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Sensitization to *Alternaria* in patients with respiratory allergy

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1. ABSTRACT

Alternaria alternata (AA) is an important mould in respiratory allergic diseases. The objective is to determine the prevalence of AA sensitization in respiratory allergic patients and to examine the annual variation of *Alternaria* spores. 824 patients between 5 and 65 years old with allergic rhinitis and /or asthma were enrolled. Prick tests were performed with two different standardized AA extracts with quantification of Alt a 1. *Alternaria* spores concentrations were provided. 151 patients (18.3%) were sensitized to AA. The patients sensitized to AA were affected more frequently by asthma when compared to the patients not sensitized to AA (chi-square test = 7.34; p = 0.003). The prevalence of AA sensitization in paediatric population was statistically significantly higher than in patients older than 13. Atmospheric levels of *Alternaria* spores showed two periods of maximum concentration: July/August and October/November. The sensitization prevalence of AA in patients with respiratory allergy is meaningful, fundamentally in paediatric patients and/or allergic asthma patients. There is a significance spore concentration of *Alternaria* in the studied area, with a seasonal behaviour.

2. INTRODUCTION

Fungal allergy is a worldwide problem (1-3). Exposure to certain fungal spores can cause human illness such as asthma. *Alternaria alternata* (AA) is considered to be one of the most frequently involved molds in reaginic-based allergic diseases. AA is a cosmopolitan organism that can be isolated in outdoor environments, where it grows on dead vegetation and can be recovered from soil samples. Nonetheless, exposure to *Alternaria* allergens can also occur indoors (4). Exposure to the airborne spores of AA can initially result in sensitization that later on may lead to the development of a fungal allergic disease.

The diagnosis of IgE-mediated allergy to AA is usually based on clinical history and skin test. Diagnosis can be supported by *in vitro* allergen-specific IgE determination. *In vivo* and *in vitro* AA allergy diagnosis is usually performed using an *Alternaria* extract containing a complex mixture of proteins, glycoproteins, polysaccharides, and other substances. The AA extracts frequently show a considerable variability due to inter-strain genomic differences, differences in culture conditions and the use of different extraction procedures

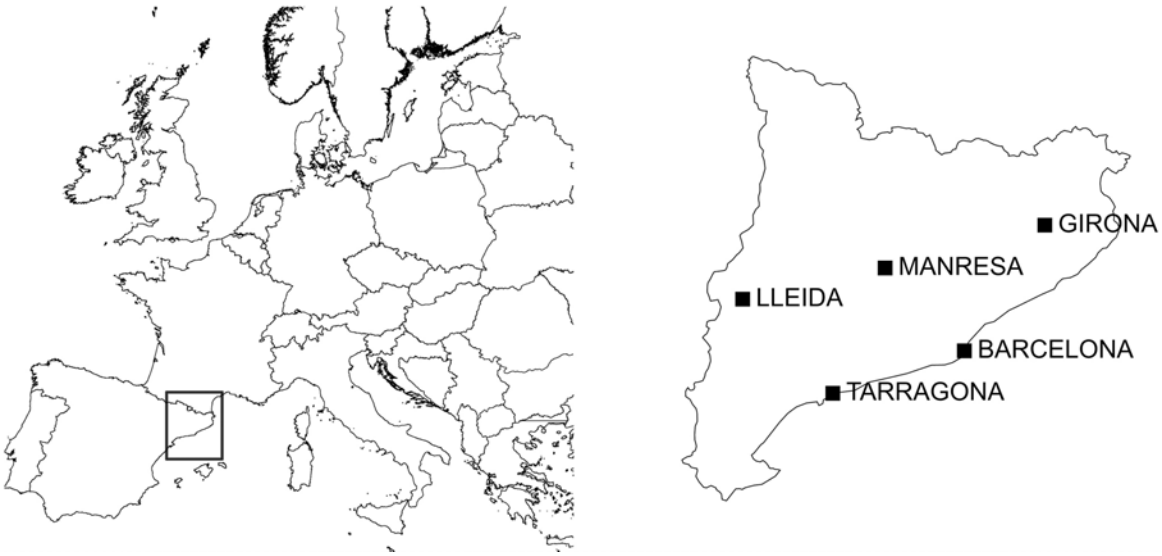

Figure 1. Geographic situation of the patients area studied with the places of centers participants. Extension of the area studied: 32.196 km^2.

(5,6). Currently, there are no standardized allergen products available in the United States(7), and the quality of commercial fungal extracts used in Europe is often low (8). More than 9 allergens have been described in AA extracts, although only 2 of them are considered major allergens (Alt a 1 and Alt a 2). Alt a 1, the most relevant allergen found in AA extract, reacts with serum IgE in 82 to 100% of AA-sensitized patients (9,10).

The real prevalence of AA sensitization in the general population or in patients with respiratory allergy is difficult to establish due to the use of un-standardized extracts in epidemiological studies also carried out with different methodologies. Two epidemiological studies based on general population reported a prevalence of sensitization to AA varying between 4.4 and 12.9% (11,12). Prevalence studies of AA sensitization in patients with respiratory allergy living in areas with similar climatic characteristics have shown great disparity with percentages ranging from 3 to 24%. (13,14). Recently published GA2LEN network results also shows marked differences in the sensitization to AA between 1.7 and 20.5% (15). The review of the epidemiological studies on the prevalence of AA sensitization so far reported reveals that they have been performed in mixed populations: people with suspected allergy from a population sample, patients tested in hospital, patients with rhinitis and /or asthma, adult population, pediatric population.

Several studies have clearly established the role of fungal sensitization in the development of symptomatic asthma. An accumulated body of evidence suggests that fungal sensitization, particularly to *Alternaria*, is associated with asthma (1,16,17) and mostly with severe and near-fatal asthma (18-20). There also reports that show that AA sensitization is more frequent in asthmatic children than in adults (1, 21-23).

The aims of our study were: 1) To determine the prevalence of AA sensitization by means of the skin prick testing using two different standardized extracts with quantification of Alt a 1 in pediatric and adult patients with suspected respiratory allergy. The results of the skin test and in vitro determination of the specific IgE were also compared to assess the concordance between the two tests and 2) to examine the annual variation of *Alternaria* spores in the atmosphere of Catalonia (Spain).

3. MATERIALS AND METHODS

3.1. Clinical study

Fourteen Allergy Units geographically representative of Catalonia (Spain) were selected to participate in this multicenter study (Figure 1). Each center carried out at least 74 evaluations according to a common experimental protocol in patients affected by rhinitis and / or asthma of suspected allergic etiology. Patients of both sexes aged 5 to 65 years, visited in the selected centers in consecutive order (7 patients per month) from January 2002 to December 2002 were enrolled. Patients on specific allergen immunotherapy and those affected by severe infections, malignant disorders or metabolic disease were excluded. Subjects with acute skin symptoms, with negative skin reactions to a histamine test or under treatment with drugs whose activity could interfere with skin prick test responsiveness were also excluded.

A questionnaire including demographic and disease data was completed by each patient included in the study. In all centers the correct use of the questionnaire as well as the consistency of clinical data and prick testing results was assessed by an allergologist . All patients or their caregivers were informed about the design and aim of the study and consented to participate. The study was

Table 1. Geographical and climatic characteristics of the located places of volumetric spore trap

Aerobiological Sampling Stations	Altitude (m.a.s.l.)	Geographical Coordinates	Mean Annual Temperature (ºC)	Annual Rainfall (mm)	Phytoclimates (25)
Barcelona	12	41º24' N, 02º11' E	16,4	593	Fresch-Tehtyc-semiarid
Bellaterra	190	41º33' N, 02º07' E	15,2	594	Fresh-Continental Oriental-semihumid
Girona	70	41º59' N, 02º60' E	15,0	740	Fresh-Continental Oriental-semihumid
Lleida	221	41º37' N, 00º37' E	15,1	385	Fresh-Tansitional-semiarid
Tarragona	20	41º07' N, 01º15' E	15,8	478	Fresh-Tethyc-semiarid

m.a.s.l: meters above sea level

approved by the Ethics Committee of the involved Institutions.

All selected patients were tested with the same standardized AA extract A with quantification of Alt a 1 (supplied by Alergia e Inmunología, ALK-Abelló, Spain) and standardized AA extract B with quantification of Alt a 1 (supplied by Bial-Aristegui, Bilbao Spain) with the aim of reducing the variability as a result of either inter-strain genomic differences or differences in culture conditions. The extracts were obtained in lyophilized form, reconstituted and distributed to all centers the same day. All selected patients were also tested with a set of the inhalant allergens most commonly used in Spain: grass mix (*Phelum pratense, Cynodon dactylon and Phragmites*) communis wall pellitory (*Parietaria judaica*), mugworth (*Artemisia vulgaris*), olive pollen (*Olea europea*), plane tree pollen (*Platanus hispanica*), Cypress (*Cupressus arizonica*), dust mites (*Dermatophagoides pteronyssinus* and *farinae*), cat and dog dander, *Aspergillus fumigatus* and *Cladosporium herbarum* species.

Diagnostic allergen extracts were placed on the volar surface of both forearms with a space between each extract of at least 3 cm. to avoid interference between allergens. Prick tests were performed by the same operator in each center, using the same sterile lancet (one lancet for each allergen tested). The drop of the extract was then wiped off one minute after the prick. Histamine (10 mg/mL) and glycerol-saline solution were used as positive and negative controls respectively. The assessment was done 15-20 minutes after the prick: a wheal of < 7 mm^2 respect to a wheal of negative control was considered as a negative reaction. On order to have a correct, uniform assessment of the skin positivity, the same allergologist who performed the in vivo tests outlined the profile of each wheal using a fine point marking pen. The mark was then transferred by an adhesive tape to the questionnaire of each *Alternaria* skin test positive subject. The area of the wheal was measured by planimetry utilizing the Prick-Film System (Inmunotek SL , Laboratorio de Alergia e Inmunología, Madrid,Spain).

To determine specific serum Ig E, 10 mL of blood (to obtain 5 mL of serum that was frozen and maintained at – 20ºC) was collected only from those subjects with a positive skin prick test to AA extract A and/or B. *Alternaria*–specific Ig E was detected by ImmunoCAP, Phadia (Uppsala, Sweden). All in vitro Ig E quantifications were performed at the end of the trial in the same laboratory using the same reagents on sera samples that had been kept frozen.

3.2. Aerobiological study

Alternaria spores concentrations during the whole 2002 year were provided by the Aerobiological Network of Catalonia (http://lap.uab.cat/aerobiologia). Sampling was made according to methodology reported elsewhere (24) similar to that recommended by the European Aeroallergen Network. Samples were obtained by means of Hirst-type volumetric spore traps: Burkard 7-day recording volumetric sampler (Burkard manufacturing Co Limited, Rickmansworth, Herdfordshire, England) or Lanzoni VPPS 2000 (Lanzoni srl, Bologna, Italy) placed on the roofs of buildings in 5 localities of Catalonia (Table 1), mostly located near the centers involved in the study. The traps were continuously working, sucking in air at a rate of 10 l/min, with the fungal spores being trapped on a Melinex strip coated with adhesive. The preparations were analyzed daily by optical microscope (600 x magnification), counting one longitudinal sweep per slide. The data obtained correspond to the mean daily values expressed as the number of spores per m^3 of air. Data are presented as total spores and as isolated *Alternaria* spores.

3.3. Data analysis

Levels of *Alternaria*-specific Ig E and wheal areas caused by AA and controls (histamine and glycerol-saline solution) were evaluated by planimetry and statistically analyzed by a specialized center not participating in this study. The reactivity of the skin prick test performed with the two allergen extracts was evaluated by comparing the wheal areas using the analysis of variance for repeated measurements (T-Student). A statistical analysis (McNemar test) was carried out to assess skin prick test positivity using the absolute value of more than 7 mm^2 as cut-off to establish the limit of positive and negative result. Correlation between the wheal area and specific Ig E serum levels was calculated using the linear correlation test. The association between both AA sensitization and the presence of asthma and AA sensitization and pediatric patients was evaluated using the Chi-square test.

4. RESULTS

4.1. Clinical study

One thousand fifty six patients were included in the study, 232 of whom were excluded because they were skin prick testing negative. Demographic and clinical characteristics of the 824 atopic patients are summarized inTable 2. One hundred fifty one patients (18.3%) were sensitive to at least one of the two AA extracts. Demographic and clinical characteristics of this group are summarized in Table 3.

Table 2. Demographic and clinical characteristics of allergic patients. All data are expressed as number of subjects and percentage (in parenthesis)

Total	**824 (100)**
Women	492 (60)
Men	332 (40)
< 14 years old	140 (17)
≥ 14 years old	684 (83)
Age range	5-65 years old
Rhinitis	516 (63)
Rhinitis and asthma	297 (36)
Asthma	11 (1)
Sensitization to House dust mite	507 (61)
Sensitization to Pollen	474 (57)
Sensitization to Epithelium	251 (30)
Sensitization to *Alternaria*	151 (18)
Sensitization to other moulds	90 (11)

House dust mite: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*; Pollen: grass mix, communis wall pellitory (*Parietaria judaica*), mugworth (*Artemisia vulgaris*), olive pollen (*Olea europea*), plane tree pollen (*Platanus hispanica*), Cypress (*Cupressus arizonica*); Epithelium: cat, dog. Other moulds: *Cladosporium herbarum, Aspergillus fumigatus.*

Table 3. Demographic and clinical characteristics of patients sensitized to *Alternaria*

Total	**151 (100)**
Women	87 (58)
Men	64 (40)
≥ 14 years old	102 (68)
< 14 years old	59 (32)
Age range	5-58 years old
Rhinitis	80 (53)
Rhinitis and asthma	71 (47)
Sensitization to House dust mite	49 (32)
Sensitization to House dust mite and Pollen	9 (6)
Sensitization to House dust mite and Epithelium	17(11)
Sensitization to House dust mite and moulds	4 (3)
Sensitization to House dust mite, epithelium and pollens	15 (10)
Sensitization to House dust mite, epithelium and moulds	2 (1)
Sensitization to House dust mite, epithelium, pollens and moulds	1 (1)
Sensitization to Pollens	26 (17)
Sensitization to Pollens and epithelium	14 (9)
Sensitization to Epithelium	6 (4)
Sensitization to only *Alternaria*	8 (5)

All data are expressed as number of subjects and percentage (in parenthesis). House dust mite: *Dermatophagoides pteronyssinus, Dermatophagoides farinae*; Pollen: grass mix, communis wall pellitory (*Parietaria judaica*), mugworth (*Artemisia vulgaris*), olive pollen (*Olea europea*), plane tree pollen (*Platanus hispanica*), Cypress (*Cupressus arizonica*); Epithelium: cat, dog. Moulds: *Cladosporium herbarum, Aspergillus fumigatus.*

The patients sensitized to AA were affected more frequently by asthma respect to the patients not sensitized to AA (chi-square test = 7.34; $p = 0.003$).

The prevalence of AA sensitization in patients between 5 and 13 years was statistically significantly higher than in patients older than 13 years (chi-square test = 31.33; $p = 0.001$).

Of the 151 patients sensitized to AA, 147 patients were positive to the two extracts AA A and B. From among of the 151 patients, one had a skin prick test positive to extract of AA B and negative to extract A; 4 patients had a skin prick test positive to extract AA A and negative to extract B. There were no statistically significant differences ($p = 0.37$) between AA extract A and AA extract B in the percentage of positive tests. Nor were significant differences in the wheal areas of the positive reactions with the AA extract A and AA extract B ($p = 0.575$).

Prick tests with the AA extracts and the level of specific Ig E were compared in 103 of the 151 patients sensitized to AA. Of these 103 patients , 67 had a specific Ig E more than 0.35 kU/L (range between 0.36 and > 100 kU/L) and 63 more than 0.70 kU/L. The range of specific Ig E was between 0.36 kU/L and more than 100 kU/L. In 36 patients (35%) the *in vitro* test did not confirm the *in vivo* test positivity. There was neither correlation between the wheal area of AA extract A and the levels of specific Ig E, nor between the wheal area of AA extract B and levels of specific Ig E.

4.2. Aerobiological study

The main results obtained in the aerobiological study are shown in Table 4. Higher levels in spore scores (annual index and mean daily spore concentrations) were reached in inland (Girona) and rural areas (Bellaterra, LLeida), with respect to coastal and urban areas (Barcelona, Tarragona). The inland and rural area of Lleida showed the highest annual levels of *Alternaria*, followed by Barcelona and Girona. Similar levels and tendencies were found in Bellaterra and Tarragona, where levels accounted for less than one third of those detected in Lleida. Figure 2 represents the annual evolution of *Alternaria* spores in the atmosphere depicted as mean weekly spores concentration. Atmospheric levels of *Alternaria* spores show two periods of maximum concentration, in July/August and in October/November.

5. DISCUSSION

This epidemiological study evaluates the prevalence of AA sensitization in a geographical area with different climatic conditions in patients with respiratory allergy, using a biologically standardized extract of the major allergen Alt a 1. Outdoor counts of Alternaria spores was also studied.

In our study the AA sensitization prevalence was 18.3% with a major percentage in patients with allergic asthma and in those younger than 14 years of age. Cutaneous tests were more sensitive to establish AA sensitization than serum specific IgE determinations. The aerobiological study shows the presence of *Alternaria* spores throughout the study period with seasonal increases in Spring and Autumn when daily peaks of spores could reach levels close to 800 spores/m3.

AA sensitization prevalence in the atopic population varies considerably according to different studies. Discrepancies in AA sensitization could be partly related to: 1. lack of standardized extracts, 2. Differences in

Table 4. Annual indexes (Sum of mean daily spore concentrations) and maximum mean daily total spores and *Alternaria* spores in the studied localities during 2002

Sampling locality	Total spores			*Alternaria* spores		
	Annual Index (Spores)	Maximum mean daily concentration (Spores/m³)	Date	Annual Index (Spores)	Maximum mean daily concentration (Spores/m³)	Date
Barcelona	284,253	5,606	14 July	12,911	412	23 July
Bellaterra	404,939	6,118	2 Aug.	11,995	375	17 Oct.
Girona	985,639	18,374	14 July	12,762	434	21 July
Lleida	388,038	6,572	17 May	27,496	767	3 Nov.
Tarragona	290,727	8,641	22 Sep.	8,123	272	3 Nov.

the study population characteristics (the most important confounding factor is the age of the patients) and 3. Differences in the performance and interpretation of skin tests. The AA prevalence found in our study (18.3%) contrasts with the 25.4% prevalence detected by Boulet et al (26) in 2,700 pediatrics and adults patients with respiratory allergy, and also with the prevalence of 9.4% reported by D'Amato et al in the European Multicenter Study (13) that includes 877 patients, from 5 to 60 years to age. In the latter study a marked geographical variation in the prevalence of AA sensitization ranging from 3% in Lisbon, Portugal, to 20% in Murcia, Spain was found. The *Alternaria* extracts used in the two studies were not biologically standardized. Corsico et al (14) using a biologically standardized extract in a multicenter study found a prevalence of AA sensitisation of 10.4 % in 2,492 patients with allergic asthma and/or rhinitis, from 1 to 73 years of age. There were also marked differences in sensitization prevalence among centers with prevalence varying from 1.9% to 29.3%, which could not be explained based on the climatic and geographic differences of the area studied. In our study, a similar prevalence was found in all 14 participating centers (data not shown) except for those where paediatric population was predominant, with higher prevalence. The prevalence interval found in the different centers was between 12 and 25%.

Two different standardized extracts with Alt a 1 (major AA allergen) quantification obtained from two different suppliers were tested in all centers in order to decrease the underdiagnosis due to problems in obtaining correct fungal extracts. For this reason, the extracts were obtained lyophilized and reconstituted, the same day of delivery to the different centres. The fact that the extract is quantified for Alt a 1 allows us to have an extract with guarantees of a high percentage of diagnosis in patients sensitized to AA. A recent study of Asturias et al demonstrated that diagnosis of AA sensitization can be simplified by using a single allergen, Alt a 1 from either natural or recombinant sources (10). Both AA extracts have shown similar results of all the 151 patients. However there were 5 where one of the extracts gave a negative result, which means that sensitization in those cases was not due to Alt a 1 but to other minority allergen, depending on the branch used, or that it disappeared during the process of the extract. This study shows the need to dispose of biologically standardized extracts containing all allergenic proteins.

The correlation between prick test results and IgE values agrees with other studies in which skin test response and seric values were compared (8,14,27). As with othe allergens, the skin test is more sensitive than specific IgE determination. Mold extracts and mold allergen molecules are rich in carbohydrates. These compounds are known to interfere with the linking of allergen molecules to solid supports employed for IgE *in vitro* analysis.

The association between AA sensitization and asthma observed in our study is in consonance with other epidemiological studies (16,17,21,28) in which fungal allergy (concretely AA) is considered a major risk factor for asthma, including severe asthma. This association is more frequently found in the paediatric population, as confirmed in our study. Currently the mechanism through the AA is a risk factor for the development of asthma are unknown but, the *Alternaria* proteases can damage the bronchi and the allergens can be more aggressive, children being more vulnerable and especially when presenting genetic condition.

The studied population is exposed to high levels of atmospheric AA spores especially in Summer and at the end of Autumn, when medium weekly concentrations are higher and maximum daily peaks are found. Although we have no knowledge of published studies showing the exposure threshold of AA spores able to sensitize predisposed subjects, these conditions are propitious to sensitize the patients. This seasonality also agrees with aerobiological studies done in other locations, where maximum *Alternaria* peaks are obtained from Spring until the end of autumn (1,29,30).

Although *Alternaria* exposure may occur outside as well as indoors, aerosampling data conducted at a time when *Alternaria* spores are prevalent in the outdoor environment indicate that spores can also infiltrate the indoor environment (1). A relationship exists between indoor fungal accumulations and outdoor weather. Higher indoor levels are noted when outdoor fungal levels increase (31). Some studies of *Alternaria* sensitization prevalence demonstrate, in geographic areas with low atmospheric *Alternaria* spore levels, that a low prevalence of *Alternaria* sensitization also exists (32,33). The number of *Alternaria* spores varies extensively depending on the climatic and geographic conditions. Another fact that demonstrates the importance of fungal atmospheric concentration are the studies that show a parallelism between asthma attack exacerbations with high fungal spore peak (3,34,35). Therefore, it is important to have a spore exposure map to give information to sensitized patients about their exposure, even though we cannot conclude data from this study about the clinical relevance of this sensitization. In function with this seasonality of *Alternaria* spores checked in former studies, patients should be enrolled in an randomized way

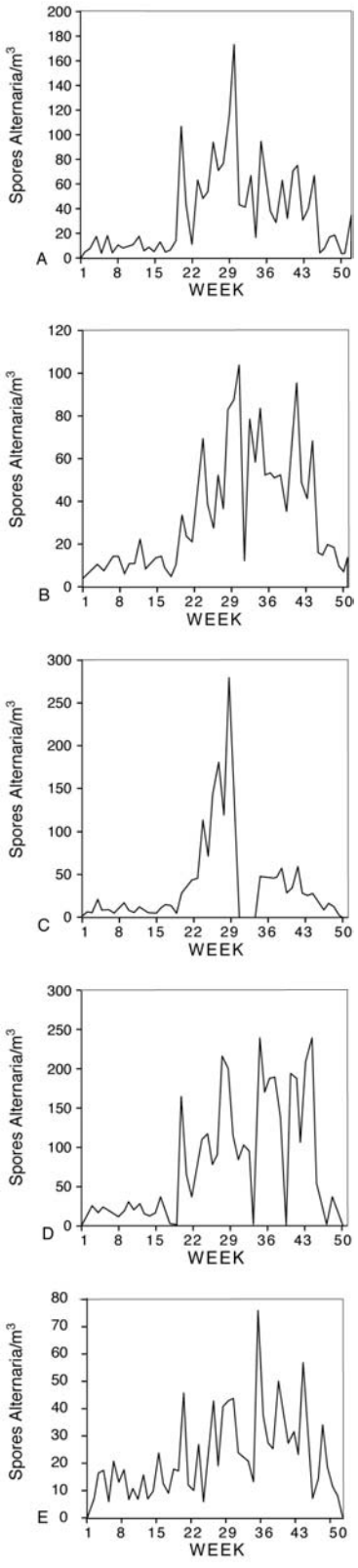

Figure 2. Seasonal variations of mean weekly Alternaria spore concentrations. A: Barcelona; B: Bellaterra; C: Girona; D: Lleida; E: Tarragona.

(patients attending the allergy department consecutively) , and they were uniformly distributed for 12 months, in order not to undervalue or overvalue the prevalence of the study, due to a seasonal influx of patients. *Alternaria* can be correlated with a seasonal-depending symptomatology, which may allow *Alternaria* allergic patients to improve by themselves or be referred, to allergy department more frequently in the high spore season.

In conclusion, data in this study reveals that the sensitization prevalence of AA in patients with respiratory allergy is meaningful, fundamentally in paediatric patients or allergic asthma patients. AA Should be considered in their diagnostic procedure using biological standardized extracts with allergen quantification of at least major allergens. There is a significance spore concentration of *Alternaria* in the studied area, with a seasonal behaviour. Further studies have to be directed in the clinical relevance of this sensitization, even in severs asthma patients. The elaboration of fungal maps with fungal spore concentration prediction are needed to make the prophylaxis and control of allergic pathology easier due to fungal spores exposure. The atmospheric concentration o AA spores does not always correlate with the concentration of Alt a 1, as the Alt a 1 concentration varies as a function of spore activity, and is highest during the germination phase. For this reason, in addition to fungal spore maps, the performance of studies on the atmospheric concentration of Alt a 1 should be recommended in order to correlate these concentrations more closely with the patients' symptoms.

6. ACKNOWLEDGEMENTS

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7. APPENDIX

Mould Allergy Study Group of the Societat Catalana d´Al.lèrgia i Immunologia Clínica (SCAIC): T. Alfaya (Hospital Santa Maria de Lleida), P. Amat (Alergocentre. Barcelona), M. Baltasar (Hospital Universitari Germans Trias i Pujol. Badalona), J. Bartra (Hospital Universitari de Girona Dr. Josep Trueta), J. Belmonte (Unitat Botànica, Universitat Autònoma de Barcelona), M. Bosque (Corporació Sanitària Parc Taulí. Sabadell), V. Cardona (Hospital Universitari Vall d'Hebró. Barcelona), M.J. Castillo (Consorci Hospitalari Terrassa), M.T. Cerdà (Hospital Universitari de Girona Dr. Josep Trueta), A. Cisteró-Bahima (Institut Universitari Dexeus. Barcelona), M. Corominas (Ciutat Sanitària i Universitària de Bellvitge. Hospitalet de Llobregat), E. Gabarra (Aerobiological Network of Catalonia), P. Gaig (Hospital Universitari de Tarragona Joan XXIII), P. García-Ortega (Ciutat Sanitària i Universitària de Bellvitge. Hospitalet de Llobregat), M. Guilarte (Hospital Universitari Vall d'Hebró. Barcelona), M. Ibero (Consorci Hospitalari de Terrassa), R. López-Abad (Hospital Universitari de Tarragona Joan XXIII), R. Llatser (Hospital Sant Pau i Santa Tecla. Tarragona), R. Lleonart (Fundació Altaia. Manresa), A. Malet (Alergocentre. Barcelona), Ll. Marquès

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Abbreviations: AA: *Alternaria alternata;* IgE: Immunoglobulin E

Key Words: *Alternaria alternata*, Alt a 1, Asthma, Rhinitis, standardized extract

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