Profilin is a marker of severity in allergic respiratory diseases

Short title: Profilin is a marker of severity in allergy

Abstract

Background: The capacity of profilin to induce allergic symptoms in patients with respiratory allergy has been questioned. In that sense, the aim of this study was to investigate the correlation between profilin exposure and induction of symptoms in a prospective case-control study.

Methods: The concentration of profilin as well pollen levels were measured. A diary score of symptoms was collected from allergic patients. Seventy-nine individuals were included in the study; 51 cases and 28 controls were positive and negative to profilin, respectively.

Conjunctival and bronchial provocation tests were performed with purified profilin (Pho d 2) in a subgroup of cases and controls.

Results: Profilin was detected in the environment in 133 days (maximum peak of 0.56 ng/m^3). A positive correlation between profilin and pollen count of *Olea* and *Poaceae* was observed. Intensity of total, nasal and ocular symptoms was statistically higher in cases than in controls ($p<0.001$). The risk of suffering symptoms was also higher in cases than in controls. The provocation test was positive in 95% of bronchial and 90% of conjunctival challenges in cases, and negative in all controls.

Conclusions: Profilin has been detected in the environment and has the ability to induce a specific allergen response. Patients sensitised to this panallergen showed more symptoms and are more likely to have symptoms. Therefore, sensitisation to profilin seems to be a marker of severity in patients with rhinoconjunctivitis and asthma mediated by pollen.

Keywords: aeroallergen, allergen quantification, profilin, provocation test, respiratory symptoms.

Introduction

Profilin is an ubiquitous family of proteins of about 12–16 kDa present in eukaryotic cells and involved in the control of actin polymerisation[1,2]. They have been reported as panallergens and their similar tertiary structure, even among taxonomically separated plant species, is the cause of their high cross-reactivity[3,4]. However, profilins are considered a minor allergens because their clinical relevance is limited[3].

The sensitisation profile of patients is highly variable (20% to 30% of patients with pollen allergy) and mainly depends on geographical distribution and other concomitant and predominant allergens[5,6]. In Central and Northern Europe, profilin sensitisation has mainly been associated with respiratory allergy to birch[7], while in Southern Europe is associated with high exposure to grasses, which sensitise up to 60% of patients[8]. On the contrary, in some areas of Australia, where ragweed pollen is predominant, 50% of patients allergic to pollen are sensitised to profilin[3].

Apart from the relevance of their sensitisation capacity, profilins have been reported as a co-factor in pollen allergy. Some authors attribute these percentages to co-recognition, or cross-reactivity[4,9,10]. On the contrary, some authors are recently questioning the lack of relevance of profilin recognized so far[11,12]. Recent studies have revealed that early sensitisation to profilin could be an early marker of predisposition to more severe allergic disease[13]. The presence of profilin-specific IgE has been associated with an increased risk of sensitisation to multiple pollens and the presence of food allergy[14] and higher risk of allergic reactions to specific immunotherapy[15].

With the aim of investigating the capacity of profilin to induce allergic symptoms in patients residing in our area of influence, the objective of this study was to measure the concentration of profilin in the environment and to establish a correlation between clinical symptoms and profilin exposure by challenging our population to a conjunctival and bronchial provocation test with purified profilin, in a prospective case-control study.

Materials and methods

Patient population

This study consists of a case-control study. The patient population consisted on patients older than 14 years who came to the Hospital Universitario Infanta Elena (Valdemoro, Madrid, Spain) for the first time, due to allergic respiratory pathology (rhinitis,

rhinoconjunctivitis and/or asthma). Over one year, all patients completed symptom diary cards.

All patients gave written consent to participate in the study. Individuals with severe atopic dermatitis, uncontrolled bronchial asthma or any other severe respiratory pathology that limits performing diagnostic tests and evaluation of the results thereof, or who declined consent, were excluded from the study.

The study protocol was approved by the Clinical Research Ethics Committee of the Jiménez Díaz Foundation (Madrid, Spain) (Number EO172011FJD).

Cases consisted of patients with rhinitis, rhinoconjunctivitis and/or asthma and sensitized to profilin while the control group included patients with rhinitis, rhinoconjunctivitis and/or asthma but negative to profilin. Serum samples from patients were collected for further studies.

***In vivo* Studies**

Skin prick tests

All individuals recruited were skin prick tested (SPT) with a standard battery of biologically standardized aeroallergens including mixture of grasses, *Lolium perenne*, *Secale cereale*, *Cynodon dactylon*, *Olea europaea*, *Cupressus arizonica*, *Platanus hybrida*, *Parietaria judaica*, *Salsola kali*, moulds (*Alternaria alternata*), mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) and animal epithelia (cat and dog), fruits including peach (peel and pulp), apple, plum, orange, melon, kiwi, banana, avocado, and fig and latex (LETIPharma, Madrid, Spain) and with purified profilin (produced under GMP conditions) from *Phoenix dactylifera* (Pho d 2, 50 μg/mL[16]) (LETIPharma).

Conjunctival allergen challenge

Conjunctival challenges were performed according to the usual technique[17] with purified Pho d 2 in 5 concentrations increasing from 0.003 μg/mL to 3 μg/mL dissolved in saline solution (0.85% NaCl, phosphate buffer 7 mM), following the recently recommended evaluation criteria[18]. Conjunctival challenges were performed in 10 cases and 5 controls outside the symptom registration period so as not to interfere with the symptom diary card.

Bronchial challenge

Specific bronchial challenge tests were performed with purified profilin[16] in serial dilutions in sterile PBS (phosphate-buffered saline) by the tidal flow method doubling

concentrations of the antigen dissolved in sterile PBS from 0.15 µg/mL to 30 µg/mL, according to the previously reported technique[19,20]. Late response with peak flow was controlled in the 24 hours after the test. Specific bronchial challenge tests were performed in 20 cases and 10 controls outside the symptom registration period in diary card.

***In vitro* studies**

sIgE and immunoblotting

Specific IgE (sIgE) to rPhl p 12 and rBet v 2 was determined (ImmunoCap, Thermo Fisher, Uppsala, Sweden).

In addition, recognition of Pho d 2 by the patients' sera was analysed by immunoblot as reported[16].

Aerobiological and clinical studies

Air sampling

A volumetric air sampler (Air Sentinel, Quan-Tec-Air Inc., Rochester, Minnesota, USA) adapted for outdoor use[21] was used for aeroallergen collection. The collector was run continuously during 2012. The sampler was placed 8.26 m above street level in Valdemoro (coordinates 40°11′53″N 3°41′50″O). Air flow was 10 m^3/h. Airborne particles were collected onto polytetrafluoroethylene membranes (Merck Millipore, Tullagreen, Ireland). Sampling time for each filter was 24 hours, which represents 240 m^3 of air per sample. Filters were replaced at approximately the same time each day. After removal, filters were sealed in plastic bags and stored at 4°C until extraction.

Filter Extraction and Allergen Quantification

The upper layer of 220 filters was separated and individually placed in tubes containing 2 mL of 0.01M PBS. Tubes were stirred until the filter was completely soaked and left for overnight extraction in a rotary mixer at 4°C. Afterwards, the content was collected and the filter discarded.

Allergen content was measured by ELISA inhibition[22]. In short, purified Pho d 2 was used as standard (from 1.95 ng to 1000 ng). Microplates were coated with profilin at 1µg/well. Samples were incubated with polyclonal anti-Pho d 2 antibodies produced in rabbit as previously reported[16] (dilution 1:30000). Allergen concentrations were extrapolated using the standard curve and were based on inhibition capacity; final results were expressed in ng/m^3 of air.

Pollen count

Aerobiological sampling was performed from January 1st to 31st December, 2012 with a Burkard pollen collector (Hertfordshire, UK) placed in Valdemoro one metre from the volumetric air sampler. Samples were examined under optic microscope with a 100X objective lens. Pollen concentrations were expressed as pollen grains/m^3 of air.

Symptom Diary Cards

Throughout the year, all the patients were given diary cards on which they recorded their conjunctival, nasal, and bronchial symptom scores according to the following scale[22,23]: 0, no symptoms; 1, mild symptoms (slight nasal obstruction, slightly runny nose, or occasional sneezing or itching of the eyes); 2, moderate symptoms (moderate nasal obstruction, moderately runny nose, some sneezing and congestion, some ocular itching, or mild asthma); 3, severe symptoms (complete nasal obstruction, almost continuously runny nose, frequent sneezing or ocular symptoms or asthma attacks).

Statistical analysis

The chi-square test (χ^2) was used to study the relationship between study variables. The Mann-Whitney Rank Sum Test was used to compare numerical numbers obtained for the different groups. Linear regression and logistic regression models were used to evaluate the relationship of variables to the intensity and presence of symptoms, respectively. Scatter plots were used and the Spearman correlation coefficient was calculated to evaluate IgE concentration and symptom severity. The software GraphPad Prims 7 (La Jolla, CA, USA) and OpenEpi (http://www.openepi.com) were used for analyses.

Results

Patient population

Seventy-nine patients (mean age 30.1±8.5 years), were included in the study: 51 positive to profilin (cases) and 28 negative to profilin (controls). The characteristics of the population are shown in Table 1.

Cases had statistically more food allergy symptoms than controls (80.4% *vs* 14.3%) (p<0.001). The main symptoms were oral allergy syndrome and the main fruit involved in symptoms was melon (p<0.001), as reported by patients (68.6% *vs* 7.1%), and correlating to wheal values obtained by SPT (52.9% vs 3.6%).

***In vivo* studies (profilin diagnosis)**

SPT

Mean value for wheal sizes induced by profilin in the 51 cases was 32.9±23.1 mm^2.

A total of 86.3% of cases presented sensitization to 3 or more pollen and only 46.4% of controls (p=0.04). Most profilin sensitized patients were sensitized to *Cynodon dactylon* (92.2%), with statistically significant differences in comparison with profilin negative patients (53.6%) (p<0.001). There was also a statistically significant correlation between sensitization to profilin and to *Platanus acerifolia* (76.5% vs 17.9%) (p<0.001) and *Parietaria judaica* (21.6% *vs* 3.6%) (p<0.05).

Conjunctival challenges

Conjunctival challenges were performed in 15 patients, who gave their consent for the test 10 cases and 5 controls, being positive in 9 of the 10 cases. The median concentration that induced the reaction was 0.3 µg/mL. All 5 controls had a negative challenge test.

Bronchial challenges

Among patients diagnosed with asthma, bronchial challenge with purified Pho d 2 was performed on the first 20 cases and 10 controls who gave their consent for the test (Table 2). Nineteen cases (95%) had a positive bronchial challenge with profilin, with amounts ranging from 0.31 to 20 µg/ml, the mean PC20 being 10.55 µg/mL (SD:11.87). A statistically significant difference was observed (P<0.001), both in the final FEV1 and in the percentage FEV1 decrease from baseline when comparing cases and controls. FEV1 decreased a mean of 24.3% for cases and 5.9% for controls. A total of 60% of cases presented additional symptoms during the test; the most common were nasal symptoms (35%), cough (20%), and palatal pruritus (20%). There was no late response in peak flow records during the 24 hours after the test.

All controls had negative bronchial challenge with purified profilin.

***In vitro* studies (sIgE and immunoblot)**

Serum samples were positive to rPhl p 12 in 38 cases (74.5%) and rBet v 2 in 42 (82.3%) (Figure 1). Values of sIgE were 4.8±10 kU/L in the case of rPhl p 12 and 6.2±10.8 kU/L for rBet v 2. Both profilins were negative in controls.

Immunoblot was performed with all sera. Forty-two of the 51 sera from cases (82.4%) showed a band of 14 kDa corresponding to Pho d 2 (1) (Figure 1). Sera that did not recognise Pho d 2 were also negative to rPhl p 12 and rBet v 2 by CAP. None of the

controls recognised the profilin in the immunoblots (data not shown). The median total IgE of patients sensitised to profilin was 264 KU/L, which was significantly higher than that for controls (91 KU/L), *P*=0.002.

Aerobiological and clinical studies

Profilin quantification on filters

A total of 220 filters were analysed. The maximum value was obtained on 10 June with 133.4 ng of profilin in the filter (0.56 ng/m^3 of air). The distribution of profilin during the year is shown in Figure 2. Profilin was detected in the environment in 133 days (36.5% of the year). For 58 days (15.9% of the year), profilin concentrations higher than 10 ng were observed in the filters (>0.04 ng/m^3 of air). The month with the highest profilin content was June, but there were also other smaller peaks in April and the end of July (Figure 2).

Correlation of amount of profilin with the pollen count of different species

Plants with pollen counts during the whole year higher than 1000 pollen grains/m^3 of air were: *Cupressaceae* (2195 pollen grains/m^3), *Olea* (2917), *Pinaceae* (1994), *Platanus* (1072), *Poaceae* (2504), and *Quercus* (12747). Others less abundant were *Amaranthaceae, Plantago* and *Fraxinus*. The profilin peak appeared some days after the pollen peak of *Olea* and *Poaceae* (Figure 2).

Patient symptoms diary

Total (Figure 3A), bronchial (Figure 3B), nasal (Figure 3C) and conjunctival (Figure 3D) symptoms were compared between cases and controls.

It was observed that mean intensity of total symptoms, along the year, was on average 0.56 points higher in cases than in controls, 95% CI (0.43,0.70) $p<0.001$ (Figure 3A); nasal symptoms 0.26 points higher in cases than in controls, CI (0.18,0.34) $p<0.001$ (Figure 3C); and conjunctival symptoms 0.27 points higher in cases than in controls, CI (0.23,0.31) $p<0.001$ (Figure 3D).

For bronchial symptoms, statistically significant differences ($p=0.020$) between cases and controls were limited to the presence of profilin (from March to September) in the environment (0.10 points; CI (0.02, 0.19) (Figure 3B).

The risk of suffering asthma was higher in cases than in controls with OR 1.32 95% CI (1.19, 1.46) ($p<0.001$). The same occurred with nasal and conjunctival symptoms, with an OR of 1.15 95% CI (1.08, 1.23) ($p<0.001$) and OR of 1.62 95%CI (1.47, 1.79) ($p<0.001$), respectively.

No correlation was detected between the intensity of these symptoms and sIgE levels to rPhl p 12 and rBet v 2.

Discussion

The ubiquitous presence of profilin makes it one of the most studied allergens. In this study profilin has been quantified in the environment and it has been related to the clinical symptoms of patients with allergy to pollen. In addition, the capacity of profilin to induce allergic symptoms has been proven, as this is the first time its capacity in the real life of patients has been analysed.

In the last few years identification of allergens in the environment has become important for allergic control of diseases and to establish a relationship with clinical symptoms[22,24,25]. According to our knowledge, previously only on one study the presence of profilin has been quantified in the environment as aeroallergen[26] with Ole e 2. In our study June showed the highest profilin concentration followed by May and April, using purified palm tree profilin (Pho d 2) as standard. As expected the highest profilin concentration correlates with the highest peaks of pollen grains in the environment, specially from grasses and olive trees, but with a few days delay between the peak of these pollens and profilin peak. In fact, the maximum profilin peak was 14 days after the peak of grasses. This could be because the characteristics of profilin require certain meteorological conditions to be detected in the environment. In this sense the profilin peak coincided with low levels of relative humidity and high temperature (data not shown).

The presence of profilin was not limited to the spring season, different concentrations were detected outside this period. This is consistent because although there are several pollens that contain profilin among their proteins, the percentage of relative profilin they contain is variable. *Lolium perenne* is the pollen with the highest percentage of relative profilin content compared to the total protein[8] (0.80). For the remaining extracts the profilin percentage is lower; olive tree (0.10), Betula (0.05), Chenopodium (0.04), Salsola (0.04) and Plantago (0.01)[8]. The clinical implications of small amounts of profilin are unknown but may enhance allergic inflammation[27].

Although the methodology for extraction and quantification of profilin in the filters rendered good results, it appears that more sophisticated methods, especially obtaining a more accurate concentration, could provide more exact data about allergen

concentration. However, the results provide a clear picture about profilin distribution over the year.

Once the presence of profilin in the environment was demonstrated, it was necessary to establish its allergenic relevance and capacity to induce allergic symptoms. Different techniques to measure sIgE to different profilins gave similar results, suggesting an appropriate selection of patients sensitized to profilin. Skin tests with purified profilin are a potent tool to select patients sensitized to these allergens. In order to confirm the skin prick test, serum samples from patients were investigated in depth by measuring profilin sensitization. All patients positive to rBet v 2 (birch profilin) by ImmunoCAP recognised Pho d 2 by immunoblot. This study revealed symptoms produced by profilin in the cases group. The results are in accordance with recently published studies confirming, the role of Phl p12 (*P. pratense* profilin) to produce symptoms *in vitro* by induction of T-cell response[28].

The ability of profilin as a respiratory allergen to produce symptoms in the conjunctival and respiratory mucosa has been poorly studied. This study is consistent with previous studies such as Nuñez et al.[29], who demonstrated this ability with positive conjunctival challenge with nPho d 2 in 65% (11/17) of patients sensitized to profilin. Ruiz-García et al.[8] observed that profilin is also capable of producing respiratory symptoms by means of bronchial challenges with nPho d 2 positive in 77% of sensitised patients. Our study confirms profilin's ability to produce an allergen-specific response locally which makes prior studies consistent and confirms that profilin should be considered as a respiratory allergen. Results showed that the concentration of profilin required to produce symptoms is much higher than that presented in the environment. After demonstrating the presence of profilins in the environment and their capacity to induce allergic symptoms, we aimed to analyse its capacity in the real life of patients. Until now, there are only a few published cases of sensitised patients[30,31]. Therefore, it is necessary to correlate the concentration of allergen in the environment with the symptoms that our patients experienced in real life, as other authors have published with other allergens[24,32]. Given that we have observed that there is profilin in the environment and that it can produce specific respiratory symptoms, it is logical to consider that this allergy could produce a summing effect on the patients' symptoms, capable of increasing their intensity or triggering symptoms during certain days when the sum of the allergens to which the patient is sensitised favours the onset of symptoms.

Recently published studies confirm that asthma-rhinitis multimorbidity is associated with IgE polysensitisation[33]. Anto et al.[34] proposed a novel allergic phenotype characterised by polysensitisation and multimorbidity, which is associated with the frequency, persistence and severity of allergic symptoms. The presence of profilin-specific IgE has been associated with an increased risk of sensitisation to multiple pollens[14]. In our study, since it was a real-life study, and most patients were polysensitised in both groups, we found that patients sensitised to profilin have a significantly higher intensity of symptoms that those not sensitised to profilin. We also observed a higher risk of presenting ocular, nasal and bronchial symptoms in a statistically significant way compared to controls. This corroborates the idea that profilin can be a marker of the severity of respiratory disease. Recent metabolomic studies could account for profilin's capacity to induce local allergic inflammation in severe phenotypes[27,35]. Only a small difference in respiratory symptoms was observed between the two groups in the presence of profilin; although the statistical significance is very low (p=0.02). Therefore, further studies will be needed to corroborate this theory.

It has been published that profilin could be a marker of evolution since sensitisation to profilin usually appears after a longer evolution time of the allergic disease and with a higher number of sensitisations[36]. This is consistent with our study where we detected a statistically significant difference in years of evolution of respiratory symptoms; this is higher in patients sensitised to profilin. This could mean that longer exposure time to allergens and longer evolution time of the disease leads to more allergens that the patients are sensitised to ranging from major to minor allergens, such as profilin. However, the opposite pattern could be the study by Asero et al.[13], who found that 16% of preschool children were already sensitised to profilin. Therefore, in this sense, prospective studies are needed to clarify whether profilin can be an early marker of severity or a marker of disease course. In our study cases had more years of rhinitis course than controls, but no statistically significant differences were observed. However, there were statistically significant differences for the years of asthma course, which was twice for profilin sensitised patients (5.8±4.9 vs 2.9±2.5 years) ($p<0.05$).

Rhinitis and asthma multimorbidity is common[37] and should be considered together. In our study we observed higher intensity and more frequency of nasal and ocular symptoms in cases than in controls. Further studies will be necessary to determine

whether an aetiological approach of this panallergen is possible with immunotherapy, as has been proposed with other panallergens such as LTP[38,39].

In summary, results demonstrate that profilin is present in the environment. This profilin is able to produce a specific allergen response at respiratory level in patients sensitised to this allergen and suffering from rhinoconjunctivitis, asthma or both. In addition, patients sensitised to this panallergen showed more symptoms and are more likely to have symptoms. Therefore, sensitisation to profilin might be a marker of severity in patients with rhinoconjunctivitis and asthma due to pollen allergy.

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463 **Table 1.** Description of the study population

464

	Total	*Cases* *Profilin positive*	*Controls* *Profilin negative*	*χ2 (p value)*
n	79	51 (64.6%)	28 (35.4%)	
Age (years±SD)	30.1±8.5	29.3±8.5	31.5±8.7	NS
Female n (%)	49(62.0%)	33(64.7%)	16/(57.1%)	NS
Respiratory symptoms				
Rhinitis	79 (100%)	51 (100%)	28 (100%)	NS
Years of evolution for rhinitis	8.6±5.1	9.2±4.9	6.9±5.4	NS
Conjunctivitis	79 (100%)	51 (100%)	28 (100%)	NS
Asthma	60 (75.9%)	38 (74.5%)	22 (78.6%)	NS
Years of evolution for asthma	4.7±4.4	5.8±4.9	2.9±2.5	p< 0.05
Sensitization to aeroallergens (SPT)				
Mites	24 (30.4%)	15 (29.4%)	9 (32.1%)	NS
Moulds	18 (22.8%)	12 (23.5%)	6 (21.4%)	NS
Epithelia	47 (59.5%)	31 (60.8%)	16 (57.1%)	NS
Pollen	79 (100%)	51 (100%)	28 (100%)	NS
Cynodon dactylon	62 (78.5%)	47 (92.2%)	15 (53.6%)	p< 0.001
Platanus acerifolia	44 (55.7%)	39 (76.5%)	5 (17.9%)	p< 0.001
Parietaria judaica	12 (15.2%)	11 (21.6%)	1 (3.6%)	p< 0.05
Sensitization to 3 or more pollen	50 (73.4 %)	44 (86.3%)	13 (46.4%)	p< 0.05
Food allergy symptoms				
Total	45 (57.0%)	41 (80.4%)	4 (14.3%)	p<0.001
OAS	40 (50.6%)	38 (74.5%)	2 (7.1%)	p<0.001
Anaphylaxis	9 (11.4%)	6 (11.8%)	3 (10.7%)	NS
Involved foods (reported by patients)				
Melon	37 (46.8%)	35 (68.6%)	2 (7.1%)	p<0.001
Watermelon	20 (25.3%)	20 (39.2%)	0 (0%)	p<0.001

Rosaceae fruits	13 (16.5%)	13 (25.5%)	0 (0%)	p<0.05
Involved foods (SPT)				
Melon	28 (35.4%)	27 (52.9%)	1 (3.6%)	p<0.001
Watermelon	2 (2.5%)	2 (3.9%)	0 (0%)	NS
Rosaceae fruits	11 (13.9%)	8 (15.7%)	3 (10.7%)	NS

Percentages calculated from the "n" in each group.

NS: non-significant.

468 Table 2. Bronchial challenge to profilin.

Cases	FEV1 baseline (L)	FEV1 post-saline (L)	FEV1 Final (L)	FEV1 decrease (%) from baseline	Last concentration of profiline (µg/ml)	PC20 (µg/ml)	Result
Case-2	3.38	3.51	2.62	25.4	5.00	7.48	Pos
Case-3	1.87	1.78	1.27	28.7	5.00	6.33	Pos
Case-12	3.00	3.01	2.35	21.0	10.00	18.67	Pos
Case-13	3.14	3.02	2.17	28.1	5.00	7.32	Pos
Case-15	2.62	2.59	2.44	5.8	20.00		Neg
Case-16	2.64	2.31	1.67	27.7	2.50	3.24	Pos
Case-17	2.35	2.34	1.84	21.4	0.31	0.43	Pos
Case-21	2.86	2.9	2.09	28.0	10.00	12.86	Pos
Case-22	2.94	2.99	2.26	24.4	1.21	1.46	Pos
Case-24	3.14	3.19	2.55	20.1	20.00	39.36	Pos
Case-26	4.25	4.33	3.46	20.0	10.00	19.77	Pos
Case-27	3.02	3.07	2.16	29.7	20.00	26.96	Pos
Case-31	4.15	4.07	3.13	23.1	2.50	2.98	Pos
Case-32	3.35	3.36	2.44	27.4	2.50	3.45	Pos
Case-33	3.17	3.02	1.94	35.8	1.25	1.27	Pos
Case-34	2.38	2.27	1.75	26.5	2.50	3.96	Pos
Case-35	4.14	4.11	3.22	21.7	20.00	34.99	Pos
Case-38	2.61	2.55	2.00	21.6	1.25	2.13	Pos
Case-48	3.25	3.21	2.44	24.0	1.25	1.19	Pos
Case-51	3.96	3.93	2.94	25.2	5.00	6.65	Pos
Mean (SD)	3.11 (0.64)	3.08 (0.68)	2.34 (0.55)	24.3 (5.9)	Median: 5.00 µg/ml	10.55 (11.87)	
Controls	**FEV1 baseline (L)**	**FEV1 post-saline (L)**	**FEV1 Final (L)**	**FEV1 decrease (%) from baseline**	**Last Concentration of profiline (µg/ml)**	**Total acumulated dose**	**Result**
Control-03	3.12	3.04	2.87	5.6	20	39.83	Neg
Control-04	3.66	3.70	3.43	7.3	20	39.83	Neg

Control-08	3.88	3.71	3.71	0.0	20	39.83	Neg
Control-11	2.69	2.79	2.80	0.3	20	39.83	Neg
Control-12	3.46	3.39	3.27	5.4	20	39.83	Neg
Control-13	4.07	4.11	3.83	7.8	20	39.83	Neg
Control-15	2.59	2.51	2.50	0.4	20	39.83	Neg
Control-23	3.78	3.80	3.32	12.2	20	39.83	Neg
Control-25	2.78	2.52	2.34	7.2	20	39.83	Neg
Control-28	4.25	4.11	3.92	4.4	20	39.83	Neg
Mean	3.43	3.37	3.20	5.1			
(SD)	(0.6)	(0.61)	(0.55)	(3.9)			

Pos=Positive result, Neg=Negative result.

Figure Legends

Figure 1: Immunoblots with the individual serum samples. Two micrograms of purified Pho d 2 were run in the solid phase. Patients sera were diluted 1:1.
sIgE to rPhl p 12 and rBet v 2 are shown for each patient and expressed in (kU/L)

Figure 2: Levels of pollen counts (*Olea* and P*oaceae*) and profilin during the period of the study. The profilin peak appeared after the maximum pollen peak of *Olea* and *Poaceae*.

Figure 3: Correlation between profilin counts and clinical symptoms (n=51 cases and n=28 controls) throughout the year. A-Total symptoms ($p<0.001$); B-Bronchial symptoms (N.S. along the year); significance limited to the presence of profilin in the environment ($p=0.02$); C-Nasal symptoms ($p<0.001$); and D-Conjunctival symptoms ($p<0.001$).