U, AE, Co, D	1.01	1.07	0.22	0.20	45.8	<0.1	0.12	<0.1	<0.1

DE-33033	6	F, Co, D	2.94	0.69	0.19	0.94	16.7	1.38	3.35	0.73	<0.1
DE-33034	13	N, R, C	4.65	6.26	<0.1	0.16	<0.1	0.16	1.04	<0.1	<0.1
DE-33035	13	AE, U, F	7.65	2.78	0.38	2.04	6.93	4.02	8.88	<0.1	<0.1
DE-33037	4	U, AE, N	6.51	2.62	<0.1	1.01	2.45	2.46	4.74	<0.1	<0.1
>0.1 kU/L*			100%	70% (n = 28)	55% (n = 22)	60% (n = 24)	60% (n = 24)	68% (n = 27)	83% (n = 33)	20% (n = 8)	19% (n = 7)

AE, Angioedema; C, conjunctivitis; Co, cough; D, dyspnea; Dph, mild dysphagia; E, emesis; F, flush; L, laryngeal edema; N, nausea; ND, not done; R, rhinitis; Tght, tightness of throat; U, urticaria; Whz, wheezing; V, vertigo.

*%positive.

TABLE E3. Walnut-allergic patients with history of anaphylaxis (group 4): age at inclusion, and IgE sensitization pattern, as determined by ImmunoCAP (kU_A/L) testing

Patient ID	Age at inclusion (y)	Extract	rJug r 1	rJug r 3	nJug r 4	rJug r 5	nJug r vicilin-H	nJug r vicilin-L	CCD	Profilin
CH-11024	17	58.0	65.8	1.69	13.4	0.76	16.2	17.0	<0.1	<0.1
CH-11025	41	11.9	6.44	3.05	<0.1	12.3	0.30	1.10	<0.1	0.22
CH-11028	39	1.24	1.46	<0.1	0.14	<0.1	0.34	0.47	<0.1	<0.1
CH-11033	31	30.6	12.6	<0.1	6.75	<0.1	11.9	22.9	<0.1	1.73
CH-11034	37	14.7	5.45	0.12	9.17	23.3	13.5	10.3	0.44	<0.1
CH-11041	32	9.77	<0.1	8.79	<0.1	90.4	0.67	1.81	0.70	<0.1
CH-11057	26	89.1	41.7	0.75	38.5	<0.1	52.8	85.2	0.18	6.10
CH-11060	26	0.97	0.58	<0.1	0.14	4.99	0.32	0.44	<0.1	<0.1
ES-22001	33	3.60	<0.1	2.67	<0.1	<0.1	<0.1	0.50	<0.1	0.17
ES-22004	53	0.55	<0.1	0.30	<0.1	<0.1	<0.1	0.34	<0.1	<0.1
ES-22008	40	0.99	<0.1	0.75	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
ES-22017	38	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
ES-22019	38	1.97	<0.1	1.62	<0.1	<0.1	0.20	0.49	0.14	<0.1
ES-22020	49	33.2	2.79	24.1	0.10	<0.1	0.51	3.44	<0.1	<0.1
ES-22021	31	0.47	<0.1	0.32	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
ES-22031	42	5.40	<0.1	4.00	<0.1	<0.1	<0.1	0.39	<0.1	<0.1
ES-22034	12	9.01	4.26	<0.1	3.09	<0.1	5.52	8.36	<0.1	<0.1
ES-22036	14	7.50	7.03	2.30	0.12	1.08	0.44	3.12	<0.1	11.5
ES-22037	8	13.9	8.90	<0.1	2.37	<0.1	5.56	10.9	<0.1	<0.1
ES-22038	4	0.45	0.43	<0.1	0.23	<0.1	<0.1	<0.1	<0.1	ND
ES-22039	4	15.2	3.13	6.22	0.82	<0.1	2.09	4.84	<0.1	<0.1
ES-22040	3	6.09	0.10	4.46	0.20	<0.1	0.46	2.02	<0.1	<0.1
DE-33003	3	>100	95.2	<0.1	7.50	3.09	9.27	40.5	0.11	0.17
DE-33004	3	45.8	26.3	<0.1	4.87	<0.1	13.3	30.0	<0.1	0.11
DE-33007	5	31.5	19.4	<0.1	2.74	68.8	7.01	18.6	<0.1	<0.1
DE-33015	9	7.90	8.48	<0.1	0.72	1.75	1.67	4.04	<0.1	ND
DE-33017	2	8.70	9.01	0.30	0.70	10.1	1.90	0.59	0.47	<0.1
DE-33018	7	21.6	11.2	11.9	8.81	20.8	0.80	4.49	<0.1	<0.1
DE-33024	15	1.92	<0.1	<0.1	0.14	1.99	0.38	2.07	<0.1	<0.1
DE-33027	2	0.70	<0.1	<0.1	<0.1	<0.1	0.20	1.00	<0.1	<0.1
>0.1 kU/L*		97% (n = 29)	67% (n = 20)	57% (n = 17)	63% (n = 19)	40% (n = 12)	77% (n = 23)	87% (n = 33)	20% (n = 6)	25% (n = 7)

ND, Not done.

*%positive.

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