Prevalence of allergic sensitization to conifer pollen in a high cypress exposure area

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ABSTRACT

Background: *Sensitization to Pinales (Cupressaceae and Pinaceae) has increased dramatically in recent years. The prevalence of sensitization in different geographic areas is related to exposure to specific pollens.*

Objectives: *To investigate the prevalence of allergy to different conifer pollens, describe the characteristics of patients with such allergy, and identify the involved allergens.*

Methods: *Patients were recruited at five hospitals near Madrid. Extracts from conifer pollen were prepared and used in skin-prick testing. Wheal sizes were recorded, and serum samples obtained from patients with positive reactions to Cupressus arizonica and/or Pinus pinea. The specific immunoglobulin E value to C. arizonica and Cup a 1 was determined. Individual immunoblots for each patient and with a pool of sera were performed. Allergenic proteins were sequenced by using liquid chromatography-tandem mass spectrometry.*

Results: *Of 499 individuals included in the study, 17 (14%) had positive skin-prick test results to some conifer pollen extracts. Sixty-four patients had positive results to C. arizonica (prevalence 12.8%) and 11 had positive results to P. pinea (2.2%). All the patients had respiratory symptoms (61.4% during the C. arizonica pollination period), and 62.9% had asthma. Approximately 86% of the patients had positive specific immunoglobulin E results to C. arizonica and 92.3% had positive results to Cup a 1. Fourteen different bands were recognized by immunoblot; the most frequent bands were those detected at 43, 18, 16, and 14 kDa. All sequenced proteins corresponded to Cup a 1.*

Conclusion: *Allergy to conifer pollen could be considered a relevant cause of respiratory allergy in central Spain. Asthma was more frequent than in other studies. We only identified Cup a 1 as involved in sensitization.*

Allergic diseases affect an estimated 10 to 25% of the population, and their prevalence is increasing continuously worldwide[1–3]. Although allergy to grass pollen is the most common seasonal respiratory allergy, sensitization to conifer tree pollen (families Cupressaceae and Pinaceae) has increased dramatically in recent years, and is currently the main cause of allergic respiratory diseases in winter, especially in North America, Japan, and Mediterranean countries,[4] particularly in central Spain.[5] The order Pinales is the main taxon involved in allergy caused by gymnosperms. This order includes six families, four of which are considered allergenic: Cupressaceae, Taxodiaceae, Podocarpaceae, and Pinaceae.[6] In North America, the most commonly involved species are from the Cupressaceae and Taxodiaceae families.[7–10] In Japan, pollinosis caused by Japanese cedar pollen is considered the most common disease.[11] In Europe, the most important genera associated with allergic diseases are *Cupressus* and *Juniperus*. To date, four allergens from *Cupressus arizonica* have been described, including the major allergen Cup a 1[12,13] and three additional ones: Cup a 2 (polygalacturonase); Cup a 3 (thaumatin); and Cup a 4 (polcalcin).[14] Homologous allergens have been described in *Cupressus sempervirens* and species of the genus *Juniperus*. Three allergens from *Pinus pinea* were included in the allergome data base: Pin pi 1 (vicilin), Pin pi 17 kDa, and Pin pi 6 kDa (albumin).[14]

Preliminary and published studies in different Mediterranean regions estimated that the prevalence of cypress allergy is between 5 and 13% according to pollen exposure.[15] Sensitization has increased over the past 3 decades, from 0.9 to 9.8% in the general population and from 9 to 35% in patients with allergy,[16] probably due to the massive popularity of these species

in gardens and hedges. Moreover, the influence of global warming tends to extend the pollination period from October to March or even until early April.[4] In contrast, the Pinaceae family is responsible for a lower prevalence, which ranges between 1.5 and 6%, and Pinaceae pollen has been considered a very poor allergen.[17]

The main clinical symptom associated with allergy to conifer tree pollen is rhinitis, often associated with disabling conjunctivitis, whereas the incidence of asthma is generally lower than in patients sensitized to other allergenic sources.[6] Occasionally, seasonal eosinophilic bronchitis has been reported.[18] Cutaneous manifestations, such as urticaria or dermatitis, were described through direct contact with pollen during tree pruning late in the year.[5] Although there are not many studies in this regard, a recent publication indicated the association of conifer pollen sensitization with lipid transfer protein syndrome.[19] To date, in Spain, there have been no studies regarding the influence of these pollens among patients with pollen allergy. The objectives of the study were to investigate the prevalence of sensitization to pollen from different Pinales species, including four Cupressaceae (*C. arizonica, C. sempervirens, Juniperus communis,* and *Libocedrus decurrens*) and two Pinaceae (*P. pinea* and *Cedrus atlantica*) to describe the characteristics of patients with allergy who reside in the central region of Spain and to identify the involved allergens.

METHODS

Patient Population

A multicenter observational prospective study was conducted at the following hospitals, all in the Madrid area in central Spain: Hospital Universitario de Getafe, Hospital Infanta Elena (Valdemoro), Hospital Infanta Cristina (Parla), Hospital del Tajo (Aranjuez), and Hospital Universitario Fundación de Alcorcón. From September to December 2011, consecutive patients, ages ≥ 14 years, who were referred to the allergy clinics of these hospitals for the first time with respiratory (rhinitis, conjunctivitis, or asthma) or cutaneous symptoms (urticaria, atopic dermatitis, or angioedema), and with clinical indications for a standard skin-prick test (SPT) to inhalant allergens were included. Rhinitis and asthma were diagnosed according to clinical history and physical findings, and, for asthma alone, spirometry to demonstrate obstruction and assess reversibility. The patients were asked about the presence or aggravation of symptoms during the first 3 months of the year and correlation with pollen counts. The study protocol was approved by the Hospital Universitario de Getafe Ethics Committee (A08-11). All the patients gave written consent to participate. Subjects without clinical indication for the performance of SPT, under

treatment with antihistamines and/or corticosteroids, or who declined consent were excluded.

Extract Preparation

Pollens from *C. arizonica, C. sempervirens, P. pinea, J. communis, C. atlantica,* and *L. decurrens* (Iber-Polen, Jaen, Spain) were collected in the Iberian Peninsula. Cupressaceae pollens, including *C. arizonica, C. sempervirens, J. communis,* and *L. decurrens,* were extracted in a 1:20 proportion (w/v) for 4 hours with ammonium bicarbonate 0.125 M. After centrifugation, the precipitate was extracted for 4 hours with NaCl 0.15 M and centrifuged, and the resulting precipitate was extracted for 4 hours with phosphate-buffered saline solution (PBS) 0.01 M. Supernatants from different steps were mixed, filtered, and freeze-dried. Pinaceae pollens, including from *P. pinea* and *C. atlantica,* were extracted in a 1:10 proportion (w/v) for 4 hours in PBS 0.01 M per NaCl 0.15 M and centrifuged. To increase protein recovery, the pellet was extracted in the same conditions for an additional 8 hours. Supernatants from the two extractions were mixed, filtered, and freeze-dried. Extracts for cutaneous tests were prepared at 5 mg/mL for Cupressaceae and 2 mg/mL for Pinaceae species according to commercially available tests (Laboratorios LETI, S.L.U., Madrid, Spain).

SPT

SPT was performed on the volar surface of the forearm with a standard battery of biologically standardized aeroallergens (Laboratorios LETI), including a mixture of grasses (*Festuca elatior, Phleum pratense, Poa pratensis, Lolium perenne,* and *Dactylis glomerata*), *Cynodon dactylon, Olea europaea, Platanus acerifolia, Plantago lanceolata, Chenopodium album, Salsola kali, Dermatophagoides pteronyssinus, Dermatophagoides farinae,* cat dander, dog dander, *Alternaria alternata, Aspergillus fumigatus* (5 mg/mL), lipid transfer protein (Pru p 3; 30 μg/mL), and profilin (Pho d 2; 50 μg/mL). Pollen, dander, and mold pricks were prepared at 30 HEP/mL (histamine equivalent prick/mL), and mite pricks were prepared at 100 HEP/mL (histamine equivalent prick/mL). SPTs were also performed with the six conifer pollen extracts in all the patients. Wheal sizes were recorded after 15 minutes, and the area was measured with PC Draft software (Microspot, Maidstone, United Kingdom); results are expressed in mm^2. Areas of >7 mm^2 were considered as positive results.

Protein Profile

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was used to determine the protein profile of the extracts. A total of 500 μg of lyophilized Cupressaceae pollen extracts and 150 μg of lyophilized Pinaceae pollen extracts were loaded per
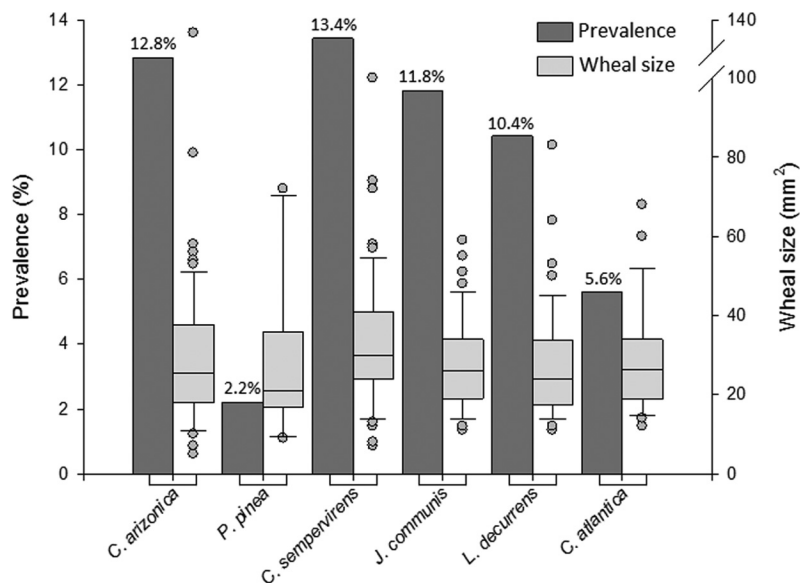
lane in SDS-PAGE gels under reducing conditions and stained with Oriole (Bio-Rad, Hercules, CA) or Coomassie blue.

Specific Immunoglobulin E

Serum samples were obtained from the patients with positive results for *C. arizonica-* and/or *P. pinea* who consented to blood collection. Specific immunoglobulin E (sIgE) to *C. arizonica* and Cup a 1 was determined by ImmunoCAP (ThermoFisher Scientific, Upsala, Sweden) by following the manufacturer's instructions; an sIgE value of >0.35 kUA/L was considered a positive result.

Allergenic Profile

Individual immunoblot for each patient was performed with *C. arizonica* extract in the solid phase. Briefly, 400 μg of lyophilized *C. arizonica* extract powder was run in SDS-PAGE, electrotransferred to an Immobilon-P membrane (Millipore, Bedford, MA), and dried at room temperature. Thereafter, membranes were incubated overnight with each individual patient's serum, in PBS. After incubation with monoclonal antihuman IgE-peroxidase (Ingenasa, Madrid, Spain), the reaction was developed with luminol (Bio-Rad) and visualized by chemiluminescence. In the same way, an immunoblot was performed with a pool of sera from the patients with positive IgE results for *C. arizonica*. In this case, 100 μg of lyophilized powder of *C. arizonica* was used in the solid phase and was run under reducing or nonreducing conditions before transference to the membrane. The pool of sera was also diluted 1:1 in PBS.

Allergen Identification

Protein bands recognized by patients' IgE by immunoblot experiments in the *C. arizonica* extract were cut from an sodium dodecyl sulfate (SDS) gel, digested with trypsin, and sequenced by LC/MS-MS (liquid chromatography-tandem mass spectrometry) (Proteomic Service, CNB, Madrid, Spain). Peptides were identified from the data base with Mascot software.[20]

Statistical Analysis

Descriptive statistical analysis were conducted for the calculation of variables. The χ^2 test or the Fisher exact test was used to study the relationship between the variables of interest. The *t*-test or Mann-Whitney rank sum test were used to compare wheal sizes and sIgE values. SigmaPlot 10.0 (Systat Software Inc., San Jose, CA) software and OpenEpi[21] were used for the statistical analysis.

RESULTS

Patient Population

A total of 499 patients were included in the study. A positive SPT result to any of the conifer pollen extracts was found in 70 individuals (14.0%) (44.3% men; mean [standard deviation] age, 38.2 ± 11.7 years). A clinical description of these patients is included in Supplemental Table 1. Of the 70 patients sensitized to conifers, 64 had positive SPT results to *C. arizonica* (12.8%) and 11 were sensitized to *P. pinea* (2.2%). A high percentage of the population was also sensitized to other conifers: 13.4% to *C. sempervirens*, 11.8% to *J. communis*, 10.4% to *L. decurrens,* and 5.6% to *C. atlantica* (Fig. 1). Fifty-four of 70 patients sensitized to conifers (77.1%) were sensitized to four or more species (Supplemental Table 1).

Among those 70 patients, all had respiratory symptoms, 43 (61.4%) during the *C. arizonica* pollination period (January to April) (Supplemental Fig. 1 *A*). Sixty-two patients (88.6%) had rhinitis, 52 (74.3%) had conjunctivitis, and 44 (62.9%) had asthma. Thirty-five
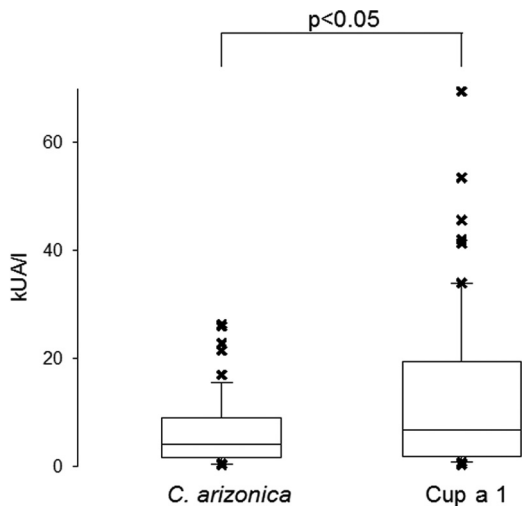
Figure 2. *Specific immunoglobulin E (sIgE) (kUA/L) to whole Cupressus arizonica extract and the individual allergen Cup a 1. Statistically significant differences were found and are shown in the graph.*

Figure 3. *Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the conifer extracts. (A) Cupressaceae extracts (500 μg) stained with Oriole; 1, Cupressus arizonica; 2, Cupressus sempervirens; 3, Juniperus communis; 4, Libocedrus decurrens. Scanning densitometry is shown at right: C. arizonica (dark green), C. sempervirens (red), J. communis (light green), L. decurrens (blue). (B) Pinaceae extracts (150 μg) stained with Coomassie; 5, Pinus pinea; 6, Cedrus atlantica. Scanning densitometry is shown at the right: P. pinea (red), C. atlantica (green).*

patients had rhinitis during the *C. arizonica* pollination period, which represented 56.5% of all the patients with rhinitis (35 of 62); 31 had conjunctivitis, which represented 59.6% of those patients with conjunctivitis (31 of 52); and 21 had asthma, which corresponded to 62.9% of the patients with asthma (21 of 44) (Supplemental Fig. 1 *A*). There were statistically significant differences in wheal sizes to *C. arizonica* ($p = 0.044$) and *C. sempervirens* ($p = 0.003$) between patients with and patients without symptoms in winter, as analyzed by the Mann-Whitney rank sum test. Ninety percent of patients were also sensitized to other pollens, with grasses (77.1%), *P. lanceolata* (62.9%), and *C. album* (62.9%) being most prevalent (Supplemental Fig. 1 *B*; Supplemental Table 1). Wheal sizes were similar for all extracts, which ranged from 33.2 ± 16.6 mm^2 for *C. sempervirens* to 27.8 ± 14.0 mm^2 for *L. decurrens* (Fig. 1). There were significant differences between the extracts with the higher and lower values, but no significant differences between any other pair of extracts.

sIgE

Sixty-five serum samples were collected from sensitized individuals. Fifty-six had positive sIgE results to *C. arizonica* (86.2%) and 60 had positive sIgE results to the major allergen Cup a 1 (92.3%) (Supplemental Table 1). The mean sIgE value was 6.5 ± 6.7 kUA/L for *C. arizonica* and 12.8 ± 14.9 kUA/L for Cup a 1 (Fig. 2). Differences were statistically significant ($p < 0.05$).

Protein Profile

SDS-PAGE of Pinales extracts is represented in Fig. 3. Cupressaceae extract profiles were similar (Fig. 3 *A*), with a band at 43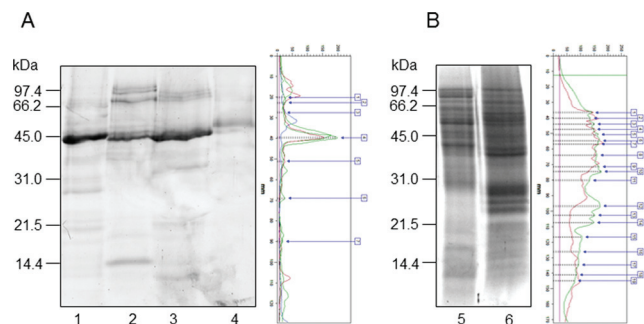 kDa (the size of Cup a 1 and its homologs) as the most prominent in all four extracts. Pinaceae extracts were different from Cupressaceae extracts, with bands from 7 to 100 kDa (Fig. 3 *B*).

Allergenic Profile

Fourteen different bands were recognized by the individual sera in the immunoblots (Fig. 4 *A*). The most recognized band was ~16 kDa (60% of individuals with a positive result). The band that corresponded to the molecular weight (MW) of Cup a 1 (43 kDa) was recognized in 41.5% of the individuals. Percentages of recognition for each band are represented in Supplemental Fig. 2. A pool of sera was prepared by mixing equal amounts of the 50 individual sera that recognized at least one band in the immunoblot. The allergenic profile of this pool of sera in reducing and nonreducing conditions is shown in Fig. 4 *B*. The most recognized bands were those at 43, 18, 16, and 14 kDa in the reducing gel, and were 43 and 16 kDa in the nonreducing gel.

Allergen Identification

Bands at 14, 16, 29, and 31 kDa from a reducing SDS gel and a 16 kDa band from a nonreducing SDS gel were cut and identified by LC/MS-MS (liquid chromatography tandem mass-spectrometry). In all cases, the identified peptides corresponded to the Cup a 1 sequence.

DISCUSSION

Allergy to Cupressaceae species has become a real problem in some specific areas of Spain[22] and other Mediterranean countries. Although the number of patients sensitized to conifer tree pollen has increased dramatically in the past few years, the real prevalence
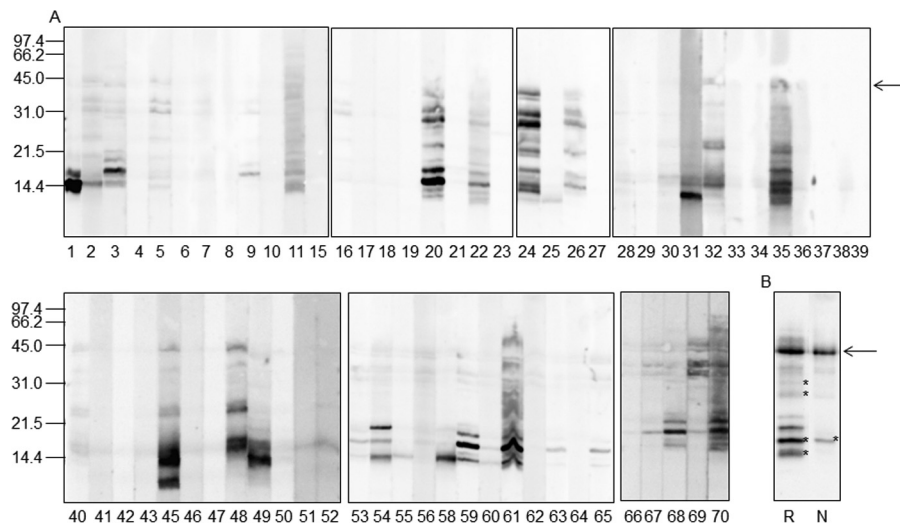
Figure 4. Immunoblot with (A) individual sera (1 to 71) or (B) a pool of sera (R = Reducing conditions; N = nonreducing conditions) to Cupressus arizonica pollen extract. Sera were used at a dilution of 1:1 in PBS. The position of Cup a 1 (43 kDa) is marked with an arrow. Sequenced bands are marked with an asterisk.

of sensitization has not been thoroughly studied to date. There is no evidence-based estimate of the prevalence of sensitization to these pollens in the adult population in Spain. In this study, the prevalence of sensitization to *C. arizonica* pollen in central Spain has been estimated at 12.8%, lower than in other Mediterranean areas (~30% in different studies from Italy)[23–25] and, surprisingly, lower than the 24.6% previously reported in 187 children from the same area.[26] However, there were differences across the participating hospitals, with the highest prevalence being 22% and the lowest being 8%. This is probably due to the distribution of cypress trees in the different towns.

Higher pollen counts have been observed in new residential areas away from the city centers, where the presence of these species as ornamental trees in hedges and parks has become widespread in the past 20 years. Although the specific number of pollen grains in an area cannot be calculated and the values can vary from the collection point to the surrounding areas, the Madrid region can be defined as a high-exposure area. During the past 5 years, peak pollen concentrations have ranged from 275 grains/m^3 in 2010 to 2010 grains/m^3 in 2014.[27] This value has been linked to a higher percentage of sensitization,[4] especially when considering that the area south of Madrid is highly industrialized and when taking into account the interaction of this pollen type with diesel particle pollution.[22] Most patients were sensitized to all Cupressaceae species used in the study (78.3% [47 of 70]), which represented a high percentage of cross-sensitization. This is probably due to the high similarity among all the Cupressaceae pollen extracts, as seen in the protein profile.

The symptoms of patients sensitized to conifers often consist of rhinoconjunctivitis, but, in recent years, asthma has been associated with conifer pollen as well.[4,28] In this study, 62.9% of patients sensitized to conifers had asthma, although only approximately half of them (47.7%) experienced asthma symptoms during the *C. arizonica* pollination period. Unfortunately, most patients were polysensitized and symptoms could be produced by additional allergens. It is difficult to find patients in the studied area who were monosensitized, and 88% of patients sensitized to pollen are polysensitized.[29] Only two patients were negative to other pollens and positive to animal dander, with symptoms in winter, which decreased the percentage of patients with asthma who were sensitized to conifer pollen to 47.1%. This percentage was still higher than those previously reported in other Mediterranean areas, *e.g.,* Italy, where only 29% of patients had asthma.[30] Although Sato *et al.*[31] found that levels of sIgE to cedar pollen were higher in patients with eosinophilic bronchitis than in patients with asthma, we did not find any significant differences between patients with asthma and patients without asthma with regard to *C. arizonica* sIgE serum levels, Cup a 1 sIgE values, allergen profile, mean wheal sizes in the SPT results, or cosensitization with different allergens, nor did we find differences between patients who were monosensitized or patients who were polysensitized to Cupressaceae. There were statistically significant differences in wheal size between patients with and those without symptoms in winter, which were higher for *C. arizonica* and *C. sempervirens.*

Although pine tree pollen has long been considered nonallergenic, the prevalence of respiratory allergy due to *C. atlantica* (5.6%) and *P. pinea* (2.2%), both of which are Pinaceae species, must be taken into account. These data were similar to the 3.2% prevalence previously described for *Pinus radiata.*[32] Although pine trees are found throughout Spain, with 22% of areas, *e.g.,* the Basque Country, covered by Pinaceae,[16] the prevalence of *P. pinea* is low, and few cases of patients who live near pine woods have been reported.[33,34] However, a

high percentage of patients who are monosensitized (60%) was described in high-exposure areas.[34] In our area of study, the amount of pine tree pollen was much lower, and, thus, it is of little clinical significance. None of the patients in this study were monosensitized to Pinaceae. This low prevalence could be related to the period of patient selection (during the first 3 months of the year) because pine tree pollination in Madrid occurs during May and June. The large size of the pine tree pollen grain could prevent its penetration into the airways. However, for pine pollen, greater allergenic potency in skin tests and sIgE levels has been reported in pollen that comes from unpolluted areas, probably related to a higher exposure to ozone, which results in greater expression of allergenic proteins.[35]

An aspect to be highlighted is that *Cupressus* sensitization is commonly associated with polysensitization. This phenomenon warrants a more in-depth investigation, not only to establish a correlation with other nontaxonomically related pollen species but also to elucidate the mechanism involved in the origin of this sensitization or the responsible allergens. All the patients with positive sIgE level results to *C. arizonica* also had a positive sIgE level to Cup a 1. IgE values to Cup a 1 were significantly higher than to the whole extract. Moreover, four patients with sIgE positive to Cup a 1 had negative titers to the whole extract, with sIgE values for Cup a 1 from 0.38 to 1.93 kUA/L. This could be explained by the fact that the amount of Cup a 1 in the ImmunoCAP complete extract was probably not enough to generate a positive signal in these patients. In this case, the selected patients would be sensitized predominantly to Cup a 1 and not to other *C. arizonica* allergens.

The allergenic profile of patients with *Cupressus* allergy is another important issue for understanding this type of allergy. In the present study, different bands were recognized by the individual sera, although Cup a 1 is the most relevant and is recognized at different MWs. We used different buffers consecutively for extraction to obtain the greatest number of proteins. Therefore, the conditions were not optimal because several bands of different MWs corresponded to Cup a 1, probably because it breaks down in the process. This allergen has a theoretical MW of approximately 37 kDa, but its glycoprotein modifications give a MW of 43 kDa in SDS.[19] In nonreducing SDS, we were able to differentiate two bands by Western blot, both corresponded to Cup a 1. In reducing SDS, proteins migrate along the gel, depending on their MW but also on their hydrophobicity, tertiary structure, and the amount of SDS bound to it.[36] In reducing SDS, more bands were detected in the *C. arizonica* extract, probably due to intermediate folding forms or protein fragmentation. Four of the recognized bands were sequenced, all of which corresponded to Cup a 1. Other *C. arizonica* allergens were described, including Cup a 3,[37] Cup a 4,[38] BP14,[39] and lipid transfer protein[19] as well as other allergens described in different Pinales species (Cha o 1, Cha o 2, Cry j 1, Cry j 2, Cup s 1, Cup s 3, Jun a 1, Jun a 2, Jun a 3, Jun o 4, Jun s 1, Jun v 1, Jun v 3).[13] None of these were identified in the sequenced bands apart from Cup a 1. This result may explain the high values of sIgE to Cup a 1 because it was the main allergen recognized by the patients in our population and undoubtedly explained sensitization in patients with allergy. Studies of the association of Cup a 1 with clinical symptoms are scarce, although a relationship between Cup a 1 content and symptoms has been found.[40] This pattern of sensitization mainly to Cup a 1 must be confirmed in a larger population. According to our data, immunotherapy treatment with Cup a 1 alone could be effective to treat patients in our population, especially if we consider that a good *C. arizonica* pollen extract is difficult to obtain. Cross-reactivity is an important factor to be considered, especially because, to date, there is limited evidence for immunotherapy with Cupressaceae pollen.[41–43] We found a significant cosensitization phenomenon among the Cupressaceae but not with the Pinaceae. These results agreed with previously published studies that reported intense cross-reactivity among different Cupressaceae species,[4] but not between Cupressaceae and Pinaceae.[16] Differences that range from pollen structure to the protein profile of the extracts could explain this low cross-reactivity.

CONCLUSION

Analysis of our data showed that allergy to conifer pollen could be considered a relevant cause of respiratory allergy in central areas of Spain. Asthma seemed to be more frequent than previously reported, and pine tree allergy should also be considered. Determination of the sIgE level to Cup a 1 seemed to be more accurate than the sIgE level to whole *C. arizonica* extract. These findings might lead clinicians to demand development of new, potent diagnostics and specific immunotherapy extracts for these pollens. To the best of our knowledge, this was the first study to provide such a detailed description of the clinical characteristics of such patients in Spain and to identify the involved allergens.

REFERENCES

1. Asher MI, Montefort S, Björkstén B, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC phases one and three repeat multicountry cross-sectional surveys. Lancet 368:733–743, 2006.
2. Bjerg A, Hedman L, Perzanowski M, et al. Decreased importance of environmental risk factors for childhood asthma from 1996 to 2006. Clin Exp Allergy 45:146–153, 2015.
3. Hastan D, Fokkens WJ, Bachert C, et al. Chronic rhinosinusitis in European underestimated disease. A GA²LEN study. Allergy 66:1216–1223, 2011.
4. Charpin D, Calleja M, Lahoz C, et al. Allergy to cypress pollen. Allergy 60:293–301, 2005.
5. Quirce S. Asthma in Alergologica–2005. J Investig Allergol Clin Immunol 19:14–20, 2009.
6. Di Felice G, Barletta B, Tinghino R, and Pini C. Cupressaceae pollinosis: Identification, purification and cloning of relevant allergens. Int Arch Allergy Immunol 125:280–289, 2001.
7. Cannington EM, Roe KL, Young PF, and Ownby DR. Sensitization to bald cypress and cross-reactivity with red cedar in northeastern Georgia. Ann Allergy Asthma Immunol 109:220–2211, 2012.
8. Rocha-Estrada A, Alvarado-Vázquez MA, Torres-Cepeda TE, et al. Airborne pollen of *Carya, Celtis, Cupressus, Fraxinus* and *Pinus* in the metropolitan area of Monterrey Nuevo Leon, Mexico. Ann Agric Environ Med 15:205–209, 2008.
9. Deane PM. Conifer pollen sensitivity in western New York: Cedar pollens. Allergy Asthma Proc 26:352–355, 2005.
10. Phillips JF, Jelks ML, and Lockey RF. Important Florida botanical aeroallergens. Allergy Asthma Proc 31:337–340, 2010.
11. Yamada T, Saito H, and Fujieda S. Present state of Japanese cedar pollinosis: The national affliction. J Allergy Clin Immunol 133:632–639.e5, 2014.
12. Aceituno E, Del Pozo V, Mínguez A, et al. Molecular cloning of major allergen from *Cupressus arizonica* pollen: Cup a 1. Clin Exp Allergy 30:1750–1758, 2000.
13. WHO/IUIS Allergen Nomenclature Sub-committee. http://www.allergen.org/.
14. Allergome. The platform for Allergen Knowledge. http://www.allergome.org/.
15. Charpin D, Calleja M, Pichot C, et al. Cypress pollen allergy [in French with English abstract]. Rev Mal Respir 30:868–878, 2013.
16. Caimmi D, Raschetti R, Pons P, et al. Epidemiology of cypress pollen allergy in Montpellier. J Investig Allergol Clin Immunol 22:280–285, 2012.
17. Gastaminza G, Lombardero M, Bernaola G, et al. Allergenicity and cross-reactivity of pine pollen. Clin Exp Allergy 39:1438–1446, 2009.
18. Bobolea I, Barranco P, Sastre B, et al. Seasonal eosinophilic bronchitis due to allergy to *Cupressus arizonica* pollen. Ann Allergy Asthma Immunol 106:448–449, 2011.
19. Sánchez-López J, Asturias JA, Enrique E, et al. Cupressus arizonica pollen: A new pollen involved in the lipid transfer protein syndrome? J Investig Allergol Clin Immunol 21:522–526, 2011.
20. Matrix science (Mascot software). http://www.matrixscience.com.
21. Open Source Epidemiologic Statistics for Public Health. http://www.openepi.com/.
22. Suárez-Cervera M, Castells T, Vega-Maray A, et al. Effects of air pollution on Cup a 3 allergen in *Cupressus arizonica* pollen grains. Ann Allergy Asthma Immunol 101:57–66, 2008.
23. Mari A, Di Felice G, Afferni C, et al. Cypress allergy: Underestimated pollinosis. Allergy 52:355–356, 1997.
24. Fiorina A, Scordamaglia A, Guerra L, et al. Prevalence of allergy to cypress. Allergy 57:861–862, 2002.
25. Ariano R, Passalacqua G, Panzani R, et al. Airborne pollens and prevalence of pollinosis in western Liguria: A 10-year study. J Investig Allergol Clin Immunol 9:229–234, 1999.
26. Feliu A, González-de-Olano D, González E, et al. A multicenter study of sensitization profiles in an allergic pediatric population in an area with high allergen exposure. J Investig Allergol Clin Immunol 23:337–344, 2013.
27. Aerobiology committee of the Spanish society of allergy and clinical immunology. http://www.polenes.com/
28. Sposato B, Liccardi G, Russo M, et al. Cypress pollen: An unexpected major sensitizing agent in different regions of Italy. J Investig Allergol Clin Immunol 24:23–28, 2014.
29. Subiza J, Pola J, Feo F, et al. Pólenes de interés en alergología en nuestro medio. In: Tratado de alergología. Ed. Ergon. Majadahonda (Madrid, Spain). 2007. Chapter 24.
30. Sposato B, and Scalese M. Prevalence and real clinical impact of *Cupressus sempervirens* and *Juniperus communis* sensitisations in Tuscan "Maremma", Italy. Allergol Immunopathol (Madr) 41:17–24, 2013.
31. Sato S, Saito J, Sato Y, et al. Clinical usefulness of fractional exhaled nitric oxide for diagnosing prolonged cough. Respir Med 102:1452–1459, 2008.
32. Harris RM, and German DF. The incidence of pine pollen reactivity in allergic atopic population. Ann Allergy 55:678–679, 1985.
33. Armentia A, Quintero A, Fernandez-García A, et al. Allergy to pine pollen and pinion nuts: A review of three cases. Ann Allergy 64:49–53, 1990.
34. Marcos C, Rodriguez FJ, Luna I, et al. Pinus pollen aerobiology and clinical sensitization in northwest Spain. Ann Allergy Asthma Immunol 87:39–42, 2001.
35. Garcia-Gallardo MV, Algorta J, Longo N, et al. Evaluation of the effect of pollution and fungal disease on *Pinus radiata* pollen allergenicity. Int Arch Allergy Immunol 160:241–250, 2013.
36. Rath A, Glibowicka M, Nadeau VG, et al. Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. Proc Natl Acad Sci U S A 106:1760–1765, 2009.
37. Cortegano I, Civantos E, Aceituno E, et al. Cloning and expression of a major allergen from *Cupressus arizonica* pollen, Cup a 3, a PR-5 protein expressed under polluted environment. Allergy 59:485–490, 2004.
38. Pico de Coaña Y, Parody N, Fuertes MA, et al. Molecular cloning and characterization of Cup a 4, a new allergen from *Cupressus arizonica.* Biochem Biophys Res Commun 401:451–457, 2010.
39. Shahali Y, Sutra JP, Charpin D, et al. Differential IgE sensitization to cypress pollen associated to a basic allergen of 14 kDa. FEBS J 279:1445–1455, 2012.
40. Benítez FM, Camacho AL, del Cuvillo Bernal A, et al. Determination of allergenic load and pollen count of *Cupressus arizonica* pollen by flow cytometry using Cup a1 polyclonal antibody. Cytometry Part B Clin Cytom 86:63–69, 2014.
41. Ventura MT, Carretta A, Tummolo RA, et al. Clinical data and inflammation parameters in patients with cypress allergy treated with sublingual swallow therapy and subcutaneous immunotherapy. Int J Immunopathol Pharmacol 22:403–413, 2009.
42. Charpin D, Gouitaa M, Dron-Gonzalvez M, et al. Immunotherapy with an aluminum hydroxide-adsorbed *Juniperus ashei* foreign pollen extract in seasonal indigenous cypress pollen rhinoconjunctivitis. A double-blind, placebo-controlled study. Int Arch Allergy Immunol 143:83–91, 2007.
43. Vervloet D, Birnbaum J, Laurent P, et al. Safety and efficacy of *Juniperus ashei* sublingual-swallow ultra-rush pollen immunotherapy in cypress rhinoconjunctivitis. A double-blind, placebo-controlled study. Int Arch Allergy Immunol 142:239–246, 2007. □