

ORIGINAL ARTICLE

Can component-based microarray replace fluorescent enzimoimmunoassay in the diagnosis of grass and cypress pollen allergy?

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Clinical & Experimental Allergy

Summary

Background Few data on the diagnostic accuracy in pollinosis of the microarray ISAC of allergens are available.

Objective We aim to comparatively analyse ISAC CRD103 with the whole-extract ImmunoCAP in grass and cypress pollen allergy, evaluating the suitability of the manufacturer's recommended cut-off points for both techniques.

Methods We studied 120 atopic patients grouped into grass and cypress pollen-allergic patients and controls based on clinical history and skin prick tests. Specific IgE against *Phleum pratense* and *Cupressus arizonica* by ImmunoCAP and ISAC CRD103 were performed on all subjects.

Results In the grass pollen group (43 allergic/26 controls), both microarray and CAP showed high sensitivity (Se) and specificity (Sp) values (ISAC: Se 97.7, Sp 92.3; CAP: Se 95.3, Sp 96.1) for recommended cut-off points. Comparing the optimal (ISAC: 0.4 ISU; CAP: 0.33 kU/L) with the recommended cut-off points within the same technique, diagnostic agreement was observed in both techniques. Thus, CAP and ISAC showed similar diagnostic performance in grass pollen allergy when using recommended cut-off points. In cypress pollen group (12 allergic/92 controls), the microarray (Se: 91.7, Sp 91.3) showed similar Se but significantly higher Sp ($P=0.034$) than CAP (Se: 91.7, Sp: 80.4) using recommended cut-off points. However, although diagnostic performance of the microarray did not change when comparing the optimal (0.82 ISU) with the recommended cut-off point, CAP improved diagnosis of cypress pollen allergy, when applying the optimal (0.66 kU/L) (CAP Se: 91.7, Sp: 89.1) instead of the manufacturer's recommended cut-off point. Thus, when the most suitable cut-off point for both techniques (ISAC: 0.3 ISU; CAP: 0.66 kU/L) is selected, microarray and CAP provide equivalent diagnoses.

Conclusions and Clinical Relevance Component-based microarray ISAC CRD103 and whole-allergen CAP showed high Se and Sp diagnosing equally grass and cypress pollen allergy. The cut-off point for each allergen should be properly applied for both techniques.

Keywords allergen microarray, component-resolved diagnosis, cut-off point, cypress pollen, grass pollen, in vitro test, specific IgE

Submitted 10 February 2011; revised 30 May 2011; accepted 2 June 2011.

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Introduction

Component-resolved diagnosis (CRD) has improved the diagnosis of pollinic patients allowing a more precise diagnosis of sensitizations [1–5]. In recent years, a micro-

array technique for the detection of specific IgE (sIgE) against recombinant or purified natural allergen components [6–9] has been commercialized. However, since its launch some years ago, few studies have been published analysing its validity as a diagnostic technique in pollinosis and its contribution to the techniques used until now. Wohrl et al. [10] analysed the sensitivity (Se) and

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specificity (Sp) of the ISAC version CRD50, for some microarrayed components from *Betula verrucosa*, *Phleum pratense* and *Artemisia vulgaris*. They concluded that both Se and Sp using an optimal cut-off point did not differ clearly from those obtained by the fluoroenzyme immunoassay ImmunoCAP. However, these data were not assessed using the manufacturer's recommended cut-off point. In addition, in recent years, the panel used in this technique has improved, increasing both the number of components per pollen and including new allergens.

Thus, we aimed to study (i) the Se and Sp, of the component microarray ISAC version CRD103 in the diagnosis of allergy to two Mediterranean pollens (*P. pratense* and *Cupressus arizonica*) by comparing the results with those obtained by the whole-extract CAP; (ii) the suitability of the manufacturer's recommended cut-off points for both techniques; and (iii) the diagnostic agreement between the two techniques selecting the most suitable cut-off point.

Methods

Patients

We studied 120 atopic patients (4–73 years old; 50% males) with rhinoconjunctivitis and/or bronchial asthma enrolled from March 2008 to May 2009 from the Clínica Universidad de Navarra in Pamplona, Spain. Patients came from different areas of Spain and all were atopic with allergic rhinoconjunctivitis and/or bronchial asthma [at least one positive skin prick test (SPT) to a battery of aeroallergens]. Allergic patients were included in the study if they had (i) an allergen-specific history (rhinitis, rhinoconjunctivitis and/or bronchial asthma) during the season of pollinization of grass pollen and/or cypress pollen and (ii) a positive SPT to the corresponding pollen, *P. pratense* and/or *C. arizonica*. Data on pollinization in different areas of Spain were checked in www.polenes.com. There was no overlap between pollinization seasons, which were, in general, May–June for grass pollen and January–March for cypress pollen. Patients showing clinical history during the season of pollinization of grass pollen and showing SPT-positive to grass pollen and to other pollen that pollinated in the same season (i.e. olive pollen) were excluded.

Controls had no pollen allergen-specific history and had negative SPT to the corresponding pollen. A subject could be considered an allergic patient or a control to one or both of the two pollens whenever the two above mentioned criteria were met. Patients who do not fulfil above mentioned criteria to be included in each group were excluded.

Skin prick tests

SPT were performed in all patients with commercial, natural extracts (ALK Abelló, Madrid, Spain) by puncture

with a standard 1 mm-tip lancet (ALK Abelló) on the volar side of the forearm. Sodium chloride (0.9%) and histamine hydrochloride (10 mg/mL, ALK Abelló) served as negative and positive controls, respectively. SPTs were read after 20 min. Weals of 3 mm diameter were considered positive as recommended by the EAACI guidelines [11]. SPTs were always performed by the same experienced nurses.

Specific immunoglobulin E against the whole extract

sIgE was determined against *P. pratense* and *C. arizonica* in both allergic patients and controls, with a fluorescence enzyme immunoassay CAP (ImmunoCAP FEIA, Phadia, Uppsala, Sweden). Values equal to or above 0.35 kU/L were considered positive as recommended by the manufacturer.

Component-resolved diagnosis microarray

Serum sIgE quantification was performed against allergenic natural (n) or recombinant (r) components from *P. pratense* (rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5, rPhl p 6, rPhl p 7, rPhl p 11, rPhl p 12) and *C. arizonica* (nCup a 1) among other allergens from different sources by the allergen microarray immunoassay ISAC version CRD103, (Phadia, Uppsala, Sweden) on all the individuals included in the study.

Serum sIgE detection by allergen microarray was performed as recommended by the manufacturers and was based on previously described protocols by Deinhofer et al. [8] and Harwanegg and Hiller [12].

As 0.3 ISU was the cut-off point recommended by the manufacturer, the microarray technique was considered to yield a positive result for cypress pollen when sIgE values against nCup a 1 were ≥ 0.3 ISU, as this is the only component from *C. arizonica* included in the ISAC CRD103 microarray, and when the results from at least one of the components included in the microarray for grasses were ≥ 0.3 ISU.

Statistical analysis

Quantitative variables were described as mean (standard deviation) or median (25th and 75th percentiles), depending on whether distribution of the data were normal or not, while qualitative variables were described as frequency (percentage). Means between the two groups were compared using the Student-*t*-test or the Mann-Whitney U-test, while χ^2 (or Fisher's exact test when needed) was used for comparing proportions.

The diagnostic accuracy of both the CAP system and microarray was evaluated by means of Se and Sp for the manufacturer's recommended cut-off point (0.3 ISU) and for the optimal cut-off point, considering which one offered the maximum total of Se and Sp while maintaining Sp values around 90%, calculated *ad hoc*.

The diagnostic accuracy of the CAP system for the two pollens and of the microarray for nCup a 1 was evaluated by means of Se and Sp for the optimum cut-off points using receiver-operating characteristics (ROC) curves plotted for each allergen separately. Se and Sp values of the microarray for grass pollen were obtained from contingency tables for different cut-off points, the same for all the microarrayed allergens of grass pollen, ranging between 0 and 1 ISU, every 0.05 ISUs. The optimal cut-off point was selected among these. Limits for confidence intervals (CI) were obtained by the standard exact method when required.

Diagnostic agreement between techniques for both manufacturer's recommended and optimal cut-off points, as well as the diagnostic performance comparing both cut-off points in each of the techniques, was analysed using the McNemar test for paired samples.

All statistical calculations were performed using the SPSS software v.15.0 (Chicago, IL, USA) and MedCalc v.9.0 (MedCalc Software, Mariakerke, Belgium) was used for the ROC analysis. *P*-values <0.05 were regarded as significant.

Results

Demographic and clinical characteristics of the patients

The grass pollen group consisted of 69 subjects (43 allergic and 26 controls) and cypress pollen group of 104 subjects (12 allergic and 92 controls). Patient characteristics are shown in Table 1. There were no significant differences between allergic and control patients with the exception of conjunctivitis, which was significantly more frequent in allergic patients than in controls. Rhinitis and conjunctivitis were the dominating symptoms in the allergic and control patients. Around a third of all allergic patients and controls reported additional bronchial asthma.

Table 1. Clinical characteristics of allergic patients and controls

	Grass pollen		Cypress pollen	
	Allergic patients	Controls	Allergic patients	Controls
Number of cases	43	26	12	92
Gender [<i>n</i> (%)]				
Male	21 (48.8)	10 (38.5)	4 (33.3)	46 (50)
Female	22 (51.2)	16 (61.5)	8 (66.7)	46 (50)
Age	29	26.5	31.5	29
[<i>m</i> (p25–p75)]	(20–37)	(17–35)	(21–44)	(19.5–38)
Symptoms [<i>n</i> (%)]				
Rhinitis	40 (93.0)	22 (84.6)	12 (100)	82 (89.1)
Conjunctivitis	38 (88.4)	15 (57.7)	12 (100)	62 (67.4)
Bronchial asthma	15 (34.9)	7 (26.9)	3 (25)	2 (34.8)

n (%), number of cases (percentage); *m* (p25–p75), median (25th and 75th percentile).

Sensitization profile

In the grass pollen group, the allergic patients showed significantly higher levels ($P < 0.001$) of specific IgE against *P. pratense* detected by ImmunoCAP [8.38 (3.47–30.70) kU/L], than controls [0.01 (0–0.05) kU/L]. In addition, grass-allergic patients were sensitized to rPhl p 1 (79.1%) and to nPhl p 4 (79.1%), followed by rPhl p 5 (69.8%), rPhl p 2 (62.8%), rPhl p 6 (58.1%), with rPhl p 12 (25.6%), rPhl p 11 (20.9%) and rPhl p 7 (11.6%) playing a minor role. None of the patients allergic to grass pollen were sensitized exclusively by means of panallergens (profilin rPhl p 12 and/or polcalcin rPhl p 7). Only one control of the grass group was sensitized to rPhl p 1 and another to rPhl p 11.

In the cypress pollen group, allergic patients showed significantly higher levels ($P = 0.002$) of specific IgE against *C. arizonica* by ImmunoCAP [2.22 (1.29–6.71) kU/L] than controls [0.04 (0.01–0.17) kU/L]. In addition, 91.7% of cypress-allergic patients and also 8.7% of controls were sensitized to nCup a 1.

Diagnostic accuracy and agreement between whole-extract CAP and ISAC CRD103 for the manufacturer's recommended cut-off point

The ISAC CRD103 showed high Se and Sp values for grass pollen and these values were similar to those for the whole allergen CAP using the manufacturer's recommended cut-off points (Table 2). In addition, there was diagnostic agreement between both techniques when the manufacturers' recommendations were followed.

The microarray showed high Se and Sp values in cypress pollen using the manufacturer's recommended cut-off point. Comparing the microarray data with the whole allergen CAP, Se was similar for cypress pollen while Sp was significantly higher for microarray cypress allergen nCup a 1 (proportions difference 10.9, 95%CI = 9.3–20.8; χ^2 , $P = 0.03$) (Table 2). These differences affect the diagnostic agreement as both techniques diagnosed statistically different ($P = 0.013$) cypress pollen allergy when the manufacturers' recommendations were followed.

Selection of the most suitable cut-off point for whole-extract CAP and ISAC CRD103

The optimal cut-off point for grass pollen microarrayed allergens in the ISAC CRD 103 technique was 0.4 ISU and 0.33 kU/L for grass pollen in CAP (Fig. 1). The Se and Sp values for both techniques, applying the optimal cut-off points, are summarized in Table 2. In order to analyse if raising the cut-off point to the optimal level produced a significant modification in the diagnosis of grass pollen

Table 2. Sensitivity (Se) and specificity (Sp) values for microarray ISAC CRD103 and CAP for the manufacturer's recommended and optimal cut-off point

	Manufacturer's recommended cut-off point		Manufacturer's recommended cut-off point	
	Microarray	CAP	Microarray	CAP
Grass pollen				
Cut-off point	0.3	0.35	0.4	0.33
Se	97.7	95.3	95.3	95.3
(95%CI)	(87.7–99.9)	(84.2–99.4)	(84.2–99.4)	(84.2–99.3)
	$P = 1$			
Sp	92.3	96.1	96.1	96.1
(95%CI)	(74.9–99.0)	(80.4–99.9)	(80.4–99.9)	(80.3–99.4)
	$P = 1$			
Cypress pollen				
Cut-off point	0.3	0.35	0.82	0.66
Se	91.7	91.7	91.7	91.7
(95%CI)	(61.5–99.8)	(61.5–99.8)	(61.5–99.8)	(61.5–98.6)
	$P = 1$			
	$P = 1$			
Sp	91.3	80.4	95.6	89.1
(95%CI)	(85.5–97.1)	(72.3–88.5)	(91.5–99.8)	(80.9–94.7)
	$P = 0.03$			
	$P = 0.47$			

The most suitable cut-off points, recommended by the authors, are presented in a darker background.

CI, confidence interval; p values show diagnostic accuracy of comparison of both techniques assessed by proportion differences (χ^2).

allergy, the diagnostic performance comparing both cut-off points was analysed within the same technique. Grass pollen CAP ($P = 1$) and microarray techniques ($P = 0.5$) showed similar diagnostic performance when we compared the recommended with the optimal cut-off point, suggesting that the manufacturer's recommended cut-off points for CAP and microarray, in grass pollen allergy diagnosis, seem to be correct.

The optimal cut-off point for cypress pollen was slightly higher in the microarray (0.82 ISU) and in the CAP (0.66 kU/L) with respect to those recommended by the manufacturer (Fig. 1). However, when the optimal and the recommended cut-off points were compared, microarrayed nCup a 1 in ISAC CRD 103 diagnosed in a similar way ($P = 0.125$), while CAP diagnosed cypress pollen-allergic patients differently ($P = 0.008$). These data suggest that, although there was no better performance of the microarrayed nCup a 1 when the optimal cut-off point is applied, CAP with cypress whole extract offered a better diagnostic performance when the cut-off point is raised to 0.66 kU/L, improving Sp.

Diagnostic accuracy and agreement between whole-extract CAP and ISAC CRD103 adjusting the cut-off points

As the most suitable cut-off point for grass pollen allergy in CAP and microarray ISAC CRD103 are those recommended by the manufacturer, both techniques showed similar diagnostic accuracy and agreement, as has been reported above.

However, given that the cut-off point for cypress whole-extract CAP should be raised to 0.66 kU/L, the comparison between the CAP and microarray techniques was assessed using the manufacturer's recommended cut-off point for the microarray, and the optimal cut-off point for the whole-extract CAP. In this case, Se and Sp values were similar (Table 2) and there was diagnostic agreement between the CAP and microarray ISAC CRD103 techniques in cypress pollen allergy.

Discussion

The Se and Sp of the component-based allergen-microarray were analysed in detecting clinically relevant

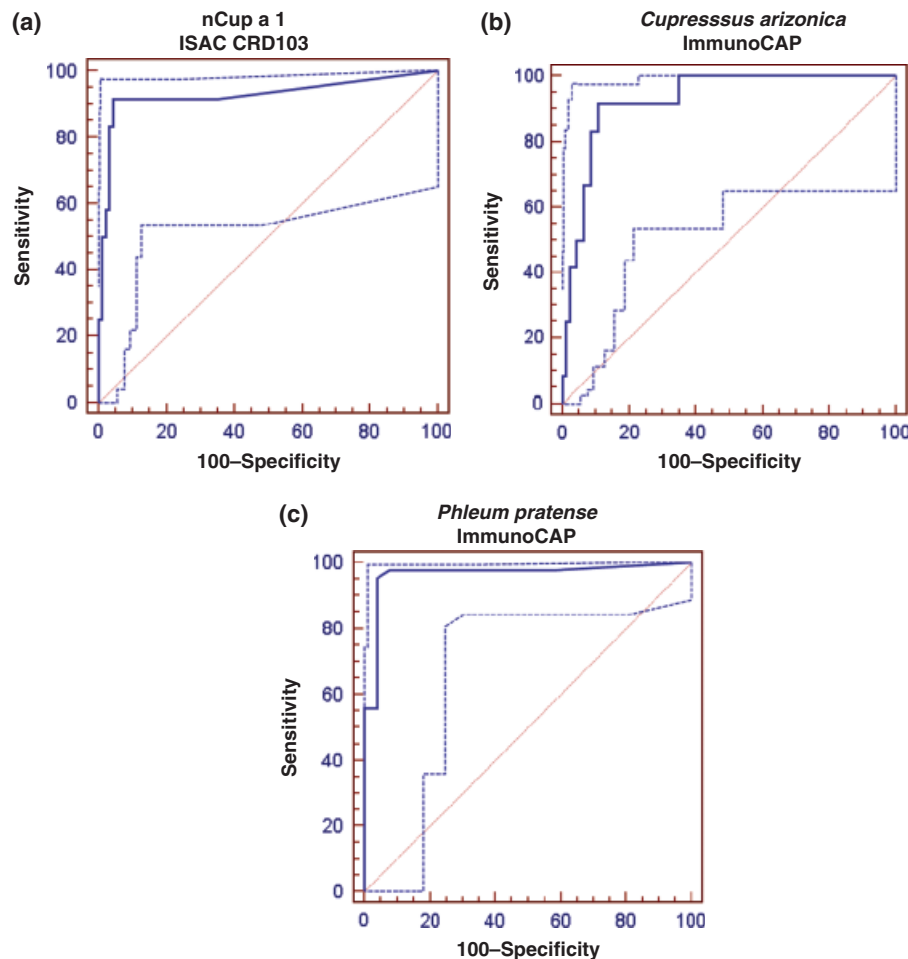


Fig. 1. Receiver-operating characteristics (ROC) curves for (a) nCup a 1 in ISAC CRD103, (b) *Cupressus arizonica* ImmunoCAP, (c) *Phleum pratense* ImmunoCAP. Continuous lines show the ROC curve and dotted lines show the 95% confidence intervals.

allergies to the aeroallergens grass and cypress pollen. This diagnostic technique was also compared with the whole-extract CAP system for these two pollens.

Our data suggest that using the manufacturer's recommended cut-off points for the microarray yields similar results to the CAP for the diagnosis of grass pollen allergy and that the microarray outperforms the CAP in the diagnosis of cypress pollen allergy as it offers greater Sp. This could be due to a subclinical sensitization of the controls that should be monitored or due to the cross-reactivity with other allergens present in the CAP, which are also found in other allergenic sources. However, 12 cypress controls showed positive results in CAP and negative results in ISAC CRD103 and only 7 out of 12 controls showed sensitization (sIgE) against the grass pollen profilin and two of them also against grass pollen polcalcin. Nevertheless, these results must be interpreted with caution, given the small number of the cypress-allergic patients included in this study.

On the basis of our results, we recommend raising CAP cypress cut-off point to 0.66 kU/L, while there was no

evidence to raise the manufacturer's cut-off point of microarrayed nCup a 1 in ISAC CRD 103 technique. Thus, both techniques provide equivalent diagnosis of cypress pollen allergy when the most suitable cut-off point is applied. Therefore, these data confirm that the whole-extract CAP and component microarray ISAC CRD103 techniques are interchangeable in terms of diagnostic capacity in grass and cypress pollen allergy. However, in our opinion, cost effectiveness requires relegating the multiplex molecular diagnosis technique only to polysensitized patients.

It is important to note, as we observed in this study, that cut-off points should be considered in such everyday techniques as the CAP in order to offer better Se and Sp values as has been described by other authors regarding specific IgE against food allergens [13]. These data confirm that the patient's clinical history should be analysed together with a positive specific IgE, in order to indicate properly an antigen-specific immunotherapy.

Different data between the two pollens studied, especially for the cut-off point, underline the importance

of analysing diagnostic validity and clinical usefulness of each allergen separately, particularly in a platform with multiple allergens such as the microarray, as has been noted by Salcedo and Diaz-Perales [14]. In fact, a recent study has reported poor performance of the ISAC CRD103 microarray in birch-derived food allergy [15], in contrast to our good results in grass and cypress pollen allergy diagnosis.

We observed high levels of Se and Sp for both grass and cypress pollen CAP, as previously reported by other authors [16–18], and also for grass pollen components and nCup a 1 in ISAC CRD103. Indeed, our results show better Sp than the previous data reported by Wohrl et al. [10] for ISAC CRD50, where grass pollen was represented by rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6 and rPhl p 7. These authors used a different analysis, using the sum of the components instead of considering at least only one of them positive and, furthermore, did not report the cut-off point they used. However, optimal Sp of the microarray ISAC version CRD50 for grass pollen allergy diagnosis was lower [Sp: 90.4 (81.2–96.0)], showing similar Se [Se: 89.4 (76.9–96.4)], than our results with ISAC CRD103, even when using only the components present in ISAC CRD50 [Se: 88.4 (78.8–97.9); Sp: 100 (86.8–100)]. In our opinion, it is important to highlight that these excellent results, especially Sp, can also stem partly from the fact that coinciding history and SPT were used as a gate keeper for inclusion of both patients and controls. However, in our study we observed that when using rPhl p 1, rPhl p 5 and nPhl p 4, 95.3% of grass-allergic patients were diagnosed in contrast to 88.4% of grass-allergic patients diagnosed using only rPhl p 5 and rPhl p 1. These data suggest that the current version of the ISAC has been improved, by adding nPhl p 4 to the microarrayed grass pollen panel as none of our patients were diagnosed with allergy to grass pollen by ISAC CRD103 exclusively using Phl p 11 and/or Phl p 12.

In addition, it is interesting to note that the only cypress microarrayed component, nCup a 1 in ISAC CRD 103 is able to diagnose 91.7% of patients allergic to cypress pollen, and the rPhl p 1, nPhl p 4 and rPhl p 5 components are able to diagnose most (95.3%) of the grass-allergic patients. These data point to the importance of a good selection of the microarrayed components, above and beyond the presence of multiple components, derived from a certain biological source. This observation has also been observed by Villalta and Asero [19] in the microarray ISAC, in which most of the profilin-sensitized patients (97.7%), could be detected by only one profilin, among the 5 profilins represented in ISAC microarray, proposing Bet v 2 as the best profilin sensitization marker.

Different authors have studied the Se and Sp values of different allergenic sources as the sum of the IgE levels against all microarrayed components belonging to that allergenic source and have compared them with the same values obtained using conventional techniques such as fluorescence enzyme immunoassays CAP system against the whole allergenic extract [6, 10, 20–22]. In our opinion, our analysis of considering a result as positive when at least one of the allergens of the studied allergenic source is positive is more useful as it is more consistent with clinical diagnosis. However, it is important to consider those patients showing positive results only against panallergens (polcalcin rPhl p 7 and profilin rPhl p 12) in order to confirm or rule out whether the sensitization is real or due to cross reactivity. In fact, one of the main advantages of the microarray in comparison with diagnostic techniques against whole extracts is its ability to detect sensitization to the pollen allergens yielding a more precise diagnosis. For this reason and given the higher financial cost of the microarray, this technique would be particularly suitable for patients with multiple sensitizations, especially when pan-allergen sensitization is suspected.

Conclusion and clinical relevance

In conclusion, allergen microarray is proposed as a reliable technique that can replace ImmunoCAP in the diagnosis of grass and cypress pollen allergy. Our data show that the manufacturer's recommended cut-off point for both techniques should be used for grass pollen allergy diagnosis. However, whereas the manufacturer's recommended cut-off for microarrayed nCup a 1 can be used to diagnose cypress pollen allergy, 0.66 kU/L for *C. arizonica* CAP should be used to achieve a better diagnosis based on the highest Se and Sp. Further studies regarding the suitability of the manufacturer's recommended cut-off points as well as the diagnostic value of each allergen should be performed to validate both ImmunoCAP and ISAC CRD103.

Acknowledgements

This study was supported partly by a grant from the Spanish Society of Allergy and Clinical Immunology Foundation. In addition, MJG, MLS, MF, GG, PG and MB are supported by grant RD07/0064 from the Spanish Research Network on Adverse Reactions to Allergens and Drugs (RIRAAF: Red de Investigación de Reacciones Adversas a Alérgenos y Fármacos) of the Carlos III Health Institute. Authors have no conflicts of interest.

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