ORIGINAL ARTICLE

# Allergy to Cypress and Olive Pollen: Clinical Phenotypes and Allergen Recognition

Alonso-Díaz de Durana MD[1], Pérez-Fernández E[2], Villalba M[3]*, Martín-Pedraza L[3], Fernández-Rivas M[4]*

[1]Allergy Unit, Hospital Universitario Fundación Alcorcón, Madrid, Spain
[2]Research Unit, Hospital Universitario Fundación Alcorcón, Madrid, Spain
[3]Biochemistry and Molecular Biology Department, Universidad Complutense, Madrid, Spain
[4]Allergy Department, Hospital Clínico San Carlos, Universidad Complutense, IdISSC, Madrid, Spain
*Both authors contributed equally to the study

J Investig Allergol Clin Immunol 2024; Vol. 34(3): 313-322
doi: 10.18176/jiaci.0917

## Abstract

*Background:* Cypress and olive pollen are the most prevalent sensitizers in the Mediterranean area. Some patients exhibit dual sensitization, which has not been well documented to date.
*Objective:* To identify the allergens involved in dual cypress and olive allergy (C+O) and to study the relationship between phenotype and allergen sensitization.
*Methods:* C+O patients were selected. Those monosensitized to olive or cypress were used as a reference. Specific IgE to whole extracts and purified allergens from olive and cypress was determined. Immunoblotting was performed to analyze IgG and IgE binding using olive polyclonal antibodies and patients' sera, respectively. Mutual immunoblotting inhibition of olive and cypress extracts and inhibition of cypress extract immunoblotting with olive allergens were performed. Multiple correspondence analysis and hierarchical cluster classifications were conducted to analyze the relationships between the clinical presentation of C+O (symptoms, seasonality) and allergen profile.
*Results:* C+O patients were clustered in 4 phenotypes. The most frequent one (58.4%) was rhinoconjunctivitis in winter (February) and spring (May), with asthma in 38% of patients. Ole e 1 and Cup s 1 were the major allergens. Proteins homologous to Ole e 1, Ole e 9, and Ole e 11 in cypress pollen were identified and shown to inhibit IgE binding to cypress extract.
*Conclusions:* The exclusive C+O results from cosensitization to Cup s 1 and Ole e 1 and cross-reactivity due to previously unreported Ole e 1–like, Ole e 9–like, and Ole e 11–like allergens in cypress pollen. Our findings point to 4 clinical phenotypes of winter and/or spring or perennial rhinoconjunctivitis with and without asthma.

Key words: Allergen. Cypress pollen. Cluster analysis. Cross-reactivity. Multiple correspondence analysis. Olive pollen.

## Resumen

*Antecedentes*: Los pólenes de ciprés y olivo son los pólenes de árboles sensibilizantes más prevalentes en el área mediterránea. Algunos pacientes presentan una doble sensibilización que aún no ha sido bien documentada.
Objetivo: Identificar los alérgenos implicados en la doble alergia a los pólenes de ciprés y olivo (C+O), y estudiar la relación entre fenotipo y sensibilización alergénica.
Métodos: Se seleccionaron pacientes con C+O. Se utilizaron como referencia sujetos monosensibilizados al olivo o al ciprés. Se determinó IgE específica frente a extractos completos y alérgenos purificados de olivo y ciprés. Se realizó inmunodetección para analizar la unión a IgG e IgE utilizando anticuerpos policlonales específicos de alérgenos de polen de olivo y sueros de pacientes, respectivamente. Se llevaron a cabo estudios de inhibición mutua de los extractos de olivo y ciprés, y de inhibición de la inmunodetección del extracto de ciprés con alérgenos de polen de olivo. Se realizaron análisis de correspondencia múltiple y clasificaciones jerárquicas de conglomerados para analizar las relaciones entre la presentación clínica de C+O (síntomas, estacionalidad) y el perfil de alérgenos.
Resultados: Los pacientes C+O se agruparon en 4 fenotipos. El más frecuente (58,4%) fue la rinoconjuntivitis en invierno (febrero) y primavera (mayo), con asma en el 38% de los sujetos. Ole e 1 y Cup s 1 fueron los alérgenos principales. Se identificaron proteínas homólogas a Ole e 1, Ole e 9 y Ole e 11 en el polen de ciprés, y estos alérgenos de olivo inhibieron la unión de IgE al extracto de ciprés.
Conclusiones: La alergia exclusiva a C+O resulta de la cosensibilización a Cup s 1 y Ole e 1, y a la reactividad cruzada debida a alérgenos homólogos de Ole e 1, Ole e 9 y Ole e 11 en ciprés no descritos previamente, y se traduce en 4 fenotipos clínicos (rinoconjuntivitis con y sin asma) con presentación en invierno y/o primavera o perenne.

Palabras clave: Alérgeno. Polen de ciprés. Análisis de conglomerados. Reactividad cruzada. Análisis de correspondencia múltiple. Polen de olivo.

**Summary box**

- **What do we know about this topic?**
  Cypress and olive pollen are the most prevalent sensitizing tree allergens in the Mediterranean area. Some patients are allergic exclusively to cypress and olive pollen allergens, although their clinical characteristics and allergen sensitization profiles have not been described.
- **How does this study impact our current understanding and/or clinical management of this topic?**
  Dual cypress and olive allergy results from cosensitization to Cup s 1 and Ole e 1 and cross-reactivity between Ole e 1, Ole e 9, and Ole e 11 and homologous allergens in cypress pollen. Four clinical phenotypes of seasonal/perennial respiratory allergy have been identified.

## Introduction

Pollen is the leading cause of allergic rhinoconjunctivitis and asthma in Spain [1]. The most allergenic pollens in the Madrid area are from grasses (*Poaceae*) and trees such as Arizona cypress (*Cupressus arizonica*), Mediterranean cypress (*Cupressus sempervirens*), olive (*Olea europaea*), and plane tree (*Platanus acerifolia*) [2,3]. Polysensitization is a common feature in patients who experience seasonal respiratory symptoms [4], with cypress and olive pollen being the most prevalent sensitizers among tree allergens [5-7]. In the last 40 years, there has been a significant increase in cypress plantation throughout the world, especially around the Mediterranean basin, for ornamental purposes and in fences. This increase, together with the pollution particles that interact with pollen grains and thus increase solubility in the air, has led to an increase in allergenicity [8,9]. The growing importance of cypress and olive tree pollen allergies in Spain, as well as geographical differences in the prevalence of sensitization, has been confirmed in the national epidemiological surveys of Alergologica performed in 2005 and 2015 [1]. Cypress and olive trees belong to different botanical families with no taxonomic relationships or overlapping seasonality. In our area, cypress pollinates during the winter months, with a peak in February, and olive pollen is released during spring, with a peak in May [3,10]. Dual sensitization to cypress and olive tree pollens without sensitization to any other pollen is not uncommon (2% of pollen allergic patients studied in our area, unpublished data) and remains stable over time (unpublished, personal communication). However, it has not been well documented in the literature to date.

Breakthroughs in molecular biology in the last 25 years have made it possible to characterize allergens from different sources. Molecular diagnosis overcomes the limitations of whole extracts in polysensitized patients [11] by identifying the responsible allergens and discriminating between genuine sensitization and cross-reactivity [12,13]. Regarding treatment, molecular diagnosis may lead to a change in the composition of the immunotherapy formerly considered appropriate according to skin prick test (SPT) results in almost 60% of patients [14,15].

The major olive pollen allergen, Ole e 1 (common olive group), as well as the homologous major cypress pollen allergens, Cup a 1 from *Cupressus arizonica* and Cup s 1 from *Cupressus sempervirens*, are responsible for genuine sensitization to the *Oleaceae* and *Cupressaceae* families, respectively. Ole e 1 shows a high degree of sequence identity within *Oleaceae* (Ole e 1–like protein). Similarly, Cup a 1 and Cup s 1, both pectate lyases, have a sequence identity higher than 95% [6-8,16]. Four groups of cypress allergens have been described and referenced in the International Union of Immunological Societies (www.allergen.org), although several other allergens have been reported [8]. Fourteen olive pollen allergens (Ole e 1 to Ole e 12, Ole e 14, and Ole e 15) have been identified [16-18]. Some of these, such as Ole e 7 and Ole e 9, behave as major allergens in areas of maximum exposure to olive pollen and are considered relevant markers of severity owing to their association with asthma [19]. Some degree of cross-reactivity between cypress and olive pollen has been attributed to panallergens [20] or to other allergens such as ß-galactosidase [21].

The aims of this study were, firstly, to determine whether double sensitization to cypress and olive pollen could be due to sensitization to specific allergens of both pollens or to cross-reactive allergens present in cypress or olive pollen. Secondly, we aimed to study the clinical phenotypes and their relationship with the allergen sensitization profile.

## Methods

### *Patients*

Patients older than 7 years of age were selected consecutively over 4 years. They had to have had respiratory symptoms of rhinoconjunctivitis and/or asthma for at least 2 years and to present positive SPT results exclusively for cypress (*C arizonica* and/or *C sempervirens*) and olive pollen. Patients with additional positive SPT results for other inhalants and those who had received immunotherapy were excluded from the study. The study was approved by the Hospital Universitario Fundación Alcorcón Ethics Committee (Ref.3/11). All the patients or their legal representatives provided their signed informed consent to enter the study.

The clinical information collected on the case report form was age, sex, symptoms of pollen allergy (rhinoconjunctivitis, asthma), seasonality (yes/no), and, in those with seasonal symptoms, the peak month of the clinical presentation (February for winter and/or May for spring) [3].

SPTs were performed with commercial extracts (ALK-Abelló) of grasses, cypress (*C arizonica* and *C sempervirens*), olive, plane tree, weeds, mites, molds, cockroach, and cat and dog dander. Histamine phosphate at 10 mg/mL and normal

saline solution were used as positive and negative controls, respectively. A wheal with a diameter at least 3 mm larger than the negative control was considered positive.

Serum total IgE was measured using nephelometry (Siemens Healthcare Diagnostics). Serum specific IgE (sIgE) to *C arizonica*, *C sempervirens*, *Olea europaea*, *Platanus acerifolia*, and *Lolium perenne* were measured using the ImmunoCAP System (Thermo Fisher Scientific). The cut-off point was 0.35 $kU_A/L$. Patients with respiratory allergy and who were monosensitized to either olive or cypress pollen were included as controls, provided their informed consent, and underwent the same clinical evaluation as the cypress+olive–allergic (C+O) participants.

### *Allergen Profiling in the Study Population*

*C arizonica* and olive pollen were purchased from Allergon-Pharmacia and extracted as described elsewhere [22]. sIgE against *C arizonica* and olive pollen extracts (20 µg) and purified allergens (0.1 µg) from olive (nOle e 1, rOle e 2, rOle e 3, nOle e 7, rOle e 9 [CtD-Ole e 9 and NtD-Ole e 9], rOle e 11, and rOle e 12), cypress (nCup s 1), and bromelain was determined using indirect enzyme-linked immunosorbent assay (ELISA). The allergens included in this study were purified by the group of Villalba et al [16], except Cup s 1, which was kindly donated by ALK-Abelló. Individual patient sera were used at a dilution of 1:10 in phosphate-buffered saline (PBS). IgE binding was detected with mouse antihuman IgE antibody (1:5000 dilution). The peroxidase reaction was developed using fresh enzyme substrate and with absorbance at 492 nm. Values with an optical density under 0.1 were considered negative.

IgE-immunoblotting assays with purified allergens (0.1 µg) or pollen protein extracts (20 µg) immobilized onto nitrocellulose membranes after SDS-PAGE were performed as follows. Membranes were incubated with individual human sera (1:10 PBS diluted) and mouse antihuman IgE monoclonal antibody (diluted 1:5000) (kindly provided by ALK-Abelló), followed by horseradish peroxidase–labeled polyclonal IgG (1:3000 diluted; Pierce). Western blots with specific polyclonal antibodies (pAb) against Ole e 1, Ole e 7, CtD-Ole e 9, NtD-Ole e 9, Ole e 10, and Ole e 11 (1:10 PBS dilution) were detected with goat antirabbit IgG horseradish peroxidase–labeled antibody (1:3000) (DAKO). The chemiluminescent signal was developed using ECL-Western blotting reagent (Amersham Bioscience) or WesternBright™ QUANTUM (Advansta) reagents. For the immunoblotting inhibition assays, individual sera or an equivolumetric pool of patients' sera were diluted in PBS (1:5) and preincubated at room temperature for 2 hours with 5 µg of the purified allergens or 500 µg of the cypress or olive extracts using PBS as a negative control. The remaining steps were as described above.

**Table.** Study Participants: Demographics, Clinical Presentation, and SPT and sIgE Results.

| | | | C+O group n=85 | C group n=21 | O group n=15 | *P* value |
|---|---|---|---|---|---|---|
| Demographic data | Age, y | Mean (SD) | 34.7 (12.7) | 45 (13.2) | 33.9 (11.1) | .012 |
| | Sex | Male | 36 (42.4%) | 8 (38.1%) | 4 (26.7%) | .512 |
| Clinical presentation | Symptoms | RC | 53 (62.4%) | 19 (90.5%) | 7 (46.7%) | .003 |
| | | Asthma | 3 (3.5%) | 0 | 4 (26.7%) | |
| | | RC+asthma | 29 (34.1%) | 2 (9.5%) | 4 (26.7%) | |
| | | Asthma with and without RC | 32 (37.6%) | 2 (9.5%) | 8 (53.3%) | .014 |
| | Seasonality | Perennial | 11 (12.9%) | 2 (9.5%) | 6 (40%) | .032 |
| | | Seasonal | 74 (87.1%) | 19 (90.5%) | 9 (60%) | |
| | | February | 11(14.9%) | 18 (94.7%) | 0 | .001 |
| | | May | 14 (18.9%) | 0 | 9 (100%) | |
| | | February and May | 49 (66.2%) | 1 (5.3%) | 0 | |
| SPT | Ratio SPT *Ca*/histamine | Median (IQR) | 0.83 (0.63-1.17) | 0.95 (0.7-1.08) | | .652 |
| | Ratio SPT *Cs*/histamine | Median (IQR) | 0.6 (0.4-0.77) | 0.71 (0.6-1) | | .035 |
| | Ratio SPT *O*/histamine | Median (IQR) | 0.6 (0.4-0.77) | | 1.71 (1.13-2.2) | .014 |
| Total IgE, IU/mL | | Median (IQR) | 85.6 (39.65-172.5) | 83.7 (49.13-209.75) | 28.2 (19-70.7) | .008 |
| sIgE | *Ca* and/or Cs | No. (% positive) | 65 (76.5%) | 20 (95.2%) | 0 | .067 |
| | *O* | No. (% positive) | 78 (91.8%) | 0 | 8 (53.3%) | .001 |

Abbreviations: C+O, cypress+olive; C, cypress; Ca, *Cupressus arizonica*; Cs, *Cupressus sempervirens*; O, olive; RC, rhinoconjunctivitis; sIgE, specific serum IgE; SPT, skin prick test.

### *Statistical Analysis*

Qualitative variables were presented as a percentage and quantitative variables as mean (SD) or median (IQR). A univariate analysis was performed to evaluate differences between the groups of study participants who were allergic to the C+O group and controls who were allergic to olive pollen (O group) or cypress pollen (C group). Qualitative variables were compared using the Pearson $\chi^2$ test or Fisher exact tests and quantitative variables using the Kruskal-Wallis test.

Multiple correspondence analysis (MCA) and hierarchical classifications were conducted to differentiate between groups of participants. MCA is a multivariate technique used to visualize the association between categorical variables through a graph and was performed to study the associations between the groups (C+O, C, and O), symptoms (rhinoconjunctivitis, asthma, seasonality), and allergen profile.

A dendrogram (hierarchical cluster analysis, Ward method) was generated to classify patients of the C+O group with a high degree of association.

The statistical analysis was performed using SPSS Statistics for Windows Version 17.0 (SPSS Inc.) and STATA 13. Statistical significance was set at $P<.05$.

## Results

### *Description of the Study Population*

The Table summarizes demographic and clinical data and SPT and sIgE results. The study included 121 patients with a clinical history and concordant SPT results: 85 participants in the C+O group (mean age, 34.7 years; males, 42%), 21 in the C group (mean age, 45 years; males, 38%), and 15 individuals in the O group (mean age, 33.9 years; males, 27%). The most prevalent respiratory symptom in the 3 groups was rhinoconjunctivitis, which was more frequently recorded in the C+O group (62.4%) and C group (90.5%) than in the O group (46.7%) ($P$=.003). Asthma with and without rhinoconjunctivitis was more frequently present in patients from the O group (53.3%) ($P$=.014). Most patients in the 3 groups had seasonal symptoms, although 40% of the O group had perennial symptoms ($P$=.032). In the C+O group, 66% of participants had symptoms in both February and May.

### *Allergen Profile*

sIgE-ELISA assays were performed in 113 patients (77 in the C+O group, 21 in the C group, and 15 in the O group).

Analyzing sIgE to purified allergens in the C+O group, we found 18 different combinations (Supplementary Table 1). The most frequent were the major allergens, Cup s 1 and Ole e 1 (27.3%), followed by monosensitization to Ole e 1 (22.1%) and to Cup s 1 (11.7%). Seven patients (9.1%) were not sensitized to any of the recombinant allergens tested.

In the C+O group, Ole e 1 and Cup s 1 were the main allergens, with a frequency of sensitization of 74% and 59.7%, respectively. Ole e 11 and CtD-Ole e 9 behaved as minor allergens, with positive results in 19.5% and 10.4%, respectively. The frequency of sensitization to other allergens was below 10% as follows: bromelain, 7.8%; Ole e 12, 3.9%;

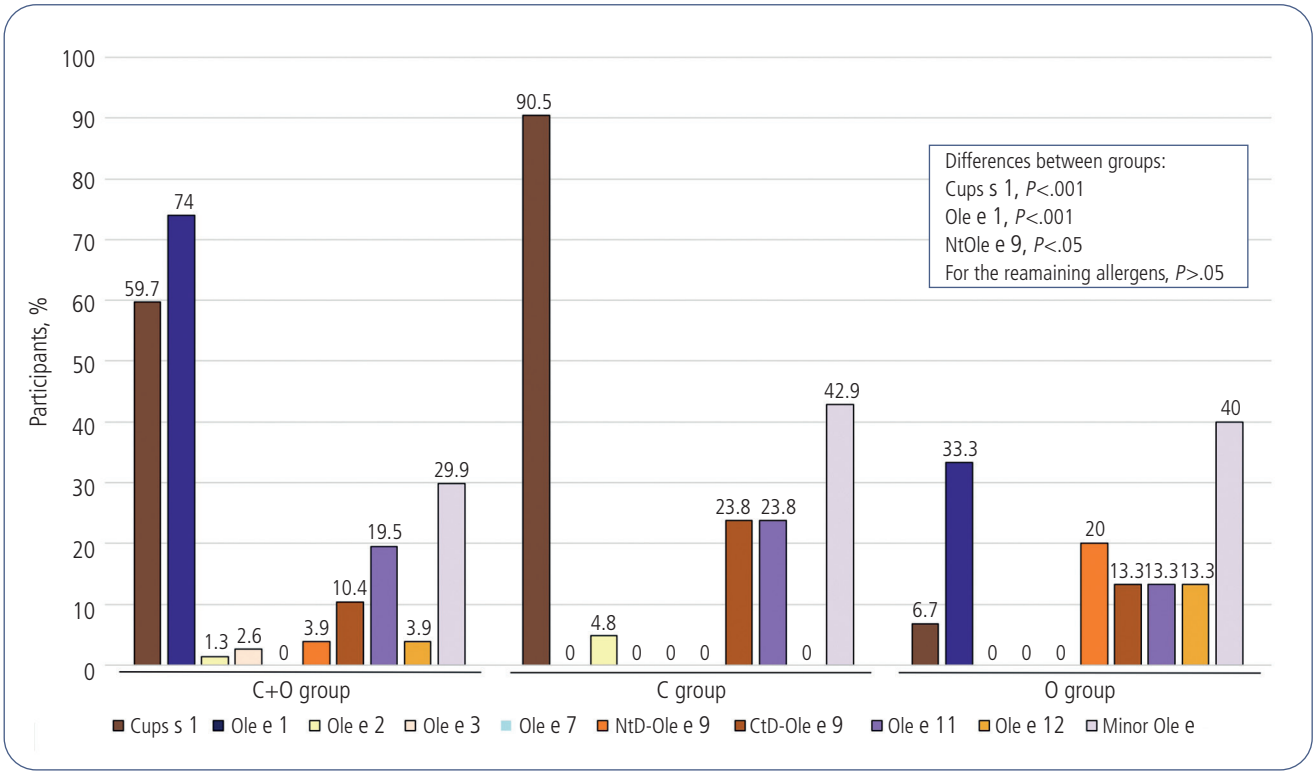



**Figure 1.** Frequency of sensitization to Cup s 1 and olive allergens in groups of patients allergic to cypress+olive (C+O), cypress (C), and olive (O) pollen. Minor Ole e, any minor olive allergen.

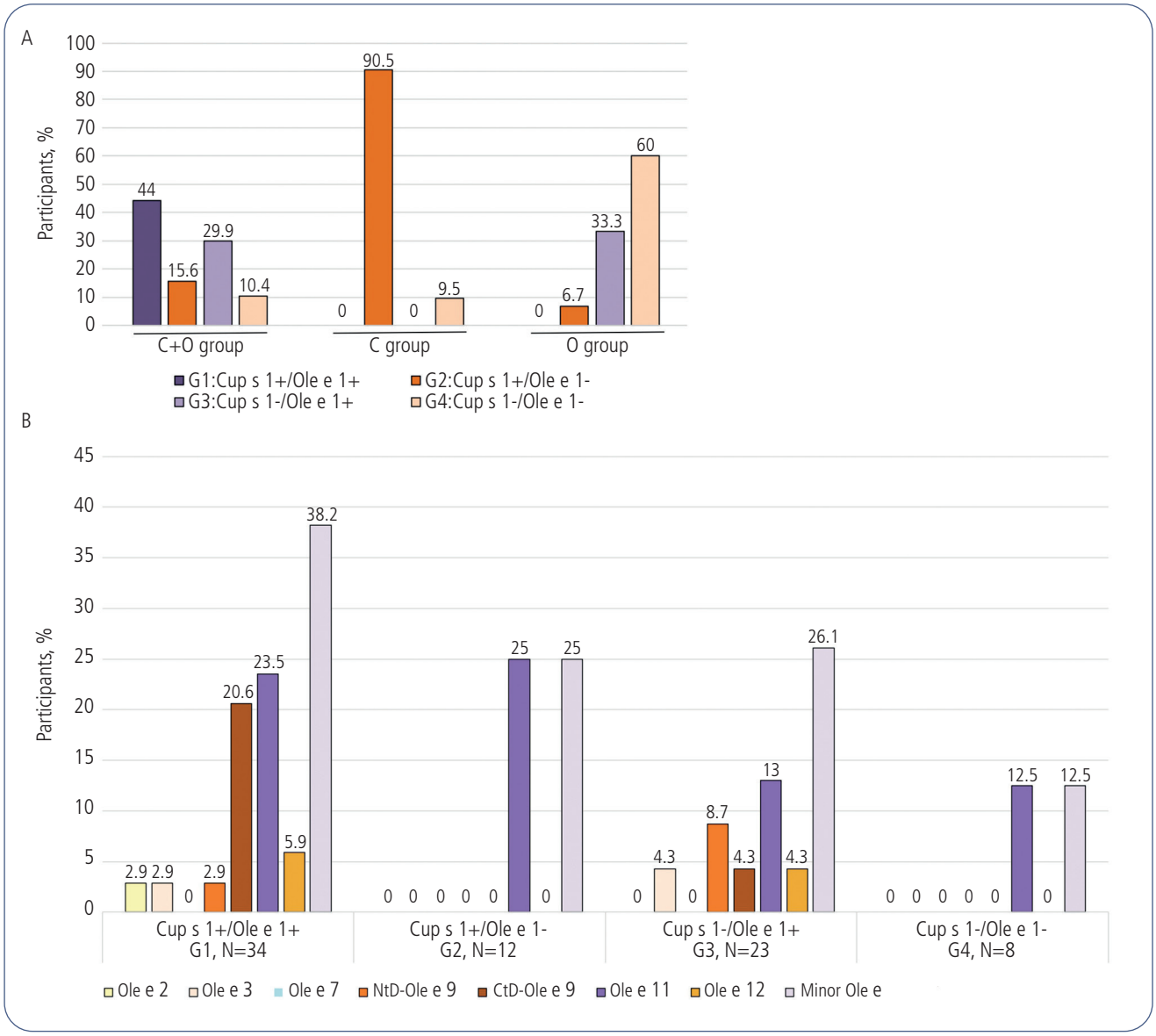



**Figure 2.** A, Subclassification of patients according to sIgE (positive/negative) to Cup s 1 and Ole e 1 in the cypress+olive group and in patients monosensitized to cypress and olive pollen. B, Sensitization profile for minor allergens in the 4 subgroups of cypress+olive–allergic patients depending on a positive or negative response to Cup s 1 and Ole e 1. Minor Ole e, any minor olive allergen sensitization percentage.

Ole e 3, 2.6%; and Ole e 2, 1.3%. In the C group, frequency of sensitization to Cup s 1 was 90.5%. In the O group, there was a strikingly low prevalence for Ole e 1 (33.3%) and NtD-Ole e 9 (20%). Statistically significant differences between the 3 groups were found for Ole e 1 (higher in C+O), NtD-Ole e 9 (higher in the O group), and Cup s 1 (higher in the C group) (Figure 1, Supplementary Table 2).

To obtain further insight into the sensitization profile, we classified patients from the 3 groups (C+O, C, and O) into 4 subgroups, defined according to the positive or negative response against the 2 major allergens, Cup s 1 and Ole e 1, as follows: G1 (Cup s 1+/Ole e 1+), G2 (Cup s 1+ / Ole e 1–), G3 (Cup s 1–/Ole e 1+), and G4 (Cup s 1–/ Ole e 1–). We found a statistically significant difference between the 3 groups ($P$<.001, Kruskal-Wallis test) (Figure 2A). Sensitization to the G1 subgroup was the most frequent (44.2%) in the C+O group, although sensitization was found to be absent in the C and O groups. In the C group, >90% of patients were exclusively sensitized to Cup s 1, and in the O group, only 1 out of 3 were sensitized to Ole e 1. We further analyzed the allergen profile in the G1 to G4 subgroups of C+O patients and found sensitization to any minor olive allergens in all 4 subgroups more frequently when the response to Ole e 1 was positive (G1, G3), but also when it was negative (G2, G4). Ole e 11 was the only allergen present in patients from the 4 subgroups (Figure 2B, Supplementary Table 3).

### *Clinical Presentation and Allergen Profile*

MCA was performed to analyze the pattern of relationships between clinical presentation and allergen

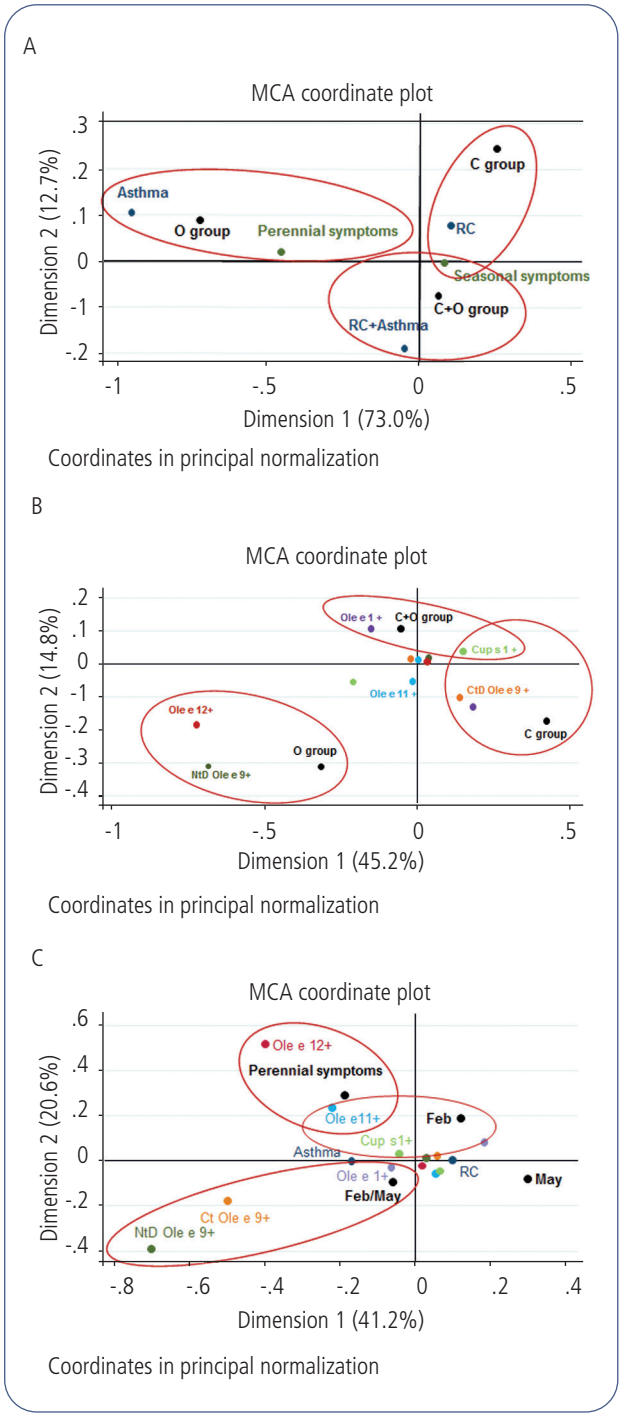



**Figure 3.** Multiple correspondence analysis (MCA) of clinical presentation and allergen profile. The distance between the variables indicates the approximate relationship between them. The distance between the variables is inversely proportional to the strength of the relationship. Circles have been added to emphasize the proximity between points. Dots with a + sign indicate allergen sensitization. Dots without a + sign indicate no sensitization.
A, Group and clinical presentation in the C+O, C, and O groups. B, Group and allergen sensitization profile in in the C+O, C, and O groups. C, Clinical presentation and allergen profile in the C+O group.
Asthma indicates asthma with and without RC; C+O, cypress+olive; C, cypress; Feb, February; F+M, February and May; O, olive; RC, rhinoconjunctivitis.

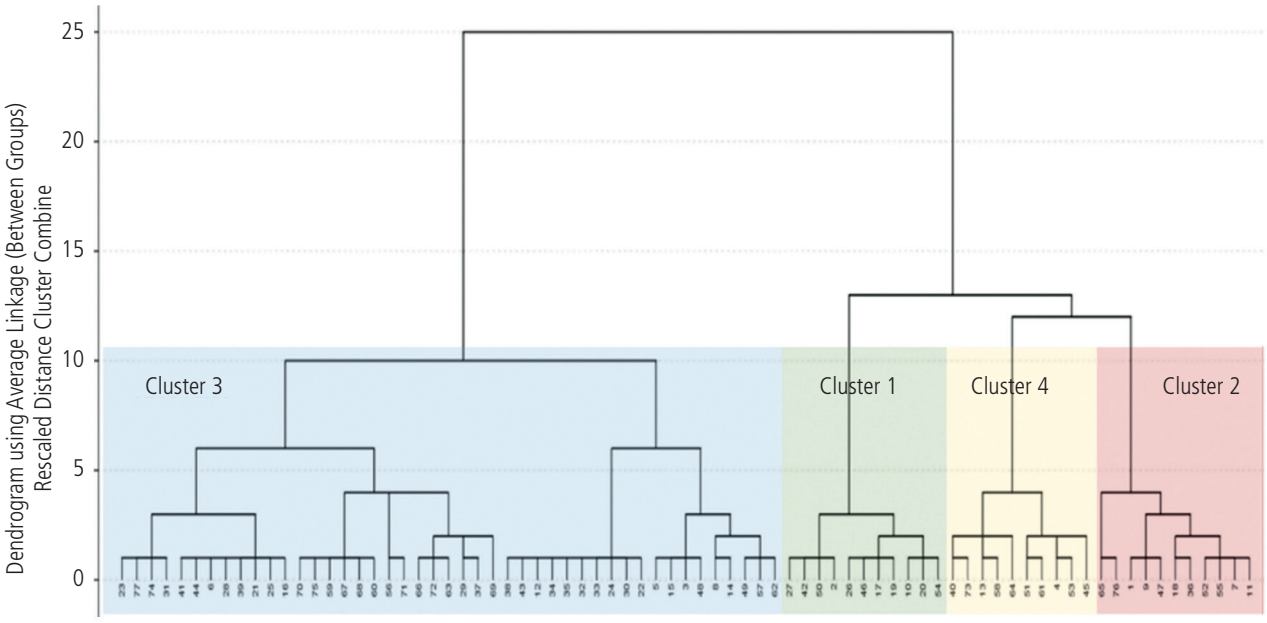
profile in the 3 study groups (Figure 3). The MCA for clinical presentation (Figure 3A) showed an association between seasonal symptoms and rhinoconjunctivitis+asthma in the C+O group, an association between asthma and perennial symptoms in the O group, and an association between rhinoconjunctivitis and seasonal symptoms in the C group. The MCA graph for allergen profile (Figure 3B) showed an association between the C+O group and sensitization to Ole e 1 and Cup s 1, sensitization to Ole e 12 and NtD-Ole e 9 and the O group, and CtD-Ole e 9 and Cup s 1 and the C group. The results of the MCA exploring clinical presentation and allergen profile in the C+O group are presented in Figure 3C. The graph shows the association between sensitization to Ole e 9 (NtD and CtD) and Ole e 1 and symptoms during February and May and between sensitization to Ole e 11 and Ole e 12 and perennial symptoms and between sensitization to Cup s 1 and Ole e 11 and symptoms during February.

The cluster analysis of participants in the C+O group generated a dendrogram. The solution that was finally adopted comprised 4 clusters (Figure 4A, 4B), defined by the seasonality of the clinical presentation ($P<.001$): cluster 1 included 11 patients (14.3%) with symptoms in February; cluster 2 included 11 patients (14.3%) with symptoms in May; cluster 3 included 45 patients (58.4%) with symptoms in February and May; and cluster 4 included the 10 patients (13%) with perennial symptoms. Sensitization to Cup s 1 and Ole e 1 appeared in all clusters, although the former was more frequent in cluster 1 (82%); sensitization to Ole e 1 was more frequent in clusters 3 (80%) and 4 (82%). No cases of sensitization to Ole e 9, Ole e 11, or Ole e 12 were found in cluster 2. The most frequent minor Ole e allergen in clusters 1 and 4 was Ole e 11 (36% and 50%, respectively), and the most frequent in cluster 3 was CtD-Ole e 9 (16%). Asthma was more frequent in cluster 4 (50% of patients).

### *IgE Immunoblots of Patients With Double Sensitization*

IgE immunoblots of olive and *C arizonica* pollen extracts performed with sera from patients from the 3 groups are shown in Supplementary Figure 1. Figure 5 shows a series of representative results that enable us to compare the allergenic profile of subgroups G1, G2, G3, and G4 of the C+O patients. Sera from subgroup G1 (24, 32, and 69) (Figure 5A) recognized a 20-kDa band corresponding to Ole e 1 and a 45-kDa band corresponding to Ole e 9, as confirmed by ELISA. The band around 14 kDa observed in sera 32 and 69 could correspond to Ole e 10, the allergen homologous to the C-terminal domain of Ole e 9, and consequently, may be associated with cross-reactivity to the whole Ole e 9 allergen. The profile of these 3 patients was similar in cypress extract, which present bands with similar molecular masses (20 kDa and 45 kDa, respectively). Sera from subgroup G2 (39, 40, and 44) only recognized Cup s 1 by ELISA (Figure 5B). However, bands of approximately 43-45 kDa were observed in cypress and olive extracts, which correspond to Cup s 1 in cypress and, possibly, to a Cup s 1–like or another unidentified allergen in olive pollen. Sera from subgroup G3 (98, 100, and 106) that recognized Ole e 1 by ELISA presented a band of around 21 kDa in olive pollen corresponding to Ole e 1 and

A




B

| | Cluster analysis (Ward Method) | | | | |
|---|---|---|---|---|---|
| | **Cluster 1 (14.33%)** | **Cluster 2 (14.33%)** | **Cluster 3 (58.4%)** | **Cluster 4 (13%)** | ***P* value** |
| Patients, N (%) | 11 (14.33%) | 11 (14.33%) | 45 (58.4%) | 10 (13%) | |
| February | 11 (100%) | 0 | 0 | 0 | |
| May | 0 | 11 (100%) | 0 | 0 | |
| February and May | 0 | 0 | 45 (100%) | 0 | |
| Perennial symptoms | 0 | 0 | 0 | 10 (100%) | |
| Asthma | 4 (36.4%) | 2 (18.2%) | 17 (37.8%) | 5 (50%) | .484 |
| Cup s 1 + | 9 (82%) | 4 (36.4%) | 28 (62%) | 5 (50%) | .165 |
| Ole e 1 + | 5 (45.5%) | 7 (63.6%) | 37 (82%) | 8 (80%) | .075 |
| NtD-Ole e 9 + | | 0 | 3 (7%) | 0 | 1 |
| CtD-Ole e 9 + | 0 | 0 | 7 (16%) | 1 (10%) | .434 |
| Ole e 11 + | 4 (36%) | 0 | 6 (13.3%) | 5 (50%) | .006 |
| Ole e 12 + | 0 | 0 | 1 (2.2%) | 2 (20%) | .095 |

**Figure 4.** A, Cypress and olive–allergic group: Allergen profile and clinical presentation of cluster analysis solution. Dendrogram (hierarchical cluster analysis, Ward method). B, Statistics of qualitative characteristics of each cluster defined by the seasonality of the clinical presentation.

bands of a similar molecular weight in cypress that could be an Ole e 1–like allergen (Figure 5C). Sera from subgroup G4 (10, 28, and 103) (Figure 5D) did not recognize either of the major allergens Cup s 1 and Ole e 1. Given that faint high-molecular-mass bands were observed in both extracts, unidentified allergens could be responsible for sensitization, although these do not include any of the allergens analyzed using ELISA.

### *Identification of Homologous Olive Pollen Allergens in Cypress Pollen Extract*

The pAbs targeting olive pollen allergens used with the cypress pollen extract in immunoblotting recognized Ole e 1, CtD-Ole e 9, Ole e 10, and Ole e 11 (Figure 5E). The pAbs against the C-terminal of Ole e 9 recognized the whole protein of 45 kDa. The pAb against Ole e 10 also recognized the

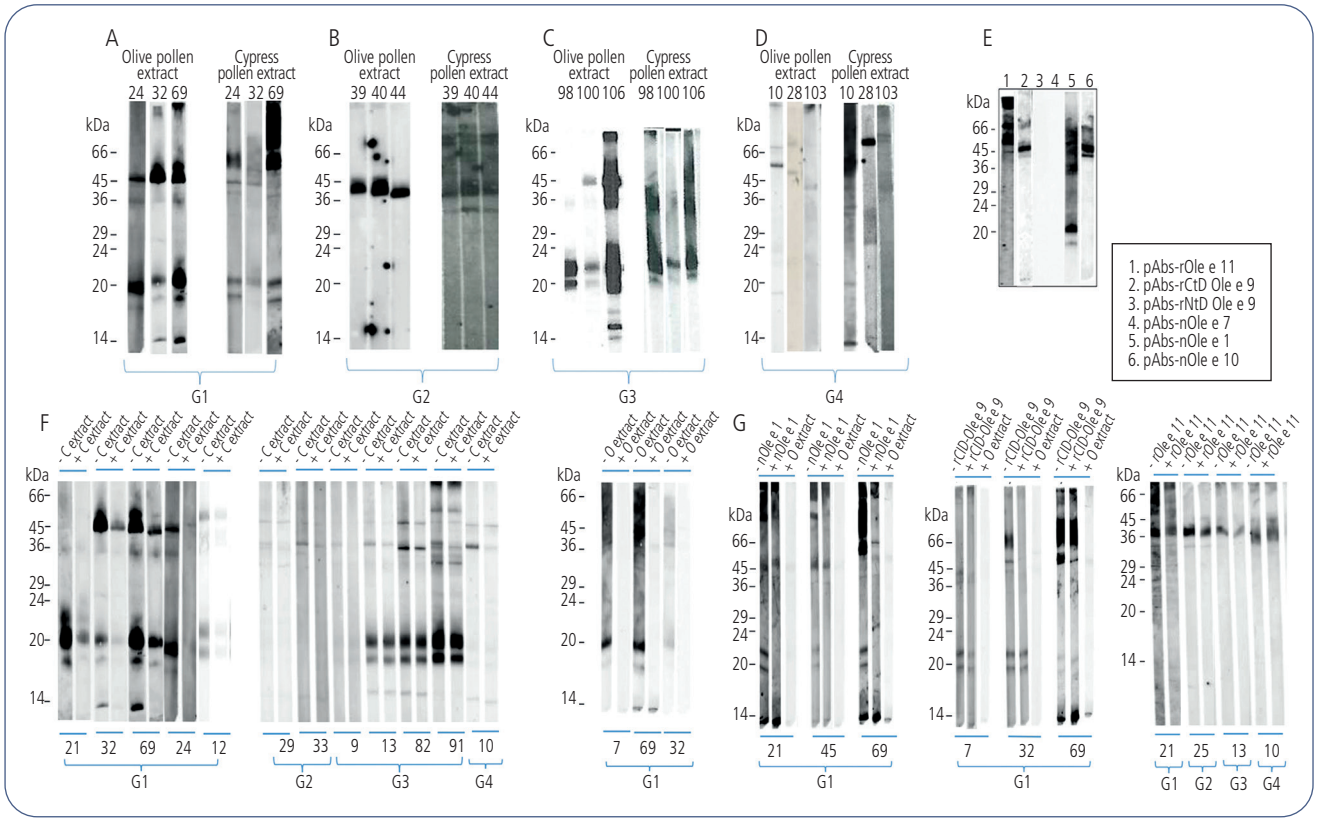



**Figure 5.** A, B, C, D, Band patterns recognized by individual C+O patients by immunoblotting assay in 4 subgroups with both olive and *Cupressus arizonica* pollen extracts; E, Immunoblotting by polyclonal antibodies (pAbs) specific to olive pollen allergens; F, IgE-inhibition assays to olive pollen extract with 500 µg of *C arizonica* pollen extract as inhibitor; G, IgE-inhibition assays to *C arizonica* pollen extract with 500 µg of olive pollen extract as inhibitor; H, Identification of Ole e 1, Ole e 9, and Ole e 11 homologs in *C arizonica* pollen extract. IgE-inhibition assays were performed with individual patients using olive pollen extract (500 µg), Ole e 1 (10 µg), Ole e 9 (10 µg), and Ole e 11 (10 µg).
G1 indicates Cup s 1+ /Ole e 1+; G2, Cup s 1+ /Ole e 1–; G3, Cup s 1–/Ole e 1+; G4, Cup s 1–/Ole e 1–; C, cypress; O, olive.

allergen Ole e 9 across the C-terminal domain homologous to this allergen. The presence of a band of around 10 kDa corresponding to Ole e 10 was not visible, possibly because of the low levels of this allergen in the cypress pollen extract.

### *Cross-Reactivity Between Olive and Cypress Pollen*

The presence of olive-homologous allergens in cypress pollen that could be responsible for cross-reactivity between both pollens was elucidated by IgE-inhibition assays, using *Cupressus arizonica* and olive pollen extracts as inhibitors (Figure 5F). Bands corresponding to Ole e 1 (20 kDa), Ole e 9 (45 kDa), and Ole e 11 (37 kDa) disappeared in the sera of the 4 subgroups (G1, G2, G3, and G4). Identification of the homologous Ole e 1–like, Ole e 9–like, and Ole e 11–like allergens in the cross-reactivity between cypress and olive were confirmed after inhibition of IgE binding of to cypress extracts with these allergens using individual sera (Figure 5G).

## Discussion

To our knowledge, we present the first clinical and molecular analysis to investigate the allergenic profile of patients with exclusive dual sensitization to cypress and olive pollen. Our novel finding that a cluster analysis identified 4 phenotypes linked to seasonal/perennial symptoms and allergen profile is a key strength of the study.

Rhinoconjunctivitis was the most frequent presentation among the 85 patients with allergy to both pollens selected for the study and among those monosensitized to cypress and olive recruited as the reference population. Cypress and olive pollen more frequently cause rhinoconjunctivitis than asthma [7]. The seasonal clinical presentation was the most frequent, especially during February and May, followed by only May and only February. In contrast, control patients allergic to cypress or olive had symptoms in February and May, respectively. Perennial symptoms were also observed in our study, as reported elsewhere for cypress- or olive-allergic patients [23,24]. These findings are consistent with the MCA and cluster analysis.

The specific and major allergens Ole e 1 and Cup s 1 were the most prevalent (74% and 59.7%, respectively). Thanks to the availability of purified allergens from olive and cypress pollen, 18 different combinations of allergens were involved in the sensitization of this population, the most frequent being that of Cup s 1 and Ole e 1, followed by monosensitization to Ole e 1 and Cup s 1. The percentages of sensitization to minor olive pollen allergens (Ole e 9, Ole e 11, Ole e 12) were low,

as expected, and very low (Ole e 2, Ole e 3) or nonexistent (Ole e 7) for panallergens. The latter result is consistent with the absence of sensitization to other pollens. The significant percentage of sensitization of Ole e 9 is noteworthy, with a higher prevalence in a previous study performed in the same area [2] and closer to the frequency in areas with high olive pollen concentrations [19,25]. Sensitization to Ole e 9 and Ole e 11 in C group patients was a striking and novel finding that suggested the presence in cypress pollen of allergens homologous to Ole e 9 and Ole e 11.

Minor olive pollen allergen profile has been associated with allergenic phenotypes such as asthma, food allergy, and atopic dermatitis [19,25].

In patients allergic to C+O, an association was found between sensitization to Ole e 9 (CtD and NtD) and to Ole e 1 and symptoms during February and May, between sensitization to Cup s 1/Cup a 1 and symptoms in February, and between sensitization to Ole e 11 and Ole e 12 and perennial symptoms. The MCA and cluster analysis results support these previously unreported associations.

Low sIgE to bromelain could suggest the absence of an impact of cross-reactive carbohydrate determinants in the study patients.

The finding of sensitization exclusively to cypress and olive pollen in the study participants supported the idea that they were either cosensitized to specific allergens of both pollens or to cross-reactive allergens not yet described. A possible implication of Ole e 1, Ole e 9, and Ole e 11 homologs was suggested by the ELISA results and confirmed by immunoblotting assays. The presence of Ole e 1, Ole e 9, and Ole e 11 homologs was first demonstrated with specific pAbs against these olive allergens in cypress pollen and confirmed by immunoblotting-inhibition assays.

In order to improve the management and optimal selection of immunotherapy for C+O patients, we propose a molecular diagnostic algorithm with the commercially available allergens Cup a 1, Ole e 1, and Ole e 9. Therefore, those patients who recognize both major allergens would be candidates for immunotherapy with both cypress and olive extracts, whereas only Cup s 1– or Ole e 1–positive patients would receive immunotherapy with cypress pollen or olive pollen, respectively. Immunotherapy should not be recommended in patients who do not recognize major allergens. For patients positive to Ole e 9, and owing to the great variability of this allergen between batches [26], an olive pollen extract in which this allergen is quantified would be the treatment of choice to achieve greater efficacy and better tolerance.

In conclusion, our results enhance current knowledge about the role of allergens in both cypress and olive allergy. Cosensitization through the major allergens Cup s 1 and Ole e 1 would explain sensitization exclusively to cypress and olive allergens (G1 group) and cross-reactivity through olive allergen homologs (Ole e 1, Ole e 9, and Ole e 11) or other, yet unknown, allergens in the other subgroups (G2, G3, and G4) that have yet to be characterized in future studies. Finally, we would like to emphasize the fact that, to ensure personalized treatment, molecular diagnosis should be complementary to the clinical approach.

## Acknowledgments

We would like to thank Sara Abián from the Complutense University of Madrid for her excellent technical support. We thank all the members of Biobank from Hospital Universitario Fundación Alcorcón for sample collection and processing.

### *Funding*

This work was supported by a grant from the Spanish Society of Allergy and Clinical Immunology (SEAIC) Foundation (Ref.2005_01).

This work was supported by the ARADyAL Research Network (RD16/0006/0009 and RD16/0006/0014), cofunded by the Ministry of Science of the Spanish Government and FEDER (European Regional Development Fund).

This work was supported by grant SAF2017-86483-R from the Ministry of Economy and Competitiveness.

### *Conflicts of Interest*

MFR reports the following: grants from the ISCIII (Spanish government), Aimmune Therapeutics, and Diater; consultancy fees from Aimmune Therapeutics, DBV, Novartis, Reacta Healthcare, and SPRIM; and lecture fees from Aimmune Therapeutics, ALK, Allergy Therapeutics, Diater, GSK, and HAL Allergy, all outside the submitted work.

The remaining authors declare that they have no conflicts of interest.

## References

1. Ojeda P, Sastre J, Olaguibel JM, Chivato T, Allergology investigators participating in the National Survey of the Spanish Society of Allergology and Clinical Immunology Alergologica 2015. Alergologica 2015: A National Survey on Allergic Diseases in the Adult Spanish Population. J Investig Allergol Clin Immunol. 2018;28:151-64.
2. Cuesta-Herranz J, Barber D, Blanco C, Cistero-Bahima A, Crespo JF, Fernandez-Rivas M, et al. Differences among pollen-allergic patients with and without plant food allergy. Int Arch Allergy Immunol. 2010;153:182-92.
3. Polen atmosférico en la Comunidad de Madrid, Direccion General de Salud Pública, Consejeria de Sanidad. Edit BOCM (Boletin Oficial de la Comunidad de Madrid) ISBN 84-451-2018-2.
4. Orovitg A, Guardia P, Barber D, Torre F de la, Rodriguez R, Villalba M, et al. Enhanced diagnosis of pollen allergy using specific immunoglobulin E determination to detect major allergens and panallergens. J Investig Allergol Clin Immunol. 2011;21:253-9.
5. Asam C, Hofer H, Wolf M, Aglas L, Wallner M. Tree pollen allergens-an update from a molecular perspective. Allergy. 2015;70:1201-11.
6. D'Amato G, Cecchi L, Bonini S, Nunes C, Annesi-Maesano I, Behrendt H, et al. Allergenic pollen and pollen allergy in Europe. Allergy. 2007;62:976-90.
7. Matricardi PM, Kleine-Tebbe J, Hoffmann HJ, Valenta R, Hilger C, Hofmaier S, et al. EAACI Molecular Allergology User's Guide. Pediatr Allergy Immunol. 2016;27 Suppl 23:1-250.

8. Charpin D, Pichot C, Belmonte J, Sutra J-P, Zidkova J, Chanez P, et al. Cypress Pollinosis: from Tree to Clinic. Clin Rev Allergy Immunol. 2019;56:174-95.
9. Asero R, Ceriotti V, Bonini M. Cypress pollen allergy in Milan: the story of an ongoing growth. Eur Ann Allergy Clin Immunol [Internet]. 2021 [cited 2023 Feb 3];53:209. Available from: http://www.eurannallergyimm.com/cont/journals-articles/1038/volume-cypress-pollen-allergy-milan-story.asp
10. Perez-Badia R, Rapp A, Morales C, Sardinero S, Galan C, Garcia-Mozo H. Pollen spectrum and risk of pollen allergy in central Spain. Ann Agric Environ Med AAEM. 2010;17:139-51.
11. Vidal C, Enrique E, Gonzalo A, Moreno C, Tabar AI, Expert Clinical Participants. Diagnosis and allergen immunotherapy treatment of polysensitised patients with respiratory allergy in Spain: an Allergists' Consensus. Clin Transl Allergy. 2014;4:36-6. eCollection 2014.
12. Barber D, Diaz-Perales A, Escribese MM, Kleine-Tebbe J, Matricardi PM, Ollert M, et al. Molecular allergology and its impact in specific allergy diagnosis and therapy. Allergy. 2021;76:3642-58.
13. Matricardi PM, Dramburg S, Potapova E, Skevaki C, Renz H. Molecular diagnosis for allergen immunotherapy. J Allergy Clin Immunol. 2019;143:831-43.
14. Sastre J, Landivar ME, Ruiz-Garcia M, Andregnette-Rosigno MV, Mahillo I. How molecular diagnosis can change allergen-specific immunotherapy prescription in a complex pollen area. Allergy. 2012;67:709-11.
15. Moreno C, Justicia JL, Quiralte J, Moreno-Ancillo A, Iglesias-Cadarso A, Torrecillas M, et al. Olive, grass or both? Molecular diagnosis for the allergen immunotherapy selection in polysensitized pollinic patients. Allergy. 2014;69:1357-63.
16. Villalba M, Rodriguez R, Batanero E. The spectrum of olive pollen allergens. From structures to diagnosis and treatment. Methods San Diego Calif. 2014;66:44-54.
17. Oeo-Santos C, Mas S, Quiralte J, Colas C, Blanca M, Fernandez J, et al. A Hypoallergenic Polygalacturonase Isoform from Olive Pollen Is Implicated in Pollen-Pollen Cross-Reactivity. Int Arch Allergy Immunol. 2018;177:290-301.
18. Segundo-Acosta PS, Oeo-Santos C, Benede S, Rios V de L, Navas A, Ruiz-Leon B, et al. Delineation of the Olive Pollen Proteome and Its Allergenome Unmasks Cyclophilin as a Relevant Cross-Reactive Allergen. J Proteome Res. 2019;18:3052-66.
19. Barber D, de la Torre F, Feo F, Florido F, Guardia P, Moreno C, et al. Understanding patient sensitization profiles in complex pollen areas: a molecular epidemiological study. Allergy. 2008;63:1550-8.
20. Weber RW. Patterns of pollen cross-allergenicity. J Allergy Clin Immunol. 2003;112:229-39; quiz 240.
21. Bistoni O, Emiliani C, Agea E, Russano AM, Mencarelli S, Orlacchio A, et al. Biochemical and immunological characterization of pollen-derived beta-galactosidase reveals a new cross-reactive class of allergens among Mediterranean trees. Int Arch Allergy Immunol. 2005;136:123-33.
22. Huecas S, Villalba M, Rodriguez R. Ole e 9, a major olive pollen allergen is a 1,3-beta-glucanase. Isolation, characterization, amino acid sequence, and tissue specificity. J Biol Chem. 2001;276:27959-66.
23. Sposato B, Liccardi G, Russo M, Folletti I, Siracusa A, Scichilone N, et al. Cypress pollen: an unexpected major sensitizing agent in different regions of Italy. J Investig Allergol Clin Immunol. 2014;24:23-8.
24. Kirmaz C, Yuksel H, Bayrak P, Yilmaz O. Symptoms of the olive pollen allergy: do they really occur only in the pollination season? J Investig Allergol Clin Immunol. 2005;15:140-5.
25. Scala E, Abeni D, Pomponi D, Paganelli R, Locanto M, Giani M, et al. Ole e 1, Ole e 7, and Ole e 9: Identifying distinct clinical subsets of olive tree-allergic patients. J Allergy Clin Immunol. 2016;137:629-31.e3.
26. Duffort O, Palomares O, Lombardero M, Villalba M, Barber D, Rodríguez R, et al. Variability of Ole e 9 allergen in olive pollen extracts: relevance of minor allergens in immunotherapy treatments. Int Arch Allergy Immunol. 2006;140:131-8.

*Manuscript received November 14, 2022; accepted for publication May 24, 2023.*

**María Dolores Alonso-Díaz de Durana**

Allergy Unit
Hospital universitario Fundación Alcorcón
C/ Budapest 1
28922 Alcorcón (Madrid), Spain
E-mail: mdolores.alonso@salud.madrid.org