
Detection of airborne Par j 1 and Par j 2 allergens in relation to Urticaceae pollen counts in different bioclimatic areas

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Background: In aerobiological studies, the *Parietaria* pollen type usually includes all *Parietaria* and *Urtica* species found in the area. Given that *Urtica* is a nonallergenic plant, the pollen counts report incomplete information on the presence of allergens in the atmosphere. Discordance between the pollen concentrations of Urticaceae and allergic symptoms has been observed in patients with pollinosis.

Objective: To compare the Urticaceae pollen counts with the Par j 1 and Par j 2 aeroallergen concentrations from 2 different Spanish geographic areas to determine the allergenic load in the atmosphere.

Methods: Hirst-type volumetric traps and Burkard Cyclone samplers were used for pollen counts and aeroallergen capture, respectively. The quantification of Par j 1 and Par j 2 allergens was performed using specific 2-site antibody enzyme-linked immunosorbent assay. Transmission electron microscopy and immunocytochemical techniques were applied to localize these allergens in the orbicules.

Results: Differences between areas and years were obtained in both pollen and aeroallergen concentrations. Despite the lower pollen counts recorded in Cartagena, higher aeroallergen concentrations were registered compared with Ourense. A lower correlation was achieved between Urticaceae pollen concentrations and aeroallergen levels, with a maximum positive significant correlation (adjusted $R^2 = 0.466$, $P < .001$). Intense labeling of Par j 1 and Par j 2 proteins was observed in the orbicules, the tapetal membrane, and the tapetal tissue remnants.

Conclusion: This method may be valuable for epidemiologic research to establish correlations between concentrations of *Parietaria* aeroallergens and clinical symptoms. Therefore, the measurement of aeroallergens should be incorporated into the aerobiological studies with clinical applications.

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INTRODUCTION

The airborne content of pollen grains is normally quantified using conventional pollen counts and microscopy identification techniques. However, discordance between atmospheric pollen concentration and allergic symptoms has been observed.¹ The presence of airborne allergen submicronic particles in the atmosphere and their association with increasing asthma and deaths due to respiratory and cardiovascular diseases have been found by different authors.² The different

processes that occur in the anther and pollen grains can provoke the presence of allergenic particles in the atmosphere. Allergens are released from pollen grains in wet conditions similar to the in vitro and in vivo germination processes.^{3,4} These allergens could also be dispersed as microdroplets and deposited on different parts of the plants, such as leaves and stems, and later dispersed again into the atmosphere.⁵ On the other hand, pollen allergens were recently localized in the tapetal tissue of *Fraxinus angustifolia* from the microsporogenesis stage to the dehiscence stage.⁶ As a result, we believe that the allergenic remnants of the tapetal membrane may be liberated into the atmosphere, provoking an increase in allergenic particles. In addition, orbicules or Ubish bodies emitted from the dehiscent anthers are also considered a possible origin of the pollen-derived paucimicronic allergenic particles.^{3,6–8}

The Urticaceae family includes a number of well-known plants, *Urtica* and *Parietaria* being the most widespread. *Parietaria* is a wind-pollinated weed that includes the most important allergenic plants in the Mediterranean countries, and *Parietaria judaica* L is 1 of the most common species of this genus found in the countryside and urban areas. *P judaica* pollen has a prevalence of 60% to 80% in Italy and

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Greece and 25% to 50% in Spain and southern France.⁹ In Europe, the sensitization to *P. judaica* is higher in the south than in the north.¹⁰ In Spain, depending on the area, the sensitization reaches between 25% and 50% in the Mediterranean area, whereas in the northwest area the prevalence is lower (6%-25%).¹¹

Allergic symptoms, including bronchial asthma, can appear in patients allergic to *Parietaria*, especially in patients monosensitized to *Parietaria*.¹² The prevalence of IgE reactivity for *Parietaria* allergens seems to depend on the geographic area where the patients live.¹³ Par j 1 and Par j 2 are the 2 main allergens that belong to the family of nonspecific lipid transfer proteins, with high homology, identical conformation, and 3 similar allergenic epitopes.¹⁴

The quantification of airborne allergens together with pollen grain counts should be performed in the control of *Parietaria* pollinosis because of its high allergenic potential, the difficulty in distinguishing its pollen grains from other non-allergenic species of Urticaceae, and the probable existence of aeroallergen paucimicronic particles. This information may be helpful to the diagnosis and treatment of patients with *Parietaria* pollen allergy, and it makes it possible to establish correlations with clinical symptoms, as well as to see whether differences among geographic areas are found. The aim of this study was to compare the Urticaceae pollen counts with the aeroallergen Par j 1 and Par j 2 concentrations in 2 different geographic areas in Spain to determine the allergenic load in the atmosphere and to establish regional and climatic characteristics between them.

METHODS

Study Area

Airborne Urticaceae pollen and Par j 1 and Par j 2 were quantified in Cartagena and Ourense, Spain. Cartagena is located in the Southeast of the Iberian Peninsula and belongs to the Mediterranean Xeric Oceanic region, and Ourense is situated in the Northwest of the Iberian Peninsula in the transition between the Mediterranean and Eurosiberian regions. The most important species present in Ourense are *P. judaica*, *Urtica dioica*, *Urtica membranacea*, and *Urtica urens*, with *U. urens* being the most scarce. In Cartagena, *Parietaria* is widely represented by 2 species, *P. judaica* and *Parietaria lusitanica*, whereas *U. dioica*, *U. membranacea*, *Urticaria pululifera*, and *U. urens* are scarcely represented.

Pollen Monitoring

In Cartagena and Ourense in 2007 and 2008, pollen sampling was performed with 1 Hirst-type volumetric trap (VPPS 2000; Lanzoni, Bologna, Spain) in each city. In both cities, the traps were located on the top of buildings at similar heights (between 10 and 20 m above ground level). The method proposed by the Spanish Aerobiological Network was followed to prepare the Melinex tape and count the captured pollen grains.¹⁵ The results of the daily mean pollen counts were expressed as pollen per cubic meter of air.

During weekends and holidays, when the allergen samples corresponded to a period of 3 aggregate days, the pollen count was calculated as the average concentration for these 3 days.

Aeroallergen Monitoring

During the same years, a Burkard Cyclone sampler (Burkard Manufacturing Co Ltd, Hertfordshire, England) was used for aeroallergen quantification. However, it was only used during the period when the highest *Parietaria* pollen levels were registered (from April to June in Cartagena and June and July in Ourense). These samplers were located beside the pollen traps in both cities. This Burkard Cyclone sampler is a continuous volumetric sampler with wind orientation that uses a single reverse-flow miniature cyclone with an air throughput of 16.5 L/min. It offers 100% efficiency sampling down to the 1.06- μ m range, decreasing to 93.82% for the size range of 0.82 to 0.75 μ m. The efficiency of particle capture in the pollen size ranges was evaluated at the Pollen Research Unit.¹⁶ Particles were collected directly into a 1.5-mL Eppendorf vial. The samples corresponded to daily periods on weekdays and to 3 aggregate days during weekends and holidays. In both cases, the allergen content was expressed as a picogram average by cubic meters of air. Airborne samples were stored at -20°C. The SPSS statistical program (SPSS Inc, Chicago, Illinois) was used to prove the possible association between Urticaceae pollen counts and Par j 1 and Par j 2 levels.

Extraction of Allergens From Airborne Samples

Airborne samples collected in 1.5-mL Eppendorf vials were extracted using a modification of the method described by Takahashi et al.¹⁷ Dry samples were extracted with 110 μ L of extraction buffer for 2 hours at room temperature. The extract was separated from the particulate matter by centrifugation at 5,000g for 10 minutes and stored at -20°C.

Quantification of Par j 1 and Par j 2

Par j 1 and Par j 2 contents in aerosol samples were quantified using a specific 2-site antibody enzyme-linked immunosorbent assay as previously described.^{18,19} Par j 1 and Par j 2 allergens, used as reference material, were purified from *P. judaica* pollen extract by affinity chromatography using a specific polyclonal rabbit antibody as previously described.¹⁹

Immunocytochemical Localization

Anthers of *P. judaica* were fixed and embedded using freeze-substitution techniques.²⁰ Sections of anthers were incubated with anti-Par j 1 and Par j 2 specific polyclonal rabbit antibodies as previously described.¹⁹ Sections were also incubated with preimmune serum to control the specific labeling of the primary antibody. Other sections were incubated omitting the primary polyclonal antibody to control the non-specific binding of the colloidal gold-conjugated antibody. They were observed using a JEOL 1010 transmission electron microscope (JEOL, Tokyo, Japan).

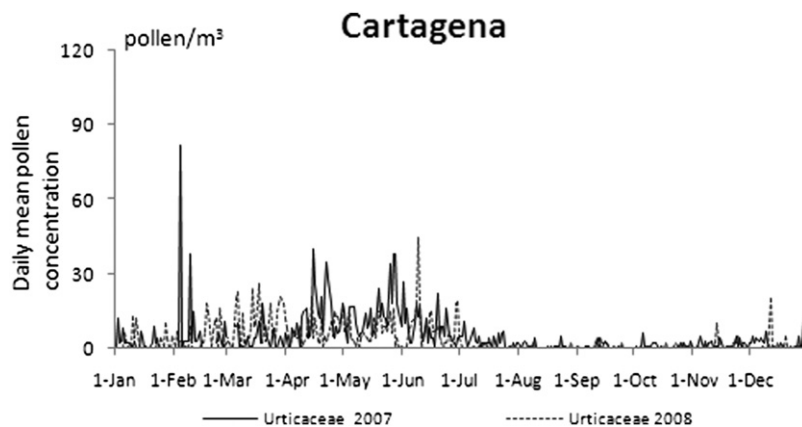


Figure 1. Urticaceae pollen concentrations in Cartagena (2007 and 2008).

RESULTS

Pollen Counts

Urticaceae pollen is detected throughout the year, but in the spring it achieves the highest pollen concentrations, with the exception of 2 peaks registered in February 2007 in Cartagena (Figure 1). Urticaceae pollen represented 7.86% and 9.4% of the total annual pollen registered in 2007 in Cartagena and Ourense, respectively, and 6.85% and 8.7% in 2008. In Cartagena, the total annual Urticaceae pollen counts were 1,693 and 1,293 pollen grains in 2007 and 2008, respectively. The SDs were 8.1 and 5.7 pollen grains in 2007 and 2008. The counts reached 82 and 45 pollens/m³ at the highest daily mean pollen concentration recorded in both years. In Ourense, the total annual Urticaceae pollen counts were 3,662 and 3,256 pollen grains in 2007 and 2008, respectively (Figure 2). The SDs of the values registered were 14.2 and 16.6 pollen grains in 2007 and 2008, respectively. The counts reached 72 and 118 pollens/m³ at the highest daily mean pollen concentration recorded in both years.

Aeroallergen Quantification

The correlation was low between concentrations of daily pollen and Par j 1 and Par j 2 in 2007 and 2008. Positive

significant correlation (adjusted $R^2 = 0.466$, $P < .001$) was only obtained between Par j 1 and Par j 2 and Urticaceae pollen concentration in 2007 in Ourense. The most important discordance between both variables was observed in Cartagena in May 2007 and in Ourense during the second fortnight in July 2007, when the Urticaceae pollen concentration stayed the same or even increased simultaneously with the decrease of the Par j 1 and Par j 2 concentrations (Figure 3).

The highest Par j 1 and Par j 2 levels were found in 2008 in Cartagena and Ourense. However, in 2007 and 2008, important differences were found between the Par j 1 and Par j 2 levels in both cities. The concentration levels were higher in Cartagena than Ourense in both years. On average, Cartagena achieved 14.09 and 46.70 pg/m³ during the study period in 2007 and 2008, respectively. On the other hand, the average concentration levels registered in Ourense in the same years were 4.25 and 31.55 pg/m³. The maximum aeroallergen concentration in Cartagena was 69.07 and 133.13 pg/m³ in 2007 and 2008, respectively, and 19.76 and 110.21 pg/m³ in Ourense. Although the high Par j 1 and Par j 2 allergen concentration levels registered in Cartagena were higher than those registered in Ourense, the pollen counts were lower in Cartagena than in Ourense.

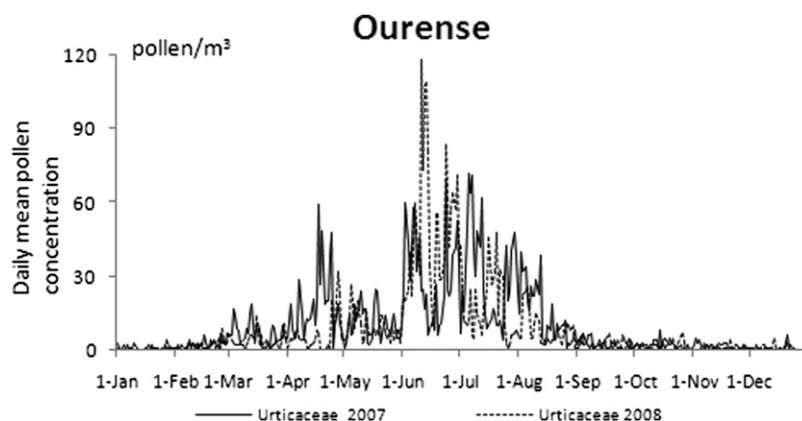


Figure 2. Urticaceae pollen concentrations in Ourense (2007 and 2008).

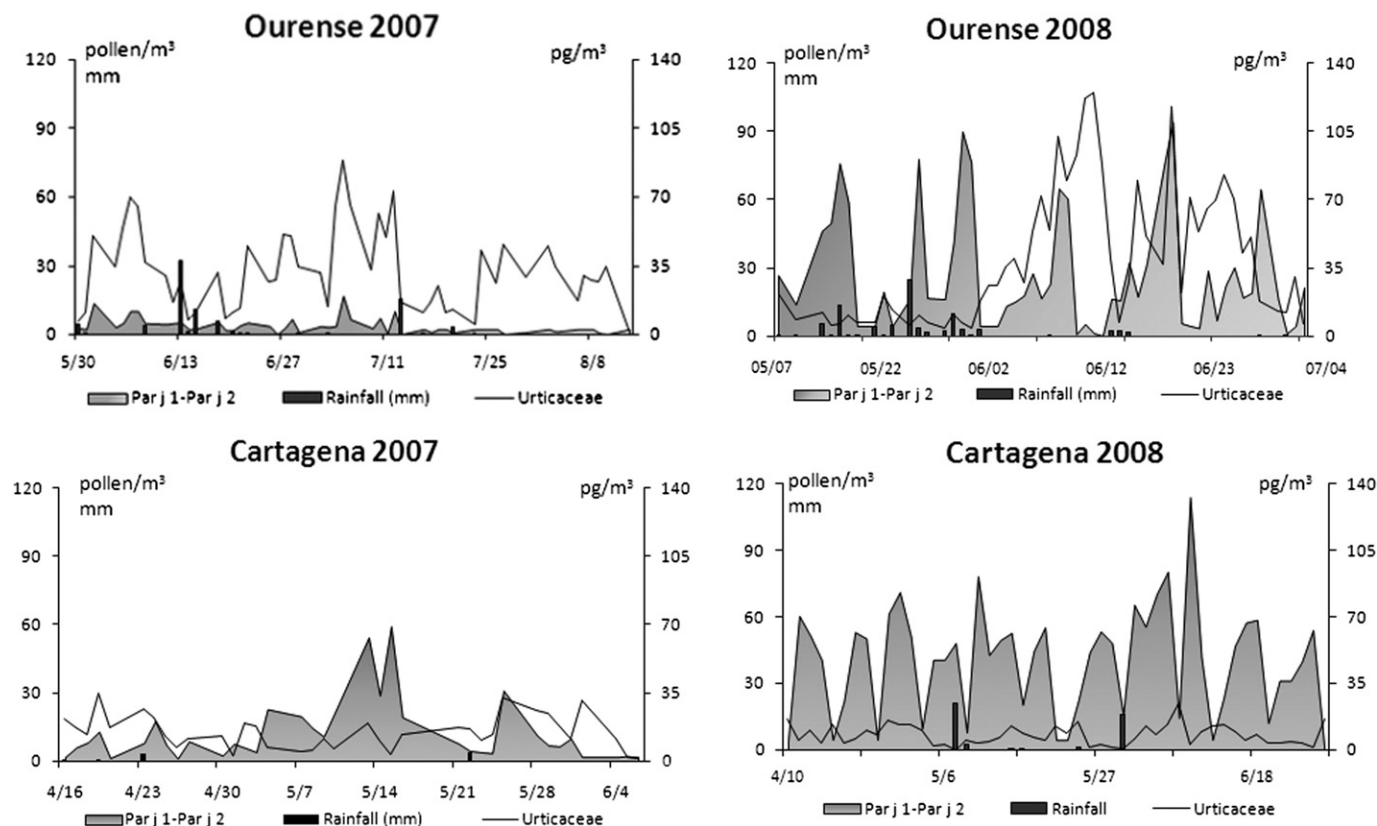


Figure 3. Par j 1 and Par j 2 concentrations, pollen counts, and rainfall in Cartagena and Ourense (2007-2008).

Despite the fact that rainy days were infrequent during the period analyzed, in general we observed that rainfall showed a greater positive influence on Par j 1 and Par j 2 allergen concentrations than on the pollen counts. In Ourense in 2008, the highest Par j 1 and Par j 2 levels coincided with continuous rainy days in which the pollen counts were low. In Cartagena in 2008, rainy days were also followed by increases in Par j 1 and Par j 2 (Figure 3).

Immunocytochemical Localization of Par j 1 and Par j 2

During pollen development and at the young pollen grain stage, the anther pollen sac showed the tapetum cells bordering the thecal cavity where the young pollen grains were situated (Figure 4A). The tapetum tissue showed intense activity forming numerous orbicules located in the tapetal membrane (Figure 4B). These orbicules were made up of a sporopollenin layer, similar to that of the exine wall, and a central electron-lucid core (Figure 4B and C). At the dehiscence stage, the developed orbicules were either situated in the tapetal membrane (Figure 4C) or free in the thecal cavity (Figure 4D). Intense labeling of Par j 1 and Par j 2 proteins were observed associated with the orbicules, the tapetal membrane, and the tapetal tissue remnants (Figure 4C and D). No significant labeling was obtained in the controls (Figure 4B).

DISCUSSION

Pollen Count vs Par j 1 and Par j 2 Airborne Concentration

Asthma and bronchial allergy symptoms are frequently observed in patients allergic to *Parietaria* pollen, but the time of pollen exposure and the time that symptoms occur often do not coincide.^{9,21} In the results shown, a weak correlation was observed between daily Urticaceae pollen counts and daily Par j 1 and Par j 2 concentrations. This dissimilarity between both variables was especially found in Cartagena at one peak in May 2007 and various peaks in 2008, as well as in Ourense at various peaks in July 2007 and May 2008. This lack of correlation for *Parietaria* pollen counts and allergen quantification is similar to that obtained in Cartagena in 2003.²² Nevertheless, the allergen quantification method used was validated for Ole e 1 in Cartagena, obtaining a significant correlation between the *Olea* pollen counts and the Ole e 1 aeroallergen concentration.²²

Different factors in relation to allergen cross-reactivity, morphology, ontogeny, and the release and dispersion of *Parietaria* pollen grains should also be considered to understand the behavior of *Parietaria* pollen and its allergens, as well as the weak relationship among them. First, the different allergenic potential

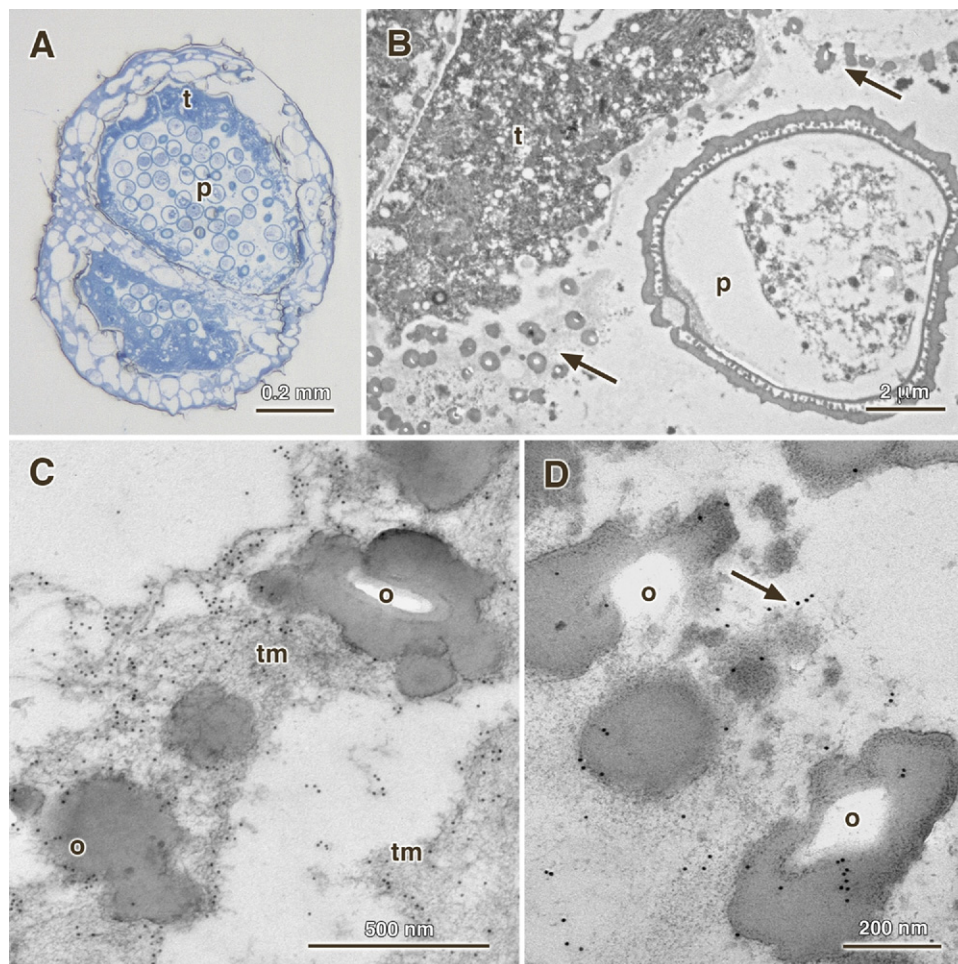


Figure 4. Sections of the *Parietaria judaica* anthers at the young pollen grain (A-C) and mature (D) stages. A, General view of the anther pollen sacs with the tapetum cells (t) bordering the young pollen grains (p). B, The tapetum cells (t) forming numerous orbicules (arrows) near the young pollen grain (p). Control of incubation without the primary antibody. Note the absence of labeling. C, Intense labeling of Par j 1 and Par j 2 proteins in the orbicules (o) and tapetal membrane (tm) at the young pollen grain stage. D, Labeled free orbicules (o) at the mature pollen grain stage. Note the labeled remnants of tapetal tissue.

of the species included in the Urticaceae pollen type is 1 of the reasons why the pollen counts do not always coincide with the allergen concentration. The allergen quantification in the atmosphere only reflects the allergenic *Parietaria* species, and the Urticaceae pollen counts include the nonallergenic *Urtica* species. Second, the dehiscence type of the anthers and the pollen wall structure explain this weak relationship. In the Urticaceae family, an explosive mechanism is involved in the pollen liberation (Suarez-Cervera, unpublished data, 2009).²³ Moreover, the pollen grains are suddenly activated once they land on the stigma or a wet medium during the first minutes of hydration, provoking the release of the allergens mostly through the dynamic pollen wall layer called the oncus.²³⁻²⁵ As a result, the release, transport, and dispersion of Urticaceae pollen in the bioaerosol are especially linked to atmospheric conditions.

The presence of orbicules in *Parietaria* pollen could also be another mechanism of pollen allergen paucimicronic par-

ticle emission. Orbicules are spheroidal structures found in the anthers of many higher plants,²⁶ which are generally found in large numbers and contain allergens.^{3,6} The abundant orbicules with significant labeling observed in the anther cavity of *P. judaica* may be another source of allergenic particles. Even though the presence of these particles has not been detected in the aerosol, they are probably involved in the dispersion of pollen, increasing the allergen levels in the air and the risk of asthma because of their optimal size for penetration into the lower airways.^{27,28}

In 2007 and 2008, increases in Par j 1 and Par j 2 levels linked to rainfall periods were observed. The number of rainy days was low in Cartagena in both years (4 in 2007 and 6 in 2008), and the rainfall registered was higher than 4 mm for only 2 days in 2008. These 2 rainy days were followed by a rapid decrease in pollen and aeroallergen concentrations. After that, the aeroallergen concentration increased faster

than the pollen counts. Moreover, in Ourense during 2008, continuous rainy days were registered from May 7 to May 30, achieving high aeroallergen concentrations on these days. Some authors argue that rain episodes result in an increase of small airborne antigenic fractions because the pollen deposited on various surfaces releases its proteins when wetted, and after drying, this material may become airborne again.^{22,29} However, an extensive survey should be performed to achieve enough data to determine this relationship.

Comparison of Allergens in Cartagena and Ourense

Despite the lower *Parietaria* pollen recorded in Cartagena in the periods studied, higher Par j 1 and Par j 2 concentrations were registered. On the contrary, in Ourense, the higher pollen concentration did not correspond to a higher allergen concentration. This dissimilarity could be explained by the different representation of Urticaceae in each city related to their distinct geographic locations. Usually *Parietaria* pollen is more frequent than *Urtica* in the coastal xeric regions (Cartagena) than in the wet inland areas (Ourense), and the prevalence of *P. judaica* is more important on the coast than in inland areas.³⁰ In Ourense, numerous nonallergenic *Urtica* species were included in the pollen counts together with the *Parietaria* species. Consequently, *Parietaria* pollen grains were better represented in the pollen counts in Cartagena than in Ourense.

In addition, the differences in contamination levels between Cartagena and Ourense could play an important role in the allergenic potential of *Parietaria*. During the spring months (May and June), the average levels of ozone (84.2 $\mu\text{g}/\text{m}^3$), particulate matter with a less than 10- μm diameter (32.6 $\mu\text{g}/\text{m}^3$), and sulfur dioxide (8.0 $\mu\text{g}/\text{m}^3$) in Cartagena³¹ can be up to 2 or 3 times higher than in Ourense, which has an average ozone level of 51.63 $\mu\text{g}/\text{m}^3$, a level of particulate matter with a less than 10- μm diameter of 13.79 $\mu\text{g}/\text{m}^3$, and a sulfur dioxide level of 4.30 $\mu\text{g}/\text{m}^3$.³² The 2 major *Parietaria* allergens (Par j 1 and Par j 2) are nonspecific lipid transfer proteins^{14,25} that belong to the pathogenesis-related protein family playing various important roles in plant metabolism.^{33–35} There is evidence of their participation in the defense mechanism of plants against external attacks,³⁶ and they are widely expressed under pollution conditions.³⁵ The more abundant allergen concentration found in the atmosphere in Cartagena could be related to the existing pollutant levels there, which may induce a higher expression of Par j 1 and Par j 2 in Cartagena than in Ourense. In addition, *Parietaria* is considered an accumulator of pollutants,³⁷ and a high concentration of trace elements has been detected in pollen and leaf samples.³⁸ All these factors probably have a significant influence on the high Par j 1 and Par j 2 allergen levels in Cartagena opposed to the low pollen grains reflected in the counts. This could explain the different levels of the prevalence of pollinosis from *Parietaria* pollen in both areas, which is higher than 50% in Cartagena³⁹ and lower than 8% in Ourense.⁴⁰

The regional climatic characteristics and the different contamination levels in the 2 regions studied may have influenced the variations in Par j 1 and Par j 2 airborne concentrations. The allergens associated with the orbicules could be responsible for increasing the presence of Par j 1 and Par j 2 allergens in the atmosphere. This information may be valuable for epidemiologic studies to establish correlations between concentrations of *Parietaria* aeroallergens and clinical symptoms.

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