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Analysis of mite allergic patients in a diverse territory by improved diagnostic tools

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Summary

Background There are few studies comparing the sensitization with mite allergens from different mite species which could potentially be the cause of allergy.

Objective To improve the diagnosis of mite allergic patients from a diverse territory in which *D. pteronyssinus*/*D. farinae* mites together with storage mites could be present in the environment.

Methods Four hundred and seventy-seven patients (both children and adults) from different regions, covering the main mite prevalent areas of Spain, were recruited. sIgE to eight allergens was measured together with SPT to whole mite extracts, level of mite allergen exposure, and specific IgG_4. BAT and CAST was performed in a subgroup of patients.

Results *D. pteronyssinus* and *L. destructor* were more prevalent in Atlantic areas, whereas *D. farinae* predominate in Mediterranean areas. About 90% of patients were sensitized to group 1 and/or group 2 allergens. Group 2 was the most prevalent, and the IgE response/intensity of sensitization in BAT was higher. sIgE to Der p 2/Der f 2 was almost fully cross-reactive, but no cross-reactivity was detected with Lep d 2. Group 1 allergens were also cross-reactive, but in some patients a species-specific response was observed. sIgE to Lep d 2 was associated with SPT results to storage mites. Sensitization to Der p 1 was more frequent in children, whereas Lep d 2 sensitization was more frequent in adults. A higher ratio IgE/IgG_4 to Der p 2 was associated with the presence of allergic asthma.

Conclusion An improved diagnosis algorithm has been established. Group 2 allergens seem to have a leading role in mite allergy, but as group 1 sensitization could be species-specific in some patients and its prevalence is higher in children, an adequate balance on major mite species and major allergens must be consider in the design of mite allergy vaccines.

Keywords component-resolved diagnosis, *Dermatophagoides*, major mites, minor mites, mite allergy, tropomyosin

Abbreviations SPT, skin prick test; BAT, basophil activation test; CRD, component-resolved diagnosis; sIgE, specific IgE; sIgG4, specific IgG4; ELISA, enzyme-linked immunosorbent assay.

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Introduction

Mites are the main cause of allergies worldwide [1]. Indeed, in regions with significant exposure they appear to be at the forefront of atopic sensitization and have proved to play an important role in the development of asthma [2, 3]. Although the pyroglyphid mites *Dermatophagoides pteronyssinus* and *D. farinae* seem to predominate, storage mites may also be important in some regions. Furthermore, some findings suggest that mite allergens might behave differently to other inhaled allergens. Thus, a significant number of mite allergens exert proteolytic activity, which has proved to have a potential direct inflammatory mechanism [4–7] in different models. Moreover, cysteine protease (as group 1 mite allergens) signalling has recently been described to have a strong TH2 up-regulation effect [8–10]. If this turns out to be the case, Der p 1 could be expected to play a leading role in the atopic march and a differential prevalence in children compared with adults.

Group 2 allergens have been shown to bind TLR4, having thus a stronger immunogenic potential [11, 12] further enhancing the complexity of mite allergy. In animal models, mites have been described to provoke direct remodelling of the airways [13, 14], thus indirectly confirming that mites behave in a different manner to other respiratory allergens. Although very few human data are available, it should be noted that Platt-Mills et al. [15] described the different behaviours of cat and mite allergens in human allergy exposure. Thus, whereas, overexposure to cat allergens leads to tolerance, in the case of mites the higher the exposure the more severe the clinical allergic condition.

The development of new diagnostic tools based on individual components, frequently leads to new methods for analysing patient sensitization dynamics. In light of this, we decided to use a component-resolved diagnostic approach to clarify the IgE reactivity profiles of mite allergic patients, and the possible role of the main mite allergen sensitization in the mite allergic respiratory disease. Following a similar approach to epidemiological studies performed in pollen-allergic populations [16, 17], we designed a study using a simplified molecular panel including Der p 1, Der p 2, Der f 1 and Der f 2 from *Dermatophagoides* mites, Lep d 2 as a marker of storage-mite sensitization [18], Blo t 5 as a marker of true *Blomia* sensitization [19] and Pen a 1/Der p 10 as markers of mite minor allergen and arthropod food-related allergy [20]. We wanted to study if different mite species or different mite allergen exposure levels lead to different prevalence of tropomyosin, as occurs with profilin prevalence and grass exposure levels [18].

This approach made full use of previous information on the different mite fauna in terms of both species and exposure levels [21, 22]. Equal populations of *D. pteronyssinus* and *D. farinae* are found in the Mediterranean region, whereas *D. pteronyssinus* prevails – *D. farinae* is almost absent in the Atlantic regions. Subtropical regions (Canary Islands) have the highest mite exposure, whereas minor mites are frequent in all Atlantic regions, but infrequent around the Mediterranean area. This heterogeneity in mite exposure provides an adequate framework to address the scientific objectives of this study.

The Advia-centaur based method used in the study, unlike CAP, does not detect low-affinity sIgE [23] and thus has enhanced specificity that would allow to discriminate better between major allergens of *Dermatophagoides* species with a high degree of homology.

Previous reports [24] have suggested, on the basis of the probability of suffering severe asthma episodes that the sIgG4 levels of untreated patients might correlate with disease severity. Therefore, our aim was to study the potential asthma protective role of sIgG4.

A total of 477 patients distributed throughout the main mite prevalent regions of Spain were included in a multi-centre design. Both paediatric and adult populations were included to analyse potential different sensitization profiles.

Methods

Patients

A total of 477 patients from 10 different clinical groups distributed throughout the Mediterranean (Valencia and Catalonia) and Atlantic regions (Canary islands, Asturias, Galicia, Andalucía, Basc Country and Navarra), in which mites are relevant allergenic sources, were included. All patients suffered from perennial rhinitis and/or asthma caused by mite sensitization {as determined by SPT– [commercial extracts from ALK-Abelló, S.A. (Madrid, Spain) of *D. pteronyssinus, D. farinae. L. destructor, B. tropicalis, G. domesticus, T. putrescentiae, A.siro* and Shrimp were used] and/or sIgE – IgE $\geq$ class 2, CAP Phadia, Uppsala, Sweden}. None had received immunotherapy in the previous 5 years and all had resided in the same location for at least the same period. All subjects provided their written informed consent and Ethics Committee approval was obtained.

Patients were included following each clinical group's routine practise.

Serum samples (5 mL) were collected from the subjects, identified with a barcode label, stored at −40°C and thawed immediately before analysis. Skin prick tests were performed in duplicate on the volar side of the arm by conventional procedures using an ALK-Abelló lancet (ALK-Abelló S.A., Hørsholm, Denmark).

Purified allergens

Der p 1 and Der f 1 were purified from acetone-precipitated mite faeces (ALK-Abelló, S.A.) using a combination of hydrophobic interaction chromatography on a HiTrap Butyl-Sepharose column (GE Healthcare, Hillerød, Denmark), metal chelate affinity chromatography on a HiTrap IMAC HP column using Cu^{2+} (GE Healthcare) and anion-exchange chromatography on a HiTrap Q-Sepharose column (GE Healthcare). Purified mite body extracts of *D. pteronyssinus* and *D. farinae* (ALK-Abelló, S.A.) were used as starting material for Der p 2 and Der f 2 purification. These allergens were purified using a combination of hydrophobic interaction chromatography on a HiPrep Phenyl-Sepharose FF column (GE Healthcare) and HiPrep Butyl-Sepharose FF column (GE Healthcare), cation exchange on a HiLoad SP cation HP column (GE Healthcare), and gel filtration on a HiLoad Superdex 75 column (GE Healthcare). Final preparations were dialysed against 10 mM NH_3HCO_4 and freeze dried.

L. destructor group 2 allergen (Lep d 2) was purified by affinity chromatography as described previously [25].

Recombinant *B. tropicalis* allergen 5 (Blo t 5) was obtained from Indoor Biotechnologies (Charlottesville, VA, USA), recombinant *D. pteronyssinus* tropomyosin (rDer p 10) was obtained from Bial-Arístegui (Bilbao, Spain) [26]. And shrimp tropomyosin (nPen a 1) was purified as described in [27].

Determination of specific IgE

Specific IgE (sIgE) was determined following the CAP method (Phadia, Barcelona, Spain) in accordance with the manufacturer's instructions.

Purified allergens (nDer p 1, nDer f 1, nDer p 2, nDer f 2, nLep d 2, rBlo t 5, rDer p 10 and nPen m 1) were biotin labelled and the level of sIgE to them tested using a reverse sandwich assay based on the ADVIA Centaur® platform (Siemens, Tarrytown, NY, USA), as described previously [16, 28].

The IgE in each sample, which obtained from 25 μL of patient serum, was immunoabsorbed onto paramagnetic particles covalently coated with anti-human IgE Abs. Non-IgE Abs were removed by washing the paramagnetic particles before incubation with the biotinylated allergen. The amount of biotinylated allergen bound to the captured IgE was estimated from the relative light units (RLU) detected after incubation with acridinium ester-conjugated streptavidin. RLUs were converted into kU/L using a separate reference assay, based on biotinylated rBet v 1 and a reference serum pool, containing a specified amount of Bet v 1 sIgE traceable to the IgE WHO reference preparation run in parallel [28]. The limit of detection of the assay was 0.1 kU/L.

Determination of specific IgG_4

Specific IgG4 against Der p 1 and Der p 2 was determined by ELISA. Briefly, ELISA plates were coated overnight at 4°C with 100 μL of monoclonal antibody anti-Der p 1 or anti-Der p 2 diluted in PBS. After blocking, the wells were incubated with an excess of *D. pteronyssinus* mite body extract. After washing, the plates were incubated with the patient's serum in different dilutions and then with a specific peroxidise-labelled monoclonal antibody G4T9 to human IgG4 [29].

Detection was performed with *o*-phenylenediamine (DAKO), and absorbance was measured at 490 nm, with a reference filter at 650 nm, after the addition of 4 N sulphuric acid. The colour developed was proportional to the serum IgG4 concentration, and its level, in arbitrary units, was determined by interpolating on the standard curve built with a previously calibrated serum pool from grass-allergic patients.

House dust collection and analysis

Dust samples were collected by the patients using their own vacuum cleaner fitted with a new dust bag as instructed (DEA-test system; ALK-Abelló, S.A.). The dust samples were sieved and weighed, and the Der p 1, Der f 1, Der 2 and Lep d 2 content was determined by ELISA based on specific monoclonal antibodies after extraction into PBS with 0.1% BSA, as described previously [21, 22].

Basophil activation test

The basophil activation test (BAT) was performed as described previously [30, 31] using blood samples from 100 patients from the Navarra and Vizcaya clinical groups. After blood cell separation, 50 μL of the patient's cell suspension was incubated with 50 μL of six concentrations of the natural purified allergens of *Dermatophagoides pteronyssinus* and *farinae* (nDer p1, nDer f 1, nDer p 2 and nDer f 2), at final concentrations of 0.2 μg/mL, 0.02 μg/mL, 2 ng/mL, 0.2 ng/mL, 0.02 ng/mL and 2 pg/mL, respectively. Appropriate allergen dilutions were prepared daily from stock solutions kept at 4°C and used in fluid phase.

To evaluate background basal values without stimulation (negative control), 50 μL of stimulation buffer (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid 20 mM, NaCl 133 mM, KCl 5 mM, CaCl2 7 mM, MgCl2 3.5 mM, bovine serum albumin 1 mg/mL, pH 7.4) containing IL3 (2 ng/mL) and heparin (10 μL) (5000 UI/

mL; ROVI, Madrid, Spain) was added to the cell suspension in another well. As a positive control, a monoclonal anti-IgE receptor antibody (Bühlmann, Allschwil, Switzerland) was used at a final concentration of 1 μL/mL.

Flow cytometric analysis of surface markers was performed using a FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), equipped with DIVA software, from Beckton Dickinson. The initial cell gate on the histogram defined by forward and side-scatter was defined by a bit map around lymphocytes. The second gate was defined around cells showing high density anti-IgE-label, thus identifying them as basophils. At least 500 basophils were assessed in each assay. The other parameter analysed for the activated basophils identified was CD63.

A cut-off point of 15% activated basophils was considered as a positive test result, being at least two times the basal value.

Antigen-specific sulfidoleukotriene production (CAST)

The instructions of the assay's manufacturer (CAST-elisa, Bühlmann Laboratories. Allschwill, Switzerland) were followed for allergen-induced sulfidoleukotriene ELISA quantification, after cell incubation under the conditions described previously for BAT [32].

Statistical methods

Pearson's Chi-square method was used when variables fitted all required assumptions and Fisher's exact test when they did not, when analysing the association between qualitative variables. When quantitative variables were categorized, the association between them was analysed through the Cochran–Armitage trend test. The correlation between quantitative variables was measured by means of the Spearmen coefficient. Logistical regression and odds ratio estimates were used to evaluate risk of suffering from lower respiratory symptoms and the risk of pan-allergen sensitization associated with the different individual tested allergens.

Results

Population description

A total of 477 patients (around 50 patients selected by each centre) were included in the study. The sample characteristics are included in Table 1. The mean age of patients was 25.6 ± 12.4 years and the mean time of evolution of allergic disease was 10.0 ± 9.0 years. The diagnosis of the severity of the rhinitis and asthma was performed according to ARIA [33] and GINA [34].

IgE reactivity to single mite allergens

Figures 1a and b show the results of sensitization (IgE-prevalence) per centre to the single mite allergens tested. As expected, both group 1 (Der p 1 and Der f 1) and group 2 (Der p 2 and Der f 2) were the most prevalent allergens and about 90% of patients were sensitized to group 1 and/or group 2. The IgE-prevalence to *D. pteronyssinus* allergens was slightly higher than to *D. farinae* allergens, except in the Mediterranean centres. The frequency of patients sensitized to group 2 (72–96% to Der p 2 and 68–94% to Der f 2) was higher than the frequency of patients sensitized to group 1 (54–84% to Der p 1 and 49–80% to Der f 1) in all centres. About 6% of patients were sensitized to group 1 but not to group 2 versus 20% of patients sensitized to group 2 but not to group 1. *L. destructor* sensitization, as determined by its major allergen Lep d 2, appeared to be more prevalent in Atlantic regions than in Mediterranean regions, whereas *B. tropicalis* (determined by its allergen Blo t 5) was only relevant in one area (Gran Canaria, Canary Islands). The prevalence of IgE-sensitization to tropomyosin (Der p 10/Pen a 1) was very low in all samples analysed. Figure 2 shows the mean sIgE value per region for the group 1 and 2 allergens. No significant differences were detected between *D. pteronyssinus* and *D. farinae* allergens, but the level of IgE to *D. pteronyssinus* allergens was slightly higher than the IgE level to *D. farinae* allergens (except in the Mediterranean area). The mean sIgE for group 2 is about 1 log unit higher than for group 1 in all centres.

Figure 3 shows the correlation between the sIgE values for *Dermatophagoides* group 1 allergens and for *Dermatophagoides/Lepidoglyphus* group 2 allergens. A significant correlation was found between Der p 1 and Der f 1 ($r = 0.842$, $P < 0.0001$). Around 16% of patients had a species-specific group 1 response, considering the standard cut-off of 0.35 kU/L. In the case of Der p 2 and Der f 2 an even stronger correlation was found ($r = 0.945$, $P < 0.0001$) with only 2% of patients having a species-specific response. There was a lack of correlation between Lep d 2 and Der p 2. The correlation between *Dermatophagoides* group 1 and 2 was statistically significant but less relevant (Der p 1/Der p 2: $r = 0.618$, $P < 0.0001$, Der f 1/Der f 2: $r = 0.518$, $P < 0.0001$), and with a non-zero y-intercept.

BAT/CAST results with mite group 1 and group 2 allergens

BAT data were obtained from the patients from Vizcaya and Navarra ($n = 100$). No significant basophil activation was obtained in seven patients with anti-human IgE receptor (positive control) therefore only 93 samples (93%) were considered. About 29% of patients had a

Table 1. Characteristic of patients (demographic and clinical data are expressed in percentages. SPT and sIgE are expressed as median)

	Age (%)		Gender (%)		Clinical diagnosis (%)			SPT (∅ > 3 mm)				sIgE (kU/L)	
Centre	≤ 14 years	≥ 15 years	Female	Male	Asthma	Asthma & Rhinitis	Rhinitis	Dpt	Dfar	Lep	Blo	Dpt	Dfar
Coruña	24	76	54	46	8	58	34	8.75	8	9.5	6	53.2	22.7
Lugo	100		30.6	69.4	10	76	14	11	11	7.5	6.3	84.9	50.2
Asturias		100	47.2	52.8	2.8	41.7	55.5	10	9	7.5	6	19.9	13.2
Vizcaya	2.1	97.9	54.2	45.8	0	18.8	81.2	8	7.5	7	4.5	74.9	50.7
Navarra	32.7	67.3	38	62	4	48	44	7.3	7	5.5	5	38.2	28.1
Barcelona		100	67.3	32.7	2	38	60	9	9.5	5	6	36.7	21.9
Valencia	4	96	60	40	0	42	58	8	8.5	5	5.8	14.8	20.8
Huelva	28	72	44	56	8	44	46	6.5	6	7	4.5	29.5	10.6
Gran Canaria	16	84	62.5	37.5	0	86	14	7	7.5	5	6.5	37.0	29.0
Tenerife	4.8	95.2	57.1	42.9	0	78	22	9	8.5	5	6.5	25.4	16.7
Global	21.9	88.1	48.5	51.5	3.6	58.7	36.7	8.5	8.3	6.4	5.7	41.5	26.4

Dpt, *Dermatophagoides pteronyssinusl*; Dfar, *Dermatophagoides farinae*; Lep, *Lepidoglyphus destructor*; Blo, *Blomia tropiocalis*; SPT, Skin prick test.

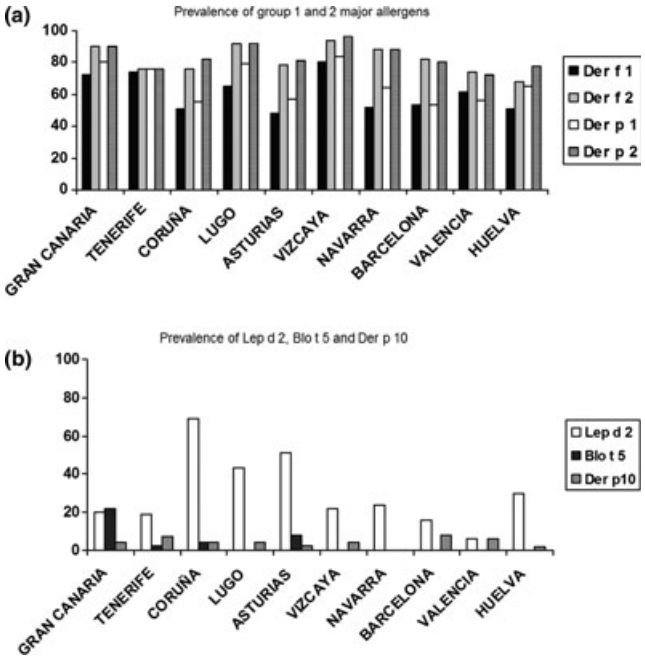

Fig. 1. IgE prevalence of allergens per centre: (a) Major *Dermatophagoides* allergens. (b) Lep d 2, Blo t 5 and tropomyosin (Der p 10).

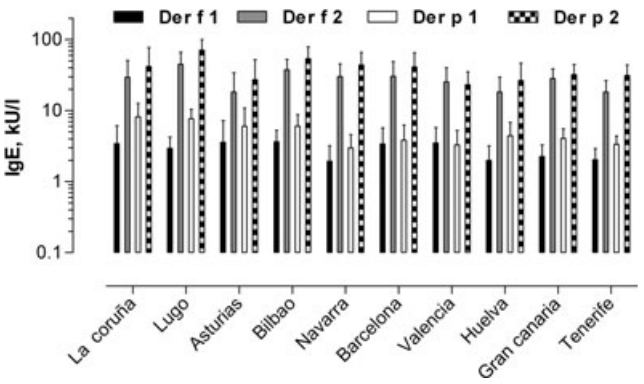

Fig. 2. Average (geometric means) of sIgE to Der p 1, Der p 2, Der f 1 and Der f 2 allergens.

negative BAT to group 1 allergens and about 12% to group 2 allergens. A total of 59 patients (63%) had a positive BAT with Der p 1, Der f 1, Der p 2 and Der f 2; 15 (16%) had a positive BAT with Der p 2 and Der f 2; 7 (7.5%) had a positive BAT with Der p 1, Der p 2 and Der f 2; 2 (2%) had a positive TAB with Der f 1, Der p 2 and Der f 2; 1 (1%) had a positive TAB with Der p 1, Der f 1 and Der p 2; 1 has a positive TAB with Der p 1 and Der f 1; 1 has a positive BAT with Der p 1; and 7 patients (7.5%) had a negative BAT result with the 4 allergens tested. Concordance between the BAT and sIgE results was obtained in 86% of cases.

As the BAT was obtained for each blood sample using several allergen concentrations, the basophil intensity of sensitization to the different allergens was also compared, by determining the allergen concentration required to reach 50% of the activation obtained, with anti-human IgE receptor. The results are shown in Fig. 4. The basophil intensity of sensitization to group 2 allergens is about 10 times higher than to group 1 allergens.

Determination of sulphidoleukotrienes release after allergen activation produced equivalent results to those obtained by BAT in terms of both prevalence and intensity of sensitization. The percentage of patients not sensitized to group 1 and group 2 allergens was about 27% and 13%, respectively, and the concentration of group 2 allergens required to obtain the 50% of the maximum sulfidoleukotriene release was about 10 times lower than that for group 1 allergens.

House dust sample allergen results

The level of Der p 1, Der f 1, Der 2 and Lep d 2 was determined in house dust samples obtained from the patients homes. The median exposure values (μg

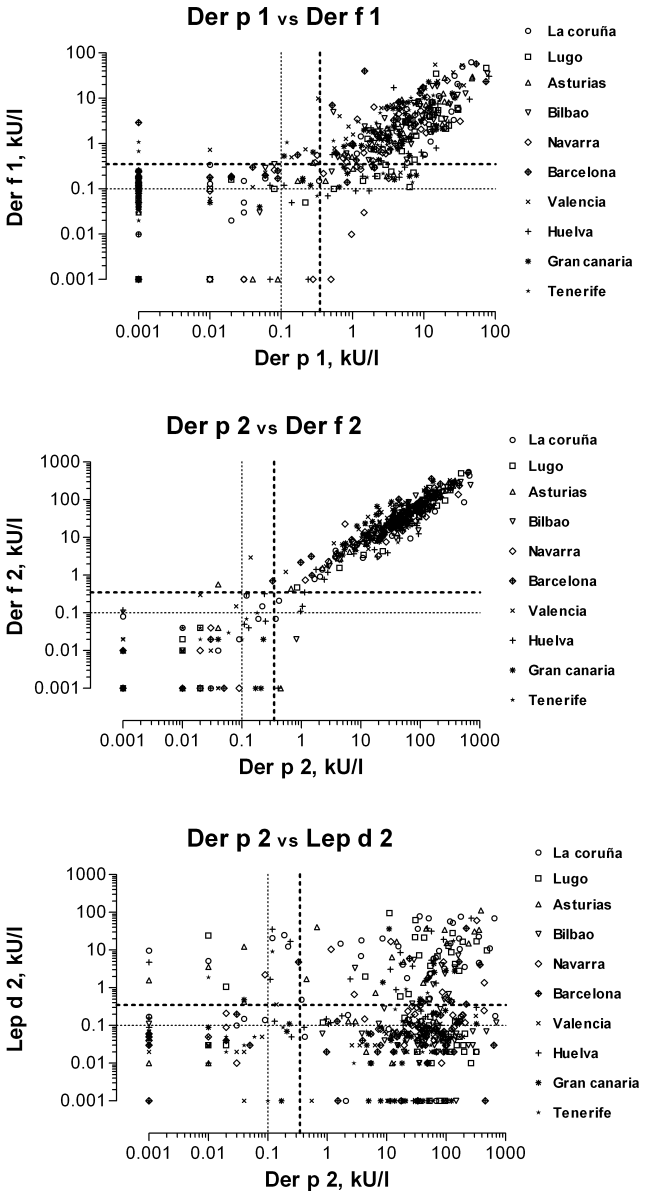

Fig. 3. Correlation between the sIgE values for *Dermatophagoides* group 1 allergens and *Dermatophagoides/Lepidoglyphus* group 2 allergens.

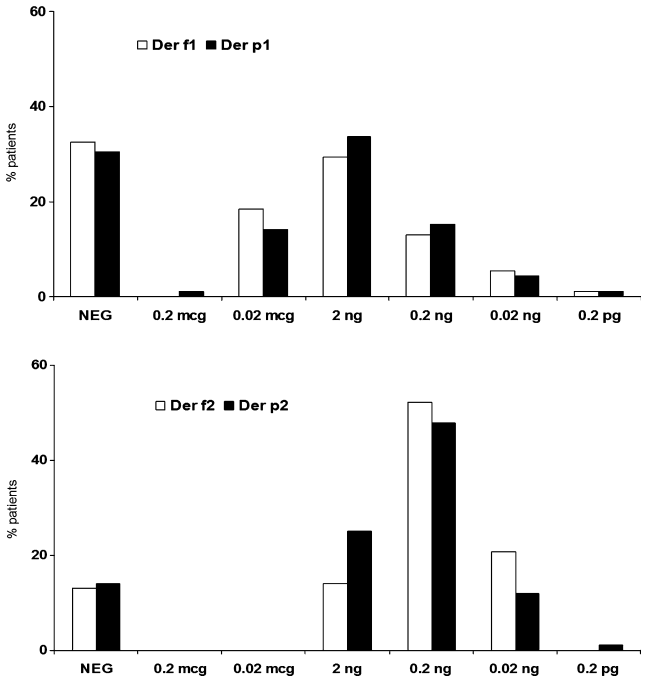

Fig. 4. Basophil intensity of sensitization to *Dermatophagoides* groups 1 and 2 allergens determined by BAT.

allergen/g of dust) were 2.7 for Der p 1, 1.0 for Der f 1, 2.3 for Der 2 and 0.5 for Lep d 2. A significant correlation ($r = 0.72$, $P < 0.0001$) was obtained for the level of Der p1 and Der 2. In all centres, except in the Mediterranean area, the level and prevalence in house dust samples of Der p 1 allergen was higher than Der f 1.

No clear correlation was obtained between the level of sIgE and the amount of allergen in house dust. However, in the case of Der p 1, the Cochran–Armitage test for trend was clearly significant ($P < 0.0006$). Patients with house dust Der p 1 levels below 0.6 μg/g show a tendency to have a negative IgE to Der p 1, whereas the situation is the opposite in the case of house dust for Der p 1 levels higher than 10 μg/g. In the case of Lep d 2, the areas with the highest IgE-prevalence also had the highest house dust Lep d 2-prevalence.

Relation between SPT and sIgE to major allergens

The SPT to *Dermatophagoides pteronyssinus* or *farinae* whole extract was exclusively associated with sIgE to group 1 and 2 allergens from these mites. However, sIgE to Lep d 2 is statistically associated with SPT results, not only to *L. destructor* but also (Table 2) to other storage mites tested in the study (*Glyciphagus, Tyrophagus, Blomia, Acarus siro*). For the SPT to *L. destructor*, the average sensitivity, determined in patients with positive IgE to Lep d 2 from the centres with *L. destructor* exposure, was 78.3%. The average specificity, determined in patients with negative IgE to Lep d 2 from centres with low *L. destructor* exposure, was 80.3%.

Relation of sensitization profile to sIgE with age and severity of allergic disease

The patients selected for studying the sIgE-age association came from three areas, located in Asturias and Galicia, with a similar profile as regards the level of exposure and type of mites, with the number of children and adults (72 and 64, respectively) being very similar. Sensitization to Der p 1 was more frequent in children than in adults (81.3% vs. 50.0%, $P = 0.0001$,

Table 2. Association between cutaneous test and sIgE

	SPT								
	Positive				Negative				
	Positive		Negative		Positive		Negative		
	N	%	N	%	N	%	N	%	*P*-value
	IgE Lep d 2				IgE Lep d 2				
D. pteronyssinus	122	87.1	275	83.8	18	12.9	53	16.2	0.3620
D. farinae	110	79.7	270	83.3	28	20.3	54	16. 7	0.3509
G. domesticus	72	60.5	74	32.2	47	39.5	156	67.8	<.0001
L. destructor	88	66.2	78	30.6	45	33.8	177	69.4	<.0001
T. putrescentiae	70	54.3	78	31.6	59	45.7	169	68.4	<.0001
B. tropicalis	60	50.0	73	30.0	60	50.0	170	69.9	0.0002
Acarus siro	56	47.1	70	27.0	63	52.9	189	72.9	0.0001
Shrimp	5	9.3	11	17.2	49	90.7	53	82.8	0.2101
	IgE Der p 1				IgE Der p 1				
D. pteronyssinus	280	88.6	117	76.9	36	11.4	35	23.0	0.001
D. farinae	268	86.7	112	73.2	41	13.3	41	26.8	0.003
	IgE Der p 10				IgE Der p 10				
Shrimp	3	42.9	13	11.7	4	57.1	98	88.3	0.0196

chi-square test), whereas sensitization to Lep d 2 was more frequent in adults than in children (63.9% vs. 45.3%, $P = 0.0297$, chi-square test). There was no statistical association between the age of patient and their sensitization to group 2 (Der p 2 or Der f 2), being the prevalence very high in both age groups.

The presence of asthma (with or without rhinitis) was also statistically associated with a higher IgE/IgG4 ratio for Der p 2 ($P = 0.037$) but not for Der p 1 ($P = 0.0883$).

Discussion

In 2004, Pittner et al. [35] defined a diagnosis algorithm for mite allergy based on CRD. Our aim was to further improve this algorithm by incorporating various new molecules that have proved to be relevant sensitization markers of minor mite species [18, 19]. Furthermore, taking advantage of the sIgE method characteristics, especially as regards differential affinity recognition [23], we aimed to evaluate differential sensitization between major mite species. To achieve our goals, we had the advantage of being able to perform the current study in a diverse mite territory.

Specific IgE responses to Der 2 allergens were higher to those elicited to Der 1 in terms of both prevalence and intensity, despite the average environmental level of group 1 and group 2 were similar. sIgE titres for both allergens differed by 1 log unit and were confirmed in independent experiments by BAT capacity and comparing our data with CAP in patients monosensitized to either Der 1 or Der 2 allergens (data not shown). These data support the relevant role of Der 2 in mite allergy. Interestingly a significant percentage of patients were found to be monosensitized to Der 2 allergens, whereas only a few of them react to Der 1 allergen only.

Der 1 prevalence was significantly higher in the paediatric sample, thereby suggesting a possible role of Der 1 in the onset of allergen sensitization, perhaps mediated by proteolytic activity specific signalling of by direct epithelia damage [4, 5]. The higher prevalence of sIgE to Der 2 in the adult population suggested a later sensitization or a loss of sIgE to Der 1. The ability of Der 2 to directly bind to TLR4[R] might be a logical explanation of this observation. The evolution of mite sensitization from group 1 to group 2 was further supported by the fact that Lep 2 prevalence is higher in the adult sample.

A lower ratio of specific IgE/IgG4 to Der 2 was significantly associated with a decrease in respiratory symptoms. This finding suggests that some patients are able to initiate the development of natural tolerance to mites and that they can be identified by a simple serological test. These patients might better respond to immunotherapeutic interventions and is complementary to that reported by Hales and cols. [24], where patients with severe asthma displayed lower sIgG4 titres.

With regards to differential major mite allergen recognition, sIgE to Der p 2 and Der f 2 was almost fully cross-reactive, whereas differential sIgE response to Der p 1/Der f 1 was observed depending on the

predominant mite specie, *D. pteronyssinus* and *D. farinae* in the Mediterranean region, and *D. pteronyssinus* in the Atlantic region. Altogether, the previous data indicate that a careful balance of major allergens, which implies a right balance of mite bodies and faecal particles, and mite species must be taken into account in the design of mite vaccination products.

Most of the patients in North Western regions of Spain were co-sensitized to both major and minor mites, whereas minor mite sensitization was scarce in the Mediterranean region. Lep d 2 and Der 2 sIgEs did not correlate, thus indicating that minor mite-induced allergy was independent of that caused by major mites, as reported previously [18].

The patients were selected on basis of a careful anamnesis and positive skin prick test or specific IgE to whole mite extracts. Classification of the patients into sensitization clusters (Table 3) shows that the molecular panel tested is able to diagnose more than 90% of patients.

Up to 78% of patients in the Mediterranean region are monosensitized to major mites, around 60% of patients in some Atlantic regions are sensitized to *Dermatophagoides* and *Lepidoglyphus*, and almost 10% are monosensitized to the minor mite.

Skin prick testing for different mites species was found to be non-specific, thus meaning that it is not possible to discriminate between minor mites because of the high cross-reactivity between them. The use of complex minor mite allergen skin prick test panels is therefore not recommended, it may lead to wrong therapeutic interventions. A previous publication [18] supports the leading role of *Lepidoglyphus destructor* in allergy to minor mites. In agreement with this previous report, Lep d 2 sIgE prevalence was found to correlate with Lep d 2 detected in dust samples. Therefore, as the Lep d 2 quantification method is specie specific, this correlation supports the proposal that *Lepidoglyphus* should be considered to be the leading minor mite in the region studied.

Tropomyosin prevalence was low (5–10%) and was not associated with either a particular mite species or mite allergen exposure levels. Moreover, a few patients were found to be exclusively sensitized to tropomyosin. Finally, sIgE to tropomyosin was only associated with positive SPT to shrimp. Taken together, these findings support the proposal that tropomyosin sensitization indicates a true food allergy independent of mite respiratory disease and that an SPT made of shrimp can be used to identify these kinds of patient. The low general prevalence to tropomyosin must, however, be noted, as should the fact that we have strong indications that multiple responses by SPT to major and minor mites are mostly owing to real and differential sensitization to such mites and not to tropomyosin interference.

Blomia allergy was only relevant in specific areas of the Canary Islands. Although Blo t 5 is a unique *Blomia* allergen, some patients with Lep d 2 sensitivity were found to react to *Blomia* by SPT, thus suggesting the existence of a Lep d 2-like allergens that might be a major allergen of *Blomia*, but have not yet been characterized.

Several practical clinical consequences arise from this study. Firstly, a panel of *Dermatophagoides, Lepidoglyphus* and shrimp is sufficient to identify and classify patients as mite sensitized by SPT. Furthermore, together with Der 1, Der 2 and Der p 10/Pen a 1 allergens, Lep d 2 must form part of any CRD approach to mite allergy. A new diagnostic algorithm for classifying mite allergic patients is proposed in Fig. 5, combining the minimum SPT and component-resolved diagnosis panel.

A second one is the relevant role of Group 2 which should be considered in the design of new vaccines. Historically Der 1 dose alone in mite allergen vaccines has been used to describe allergen dose, but our data

Table 3. Mite-sensitization clusters in the regions studied

	La Coruña	Lugo	Asturias	Vizcaya	Navarra	Gran Canaria	Tenerife	Barcelona	Valencia	Huelva
D	22.5	59.3	40.5	74.0	74.0	62.0	73.8	70.0	84.0	61.2
D + L	59.1	36.7	35.1	22.0	20.0	14.0	16.7	16.0	2.0	24.5
L	8.2	2.0	8.1		2.0		2.4			6.1
B + D			2.8			16.0				
B + D + L	4.1		5.4			4.0				
B + L						2.0				
Negative	6.1	2.0	8.1	4.0	4.0	2.0	7.1	14.0	14.0	8.2
Total (%)	100	100	100	100	100	100	100	100	100	100
N	49	49	37	50	50	50	42	50	50	49

D, *Dermatophagoides* (pteronyssinus and/or farinae); L, *Lepidoglyphus* destructor; B, *Blomia tropicalis*.

The figures include the percentage of patients sensitized to each mite or group of mites. For example, in the first column, 22.5% of patients were exclusively sensitized to *Dermatophagoides*, 59.2% to *Dermatophagoides* and *Lepidoglyphus*, 8.2% to *Lepidoglyphus* exclusively, 4.1% to *Blomia, Lepidoglyphus* and *Dermatophagoides* being 6.1% negative to any major mite allergen (but some of these patients were sensitized to tropomyosin).

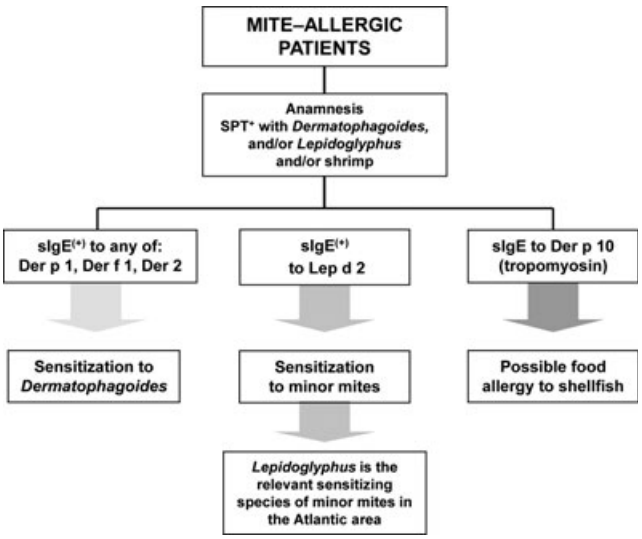

Fig. 5. Diagnostic algorithm for mite allergic patients. D, *Dermatophagoides*; L, *Lepidoglyphus*; B, *Blomia*; T, tropomyosin.

suggest that knowing Der 2 dose of allergen vaccines is equally important for analyzing the dose-effect in mite-related clinical trials and that a careful balance of major allergens must be assessed. Finally, there might be simple serological determinations that might allow identifying patients with lower disease severity. Probably there is a natural progression of the disease, suggested by the differential adult and paediatric allergen prevalence supporting that early intervention strategies are advisable.

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