ORIGINAL ARTICLE

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

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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/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 mg/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 h1, rAra h2, rAra h3, rAra h8, rAra h9, rCor 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 h1, rAra h2, rAra h3, rAra h8, rAra h9, 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 h9 (peanut), Cor a 8 (hazelnut), and Jug r 3 (walnut) was measured. Specific IgE against Ara h9, 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 kUA/L, and values of 0.35 kU/L and greater were considered positive.

Specific IgE against nAra h6 (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 μg/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)                   | 10                          | 11 (25)                     |
| Anaphylaxis                 | 10 (25.6)                   | 10 (27.7)                   | 11 (25)                     |                             |                             |
| sIgE against nut, median (25-75 percentile), kU/L |                       |                             |                             |                             |                             |
| Positive Pru p 3 enriched peach extract SPT, No. (%) | 1.31 (0.75-5.9)            | 1.96 (1.03-7.18)            | 1.73 (1-4.81)               | ND                          | ND                          |
| 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 $\chi^2$ 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,
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 Prp 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...
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 \( P = .011 \)). 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 \( P = .247 \)) and walnut-allergic peach-sensitized patients (ISAC 85.4% [35/41] vs ImmunoCAP 87.8% [36/41], \( P = .746 \)). 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...
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/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.

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.
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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