

**Allergy to cypress and olive pollen: Clinical phenotypes and allergen recognition****Brief running title:** Allergy to cypress and olive pollen

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## Abstract

**Background:** Cypress and olive pollen are the most prevalent sensitizer trees in the Mediterranean area. Some patients exhibit a dual sensitization which has not been well documented yet.

**Objective:** To identify the allergens involved in the dual cypress and olive allergy (C+O) and study the relationship between phenotype and allergen sensitization.

**Methods:** C+O patients were selected. Monosensitized subjects to olive or cypress were used as reference. Specific IgE to whole extracts and purified allergens from olive and cypress were performed. Immunoblotting was done 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 C+O clinical presentation (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 subjects. Ole e 1 and Cup s 1 were the major allergens. Homologous proteins to Ole e 1, Ole e 9 and Ole e 11 in cypress pollen were identified and these olive allergens inhibit IgE-binding to cypress extract.

**Conclusions:** The exclusive C+O allergy results from co-sensitization to Cup s 1 and Ole e 1, and to cross-reactivity due to Ole e 1-like, Ole e 9-like and Ole e 11-like allergens not described previously, and translates into 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 alérgica.

**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 trees in the Mediterranean area. There are patients with exclusive cypress and olive pollen allergy, but 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 co-sensitization to Cup s 1 and Ole e 1, and to cross-reactivity of Ole e 1, Ole e 9, and Ole e 11 with homologous allergens in cypress pollen and translates into 4 clinical phenotypes of seasonal/perennial respiratory allergy.

## INTRODUCTION

Pollen is the leading cause of both allergic rhinoconjunctivitis (RC) and asthma in Spain[1]. The most allergenic pollen in the Madrid area are from grasses (*Poaceae*), and from 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 with seasonal respiratory symptoms[4] with cypress and olive pollen being the most prevalent sensitizers among trees[5–7]. In the last 40 years, there has been a significant increase in cypress plantation worldwide, especially around Mediterranean basin, for ornamental purposes and in fences, that together with the pollution particles that interact with pollen grains and increases its solubility in the air, has increased the allergenicity[8,9]. The growing importance of cypress and olive tree pollen allergies in Spain, as well as geographical differences in their prevalence of sensitization, have been confirmed in the national epidemiological surveys of Alergologica performed in 2005 and 2015[1]. The cypress and the olive trees belong to different botanical families without taxonomic relationships or overlapping seasonality. In our area, cypress pollinates during 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 trees 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) but, it is not well documented in the literature to date.

In the last twenty-five years a great breakthrough in molecular biology has taken place, with the characterization of allergens from different sources. Molecular diagnosis solves the limitations of whole extracts in polysensitized patients[11], by identifying the responsible allergens and discriminating between genuine sensitization or cross-reactivity[12,13]. Regarding treatment, molecular diagnosis may lead to a change in the composition of the immunotherapy (IT) formerly considered appropriate according to the skin prick test (SPT) results in almost 60% of patients[14,15].

The olive pollen major allergen, Ole e 1 (common olive group) and both cypress pollen major allergenic homologous allergens, Cup a 1 from *Cupressus arizonica* (Ca) and Cup s 1 from *Cupressus sempervirens* (Cs) detect genuine sensitization to the *Oleaceae* and *Cupressaceae* families, respectively. Ole e 1 shows 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%[6,7,8,16]. Four groups of cypress allergens have been described and referenced in IUIS (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 them, such as Ole e 7 and Ole e 9, behave as major allergens in areas of maximum exposure to olive pollen, and they are considered relevant markers of severity due to its association with asthma[19]. Some degree of cross-reactivity between cypress and olive pollen were attributed to panallergens[20] or to other allergens such a beta-galactosidase[21].

The aims of this study were firstly to determine if the double sensitization to cypress and olive pollen could be due to sensitization to specific allergens of both pollen, 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 for 4 years. They had to refer respiratory symptoms of RC and/or asthma for at least two years and had to present positive skin prick tests (SPT) exclusively to cypress (Ca and/or Cs) and olive pollen. Patients with additional positive SPT to other inhalants and those who had received IT 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 signed informed consent to enter the study.

The following clinical information was collected in a case record form: age, sex, symptoms of pollen allergy (RC, asthma), seasonality (yes/no) and in those with

seasonal symptoms the peak month (February for winter and/or May for spring) of the clinical presentation[3].

SPT were performed with commercial extracts (ALK-Abelló, Madrid, Spain) of grasses, cypress (*Ca and Cs*), 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 *nephelometric* method (Siemens Healthcare Diagnostics, Germany). Serum specific IgE (sIgE) to *Ca*, *Cs*, *Olea europaea*, *Platanus acerifolia* and *Lolium perenne* were measured with the ImmunoCAP System (Thermo Fisher Scientific, Uppsala, Sweden). The cut-off point was 0.35 kU<sub>A</sub>/L. Patients with respiratory allergy and monosensitized to either olive or cypress pollen were included as controls, provided informed consent, and were submitted to the same clinical evaluation as the cypress+olive study subjects.

#### **Allergen profiling in the population studied**

*Ca* and olive pollen was purchased from Allergon-Pharmacia (Sweden) and extracted as described[22]. sIgE against *Ca* 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 were performed by indirect ELISA. Allergens included in this study have been purified by the group of Villalba [16] except Cup s 1 kindly donated by ALK-Abelló (Madrid, Spain). Individual patient sera were used at dilution 1:10 in PBS. IgE binding was detected with mouse anti-human IgE antibody (1:5000 dilution). Peroxidase reaction was developed using fresh enzyme substrate and measuring absorbance at 492 nm. Values under 0.1 optical density (OD) were considered negative.

IgE-immunoblotting assays to 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), with mouse anti-human IgE monoclonal antibody (diluted 1:5000) kindly provided by ALK-Abelló (Madrid, Spain), followed by horseradish

peroxidase-labeled polyclonal IgG (1:3000 diluted; Pierce, Rockford, Illinois). 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, Ole e 11 (1:10 PBS dilution) were detected by goat anti-rabbit IgG horseradish peroxidase-labeled antibody (1:3000) (DAKO, Glostrup, Denmark). Chemiluminiscent 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 pre-incubated at room temperature for 2 hr with 5 µg of the purified allergens or 500 µg of the cypress or olive extracts, using PBS as negative control. The rest of the steps were as described above.

### **Statistical analysis**

Qualitative variables were presented as a percentage and quantitative variables as mean and standard deviation (SD), or median and interquartile range (IQR, Q1-Q3). A univariate analysis was performed to analyze differences between the groups of study subjects allergic to cypress and olive (C+O group), and controls allergic to olive (O group) and cypress (C group) pollen. Qualitative variables were compared with Pearson chi-squared test or Fisher's exact tests and quantitative variables with Kruskal Wallis non-parametric test.

Multiple correspondence analysis (MCA) and hierarchical classifications were conducted to discriminate groups of subjects. MCA is a Multivariate technique to visualize the association among categorical variables through a graphic and was performed to study association between groups (C+O, C and O), symptoms (RC, asthma, seasonality) and allergen profile.

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

Statistical analysis was performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and STATA 13. Significant level was set at  $P < .05$ .

## **RESULTS**

### ***Description of the population under study***

Demographic and clinical data together with SPT and sIgE results are summarized in Table 1. The study included 121 patients with clinical history and concordant SPT: 85 subjects in the C+O group (mean age 34.7 yrs, males 42%), 21 in the C group (mean age 45 yrs, males 38%) and 15 individuals in the O group (mean age 33.9 yrs, males 27%). The most prevalent respiratory symptom in the three groups was RC, more frequently presented in the C+O (62.4%) and C (90.5%) groups, than in the O group (46.7%) ( $P=0.003$ ). Asthma with and without RC was more frequently present in patients from O group (53.3%) ( $P=0.014$ ). Most patients in the three groups had seasonal symptoms, although 40% of O group had perennial symptoms ( $P=0.032$ ). In the C+O group 66% of subjects had symptoms in both February and May.

### **Allergen profile**

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

Analyzing sIgE to purified allergens in C+O group, we found 18 different combinations (Supplementary Table 1). The most frequent was both major allergens, Cup s 1 and Ole e 1 (27.3%), followed by the single sensitization 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 19.5% and 10.4% positive results, respectively. Sensitization to other allergens was below 10%: bromelain (7.8%), Ole e 12 (3.9%), Ole e 3 (2.6%), and Ole e 2 (1.3%). In C group, the sensitization to Cup s 1 was 90.5%. In O group, there was a strikingly low prevalence for Ole e 1 (33.3%), along with 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 O group) and Cup s 1 (higher in C group) (Figure 1, Supplementary Table 2). To get further insight in the sensitization profile, patients of the 3 groups (C+O, C and O) were subclassified in 4 sub-groups, defined according to the positive or negative response against the two major allergens, Cup s 1 and Ole e 1: 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 (Kruskal Wallis test,  $P<.001$ ) (Figure 2A). The sensitization to G1 subgroup was the most frequent (44.2%) in C+O group but absent in C or O groups. In C group >90% were exclusively sensitized to Cup s 1, and in the monosensitized to olive only 1 out of 3 were sensitized to Ole e 1. We have further analyzed the allergen profile in the G1 to G4 subgroups of C+O subjects and found that the sensitization to any minor olive allergens is presented in all four subgroups more frequently when response to Ole e 1 was positive (G1, G3), but also when negative (G2, G4). Ole e 11 was the only allergen present in patients from the four subgroups (Figure 2B, Supplementary Table 3).

### ***Clinical presentation and allergen profile***

MCA analysis was performed to analyze the pattern of relationships between clinical presentation and allergen profile in the three study groups (Figure 3). The MCA for clinical presentation (Figure 3A) showed an association of seasonal symptoms and RC+asthma in the C+O group, whereas O group presented an association with asthma and perennial symptoms, and C group with RC and seasonal symptoms. The MCA graph for allergen profile (Figure 3B) showed the association between C+O group and sensitization to Ole e 1 and Cup s 1, while in the O group sensitization to Ole e 12 and NtD-Ole e 9 appeared, and CtD-Ole e 9 and Cup s 1 in the C group. MCA exploring clinical presentation and allergen profile in the C+O group is presented (Figure 3C). The graph shows the association between sensitization to Ole e 9 (NtD and CtD) and Ole e 1 with symptoms during February+May; sensitization to Ole e 11 and Ole e 12 with perennial symptoms and sensitization to Cup s 1 and Ole e 11 with symptoms during February.

In cluster analysis of subjects of the C+O group, a dendrogram plot was generated and the final adopted solution was constituted by 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, 11 patients (14.3%) with symptoms in May were in cluster 2, cluster 3 included 45 subjects (58.4%) with symptoms in February and May, and cluster 4 the 10 patients (13%) with

perennial symptoms. Cup s 1 and Ole e 1 sensitizations were presented in all clusters, but the former was more frequent in cluster 1 (82%) and Ole e 1 in clusters 3 (80%) and 4 (82%). No cases of Ole e 9, Ole e 11 or Ole e 12 were found in cluster 2. The more frequent minor Ole e allergen in Cluster 1 and 4 was Ole e 11 (36% and 50%, respectively), and CtD-Ole e 9 in cluster 3 (16%), Asthma was more frequent in cluster 4, presented by 50% of patients.

### ***IgE immunoblots of patients with double sensitization***

IgE immunoblots of olive and *Ca* pollen extracts performed with sera of subjects from C+O, C and O groups are shown in Supplementary Figure 1. In order to compare the allergenic profile of the subgroups G1, G2, G3 and G4 of C+O patients, some representative results are presented in Figure 5. Sera from subgroup G1 (24, 32 and 69) (Figure 5A) recognized a band of 20 kDa that corresponds to Ole e 1, and a band of 45 kDa corresponding to Ole e 9 as was confirmed by the ELISA assays. The band around 14 kDa observed in sera 32 and 69 could correspond to Ole e 10, the homologous allergen to the C-terminal domain of Ole e 9 and consequently with cross-reactivity to the whole Ole e 9. The profile of these three patients is similar in cypress extract, which present bands with similar molecular masses (20 kDa and 45 kDa, respectively). Sera of 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 maybe to a Cup s 1-like or another unidentified allergen in olive pollen. Sera of subgroup G3 (98,100,106) with recognition of Ole e 1 by ELISA, present a band around 21 kDa in olive pollen that corresponds to Ole e 1, and bands of a similar MW in cypress that could be an Ole e 1-like allergen (Figure 5C). Sera of subgroup G4 (10, 28 and 103) (Figure 5D) did not recognize any of the major allergens Cup s 1 and Ole e 1. Faint high molecular mass bands were observed in both extracts, so unidentified allergens could be responsible for sensitization, although they are not any of the analyzed by ELISA.

### ***Identification of homologous olive pollen allergens in cypress pollen extract***

The pAbs directed to olive pollen allergens were used with the cypress pollen extract in immunoblotting, obtaining positive recognition with 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 can recognize the whole protein of 45 kDa. The pAb against Ole e 10 also recognized the 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 is not visible maybe due to 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 of cross-reactivity between both pollen was elucidated by IgE-inhibition assays, using Ca 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 subgroups G1, G2, G3 and G4 selected. Identification of homologous allergens Ole e 1-like, Ole e 9-like and Ole e 11-like in the cross-reactivity between cypress and olive were confirmed after inhibiting the IgE-binding capability to cypress extracts with these allergens using individual sera (Figure 5G).

## **DISCUSSION**

This study is, to the best of our knowledge, the first clinical and molecular analysis to investigate the allergenic profile of patients with exclusive dual sensitization to cypress and olive pollen. An important novelty and strength of this study is that a cluster analysis has identified 4 phenotypes linked to seasonal/perennial presentation of the symptoms and allergen profile.

From the 85 patients with allergy to both pollen selected for the study, RC was the most frequent presentation as well as in monosensitized to cypress and olive populations recruited as reference. Cypress and olive pollen cause more frequently RC 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 have been also observed in our study, as previously other papers reported in cypress or olive allergic patients[23,24]. These findings are consistent with the MCA and cluster analysis. 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, being the most frequent that of Cup s 1 and Ole e 1 followed by the single sensitization to Ole e 1 and Cup s 1. The percentages of sensitization to minor olive pollen allergens were low (Ole e 9, Ole e 11, Ole e 12) as expected, and very low (Ole e 2, Ole e 3) or inexistent (Ole e 7) to panallergens. The latter result agrees with the absence of sensitization to other pollen. Of note the significant percentage of sensitization of Ole e 9, with a higher prevalence than previously found in a study performed in the same area [2] and closer to the frequency in areas with high olive pollen concentration[19,25]. It is striking the sensitization to Ole e 9 and Ole e 11 in cypress pollen monosensitized patients, which has not been previously described, and suggested the presence in cypress pollen of allergens homologous to Ole e 9 and Ole e 11.

It is known that minor olive pollen allergen profile has been associated with allergenic phenotypes such as asthma, food allergy and atopic dermatitis[19,25]. In C+O allergic patients, an association was found between Ole e 9 (CtD and NtD) and Ole e 1 sensitization and symptoms during February+May, Cup s 1/Cup a 1 sensitization and symptoms in February, and Ole e 11 and Ole e 12 sensitization and perennial symptoms. MCA and Cluster analysis results support these interrelationships never described before.

Low sIgE to bromelain could suggest the absence of impact of Cross-reactive Carbohydrate Determinants (CCDs) recognition in our patients.

The finding of an exclusive sensitization to cypress and olive pollen in our study subjects supported the idea that they were either co-sensitized to specific allergens of both pollen or to cross-reactive allergens not yet described. A possible implication of Ole e 1, Ole e 9 and Ole e 11 homologues was suggested by the ELISA results and confirmed by immunoblotting assays. The presence of Ole e 1, Ole e 9, and Ole e 11 homologues have been firstly 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 IT for patients allergic to both C+O pollen, 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 IT with both cypress and olive extracts, whereas only Cup s 1- or Ole e 1-positive patients would receive IT with cypress pollen or olive pollen, respectively. In patients with no recognition of major allergens, IT should not be recommended. For patients positive to Ole e 9 and due 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 a greater efficacy and better tolerance.

In conclusion, our results contribute to enhance the knowledge about the role of allergens in both cypress and olive allergy. Co-sensitization through major allergens Cup s 1 and Ole e 1 would explain both cypress and olive allergy exclusive sensitization (G1 group) and cross-reactivity through olive allergen homologues (Ole e 1, Ole e 9 and Ole e 11) or other yet unknown allergens in the other groups (G2, G3 and G4) which need to be characterized in future studies. Finally, we would like to emphasize the fact that, to achieve a personalized treatment of the patient, molecular diagnosis should be complementary to the clinical approach.

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### **Conflicts of Interest**

MFR reports grants from ISCIII of 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 of them outside the submitted work.

All other authors declare that they have no conflicts of interest.

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## TABLE LEGENDS

**Table.** Study subjects: demographics, clinical presentation, SPT and sIgE results.

			<b>C+O group</b>	<b>C group</b>	<b>O group</b>	<b>P value</b>	
			<b>N= 85</b>	<b>N= 21</b>	<b>N=15</b>		
<b>Demographic Data</b>	<b>Age, yr</b>	Mean (SD)	34.7 (12.7)	45 (13.2)	33.9 (11.1)	.012	
	<b>Sex</b>	Male	36 (42.4%)	8 (38.1%)	4 (26.7%)	.512	
<b>Clinical Presentation</b>	<b>Symptoms</b>	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%)		
	<b>Seasonality</b>	Asthma w/ and w/o RC	32 (37.6%)	2 (9.5%)	8 (53.3%)	.014	
		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
May	14 (18.9%)	0		9 (100%)			
Feb+May	49 (66.2%)	1 (5.3%)		0			
<b>SPT</b>	<b>R SPT Ca/ histamine</b>	Median (Q1-Q3)	0.83 (0.63 - 1.17)	0.95 (0.7 - 1.08)		.652	
	<b>R SPT Cs/ histamine</b>	Median (Q1-Q3)	0.6 (0.4 - 0.77)	0.71 (0.6 - 1)		.035	
	<b>R SPT O/ histamine</b>	Median (Q1-Q3)	0.6 (0.4 - 0.77)		1.71 (1.13 - 2.2)	.014	
<b>Total IgE (IU/ml)</b>		Median (IQR)	85.6 (39.65 - 172.5)	83.7 (49.13 - 209.75)	28.2 (19 - 70.7)	.008	
<b>sIgE</b>	<b>Ca and/or Cs</b>	N (% positive)	65 (76.5%)	20 (95.2%)	0	.067	
	<b>O</b>	N (% positive)	78 (91.8%)	0	8 (53.3%)	.001	

C+O, Cypress+Olive; C, Cypress; O, Olive; Ca, *Cupressus arizonica*; Cs, *Cupressus sempervirens*; RC, rhinoconjunctivitis; R, ratio; sIgE, specific serum IgE; SPT, Skin Prick Test; yr, year; w/ and w/o: with and without

## FIGURE LEGENDS

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

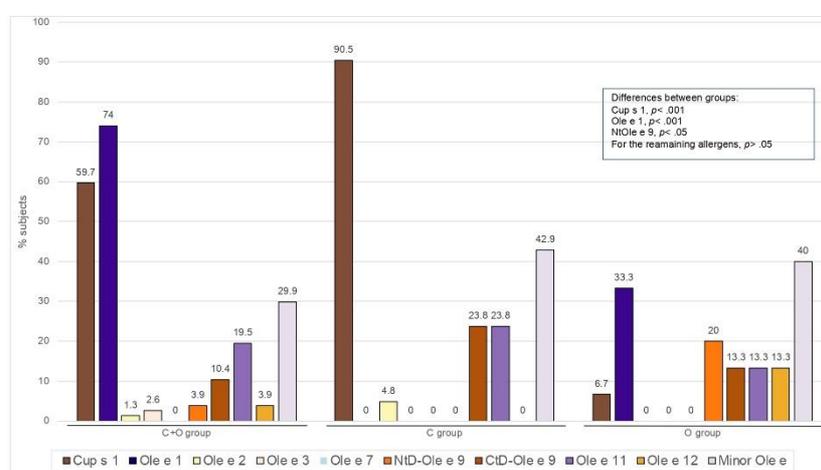
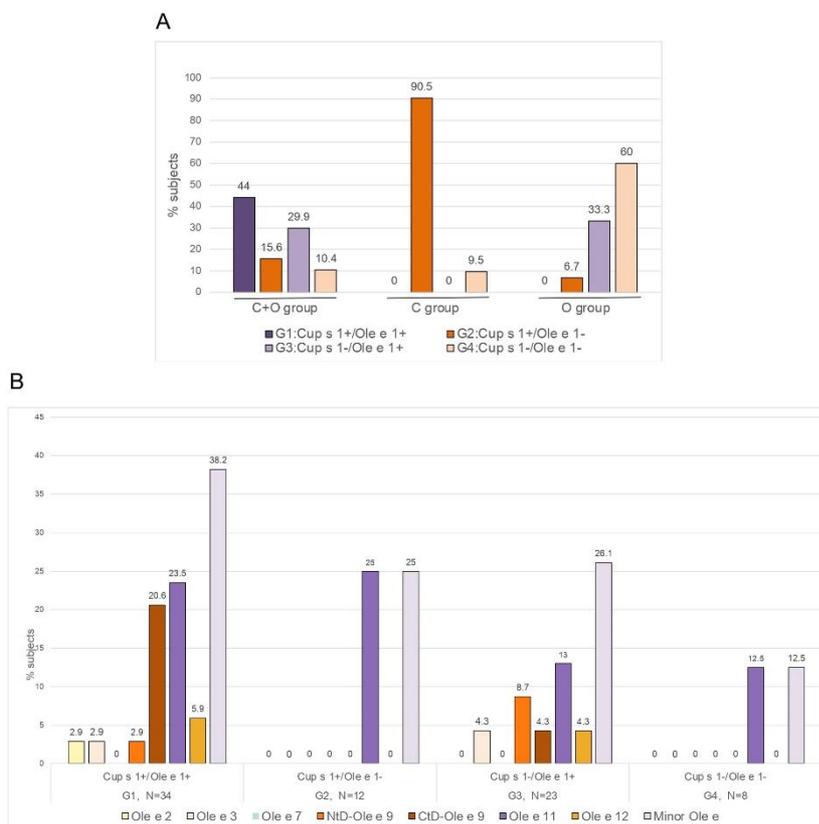


Figure 1\_Alonso et al.

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

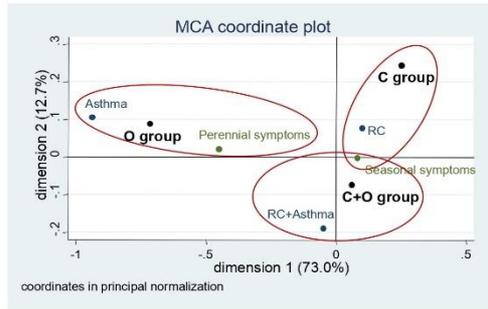


**Figure 3.** Multiple Correspondence Analysis (MCA) of clinical presentation and allergen profile. Distance between variables indicates the approximate relation between them. The distance between variables is inversely proportional to the strength of the relation. Circles have been added to emphasize the proximity between points. Dots with a sign + mean allergen sensitization. Dots without a sign + mean no sensitization.

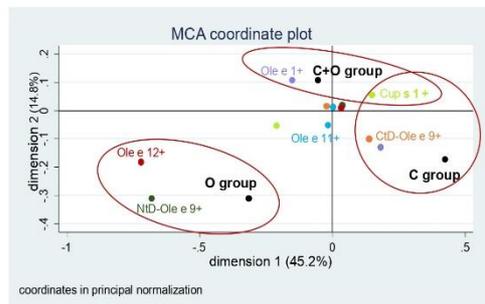
A, Group and clinical presentation in the C+O, C and O groups; B, Group and allergen sensitization profile in in the C+O, C and O groups; C, Clinical presentation and allergen profile in C+O group.

Asthma, asthma with and without RC; C+O, Cypress+Olive; C, Cypress; Feb, February; F+M, February and May; O, Olive; RC, rhinoconjunctivitis.

A



B



C

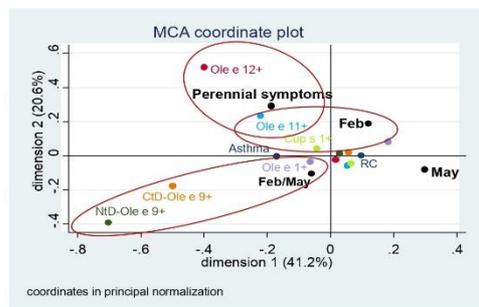


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**Figure 4.** A, Cypress and olive allergic group: Allergen profile and clinical presentation of cluster analysis solution. Dendrogram (hierarchical cluster analysis, Ward method). B, Statistics of qualitative characteristics of each cluster defined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

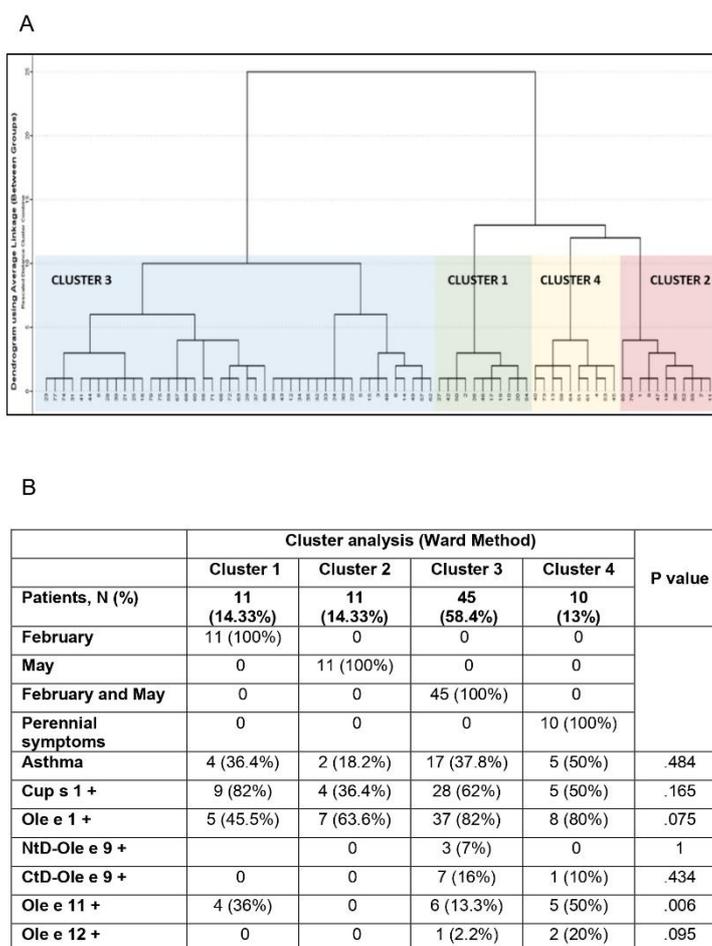


Figure 4\_Alonso et al

**Figure 5.** A, B, C, D: Band patterns recognized by individual C+O patients by immunoblotting assay in 4 subgroups with both Olive and *C.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 homologues 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, 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.

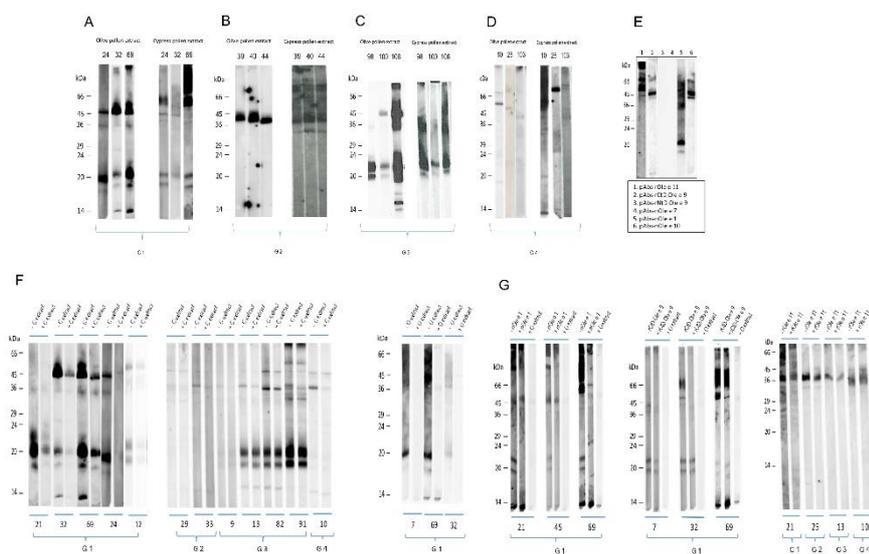


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