

Is Microarray Analysis Really Useful and Sufficient to Diagnose Nut Allergy in the Mediterranean Area?

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■ Abstract

Background: Component-based diagnosis on multiplex platforms is widely used in food allergy but its clinical performance has not been evaluated in nut allergy.

Objective: To assess the diagnostic performance of a commercial protein microarray in the determination of specific IgE (sIgE) in peanut, hazelnut, and walnut allergy.

Methods: sIgE was measured in 36 peanut-allergic, 36 hazelnut-allergic, and 44 walnut-allergic patients by ISAC 112, and subsequently, sIgE against available components was determined by ImmunoCAP in patients with negative ISAC results. ImmunoCAP was also used to measure sIgE to Ara h 9, Cor a 8, and Jug r 3 in a subgroup of lipid transfer protein (LTP)-sensitized nut-allergic patients (positive skin prick test to LTP-enriched extract). sIgE levels by ImmunoCAP were compared with ISAC ranges.

Results: Most peanut-, hazelnut-, and walnut-allergic patients were sensitized to the corresponding nut LTP (Ara h 9, 66.7%; Cor a 8, 80.5%; Jug r 3, 84% respectively). However, ISAC did not detect sIgE in 33.3% of peanut-allergic patients, 13.9% of hazelnut-allergic patients, or 13.6% of walnut-allergic patients. sIgE determination by ImmunoCAP detected sensitization to Ara h 9, Cor a 8, and Jug r 3 in, respectively, 61.5% of peanut-allergic patients, 60% of hazelnut-allergic patients, and 88.3% of walnut-allergic patients with negative ISAC results. In the subgroup of peach LTP-sensitized patients, Ara h 9 sIgE was detected in more cases by ImmunoCAP than by ISAC (94.4% vs 72.2%, $P < .05$). Similar rates of Cor a 8 and Jug r 3 sensitization were detected by both techniques.

Conclusions: The diagnostic performance of ISAC was adequate for hazelnut and walnut allergy but not for peanut allergy. sIgE sensitivity against Ara h 9 in ISAC needs to be improved.

Key words: Hazelnut. Lipid transfer protein. Microarray. Peanut. Walnut.

■ Resumen

Introducción: La utilidad clínica del diagnóstico por componentes no ha sido evaluada en el estudio de la alergia a frutos secos (FS).

Objetivo: Evaluar la capacidad diagnóstica de una micromatriz comercial de proteínas alérgicas en la alergia a cacahuete, avellana y nuez.

Métodos: Se determinó la sIgE en pacientes alérgicos a FS mediante la micromatriz ISAC 112, e ImmunoCAP en los pacientes con sIgE negativa frente a los componentes de ISAC. Además, se realizó ImmunoCAP frente a Ara h 9, Cor a 8 y Jug r 3 en un subgrupo de pacientes sensibilizados a LTP. La sIgE detectada por ImmunoCAP fue comparada con los rangos de ISAC.

Resultados: La mayoría de los alérgicos a cacahuete (66,7%), avellana (80,5%) y nuez (84%) estaba sensibilizados a su LTP. Sin embargo, no se detectó sIgE frente a los componentes de ISAC en el 33,3% de alérgicos a cacahuete, 13,9% de alérgicos a avellana y 13,6% de los alérgicos a nuez. El ImmunoCAP permitió detectar sIgE a Ara h 9 en 61,5%, Cor a 8 en 60% y Jug r 3 en 83,3% de los ISAC negativo. En el subgrupo LTP, ImmunoCAP (94,4%) fue superior a ISAC (72,2%) en la detección de sIgE a Ara h 9 ($p < 0,05$). La sIgE frente a Cor a 8 y Jug r 3 fue detectada de forma similar por ambas técnicas.

Conclusiones: La micromatriz ISAC es adecuada para el diagnóstico de alergia a avellana y nuez. La sensibilidad del componente Ara h 9 de ISAC debe ser mejorada.

Palabras clave: Avellana. LTP. Micromatriz. Cacahuete. Nuez.

Introduction

Tree nuts are considered to be one of the most allergenic plant foods [1] and are an important cause of anaphylaxis [2]. Peanuts are a member of the Leguminosae family, and grow underground, unlike other nuts such as walnuts. They are, however, widely consumed and frequently found together with other tree nuts such as hazelnuts and walnuts. For practical purposes, peanuts will be considered tree nuts for the purpose of this study.

The use of component-resolved diagnosis (CRD) has improved the diagnosis of food allergies, as different allergenic profiles have been reported to be related to particular types of clinical reactivity [3-5]. Furthermore, differences in allergenic profiles have been described for different geographical regions [6,7]. Lipid transfer proteins (LTPs) are the main allergens in tree nut allergy in the Mediterranean area [6,8,9], although it has been suggested that LTPs might also be markers of peach sensitization in the absence of clinical allergy [8,10]. In this scenario, multiplex platforms for the quantitative

determination of specific IgE (sIgE) are useful, since sIgE against LTPs and other nut components can be measured in a single test.

It is accepted that CRD, through protein microarrays, offers the possibility of analyzing sIgE against multiple allergens, bringing the allergist closer to more individualized diagnoses and thus allowing a more tailored approach to allergy treatment and management [11]. However, despite the widespread use of CRD in clinical practice, few studies have analyzed the clinical utility of the commercially available microarray platform—ImmunoCAP ISAC CRD112—in nut allergy [12-14], and low specificity has been identified for certain nut components such as Jug r 2 [15].

The objective of this study was to evaluate the diagnostic performance of ImmunoCAP ISAC CRD112 (ISAC) (Thermo Fisher Scientific) in the detection of sIgE against peanut, hazelnut, and walnut allergen components in a Mediterranean area. We also sought to evaluate whether measurement of molecular components not included in ISAC might improve the diagnosis of nut allergy in our area.

Material and Methods

Patients

We enrolled 39 peanut-allergic, 36 hazelnut-allergic, and 44 walnut-allergic nonpediatric patients (aged ≥ 14 years) with clear IgE-mediated allergic symptoms, a positive skin prick test (SPT), and positive sIgE determination (≥ 0.35 kU_A/L ImmunoCAP, Thermo Fisher Scientific) to the tree nut(s) triggering their allergic reaction. A detailed clinical history and a complete questionnaire were obtained from each patient regarding demographic characteristics, atopic history, and food habits. Symptoms were categorized into oral allergy syndrome, systemic symptoms, and anaphylaxis. When patients experienced more than 2 types of reactions with the same nut, the reaction associated with the severest symptoms was recorded as the most serious reaction. Eighty-one controls (37 with rhinitis and/or bronchial asthma and sensitized to dust mites but not to plant foods or pollen and 44 nonatopic controls) were also enrolled. Enrolment was performed in 14 hospitals across Spain. The study was approved by the ethics committee of Clinica Universidad de Navarra (045/2011) and this approval was supported by the ethics committees at the rest of participating hospitals.

Skin Prick Tests

SPTs were performed in all participants with commercial extracts of peanut, hazelnut, walnut, and peach (30 μ g/mL of Pru p 3) (ALK Abelló). Sodium chloride (0.9%) and histamine hydrochloride (10 mg/mL, ALK-Abelló) served as negative and positive controls, respectively. Wheals of 3 mm in diameter were considered positive, as recommended by the European Academy of Allergy Clinical Immunology guidelines [16].

Multiplex Specific IgE

Specific IgE against rAra h 1, rAra h 2, rAra h 3, rAra h 8, rAra h 9, rCor a 1.0410 (from now on referred as Cor a 1), rCor a 8, nCor a 9, rJug r 1, nJug r 2, nJug r 3, and rPru p 3, among

other components, was measured using ISAC in accordance with the manufacturer's instructions. Specific IgE values were expressed in ISAC standard units (ISU), with values of 0.3 ISU or greater considered positive. As the technique is semi-quantitative, ISU values were grouped into established ranges (<0.3 ISU, not detectable; ≥ 0.3 to <1 ISU, low; ≥ 1 to <15 , moderate; and ≥ 15 , very high) following the manufacturer's instructions.

In nut-allergic patients with a negative result to the corresponding nut components in ISAC, sensitization to panallergens and other allergen families (LTPs, profilins, PR-10 proteins, 2s albumin, 11s globulin, and 7s globulin) was also analyzed in ISAC.

Single Specific IgE Against Nut Components

In allergic patients with negative results to nut components in ISAC, sIgE against rAra h 1, rAra h 2, rAra h 3, rAra h 8, rAra h 9, rCor a 1, rCor a 8, nCor a 9, rCor a 14, Jug r 1, and rJug r 3 was measured by fluorescence enzyme immunoassay (ImmunoCAP, Thermo Fisher Scientific) following the manufacturer's instructions. Moreover, in peanut-, hazelnut-, and walnut-allergic patients with a positive SPT to Pru p 3 enriched peach extract, sIgE against Ara h 9 (peanut), Cor a 8 (hazelnut), and Jug r 3 (walnut) was measured. Specific IgE against Ara h 9, Cor a 8, and Jug r 3 was also determined by ImmunoCAP in 30 randomly selected controls (15 atopic and 15 nonatopic). Specific IgE values were quantified in kU_A/L, and values of 0.35 kU_A/L and greater were considered positive.

Specific IgE against nAra h 6 (ETSIA, Universidad Politécnica de Madrid)—an allergenic component not available in ImmunoCAP—was determined by direct ELISA as previously reported [17] in peanut-allergic patients with negative sIgE to peanut components in ISAC. The test was performed using patient sera at the corresponding dilution and rabbit anti-IgE antibody (dilution 1:5000; Biosource). Proteins were coated at 5 μ g/mL in PBS blocking solution (0.1%) (commercial digested casein SIGMA) without a solid

Table. Clinical and Demographic Characteristics of Patients and Controls

	Peanut-Allergic Patients	Hazelnut-Allergic Patients	Walnut-Allergic Patients	Nonatopic Controls	Atopic Controls
Individuals, No.	39	36	44	44	37
Age, mean (SD), y	29.7 (9.1)	29.6 (8.7)	31.1 (9.5)	47.3 (15.2)	39.6 (14.2)
Male sex, %	28.2	27.7	36.4	25	37.8
Symptoms caused by triggering nut, No. (%)					
Oral allergy syndrome	13 (33.3)	13 (36.1)	17 (38.6)	0	0
Systemic symptoms	16 (41)	13 (36.1)	16 (36.4)		
Anaphylaxis	10 (25.6)	10 (27.7)	11 (25)		
sIgE against nut, median (25-75 percentile), kU _A /L	1.31 (0.75-5.9)	1.96 (1.03-7.18)	1.73 (1-4.81)	ND	ND
Positive Pru p 3 enriched peach extract SPT, No. (%)	33 (84.6)	32 (88.9)	38 (86.4)	0	0

Abbreviations: ND, not determined; sIgE, specific IgE; SPT, skin prick test.

phase. Bovine serum albumin as a solid phase or a serum pool from healthy individuals was used as a negative control in the different studies and optical density (OD) values greater than mean [OD] + 3 x SD with respect to the negative control were considered positive. Serum dilution was determined by titration curves.

Statistical Analysis

Quantitative variables were described as means (SD) or as medians and interquartile ranges (25-75 percentile) when data distribution was not normal. Normality was assessed by the Shapiro-Wilk test. Qualitative variables were described as frequencies (percentages), and proportions were compared using the χ^2 test. All statistical analyses were performed using Stata/IC 12.0. Differences were considered statistically significant when the *P* value was less than .05.

Results

Nut-Allergic Patients

We enrolled 66 patients with IgE-mediated allergic symptoms to peanut (n=39), hazelnut (n=36), or walnut (n=44) with a positive SPT and sIgE to the corresponding tree nut(s). Eighteen patients were allergic to the 3 nuts, and 46 (69.7%) were also allergic to peach. The demographic and clinical data for the patients and the 37 atopic and 44 nonatopic controls are summarized in the Table.

Protein Microarray Sensitization Profile of Nut-Allergic Patients

The ISAC microarray results showed that 66.6% of peanut-allergic patients, 80.5% of hazelnut-allergic patients,

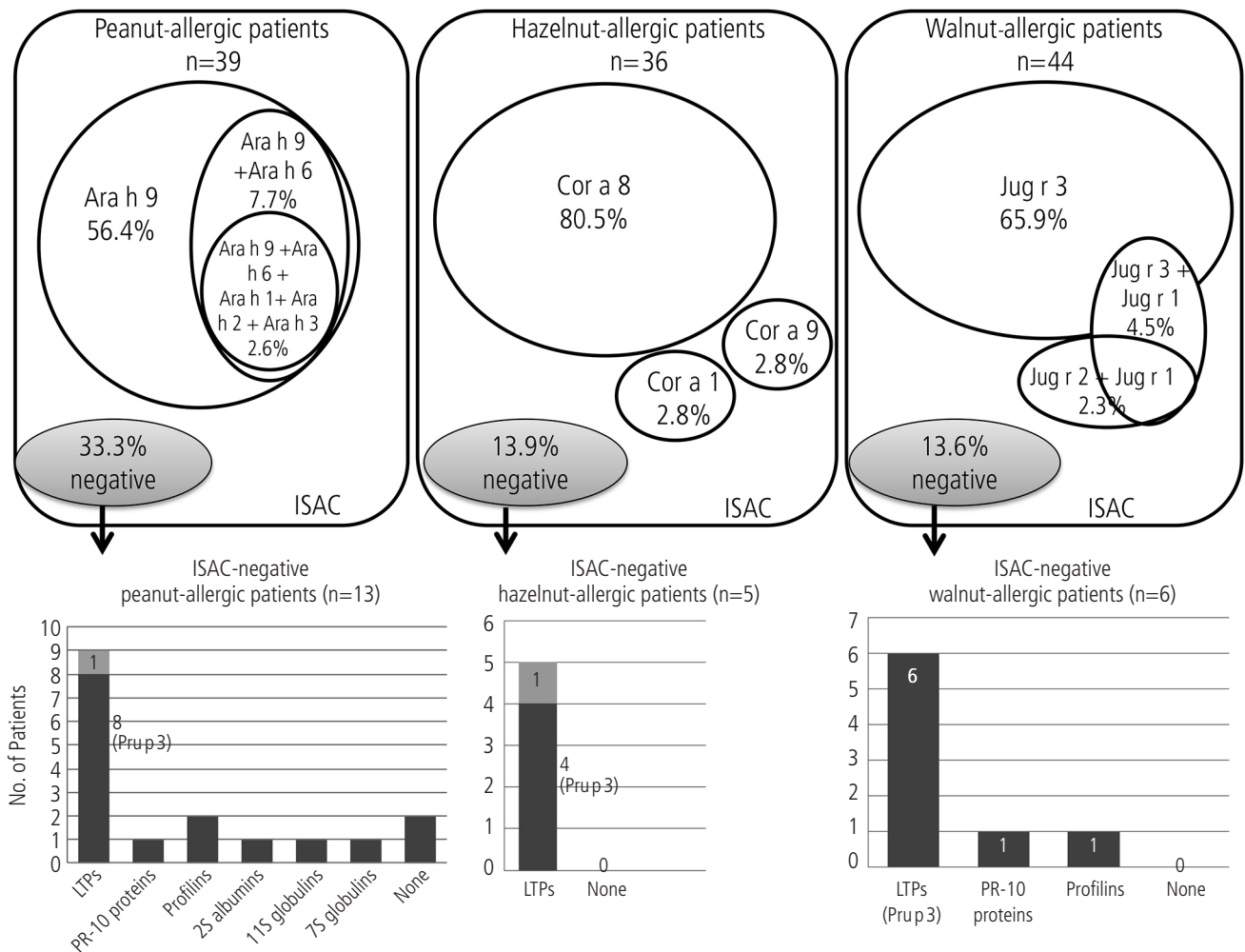


Figure 1. Sensitization profile of nut-allergic patients according to ImmunoCAP ISAC CRD112. Upper panel, summary of ISAC data regarding sensitization to nut allergens. Lower panel, summary of ISAC data regarding sensitization to panallergens and allergen families different from nuts. The number of patients sensitized to Pru p 3 is shown in dark grey. LTP indicates lipid transfer protein.

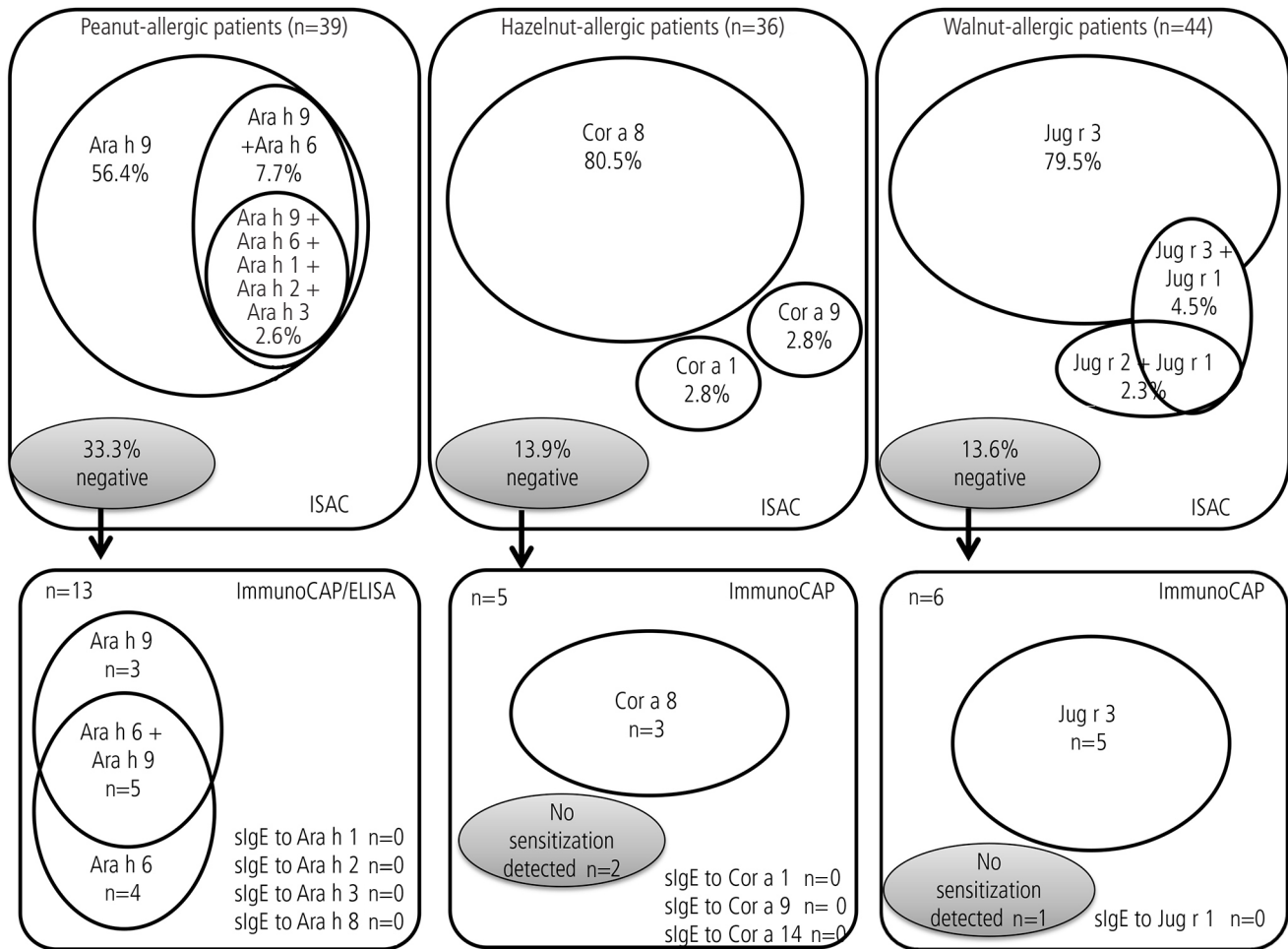


Figure 2. Sensitization profile of nut-allergic patients according to ImmunoCAP. Upper panel, summary of ISAC data regarding sensitization to nut allergens. Lower panel, summary of ImmunoCAP data regarding sensitization to nut components in nut-allergic patients with negative results to nut components in ISAC. sIgE indicates specific IgE.

and 70.4% of walnut-allergic patients were sensitized to the corresponding LTP (Ara h 9, Cor a 8, and Jug r 3, respectively) and that a small proportion of patients were also sensitized to other allergens. One third of peanut-allergic patients ($n=13$), 13.9% of hazelnut-allergic patients ($n=5$), and 13.6% of walnut-allergic patients ($n=6$) did not show sIgE against any of nut components in ISAC. Figure 1 summarizes the sensitization profiles of nut-allergic patients according to the protein microarray results. None of the controls were sensitized to peanut components; 1 was sensitized to Cor a 8, 1 to Jug r 2, and 2 to Jug r 3.

Other Sensitizations in Nut-Allergic Patients With Negative ISAC Results

Panallergen sensitization (profilin, PR-10 proteins, 2s albumin, 11s globulin, and 7s globulin) was assessed in nut-allergic patients with negative results to the nut components in ISAC.

Sensitization to LTPs was common among nut-allergic patients without detectable sensitization to the triggering

nut in ISAC. This sensitization was detected in 9 of the 13 patients with peanut allergy, in 5 of the 5 patients with hazelnut allergy, and in 6 of the 6 patients with walnut allergy. Most of the patients were sensitized to Pru p 3, and only a few were sensitized to PR-10 proteins (1/13 peanut-allergic and 1/6 walnut-allergic patients), profilins (2/13 peanut-allergic and 1/6 walnut-allergic patients), or storage proteins (1/13 peanut-allergic patients to 2s albumin, 1/13 peanut-allergic patients to 11s globulins, and 1/13 peanut-allergic patients to 7s globulin). Only 2 peanut-allergic patients showed no sensitization to peanut components or panallergens/storage proteins by ISAC. These results are summarized in Figure 1.

Sensitization as Assessed by Alternative Techniques in ISAC-Negative Nut-Allergic Patients

sIgE against Ara h 1, Ara h 2, Ara h 3, Ara h 8 and Ara h 9 using ImmunoCAP and Ara h 6 using ELISA was measured in the 13 peanut-allergic patients in whom peanut sensitization was not detected by ISAC. ImmunoCAP showed sIgE against Ara h 9 in 8 of these patients. Of these 8 patients, 5 were also

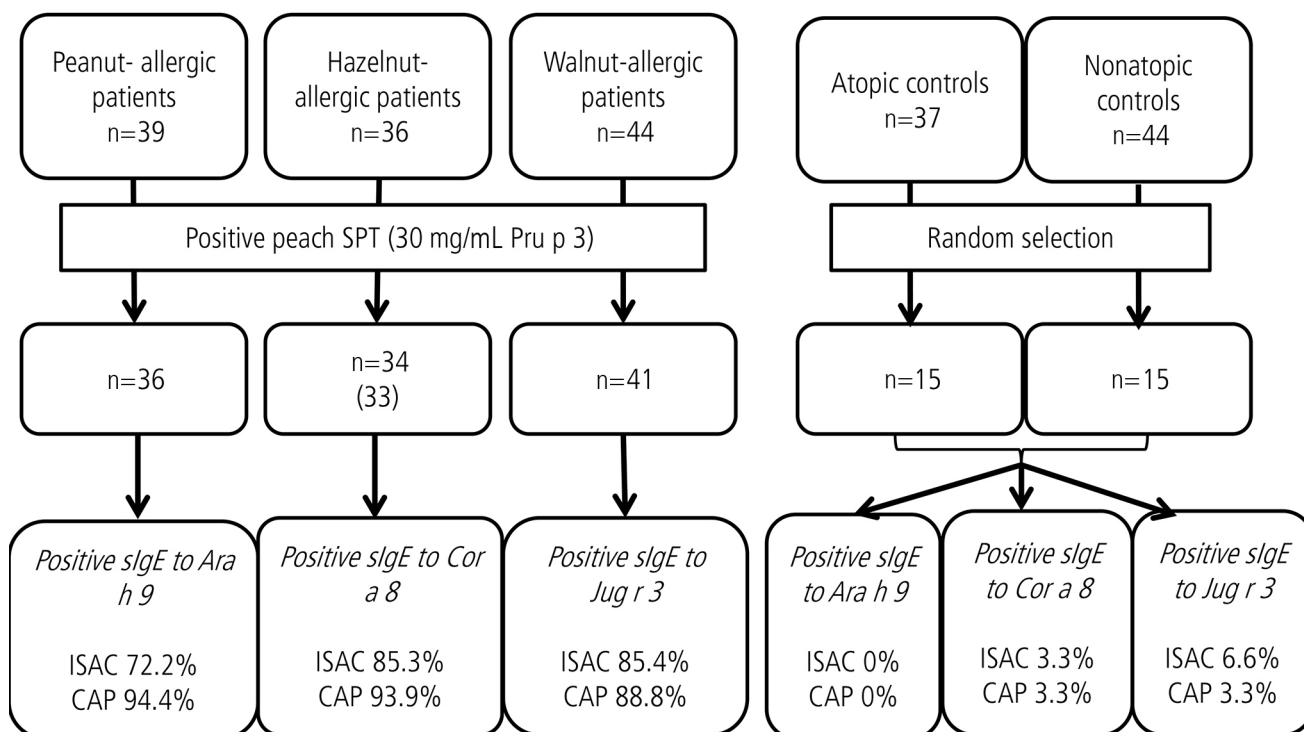


Figure 3. Specific IgE against nut LTPs according to CAP and ISAC in peach-sensitized peanut-, hazelnut-, walnut-allergic patients. Patients with serum available for ImmunoCAP determination are shown in brackets. SPT indicates skin prick test; sIge, specific IgE.

sensitized to Ara h 6 according to ELISA. Four of the 13 patients were only sensitized to Ara h 6 according to alternative techniques. No sensitization was detected to Ara h 1, Ara h 2, Ara h 3, or Ara h 8 by ImmunoCAP in any of the ISAC-negative peanut-allergic patients.

sIgE against Cor a 1, Cor a 8, Cor a 9, and Cor a 14 was determined by ImmunoCAP in the 5 hazelnut-allergic patients in whom hazelnut sensitization was not detected by ISAC. Three patients were found to be sensitized to Cor a 8. No sensitization was detected for Cor a 1, Cor a 9, or Cor a 14 by CAP in any of the 5 ISAC-negative hazelnut-allergic patients.

sIgE against Jug r 1 and Jug r 3 was measured by ImmunoCAP in the 6 walnut-allergic patients in whom no walnut sensitization was detected by ISAC. ImmunoCAP showed 5 of the patients to be sensitized to Jug r 3 and none of the patients to be sensitized to Jug r 1. These data are summarized in Figure 2.

Diagnostic Performance of the ISAC Microarray for Nut LTPs

In order to assess the diagnostic performance of peanut, hazelnut, and walnut LTPs in ISAC, LTP-sensitized nut-allergic patients were selected from our sample together with 30 controls (15 nonatopic controls and 15 atopic dust mite-allergic patients without plant allergen sensitization) randomly selected from the control group. LTP sensitization was defined as a positive SPT to Pru p 3 enriched peach. Serum sIgE against peanut, hazelnut, and walnut LTPs was measured by ImmunoCAP. The flow chart of this subgroup is presented

in Figure 3. sIgE against Ara h 9, Cor a 8, and Jug r 3 using ImmunoCAP and the ISAC microarray was measured in all patients and controls and then compared.

Serum sIgE against Ara h 9 was detected in more peanut-allergic peach-sensitized patients by ImmunoCAP (94.4%, 34/36) than by ISAC (72.2%, 26/36) (2-tailed $P=0.011$). However, sIgE to Cor a 8 and to Jug r 3 was detected to a similar degree by both techniques in hazelnut-allergic peach-sensitized patients (ISAC 85.3% [29/34] vs ImmunoCAP 93.9% [31/33], 2-tailed $P=0.247$) and walnut-allergic peach-sensitized patients (ISAC 85.4% [35/41] vs ImmunoCAP 87.8% [36/41], $P=0.746$). Few controls showed sIgE against Ara h 9 (0/30 by ISAC and by ImmunoCAP), Cor a 8 (1/30 by ISAC and by ImmunoCAP), or Jug r 3 (2/30 by ISAC and 1/30 by ImmunoCAP).

Specific IgE against nut LTPs using ImmunoCAP was compared with sIgE ranges obtained using ISAC. The data are shown in Figure 4.

Discussion

We confirmed in a well-defined group of patients allergic to peanut, hazelnut, and walnut that LTP is the main allergen in nut allergy, as has been previously reported in the Mediterranean area [6-8,10]. It is important to highlight that this study did not include children. This exclusion criterion may explain why only a few of the individuals analyzed were sensitized to common major allergens (storage proteins), since sensitization profiles have been reported to differ according to age [18-20]. The differential profile we observed

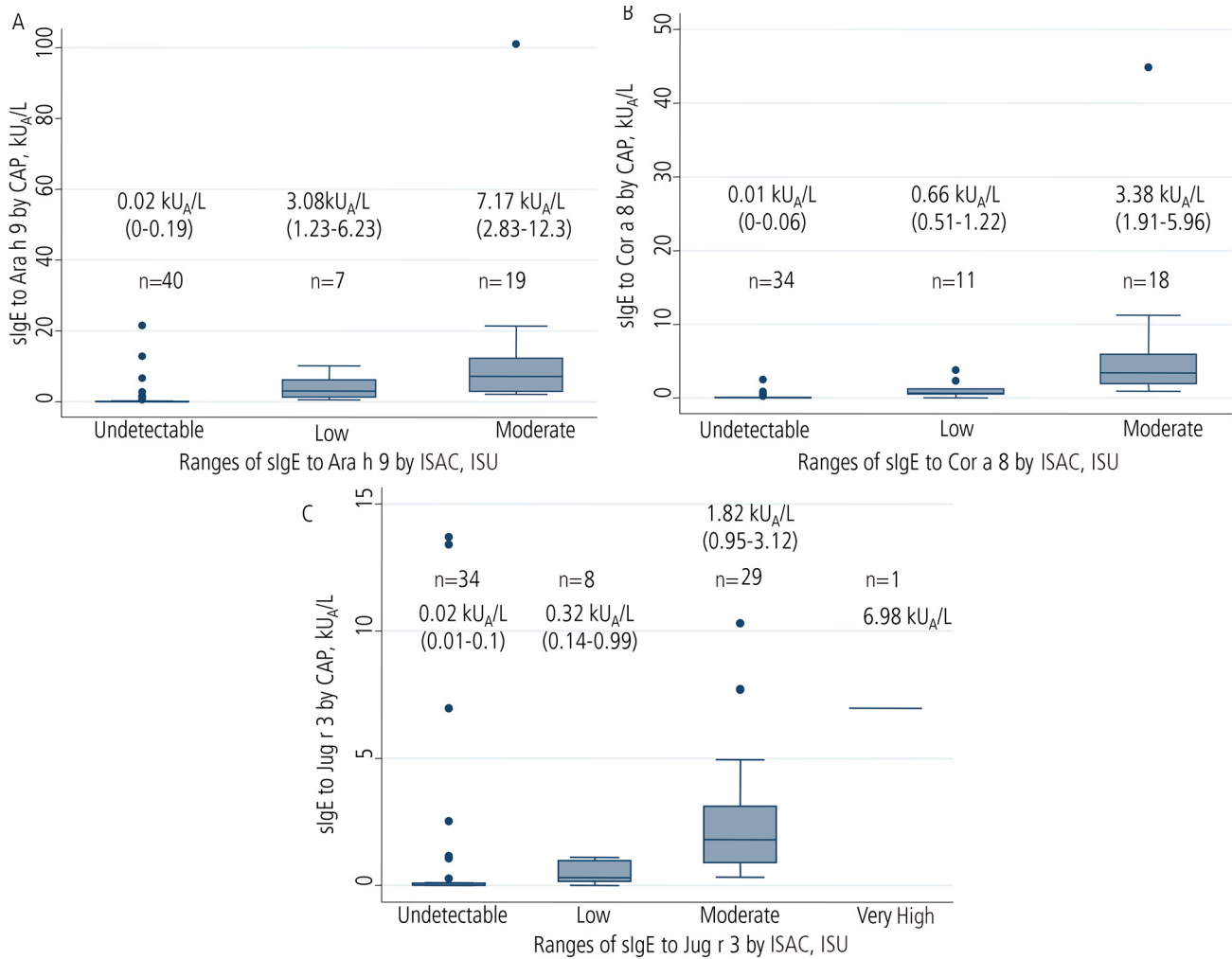


Figure 4. Specific IgE (sIgE) against nut lipid transfer proteins by CAP regarding ISAC sIgE ranges in peach-LTP-sensitized peanut-, hazelnut-, walnut-allergic patients.

with regard to other series may also have been influenced by geographic location [6,20].

We also observed that not all nut-allergic patients were diagnosed by the ISAC protein microarray, since 33.3% of peanut-allergic patients (13/39), 13.9% of hazelnut-allergic patients (5/36), and 13.6% walnut-allergic patients (6/44) did not show any sIgE against proteins from the nut responsible for their symptoms. Interestingly, Ara h 9 sensitization was detected by the consolidated ImmunoCAP in 8 of the 13 peanut-allergic patients with ISAC-negative results, suggesting that ISAC performs poorly in the detection of peanut LTP sensitization.

To assess the diagnostic performance of ISAC for the detection of sIgE to peanut, hazelnut, and walnut LTPs, we selected a subgroup of LTP-sensitized nut-allergic patients and controls. Based on the sIgE results from ImmunoCAP and ISAC against Ara h 9, Cor a 8, and Jug r 3 in this sample we can conclude that the sensitivity of the peanut LTP Ara h 9 needs to be improved in the ISAC platform, since ImmunoCAP outperformed ISAC in diagnosing cases of sensitization to Ara h 9 in peanut-allergic patients. Furthermore, this low

diagnostic capacity in ISAC cannot be explained by low levels of sIgE, since according to ISAC, considerable sIgE values as determined by ImmunoCAP (including values >20 kU_A/L for Ara h 9) were recorded as undetectable (Figure 4A). Moreover, these discrepancies in sIgE values between ISAC and ImmunoCAP were also observed for Cor a 8 (Figure 4B) and Jug r 3 (Figure 4C). In the case of Jug r 3, the recombinant form is used in ImmunoCAP, while the natural form is used in ISAC. However, in the case of Ara h 9 and Cor a 8, according to the manufacturer's information, the recombinant forms of both these proteins are used in both tests. These data suggest that Ara h 9 and Cor a 8 underwent a different folding process in the ISAC slide than in the ImmunoCAP polymer and this may have affected IgE binding.

Because Pru p 3 shows high cross-reactivity with Ara h 9 [21] and Jug r 3 [9], in addition to significant cross-reactivity with Cor a 8 [22], in the Mediterranean area, the inclusion of this LTP in nut CRD diagnosis by ISAC helps to detect otherwise ISAC-negative patients. In fact, in ISAC, positive sIgE against specific nut components in addition to Pru p 3 diagnosed 87.2% (34/39) of all peanut-allergic

patients, 97.2% (35/36) of all hazelnut-allergic patients, and 100% (44/44) of all walnut-allergic patients compared with positive sIgE against specific nut components only (peanut, 26/36 [72.2%], hazelnut, 31/36 [86.1%], walnut, 38/44 [86.4%]). Although it has been suggested that a limited set of well-validated sensitization markers rather than a panel of components for CRD can facilitate allergy diagnosis [23], Pru p 3 sensitization does not determine clinical reactivity to nuts as has been previously reported [10]. In conclusion, after confirming that LTP is the main allergen in peanut-, hazelnut-, and walnut-allergic patients in our area, we demonstrated that the sensitivity of the ISAC protein microarray can vary between certain allergens. In particular, the sensitivity of certain LTPs, such as the peanut LTP Ara h 9, needs to be improved in ISAC.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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