# Degree of Olive Pollen Exposure and Sensitization Patterns. Clinical Implications

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**Abstract**

*Background:* Very high levels of exposure to olive pollen in the south of Spain lead to differential allergen sensitization profiles. Therefore, new approaches to allergen standardization, diagnosis, and vaccination are necessary.
*Methods:* Quantification of minor allergens in extracts, component-resolved patient diagnosis, and IgG4 individual allergen responses were used to evaluate new strategies in the management of olive pollen allergy.
*Results:* Allergen variability observed between different olive cultivars can be used to identify suitable allergen sources that can be combined to yield consistent allergen extracts for diagnosis and immunotherapy. Component-resolved diagnosis can provide a better patient classification. IgG4 levels to major allergens increase significantly, whereas specific IgG4 to minor allergens does not seem to increase, at least during the early phases of immunotherapy.
*Conclusion:* Patients exposed to extreme olive pollen levels display a different severity of allergy from those exposed to normal levels, which makes it necessary to follow a different clinical approach. The use of component-resolved diagnosis, better standardized allergen extracts, and new efficacy monitoring techniques will lead to a significant improvement in the management of olive allergy disease.

Key words: Olive pollen. Minor allergen. Immunotherapy. Molecular profile.

**Resumen**

*Introducción:* Los elevados niveles que alcanza el polen de olivo en zonas del sur de España causan que el perfil de sensibilización de los pacientes alérgicos al polen de olivo sea diferente. Este hecho lleva aparejada la necesidad de una nueva aproximación para el diagnóstico y para la estandarización de extractos alergénicos para inmunoterapia.
*Métodos:* La cuantificación de alérgenos menores en los extractos, el diagnóstico del paciente mediante el uso de un panel de alérgenos de olivo y la medida de los niveles de IgG4 a componentes individuales forman parte de un panel de metodologías para definir nuevas estrategias terapéuticas.
*Resultados:* La variabilidad alergénica observada entre distintos cultivares de olivo puede usarse para lograr una combinación que permita extractos reproducibles con contenido de alérgenos menores controlado. El diagnóstico por componentes permite una mejor clasificación del paciente alérgico. En un estudio retrospectivo, mientras que la IgG4 al alérgeno mayor de olivo se incrementa de modo significativo en el curso de la inmunoterapia, la IgG4 a alérgenos menores no parece incrementarse al menos durante la fase inicial de la inmunoterapia.
*Conclusiones:* Los pacientes expuestos a niveles extremos de polen de olivo presentan una mayor severidad clínica respecto a aquellos expuestos a niveles inferiores. Esto hace necesario un enfoque clínico diferente. El uso del diagnóstico por componentes, la mejor estandarización de extractos y nuevos métodos para evaluar la eficacia llevará aparejado una mejora significativa en la práctica clínica.

Palabras clave: Polen de olivo. Alérgeno minoritario. Inmunoterapia. Perfil molecular.

## Introduction

Olive pollinosis is the leading seasonal allergic disease in some regions of southern Europe [1-4]. In extreme areas, almost half of the territory is covered with olive trees. Thus, olive pollen counts as high as 14 000 grains/m$^3$, the highest in the world, have been reported during pollination seasons. High levels of exposure can cause minor allergens to become major for allergic patients in these regions [5]. Patients sensitized to some minor allergens, such as Ole e 9, and particularly

Ole e 7, have recently been shown to have an increased risk of adverse reactions during immunotherapy [6] and a more severe condition, with a 100% increase in the prevalence of asthma [7]. In fact, while the prevalence of Ole e 1, the major olive allergen, seems to stabilize at 80% and median sIgE values are around 10 kU/L in olive-growing areas, the prevalence of Ole e 7 and Ole e 9 and sIgE values increase progressively along the pollen gradient. In extremely exposed areas, Ole e 7 (but not Ole e 9) is independent of Ole e 1, with 40% of Ole e 1-negative patients being sensitized to Ole e 7 [7]. These findings show that allergen extracts for immunotherapy must be standardized by assessing the content of relevant minor allergens [8].

These studies stress the need for new improved approaches to allergen standardization, olive allergy diagnosis, and therapy for olive-allergic patients living in highly exposed areas. The objectives of the present study were to investigate the patient sensitization profile in highly exposed areas, to assess the allergen concentration of olive cultivars, and to monitor specific immunoglobulin levels during immunotherapy.

## Material and Methods

### Study Population

Patients diagnosed with olive pollinosis by normal diagnostic procedures prior to immunotherapy (control group) or after immunotherapy were studied. All subjects provided written informed consent.

Patients' data were collected according to standard clinical practice. In order to guarantee blinded analysis of serum samples and clinical data, each patient was identified by means of a numeric bar-coded label.

### Preparation of Olive Pollen Extracts

Different batches of olive pollen from different Spanish cultivars were collected. Olive pollen was extracted at a 1:10 (w/v) ratio in phosphate buffer (pH 6.5) with magnetic stirring, at 4°C for 90 min. The soluble fraction was separated by centrifugation at 22 000*g* for 20 min at 4°C and filtered through 0.22-μm filters (Sartorius, Göttingen, Germany).

### Allergenic Activity of Olive Pollen Extracts

Allergenic activity of olive pollen extracts was determined by the radioallergosorbent test (RAST). Paper disks were activated with cyanogen bromide and sensitized with an olive pollen in-house reference extract following procedures described elsewhere [9]. A pool of human sera from allergic patients living in highly exposed areas was used in the experiments. A 50-μL volume of a 1/3 dilution of the serum pool was added to each well of a 96-well microtiter plate (Costar, Corning Life Sciences, Cambridge, Massachusetts, USA) and incubated together with the same volume of 3-fold serial dilutions of samples (olive pollen extracts) and in-house reference extract for 30 min at 37°C. One allergen disk per well was then added and incubated for another 3 to 4 hours at room temperature. After washing the disks 3 times with 0.1% Tween 20 in phosphate buffered saline (PBS), about 125 000 cpm/well of $^{125}$ I-labeled anti-human IgE mAb HE-2 was added and incubated overnight at room temperature. Finally, the disks were washed and the bound radioactivity was measured in a gamma counter. The allergenic activity of olive extracts was expressed in BU/mL compared with the in-house reference, which had been previously calibrated in BU/mL by a skin prick test [10].

### Ole e 1 Enzyme-linked Immunosorbent Assay

Ole e 1 was quantitated using a 2-site solid-phase enzyme-linked immunosorbent assay (ELISA), adapted from the radioimmunoassay described by Lombardero et al [11]. Briefly, ELISA plates (Costar, Corning life Sciences, Cambridge, Massachusetts., USA) were coated overnight at 4°C with 100 μL of anti-Ole e 1 mAb OL7 at 5 μg/mL in PBS. After blocking with PBS-BSA-Tween for 30 min at room temperature, wells were sequentially incubated with samples and references, biotin-labeled anti-Ole e 1 mAb OL2 (1/1000 dilution), and streptavidin-peroxidase (1/1000 dilution, Amersham Biosciences, GE Healthcare). All incubations were carried out for 1 h at room temperature with intermediate washes with 0.1% Tween 20 in PBS between successive steps. Finally, the wells were incubated in the dark at room temperature with peroxidase substrate buffer (0.012% $H_2O_2$, 0.66 mg/mL o-phenylenediamine, OPD; Dako, Glostrup, Denmark) for 30 min. The reaction was then stopped with 50 μL of 2M $H_2SO_4$ and the optical density was measured at 490 nm with a 650-nm reference filter. Assays were performed in duplicate. PBS-BSA-Tween was used as a negative control. The Ole e 1 content of the samples was obtained by interpolating from a standard curve (range 0.435-0.006 μg/mL) constructed with serial 2-fold dilutions of a reference with a known Ole e 1 concentration.

### Ole e 9 Enzyme-linked Immunosorbent Assay

ELISA plates (Costar, Corning Life Sciences, Cambridge, Massachusetts, USA) were coated overnight at 4°C with 100 μL of anti-Ole e 9 mAb 18.1 at 10 μg/mL, in PBS. After blocking with PBS-BSA-Tween, wells were sequentially incubated with samples and references, antiolive pollen extract rabbit serum (1/1000 dilution), and goat antirabbit immunoglobulin antibodies conjugated with horseradish peroxidase (1/10 000 dilution; Calbiochem, San Diego, California, USA). Samples, controls and reagents were diluted in PBS-BSA-Tween, and all incubations were carried out for 1 h at room temperature with intermediate washes with 0.1% (v/v) Tween 20 in PBS between successive steps. Detection was accomplished with OPD, as described for the Ole e 1 ELISA. Assays were performed in triplicate. PBS-BSA-Tween was used as a negative control. The Ole e 9 content of the samples was obtained by interpolating from a standard curve constructed with 12 serial 2-fold dilutions of affinity-purified Ole e 9, starting from 5.2 μg/mL.

### Serum Samples

Serum samples were collected from the subjects and identified by the corresponding bar-coded label. They were stored at – 40°C and thawed immediately before use.

Table IA: Individual Allergen Content of Five Batches of Olive Pollen Collected in Spain From Different Olive Varieties. IB: Biological Activity/Allergen Content Ratio for the 5 Batches.

**A**

| Pollen Batch | Variety | Place | Pollen Season | μg Ole e 1/g Pollen | μg Ole e 7/g Pollen | μg Ole e 9/g Pollen |
|---|---|---|---|---|---|---|
| 1 | Arbequina | Córdoba | 2006 | 5063 | 2335 | 1653 |
| 2 | Lucio | Granada | 2006 | 69532 | 2280 | 1368 |
| 3 | Sylvestris | Jaén | 2006 | 11370 | 2002 | 1997 |
| 4 | Blanqueta | Granada | 2006 | 39310 | 806 | 2044 |
| 5 | Mixture | Granada/Jaén Córdoba/Málaga/Almería | 2006 | 50240 | 1473 | 1648 |

**B**

| Pollen Batch | μg Ole e 1/100 BU | μg Ole e 7/100 BU | μg Ole e 9/100 BU |
|---|---|---|---|
| 1 | 36.5 | 16.8 | 11.9 |
| 2 | 59.1 | 1.9 | 1.16 |
| 3 | 17.0 | 3.0 | 2.99 |
| 4 | 43.4 | 0.9 | 2.26 |
| 5 | 74.4 | 2.2 | 2.44 |

## *Allergens for IgE Determination*

The following allergens were included: *Phleum pratense* nPhl p 1 and nPhl p 5 [12], *Olea europaea* nOle e 1, nOle e 7, and r-Ole e 9 [13-16]. The panallergens were profilin from apple (a mixture of two isoforms of r-Mal d 4 [17]) and polcalcin from *Chenopodium album* pollen, rChe a 3 [18].

## *Specific IgE*

sIgE to the different allergens was tested on the ADVIA Centaur® platform (Bayer HealthCare Diagnostics Division, Tarrytown, New York, USA). The sIgE assay is based on a reverse-sandwich assay. The platform is a continuous and fully automated system. Bar-coded controls and samples are loaded onto the apparatus and the system runs 120 tests per hour. The methodology has been described elsewhere for whole allergen extracts [19]. Single allergens were biotinylated with NHS-LC-biotin (Pierce, Rockford, Illinois, USA) at a 7:1 (w/w) ratio following the manufacturer's instructions. Dose-response curves of biotinylated allergen were obtained and the optimal allergen dose per test was selected. This dose ranged from 10 ng to 40 ng of allergen per test. Biotinylated allergens were stored 50%-glycerinated at about – 20°C. Prior to the assay, allergens were diluted to the optimal dose and used. We did not observe any stability problems during the storage of allergens under these conditions. The amount of sIgE, in kU/l, in the serum samples was determined by a 2-point calibration system. A separate "reference" assay was simultaneously performed alongside the sIgE assay. The assay used recombinant Bet v 1 as a standard biotinylated allergen, a high calibrator (ie, a serum pool with a specified amount of Bet v 1 sIgE that is traceable to the total WHO IgE reference preparation), and a low calibrator, which was an IgE-depleted serum sample. Reference reagents were obtained from ALK-Abelló A/S (Stenløse, Denmark).

The sample dose was calculated by applying the formula:

$$D = D_{hical}[(B–B_0) / (B_{hical}–B_{local})]$$

where D is the sample dose to a single allergen in kU/L, $D_{hical}$ is the dose of high calibrator in kU/L, B is the sample response (relative light units) to the single allergen, $B_0$ is the low calibrator response to the single allergen, and $B_{hical}$ and $B_{local}$ are responses of high and low calibrators to Bet v 1.

All samples were run in duplicate and confirmed in triplicate when necessary (CV above 12% for positive samples, and above 20% for negative samples). For reference samples, 5 replicas were run. The detection limit of the method was 0.1 kU/L [19]. A cut-off value of 0.35 kU/L was used following the standard criterion.

Dilution recovery experiments were performed as described elsewhere [19] to evaluate the assay's dynamic range with the different single allergens and the degree of parallelism between them and the reference Bet v 1 allergen. A linear response was obtained with the different single allergens from doses as high as 400 kU/L. Parallel responses were obtained with the single allergens and Bet v 1, thus validating the use of the 2-point calibration system for dose determination.

## *Ole e 7 Determination*

Ole e 7 was quantitated using inhibition of the Ole e 7 IgE immunoassay. The same procedure was used as for the sIgE assay, except that the serum sample was always a pool of human sera with a high titer of Ole e 7 IgE. Biotin-labeled

Ole e 7 at the optimal dose was used as the allergenic reagent after mixing with serial dilutions of olive pollen extract or reference extract. The amount of Ole e 7 in the olive pollen extract was determined by interpolating on the standard curve built with the reference extract of the known Ole e 7 concentration (range: 0.001 μg Ole e 7/mL to 1 μg/mL).

### *Specific IgG4*

Specific IgG4 was determined by ELISA. Briefly, the allergen (Ole e 1 or Ole e 9) was bound to a specific monoclonal antibody previously adsorbed to the solid phase, as indicated in the Ole e 1 and Ole e 9 ELISAs. It was later incubated with the patient's serum and then with a specific monoclonal antibody to human IgG4 labeled with peroxidase. Detection was by OPD as described for the Ole e 1 ELISA. The color developed was proportional to the serum sIgG4 concentration, and its level in arbitrary units was determined by interpolating on the standard curve built with a previously calibrated serum pool from grass-allergic patients.

## Results

### *Cultivar Variability*

The Ole e 1, Ole e 7, and Ole e 9 contents of different pollen batches obtained from single cultivars and a mixture of different cultivars are shown in Table IA. The main difference is in the amount of Ole e 1 content, whereas Ole e 7 and Ole e 9 vary less from one cultivar to another. Biological activity determined by RAST inhibition is mainly related to the main IgE-binding component (Ole e 1). Thus, as shown in Table IB, the technique is relatively insensitive to variations between minor allergens. While the Ole e 1/allergenic activity ratio varies 4-fold, the corresponding minor allergen ratios vary up to 19-fold from one batch to another.

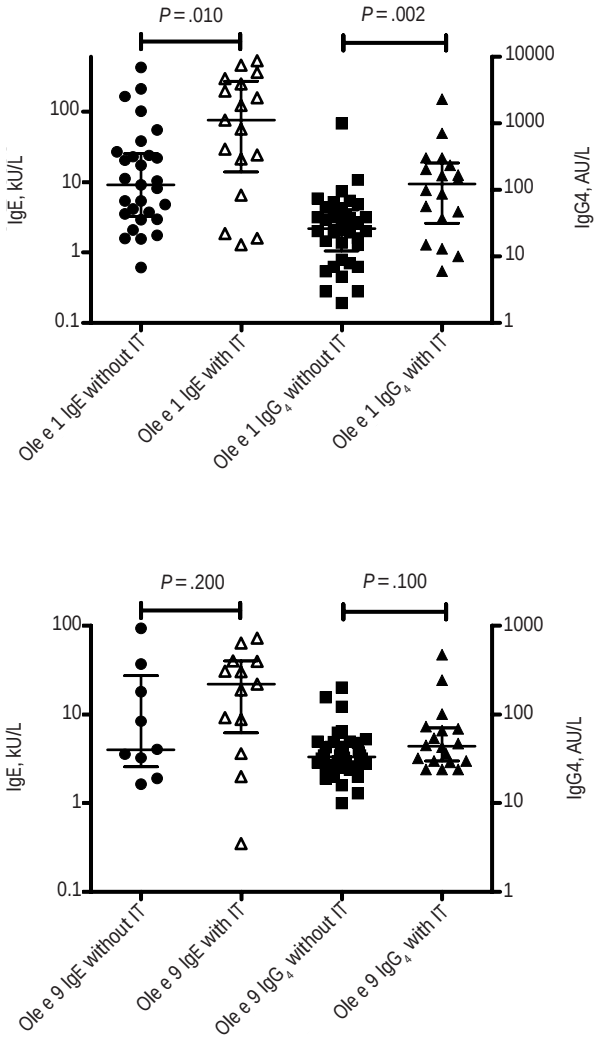
### *Patient Variability*

The molecular profile of patients from Córdoba, a highly exposed area in the south of Spain, is shown in Table II. Patients included were diagnosed as olive-allergic by routine procedures, which did not include molecular screening.

In a homogeneous geographical area, there is a full array of sensitization patterns. A significant number of patients, such as case number 40, were wrongly classified as olive-allergic. In other cases, such as number 17, monosensitized olive-allergic patients were also considered to be allergic to grass. As for olive allergens, 50% of the patients only reacted to Ole e 1, which is normal in olive pollinosis in areas with olive pollen counts below 1000 grains/$m^3$. Ole e 9 sensitization always coincided with Ole e 1, whereas in 5% of cases Ole e 7 was the only olive positive allergen detected.

### *Immunological Changes During Specific Vaccination*

sIgE and sIgG4 against Ole e 1 and Ole e 9 were measured in a population of patients undergoing specific allergy vaccination and compared with the values of untreated patients.




sIgE and sIgG4 levels to Ole e 1 and Ole e 9 in patients who have had specific allergy vaccination compared with a control group of nonvaccinated patients.

The results are shown in the figure. A significant change was detected in the immunoglobulin levels of Ole e 1 but not of Ole e 9. The formulation used for vaccination was a mixture of olive and grasses (50% v/v) in all but 4 cases.

## Discussion

Olive allergy is the main seasonal allergic disease in the south of Spain. The extremely high olive pollen counts in this area often cause a severe allergic reaction. Patients show a different allergy profile, as determined by molecular diagnosis, when compared with patients living in areas with lower pollen counts, where Ole e 1, the major olive allergen, seems to be the only relevant allergen involved in olive sensitization. Normal standardization methods are based on the concentration of the main IgE-binding molecules, in this case Ole e 1. However,

Table II. sIgE Values (kU/L) of Allergic Patients Diagnosed by Conventional Procedures as Being Sensitized to Olive Pollen.

| PATIENT | Ole e 1 | Ole e 7 | Ole e 9 | POLCALCIN | PROFILIN | Phl p 1 | Phl p 5 |
|---|---|---|---|---|---|---|---|
| 1 | 12.67 | 0.05 | 0.00 | 0.00 | 0.09 | 44.57 | 0.13 |
| 2 | 4.62 | 2.38 | 0.00 | 0.01 | 0.11 | 0.00 | 0.00 |
| 3 | 2.22 | 0.03 | 0.08 | 0.05 | 0.10 | 6.45 | 2.08 |
| 4 | 12.53 | 0.03 | 0.01 | 0.00 | 0.00 | 33.04 | 0.08 |
| 5 | 27.42 | 7.53 | 16.97 | 3.10 | 3.40 | 0.00 | 0.06 |
| 6 | 0.43 | 0.02 | 0.03 | 0.00 | 4.24 | 16.39 | 54.43 |
| 7 | 4.72 | 0.03 | 0.02 | 0.00 | 0.03 | 0.00 | 0.01 |
| 8 | 151.98 | 0.15 | 4.09 | 0.02 | 0.01 | 26.59 | 0.07 |
| 9 | 173.00 | 27.42 | 12.12 | 0.04 | 0.04 | 0.00 | 0.00 |
| 10 | 4.40 | 2.06 | 1.41 | 0.05 | 0.84 | 30.13 | 45.17 |
| 11 | 10.93 | 0.05 | 0.05 | 0.01 | 0.07 | 9.84 | 0.04 |
| 12 | 11.62 | 0.02 | 0.00 | 0.00 | 0.01 | 2.26 | 19.98 |
| 13 | 0.22 | 34.66 | 0.01 | 0.00 | 0.07 | 0.02 | 0.03 |
| 14 | 0.27 | 0.10 | 0.00 | 0.00 | 0.01 | 6.06 | 0.98 |
| 15 | 16.83 | 30.19 | 15.09 | 0.00 | 0.01 | 0.44 | 0.22 |
| 16 | 8.08 | 0.06 | 0.02 | 84.32 | 0.00 | 45.44 | 85.43 |
| 17 | 39.21 | 3.75 | 5.59 | 49.34 | 0.00 | 0.00 | 0.01 |
| 18 | 153.95 | 0.30 | 0.08 | 0.02 | 0.01 | 0.00 | 0.03 |
| 19 | 53.52 | 42.26 | 0.03 | 0.01 | 6.64 | 0.02 | 0.11 |
| 20 | 20.15 | 9.93 | 0.03 | 0.00 | 0.00 | 0.48 | 0.00 |
| 21 | 86.35 | 6.87 | 0.04 | 12.33 | 3.77 | 2.37 | 6.16 |
| 22 | 75.66 | 0.15 | 0.07 | 0.02 | 3.33 | 0.00 | 0.03 |
| 23 | 112.25 | 9.07 | 60.96 | 0.03 | 3.23 | 0.24 | 0.33 |
| 24 | 562.36 | 0.47 | 0.33 | 0.04 | 0.10 | 0.00 | 0.02 |
| 25 | 40.77 | 0.06 | 0.01 | 0.00 | 0.04 | 24.39 | 0.03 |
| 26 | 60.34 | 0.08 | 0.07 | 0.03 | 0.19 | 3.02 | 0.04 |
| 27 | 0.51 | 0.05 | 0.01 | 0.00 | 0.06 | 15.66 | 27.29 |
| 28 | 166.08 | 0.36 | 66.09 | 0.00 | 0.05 | 5.93 | 0.06 |
| 29 | 1.00 | 0.01 | 0.02 | 0.01 | 0.20 | 6.45 | 14.38 |
| 30 | 0.70 | 0.01 | 0.00 | 0.01 | 0.07 | 0.00 | 0.00 |
| 31 | 0.20 | 3.64 | 0.10 | 0.00 | 0.04 | 0.00 | 0.01 |
| 32 | 4.04 | 0.02 | 0.02 | 0.00 | 0.08 | 0.26 | 0.08 |
| 33 | 1.12 | 0.10 | 0.02 | 0.00 | 0.07 | 0.59 | 0.03 |
| 34 | 24.94 | 0.07 | 0.03 | 0.00 | 13.53 | 58.99 | 50.30 |
| 35 | 12.36 | 0.77 | 0.05 | 0.00 | 17.07 | 11.98 | 3.55 |
| 36 | 0.02 | 26.94 | 0.03 | 0.01 | 0.08 | 1.01 | 0.02 |
| 37 | 13.35 | 2.02 | 5.48 | 0.38 | 9.59 | 0.76 | 0.73 |
| 38 | 64.07 | 31.71 | 0.08 | 0.01 | 0.06 | 2.71 | 0.01 |
| 39 | 0.46 | 0.88 | 0.03 | 0.00 | 0.04 | 0.00 | 0.00 |
| 40 | 0.00 | 0.01 | 0.00 | 0.00 | 14.96 | 15.85 | 63.32 |

sensitization to minor allergens has been associated with more severe disease [6,7]. It is clear that new strategies must be developed for allergen standardization, patient diagnosis, and therapy.

Olive trees are propagated by cuttings. The trees live for a long time, often several hundred years, and most of the trees in one area generally proceed from the same genetic background that determines olive fruit properties and is used to name the cultivar. Extreme variability of olive pollen allergenicity has been observed. In 1990, Barber et al [20] pointed out the extreme allergenic differences between pollens obtained from different cultivars. This was attributed to the variability of the major IgE-binding component. In the present study, we confirm that this variation could be attributed to a large variation in the Ole e 1 concentration. As biological activity is linked to the major allergen concentration, routine standardization based on major allergen content and biological activity might lead to a marked variability in minor allergen concentration. The concentration of Ole e 9 has also been reported to vary several hundred times between different pollen batches [5]. Therefore, it is clear that both major and minor allergen quantitation must be an integral part of the standardization of olive allergy vaccines, and that natural variability in allergen content can be used to search for adequate combinations to ensure a controlled allergen composition of vaccines.

Patients living in areas with high pollen counts display a complex allergen sensitization profile [21]. In Córdoba, more than 90% of patients are allergic to olive, grasses, or both. Nevertheless, when component-resolved diagnosis is added to routine diagnostic procedures, around 30% of the immunotherapy formulations are changed, with an increase in the use of single-component vaccines instead of combination vaccines. To date, there are no convincing clinical trials using combination products. In the present study, we evaluated sIgE and sIgG4 to Ole e 1 and Ole e 9 in patients undergoing immunotherapy. The increase in IgG4 levels is considered to be a surrogate marker of efficacy, indicating the progression in tolerance. Interestingly, while sIgG4 titers to Ole e 1 increase during vaccination, Ole e 9 levels remain unchanged.

The fact that almost all patients included were treated with a combination product suggests that suboptimal minor allergen doses were used and stresses the need for optimal therapy for these patients. In a similar study comparing 2 allergy vaccines, Rosi et al [22] reported a dose-dependent increase in IgG4 to minor allergens.

Olive allergy represents a unique allergy model where a sharp exposure gradient leads to different clinical allergy profiles. This should enable us to develop more specific approaches to diagnosis and therapy that can increase the quality of life of allergic patients.

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