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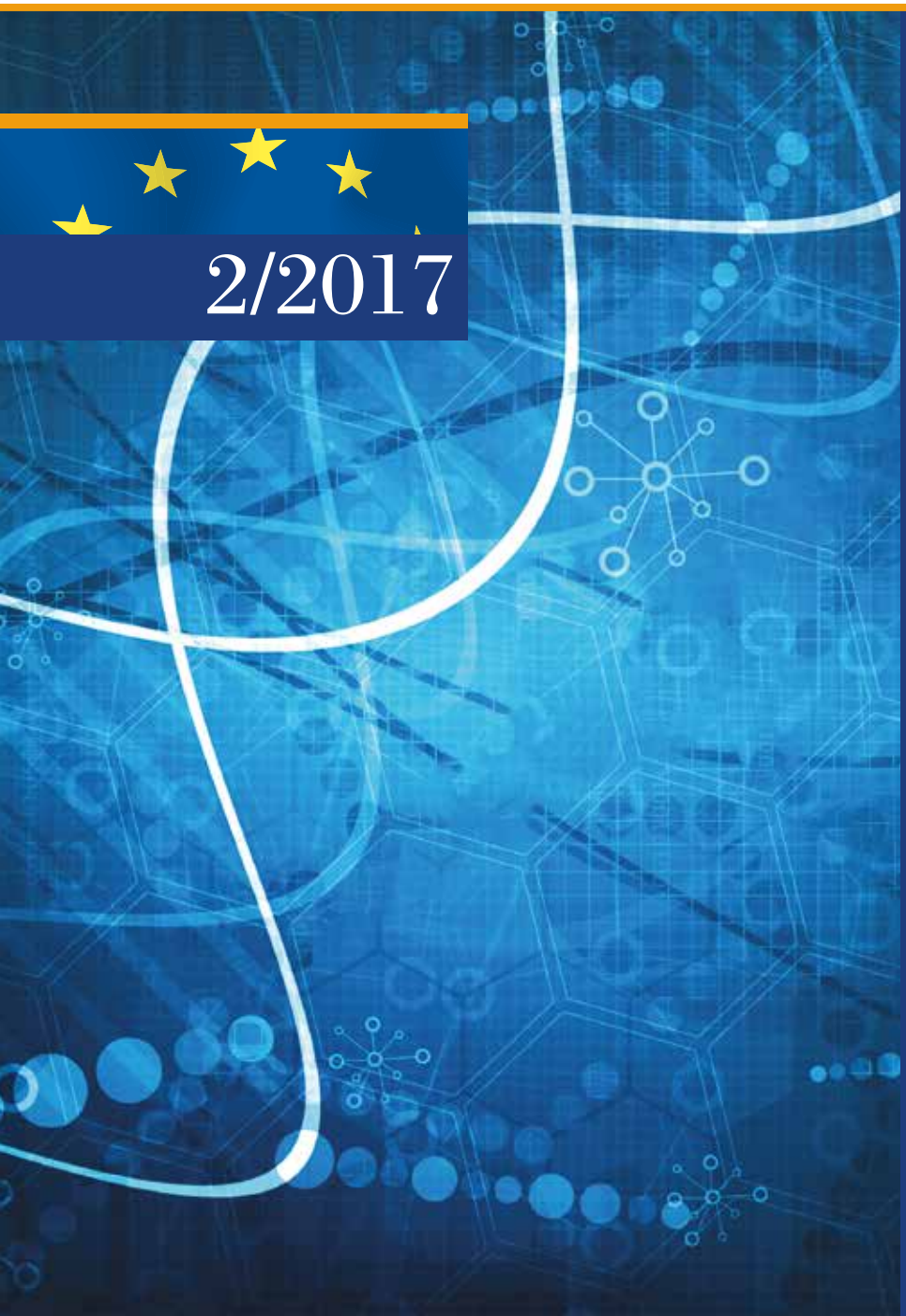

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M.T. Ventura[1], R. Rodriguez-Perez[2], M.L. Caballero[3], M. Garcia-Alonso[2], L. Antonicelli[4], R. Asero[5]

IgE, IgG_1 and IgG_4 response to specific allergens in sensitized subjects showing different clinical reactivity to *Anisakis simplex*

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Key words

Anisakis simplex, Food allergy; Immune response; Allergens; Immunoglobulins

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Summary

Background. Anisakis simplex *hypersensitive subjects may be sensitized without clinical allergy, or experience acute symptoms or chronic urticaria induced by raw fish. We studied whether the 3 subgroups differ in IgE, IgG_1 or IgG_4 reactivity to specific* Anisakis simplex *allergens.* ***Methods.*** *28* Anisakis simplex*-hypersensitive adults, 11 with acute symptoms, 9 with chronic urticaria, and 8 sensitized were studied. IgE, IgG_1 and IgG_4 to rAni s 1, 5, 9 and 10 were sought by ELISA. IgE and IgG_4 to nAni s 4 were determined by WB.* ***Results.*** *IgE to Ani s 1, 4, 5, 9, and 10 were found in 8, 3, 2, 5, and 9 sera, respectively. Nine sera did not react to any allergen. IgG_1 to Ani s 1, 5, 9, and 10 were detected in 5, 16, 14, and 4 sera, respectively. Four sera did not react to any of the 4 allergens. IgG_4 to Ani s 1, 4, 5, 9, and 10 were detected in 10, 0, 2, 6 and 1 sera, respectively. Fifteen subjects did not react to any of the 5 allergens. On ELISA sensitized subjects showed lower IgE and IgG_1 levels than patients. IgG_4 levels were highest in the sensitized group. The prevalence of IgE, IgG_1 or IgG_4 reactivity to any of the studied allergens did not differ between the 3 subgroups.* ***Conclusion.*** *The clinical expression of* Anisakis simplex *sensitization does not seem to depend on IgE reactivity to a specific allergen of the parasite, nor on the presence of IgG antibodies possibly related with blocking activity.*

Introduction

Anisakiasis is a human parasitic infection of the gastrointestinal tract caused by the consumption of raw or undercooked seafood containing live larvae of the nematode *Anisakis simplex.* Many countries require all types of fish with potential risk intended for raw consumption to be previously frozen to kill parasites. However, despite the mandatory rules about the correct processing procedures required to abate the nematode present in fish for human consumption, anisakiasis occurs all over the world, due to the ingestion of the live larval stage 3 parasite found in raw or undercooked marine fish that has not undergone preventive freezing (1). Most cases are reported from Japan, but also some areas of Spain (2-4) and Italy (5) can be considered endemic for the disease. It is generally accepted that sensitization to *Anisakis simplex* follows the ingestion of raw, traditional marinated, or poorly cooked fish (6).

Three different subsets of *Anisakis simplex* hypersensitive patients can be encountered in the endemic areas: (a) patients with a history of acute systemic symptoms such as urticaria, angioedema, asthma and/or anaphylaxis upon ingestion of raw fish; (b) patients with chronic urticaria who eat marinated fish almost on a daily basis that improve after a fish-free diet (7,8); and (c) sensitized subjects who do not report any symptom despite the

frequent consumption of raw fish-based dishes. Whether such different clinical response depends on the level and type of immune response to specific *Anisakis simplex* allergens is unclear. IgG_4 antibodies have been reported to exert an IgE-blocking activity, particularly after allergen specific immunotherapy, as a result of the activities of regulatory T cells, and have been associated with the induction of immunological tolerance upon prolonged exposure to antigen (9,10), whereas IgG_1 response is the one found in normal individuals after exposure to non-self-proteins. In the present work sera from individuals belonging to the three subsets of *Anisakis simplex* hypersensitive patients have been studied, in order to detect possible differences in their humoral immune response to different *Anisakis simplex* allergens.

Patients and methods

Patients

Sera from 28 adult subjects, all sensitized to *Anisakis simplex*, as shown by unequivocally positive SPT with commercial extract of the parasite (ALK-Abellò, Madrid, Spain; 2 mg protein/ml) were used in this study. The age of study patients ranged between 18 and 77 years (mean 55; median 59), with no difference between the three subgroups. Twenty out of 28 were females; no difference in gender distribution between the subgroups was observed. Eleven patients had a history of acute symptoms (defined as the appearance of urticaria / angioedema or anaphylaxis) shortly after the ingestion or raw or marinated fish. Nine had a history of chronic urticaria (defined as the occurrence of wheals with or without angioedema on most days of the week for more than 6 weeks) that had completely and persistently resolved following the exclusion of raw or marinated fish from their diet. Eight subjects were sensitized to *Anisakis simplex* but were completely symptomless, even following the ingestion of raw or marinated fish. In order to include only patients primarily sensitized to *Anisakis simplex* and to avoid the interference of cross-reacting allergens of invertebrates, such as tropomyosin, patients positive on skin prick testing (SPT) with shrimp were excluded. Patients sensitized to fish were excluded as well. Sera from chronic urticaria patients were collected before the start of the fish-free diet. All patients underwent SPT with a large series of commercial extracts of both airborne (Pollens [grass, ragweed, mugwort, pellitory, plantain, birch, olive and cypress], moulds, mites, and cat and dog dander) and food allergens. In the acute group, 2 patients scored positive for mites, 1 for cypress pollen, and 2 for food (peanuts in both cases). In the chronic group, 3 patients scored positive for mites, 2 for pollens (both pellitory), and 3 for food (celery, maize, and egg, respectively). In the sensitized group, 3 patients scored positive for mites, 2 for pollens (both cypress and olive, one for pellitory), and 2 for food (peach and maize, respectively).

In vitro tests

Detection of specific IgE to whole *Anisakis simplex* extract
All study participants underwent the detection of IgE specific for whole *Anisakis simplex* extract by ImmunoCAP (ThermoFisher-Phadia, Uppsala, Sweden); results were expressed in kUA/L, and values > 0.35 kUA/L were considered positive.

Anisakis simplex allergens
rAni s 1 was cloned in the pPIC9 vector in the yeast *Pichia pastoris* (both kindly supplied by Dr Gabriel Salcedo from ETSIA, Madrid) and purified from the culture medium by a two steps chromatography procedure. The dialysed extracellular medium of the culture was first fractionated by cation-exchange. Subsequently, the fraction containing rAni s 1 was dialysed, freeze-dried and then separated by RP-HPLC (11). rAni s 5, rAni s 9 were obtained following the same work flow; briefly, they were cloned into the plasmid expression vector pET46 EK/LIC (Novagen, Merck KGaA, Darmstadt, Germany) that produces the protein with a histidine N-terminal tag. The resultant plasmids were transferred into E. coli BL21 Star (DE3) One Shot (Invitrogen, Carlsbad, CA, USA). Recombinant proteins were intracellularly expressed as a 6-His tagged proteins and then purified from bacterial lysate. They were purified from the soluble protein fraction using HisTrap HPcolumn (GE Health-

Table 1 *- Nature of the allergens produced and used in the study.*

Allergen tested	Source	Purified	Reference
rAni s 1	recombinant in Pichia pastoris	Cation exchange, Reversed phase HPLC	11
rAni s 5	recombinant in Escherichia coli (BL21)	Ni- Affinity cromatography	27
rAni s 9	recombinant in Escherichia coli (BL21)	Ni- Affinity cromatography	12
rAni s 10	recombinant in Escherichia coli (KRX)	Ni- Affinity cromatography	13
nAni s4	natural, extracted from A. simplex L3	Ethanol fractionation extract enriched	28

care Bio-Sciences AB) following manufacturer's specifications (12,13). Regarding rAni s 10, it was cloned into the plasmid expression vector pET46 EK/LIC, as rAni s 9 and rAni s10, but expressed in *Escherichia coli* E. coli KRX (Promega, Madison WI, USA) following manufacturer's specifications. Then it was purified from the soluble protein fraction using HisTrap HPcolumn, as for rAni 5 and rAni s9 (14) (**table 1**).

To obtain natural Ani s 4 (nAni s 4), *Anisakis simplex* L3s (1.5 g) extracted from hake muscle (*Merlucius merluccius*) were ground in PBS (5 ml) with a mortar and pestle and the mixture was centrifuged at 4,000 g for 15 min. The supernatant obtained was mixed with 5 ml of ethanol, incubated for 30 min on ice and centrifuged in the same conditions. Again, the supernatant was mixed with ethanol to increase the ethanol concentration to 66%, incubated for 30 min on ice and centrifuged. The supernatant was discarded and the pellet was re-suspended in 500 µl of dH_2O (15) (**table 1**).

Detection of specific IgE, IgG_1, and IgG_4 to rAni s 1, rAni s 5, rAni s 9 and rAni s 10 by ELISA

Polystirene 96-well plates (Costar 3590, Corning, NY, USA) were coated for 2 h at 37 ºC with 100 ml of recombinant allergen at 10 mg/ml in carbonate buffer pH 9.6. The coated wells were blocked with 1% BSA in PBS for 30 min at 37 ºC, and then incubated overnight at room temperature with 100 ml of patient's serum (1/4 dilution in 1% BSA, 0.05% Tween-20, in PBS). After washing with 0.05% Tween-20 in PBS, wells were incubated for 1 h at room temperature with peroxidase-labelled anti human IgE (SouthernBiotech, Birmingham, AL, USA; 1/2000 dilution), or peroxidase-labelled anti human IgG_1 (SouthernBiotech; 1/8000 dilution), or peroxidase-labelled anti human IgG_4 (SouthernBiotech; 1/1000 dilution). Plates were washed again and, and then developed with 100 ml of TMB-turbo ELISA substrate (Thermo Scientific, Rockford, IL, USA). The reaction was stopped after 30 min with 10 ml of 2N H_2SO_4, and the optical density (OD) was measured at 450 nm. Assays were performed in duplicate. Blocking buffer was used as negative control. For data analysis, sera were considered positive if OD was more than arithmetic mean plus 3SD (standard deviation), calculated from the results of 25 control sera from non-atopic individuals tested for IgE, IgG_1 and IgG_4. The cut-off values for IgE, IgG_1 and IgG_4 determinations were 0.14 for rAni s 1 and 0.12 for rAni s 5, rAni s 9 and rAni s 10.

Detection of IgE and IgG_4 to nAni s 4

Specific IgE and IgG_4 against nAni s 4 were determined by western-blotting with an extract enriched in this allergen. Extract containing nAni s 4 (5 µg), was fractionated by 16% SDS-PAGE and then separated proteins were transferred onto nitrocellulose membranes by diffusion (Nitro-Pure supported, 0.45 µm, GE Osmonics Labstore, Minnetonka, MN, USA) to perform a western-blotting.

The membranes were washed and blocked with 3% Nonidet NP-40 (Amresco, Solon, OH, USA) in PBS for 30 minutes. After that, they were incubated overnight with individual sera from the patients (1:10 dilution). Specific IgE or specific IgG_4 detection was carried out with monoclonal anti-human-IgE (1:1000) (Ingenasa, Madrid, Spain) or monoclonal anti-human IgG_4 (1:000) (Sigma-Aldrich, St Louis, MO, USA), respectively. Subsequently, membranes were incubated with alkaline phosphatase-labelled goat anti-mouse antibody (1:2500) (Sigma-Aldrich, St Louis, MO, USA). Finally, the signal was visualized with the alkaline phosphatase 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/4-nitroblue tetrazolium (NBT) system (Amresco) for 30 min.

Statistics

Qualitative variables have been summarized with their relative frequency. Pearson $\chi 2$ test was applied for the bivariate comparison of proportions. Likelihood test of independence was used when the application conditions of the $\chi 2$ test were not met. The Kruskal-Wallis nonparametric test was used for the comparison of quantitative variables in independent samples.

Probability values < 0.05 were considered statistically significant. Data analysis was performed using the software SPSS 15.0 (SPSS Inc., Chicago, IL).

Results

Results are summarized in **table 2**. All study participants but 2 (both with a history of chronic urticaria) showed circulating IgE to whole *Anisakis simplex* extract tested by ImmunoCAP; one of those two negative subjects reacted to Ani s 10 on ELISA. The three subgroups of patients studied (sensitized, acute and chronic) did not show any statistically significant difference in terms of *Anisakis*-specific IgE levels tested by ImmunoCAP. The two subjects showing the highest levels of IgE to *Anisakis simplex* extract belonged to the subgroup showing symptomless sensitization.

IgE specific for rAni s 1, nAni s 4, rAni s 5, rAni s 9, and rAni s 10 were found in 8, 3, 2, 5, and 9 sera, respectively. Nine sera did not show IgE reactivity to any of the studied allergen components.

IgG_1 to rAni s 1, rAni s 5, rAni s 9, and rAni s 10 were detected in sera from 5, 16, 14, and 4 subjects, respectively. Sera from 4 subjects did not show IgG_1 reactivity to any of the 4 allergens. IgG_1 to rAni s 5 and rAni s 9 were frequently detected in the absence of the corresponding IgE response.

IgG_4 to rAni s 1, nAni s 4, rAni s 5, rAni s 9, and rAni s 10 were detected in 10, 0, 2, 6, and 1 sera respectively. Sera from 15 subjects did not show IgG_4 reactivity to any of the 5 allergens

Table 2 - *Detection of IgE, IgG_1, and IgG_4 against different Anisakis simplex allergen proteins. Positive values are highlighted in bold.*

					rAni s 1			nAni s 4		rAni s 5			rAni s 9			rAni s 10		
	Code	**sex**	**age**	**CAP**	**IgE**	**IgG1**	**IgG4**	**IgE**	**IgG4**	**IgE**	**IgG1**	**IgG4**	**IgE**	**IgG1**	**IgG4**	**IgE**	**IgG1**	**IgG4**
	A1	M	77	75.00	**1.13**	**0.88**	**0.93**	**+**	-	**0.43**	**0.14**	0.03	**1.15**	**1.60**	**0.86**	0.04	0.02	0.03
	A2	F	65	3.45	0.00	0.13	0.13	-	-	0.02	0.03	0.01	0.01	0.04	0.04	0.00	0.07	0.01
	A3	F	37	16.0	0.02	0.06	0.02	-	-	0.06	0.09	0.00	**0.91**	**1.65**	**0.74**	0.03	0.06	0.01
	A4	F	49	3.2	0.01	0.11	0.10	-	-	0.05	**0.44**	0.00	0.01	0.10	0.06	0.00	0.09	0.02
	A5	F	55	1.01	0.02	0.05	0.02	-	-	0.01	0.08	0.07	0.00	**0.41**	0.08	0.04	0.02	0.05
Acute	A6	F	75	1.12	0.02	0.13	**0.72**	-	-	0.00	**0.67**	0.05	0.00	0.12	0.05	0.06	0.02	0.01
	A7	F	60	2.65	0.01	0.08	0.10	-	-	0.01	**0.42**	0.04	0.00	0.08	0.10	**0.14**	0.06	0.10
	A8	F	54	15.60	**1.08**	0.13	**1.08**	-	-	**0.27**	**0.74**	**0.91**	0.00	**0.19**	0.05	0.03	0.03	0.03
	A9	F	24	5.60	0.00	**0.27**	0.03	-	-	0.01	**1.34**	0.02	0.00	0.10	0.02	**0.17**	0.06	0.00
	A10	M	18	0.36	0.00	0.08	0.05	-	-	0.00	**0.21**	0.02	0.00	0.10	0.04	**0.20**	0.05	0.00
	A11	M	26	0.36	0.00	**0.17**	0.04	-	-	0.01	**0.34**	0.02	0.00	**0.51**	0.04	0.00	0.09	0.01
	S1	F	75	40.80	0.00	0.05	0.13	**+**	-	0.01	**0.23**	0.01	0.00	**0.28**	0.02	0.02	0.06	0.08
	S2	F	72	0.36	0.00	0.01	0.05	-	-	0.00	0.10	0.03	0.00	**0.21**	0.03	0.02	0.03	0.02
	S3	F	62	19.40	**0.95**	0.06	**1.07**	**+**	-	0.01	0.05	0.02	**0.31**	**0.45**	**0.61**	0.00	**0.16**	0.02
Sensitized	S4	M	49	17.30	0.00	0.09	**1.12**	-	-	0.09	**0.33**	**0.68**	0.00	0.05	0.07	**0.15**	**0.14**	**0.37**
	S5	M	22	3.30	0.00	**0.19**	0.02	-	-	0.00	0.04	0.06	0.00	0.04	0.03	**0.14**	0.08	0.10
	S6	M	62	93.00	0.05	0.08	0.07	-	-	0.00	0.06	0.08	**0.33**	**1.32**	**0.84**	0.08	0.04	0.01
	S7	F	53	90.00	**0.55**	**0.30**	**0.86**	-	-	0.00	0.11	0.05	0.00	0.03	**0.25**	0.09	0.08	0.10
	S8	F	71	14.00	0.00	0.03	**0.17**	-	-	0.01	0.10	0.07	0.00	0.03	0.08	**0.13**	0.01	0.10
	C1	F	72	13.14	**0.95**	0.09	**1.04**	-	-	0.00	**0.36**	**0.13**	0.00	0.05	0.10	0.09	0.08	0.05
	C2	F	76	2.96	**0.20**	0.07	**1.08**	-	-	0.00	0.08	0.02	0.00	0.02	0.02	**0.17**	0.03	0.01
	C3	F	30	0.00	0.01	0.07	0.04	-	-	0.00	0.04	0.02	0.00	**0.58**	0.04	**0.17**	0.04	0.00
Chronic	C4	F	44	0.73	0.01	0.08	0.05	-	-	0.01	**0.50**	0.03	0.00	**0.71**	0.06	**0.14**	0.02	0.00
	C5	F	67	8.74	**0.57**	0.12	0.11	-	-	0.01	**0.78**	0.01	**0.22**	**0.39**	0.10	0.07	**0.37**	0.01
	C6	F	65	0.75	0.01	0.08	0.09	-	-	0.01	**0.36**	0.07	0.00	**0.70**	**0.26**	0.07	0.04	0.08
	C7	F	75	3.41	0.08	0.08	**0.15**	-	-	0.01	**0.39**	0.05	0.00	0.03	0.03	0.04	0.03	0.02
	C8	M	57	40.50	**0.77**	0.05	0.02	-	-	0.02	**0.19**	0.11	0.00	0.09	0.04	0.08	**0.16**	0.03
	C9	M	43	0.00	0.01	0.11	0.00	-	-	0.00	0.07	0.00	0.00	**0.18**	0.01	0.00	0.01	0.01

A1-11: patients with a history of acute reactions. S1-8: Subjects sensitized to *Anisakis simplex* without any clinical symptom. C1-9: patients with *Anisakis simplex*-induced chronic urticaria.
CAP values to whole *Anisakis simplex* extract are expressed as kUA/L (n.v. < 0.35). The cut-off values were 0.14 for rAni s 1 and 0.12 for rAni s 5, rAni s 9 and rAni s 10.

studied. An IgG_4 response in the absence of the corresponding IgE response was found in 7 instances (4 rAni s 1, 1 rAni s 5, 2 rAni s 9) (**table 3**).

We compared the specific IgE values tested by ImmunoCAP and by ELISA with recombinant allergens. The main differences were observed in the sensitized group, since the CAP values were much higher (and dispersed) than in the acute and chronic patients. On the contrary, when we analysed the specific IgE levels considering at least the highest positive of the four allergens tested by ELISA, we found that specific IgE levels were lower among the sensitized patients than among the acute or the chronic patients (**figure 1**, panels A and B). Nonetheless , a correlation coefficient after Pearson calculated for ImmunoCAP values and the sum of the ELISA results for each individual sample showed a weak, albeit statistically significant, correlation ($r= 0.4$; $p< 0.05$).

We analyzed the specific IgE levels considering at least one positive of the four allergens tested by ELISA, and we found that specific IgE levels were lower among the sensitized patients than among the acute or the chronic patients. A similar pattern was observed for IgG_1, with the highest levels observed among the acute patients. Regarding IgG_4 levels, the tendency was the opposite, being the highest levels present in the sensitized group and the wider range of values among the chronic patients (Figure 1, panels B to D). However these differences did not reach the significance level.

The 3 groups did not show any statistically significant difference in the prevalence of either IgE or IgG reactivity to any of the studied allergens.

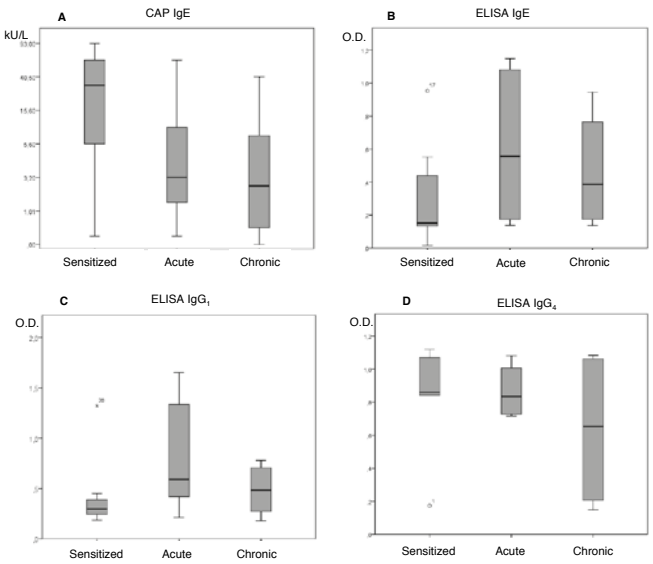
Table 3 *- Allergen detection comparison between Anisakis simplex-induced acute or chronic pathology and Anisakis simplex sensitized individuals.*

		Sensitized n = 8	Acute n = 11	Chronic n = 9	p value
rAni s 1	IgE	2 (25%)	2 (18%)	4 (44%)	0.425
	IgG_1	2 (25%)	3 (27%)	0 (0%)	0.111
	IgG_4	4 (50%)	3 (27%)	3 (33%)	0.589
nAni s 4	IgE	2 (25%)	1 (9%)	0 (0%)	0.186
	IgG_4	0 (0%)	0 (0%)	0 (0%)	--
rAni s 5	IgE	0 (0%)	2 (18%)	0 (0%)	0.137
	IgG_1	2 (25%)	8 (72%)	6 (66%)	0.086
	IgG_4	1 (12%)	1 (89%)	1 (11%)	0.971
rAni s 9	IgE	2 (25%)	2 (18%)	1 (11%)	0.752
	IgG_1	4 (50%)	5 (45%)	5 (55%)	0.904
	IgG_4	3 (37%)	2 (18%)	1 (11%)	0.406
rAni s 10	IgE	3 (37%)	3 (27%)	3 (33%)	0.891
	IgG_1	2 (25%)	0 (0%)	2 (22%)	0.109
	IgG_4	1 (12%)	0 (0%)	0 (0%)	0.273
At least one positive	IgE	7 (87%)	6 (54%)	6 (66%)	0.284
	IgG_1	7 (87%)	10 (90%)	8 (88%)	0.971
	IgG_4	5 (62%)	4 (36%)	4 (44%)	0.521

Discussion

IgE plays a fundamental role in allergic diseases since it is responsible of allergic symptoms, whereas IgG_4 immunoglobulin has been associated with the suppression of IgE-dependent immediate hypersensitivity reactions which occur, for instance,

Figure 1 *- Comparison between Ig levels to recombinant Anisakis simplex allergens in sensitized, acute and chronic patients. A: sIgE CAP assay, B: sIgE ELISA with recombinant Anisakis simplex allergens assay, C: $sIgG_1$ ELISA with recombinant Anisakis simplex allergens assay and D: $sIgG_4$ ELISA with recombinant Anisakis simplex allergens assay. The ELISA value chosen for each patient corresponds to the highest positive of the allergens tested (rAni s 1, rAni s 5, rAni s 9 or rAni s 10).*



during helminthic infections. In this sense, it has been proposed that the regulation of isotype switching is an important checkpoint in the development of clinical allergy versus tolerance, and that IgG_4 may act by blocking IgE access to the antigen (16). According to this theory, our initial hypothesis was that both patients with chronic urticaria and sensitized but asymptomatic subjects might show a higher prevalence of specific IgG_4 response and lower levels of specific IgE than patients with a history of acute symptoms following the ingestion of raw fish. In this sense, Daschner et al. (8) found that patients with both chronic urticaria and *Anisakis simplex* sensitization show a clear positive response to a two-month fish-free diet particularly if they show specific IgG_4 levels to the parasite. The same group detected a higher IgG avidity among patients with gastro-allergic anisakiasis (GAA, corresponding to our "acute" subset) than in patients with *Anisakis simplex* sensitization-associated chronic urticaria, whereas the former subgroup showed a statistically non-significant trend to a lower IgE avidity (17). Further, they found higher IgG_4 and IgE levels to *Anisakis simplex* crude extract and higher IgG_4 levels to both Ani s 1 and Ani s 7 in patients with GAA (18). In the present study we found that among the three subsets studied (sensitized, acute, or chronic)

sensitized subjects showed the lowest specific IgE levels tested by ELISA and the highest levels of specific IgG_4; chronic patients showed the widest range of values. We observed a similar pattern when we compared the specific IgE and specific IgG_1 responses, with the highest levels observed among acute patients (**figure 1**) although the tendency observed did not reach the significance level, maybe due the low number of patients studied.

Another interesting finding was the different distribution of specific IgE determined by ImmunoCAP or by ELISA among the three subsets of patients. On ImmunoCAP, the highest values were found among sensitized, asymptomatic subjects, which may seem illogical from an immunological point of view. A possible explanation might be that ImmunoCAP reflects the presence of IgE to *Anisakis simplex* proteins that, although able to fix IgE, are less active in triggering allergic symptoms; another explanation might be that, since the assay is performed with a whole extract of the parasite, it reflects sensitizations that are not primarily directed to *Anisakis simplex*. In this sense, cross-reactions with crustaceans (19), mites (20) and other nematodes like Ascaris (21) have been reported. Another fact to take into account is the detection of carbohydrate-type determinants of parasite antigens; in a group of sensitized Spanish patients, sIgE CAP values dropped by 27.44% on average (range 0-48.62%) after CAP inhibition assay with bromelin as source of CCDs (22). This means that CCDs may interfere with accuracy when detecting clinically relevant sIgE, and to make things even worse, the magnitude of the interference may differ from one serum to another. This could explain the more dispersed data of sIgE CAP when compared with ELISA performed with recombinant allergens. Furthermore, the higher specificity for the diagnosis of rAni s 1, rAni s 5, rAni s 9 and rAni s 10 compared with ImmunoCAP has been demonstrated in a Spanish population (22), where this set of allergens diagnoses up to 99.99% of the allergic population. In other populations some other allergens might be included in order to cover the whole population for diagnostic purposes. These observations are in keeping with a very recent study of respiratory allergy showing that the levels of IgE specific for extracts were higher than the levels of IgE specific for the corresponding components (23)

Although a statistically significant correlation between ImmunoCAP and the sum of ELISA results was found, the ELISA with recombinant allergens seems more specific than the ImmunoCAP. This is consistent with the results of our previous study (24) in which sensitized subjects showed lower IgE levels than patients. If specific IgE were really low among sensitized (clinically asymptomatic) subjects, one might speculate that the IgE response to *Anisakis simplex* is normal or even protective, as has been suggested for honeybee venom (25). The repeated exposition to the parasite would lead to an increase in both IgE and IgG_4 without a clear predominance between them; this might not be necessarily the expression of a severe disease, but rather of a long-term interaction between host and parasite with the occurrence of an unstable balance (26,27) whose clinical expression could be represented by patients with *Anisakis simplex* sensitization-associated chronic urticaria. The altered intestinal permeability and its reversibility following a fish-free diet (28,29) might be the expression of this instable equilibrium in the intestinal tract. In this scenario, the more severe allergic / anaphylactic reactions might reflect an interaction between the allergen and specific IgE that occurs in patients in whom conditions triggering anaphylaxis coexist (30); obviously, such conditions could not be predicted on the basis of either specific IgE (31) or IgG_4 (32) levels. In fact, in the three subsets studied here specific IgE and IgG_4 were largely overlapping and do not allow a clear distinction between the different clinical conditions. This scenario does not seem to fit with the protective role of specific IgG_4 levels such as that observed in beekeepers (25); however, in that model the dose of allergen as well as the timing of stings are known, whereas both these conditions are largely unknown in the long-term interaction between the immune system and *Anisakis simplex*.

Of course, we cannot exclude at all that the results of the present study are due to the low numbers of subjects studied within each group; however, the two highest levels of *Anisakis simplex* IgE were detected in asymptomatic but sensitized subjects. In conclusion, these data suggest that the clinical expression of *Anisakis simplex* sensitization depends on factors other than the mere presence of specific IgE. Further, in keeping with the model of food allergies (33), our observations suggest that IgG do not seem to exert any "blocking" or preventive effect in subjects sensitized to the parasite.

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Static and elevated pollen traps do not provide an accurate assessment of personal pollen exposure

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Pollen, exposure, expology, environment, allergy

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Summary
Background. *Volumetric pollen traps are commonly used to assess pollen exposure. These traps are well suited for estimating the regional mean airborne pollen concentration but are likely not to provide an accurate index of personal exposure. In this study, we tested the hypothesis that hair sampling may provide different pollen counts from those from pollen traps, especially when the pollen exposure is diverse.* ***Methods.*** *We compared pollen counts in hair washes to counts provided by stationary volumetric and gravimetric pollen traps in 2 different settings: urban with volunteers living in short distance from one another and from the static trap and suburban in which volunteers live in a scattered environment, quite far from the static trap.* ***Results.*** *Pollen counts in hair washes are in full agreement with trap counts for uniform pollen exposure. In contrast, for diverse pollen exposure, .individual pollen counts in hair washes vary strongly in quantity and taxa composition between individuals and dates. These results demonstrate that the pollen counts method (hair washes vs. stationary pollen traps) may lead to different absolute and relative contributions of taxa to the total pollen count.* ***Conclusions.*** *In a geographic area with a high diversity of environmental exposure to pollen, static pollen traps, in contrast to hair washes, do not provide a reliable estimate of this higher diversity.*

Introduction

Seasonal allergic rhinitis is a widespread disease that occurs around the world. According to International Study of Asthma and Allergies in Childhood (ISAAC), the 12-month prevalence of this disease ranges from 10 to 20% in developed countries (1). At a population level, exposure to types of pollen is measured by volumetric (2) pollen static traps. However, this technique is not accurate for personal measurements, because elevated static trap only provides a rough estimate of exposure over a large geographical area (3). Static traps are usually set up on the tops of buildings and are unlikely to sample the heaviest pollens, which remain close to the ground level. To address this issue, static gravimetric traps (4) have been developed that provide a better sampling of local pollens if they are located close to the ground. However, these traps are stationary and cannot account for the diversity of environmental exposure encountered by a given individual across time and locations. Because hair is a natural filter that is close to the respiratory tract, it has the potential to provide a much better estimate of personal exposure. Hair has already been used in the field of forensic medicine (5) to investigate murders and generate hypotheses about the places a victim had visited. It has also been used in other fields of environmental toxicology to assess cumulative exposure (6). In this study, we compared the results of the "Pollen Counts in Hair Washes" (PCHW) method to the counts provided by stationary volumetric and gravimetric pollen traps in 2 different settings (a large city and a suburban environment). The objective of the study was to know whether a static pollen trap can provide a reliable estimate of personal pollen exposure. The absolute quantities of pollen counts and the relative quantities of pollen from specific taxa were analyzed.

Methods

Study group

Twenty volunteers working in a hospital setting were selected from 2 urban and suburban areas with contrasting environmental conditions in southern France. They were 13 females and 7 males, 15 to 58 years old with a mean age of 33 +/- 9 years. They were outdoors for a mean (S.D.) weekly duration of 17.7 +/- 4.2 hours. The first set comprised 11 people living in the same district of the city of Marseille (these individuals were identified M1 to M11). Due to the restricted area (the max distance from their homes to the static traps was less than 1 km), we hypothesized that these individuals experienced similar individual pollen exposures. Furthermore, they all worked at the University hospital, where the Hirst trap is located. They all lived less than 1 km away from the hospital. A second set of 9 people was chosen from the Valence area (identified as V1 to V9) and who lived up to 20 km from the city center; these individuals lived in diverse environmental conditions, and they most likely experienced different pollen exposures (**figure 1**).
All volunteers were asked to wash their hair once a week on 5 occasions, from 4th February to 9th March 2008 in Marseille and from 11th February to 9th March 2008 in Valence. These time periods correspond to the pollination of *Cupressaceae,* the major pollinating taxon in southern France, which accounts for most of the total annual pollen count.

Pollen Counts

Pollen counts in traps
At both sites, atmospheric pollen was counted using the volumetric spore-trap Hirst method (Hirst, 1952). These 2 volumetric spore-traps (located on the tops of buildings) were the 2 permanent pollen traps used in Marseille and Valence, respectively, by the National Aerobiology Monitoring Network (RNSA). Results were expressed as average of 7 daily slides. Another type of static trap was used, namely the Cour trap. The Cour trap is not usually used as a gravimetric trap, but it was used in this study on a horizontal holder, without any suction. This type of receptor is frequently used in agronomy and pollen allergy studies (Katelaria et al; 2004), allowing the sedimentation of pollen through horizontal filter composed of hydrophilic gauze with a 400 cm^2 sampling surface. Cour traps were installed for the study in Marseille and 10 km away from Valence (**figure 1**). They were situated 1.2 meters above the ground in order to trap nearby pollens. Results were expressed as the total pollen caught along a week.

Pollen counts in hair washes
Volunteers were instructed how to wash their hair. It was mentioned that they should employ a sustained and careful massaging of the scalp. Furthermore, hair characteristics were collected and taken into account in the data analysis
Participants were asked not to wash their hair in between the experimental washings. One 1.5-liter bottle is sufficient to wash hair (2 liters for long hair). A plastic mineral water bottle or a well-cleaned soft drink bottle can be used, and tepid water is preferable. A personal preliminary observation has shown that cold tap water does not contain any pollen. The hair was wetted with 0.5 liters before shampooing, and 1 liter was used to completely rinse the hair after shampooing. The same brand of shampoo was used for all hair washes. The entire 1.5 liters was collected in a basin which had been previously cleaned using clear water, poured into the bottle and transported as soon as possible to the laboratory. If immediate transport was impossible, a thymol crystal was added to prevent contamination or microbial growth. The liquid was centrifuged, and the pellet was treated under heat overnight in a 20% potash solution, then for 10 minutes in 30% HCl, then acetolyzed and stained by fuchsine, and the pellet was suspended again in 80% glycerin and analyzed with a microscope on a microscope slide at 400X or 630 X magnification. Pollens were not altered by hair washing as there was no significant pollen fragmentation following the above-mentioned treatment (7).
The results were expressed as the number of pollen grains/m^3 (Burkard trap), the number of pollen grains/cm^2 (Cour trap), or the number of pollen (grains/wash) for each taxon and overall, averaged over the week, as is commonly done by aerobiologic networks. Hair washing occurred once a week, at the same time of day in the late afternoon. Personal hair characteristics of each participant were recorded: length (long vs. short), texture (curly vs. stiff), and thickness (thick vs. fine)

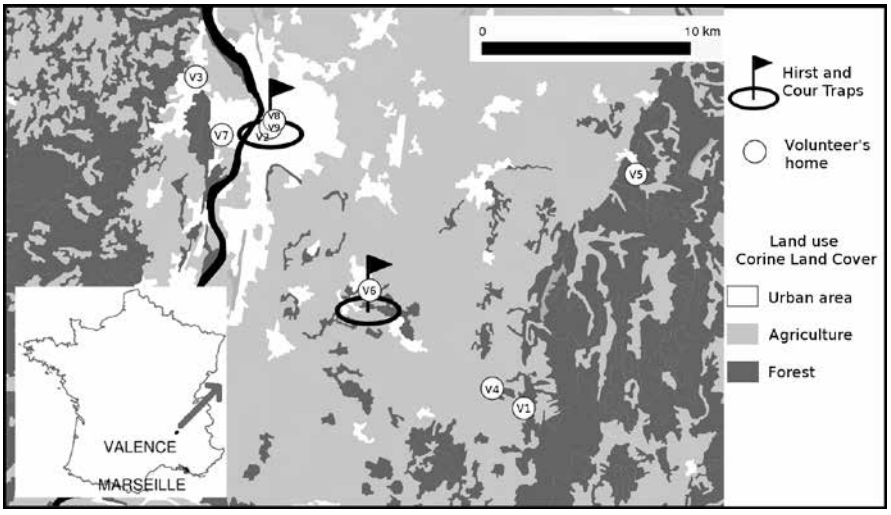
Diaries

Volunteers were asked to record in a diary whether they moved to places where they could get exposed to unusual vegetation

Statistical analysis

Pollen data correspond to averages for an entire week. Week 1 to week 5 refer to an experimental period, not to a calendar date.
At both sites, Pearson correlation coefficients were computed between the PCHW mean values from all volunteers and the pollen counts from the Hirst or Cour traps to estimate the concordance among pollen counts over the whole study period. The distribution of the variables was normal, allowing to use such a statistics.
The variability of PCHW within and among volunteers was graphically illustrated using boxplots.
The effect of "individual" (volunteer) and "date" (week within pollination period) on the prediction of pollen counts on hair washes (PCHW) from the Hirst pollen trap counts was analyzed using the following generalized linear model (8):

Figure 1 *- Location of volunteers' houses and pollen receptors involved in the assessment of individual pollen exposure in Valence vicinity using the "Pollen Counts in Hair Washes" (PCHW) method. Volunteers from the Valence sample experimented heterogeneous environmental conditions, as indicated by land use data from Corine Land Cover. In Marseille all volunteers lived in a restricted urban area.*



Log (PCHW) ~ log (Burkard) + date + individual (I)

Due to overdispersion, the quasi-Poisson distribution was used as the link function.

We estimated the effect of the "pollen count method" (Hirst, Cour or PCHW) and the effect of personal pollen exposure on the relative contribution of *Cupressaceae* to the total pollen counts using the following generalized linear model:

*Cupressaceae Rate ~ trap-type * date + individual (in PCHW)* (II)

Due to overdispersion, we selected the quasi-binomial distribution as the link function.

In Valence, 2 other taxa (*Alnus* and *Fraxinus*) also produce substantial amounts of pollen grains. Here, the pollen frequency patterns were analyzed using a multinomial logit model. The pollen counts from *Cupressaceae*, *Alnus*, *Fraxinus* and the cumulative pollen counts from all other taxa were taken as the 4 categorical variables and analyzed by the following model with *Cupressaceae* as the baseline:

$$\log\left(\frac{\pi_t}{\pi_c}\right) = \beta_{0t} + \text{trap} - \text{type} + \text{date} \quad \text{(III)}$$

where π_t is the probability of collecting pollen from taxon t (excluding *Cupressaceae*) and π_c is the probability of collecting pollen from *Cupressaceae.*

The significance of the effects of trap-type and date were tested using submodels. The differences in the models' deviances were compared to the chi-square distribution.

All statistical analyses were performed using the R statistical software (R Foundation for Statistical Computing, Vienna, 7) with the glm and multinom function from the net package (9). Analysis of mean pollen concentrations according to hair characteristics was performed using an analysis of variance (ANOVA), with 3 controlled variables.

Results

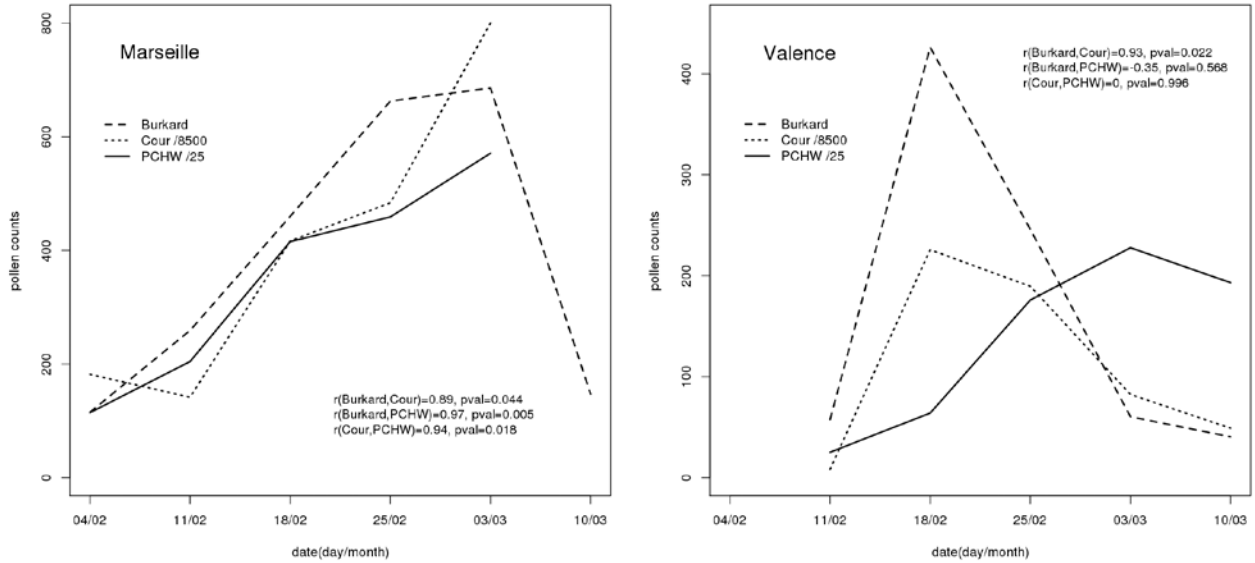
Correlation between pollen trap counts and PCHW

Pollen trap concentrations over the 5-week study period confirmed that this period corresponded to the time of *Cupressaceae* pollination (**figure 2**). In Marseille, *Cupressaceae* pollen counts from the 2 stationary pollen traps (Burkard and Cour) increased weekly from week 1 to week 5, while in Valence the maximum pollination occurred in weeks 3 and 4. At both sites, the 2 stationary traps were in good qualitative accordance with each other. The mean PCHW was in good accordance with the pollen trap assessments in Marseille, but the correlation between these methods was very poor in Valence. The *Cupressaceae* pollen peak derived from the mean PCHW values occurred two weeks after the pollen peaks from the 2 pollen traps.

The individual *Cupressaceae* PCHW exhibited uniform patterns among volunteers in Marseille, but very different trends were observed in Valence. Similar results were observed for the total pollen concentrations from all species. The boxplot of the individual PCHW illustrates the large diversity of pollen exposure amongst volunteers in Valence relative to Marseille (**figure 3**).

In Marseille, the curve displaying individual PCHW for total pollen counts was parallel to the one obtained by Hirst and

Figure 2 *- Cupressaceae pollination dynamics in February and March 2008 in Marseille and Valence, estimated through pollen counts from the volumetric Hirst pollen trap, the gravimetric Cour trap, and the new hair washes method (PCHW).*



Cour traps (**figure 2**) and did not vary significantly according to either the individual or the time (**table 1**).

Diary analysis

In Valence, the PCHW was not related to pollen trap counts, but instead varied significantly between individuals. This individual-based effect is mainly due to the very high pollen counts measured with two volunteers (V5 and V6) at weeks 5 and 6. More than one quarter of these high pollen counts were due to *Buxus* pollen, and the same analysis for *Cupressaceae* pollen counts led to a slightly reduced individual effect (p value = 0.03). Furthermore, hair washes performed by a volunteer who lived in an area heavily contaminated by *Ambrosia* found that those pollens represented

Figure 3 *- Variability of individual pollen counts, including all taxa, in hair (PCHW) in Marseille (white boxes) and Valence (grey boxes), over the entire study period.*

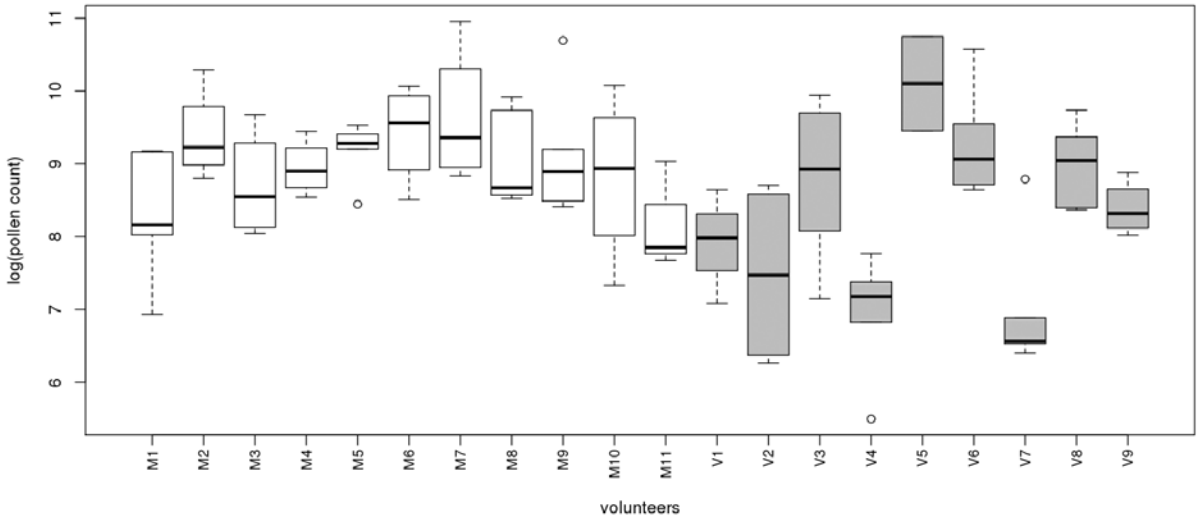


Table 1 *- Analysis of the PCHW values according to Hirst trap counts, individual (volunteer) and date of sampling in Marseille (a) and Valence (b) using the generalized linear model (I). Df: degree of freedom.*

(a)	Df	Deviance	Resid.Df	Resid.Dev	F	Pr(>F)
NULL			46	31892		
log(Hirst)	1	5334.4	45	26558	8.68	0,00607[2]
date	4	1725.0	41	24833	0.70	0.596
Individual	10	7550.2	31	17283	1,23	0.312
(b)						
NULL			38	14453		
log(Hirst)	1	271.8	37	14181	1.14	0.296
date	4	1989.1	33	12192	2.09	0.113
Individual	8	6076.2	25	6116	3.19	0.012[1]

[1]p value < 5%, [2]p value < 1%

43% of total, although the study was done in wintertime. A female participant who lived in the maquis had a level of *Buxus* pollens that was 25.9% of total. For an individual living in an area with many mimosa trees, those pollens represented 12.6% of total in her hair washes. Lastly, an individual who was exposed to lime trees had a 3% content of this pollen, whereas this percentage was less than 0.1% for the other washes from the same individual.

Contribution of Cupressaceae to the total pollen count

Analysis of the proportion of *Cupressaceae* pollen within the total counts confirmed the stronger pollination dynamics in Valence than in Marseille, where the proportion of *Cupressaceae* pollen was consistently high (90% versus 60% in Valence). The variability of the *Cupressaceae* pollen contribution among the count methods appears to be moderate, despite the fact that the proportion of *Cupressaceae* assessed by PCHW in Marseille was less than the proportion determined by the 2 stationary traps. In Valence, the Hirst trap that was installed in the city center produced a lower estimate than those of the Cour trap or the PCHW method.
According to the results obtained using model II, the *Cupressaceae* pollen rate was significantly affected by the date at which hair washes were performed, and the pollen count method at both sites (**table 2**). In Valence, the interaction between these two factors was also highly significant, and was mainly due to the high proportion of *Cupressaceae* pollen collected in week 2 (18 to 25 February) in the 2 stationary traps (79% and 75% in the Hirst and Cour traps, respectively, versus 27 to 47% in PCHW). Removing the week 2 counts produced results that were quite different: the *Cupressaceae* pollen contribution no longer depended on the trap type, and the interaction between the trap type and the date was reduced to a level similar to that observed in Marseille. At both sites, the variability of the *Cupressaceae* pollen contribution among volunteers was low.

Multi-species pollen pattern in Valence

According to the multinomial analysis (model III), the trap type and the date of pollen collection significantly affected the relative quantities of pollen from the different taxa.
When assessed over the 5-week period, the *Alnus* / *Cupressaceae* pollen ratio was 2.15 time higher for PCWH than for Hirst traps. Contrasting results were observed for the *Fraxinus* / *Cupressaceae* pollen ratio (0.39 times lower). Removing week 2 from the multinomial analysis decreased the PCWH *Fraxinus* / *Cupressaceae* pollen ratio to one quarter of the value derived from the Hirst trap. In contrast, for *Alnus* and the other taxa, the ratios obtained with the PCWH and Hirst traps were similar. PCHW indicated a higher proportion of *Alnus* pollen (and pollen from other species) for week 2 only, while the proportion of *Fraxinus* pollen indicated by the Hirst trap was always higher than those of PCWH.

Contribution of hair characteristics

The data in **table 3** demonstrates that, although mean PCHW values were higher in subjects with long and curly hair, the differences were not statistically significant.

Discussion

To the best of our knowledge, this is the first study comparing hair washes to static pollen traps as methods for pollen sampling.

Table 2 *- Analysis of the Cupressaceae pollen contribution to the total pollen counts in Marseille (a) and Valence (b1 and b2): effects of pollen count methods (Hirst, Cour and PCHW), individual and date. GLM (II).*

(a)	Df	Deviance	Resid. Df	Resid. Dev	F	Pr (> F)
NULL			56	7041.2		
trap-type	2	2244.48	54	4796.7	24.04	1.329e-07[3]
date	4	1904.20	50	2892.5	11.47	6.873e-06[3]
trap-type[1] date	8	807.50	42	2085.0	2.43	0.035[1]
individual (in PCHW)	10	894.38	32	1190.6	2.16	0.048[1]
(b1, all weeks)						
NULL			48	6840.5		
trap-type	2	848.17	46	5992.4	16.97	1.924e-05[3]
date	4	2146.90	42	3845.5	21.48	6.307e-08[3]
trap-type[1] date	8	2766.21	34	1079.2	13.84	1.334e-07[3]
individual (in PCHW)	8	403.89	26	675.4	2.02	0.084
(b2, without week from 18 to 25 February)						
NULL			38	3427.5		
trap-type	2	189.08	36	3238.4	3.22	0.063
date	3	1738.47	33	1500.0	19.76	4.595e-06[3]
trap-type[1] date	6	526.52	27	973.4	2.99	0.03[1]
individual (in PCHW)	8	403.45	19	570.0	1.72	0.1581

[1]p value < 5%, [2]p value < 1%, [3]p value < 0.1%

Table 3 *- Mean (+/- S.D.) PCWH according to hair characteristics.*

	Mean	Standard deviation	F	p
Long hair	41,932	45,671		
			1.19	0.19
Short hair	19,822	47,352		
Curly hair	41,993	56,959		
			3.79	0.06
Stiff hair	9,405	11,543		
Thick hair	28,711	15,155		
			0.05	0.95
Fine hair	29,867	14,811		

The limitation of the study was that it did not take into account all anthropogenic variables such as jobs and daily activities.

Pollens are not altered by hair washing. There are indeed other instruments dedicated to evaluate personal pollen exposure, such as nose filters or individual portable air samplers, although they are not convenient for the former and they only allow for a very limited sampling of airflow for the latter.

The data from Marseille showed that the pollen counts from hair washes were highly correlated to the pollen counts of the stationary traps. For individuals who were living in the same neighborhood and who were subjected to similar pollen exposures, the variability in the PCHW results could not be explained by inter-individual variability over the 5-week study period. Rather, this variability was dependent on the relationship between the time of sampling and the local concentration of pollens.

In Valence, participants were selected from a large area that was located 20 km from the city center. The pollen counts from the hair washes exhibited large qualitative and quantitative diversity and, over the 5-week study period, had a non-significant correlation with the pollen counts from the static traps located in the city center. There was a large intra-individual variation due to daily activities, especially walking in a forested area where huge quantities of *Buxus* and *Quercus* pollen can be deposited in the hair.

Thus in Marseille, both static traps and hair washes were representative of exposure whereas, in Valence, static traps did not provide a valid estimate of exposure because volunteers move

and travel back and forth between their homes and the city center. This is so because pollen counts from hair washes include on the one hand pollen of large size, like *Mimosaceae*, which cannot be collected by a static trap positioned on a roof, and on the other hand pollen released in places such as forests or open spaces. Such large-sized pollens are likely to contribute to clinical symptoms, provided that the patient is sensitized to them.

The number of pollens collected by hair washes can indeed be influenced by many uncontrolled factors. This is part of the inter-individual variation which was taken into account in the statistical analyses. Differences obtained under those circumstances were all the more significant as the inter-individual, as well as the intra-individual variations, tend to bias the association towards the null value.

To assess background pollen concentrations, we used Hirst traps and Cour traps. The French National aerobiological network, which provided us the pollen counts, exclusively uses Hirst traps. Rotorod is not used in France. Cour traps located at 1.2 m above the ground were dedicated to assessment of nearby exposure. A gravimetric trap does not by itself provide a better sampling of local pollens, but it does so if it is located close to the ground.

In Marseille, the proportion of *Cupressaceae* pollen in the total pollen count when determined by PCHW was significantly lower than the estimates based on the Hirst trap. This result suggests that a significant portion of the *Cupressaceae* pollen came from distant sources, such as domestic hedges in periurban areas or windbreaks in agricultural areas.

Analysis of the proportion of *Cupressaceae* pollen in Valence revealed unexpectedly high values for week 2 from the two stationary traps. It appears that the large amount of *Cupressaceae* pollen came from regions where this taxon was heavily pollinating. In the south of France, *Cupressaceae* are mostly found in the lower part of the Rhône Valley and in coastal plains. Pollination usually occurs from mid-February to late March and follows south-to-north dynamics that are controlled by daily temperatures. Valence is located in the northern part of the *Cupressaceae* area, and pollination in this area generally occurs 2 to 3 weeks later than in Marseille.

Analysis of the wind directions and intensities that occurred during the 5-week study period revealed that there were 3 days with southerly winds during week 2:2 of these days had moderately intense winds, and one of these days had strong winds. Thus, the high quantity of Cupressaceae pollen came from the southern part of the Rhône Valley and was transported there by the wind.

Interestingly, it should be noted that this peak was observed in the 2 stationary pollen traps but not in PCHW. Consequently, this long-distance pollen transportation was also likely responsible for the low correlation between the pollen counts from the 2 traps and those from PCHW. Additional experiments should be conducted to determine if pollen transported over long distances always contributes little to individual exposures.

According to our results, the pollen trap type may also affect the determination of the relative contributions of pollen from different taxa. In Valence, the differences observed in week 2 were due to the large *Cupressaceae* pollen contribution. The data from the 4 other weeks revealed that the Hirst trap indicated a higher proportion of *Fraxinus* pollen. This result must be confirmed by considering the location of the pollen sources relative to the locations of the pollen traps, as well as the morphological and aerodynamic characteristics of pollen from the different taxa.

In conclusion, our study provides evidence that in a geographical area with a high diversity of environmental exposure to pollen, pollen counts from static traps do not provide a valid estimate of the diversity of pollen exposure. Pollen counting in hair samples cannot currently be recommended as a suitable technique for assessing personal exposure because its precision and reproducibility has not yet been established. The results presented here are of clear interest to practicing allergist as they highlight the limitations of pollen counts provided by static pollen traps, thus underscoring the necessity to design new devices for assessing personal pollen exposure.

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Pediatric eosinophilic esophagitis in Portugal

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Key words

Eosinophilic esophagitis; allergy; children; aeroallergens; food allergens

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Summary

Eosinophilic esophagitis (EoE) is an increasingly frequent diagnosis in our clinical practice, mainly in pediatric age. Allergic responses to food and aeroallergens have been increasingly implicated in the etiology of this disease. We describe a retrospective data analysis of pediatric EoE patients followed in our Immunoallergology Department.

Of the 25 children (22 male, average 10.8 years), 88% had prior history of rhinoconjunctivitis, 76% asthma, 48% eczema and 36% food allergy. After evaluation, we identified in 76% and 92% of patients food and aeroallergen sensitization, respectively; 68% had simultaneously food and inhalant sensitization and 96% had at least one positive test to aeroallergens or food allergens. The first (44%) and the most frequent (56%) symptom was dysphagia. The time between symptoms onset and the EoE diagnosis averaged 18.6 ± 29.4 months. A multidisciplinary approach is needed for a correct evaluation, intervention and follow-up of these patients.

Introduction

Eosinophilic oesophagitis (EoE) is a chronic, immune-mediated inflammatory disease of the esophagus, characterized simultaneously by clinical symptoms related to oesophageal dysfunction and histological eosinophil-predominant inflammation and infiltration of the oesophageal epithelium (1).

First described in 1978 by Landres et al (2) and initially thought to be rare, it has taken a dramatic shift in prevalence over the past decades. Its rapidly increasing incidence has been shown in Europe and the USA and has recently been reported throughout the world (3-11).

Currently, studies in Western Countries point to a prevalence of 43 to 55 patients per 100,000 inhabitants, making it the second leading cause of chronic oesophagitis, after gastro-esophageal reflux disease (GERD) and the most frequent cause of dysphagia in young patients (12). In fact, between 5% and 15% of patients undergoing endoscopic evaluation for dysphagia will be diagnosed as having EoE and more than 50% of patients presenting in an emergency room with food impaction are now diagnosed with EoE (9,12-16).

In terms of pathophysiology, EoE is believed to be an immune-mediated allergic process of an unknown etiology. In fact, given the dramatic epidemiologic shift regarding EoE, allergen exposure has been postulated to play a role; a hypothesis compatible with it being a predominantly T helper-2 (Th2) lymphocyte driven disorder, with an increase in mucosal eosinophils, mast cells and basophils. Additionally, the basal cell proliferation found in these patients, with subepithelial remodeling and deposition of collagen, may contribute to the esophageal dysmotility found in EoE patients; explaining why the development of peristaltic dysfunction felt as dysphagia may occur so early in the disease course (17-29).

A growing number of papers show not only that patients with EoE have high frequencies of previous atopic eczema, food and

respiratory allergies, but also high frequencies of sensitization to food and aeroallergens (30-35).

Material and methods

We performed a retrospective analysis of patient's files who were evaluated at the Food Allergy Consultation in our Immunoallergology Department from February 2009 to March 2014, and who had received the EoE diagnosis according to the 2011 consensus guidelines (1). Additional inclusion criteria were: all patients were under 18 years of age, all were symptomatic at the initial evaluation at their first Food Allergy Consultation and all had performed diagnostic esophageal endoscopy with biopsy. A total of 25 patients fulfilled the criteria.

Patients were subsequently characterized according to: demographic data; prior history of allergic disease; clinical, laboratorial (peripheral eosinophilia and total IgE), endoscopic and histological features; sensitization profile (specific IgE, skin prick tests, prick-prick tests and patch tests) and evolution throughout their follow-up.

All patients were submitted to skin prick tests (SPT) with commercial extracts (Bial-Aristegui®, Bilbao, Spain) of inhalant (*Dermatophagoides pteronyssinus, Dermatophagoides farinae, Acarus siro, Blomia tropicalis, Euroglyphus maynei, Glycyphagus domesticus, Lepidoglyphus destructor, Tyrophagus putrescentiae,* dog fur, cat fur, feathers, *Aspergillus fumigatus, Alternaria alternata, Cladosporium herbarum, Cynodon dactylon, Gramineae* mix, *Phleum pratense*, *Olea europaea, Artemisia vulgaris, Platanus, Plantago*) and food allergens (*Pho d 2, Pru p 3*, shrimp, tropomyosin, cow's milk, α-*lactoalbumin*, β-*lactoalbumin*, *casein*, egg yolk, egg white, ovomucoid, ovalbumin, wheat, soy, peanut, walnut, hazelnut, almond, pistachio, cashew, porc, beef, turkey, hake, cod). Additional extracts for food allergens were used, when there was a high clinical suspicion (based on the individual clinical history) of an additional possible food sensitization.

Prick-prick tests were performed for all negative food allergens on the SPT battery, and to the foods to which there was a high clinical suspicion of being a possible culprit.

Specific IgE was performed for all the food allergens investigated on the SPT battery and the prick-prick tests.

Patch tests were performed using a whole food sample on the patients back. The patches were left in place for 48 hours, after then they were removed and an initial reading was taken one hour later. The final reading was taken a further 48 hours later.

All EoE patients performed a standard patch test battery with native food (milk, egg yolk and white (raw and cooked), wheat, corn, peanut, walnut, cashew, pistachio, hazelnut, pinion, cooked beef, cooked chicken, cooked porc, cooked turkey, cooked shrimp, cooked cod, soy). Also additional patch tests were performed for foods to which there was a high clinical suspicion based on each clinical history.

IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. was used for statistical analyses of the data: relative frequencies, averages, standard deviation, mediums and binomial tests were performed.

Results

25 patients with EoE diagnosis in pediatric age were included in this study. Their characteristics and correspondent statistical analyses appear in **table 1** and **figure 1**.

Figure 1 *- Comparison of EoE symptoms by patient age. Green bar: children up to 6 years, yellow bar: children 6-12 years, and blue bar: children 12-18 years of age.*

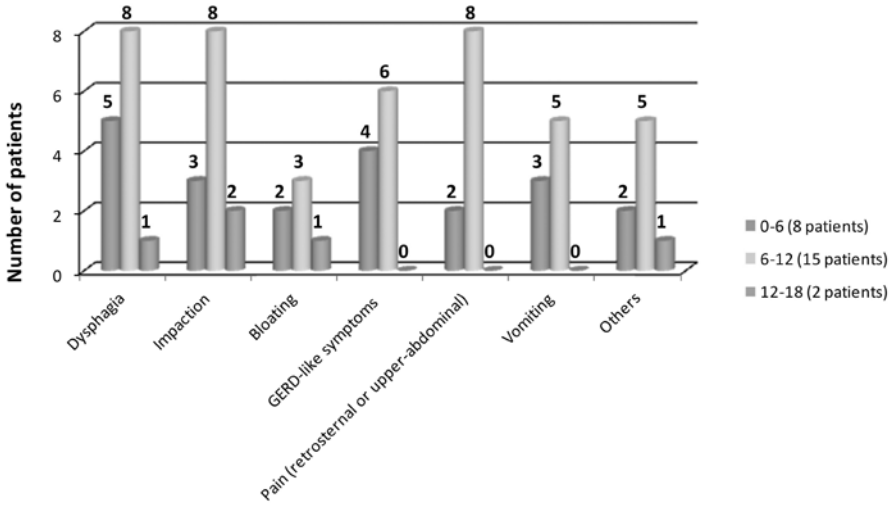

Table 1 *- Characterization of the pediatric population and statistical analyses of its binomial variables.*

Binomial variable		**Positive test in children**	
		Number of patients (%)	**p-value (binomial test)**
Male gender		22 (88)	**< 0.01**
Atopy prior to EoEd	Food allergy	9 (36)	NS
	Eczema	12 (48)	NS
	Asthma	19 (76)	**< 0.01**
	Rhinoconjunctivitis	22 (88)	**< 0.01**
	At least 1 of the 4 previous	24 (96)	**< 0.01**
First symptom	Dysphagia	11 (44)	NS
	GERD-like symptoms	4 (16)	NS
	Vomiting	3 (12)	NS
	Bloating	3 (12)	NS
	Impaction	2 (8)	NS
	Pain (retrosternal or upper-abdominal)	1 (4)	NS
Symptoms	Dysphagia	14 (56)	NS
	Impaction	13 (52)	NS
	GERD-like symptoms	10 (40)	NS
	Pain (retrosternal or upper-abdominal)	10 (40)	NS
	Vomiting	8 (32)	NS
	Others (anorexia, lower abdominal pain, chocking, food refusal)	8 (32)	NS
	Bloating	6 (24)	**< 0.01**
Positive Specific IgE	Mites	17 (68)	NS
	Food	16 (64)	NS
	Pollens	13 (52)	NS
	Animal epitheliums	7 (28)	**< 0.01**
	Fungi	3 (12)	**< 0.01**
Positive SPT and prick-prick test	Mites	19 (76)	**< 0.01**
	Food	16 (64)	NS
	Milk	8 (32)	NS
	Shellfish	7 (28)	**< 0.01**
	Egg	5 (20)	**< 0.01**
	Cereal	4 (16)	**< 0.01**
	Nuts	4 (16)	**< 0.01**
	Fish	3 (12)	**< 0.01**
	Meat	3 (12)	**< 0.01**
	Fruits	2 (8)	**< 0.01**
	Soy	0 (0)	**< 0.01**
	Pollens	14 (56)	NS
	Animal epitheliums	8 (32)	NS
	Fungi	6 (24)	**< 0.01**

Binomial variable		Positive test in children	
		Number of patients (%)	p-value (binomial test)
Positive patch test	Shellfish	12 (48)	NS
	Meat	6 (24)	**< 0.01**
	Egg	4 (16)	**< 0.01**
	Cereal	3 (12)	**< 0.01**
	Nuts	2 (8)	**< 0.01**
	Fish	2 (8)	**< 0.01**
	Cephalopods	1 (4)	**< 0.01**
	Soy	0 (0)	**< 0.01**
Endoscopy findings	Normal	1 (4)	**< 0.01**
	Furrows	20 (80)	**< 0.01**
	White plaques	10 (40)	NS
	Narrowing	8 (32)	NS
	Erosive esophagitis	7 (28)	**< 0.01**
	Rings	4 (16)	**< 0.01**
	Stricture	0 (0)	**< 0.01**
Histology findings	15-40 eos / HPF (PEC)	18 (72)	**< 0.01**
	> 40 eos / HPF (PEC)	7 (28)	**< 0.01**
	Microabscesses	8 (32)	NS
Food sensitization		19 (76)	**< 0.01**
Aeroallergens sensitization		23 (92)	**< 0.01**
Food and/or Aeroallergen sensitization		24 (96)	**< 0.01**

SPT - Skin prick test, Eos - eosinophils, HPF - High power field, PEC - peak eosinophil count, NS - not significant p-value ($p > 0.01$)

22 (88%) were male, with a statistical significant preponderance for the male gender ($p < 0.001$). The minimum age, at the time of this study, was 4 years old and the maximum 16, with an average age of 10.8 ± 3.4 years and a median of 11 years.

The average age at EoE diagnosis was 6.8 ± 3.6 years (91 ± 45.9 months), ranging from 1 year (13 months) to 14 years (171 months). EoE diagnosis was always confirmed after oesophageal biopsy, which was performed within three months prior or after the first Food Allergy Consultation. Diagnosis was reached prior to the Food Allergy Consultation when patients were first referred to a Pediatric Gastroenterology Consultation, performed the biopsy, and then were referred the Food Allergy Consultation. Diagnosis was reached after the Food Allergy Consultation when patients were first referred to a Food Allergy Consultation and then, referred to a Pediatric Gastroenterology Consultation to perform a oesophageal biopsy.

After diagnosis, the subjects had an average follow-up time at our Food Allergy Consultation of 25.5 ± 12.3 months, with the shortest follow-up time being 5 months and the longest 45 months.

The time elapsed between symptoms onset and the time of diagnosis averaged 18.6 ± 29.4 months, with a minimum time corresponding to 1 month and the maximum 133 months.

We searched for pre-existing allergic disease: 22 (88%) had prior history of allergic rhinoconjunctivitis ($p < 0.01$), 19 (76%) of asthma ($p < 0.01$), 12 (48%) of eczema and 9 (36%) had a history of food allergy (9 to milk, 2 to egg, 1 to fish and 1 to nuts). Overall, 96% had at least one prior atopic condition ($p < 0.01$).

Clinically, all patients had at least one symptom attributable to oesophageal dysfunction. The most prevalent were dysphagia in 14 (56%), food impaction in 13 (52%) patients, GERD-like symptoms in 10 (40%) and epigastric / retrosternal pain in 10 (40%). The most frequent onset symptom was dysphagia (44%).

Total IgE and peripheral eosinophil count were analyzed and a great variability was found. Total IgE averaged 621 ± 373 kU/L

(170-1830) and peripheral eosinophils averaged 532 ± 714 cells / mL with 3 patients having eosinophils count over 1000 cells/mL. The most frequent positive specific IgE were to mites (68%), food (64%) and pollens (52%).

Positive skin prick revealed sensitization to mites (76%), food (64%) and pollens (56%). Positive patch tests were positive to shellfish (48%), meat (24%), egg (16%) and cereal (12%).

Overall, in our pediatric population, 23 (92%) had at least one positive test to aeroallergens ($p < 0.01$), 19 (76%) had at least one positive test to food allergens ($p < 0.01$), 17 (68%) had at least one positive test to aeroallergens and simultaneously had at least one positive test to food allergens, and a total of 24 patients (96%) had at least one positive test to aeroallergens or food allergens ($p < 0.01$).

At EoE diagnosis, the most common endoscopic finding were furrows (80%) and white plaques (40%). The biopsies revealed in 7 (28%) patients > 40 eos/HPF and in 8 (32%) patients microabscesses.

The allergy tests were used to select the exclusion diet, and oral fluticasone (MDI in a dose of 250-500 mcg/day) was prescribed during a 6 month period. After six months, all patients repeated endoscopy and biopsies; after this time there was histological normalization in 8.3% of pediatric patients and clinical improvement in all of them.

Discussion

EoE is becoming a more frequent diagnosis. However, published EoE data from Portugal is still lacking. The closest available data comes from Spain and it may not be an exact match to the Portuguese population. There are differences in patient characteristics and clinical manifestations from reports from countries with similar diet and geographical location, as Spain (31,33,36), France (38) and Italy (39). One of the essential aspects of this study is the description of clinical and allergological characteristics of a Portuguese pediatric population with EoE.

Our study shows a statistically significant preponderance for the male sex with a M/F ratio of 7/1, a much higher ratio than that present in other studies (22,31,37,40-42), which indicate a 3/1 ratio. This difference may be explained by our small sample size. Nevertheless, the male gender seems to be highly associated with the development of EoE.

No conclusion could be found about the age of EoE diagnosis in our study, although most of our patients were diagnosed between the ages of 6 and 18 years of age; similar to other studies: 7.4 ± 3.8 years found by Rezende et al (30) or the 9 ± 3.8 years found by Lucendo et al (31). This age range may indicate that EoE is not a disease characteristic of toddlers, as it is of older children. This may be explained by an immature immune system in toddlers.

The time elapsed from symptom onset to diagnostic endoscopy, averaged 25.44 ± 12.30 months, with 23 patients (92%) being diagnosed between the ages of 6 and 18 years-old. Nonetheless, 48% were diagnosed within the first 6 months and 76% within the first 2 years of disease. This delay in diagnosis may be explained by the lack of awareness of this disease and the undervaluation of symptoms, especially in younger children with more unspecific symptoms. These results are in line with the average 28 months described by Lucendo et al (31) in Spain; but still far beyond the average of 3.9 months described by Sorser et al (43).

In line with previous studies, we have documented a high prevalence of pre-existing atopic diseases in our patients, with almost all (96%) having at least 1 prior atopic condition. This high prevalence is also found in other series, even though our population had a particularly high rate (**table 2**). As for food allergy prior to EoE diagnosis, the main allergen was milk; which is in consonance with the high prevalence of milk allergy in the pediatric age and might not be directly related with the posterior development of EoE.

The sensitization profile supports the numerous studies stating the high prevalence of allergic sensitization in EoE patients. Our patients showed a greater aeroallergen (91.7%) than food sensitization (75%). Whether the high prevalence is due to the high concomitant frequency of respiratory allergic diseases or is part of the pathophysiological process of EoE development, is a matter requiring further study.

Clinically, dysphagia was the most frequent symptom (56%), with impaction (52%) the second most frequent. It is important to point out the high number (32%) of pediatric patients who referred more unspecific symptoms (anorexia, lower abdominal pain, chocking, food refusal); which may indicate a difficulty for younger children to correctly explain and complain about what they are feeling, and which may delay the diagnosis in some cases (**table 2**). These findings highlight the importance of a thorough medical history, and the importance of inquiring the parents about coping mechanisms of children.

We have also found that many patients' parents confounded the results, pointing mainly to more exuberant symptoms, as vomiting or impaction. However, when questioned more thoroughly, it was possible for most of them to pin point previous undervalued symptoms of dysphagia (both by the parents and the children).

In this pediatric population, we found a high percentage of abnormal endoscopies, whose main abnormalities were furrows ($p < 0.01$) and white plaques. These alterations also seem to be fairly frequent in others studies (**table 2**), making it important to suspect and discard EoE when they are seen. Our results had a very small number of normal endoscopies (4%), albeit with disease, compared to other studies. But is never too much to underline the importance of excluding EoE simply on the basis of a normal endoscopy and the need for biopsies. Histologically, we found an

Table 2 - *Comparison of EoE characteristics between studies.*

	Current study (25 children)	**(30) (35 children)**	**(22) (381 children)**	**(43) (103 children)**	**(56) (30 children + adults)**	**(31) (705 children + adults)**	**(37) (43 children + adults)**
Rhinoconjunctivitis	88%	74.2%	X	57.4%	43.3%	47.4%	72%
Asthma	76%	60%	X	36.85	50%	32.8%	
Eczema	48%	42.8%	X		13.3%	6%	7%
Food allergy	36%		X	46%	X	25.7%	49%
At least 1 of the 4 previous	96%	X	53%	X	93.3%	61.8%	83.7%
Dysphagia	56%	28.5%	18.1%	X	X	54.9%[1]	19%
Impaction	52%	11.4%	X	X	X	26.3%[1]	9%
Bloating	24%	X	X	X	X	X	X
GERD-like symptoms	40%	X	X	X	X	7.4%[1]	X
Pain (retrosternal or upper-abdominal)	40%	28.5%	49.9%	X	X	15.8%	X
Vomiting	32%	71.4%	57.2%	X	X	24.6%[1]	X
Normal	4%	2.8%	32%	X	3.3%	27.9%	X
Stricture	0%	X	X	X	X	X	X
Furrows	80%	60%	41%	X	X	X	X
Rings	16%	22.8%	12%	X	X	50.6%	X
Narrowing	32%	X	X	X	X	X	X
Erosive esophagitis	28%	X	X	X	X	X	X
White plaques	40%	68.7%	15%	X	X	X	X
Food sensibilization present (specific IgE, prick, prick-prick or patch test)	76%	45.75	X	X	X	X	53.6%
Aeroallergens sensibilization present (specific IgE, prick or prick-prick)	92%	X	X	X	X	X	74.4%
Food and/or Aeroallergen sensibilization (specific IgE, prick, prick-prick or patch test)	96%	77.1%	X	X	X	X	X

[1]These results are for the children in this study alone, and not the 705 children and adult cohort.
X - value not present in the original articles.

unusually high number of microabscesse formation (32%) which may indicate a more exuberant or prolonged disease and which may explain why so many endoscopies were abnormal.

The allergic profile revealed a very high frequency aeroallergen (92%, $p < 0.01$) and food sensitization (76%, $p < 0.01$). These high numbers of aeroallergen sensitized patients suggest that these allergens may contribute to the etiology of this disease.

Another interesting finding was the high prevalence of positive patch tests. In the 2011 EoE guidelines, it was reviewed that 30 to 95% of patients may have a positive patch test. However, by far the most common positive test was to shellfish (48%). The meaning of this is still unclear and further studies are needed to determine the true prevalence of shellfish allergy in EoE patients. In our pediatric population, some of the children had never knowingly eaten shellfish but presented positive patch and/or prick tests. This could be due to a previous exposure to shellfish (accidental, due to cross-contamination or to hidden allergens in processed foods) (44,45). However, a different route has been proposed to account for this sensitization. In a 2003 study, a population of orthodox Jews with perennial allergic rhinitis and

dust mite hypersensitivity (and who are prohibited by religious dietary laws from eating shellfish), was found to have a positive sensitization to shrimp (46). The term "mite-crustaceans-molluscs syndrome" is sometimes used to describe clinically relevant cross-reactivity between crustacean and dust mites (47-50).
Considering this, when implementing the exclusion diet in these patients, exclusion of shellfish seemed beneficial. The authors suggested this approach to the patients for the following reasons: 1) a six-food elimination diet (which excludes shellfish from the patients diet) has shown to be effective in EoE both in adult and pediatric patients (51-53); 2) targeted dietary elimination therapy has also shown to be an effective option for in some patients with EoE (54) and given that shellfish sensitization had been shown in several of our patients, exclusion of shellfish in these patients was part of the targeted elimination diet; 3) recommendation to avoid specific foods and awareness of the importance of label reading might be helpful in preventing accidental exposures attributed to failure to read labels (a cause of accidental exposures in food-allergic patients) (55).
No positive patch tests were found for milk, suggesting that milk allergy in our EoE pediatric population seems to be exclusively mediated by IgE dependent mechanisms.

Conclusions

Dysphagia is not only the most frequent symptom, but also the most frequent first symptom in our patients.
Furrows and white plaques are the most frequent endoscopic findings and, when present, EoE should always be excluded. However, even in a normal esophagus, biopsies should be made as it does not exclude the presence of EoE.
There is still a considerable delay between symptom onset and EoE diagnosis, which given the high morbidity of this disorder highlights the need for further awareness and early diagnosis.
We confirmed the high prevalence (96%) of sensitization in pediatric EoE patients; with 92% with aeroallergen sensitization and 76% with food sensitization.
Patch tests seem to be an important part of the allergological evaluation, allowing for the identification of additional food sensitizations not detected by specific IgE, SPT or prick-prick tests. In our population, we found positive patch tests in 56% of the children, but further investigation is needed to understand the pathophysiological implications of this high frequency of food sensitization observed in patch tests.
A conjoined approach by different specialties to this disease is needed for a correct evaluation, intervention and follow-up of these patients.
This study gives important information about the characteristics of EoE pediatric patients in Portugal; nonetheless, further data is needed for a better understanding of this disease.

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Vitamin D levels and allergic diseases. An italian cross-sectional multicenter survey

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Key words

Vitamin D; allergic diseases; bronchial asthma; allergic rhinitis; atopic dermatitis; house dust mite; allergens

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Summary

Background. *During the two last decades, the interest in the role of Vitamin D (VD) in allergic disease has increased. Apart from the well-known actions of VD in bone metabolism, recent studies suggested its possible role as an immune-modulator in allergy.* ***Objective.*** *This study, conducted over the Italian territory, evaluated the possible correlations between VD serum level and diagnosed allergic diseases (rhinitis / asthma, food allergy, atopic dermatitis). Thus, VD was assessed in patients with physician-diagnosed allergic diseases.* ***Methods.*** *The study was carried out in hospital- and private practice-based setting between October 2012 and March 2013, and 18 Centers participated. Only adult patients, with at least one positive skin prick test were included. The diagnostic procedures and the data collection were standardized among the centers. VD levels were assayed by the same laboratory test.* ***Results****. Three hundred and nine patients were enrolled (132 male, mean age 37.5 ± 17 years). Of them, 40% reported a positive family history for allergies (asthma / rhinitis). Rhinitis plus asthma was present in 47% of patients, atopic dermatitis in 15%, and a consistent clinical history of food allergy associated with positive skin tests was present in 25% of subjects. There was no significant association between VD level and age, sex, family history, rhinitis, or food allergy. VD levels were overall lower in patients with asthma and rhinitis, but without statistical significance. A significant difference in VD levels was detected between patient with or without atopic dermatitis. VD was not related to seasonal allergens, whereas a significant negative correlation was seen for house dust mite and dog dander.* ***Conclusion****. Our data, derived from a cross-sectional study involving only allergic patients, agree partially with the current literature. Nonetheless, the association between VD levels and allergies appeared weak. Studies involving larger samples would be required to better define the association between VD and allergies.*

Introduction

During the two last decades, there was a renewed scientific interest in the Vitamin D (VD) system, since new observations suggested its functional role as an immunomodulator (including allergic diseases and asthma) (**1-4)**. Cholecalciferol, and its metabolites, are more properly hormones. Ultraviolet radiations determine, in the skin, the conversion of 7-dehydrocholesterol into cholecalciferol. Subsequently, liver enzymes hydroxylate it to 25-hydroxy-cholecalciferol, that is usually named and assayed as VD. The second hydroxylation, necessary for having an active hormone, occurs in kidney, where VD is converted in the active form (1-25 hydroxyVD, calcitriol) (5,6). Based on 25(OH) VD levels, subjects are categorized as: deficient (< 20 ng/mL), insufficient (≥ 20 and < 30 ng/mL), or sufficient (≥ 30 ng/mL). VD is well known to be essential for calcium resorption and bone metabolism in general. Apart this, VD seems to play also a relevant role in the general function / regulation of immune system, especially concerning lymphocyte activation, antigen receptor functioning and signaling pathways (7,8). On this background it was suggested that VD could affect the onset and course of allergic diseases and, may play therefore also a preventative role (9,10).

Indeed, in allergic diseases, the clinical studies so far available have provided conflicting results. In addition to serum levels of VD, other factors may play a crucial role in the development of allergies and asthma, including environmental and genetic factors. Also, the interventional studies with VD in patients with immune-mediated diseases did not provide conclusive results (11-13). The extent of involvement of vitamin VD-dependent and VD-independent pathways in homeostasis and regulation of immune system in diseases still needs to be explored. Some studies reported proofs of the role of VD in allergy. For this reason, we undertook a cross-sectional real-life study to evaluate if VD serum level is related to nature and severity of disease in patients with ascertained allergies.

Methods

The study was conducted in hospital- and private practice-based settings among Italian allergists between October 1st 2012 and March 30th 2013. Eighteen individual Centres participated in this survey, all adhering to AAITO (Italian Association of Territorial and Hospital Allergists). Each centre was required to collect demographic (age, sex familiar history etc.) and clinical data (severity and duration of symptoms) from at least 20 patients firstly referred for suspected allergic diseases, that had to be confirmed by the physicians themselves by the standard diagnostic procedures. Thus only diagnosis-naïve patients were included. The study was approved by, or simply notified to local ethic committees, as per Italian law, since the study was observational. Adult patients (> 18 years), with at least one positive skin prick test (see below) were included. Asthma and/or rhinitis were diagnosed according to current guidelines (14,15). Allergic rhinitis required the presence of nasal obstruction, sneezing, itching and rhinorrhea in various combinations, associated to concordant allergen exposure. Asthma was diagnosed on clinical basis and by functional respiratory tests. Atopic dermatitis was clinically diagnosed (distribution of lesions, history, onset) (16). Food allergy / sensitization diagnosis required, in addition to a suggestive clinical history, the positivity to commercial extracts and/or prick by prick with fresh food, and/or in selected cases to oral food challenge. For inhalant allergens a diagnostic panel of commercial standardized extracts was used in all Centres, including: mites (*Dermatophagoides pteronyssinus* and *farinae)*, grass mix, *Parietaria*, olive, birch, cypress, ragweed, cat/dog dander, *Alternaria*. Family history, demographic and clinical data were all included in a standardized database. Patients having concomitant systemic diseases, such as malignancies, celiac disease, autoimmune disorders or immunodeficit were not included. The serum level of VD was assayed at all centres by commercial ELISA kits, which limit of detection was 2 ng/mL. Samples were always run in duplicate with quality control samples to ensure the validity of results. This assay is regarded as the best representation of VD metabolic status, reflecting the total VD from dietary intake, sunlight exposure and conversion from adipose tissue in the liver.

Demographic variables were reported by descriptional analysis. VD levels were categorized within quartiles: 1: 1-14 ng/mL; 2: 15-22 ng/mL; 3: 23-36 ng/mL; 4: > 37 ng/mL (17). The categorization into quartiles was made to ease the interpretation of results regarding dichotomous outcome variables and to be able to directly compare odds ratios between highest and lowest levels of serum 25(OH)D. A multivariated logistic regression model was applied, with results expressed as Odds Ratio (OR) and confidence interval 95%. All analyses were two-tailed to reject the null hypothesis, with a threshold of $p = 0.05$. Statistical analyzes were performed using STATA software for personal computers (Stata Statistical Software 12.0, 2012; Stata Corporation, College Station, Texas, USA).

Results

Three hundred and nine patients (132 male, 177 female, mean age 37.5 ± 17.0 years) were assessed (**table 1**). Of them, 40% reported a positive family history for respiratory allergy (in one or both parents). Rhinitis was present in 84% patients and rhinitis plus asthma in 47%. Atopic dermatitis accounted for 15% of subjects, and food allergy / sensitization was diagnosed in 25% (32 patients, 20 of them with positive open food challenge). **Table 2** shows the distribution of patients according to VD level

Table 1 *- Demographic and clinical characteristics of the patients' population (n = 309).*

Sex, *n* (male/female)	132/177
Age years mean (SD)	37.5 ± 17.0
Positive atopy family history *n* (%)	128 (40%)
Residence: Urban/Rural	169/134
Allergic rhinitis *n* (%) *Mild* *Moderate-severe*	268 (84%) *81 (30%)* *187 (70%)*
Bronchial asthma alone *n* (%)	42 (13%)
Rhinitis plus asthma *n* (%)	151 (47%)
Atopic dermatitis *n* (%)	45 (15%)
Food allergy / sensitization *n* (%)	77 (25%)

Figure 1 *- Distribution of patients with bronchial asthma (left), allergic rhinitis (center) and atopic dermatitis (right) according to VD quartiles.*

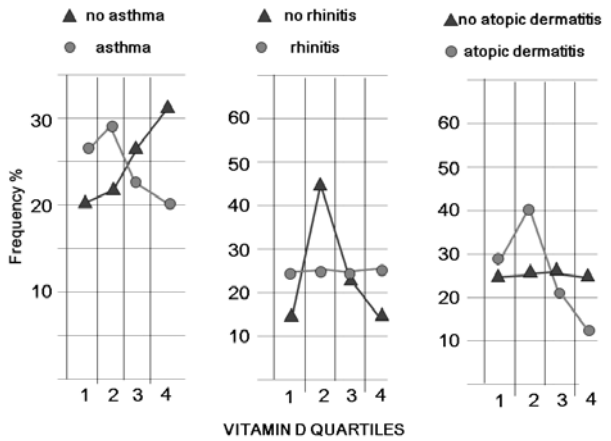
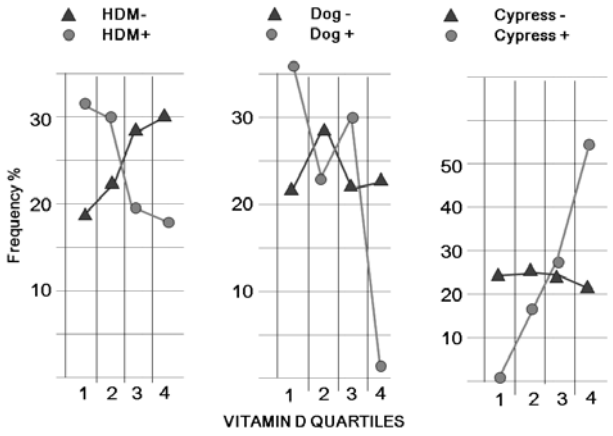


Figure 2 *- Distribution of patients with house dust mite sensitization (left), dog dander sensitization (center) and cypress sensitization (right) according to VD quartiles.*



quartiles in the population. There was no significant association between VD level, age, family history, rhinitis or food allergy (not shown). VD levels were overall lower in patients with asthma, but without statistical significance (p = .113) (**figure 1**), and the same was observed in patients with rhinitis (**figure 1**). A significant difference, according to VD levels, was detected between patient with or without atopic dermatitis (**figure 1**) (OR = 1.55, p = 0.042). VD level resulted to be not related to the sensitization to the most common seasonal allergens (grasses, *Parietaria*, olive, birch, not shown), except for cypress (OR = 0.36, p = 0.001) (**figure 2**). A significant inverse correlation was also seen for house dust mite (OR = 1.40, p = 0.005) and dog dander (OR = 1.49, p = 0.008) (**figure 2**). No difference was observed between the patients with VD < 20 ng/mL and those with higher serum concentrations according to their predominant clinical disease or sensitization. No statistical correlation could be detected even selecting those patients with ascertained food allergy (positive open oral challenge).

Table 2 *- Distribution of patients according to VD level quartiles (see text) in the population.*

Quartile	Q1	Q2	Q3	Q4	Total	P value
Age range						
18-29	27 (25.0%)	33 (30.8%)	24 (22.0%)	23 (21.5%)	107 (100%)	NS
30-49	30 (23.6%)	33 (25.9%)	29 (22.8%)	35 (27.5%)	127 (100%)	NS
50-76	19 (25.0%)	16 (21.0%)	23 (30.6%)	17 (22.6%)	75 (100.0%)	NS
Total	76	82	86	75	309	NS

Discussion

The data collected in the present study are only in part in agreement with the current literature. In fact, a positive / negative significant correlation between serum levels of VD and the presence of respiratory allergic disease could be not clearly demonstrated, and this was especially true for seasonal allergens. Indeed, our data would suggest some correlation between 25-hydroxyvitamin D levels and the immune response to perennial allergens, such as house dust mites and dog dander specifically. Our data remain partially in agreement with the results of previous studies (1,2,18). In particular, pediatric patients with asthma and allergic rhinitis and positive specific IgE to dust mite, had significantly lower VD levels than children with negative dust mites specific IgE (18). This would confirm a possible role of VD in the regulation of the immune system in allergies. On the other hand, our data cannot support the concept that VD might be useful as preventive treatment or as an adjunct to allergen immunotherapy as previously described (19,20,21), since this was not an interventional trial. The relatively small sample size could explain in part the weakness of correlations. Also, other potential confounding factors (e.g. time spent outside, type of allergic sensitization, physical exercise etc.) must be considered. Since those factors strictly interplay and overlap, it was not possible taking into account all of them, so that we opted for a more simplified and "clean" model.

Respiratory allergies such as asthma and allergic rhinitis, according to our study, did not show a significant correlation with low levels of serum 25-hydroxyvitamin D, although lower levels of VD in patients with asthma than non-asthmatics were noticed. This would be in agreement with the previously hypothesized role of VD in the development of innate and adaptive immunity, especially against infections (22). The present study demonstrated a significant difference between patients with or without atopic dermatitis, according to VD levels. It is also true that patients with atopic dermatitis have genetically-controlled risk factors that affect the barrier function. As the pathogenesis of atopic dermatitis involves a complex interplay of epidermal barrier dysfunction and dysregulated immune response, and VD is involved in both processes, it is reasonable to expect that VD levels could be associated with atopic dermatitis' risk or severity. This is in agreement to what shown in other studies (23-25). Of course, case-control studies comparing allergic and non-allergic subjects, and cross-comparing those with normal or low levels of VD are needed to identify significant differences. Of note, this study was not aimed at comparing healthy and diseased subjects, but at evaluating only patients with ascertained allergic diseases with regard to their VD serum level.

In conclusion, this study suggested that VD could have some role in atopic dermatitis and asthma, with a particular link with dust mite sensitization. Since most allergies start in childhood, VD deficiency or insufficiency in childhood might influence initiation of allergy targeting unclear and little known immunologic aspect of the disease (26).

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Prescriptive appropriateness using inhalant and food allergen panels: a comparison between General Practitioners' and Allergists' prescription in Genoa (Italy)

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Key words

allergen-specific IgE; panels; serum; appropriateness; prescription; specialist; general practitionnaire

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Summary

Background. *Prescriptive appropriateness is an actual claim in healthcare, and it also concerns in vitro tests used in the allergy work-up, such as the serum allergen-specific IgE (sIgE) assay. In the Liguria Region, two panels were defined (for inhaled and food allergens) including 12 allergens. Their composition changed over time.* ***Objectives.*** *The aims of the present retrospective study were: i) to evaluate the percentage of positive tests, and ii) to compare the findings of sIgE assay on the basis of the general practictioners' (GPs) or specialist' prescription, considering both the old panels and the new panels.* ***Methods.*** *This retrospective study considered a population of adult patients, which consisted of 2368 subjects (68% females; mean age 50 years; age range: 10-103 years). Serum sIgE were measured by ImmunoCap system.* ***Results.*** *The percentages of positive tests were very low for food allergens and low for inhaled ones (ranging between 5% to 35%). There was change of prevalent prescriptor with new panels.* ***Conclusions.*** *This study underlines the relevance of prescriptive appropriateness in the allergy work-up. The sIgE assay should be limited to those allergens that have a clinical relevance, based on clinical history.*

Introduction

Allergic disorders have an impressive prevalence: up to 40% of the general population (1,2). Sensitization is the signature of the impaired immune response to allergen(s) in allergic patients, such as the on-going production of allergen-specific IgE. Sensitization is the condition necessary (but not sufficient) for diagnosing allergy. In fact, allergy is formally documented when symptoms appear after exposure to sensitizing allergen, otherwise sensitization has no clinical relevance. Thus, a sensitization has to be always interpreted during the allergy work up.

Sensitization can be demonstrated *in vivo* (by skin prick test, SPT) or *in vitro* (by serum allergen-specific-IgE (sIgE) assay): the last is usually more precise, and well-defined serum IgE cut-offs have been associated with likely allergy diagnosis and clinical severity (3,4,5). On the other hand, serum sIgE assay is expensive, and the interpretation needs specific knowledge, mainly concerning molecular components (6,7). In this regard, the convenience of using laboratory resource is extremely timely and indispensable. For a long time, the need of defining the prescriptive appropriateness in laboratory utilization has been acknowledged an impellent requirement. So, a long time ago van Walraven and Naylor performed a systematic review of published studies, that measured inappropriate laboratory use and methodological criteria, including implicit and explicit ones (8). They concluded that many studies confirmed an inappropriate use of laboratory tests. This issue is always more mandatory in light of narrowed healthcare budget and the global concept of appropriateness of care is up-to-date (9). In this context, it is necessary that the prescription of lab test is based on appropriateness criteria.

On the basis of this premise, the Italian Health Ministry issued a decree (DM 9 December 2015) that defined the "conditions of dispensation and prescriptive appropriateness indications for a series of health services for outpatients, including tests for al-

lergy diagnosis, namely skin prick test and sIgE assay. Thus, SPT was defined first level test in the allergy work up and should be prescribed only by the specialist. However, sIgE assay should be prescribed as second level, such as confirmatory test, when SPT cannot be carried out or is not thorough, also it may be prescribed only by the specialist. As expected, this decree has raised many objections, mainly by General Practictionnaires (GPs) and family paediatricians who felt a limitation of their professional autonomy. Therefore, the Ministry defined a series of indications for its application at the end of March 2016. This document states that the application is presently on an experimental stage, so both GPs and family paediatricians may prescribe a basic test, consisting of no more than 12 allergen-specific IgE.

On the other hand, the Liguria Region some years ago defined two panels of 12 allergen-specific IgE (for inhaled or food allergens) that could be prescribed by both GPs and specialists. Later, the composition of both panels was revised, including some molecular components.

On the basis of these considerations, the aims of the present retrospective study were: i) to evaluate the percentage of positive tests, and ii) to compare the findings of sIgE assay on the basis of the GPs or specialist prescription, considering both the old panels and the new panels.

Materials and methods

Patients

This retrospective study considered a population of adult patients, which consisted of 2368 subjects (68% females; mean age 50 years; age range: 10-103 years). The patients were sent by GPs or specialists to the Laboratory Medicine Service of the University-Hospital San Martino of Genoa (Italy) for serologic assessment, as they suffered from complaints suggestive for respiratory and/or food allergy.

The old panels were in effect from January 2007 to May 2014. The new panels have been introduced in the clinical practice since June 2014.

The old inhaled panel included: *Dermatophagoides pteronyssinus* (D1), *Dermatophagoides farinae* (D2), *Cynodon dactylon* (G2), *Lolium perennis* (G5), *Alternaria alternata* (M6), birch (T3), hazelnut tree (T4), olive tree (T9), *Parietaria officinalis* (W19), dog (E5), and *Ambrosia trifida* (W3). The old food panel included: egg white (F1), milk (F2), fish (F3), wheat (F4), shrimp (F24), tomato (F25), egg yolk (F75), a-Lactoalbumin (F76), casein (F78), and hazelnut (F17).

The new inhaled panel includes: *Artemisia absinthium* (W5), *Parietaria officinalis* (W19), *Cupressus sempervirens* (T23), olive tree (T9), cat (E1), dog (E5), *Alternaria alternata* (M6), *Dermatophagoides pteronyssinus* (D1), Bet v 1 (T215), Bet v 2 (T216), Pru p 3 (F420), *Phleum pratense* (G6). The new food panel includes: milk (F2), fish (F3), wheat (F4), peanut (F17), soybean (F14), hazelnut (F17), shrimp (F24), egg white (F1), Pru p 1 (F419), Pru p 3 (F420), Pru p 4 (F421), and Bet v 2 (T216).

All patients gave the written informed consent, and the Review Board of the IRCCS-AOU San Martino-IST approved the procedure.

Assay

Serum levels of specific IgE were detected by the IFMA procedure (ImmunoCAP, Thermo Fisher Scientific, Uppsala, Sweden) in peripheral blood samples from patients. Serum was collected into gel-separator tubes, centrifuged and stored at -20 °C until analysis. Measurement of circulating specific IgE antibodies was performed according to manufacturer's instructions (10). Specific IgE concentrations were expressed in kUA/L according to the traceable calibration to the 2nd IRP WHO for Human IgE, and 0.35 kUA/L has been considered as a cut-off for defining positivity, such as sensitization (11).

Analytical quality control was performed both by using an internal quality control (Immunocap Specific IgE Control LMH, Thermoscientific, Uppsala, Sweden) and by participating to an external quality assessment scheme (UK NEQAS, Herries Road Sheffield).

Statistical analysis

Numbers were analysed by C^2 test. A p-value < 0.05 was considered as statistically significant. Data were analyzed using Stata statistical package version 13.1 (StataCorp, College Station, TX, USA).

Results

Old panels

Inhaled allergens: the test was performed in 847 patients. The panel was requested more frequently by GPs (96%) than by specialists (4%). **Figure 1** reports the percentages of positivity for the single allergens considering the prescription by GPs or specialists. There were significant differences between GPs' and specialists' prescriptions, such as positive results were more common for specialists' prescriptions, for hazelnut tree ($p < 0.0001$), olive tree ($p < 0.0001$), *Parietaria officinalis* ($p < 0.0001$), dog ($p < 0.0001$), and *Ambrosia trifida* ($p = 0.04$).

Food allergens: the test was performed in 1187 patients. The panel was requested more frequently by GPs (99%) than by specialists (1%). **Figure 2** reports the percentages of positivity for the single allergens considering the prescription by GPs or specialists. There was no significant difference between GPs' and specialists' prescriptions for all tested allergens.

Figure 1 *- New and old panels for inhaled allergens, considering the prescription made by the GP or the specialist.*

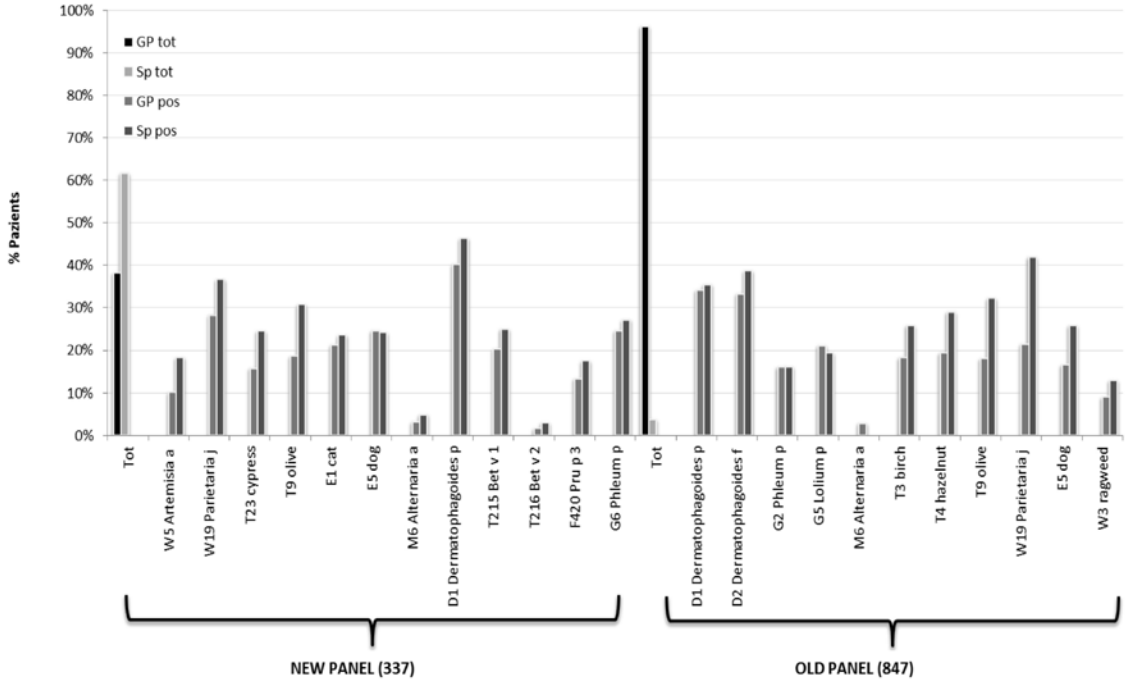
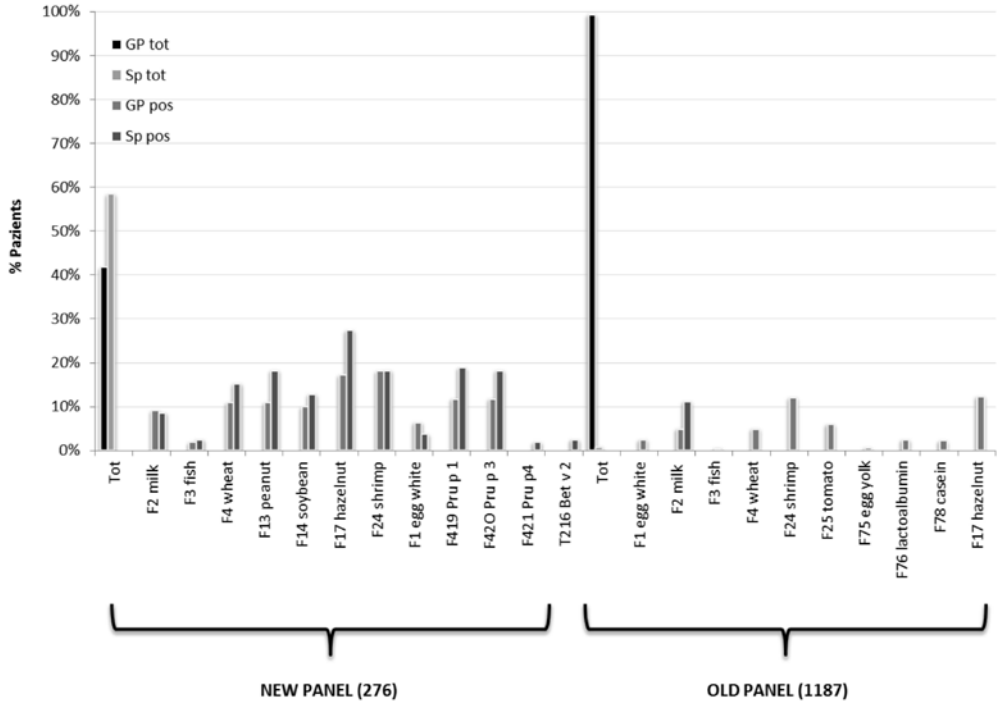


Figure 2 *- New and old panels for food allergens, considering the prescription made by the GP or the specialist.*



New panels

Inhaled allergens: the test was performed in 337 patients. The panel was requested more frequently by specialists (62%) than by GPs (38%). **Figure 1** reports the percentages of positivity for the single allergens considering the prescription by GPs or specialists. There was significant difference between GPs' and specialists' prescription, such as positive results were more common for specialists' prescriptions, for Pru p 3 ($p = 0.0006$) alone.
Food allergens: the test was performed in 276 patients. The panel was requested more frequently by specialists (60%) compared with GPs (40%). **Figure 2** reports the percentages of positivity for the single allergens considering the prescription by GPs or specialists. There was no significant difference between GPs' and specialists' prescriptions for all tested allergens.

Discussion

sIgE is usually envisaged as the main biomarker for the allergic phenotype, as allergic disorders are paradigmatically characterized by an IgE-mediated inflammation. IgE measuring is a common way to work up allergy.
The present study aimed to investigate the percentage of positive tests and the comparison of the findings considering the GPs' or specialists' prescription, both for the old panels and the new panels defined by the Liguria Region.
The study was conducted on a large cohort of subjects referring to serologic assessment for suspected respiratory or food allergy. Firstly, this study demonstrated that there was a relevant difference between the number of prescriptions by GPs or specialists: the ratio between GPs' and specialists' prescriptions was initially disproportionate in favor of GPs, but then it inverted using the new panels.
The percentages of positive tests were very low for food allergens, mainly for old panels, and low for inhaled allergens. These findings denote a scarce appropriateness in using predefined panels. In this regard, it is noteworthy to consider the reimbursement price: € 71.18 for extract allergens (up to 12 allergens) and € 9.92 for each molecular component. On the other hand, the productive cost ranges between € 12 and 15. So the use of panels is in and of itself unprofitable, but considering the present outcomes it seems inappropriate. In fact, an efficacy of 10-30% of panels does not justify their prescription. This consideration agrees with a recent document provided by Italian society of allergy, asthma and clinical immunology (SIAAIC) that reported a list of identified 5 most inappropriate allergological procedures (12). More recently, a document has been published to improve the appropriateness in the field of respiratory allergy suggesting a direct interaction between allergists and policy makers / institutions (13).
Anyway, the current study had some limitations: it was retrospectively conducted on a selected patient population sample, subjects referring for serologic assessment, there was no follow-up, and there are no clinical data. This issue is particularly relevant, as sensitization does not always correspond to allergy: this fact probably further reduces the percentages of subjects really "positive" to tests, such as allergic. In addition, this study did not consider possible confounding factors, such as smoking status, parasite infestation, environmental exposures, seasonal variations, and number of co-sensitizing allergens. Therefore, there is need to conduct cohort studies and long-term follow up trials to confirm these preliminary findings. However, the strength of the present study is represented by the large size of the sample: higher than in the other studies.
In conclusion, this study underlines the relevance of prescriptive appropriateness in the allergy work-up. The sIgE assay should limited to those allergens that are clinically relevant.

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Selective hypersensitivity to cefazolin and contribution of the basophil activation test

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Key words
basophil activation test; cefazolin; R1 side chain; selective hypersensitivity.

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Summary
The authors present 2 case reports of selective cefazolin hypersensitivity: a 49 year-old woman with a history of two perioperative reactions (urticaria and severe anaphylaxis) after the use of rocuronium, propophol and cefazolin; a 36 year-old pregnant woman who developed facial erythema, lips angioedema and hypotension immediately after administration of ropivacain, sufentanil, cefazolin, oxytocin and ephedrine. In both cases, intradermal skin tests were positive for cefazolin. A basophil activation test was performed for cefazolin, which was positive in one patient. Oral challenge tests with penicillin, amoxicillin and other cephalosporins were negative. This selective hypersensitivity to cefazolin may be associated with a R1-side chain different from other beta-lactams.

Introduction

The prevalence of hypersensitivity to cephalosporins is increasing due to the rising number of prescriptions (1,2). Several studies suggest that the R side chain of cephalosporins is the preferential way of hypersensitivity, thereby explaining the cross-reactivity between cephalosporins and other βlactams; however, the chemical structure of the antigenic determinants is not yet fully understood. In particular, the aminopenicillins have the same R-group side chains as well as some of the first- and second-generation cephalosporins. The highest cross-reactivity rate documented is around 27%, with cefadroxil, which has also the same R-group side chain as amoxicillin (3,4). Concerning cefazolin, the selective hypersensitivity seems to be the preferential presentation because the R1 side chain is different from the other cephalosporins; also, due to its parenteral use, hypersensitivity reactions are often immediate and severe (5-8).

It is well documented that approximately 40% of perioperative anaphylaxis related to drugs is due to cephalosporins prophylactic administration, thereby being extremely important to hold a high level of suspicion and notify all cases (9).

The drug allergy diagnosis workup for β-lactam hypersensitivity includes skin testing and drug challenge tests. Two main in vitro methods are used to confirm this type of immediate allergy: eEvaluation of specific IgE antibodies by immunoassay, in the serum, and the basophil activation test (BAT) upon incubating the blood sample with different concentrations of the drug. All these techniques are accepted to be complementary, although in those cases with severe reaction the in vitro tests may be the alternative for the diagnostic evaluation. The specificity of these methods is acceptable, but the sensitivity needs to be improved, especially in those patients with a clinical history of anaphylaxis and negative skin tests. Since several cases with negative skin tests and positive specific IgE have been reported, it is advisable

to perform this assay in addition to skin testing, in order to improve the sensitivity of the overall workup.
The basophil activation test (BAT) may add an additional contribution in those cases of immediate and severe allergy and negative skin tests, since it could avoid a risky drug challenge.
The authors describe two patients with immediate drug reactions, whose drug allergy workup including basophil activation test, confirmed as being selective cefazolin hypersensitivity reactions.

Case Report 1

The first patient is a forty-nine year-old woman with a past history of morbid obesity and several hospitalizations due to surgery in this setting. In 2008, three hours following a gastric banding procedure she developed an acute generalized urticaria. In 2009, she undergone revision gastric banding surgery, with no reaction documented during entire procedure. In 2012, approximately 15 minutes after the anesthetic induction for sleeve gastrectomy, she developed a IV-grade anaphylaxis which required immediate treatment with epinephrine, hydrocortisone, fluids, and subsequent mechanical ventilation was necessary in intensive care unit. The common drugs used in both interventions with documented reaction were rocuronium, propophol and cefazolin. She had no previous history of any drug allergies and was referred to our Immunoallergology clinic for evaluation.
The drug allergy diagnosis workup was performed after obtaining informed consent. Specific IgEs were negative for beta-lactams. Skin prick tests with all the drugs involved were negative, and the intradermal test with cefazolin was immediate positive at the concentration of 0.1 mg/mL. In order to test tolerance to other β-lactams, open drug challenges for penicillin, amoxicillin and other cephalosporins (cefuroxime and ceftriaxone) were performed, and the immediate and delayed responses negative. The basophil activation test was positive with cefazolin in the two concentrations used, with a percentage of activated basophils of 15.86% and 9.79% and a stimulation index of 10 and 6 respectively (**figure 1**).

Case Report 2

A thirty-six year-old woman, 38 weeks pregnant and otherwise healthy, was submitted to epidural block with ropivacain and sufentanil for cesarean section in March 2010. Immediately after the administration of cefazolin, oxytocin and ephedrine intravenous bolus, she developed a facial rash, lips angioedema and hypotension. She was given intravenous fluids, hydrocortisone and clemastine, with reversal of symptoms. No previous history of drug allergy had been documented, and the patient was referred to our Immunoallergology clinic for evaluation.
The drug allergy diagnosis workup was performed after obtaining informed consent. Specific IgEs were negative for beta-lactams. Skin prick and intradermal tests were negative for all drugs used, except the intradermal for cefazolin which was positive at 1 mg/mL (immediate response). Open oral challenge tests with penicillin, amoxicillin and other cephalosporins (cefuroxime and ceftriaxone) were performed and the immediate and delayed results were negative. The basophil activation test was negative with cefazolin. This patient has tolerated several treatments with cefuroxime after this evaluation.

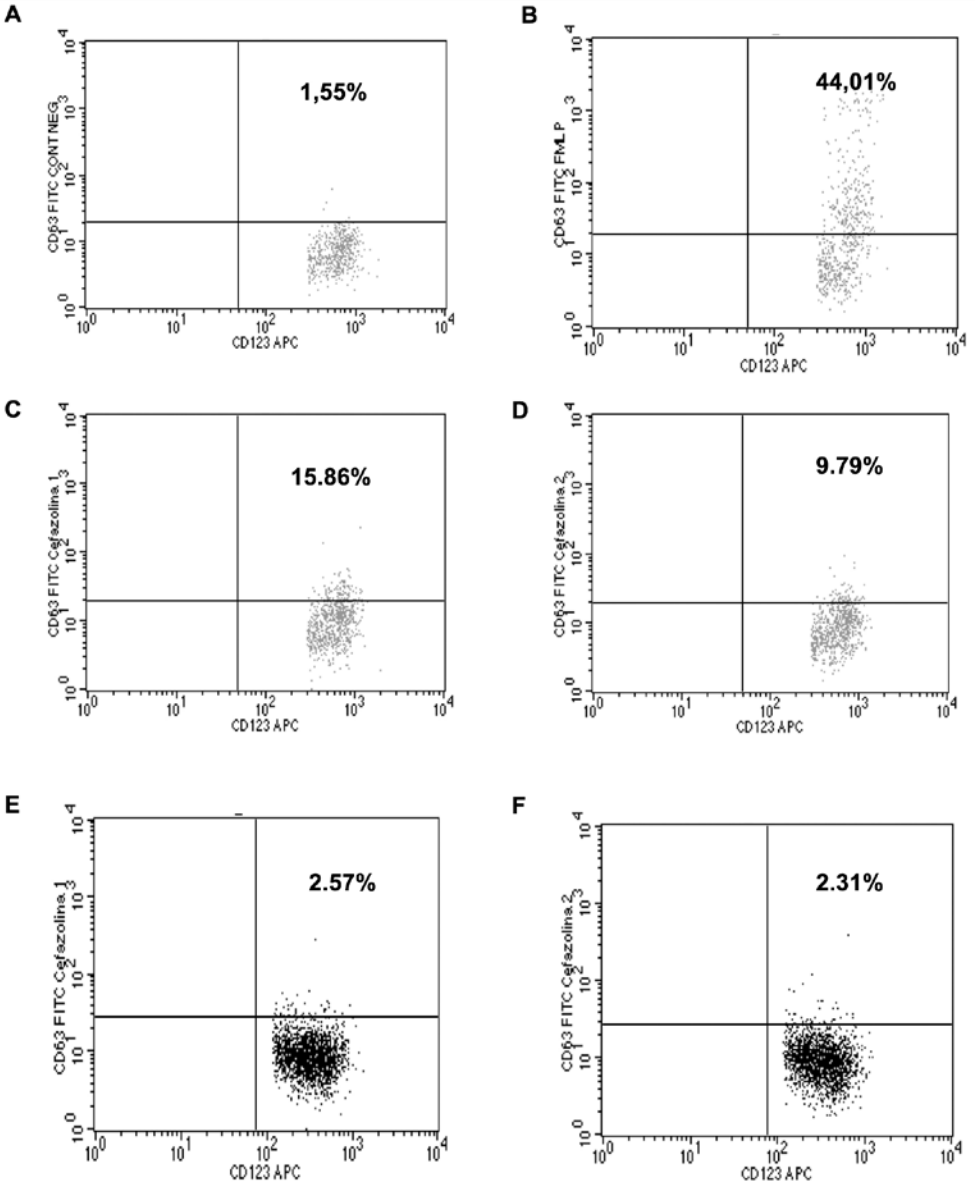
Discussion

The drug allergy diagnosis workup performed in these two cases confirmed a selective hypersensitivity to cefazolin with tolerance to other beta-lactam antibiotics, thereby cross-reactivity was ruled out. This reactivity pattern is most probably associated with the R1 side chain, which is different in cefazolin when compared to other cephalosporins.
A study by Pipet et al. (10) assessed patients with suspected hypersensitivity to cefazolin during the perioperative period. Patients were selected from the "Drug Allergy and Hypersensitivity Database" (DAHD). Among 4200 notifications, cefazolin was the suspected culprit drug in 25 cases. Allergy diagnosis to cefazolin was confirmed in ten patients, and reactions were classified as severe and immediate: anaphylactic shock in 6 patients, anaphylaxis in 2 patients, urticaria and angioedema in 2. Only one patient with hypersensitivity to cefazolin had positive skin tests for other beta-lactams. In this series, the prevalence of allergy was approximately 0.2%, which was lower when compared with other studies (6,11). Reactions are generally documented as immediate and severe mainly due to its administration in bolus. Although there may be false negatives, the skin tests seem to have a high predictive value. On the other hand, a study of Weber (11) documents that the hypersensitivity to cefazolin is extremely rare and the majority of patients were also sensitized to other beta-lactams.
The basophil activation test performed in this study was validated with a healthy control under the same conditions. It is well documented that, the shorter the time interval between the reaction and the allergy diagnosis workup, the more likely will be to get a positive BAT. This may have a major role in our study since the positive BAT corresponded to the most recent reaction (2 years before) as opposed the patient who had reacted five years before (12). Although there is no fully established cutoff for BAT positivity, we followed the published criteria for positivity in drug allergy: percentage of activated basophils > 5% and a stimulation index > 2 (13).
Concerning the reactions to betalactams, several studies have shown the sensitivity of the technique to be around 50%, with a specificity of 93%. Interestingly, in patients with negative skin tests the sensitivity of BAT can be as high as 60% when the immunoassay is also positive, and 14% when it is negative and the drug challenge test is positive (14).

Figure 1 - *Basophil activation test: Patient 1 vs Healthy control*
The B*AT was performed using FLOW2 CAST® kit, modified (Bühlmann Laboratories AG) according to the manufacturer's instructions. Testing for each patient was done by using separate tubes as follows:*

Patient 1
A - patient background (negative control)
B - positive control (stimulation with formyl-Met-Leu-Phe (FMLP)
C - cefazolin 1/40 of initial concentration (100 mg/mL), and
D - cefazolin 1/160 of initial concentration (100 mg/mL);

Healthy Control
E - cefazolin 1/40 of initial concentration (100 mg/mL)
F - cefazolin 1/160 of initial concentration (100 mg/mL).
Basophils were identified by CD123bright / side scatter and activation presented according to % of CD63 expression.



We might overall speculate that hypersensitivity to this cephalosporin is rare. Although the hapten determinants of cephalosporins are still unclear, we may presume that R1 is the major side chain involved, being on the basis of cross-reactivity. Cefazolin has a particular R1 side chain consisting of a heterocycle linked to an amide function by a methylene group (CH2).

The notified cases are scarce and insufficient to draw concrete conclusions or predict the existence of cross-reactivity, however it is generally possible to define three different patterns of sensitivity. In descending order of frequency, we may find: patients with selective allergy to cefazolin; patients with allergy to cephalosporins, but who tolerate amoxicillin; patients with allergy to several beta-lactams, due to hypersensitivity to the beta-lactam ring. A study of Romano et al. (2015) that involved one hundred patients with suspected cephalosporin allergy suggested that cephalosporin hypersensitivity doesn't seem to be a transversal class hypersensitivity. Oral challenges were performed with cephalosporins with different side-chains from the culprit drug, and were well tolerated. Therefore, it is important that patients who need alternative treatment should be prescribed with cephalosporins with different side-chain determinants different from those of the culprit drugs (15,16).

According to the literature not all studies are concordant, and this is mainly due to the different sensitivity pattern which varies according to the studied population and time of data collection. It is important to alert possible non-notified cases and refer them to Allergy Units, since the complete workup of beta-lactam is essential due to the relative predominance of selective hypersensitivity, and these patients might tolerate other beta-lactams.

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Successful Omalizumab treatment in HIV positive patient with chronic spontaneous urticaria: a case report

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Key words

Omalizumab; chronic spontaneous urticaria; HIV-positive

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Summary

We described a case of a 56 year old homosexual HIV positive man who presented a history of CSU since one year (2012). All the allergologic, immunologic and microbiologic tests to evaluate the pathogenesis of wheals resulted negative. Therefore in June 2015 we decided to start therapy with Omalizumab while the patient kept on effective antiretroviral therapy with 310 cells/mm3 TCD4 counts and undetectable HIV viremia. After two monthly subcutaneous injection of 150 mg of Omalizumab the patient had no more urticarial symptoms. UAS7 (Urticaria Activity Score over 7 days) and Cu-Q2oL (chronic urticarial quality of life questionnaire) dropped respectively to 14 from 42 and to 0 from 40 with increase of TCD4 counts while viral load remained undetectable. In November 2015, i.e. 4 months after the end of Omalizumab therapy, the patient was still asymptomatic with persistent effective immune-virological response to antiretroviral therapy. This case report confirms the excellent tolerability and efficacy of anti-IgE therapy in the treatment of spontaneous chronic urticarial even in an immunodepressed patient for HIV infection. Omalizumab therapy shows a remarkable clinical success and had no effect on peripheral TCD4 counts and HIV viral load.

Chronic spontaneous urticaria (CSU), defined as episodic or daily hives lasting for 6 weeks, is a common skin condition that affects 1-3% of people in western countries (1-3) and can cause severe handicap and impairment of quality of life (4,5).

The pathogenesis of CSU is unknown, even if an autoimmune process has been proposed for a subset of patients (6). In particular, IgG auto antibodies to the α-subunit of the high-affinity IgE receptor (FcεRI) (7,8) or naturally occurring IgG anti-IgE autoantibodies (9) have been described in approximately the 35-40% and 5-10% of CSU patients respectively. Although the real pathogenetic role of these auto antibodies remains controversial, they are thought to participate in the pathogenesis by directly activating skin mast cells degranulation in a complement-dependent manner that generates urticaria (10).

Further investigation has shown that circulating basopenia, their altered IgE receptor-mediated degranulation (11), activation of coagulation and fibrinolysis cascade (prothrombin fragment F1+F2, activated factor VII and D-dimer) (12,13) in addiction to inflammatory biomarkers (IL-6 and PCR) (14,15) characterize severe disease exacerbations in CSU.

The real incidence of CSU in HIV infected patients is not known, although it should be similar to the general population. Acute urticaria has been reported as initial manifestation of human immunodeficiency virus (HIV) infection (16,17).

In HIV infection, increased serum IgE levels is associated with an expression of an imbalance between a Th1 and Th2 cytokine profile (18) and an abnormal T cell regulation of antibody synthesis by B cells. Elevated IgE levels during HIV infection is also been correlated to disease progression with advanced HIV diseases and lower peripheral TCD4+ T cells counts (19-21).

The urticaria therapeutic approach in HIV positive patients is similar to that of immune-competent patients, but from the pharmacokinetic point of view, some medications used in the

treatment of urticaria, such as steroids or cyclosporine, may cause clinical significant drug interactions with antiretroviral.
Omalizumab is a humanized murine anti-IgE antibody that has proven to be effective in the treatment of recalcitrant chronic urticaria (22,23) and it has been recommended in EAACI/WAO guidelines as add-on treatment for CSU in patients with inadequate response to H1-antihistamines (24,25).
Omalizumab, by virtue of its ability to deplete IgE, attenuates the multiple effects of IgE to maintain and enhances mast cell activities. Therefore, it reduces the ability of mast cells to release inflammatory mediators in CSU (26).
So far, in the literature there are no data about the effects of Omalizumab therapy in the HIV-positive population, although there are numerous examples of the effectiveness and lack of toxicity of the use of other biological therapy, generally speaking (27).
We describe a case of a 56 year old homosexual male. He presented a history of CSU since 2012, clinically diagnosed by two allergists in two different hospitals, with various accesses to emergency room for acute worsening. Skin prick tests for food allergens resulted negative, and no history of urticaria induced by cholinergic factors, heat, cold, water, sun and vibration emerged from the anamnesis.
He had previously been treated with short course of steroids (Prednisone, Triamcinolone and Methylprendisolone) and antihistamines (Cetirizine and Rupatadine) with only temporary resolution of wheals but subsequent recurrence of hives. In August 2013 he was hospitalized in our Department for pneumonia and latent syphilis infection. He was treated with Ceftriaxone plus Azithromycin with complete pneumonia resolution. Simultaneously latent syphilis infection was treated with diaminocillin intramuscular with subsequent RPR (Rapid Plasma Reagin) antigen negativity. HIV test was performed and resulted positive. The peripheral TCD4+ count was 113 cell/mm^3 (4%) and viral load (VL) was 90,110 copies/ml. In September 2013 he started antiretroviral therapy (HAART) with Tenofovir / Emtricitabine, Darunavir / Ritonavir, simplified in August 2014 with Epivir plus Darunavir / Ritonavir. Immunological and virological examination in February 2015 showed TCD4 299 cell/mm^3 (11.3%) and VL undetectable (< 37 copies/ml). Despite the use of antihistamine (up to four-fold) and steroids, the urticaria persisted and the patient came to our Immunological and Allergological Department. The blood tests resulted normal, except for the presence of weak positivity for ANA (1:160 speckled) and a MGUS (0.370 mg/dl) that were evaluated as non-specific results. The parasitological examinations of stools and serology for hepatitis B, A and C were negative, and total IgE were 761 KUI. The patient has been studied in another hospital with food skin prick test (negative). No intake of NSAIDs or other drugs was reported. Helicobacter pylori and thyroid autoimmunity screening resulted negative. The (Urticaria Activity Score over 7 days) UAS7 resulted 42, and (chronic urticaria quality of life questionnaire) Cu-Q2oL was 49. In June 2015, after we had obtained an informed written consent, Omalizumab 150 mg was administered subcutaneously and monthly. After only two monthly injections of Omalizumab the patient had no more urticaria symptoms and all the therapy for the treatment of urticaria was stopped. The UAS7 became 14 and the Cu-Q2oL 0, and these parameters remained stable several months later (**table 1**). During Omalizumab therapy the patient kept on antiretroviral therapy with Epivir and Darunavir / Ritonavir, and at the end of anti-IgE therapy the peripheral TCD4 count showed 326 (13.2%) cells/mm^3 and VL remained undetectable.

Table 1

Date	VL copies/ml	TCD4 cell count	TCD4 cell %	UAS7	Cu-Q2oL	Comment
August 2013	90.110	113	4%	NA	NA	start HAART[1]
February 2015	< 37	299	11%	42	49	keep on HAART[2]
June 2015	< 37	310	10.5%	42	49	keep on HAART[2] start Omalizumab
July 2015	< 37	326	13.2%	14	0	keep on HAART[2] stop Omalizumab
November 2015	< 37	307	13.4%	14	0	keep on HAART[2]

VL: viral load; TCD4: T limphocytes CD4+; UAS7: Urticaria Activity Score over 7 days; CuQ2oL: chronic urticaria quality of life questionnaire; HAART: antiretroviral therapy.
[1]Tenofovir / Emtricitabine (245/200 mg) + Darunavir / Ritonavir (800/100 mg);
[2]Epivir (300 mg) + Darunavir / Ritonavir (800/100 mg): NA: not available

In November 2015 (4 months after the end of anti-IgE therapy) the patient remained asymptomatic with persistent immuno-virological response and complete healing of wheals after only two injections of Omalizumab.

The two most important concerns for the potential use of Omalizumab in HIV positive patients are the lack of information on the response to therapy and the potential effect on viral replication and decline on TCD4+ counts.

The findings of the current case report indicate for the first time that Omalizumab could be safely and rapidly effective in the treatment of chronic spontaneous urticaria in HAART treated virologically-suppressed HIV seropositive patients.

In particular, during and after Omalizumab treatment the viral load remained undetectable and the TCD4+ cells counts kept improving (see **table 1**). The rapid and effective response of hives to a short course of Omalizumab therapy is a very particular aspect of this case report. It could give a role to antiretroviral therapy in the improvement of urticaria, but this seems unlikely due to the persistence of wheals despite two years of virologically effective antiviral therapy (from September 2013 until June 2015). Skin and mucosal tissue disorders are common during HIV infection (28) and several HIV products like gp 120, gp 41, Tat, and Nef induce human basophil chemotaxis with chemokine receptors such as CCR3 and CXCR4 (29-32). Moreover, a population of basophil / mast cell precursors in peripheral blood of allergic donors can be infected in vitro by HIV-1, and patients with AIDS have HIV-1 infected basophil / mast cell precursors in their peripheral blood (33). However, the roles of basophils in the development of HIV-related skin disorders and/or diseases progression have yet to be established in vivo. Whereas that elevated IgE concentration is a marker of Th2 activation and is associated with HIV disease progression, we could hypothesize that Omalizumab reducing IgE pool in the immune system could also play a role in improving immune function during HIV disease.

In conclusion this case report, confirming the excellent tolerability of anti-IgE therapy, showed that Omalizumab in HIV antiretroviral successful treated people should be administered in safety without modification of viral load and peripheral TCD4+ cells. However, the safety and the immunological efficacy of treatment with Omalizumab in HIV infection need further randomized controlled trials.

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Can dog allergen immunotherapy reduce concomitant allergic sensitization to other furry animals? A preliminary experience

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Key words
Allergen immunotherapy; allergic rhinitis; allergic sensitization; bronchial asthma; dog; dog allergen; furry animals; hypersensitivity

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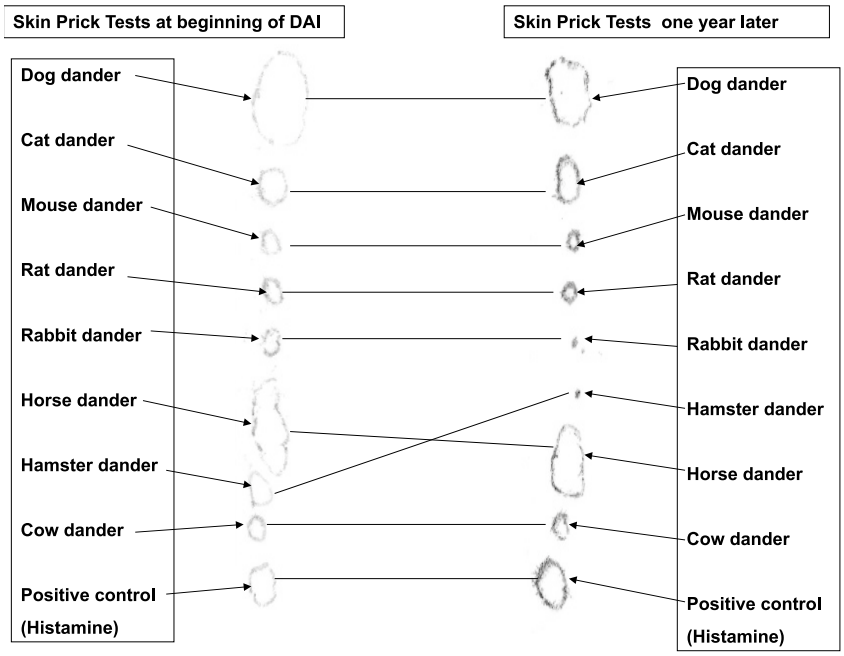
Summary
It has been shown that allergen immunotherapy (AIT) is effective in reducing symptoms of allergic asthma and rhinitis. Data on the efficacy are less convincing with regard to AIT for allergens of common pets (cats/dogs).
We describe a case of dog allergy in which we explored if dog AIT (DAI) could reduce a concomitant allergic sensitization to other allergens of furry animals. Our case demonstrates the efficacy of sublingual DAI on SPTs, symptom score, and spirometric responses despite persistent exposure to dog allergens at home in a patient sensitized, but not exposed, to several other furry animals. Moreover, this is the first report suggesting that DAI is able to reduce SPTs responses not only to dog, but also to other furry animals such as rabbit, horse, mouse, rat, hamster, cow.
We recommend an accurate anamnesis and diagnosis of dog allergy before prescribing DAI. In particular, the use of ImmunoCAP ISAC is essential to verify the presence of IgE to lipocalins / albumins belonging to other furry animals.
Obviously further studies carried out by using different DAI schedules, allergen amount and time of re-evaluation, laboratory procedure should be performed to confirm our findings.

To The Editor

International Consensus Reports have shown that allergen immunotherapy (AIT) is effective in reducing symptoms of allergic asthma and rhinitis, improving the quality of life of allergy sufferers and potentially modifying the underlying course of disease (1). Relevant allergens are the major contributors to the safety and efficacy of the allergenic extracts used for AIT. Most of the currently available data address mites, selected pollens, and animal dander. On the other hand, less is known concerning the efficacy and safety of mould or cockroach allergens. Double-blind, placebo controlled trials with both subcutaneous AIT (SCIT) and sub-lingual AIT (SLIT) in patients with perennial dust mite allergic asthma have provided more robust evidence of efficacy. The literature data are less convincing with regard to AIT for allergens of common pets (cats/dogs) (1). In particular, Smith and Coop (2) reviewed medical literature on dog AIT (DAI) in patients with hypersensitivity to dog. They demonstrated the poor and conflicting results of clinical efficacy, correlated with the poor-quality extracts and the inherent complex allergenic profile of dog materials. However, we believe that further important factors can be involved. For example, it

Figure 1 *- Wheals of SPTs for animal allergens at the beginning and one year after DAI. The profile of the wheals was outlined using a fine-point marking pen and transferred by adhesive tape onto patient's form.*



has been shown that allergic sensitization to common pets, and likely to other furry animals, can be induced by both direct and indirect exposure. In fact, it has been widely recognized that cat and dog allergens should be considered as ubiquitous, since they are found not only in indoor environments containing these animals but also in other indoor private / public places where cats/dogs have been never kept (3,4). In Naples area, less than fifty percent of patients sensitized to cats/dogs or other animals such as horses, rats, mouse, rabbits, hamsters and cows are directly exposed, whereas a significant percentage of subjects are indirectly or not exposed (3). A plausible explanation for allergic sensitization in these last cases is a cross-reaction mechanism involving some families of allergenic proteins such as lipocalins [the major allergenic materials derived from dog (Can f 1-2), cattle (Bos d 2), horse (Equ c 1), rat (Rat n 1), mouse (Mus m 1), guinea pig (Cav p 1), rabbit (Ory c 1), hamster (Pho s 21)] and serum albumins (SA) (5,6). Moreover, we have shown, by using an *in vivo* (skin prick test) and *in vitro* model (the micro-array technique ImmunoCAP ISAC), that exposure and allergic sensitization to common pets may increase the risk of developing sensitization to other furry animals (allergic phenotype?) (7,8). It is likely that the concomitant sensitization to lipocalins and/or serum albumins of other furry animals, especially in those patients directly exposed, could be the crucial condition which determines the efficacy of DAI in dog-sensitized individuals (9). The availability of molecular-based diagnosis (CRD) introduced the possibility of better targeted prescription of AIT because it might be useful for excluding cross-reactive allergens. In fact, it has been demonstrated that AIT has to be presently considered a prototype of so-called "Precision Medicine" (10) because CRD helps to improve the selection of the allergen product for AIT of an individual patient.

Since, at the best of our knowledge, no studies have assessed the relationship between DAI and sensitization to other furry animals, we describe briefly a case of dog allergy in which we explored if DAI could reduce a concomitant allergic sensitization to other furry animals as assessed by using skin prick test (SPTs). A 38 year old man sensitized to dog, cat, rabbit, horse, mouse, rat, hamster, cow and *Parietaria* allergens, underwent sublingual DAI (ALK Group Milan Italy) because he refused to relocate his dog for family reasons. He had declared no direct or significant indirect exposure to cat and other furry animals. Diagnosis was done by using SPTs, evaluating specific IgE (CAP System and ImmunoCAP ISAC), symptom score and spirometry. One year after having begun DAI, we re-evaluated SPTs and found a significant reduction of wheal diameters (expressed in mm) of dog, horse, cow, mouse, rat, abolished skin response to rabbit / hamster, and no significant change of wheal diameters of cat (**figure 1**). This last finding can be easily explained because the main cat allergen Fel d 1 does not belong to lipocalins' family and, in

Table 1 *- Clinical and diagnostic data at the beginning and one year after DAI.*

At the beginning of DAI	**One year after DAI**
SPTs (wheal diameters expressed in mm)	SPTs (wheal diameters expressed in mm)
Dog allergen: 13 x 15	Dog allergen: 9 x 10
Cat allergen: 6 x 6	Cat allergen: 5 x 7
Mouse allergen: 4 x 4	Mouse allergen: 2 x 2[1]
Rat allergen: 4 x 4	Rat allergen: 2 x 2[1]
Rabbit allergen: 4 x 4	Rabbit allergen: 0 x 0 (no SPT response)
Hamster allergen: 5 x 6	Hamster allergen: 0 x 0 (no SPT response)
Horse allergen: 8 x 16	Horse allergen: 7 x 11
Cow allergen: 4 x 4	Cow allergen: 3 x 3
Positive control (Histamine): 6 x 7	Positive control (Histamine): 7 x 7
(T-paired test results: t = 2.4062; p = 0.0470. This difference is considered statistically significant.)	
Serological data	
Evaluation total IgE: 263 kUL (n.v. < 100 kUL)	n.a.[2]
Evaluation specific IgE (CAP System) (value)	
Dog epithelia: 51.1 kUL (Very high)	n.a.
Cat allergen: 7.27 kUL (High)	n.a.
Mouse allergen: 2.19 kUL (Moderate)	n.a.
Rat allergen: 1.28 kUL (Moderate)	n.a.
Rabbit allergen: 0.15 kUL (Low)	n.a.
Hamster allergen: n.a	n.a.
Horse allergen: 2.24 kUL (Moderate)	n.a.
Cow allergen: 2.37 kUL (Moderate)	n.a.
Evaluation specific IgE (ImmunoCAP ISAC) (value)	
rCan f 1 (Lipocalin): 36 ISU-E (Very high)	n.a.
rCan f 2 (Lipocalin): 12 ISU-E (High)	n.a.
rCan f 5 (Arginin esterase): 1.9 ISU-E (Moderate)	n.a.
rEqu c 1 (Lipocalin): 3.5 ISU-E (High)	n.a.
rFel d 1 (Uteroglobulin): 0.6 ISU-E (Low)	n.a.
nCan f 3 (Serum albumin): 22 ISU-E (Very high)	n.a.
nEqu c 3 (Serum albumin): 3.5 ISU-E (High)	n.a.
nFel d 2 (Serum albumin): 18 ISU-E (Very high)	n.a
Fel d 4 (Lipocalin): < 0.3 ISU-E (Negative)	n.a.
Mus m 1 (Lipocalin): < 0.3 ISU-E (Negative)	n.a.
Bos d 1 (serum albumin): < 0.3 ISU-E (Negative)	n.a.
(Statistical evaluation not applicable)	
Symptom score (Nov 2012 - Mar 2013)	Symptom score (Nov 2013 - Mar 2014)[3]

At the beginning of DAI	One year after DAI
ANOVA test for all nasal / bronchial symptoms (running nose, stuffy nose, nasal congestion, itchy nose, sneezing, cough, wheezing, dyspnea) and following multiple rank test:	
F = 93.53; P < 0.001	
Multiple rank test has been carried out by using LSD of Fisher.	
Significant statistical difference for: running nose, stuffy nose, sneezing and dyspnea.	
Spirometric evaluation[4]	
Once a month from Nov 2012 to Mar 2013	Once a month from Nov 2013 to Mar 2014
Mild obstruction at lower airways:	Mild obstruction at lower airways:
(in 4 out of 5 monthly controls)	(in 1 control, the remain 4 controls: normal values)

[1]Values below usual limits of positivity (3 x 3 mm)
[2]n.a. = not available
[3]These periods have been chosen because out of *Parietaria* pollen season.
[4]These periods have been chosen because out of *Parietaria* pollen season.
SPTs = Skin Prick Tests
LSD = Least Significant Difference

the case of our patient, for the high presence of IgE against nFel d 2 (cat SA). Nasal and bronchial symptom scores showed a statistical significant difference, spirometric evaluations showed normal values in 4 out of 5 controls (see additional materials for details). Unfortunately, the patient refused to continue DAI and undergo the second blood sample, so we have no data on specific IgE levels to dog and other animals.

Our case demonstrates the efficacy of sublingual DAI on SPTs, symptom score, and spirometric responses despite persistent exposure to dog allergens at home in a patient sensitized, but not exposed, to several other furry animals. This is the first report suggesting that DAI is able to reduce SPTs responses not only to dog, but also to other furry animals such as rabbit, horse, mouse, rat, hamster, cow. Since our patient denied any direct / indirect contact with all animals (excluded the dog), perhaps the clinical influence of this finding could be negligible for him. We cannot exclude that, in patients sensitized and exposed to several animal species, the clinical effectiveness of DAI may be of great extent. The limitations of our study, carried out in "real life" by using commercially available extracts, are related with the time of re-examination and the lack of serological data, merely due to the patient's decision.

In conclusion, our preliminary experience on DAI suggests that this therapeutic approach is effective on symptoms related to dog allergy, but could be potentially useful also to reduce allergic sensitization to other animal allergens as assessed by SPTs. We recommend an accurate anamnesis and diagnosis of dog allergy before prescribing DAI. In particular, the use of ImmunoCAP ISAC is essential to verify the presence of IgE to lipocalins / SA belonging to other furry animals (11), as these allergens are likely to stimulate patient's airways inducing clinical symptoms. Obviously, further studies carried out by using different DAI schedules, allergen amount and time of re-evaluation, an adequate number of patients and laboratory evaluation should be performed to confirm our findings.

Summary statement

Our preliminary experience suggests that the effects of dog allergen immunotherapy could be potentially useful also to reduce allergic sensitization to other animal allergens as assessed by SPTs.

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