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Pollen allergenic potential nature of some trees species: A multidisciplinary approach using aerobiological, immunochemical and hospital admissions data[☆]

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ABSTRACT

Trees are considered producers of allergenic pollen. The aims of this work were to characterize the aerobiology of the *Platanus*, *Acer*, *Salix*, *Quercus*, *Betula* and *Populus* pollen, linking it with monthly emergency hospital admissions and to identify the different reactivity levels in sensitized patients. This information would be of great importance to evaluate the convenience of changing the inventory of pollen producer trees related to the risk of allergenic reactions. The study was conducted in Porto, Portugal, from 2005 to 2007. Airborne pollen was sampled using a Hirst-type volumetric trap. The antigenic and allergenic properties of *Acer negundo*, *Betula pendula*, *Platanus occidentalis*, *Populus hybrida*, *Quercus robur* and *Salix babylonica* pollen, collected in public gardens or sidewalks, were investigated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunological techniques using polysensitized-patient sera. Monthly hospital admissions of asthma or dyspnea related with respiratory diseases were obtained from the Emergency Room database of Hospital Geral de Santo António. Tree pollen and hospital admissions were positively correlated. Tree pollen peaked in March which coincides with the hospital admissions maximum. The highest binding affinity was observed with *A. negundo*, *S. babylonica* and *P. occidentalis* pollen extracts and the lowest with *P. hybrida*. Consistently, *Acer* and *Platanus* maximum airborne pollen concentrations were observed during March attaining levels considered moderate to high risk for allergenic reactions. Prominent bands with approximately 71, 35, 31, 22, 19, 16, 14, 13 and 11 kDa were revealed. A 52 kDa band was shared by all analyzed sera. High levels of airborne pollen and emergency hospital admissions were related. High binding affinity of specific IgE to pollen extracts of the most abundant tree pollen present in the atmosphere was observed. Patient sera revealed multiple similar allergenic bands shared by the different extracts. This multidisciplinary approach is useful in day-to-day medical practice to help in diagnostic, therapeutic and allergy alerting system adjusting.

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1. Introduction

Respiratory allergic diseases related with atmospheric pollen content are increasing in prevalence and severity. This has been particularly observed in several industrialized countries especially related with the cities' resident's lifestyle and the raise in air pollution (D'Amato, 2002; D'Amato et al., 2005). Nowadays, it is estimated that the prevalence of respiratory allergy due to pollen

reaches up to 40% (D'Amato et al., 2007), with serious implications on the economy, due to the absence from work, and particularly on the healthcare system, due to high resources allocation to treatment and preventive management programmes.

Several aerobiological studies have been performed for the elaboration of pollen calendars that are indicative of the allergenic potential present in the atmosphere and provide an estimated date for the flowering season of plants that produce allergenic pollen grains (D'Amato et al., 2007). Also, the classification of the most allergenic pollen types through immunological studies (D'Amato and Liccardi, 2002) and works relating the regional pollen exposure with in vivo percutaneous reactivity to specific pollen extracts (Bass et al., 2000; Gonianakis et al., 2006; White et al., 2005) or with allergic respiratory hospital admissions

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(Carracedo-Martinez et al., 2008; Héguy et al., 2008; Tobías et al., 2003; Zhong et al., 2006) have been widely studied. All this information is useful for clinicians, in order to adequate the standard skin-prick test battery or the intradermal tests with extracts to the regional pollen spectrum, for effective immunotherapy or other treatment procedure. It can also be helpful for patients in taking prophylactic measures such as the planning of outdoor activities, contributing to an improvement on their life quality.

In urbanized environments, the absence of abandoned soils, where weeds or grasses might develop, and the predominance of frequently cared and maintenance gardens by government authorities or homeowners, lead to the elimination of many spontaneous weeds and grasses. Furthermore, the plantation of trees for ornamental purposes is increasing, both in city avenues, street-walks and in public parks, with great allergenic impact upon the health of the residents. While the existence of a relationship between grasses and weeds airborne pollen concentration and hospital admission or exacerbation of respiratory allergic symptoms has been demonstrated (Atkinson and Strachan, 2004; Erbas et al., 2007), only recently attention has been drawn to tree pollen allergy (Mothes et al., 2004) and few studies have been conducted in order to investigate its impact on respiratory allergies. Therefore, the aims of this study were to characterize the aerobiological behavior of airborne pollen from several trees in the city of Porto (Portugal), to identify the different reactivity levels in sensitized patients and to relate the seasonal airborne pollen concentration with the monthly asthma or dyspnea related with respiratory disease emergency hospital admissions. This information would be of great importance to evaluate the convenience of changing the inventory of pollen producer trees related to the risk of allergenic reactions.

2. Methods

2.1. Study area

This research was performed in the second largest Portuguese city—Porto with approximately 263,000 inhabitants and a population density of 5787 inhabitants/km². The annual mean temperature ranges between 14 and 16 °C. The average annual number of frost days (minimum temperature <0 °C) ranged between 2 and 6 while the number of hot days (maximum temperature >35 °C) was lower than 5 days. Annual mean relative air humidity is between 75% and 80%, and mean annual total precipitation ranges between 1000 and 1200 mm (Miranda et al., 2001).

2.2. Aerobiological sampling

Airborne pollen was performed from 2005 to 2007, using a 7-day Hirst-type volumetric trap (VPPS-2000, Lanzoni) set on the roof of the Faculty of Sciences in Porto (41°11'N, 8°39'W), approximately 20 m above ground level, and calibrated to sample air at 10 l/min. Pollen grains were trapped on a 7-day Melinex tape coated with silicone oil, which was then cut into daily segments and mounted on the slides with a glycerol jelly media. The daily mean concentration of the number of pollen grains was carried out using an optical microscope (DMLB, Leica) with 400× magnification along four full lengthwise traverses. Pollen counts were expressed as the sum of the number of pollen grains per cubic meter of air for a 24 h period.

The pollen types considered in this study were the ones belonging to *Acer*, *Betula*, *Platanus*, *Quercus*, *Salix* and *Populus* trees, which are allergenic pollen producers according to the Portuguese Aerobiology Network (www.rpaerobiologia.com). The main pollen season was determined using a non-linear logistic regression model fitted to the values of the accumulated sum of the daily airborne pollen concentration. The beginning and ending dates of the main pollen season correspond to the thresholds where the daily difference between the pollen emission model and its superior and inferior asymptotes was significant (Ribeiro et al., 2007).

2.3. Pollen samples

The pollen from *Acer negundo*, *Betula pendula*, *Platanus occidentalis*, *Populus hybrida*, *Quercus robur* and *Salix babylonica* was collected in Porto region in public gardens or sidewalks, during the flowering period. The trees selected were easy to access, preferably young, free standing, with abundant branches and presented flowers with dehiscent anthers. One tree per each species was sampled and the catkins were randomly collected from all quadrants of the tree in different branches until a 30 cm × 10 cm box was filled.

After separation of extraneous materials, the anthers were dried at 24 °C, gently crushed and the pollen thus released was passed through different grades of sieves to obtain pure pollen. Pollen samples were stored at −20 °C.

2.4. Patient sera

Eight patients previously designated as polysensitized to tree protein extracts assessed by skin-prick tests were chosen. Their sera were collected having specific IgE values between low and very high (Table 1). One control patient sensitized to different allergenic sources other than trees was included in the study. Sera were separated from whole blood, collected by venipuncture, and allergen-specific serum IgE were measured with a standard ImmunoCAP™ test (ImmunoCAP™-specific IgE, Phadia AB) and expressed as kilo arbitrary unit/L (kU/L), according to the manufacture's instructions.

2.5. Protein extraction and estimation

Dry pollen was suspended in 1:20 ratio in phosphate buffer saline at pH 7.4 (w/v) at 4 °C. Soluble proteins were extracted in the same buffer by continuous stirring for 4 h. The suspension was then centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was dialyzed against bidistilled water in vialing dialysis tubing (cut off point of 7000 Da) for 24 h at 4 °C with frequent changes of bidistilled water. After dialysis, the extract was centrifuged again for 30 min at 4 °C. The supernatant was then filtered through a 0.45 µm millipore filter. The soluble protein content of all the pollen extracts was quantified colorimetrically with Coomassie Protein Assay Reagent (Pierce) by the Bradford method (1976).

2.6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli (1970) in 12.5% (w/v) polyacrylamide gels under non-reducing conditions and the proteins were visualized by Coomassie Brilliant Blue R-250 staining. The molecular mass of protein bands was estimated by comparison with protein markers (Bio-Rad Laboratories). The antigenic profile was studied using a Molecular Imager GS800 calibrated densitometer (Bio-Rad Laboratories) and Quantity One 1-D Analysis, v 4.6 (Bio-Rad Laboratories) software.

2.7. Immunoblots

Protein bands separated onto SDS-PAGE were electrophoretically transferred on a blot apparatus (TE22 Mighty small transphor unit-GE Healthcare) to a nitrocellulose membrane. Transfers were carried out in a solution of 192 mM glycine, 25 mM Tris and 20% methanol (v/v) (transfer buffer) during 1 h at 200 mA. The membranes were blocked during 45 min with 1% BSA (w/v), 0.1% goat serum (v/v), 5% non-fat dry milk (w/v) in 20 mM Tris, 150 mM NaCl (TBS) and 0.1% Tween 20 (blocking buffer) and incubated overnight at 4 °C with patient sera diluted 1:12 in blocking buffer, carried out overnight at 4 °C and after washing bound antibodies were revealed with mouse anti-human IgE peroxidase (Southern

Table 1

Allergen-specific serum IgE from eight polysensitized patients' (A–H) to protein extracts (tx1, t3, t9, t7, t14, t16, t18) and expressed as kilo arbitrary unit/L (kU/L).

Patient sera	tx1 (ku)	t3 (ku)	t9 (ku)	t7 (ku)	t14 (ku)	t16 (ku)	t18 (ku)
A		2.3					
B					0.72		0.67
C			2.79				
D	27.8						
E	0.58						
F					0.42		
G			0.71	0.67	0.46	0.49	
H		2.2	3.85	3.82	1.85	1.05	2.08

tx1, *Acer negundo*, *Betula verrucosa*, *Quercus alba*, *Ulmus americana*, *Juglans californica*; t3, *Betula verrucosa*; t9, *Olea europaea*; t7, *Quercus alba*; t14, *Populus deltoides*; t16, *Pinus strobus*; t18, *Eucalyptus* spp.

Biotechnology Associates) diluted 1:2000 in TBS-T, during 30 min. In all cases, an ECL Western blotting kit (GE Healthcare) was used as substrate. The chemiluminescence signal was exposed to AGFA medical X-ray film and developed by Fuji medical film processor model FPM 100A.

2.8. Emergency hospital admissions

Daily hospital admissions' information concerning patients' origin, age, type of allergic disease (asthma or dyspnea having as final diagnosis respiratory disease) and the medication applied (antiasthmatic medication and other systemic drugs for obstructive illness), classified according to the Manchester Triage System, were obtained from the Emergency Room database of the Hospital Geral de Santo António in Porto from 2005 until 2007. Information concerning the residence location, age, type of allergic disease and medication applied to the patient was collected.

Monthly number of hospital admissions and monthly total pollen of each tree-type sampled were correlated, using the Spearman correlation coefficient, at a significance level of 5%.

3. Results

During the 3-year study, a total of 30,945 pollen grains/m³ were sampled, mainly distributed during spring and early summer. The major pollen sampled belongs to weeds followed by trees and grasses, representing, respectively, on average 32%, 24% and 12% of the annual pollen spectrum sampled.

The pollen of the studied trees was present in the atmosphere of Porto from mid-winter (January) until early spring (April) being the major pollen sampled during this period, representing 36% of the pollen sampled.

Interannual variations in the start and end dates and in the duration of the MPS of the trees studied were observed. The pollen of *Salix* was sampled at the end of February, while the pollination period of *Acer*, *Platanus* and *Populus* started in March, persisting until April. For these trees, the maximum pollen concentration was observed during March. During April was also observed the start of *Betula* and *Quercus* pollination period. The average duration of the main pollen season varied between 12 days (*Betula* or *Populus*) to 40 days (*Quercus*) (Table 2).

Concerning the variations of the annual pollen concentration from each tree species a similar trend was observed, except for *Acer*. The lowest annual concentrations were sampled in 2006 while the highest in 2007 (Fig. 1).

The results of 12.5% SDS-PAGE of total soluble proteins from the tree pollen extracts demonstrated several bands ranging from 13 to 97 kDa after Coomassie staining of the gel (Fig. 2).

The allergic patient sera analyzed against saline protein extracts obtained from the tree pollen revealed more IgE reactivity with *A. negundo*, *S. babylonica* and *P. occidentalis* while *B. pendula* and *P. hybrida* showed reduced reactivity in all sera (Fig. 3). Control subject showed no reaction to the trees' extracts.

The pollen-allergen patients' pool of sera revealed distinct bands shared by the different extracts: a band with a molecular weight of approximately 52 kDa (*P. occidentalis*, *A. negundo*, *S. babylonica*, *Q. robur*, *B. pendula* and *P. hybrida*); two bands with molecular weights of approximately 35 and 31 kDa (*P. occidentalis*, *A. negundo*, *S. babylonica* and *B. pendula*); and two other bands with molecular weights of approximately 19 and 16 kDa (*P. occidentalis*, *A. negundo* and *S. babylonica*) (Fig. 3). Furthermore, the *A. negundo* extracts raised against the seven patients sera also showed bands with molecular weights of approximately 71 and 14 kDa. For *P. occidentalis* and *S. babylonica* extracts, only one patient presented reactivity to a 13 kDa molecular weight protein band while for *Q. robur* extracts one patient showed reactivity to two protein bands with molecular weights of approximately 22 and 11 kDa. One patient sera presented reactivity to a band with a molecular weight of approximately 14 kDa with *B. pendula* extract.

A total of 1433 daily hospital admissions diagnosed with asthma or dyspnea related with respiratory disease were recorded. The sample ages ranged between 13 and 99 years old (average of 61 years), being 25% of the patients younger than 47 years and 75% younger than 76 years. Approximately two-thirds (67%; $n = 960$) were females. The majority of the patients (69%; $n = 989$) presented dyspnea related with respiratory disease and 31% ($n = 444$) asthma.

The annual dynamic observed along the 3-year study was a decrease in hospital admissions from April until July to rise again during August until March. In 2005 and 2006, a seasonal annual peak of hospital admissions was observed during March. In December 2006 and January 2007, an admission peak was observed.

The monthly admissions were significantly positive correlated with the monthly airborne pollen concentrations of *Acer* ($r = 0.92$; $p < 0.01$), *Platanus* ($r = 0.81$; $p < 0.01$), *Populus* ($r = 0.72$; $p < 0.05$) and *Quercus* ($r = 0.54$; $p < 0.05$). Although not statistically significant, the hospital admissions were also positive correlated with the other three tree pollen types studied and with the total tree airborne pollen concentration.

4. Discussion

In Porto, the total maximum airborne pollen concentrations were found during March–April. The same pattern was also found in Lisbon (Portugal's capital), but was different from Coimbra, Évora and Portimão that presented the highest concentrations during May mainly due to the main pollination period of grasses (Caeiro et al., 2007). This pattern may be related to the higher degree of urbanization in Porto and Lisbon where the vegetative landscape of the street-walks and parks consists predominantly of ornamental trees with dominant pollination between March and April.

Table 2

Main pollen season (MPS) duration (D), beginning (x_i), peak pollen concentration (PI) and ending (x_s) dates estimated using a non-linear logistic regression model fitted to the values of the accumulated sum of the daily airborne pollen concentration of the six tree species studied in Porto from 2005 to 2007.

Pollen	MPS (Julian days)			PI	
	x_i	x_s	D	Day	Pollen/m ³
2005					
<i>Acer</i>	74	92	18	83	122
<i>Betula</i>	95	121	26	108	102
<i>Platanus</i>	77	89	12	83	454
<i>Quercus</i>	95	135	40	115	92
<i>Salix</i>	60	94	34	77	16
<i>Populus</i>	76	88	12	82	27
2006					
<i>Acer</i>	70	88	18	79	63
<i>Betula</i>	107	119	12	113	56
<i>Platanus</i>	83	99	16	91	198
<i>Quercus</i>	101	135	34	118	27
<i>Salix</i>	73	93	20	83	6
<i>Populus</i>	88	100	12	94	6
2007					
<i>Acer</i>	72	94	22	83	179
<i>Betula</i>	99	114	15	107	133
<i>Platanus</i>	77	99	22	88	508
<i>Quercus</i>	88	116	28	102	100
<i>Salix</i>	58	90	32	74	16
<i>Populus</i>	70	98	28	84	33

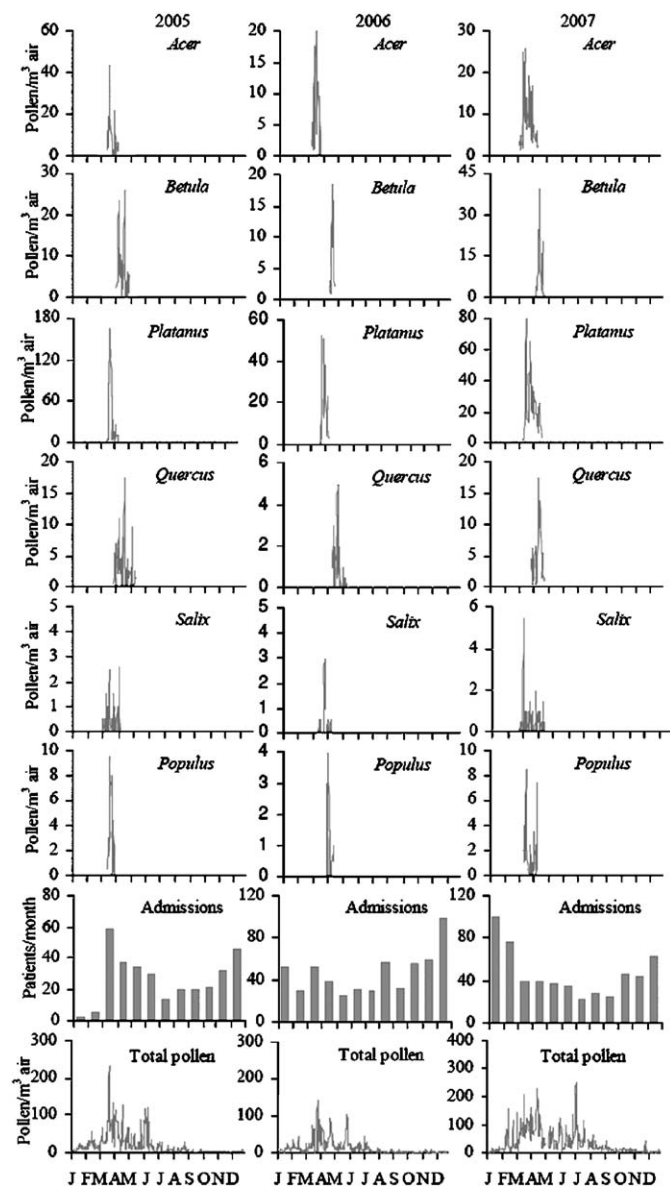


Fig. 1. Daily distribution of airborne pollen concentration from *Acer*, *Betula*, *Platanus*, *Quercus*, *Salix*, *Populus* and total pollen and monthly emergency hospital admission, concerning patients with asthma or dyspnea having as final diagnosis respiratory disease, in Porto from 2005 to 2007.

The peak of airborne pollen concentration observed in the city of Porto coincides with the flowering season of almost all trees studied (with exception for *Salix*). These trees are considered common allergenic pollen producers (Bousquet et al., 2008; D'Amato et al., 2007) having an anemophilous pollinization, with the production of a great amount of pollen and a rapidly release in the beginning of the main pollen season, being the maximum airborne concentrations achieved a few days latter. Their short main pollen season, when compared for instance with grasses or some weeds such as *Parietaria* that are present in the atmosphere for many months (Abreu and Ribeiro, 2005; Ribeiro et al., 2007), diminishes the exposure period to the allergenic reaction inducer. However, the highest airborne pollen levels attained for *Acer*, *Platanus* or *Betula* were superior to 30 and even 60 pollen grains/ m^3 that are considered, according to the Portuguese Aerobiology Network, moderate and high risk for allergenic reactions. Also, patients' sensitization in skin-prick tests to tree pollen from the

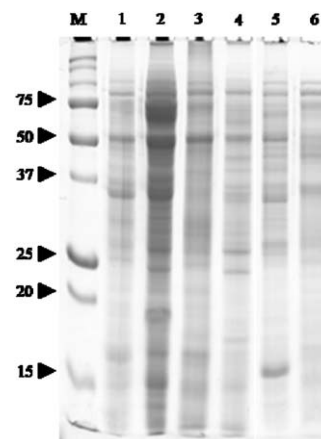


Fig. 2. Results of 12.5% SDS-PAGE gel of total soluble proteins from the tree pollen extracts from *Platanus occidentalis* (lane 1), *Acer negundo* (lane 2), *Salix babylonica* (lane 3), *Quercus robur* (lane 4), *Betula pendula* (lane 5) and *Populus hybrida* (lane 6). Molecular weight markers (lane M). Several bands ranging from 13 to 97 kDa are revealed after Coomassie blue staining of the gel.

six species analyzed has been reported in several studies (Gonianakis et al., 2006; Loureiro et al., 2005; Samolinski et al., 1996).

All patients showed high binding affinity to specific IgE of *A. negundo* extract and shared different bands with molecular weights around 71, 52, 35, 31, 19, 16 and 14 kDa. These results suggest that *A. negundo* pollen contains multiple potentially allergenic proteins. The hypersensitization to *Acer* pollen was previously reported in New York in a study testing serologically 371 allergy patients for hypersensitization towards tree pollens (Lin et al., 2002). However, the protein allergenic profile of *Acer* is not yet characterized, as far as we know. Nevertheless, in our study this tree pollen extract revealed more IgE reactivity in tested sera and together with the fact that *Acer* trees are widely spread throughout the region of Porto, emitting great amount of pollen to the atmosphere, as observed through the aerobiological study, makes this species potentially inducer of pollinosis in atopic inhabitants of the studied region.

In our study, it was observed that the tested patients' sera revealed similar bands shared by the different extracts. The negative results obtained in the control serum indicate the absence of non-specific binding.

All eight allergic patient sera analyzed against saline protein extracts of the six trees studied shared a major protein band with an estimated molecular weight of 52 kDa. Nevertheless, this pollen protein molecular characterization is still unidentified.

Also, the pool of sera revealed in *P. occidentalis* extracts one band similar to Pla a 1 (19 kDa: a putative invertase inhibitor) (Asturias et al., 2006). A 19 kDa band was also revealed in *A. negundo* and *S. babylonica*. In *B. pendula*, two prominent bands analogous to Bet v 2 (14 reporting to profilin) and Bet v 6 (35 kDa: an isoflavone reductase) were observed (Mothes et al., 2004). The 35 kDa band was also revealed in *A. negundo*, *P. occidentalis* and *S. babylonica*. Finally, a protein band, with molecular weight around 16 kDa, comparable to an allergen described in Ole e 5 (a superoxide dismutase) was also observed in *A. negundo*, *P. occidentalis* and *S. babylonica* while in *Q. robur* extracts, one patient showed reactivity to one protein band with molecular weight similar to an allergen described in Cas s1 (22 kDa: PRP). These bands sharing could indicate the existence of cross-reactivity among pollens since allergic cross-reactivity to tree pollen from various plant orders has been reported (Mothes et al., 2004). However, with our results, we cannot demonstrate this fact

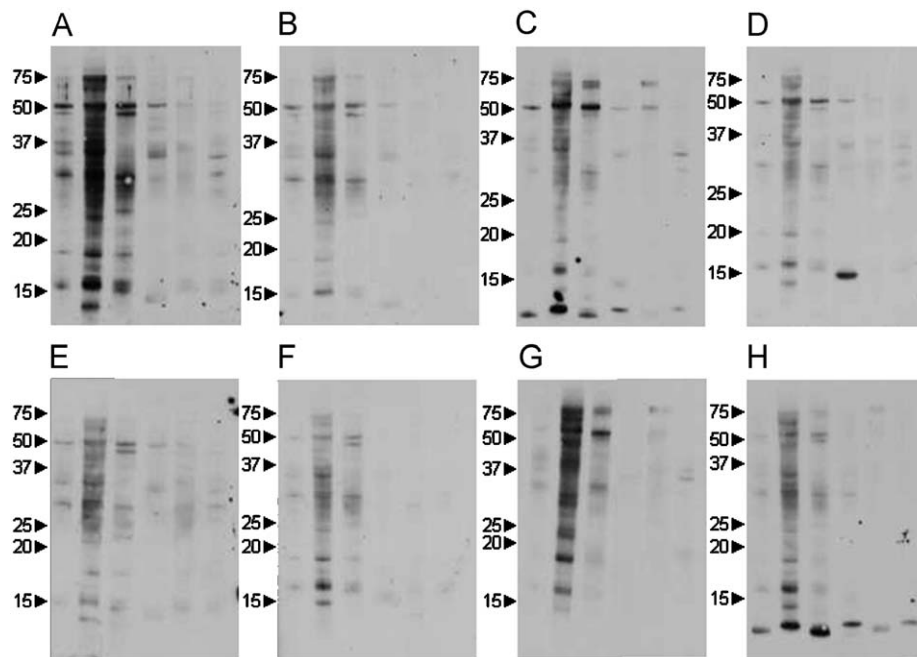


Fig. 3. Tree-sensitized sera of eight patients, previously designated as polysensitized to tree protein extracts, with specific IgE values between low and very high immunoblots (A–H). Lanes 1–6 contain, respectively, *Platanus occidentalis*, *Acer negundo*, *Salix babylonica*, *Quercus robur*, *Betula pendula* and *Populus hybrida* pollen extracts. The allergic patient sera analyzed against saline protein extracts obtained from the tree pollen IgE reactivity is showed.

because it would have to be performed inhibition studies or use recombinant allergens. Nevertheless, the binding of similar molecular weight proteins in the patients' sera to the different pollen extracts can indicate the existence of a similar pollen allergenic profile.

The selection of patients to receive allergen-specific immunotherapy is presently performed based on clinical criteria, provocation or serological testing with pollen extracts. For an exact allergen-specific immunotherapy, use the allergen pollen type must be identified. Therefore, it is advised the use of major allergen markers to confirm the sensitization (Kazemi-Shirazi et al., 2002; Mothes et al., 2004). In our study, the patients (A, B, C, F and G) that have not been tested with tx1 mixture (which includes *Acer*) presented higher IgE reactivity to *Acer* pollen extracts compared with the reactivity obtained with the extract for which they were tested in clinical trials. It was also observed in Central Africa patients who were never exposed to birch pollen exhibited positive IgE reactivity to this pollen due to profilin sensitization (Westritschnig et al., 2003) showing that they were sensitized against some other pollen containing the same allergens (Mothes et al., 2004). These examples show the importance of conducting a multidisciplinary study involving aerobiological data since it allows the identification of the regional airborne allergenic potential and the pollen types which occur in higher concentrations helping the adjust of clinical trials to each particular region.

The immunological study revealed higher binding affinity to specific IgE for *A. negundo*, *S. babylonica* and *P. hybrida*. These species' highest airborne pollen concentrations were observed during March, the same month where the highest hospital admissions were observed. It is widely known that high airborne pollen release seasons are often related with exacerbation of allergenic symptoms. Asthma hospital admissions can also be influenced by many variables, not all having a periodical pattern origin such as pollution level or weather conditions. The hospital admissions peak registered in December 2006 and January 2007 can be related with an influenza epidemic. The March admissions

peak observed in previous years was outside the influenza season and can be related with the high airborne tree pollen content. However, the simple relationship between hospital admissions and airborne pollen concentration must be interpreted with caution. Nevertheless, the highly significant correlation coefficient obtained in our study between these two variables is an important indicator of the influence of tree pollen on the exacerbation of allergenic respiratory symptoms.

This work reports an example how the combination of aerobiological, immunochemical and clinical studies is useful in medical practice, such as in choosing the standard skin-prick test battery or the intradermal tests and in adjusting allergy alerting systems. It is also important for architectural landscape planning, in order to avoid the use of allergenic pollen producers species.

Finally, this multidisciplinary approach can also be important to evaluate the convenience of changing the inventory of trees and thresholds of pollen concentration related to the risk of allergenic reactions. Aeroallergen sensitization prevalence deeply depends on intrinsic characteristics of a given population, with the airborne pollen concentration thresholds varying widely from region to region and a consensus is still far from being achieved. Although the results obtained in our work do not enable an exact establishment of threshold values, they point out to the importance of enhancing further studies and the awareness for some tree species, as *Acer*, *Salix* or *Platanus* increasingly used for ornamental purposes, to the level of *Olea* or *Cupressus*, which are considered the most allergenic tree pollen producers in the Mediterranean regions (D'Amato et al., 2007).

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