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Printed in June 2010

The contents of this Journal are indexed in PubMed - U.S. National Library of Medicine and Embase/Excerpta Medica

# European Annals of Allergy and Clinical Immunology

THE OFFICIAL JOURNAL OF AAITO -  
ASSOCIAZIONE ITALIANA ALLERGOLOGI IMMUNOLOGI TERRITORIALI E OSPEDALIERI

*Original articles*

Prospective study of sensitization and food allergy to flaxseed in 1.317 subjects

S. FREMONT, D.A. MONERET-VAUTRIN, P. FRANCK, M. MORISSET, A. CROIZIER, F. CODREANU, G. KANNY

103

The biological potency of different extracts for sublingual immunotherapy assessed by skin prick tests

R. ROSSI, G. MONASTEROLO, G. PASSALACQUA

112

Long-lasting effect of a monophosphoryl lipid-adjuvanted immunotherapy to parietaria. A controlled field study

A. MUSARRA, D. BIGNARDI, C. TROISE, G. PASSALACQUA

115

The prevalence of nasal polyps and the corresponding urinary LTE4 levels in severe compared to mild and moderate asthma

C. MICHELETTO, M. VISCONTI, F. TREVISAN, S. TOGNELLA, S. BERTACCO, R.W. DAL NEGRO

120

*Letter to the Editor*

Cypress pollen does not cross-react to plant-derived foods

R. PANZANI, R. ARIANO, G. MISTRELLO

125

*Case report*

Respiratory allergy induced by exclusive poly-sensitization to serum albumins of furry animals

G. LICCARDI, B. DENTE, P. RESTANI, G. SENNA, P. FALAGIANI, C. BALLABIO, G. D'AMATO

127



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# Prospective study of sensitization and food allergy to flaxseed in 1,317 subjects

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## KEY WORDS

Flaxseed allergy, industrial processing, cross-reactivity, CCD, extruded flaxseed

## SUMMARY

**Background:** Foods containing flaxseed proteins rich in polyunsaturated fatty acids are new on the market. **Objectives:** In a population of patients attending the allergology department, we evaluated the frequency of sensitization to flaxseed, characterized allergens and looked for modifications related to industrial processing. **Methods:** Natural, heated and extruded flaxseeds were tested using prick-in-prick tests (PIP using the fresh seed), SDS PAGE, immunoblots, immunoblot inhibition and Fourier Transform Infrared (FTIR) spectroscopy. **Results:** PIP tests to natural flaxseed were positive in 5.8% of the 1,317 patients. 73 of 77 PIP-positive patients were atopic. There was cross-reactivity with five seeds: peanut, soybean, rapeseed, lupine and wheat, and with rape pollen. Immunoblot inhibition by bromelain confirmed the presence of specific IgE to cross-reactive carbohydrate determinants (CCD). 0.15% of this population presented with food allergy to flaxseed and positive PIP to heated and extruded flaxseed. Two sera showed that clinically relevant allergens in industrial products had MW between 25 and 38 kDa. Sensitization to processed flaxseed characterized only the allergic subjects. FTIR spectroscopy showed major modifications in  $\beta$  and  $\alpha$  structures following industrial processing. **Conclusion:** Positive prick tests to natural flaxseed were mainly due to cross-reactions. Flaxseed allergy is rare and could be detected by PIP to heated extruded flaxseed. Increasing consumption calls for monitoring of clinical risk.

## Introduction

Food allergy is a public health issue and any new food appearing on the market should be carefully monitored. Amongst health foods, omega-3 polyunsaturated fatty acids (PUFA) have a preventive effect on cardiovascular disease. Flaxseed contains PUFA. PUFA exert a protective role in atopic dermatitis and reduce the prevalence of

atopy (1-6). Flaxseed given to horses with *Culicoides* species sensitivity significantly reduced the size of prick test wheal areas (7).

The recommended ratio of omega 6/omega 3 is around 1/5, but this is not obtained and other sources of supplementation are investigated, either of animal origin: fish (particularly sardines and tuna), or of vegetable origin (rapeseed oil, olive oil, blackcurrant seed oil, etc.) (8). A

**Abbreviations:** PUFA (Polyunsaturated Fatty Acids); PIP (Prick-in-prick) = prick-prick using the fresh seed; FTIR spectroscopy (Fourier Transform Infra Red spectroscopy); CCD (Cross-reactive Carbohydrate Determinants); DBPDFC (Double Blind Placebo Controlled Food Challenge); MW (Molecular Weight)

current strategy is to add varieties of flaxseed (*Linum usitatissimum*) rich in omega 3 into cereals. Detoxified flaxseed flour is now used in human food, but there are few data concerning the risk of food allergy. Only a few cases of food allergy with flaxseed or to flaxseed oil used as a laxative have been published (9-12).

It would therefore seem useful to envisage allergic risk related to novel food, such as that reported for the Micronesian nut Nangai (*Canarium indicum*) (13). The potential allergenicity of a new food has to take into account possible modifications of the natural food by food technologies (14). The primary objective of this study was to evaluate the prevalence of sensitization and allergy to natural flaxseed flour in 1,317 patients consulting for diverse allergic disorders. The secondary objectives were to study cross reactivity of flaxseed with other seeds (soybean, lupine, rape) and to compare sensitization to natural flaxseed with flaxseed found in food after transformation by two industrial methods: heating (heated flaxseed) then extrusion (extruded flaxseed).

## Material and methods

### *Studied population*

The prospective study was conducted over 18 months in 1,317 patients attending the allergology department (Internal Medicine, Clinical Immunology and Allergology, Nancy University Hospital Center). All subjects were routinely interviewed to detect consumption and possible food allergy to flaxseed and underwent prick tests to 12 standard airborne allergens. When food allergy was suspected, the routine diagnosis relied on PIP to 25 standard food allergens, extending to other allergens when required (9).

These patients were classified in two groups:

The first group comprised 603 non-atopic patients without any anamnesis of atopic disease, and negative prick tests to 12 airborne allergens. These patients consulted for allergy to Hymenoptera, drug-related adverse reactions, non-allergic rhinitis.

The second group comprised 713 atopic patients with atopic dermatitis, food allergy, allergic rhinitis or allergic asthma, confirmed by prick tests with at least one reference airborne or standard food allergen.

### *Material*

Airborne allergens were Allerbio extracts (Varennes en Argonne)

The natural flaxseeds were obtained from a health food shop that guaranteed the absence of pesticides and other

products used in traditional agriculture. These products are commonly found in human food. They were stored at  $-80^{\circ}\text{C}$ . They were ground to powder and mixed into a saline solution for prick tests.

Flaxseed flour was supplied by Valorex (35210 Combour-tillé, France). It contained eight varieties of *Linum usitatissimum*. Seeds were steamed at a temperature not exceeding  $100^{\circ}\text{C}$ . Extruded flaxseed was obtained from heated flaxseed that had been processed in an extruder (patented manufacturing technique). These products are used by the food industry.

### *Prick tests*

Basal skin reactivity was assessed by prick tests to 9% codeine and saline solution (negative control).

The 12 standard airborne allergens were: *D. pteronyssinus*, cockroach, *Alternaria alternata*, cat and dog epithelia, latex, grass pollen, birch pollen, *Artemisia* pollen, plantain pollen, ash pollen, rape pollen (extracts from Allerbio, Varennes en Argonne, France).

24 standard food allergens were tested by PIP (prick-prick using the fresh seed) (15). They were: milk, egg, fish, wheat flour, rye, celery, carrot, kiwi, peanut, hazelnut, walnut, Brazil nut, almond, sesame, buckwheat, crab, green peas, lentils, chicken, pork, garlic, onion, mustard, potato. Other allergens often tested were: various seeds (natural flaxseed, poppy seed etc.), tomato, Prunoideae, maize, soy, baker's yeast, spices, molluscs, beef and other meats.

Six patients consented to undergo PIP to Valorex products: natural, heated and extruded flaxseed flour.

Criteria for positivity was 3 mm on the condition that codein control be positive and saline solution be negative.

### *Specific IgE*

Specific IgE were assayed using Cap System RIA (Pharmacia, Uppsala, Sweden). Tests were performed on sera remaining from the blood samples from 26 patients with positive prick tests to flaxseed.

### *DBPCFC*

They were performed according to the procedure conducted in the department since 1990. Briefly, flaxseed flour from Valorex was mixed into stewed apple. Six doses were given at 20 minutes intervals: 5 mg – 10 mg – 50 mg – 150 mg – 285 mg - 465 mg. A further dose increase rose to 7,110 mg. Pulse, blood pressure peak flow rate, aspect of the skin and oral mucosa were evaluated.

#### *Preparation of protein extract*

Seed mixtures were prepared using one gram of seeds ground by Ultraturrax and one ml of buffer at pH 8 (0.1 M Tris, 0.14 M NaCl, 2 mM EDTA, 0.05% Triton, 0.05% Deoxycholate, 0.1% Nonidet P40). Bromelain extract was prepared by dissolving one gram of bromelain (Sigma, France) in 3 ml of PBS. After incubation overnight at 4°C while shaking, each mixture was centrifuged at 3,500 rpm for 15 min. The protein concentration of the supernatant, measured using the pyrogallol red method, was 10 mg/ml (natural flaxseed), 6 mg/ml (heated flaxseed), 3 mg/ml (extruded flaxseed), 11 mg/ml (soybean), 5 mg/ml (peanut), 49 mg/ml (lupine), 3 mg/ml (rapeseed), 4 mg/ml (wheat), 39 mg/ml (bromelain). Rapeseed pollen extract was supplied by Allerbio (Varennes en Argonne, France). After preparation, the extracts were immediately aliquoted and stored at -20°C.

#### *RAST inhibition assay*

RAST inhibition of flaxseed was conducted using the serum of the 14 patients with flaxseed-specific IgE higher than 1 kU/L. It was performed at a dose of 1.50 mg/mL of protein with each of the following extracts: peanut, soybean, wheat, rapeseed, rapeseed pollen, lupine, bromelain, natural flaxseed, heated flaxseed and extruded flaxseed.

Inhibition mixtures, containing 25 µL of the patient's serum and 25 µL of the test allergen extract, were first incubated with continuous stirring for 30 minutes at room temperature and subsequently incubated with the CAP allergen support before addition of the detecting reagents and measurement of the resulting radioactivity. A mixture of patient's serum and PBS served as the control mixture. All the experiments were carried out using the Pharmacia CAP System. The inhibition percentage was calculated as follows:  $1 - (\text{cpm bound to inhibiting mixture} / \text{cpm bound to control mixture})$ . The limit of positivity is currently 30% of inhibition. Inhibition tests could not be done in duplicate nor at several dilutions due to lack of serum. Because a single concentration of inhibitor was tested, the limit of positivity was raised to 40% of inhibition.

#### *Fourier Transform InfraRed (FTIR) spectroscopy*

Each type of seed (i.e. natural flaxseed, heated flaxseed, extruded flaxseed) was ground with KBr. Each KBr pellet was analysed using FTIR (Mattson, Unicam). The protein structure was studied by second derivative spectrophotometry between the wave-numbers 1,600-1,720

cm<sup>-1</sup>, 1,500-1,600 cm<sup>-1</sup> and 1,200-1,350 cm<sup>-1</sup>, corresponding to the absorption domains of the primary, secondary and third amide function of proteins.

#### *SDS-PAGE and immunoblotting*

Two sera were selected because of the high level of specific IgEs (8.5 and 11 kU/L): serum from patient 6 (sensitized patient) and serum from one allergic patient (case 2), respectively. Negative control immunoblots were tested using either an aqueous solution containing 1% BSA or the sera from 2 non-allergic patients.

Electrophoresis was conducted as previously described (14). Briefly, 4 µg of each protein extract (natural flaxseed, heated flaxseed, extruded flaxseed) were denatured and loaded on an 8-25% polyacrylamide gradient gel (Phast-System Pharmacia).

A molecular weight standard consisting of biotinylated proteins (Covalab, Dako) was also run on the gels.

After migration, the gel was either stained with Coomassie brilliant blue or transferred to a cellulose membrane with 0.45 µm pores. The Western blot was conducted as previously described (Franck 2002). Briefly, the membrane was incubated at room temperature for 1 hour in 5% bovine serum albumin, washed, incubated overnight with the serum, washed and incubated for 1 hour in a 1:10,000 dilution of peroxidase-conjugated human anti-IgE antibody (Dako) in 1% BSA, 0.05% Tween in PBS. After washing, the membrane was immersed in a solution of luminol in the presence of H<sub>2</sub>O<sub>2</sub> (Covalab, Dako) directly on a Kodak Digital Science 1D image analyzer. An image was acquired after an exposure time of 6.6 minutes (20 captures). In order to visualize the molecular weight standards, the membrane was incubated for 15 minutes in the presence of peroxidase-conjugated avidin (diluted 1:2,000) and revealed as described above.

#### *Immunoblot inhibition*

As a sufficient quantity of serum was required, immunoblot inhibition was conducted using only one serum, that of patient 6.

A volume of 500 µl of serum was pre-incubated overnight at 4°C in the presence of 500 µl (2.5 mg of protein) of the peanut extract. As described above, an immunoblot was carried out with this pre-blocked serum. A mixture of the patient's serum and PBS served as the control mixture. A positive control was conducted using flaxseed extract as an inhibitor.

## Results

### Clinical data

PIP tests to flaxseed were performed in 714 patients with food allergy or respiratory allergy (360 children aged under 15 years, 354 adults) and in 603 non-atopic patients (213 children, 390 adults). There was 12.2% prevalence of sensitization in atopic children and 8.2% in atopic adults. No sensitization was detected in 213 non-atopic children and the prevalence of 1% of sensitization characterized 390 non-atopic adults (Tab. 1).

Among the 77 patients sensitized to flaxseed, 73 were atopic. Of 44 children, 39 had food allergy and 31 were sensitive to airborne allergens, including 30 to pollens. Of 33 adults, 10 had food allergy, 21 had respiratory allergy or atopic dermatitis and were sensitive to airborne allergens

(including pollens). The study of associated sensitizations in atopic children and adults sensitized to flaxseed proteins, showed polysensitization to airborne allergens and food allergens in nearly all cases (Tab. 2). Standard airborne allergens were predominant: grass pollen, birch pollen, ash pollen, plantain pollen, Artemisia pollen and dog and cat epithelia.

Two cases of food allergy to flaxseed were diagnosed. Case 1 was a 41-year old female with atopic dermatitis and asthma, allergic to birch, grass, ash, pollens, cat epithelia, latex, polysensitized to avocado, banana, sweet pepper, buckwheat. She experienced laryngeal angio-edema after eating 13 g of flaxseed. The prick test was positive (10.5 mm) and flaxseed RAST was at 0.51 kU/L. The DBPCFC was positive at the cumulative dose of 265 mg, eliciting generalized urticaria.

**Table 1** – Sensitization to flaxseed assessed by PIP to natural flaxseed

	Non-atopic	Evolutionary atopic diseases	Total
Children	0/213 (0%)	44/360 (12.20%)	44/573 (7.68%)
Adults	4/390 (1.02%)	29/354 (8.2%)	33/744 (4.44%)
Total	4/603 (0.66%)	73/714 (10.2%)	77/1317 (5.84%)

**Table 2** – Sensitizations associated to flaxseed sensitization in atopic children and adults

	Airborne allergens (12)			Food allergens (24)	
	Atopic <15 years	Atopic >15 years		Atopic <15 years	Atopic >15 years
Grass	55%	100%	Pulses*	51%	16%
Artemisia	26%	35%	Nuts*	76%	32%
Rape	16%	23%	Cereals*	16%	40%
Plantain	29%	29%	Egg (white, yolk)	38%	12%
Birch	42%	59%	Sesame	16%	20%
Ash	42%	53%	Fish	17%	8%
<i>Alternaria alternata</i>	19%	12%	Kiwi	12%	12%
Dog	32%	53%	Milk	12%	0
Cat	45%	47%	Potato	0	12%
Cockroach	6%	6%	Mustard	4%	12%
<i>D. pteronyssinus</i>	32%	76%	Garlic	2%	12%
Latex	0%	18%	Apiaceae*	0	12%
			Buckwheat	0	8%
			Meats*	2%	8%
			Crustaceans (crab)	2%	0

Explanatory note: Pulses = green peas, lentils; Meats = chicken, pork; Apiaceae = carrot, celery; Cereals = wheat, rye; Nuts = hazelnut, walnut, peanut, Brazil nut, almond

**Table 3** - Compared allergenicity of natural flaxseed, heated flaxseed and extruded flaxseed in a flaxseed-allergic patient (FA) and five sensitized patients (S)

Clinical status	Sex	IgE Flaxseed kU/L	Codeine (mm)	Flaxseed (mm)	PIP wheal diameter (mm)		
					Natural flaxseed flour (mm)	Heated flaxseed (mm)	Extruded flaxseed (mm)
FA	F	0.51	3	10	10.5	15.5	13.5
S	M	8.8	3	5	6	0.5	0
S	M	3.7	2.5	4	3	1	1
S	F	<0.35	5	3	6	0	0
S	M	<0.35	5	4	5	2	0
S	M	<0.35	1.5	5.5	5	0	0

Case 2 was a 40-year old female with past history of asthma, presenting recurrent systemic reactions (generalized urticaria, gastric pain and conjunctivitis after eating bread containing flaxseed. The prick test was positive (10.5 mm) and elicited conjunctivitis. Flaxseed RAST was at 11 kU/L. An oral challenge was not performed because of the syndromic reaction to prick test.

Globally 63% of patients sensitized to flaxseed had some kind of food allergy, 67% were sensitized to pollens and 0.15% had food allergy to flaxseed. It should be noted that case 2 had positive PIP to heated and extruded flaxseed, whereas five sensitized patients had negative results (Tab. 3).

Flaxseed-specific IgE were determined for 26 cases of sensitization detected by PIP. They were present in 16 cases. Amongst these, 14 sera with flaxseed-specific IgE ranging from 1 to 11 kU/L were analysed by RAST inhibition assays with extracts of soybean, peanut, lupine, wheat and rape. Inhibition reached significant levels up to 78%, and was homogenous for the five seeds tested and for rape pollen (Tab. 4). The comparison of the rate of rapeseed inhibition and rape pollen inhibition may indicate common allergens between seeds and pollens. Out of the 14 sera, 11 had specific IgEs to bromelain. Bromelain consistently and impressively inhibited flaxseed RAST. However, testing several concentrations of inhibitor would have improved the quality of the results.

A study was carried out in 6 subjects comparing natural, heated and extruded flaxseed flour reactivity by PIP. The flaxseed allergic patient had positive heated and extruded flaxseed PIP, while sensitized patients had negative PIP (Tab. 3). RAST inhibition of flaxseed using natural, heated and extruded flaxseed showed significant inhibition with 12/14 sera, i.e. not for patients 5 and 9 who showed inhibition percentages ranging between 14 and 20% (Tab.

5). Patient 9 had no food allergy and was sensitized only to pollens (birch and grass). The fact that we observe a positive result to flaxseed and a negative inhibition of this Cap by flaxseed might indicate a very low affinity of IgE to flaxseed. A technical bias cannot be excluded. We postulate that there was cross reactivity between flaxseed and pollens and the high rate inhibition by bromelain may indicate the presence of anti CCD IgEs. Besides, in Table 4, no inhibition at all is observed by any food. Case 5 had cashew allergy and inhibition of flaxseed RAST was obtained by all other seeds than flaxseeds (Tab. 4). The meaning of this discrepancy is not clear.

#### SDS-PAGE

A similar electrophoresis pattern was observed with the extracts from natural, heated and extracted flaxseed (Fig. 1). For each sample, three protein fractions with molecular weights of 14, 25 and 35 kDa were stained.

#### Western blot

The immunoblot conducted using the serum of patient 6 (flaxseed sensitized patient, RAST= 8.5 kU/L) showed several bands from 5 to 40 kDa, present in natural and processed flaxseed (heating and extrusion). In extruded flaxseed flour, only one band of 30-35 kDa appeared (Fig. 2). The immunoblot from case 2 (flaxseed-allergic patient, RAST= 11 kU/L) showed two bands at 25 and 38 kDa (natural flaxseed) and one band (38 kDa) with heated and extruded flaxseed. Control tests performed using a BSA solution or sera from two non-allergic patients were negative.

#### Blot inhibition

Incubation of serum from patient 6 with peanut extract showed complete inhibition of the blot. The same result

**Table 4** - RAST inhibition of flaxseed using different extracts in the 14 sera of sensitized patients with RAST values exceeding 1 kU/L

Patient	Clinical status	Flaxseed PIP (mm)	Sex	Age (years)	Flaxseed RAST (kU/L)	Peanut inhibition	Soybean inhibition	Wheat inhibition	Rape seed inhibition	Rape pollen inhibition	Lupine inhibition	Bromelain RAST (kU/L)	Bromelain inhibition
1	Peanut allergy	13.5	F	10	10.9	69%	56%	70%	30%	21%	60%	2.02	90%
2	Wheat flour allergy	4	M	47	2.9	62%	60%	70%	35%	40%	60%	7.87	85%
3	Peanut allergy	4	M	11	10.9	54%	30%	34%	57%	48%	42%	0.67	89%
4	Peanut allergy	4	M	11	2.22	62%	35%	65%	49%	47%	48%	1.88	90%
5	Cashew allergy	4.5	M	11	2.86	53%	54%	33%	56%	57%	50%	1.11	92%
6	Peanut allergy	2.5	M	11	8.54	71%	50%	74%	52%	32%	55%	1.07	92%
7	Peanut allergy	7	M	6	10	60%	82%	70%	73%	55%	85%	1.5	85%
8	Atopic dermatitis	4	M	3	2.68	57%	63%	51%	60%	40%	60%	< 0.35	80%
9	Atopic dermatitis	5	M	58	7.78	5%	3%	2%	0%	0%	5%	< 0.35	93%
10	Peanut allergy	5	M	11	1.14	70%	40%	78%	53%	45%	50%	1.98	47%
11	Wheat flour allergy (cured)	5	M	12	3.24	62%	45%	32%	57%	40%	55%	7.54	76%
12	Peanut allergy	3	F	6	4.46	68%	56%	61%	55%	41%	62%	< 0.35	91%
13	Peanut allergy	4	M	9	1.4	55%	50%	45%	55%	39%	53%	1.9	75%
14	Peanut allergy	3	M	7	8.06	62%	55%	55%	50%	35%	60%	1.2	92%

was obtained using a protein extract of natural flaxseed, tested as a positive control (Fig. 3).

#### FTIR

Analysis by Fourier Transform Infrared (FTIR) spectrometry showed a modification in the spectrum at the 1,317 cm<sup>-1</sup> and 1,650 cm<sup>-1</sup> regions (alpha structure) and at the 1,242 cm<sup>-1</sup>, 1,620 cm<sup>-1</sup> and 1,640 cm<sup>-1</sup> regions (beta structures), after heating. Major changes between 1,560 and 1,570 cm<sup>-1</sup> were observed after heating (Fig. 4).

#### Discussion

Flaxseed (*Linum usitatissimum*) belongs to the Dicotyledone family. Although the risk of sensitization has already been

reported (16), clinical case reports are rare, and related to other seeds (in bread) or to flaxseed oil used as a laxative (11, 12). The presence of specific IgE has been demonstrated by RAST confirming positive prick tests (10). Cases of occupational asthma have been reported (17, 18).

Flaxseeds are rich in protein: 20% to 49% of the seed (Viking cultivar). 40% are albumins, 40% are globulins, 6.6% are prolamins and 13.5% are glutelins. The storage proteins are essentially of the legumin family (19).

Allergenicity to flaxseed has been studied in two cases of allergy to flaxseed, corresponding to reactive bands at 38, 35, 30, 22 and 20 kDa (11). Under non-reducing conditions, proteins of 150-175 kDa bind intensively to IgE, showing that there are many potentially allergenic subunits (10, 12). A 56 kDa allergen was identified in one

**Table 5** - Cross-reactivity evaluated by RAST inhibition of flaxseed by extracts of natural, heated and extruded flaxseed in the 14 sera with flaxseed RAST higher than 1 kU/L

Patients	Flaxseed RAST (kU/L)	Natural flaxseed inhibition (%)	Heated flaxseed inhibition (%)	Extruded flaxseed inhibition (%)
1	10.9	63	69	72
2	2.9	60	62	70
3	10.9	41	37	40
4	2.22	62	66	55
5	2.86	14	21	32
6	8.54	60	75	77
7	10	79	90	89
8	2.68	48	49	61
9	7.78	20	30	28
10	1.14	65	74	66
11	3.24	52	59	63
12	4.46	61	65	67
13	1.4	55	59	63
14	8.06	60	63	70

case and could be a dimer corresponding to the 28 kDa subunit. A malate dehydrogenase would be one candidate (12). It is possible that the band we observed at 25 kDa corresponded to this protein.

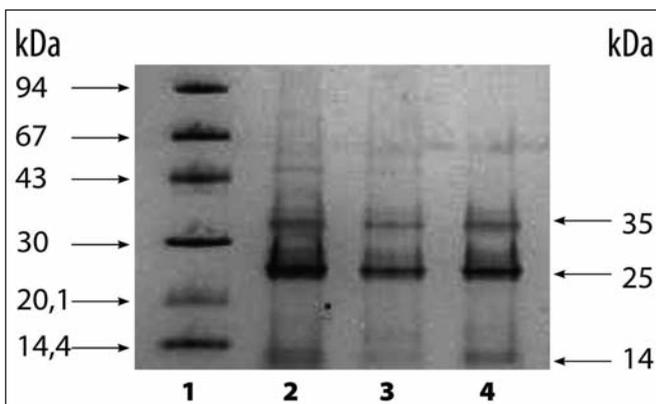
In this population of 1,317 patients consulting in the allergology department, 5.8% were sensitized to natural flaxseed. 54% of the 1,317 patients presented with atopic disease (food or respiratory allergy). Extrapolation to the French population, on the basis of an overall rate of 5% to 10% for atopic disease (respiratory allergies, atopic dermatitis, food allergies), produces an estimated prevalence of flaxseed sensitiza-

tion in the French population ranging from 0.54% to 1.08%. However, allergy to flaxseed is extremely rare (2 cases out of 1,317), corresponding to an estimated 0.01 to 0.02% of the population (1 allergic patient for 6,000 inhabitants).

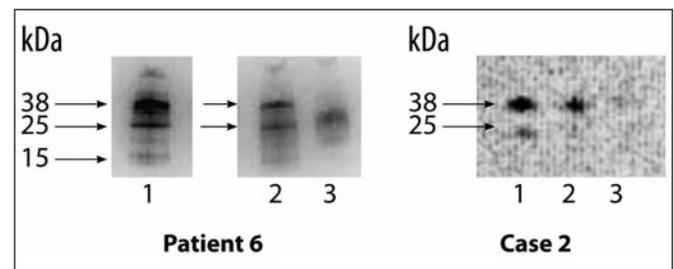
The frequency of positive prick tests to multiple airborne and/or food allergens indicates possible cross-reactions that could explain positive prick tests to flaxseed. There was marked positivity of RAST inhibition assays with different seeds. There was complete immunoblot inhibition to flaxseed by a peanut extract. This could indicate that part of the cross-reaction is related to the storage proteins that represent a large percentage of the total proteins. The protein composition of flaxseed, because of shared homologous proteins with the other seeds (lupine soy, rape seed wheat), explains the inhibition of flaxseed

**Figure 1** - SDS-PAGE (8-25%) of different protein extracts stained with Coomassie brilliant blue

Band 1: standard MWs  
Band 2: natural flaxseed  
Band 3: heated flaxseed  
Band 4: extruded flaxseed

**Figure 2** - Immunoblot carried out with one serum from a sensitized patient (patient 6) and one serum from a flaxseed-allergic patient (case 2)

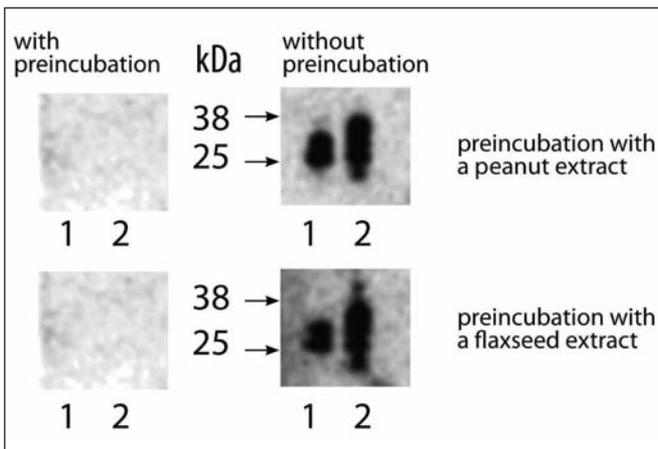
Band 1: natural flaxseed  
Band 2: heated flaxseed  
Band 3: extruded flaxseed



**Figure 3** - Inhibition of immunoblots conducted with the serum of a sensitized patient (Patient 6) with or without pre-incubation of serum with a peanut extract, or with a natural flaxseed extract.

Band 1: extruded flaxseed

Band 2: natural flaxseed



RAST by these seeds. However, further details cannot be discussed because of the use of a single dose inhibition. The frequency of pollen polysensitization and the constant RAST inhibition of flaxseed by bromelain suggest that cross-reactive carbohydrate determinants (CCD) are involved in these cross-reactions. In three cases, a discrepancy between the positive inhibition by bromelain and the negative RAST to bromelain might point to poor availability of CCD on the RAST matrix

Anti-CCD antibodies are relatively frequent, particularly in patients sensitized to pollen and hymenoptera venom (20-22). The clinical relevance of anti-CCD IgE is much discussed (23, 24). In general, these antibodies do not seem to be involved in food allergy (25). Besides, prick tests to vegetable glycosylated proteins are negative when the sensitization is exclusively directed towards CCD. In patients having positive PIP to natural flaxseed, negative PIP with heated and extruded products are observed. Indeed, FTIR spectrometry confirmed the profound modification of alpha and beta structures after heating and extrusion. These data support the hypothesis of sensitization to conformational epitopes.

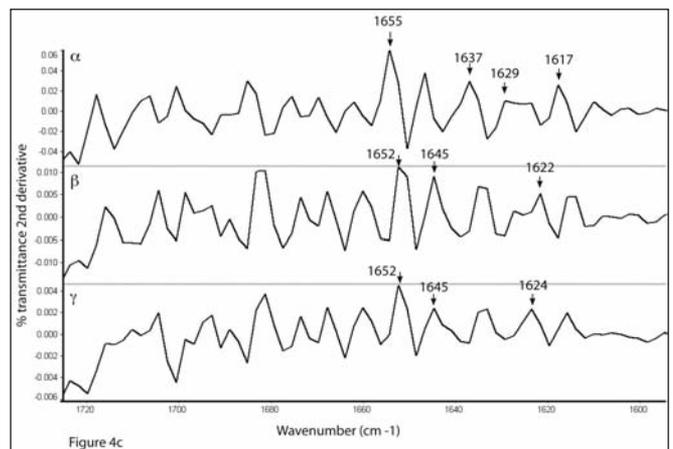
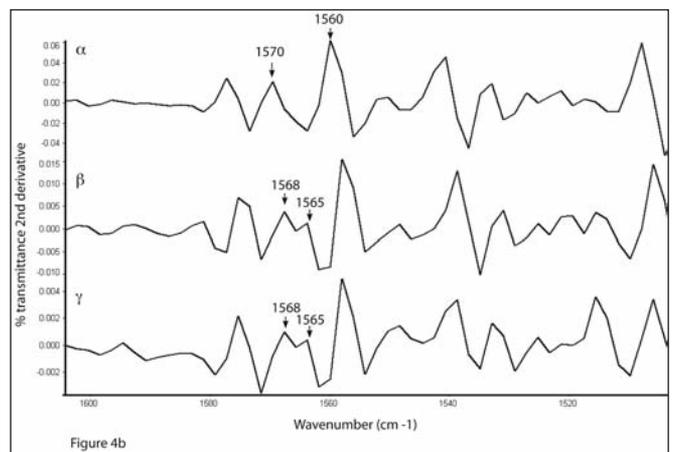
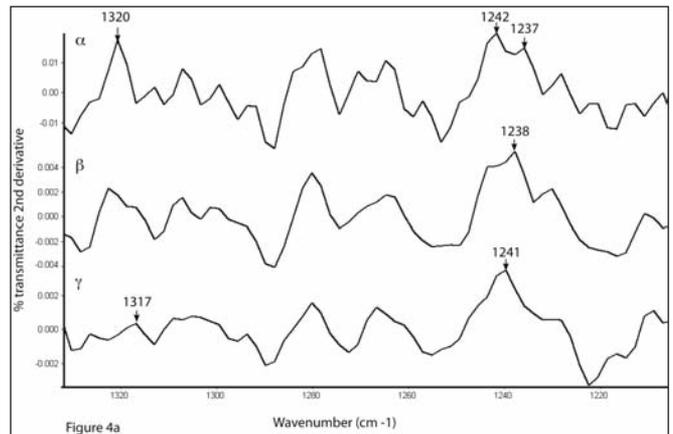
The patient allergic to flaxseed (case 2) had positive PIP tests with heated and extruded flaxseed. Immunoblot testing confirmed IgE binding to a 38 kDa band present in both heated and extruded flaxseed. Hence the basis of allergenicity could be related to thermostable peptides that

**Figure 4** - Fourier Transformed InfraRed (FTIR) spectroscopy Comparison of transmittance second-derivative spectra in the amide I (1,600-1,720 cm<sup>-1</sup>), amide II (1,500 -1,600 cm<sup>-1</sup>), and amide III (1,200 -1,350 cm<sup>-1</sup>) regions of natural, heated and extruded flaxseed.

$\alpha$ : Natural flaxseed

$\beta$ : Extruded flaxseed

$\gamma$ : Heated flaxseed



resist extrusion. This justifies the hypothesis of clinically relevant linear epitopes.

To conclude, the relative frequency of skin sensitization, demonstrated by PIP to natural flaxseed, seems to mainly reflect cross-reactivity, and corresponds to allergens that are not clinically relevant. These may be related to CCD in vegetable proteins, and/or to homologies between storage proteins in seeds. The latter would have to be studied. Only two out of 77 patients with positive PIP to natural flaxseed were allergic to flaxseed, documenting the assertion that PIP to natural flaxseed are of no use for diagnosis of flaxseed allergy. If flaxseed allergy is suspected, PIP should be carried out with heated flaxseed or extruded flaxseed obtained from industry.

Given the nutritional benefits of flaxseed, it is becoming increasingly popular in food preparations, indicating that structures like the Allergy Vigilance Network will have to monitor the potential clinical risk. No other case has been declared out of the 812 anaphylactic food reactions reported over the last 7 years (26). Studies of allergenicity of new foods have to include these natural foods but moreover must take into account the modifications of allergenicity induced by various food technologies.

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# The biological potency of different extracts for sublingual immunotherapy assessed by skin prick tests

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## KEY WORDS

*Sublingual immunotherapy;  
biological potency; skin prick test*

## SUMMARY

*The standardization of allergen extracts is of primary relevance to the clinical efficacy. Biological standardization procedures are widely used in the commercial production of vaccines. We tested, in grass-allergic patients, the potency of three different grass extracts for sublingual immunotherapy by means of skin prick tests. Specific IgE against Phl p 1, 2, 4, 5, 7, 11 and 12 were also assayed. Allerslit® and Sublivac® were directly applied as skin test. Grazax®, was prepared by dissolving two tablets in 2mL saline. Thirty-three subjects (mean age 38.8) were studied. The skin response was significantly different among extracts, decreasing from Allerslit to Grazax ( $t$  test  $<0.01$ ), but all the extract produced a skin response greater than histamine. All the subjects had specific IgE to Phl p 1 and Phl p 4 but 24% did not have specific IgE to Phl p 5. In those subjects the skin response to the three extracts did not differ from that of Phl p 5-positive subjects. Our findings confirm that there is a variability in the biological potency among different extracts. In addition, the standardization of grass extracts based on Phl p 5 only, may be insufficient in some cases.*

## Introduction

Sublingual Immunotherapy (SLIT) is now recognized as an effective treatment for respiratory allergy (1). It is widely used in most European countries, where numerous different products are commercially available. Due to the large heterogeneity of the commercial preparations, in term of doses and allergen content, one of the important aspects of SLIT still remains the standardization of products. The standardization, which is the reproducibility of the extracts, is mainly related to the concentration or content of the major allergen(s). This problem is of great clinical relevance for two main reason. First, the clinical efficacy and the safety of an extract is at a certain extent dose-dependent, as clearly shown in the recent dose-finding tri-

als with grass extracts (2, 3). Second, the knowledge of the exact allergenic content of each product would allow comparisons among products, a better definition of the dose-response aspects and would also provide a support for investigating the mechanisms (4). As usually done in the biological standardization procedures, the overall potency of an allergenic extract can be roughly evaluated by means of skin prick test. In fact, the biological standardization, which is based on skin reactions is still largely used among manufacturers, although for many extracts the content of allergen(s) in micrograms is currently available.

We performed an evaluation of the biological potency of three commercial extracts for SLIT, by means of skin prick tests (SPT), in subjects sensitised to grasses who were also evaluated by component resolved diagnosis.

## Methods

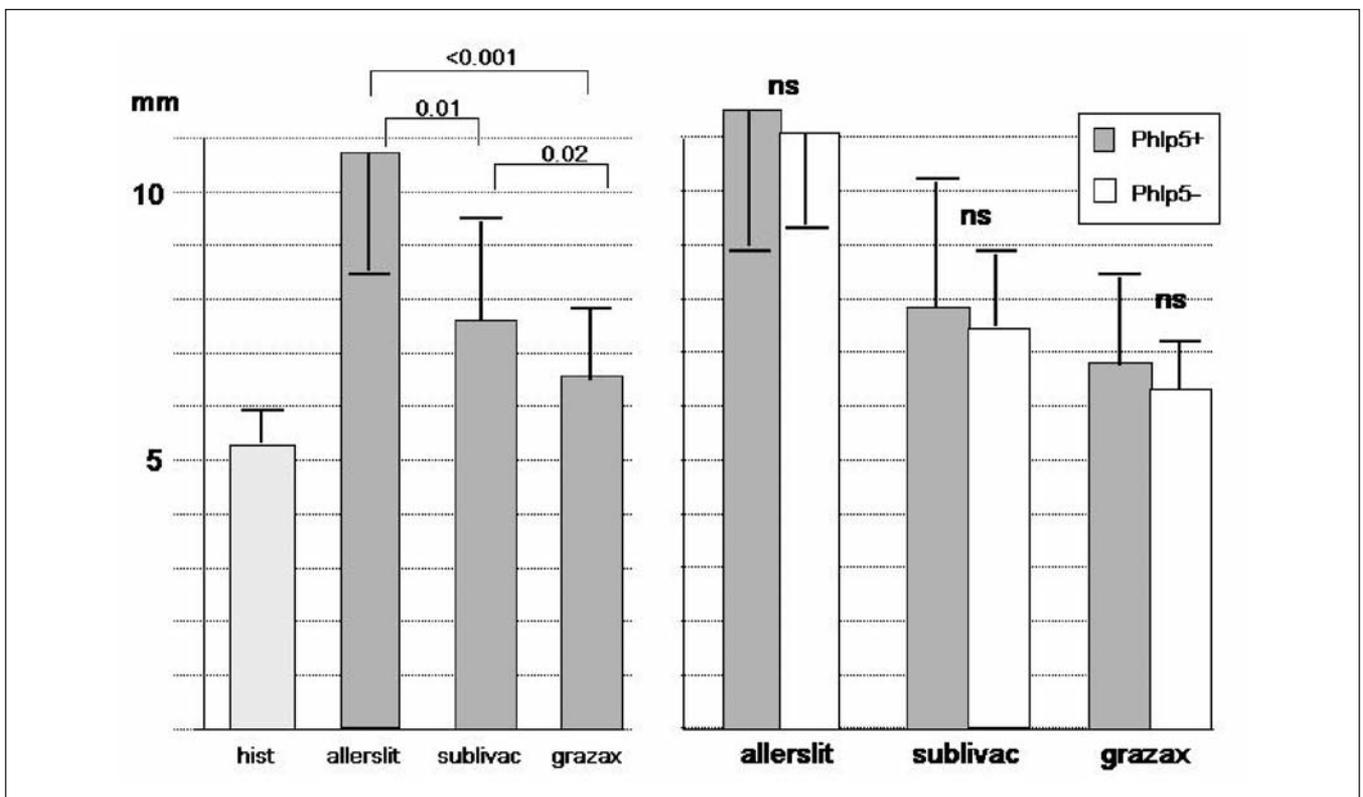
Adult patients with seasonal asthma and/or rhinoconjunctivitis underwent the standard diagnostic procedure including clinical history, examination, skin prick tests with commercial extracts (Stallergens Italy, Lainate, Milan), and total IgE assay. Those patients with sensitisation to grass pollen ad eligible for grass-specific immunotherapy underwent further investigations, as follows. An ImmunoCAP assay (Phadia SrL) was performed according to the manufacturer's instruction, to detect the presence of specific IgE to Phl p 1, 2, 4, 5, 7, 11 and 12. The results of the assay were expressed in kUA/l. Additional skin tests were performed with three commercial extracts: Allerslit (Allergopharma, D), Grazax (Alk-Abellø, DK) and Sublivac (Hal Allergy, NL). Allerslit (40 µg/mL Phl p 5) and Sublivac (1,000 AU/mL) are prepared as solution, thus they were used as in a standard skin test. Grazax, that is a tablet formulation, was prepared by dissolving two 75000 SQ-T tablets in 2 ml of saline. This produced a solution containing 15 µg/mL Phl p 5. The fresh solution was

then used within 12 hours. The results were expressed as the mean of the major diameter of the wheal plus its orthogonal. Histamine HCl 0.1% and saline were used as positive and negative controls.

## Results

Thirty-three subjects (17 male, age range 9-62 years, mean age 38.8) were studied. Of them, 22 had rhinoconjunctivitis alone and 11 had also asthma. Their mean total IgE level was  $271 \pm 32$  kU/L. The results of the skin prick with the three SLIT extracts are reported in figure 1 (left). It could be noticed that the skin response was significantly different among extracts, in decreasing order from Allerslit to Grazax (t test  $<0.01$ ). On the other hand, the skin response was significantly greater than with histamine for each of the three extract ( $p < 0.05$ ). The percentages of patients with positive specific IgE to each grass pollen allergen, as assessed by the component resolved diagnosis, was as follows: Phl p 1 =100%, Phl p 2

**Figure 1** - Left: mean and SD wheal diameter with the three extracts in the whole population (n=33). Right: mean and SD wheal diameter with the three extracts in patients with (n=25) or without (n=8) specific IgE to Phl p 5



= 66%, Phl p 4 =100%, Phl p 5 =76%, Phl p 6 =66%, Phl p 7 =3%, Phl p 11 = 50%, Phl p 12= 45%. Of note, 8/33 (24%) patients had undetectable specific IgE to Phl p 5, but their skin response to the three extracts did not differ from that of Phl p 5 positive subjects, as shown in figure 1 (right).

## Discussion

Overall, our results confirm that some differences in the biological potency exist among commercial extracts. This probably reflects the largely variable content of proteins among products, as recently shown in comparison studies (5, 6). This should be true also for sublingual vaccines under investigation and their content in relevant grass pollen allergens. This fact may have consequences in terms of immunological and clinical response, as well as in terms of possible adverse reactions (4). It is true that the preparation of the solution from the Grazax tablets may have introduced an artefact, since the resulting solution does not exactly reproduce the concentration of allergens in the tablets. Nevertheless, since the volume of a tablet is approximately one mL, the error cannot be expected to be great. The IgE sensitisation to the major allergens largely contribute to patients' skin reactivity since all the patients had specific IgE to Phl p 1. More interestingly, 24% of the patients did not have specific IgE to Phl p 5, that is considered a major allergen (7, 8), but their skin reactivity was not decreased. The concomitant presence of other antigenically relevant proteins in the extracts may reasonably explain the observation. Nevertheless, almost all the commercial products are standard-

ized according to their content in Phl p 5. As a consequence, the standardization based on only one allergenic protein may be considered imprecise or incomplete, since several proteins are involved in the sensitisation and, therefore, intervene in the mechanism of action of immunotherapy.

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# Long-lasting effect of a monophosphoryl lipid- adjuvanted immunotherapy to parietaria. A controlled field study

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## KEY WORDS

*Monophosphoryl lipid adjuvant, immunotherapy, long lasting effect, rhinitis, asthma.*

## SUMMARY

**Background:** The clinical efficacy of Monophosphoryl lipid A-adjuvanted immunotherapy (MPLA-SCIT) is ascertained, but there are no data on its possible long-lasting effect. We assessed in a real-life setting the persistence of the clinical effect five years after discontinuation. **Methods:** Patients with parietaria-induced respiratory allergy and fulfilling the criteria for immunotherapy prescription were evaluated at baseline, after the third year of MPLA-SCIT and five years after discontinuation. Visual analog scores, severity of the disease, pulmonary function and skin reactivity were assessed. Matched subjects who refused immunotherapy served as controls. **Results:** Twenty nine patients received MPLA-SCIT and 28 were the control group. There was a significant clinical improvement, as assessed by VAS only in the active group after 3 years that remained significant at 5 years versus baseline and controls. The distribution of severity of rhinitis was overall decreased at 3 and 8 years as well. The number of patients with conjunctivitis in the active group decreased from 19 to 6 at the end of the treatment and to 9 after 5 years. There was also a decrease in the number of patients with asthma symptoms (from 6 to 2 to 4), which doubled in the control group. A significant reduction in the wheal of the Parietaria skin test was seen in the active group at the end of the treatment ( $9.5 \pm 2.1$  mm VS  $6.4 \pm 2.6$  mm;  $p = .01$ ), but this reduction was lost at the 5-year. No relevant change was overall detected in pulmonary function. **Conclusion:** MPLA-SCIT is effective, and the clinical efficacy is maintained after 5 years of discontinuation.

## Introduction

Allergen specific immunotherapy (SIT) is widely used in the management of respiratory allergy, and the subcutaneous administration (SCIT) has represented the standard treatment for one century (1). During the last twenty years, numerous attempts were made to improve both the safety of SCIT (e.g. by the chemical modification of allergens to obtain allergoids)(2), or by changing the modality of administration (e.g. intralymphatic injection) (3). Another field of research was aimed at improving the

efficacy, to obtain favourable clinical effects with smaller amounts of allergens. One of this approaches involves the administration of adjuvants together with the allergen. Adjuvants are non-immunogenic molecules that enhance the immunological effects of the antigen, but to be suitable for the human use, they should obviously be non toxic and non irritating. The monophosphoryl lipid A (MPLA), derived by the cell wall of a non-pathogenic Salmonella, was identified as an ideal candidate for this use. MPLA was demonstrated capable to enhance the Th1 response, and to increase the response to allergens

(4-6). Therefore, a MPLA-adjuvanted immunotherapy was prepared, tested in clinical trials (7, 8) and subsequently commercialised. Nowadays, MPLA-SCIT is widely used in routine clinical practice in many European countries.

In addition to the well-known clinical effects, namely the reduction of symptoms and/or medication intake, specific immunotherapy possesses additional properties, including the prevention of the onset new sensitisations and of the development of asthma. One of the most important additional properties is the long-lasting effect, that is the persistence of the clinical benefit for several years after discontinuation (9). The long-lasting effect can be interpreted as the result of a profound immunological modification of the immune response, with a Th1 skew partly mediated by allergen-specific T regulatory cells (10). The persistence of the clinical benefit has been consistently confirmed for the traditional SCIT in several trials (11), but there is so far no data on MPLA-SCIT. Based on this, we performed a prospective controlled trial to assess if MPLA-adjuvanted immunotherapy exerts a long-lasting effect.

## Methods

The trial was designed as prospective, open, and nonrandomized. Patients with respiratory allergy due to Parietaria were prescribed a 3-year course of MPLA-SCIT. They were assessed for clinical characteristic at baseline, at the end of immunotherapy and 5 years later. A matched group of patients who refused SCIT served as controls.

Inclusion criteria were those recommended by guidelines for the prescription of immunotherapy: i) mild persistent or moderate/severe rhinitis (12) with or without asthma, ii) proven sensitisation to parietaria (assessed by skin prick test or CAP RAST), iii) presence of symptoms during the Parietaria season. Exclusion criteria were severe or uncontrolled asthma, mild intermittent rhinitis, previous courses of specific immunotherapy for Parietaria. Patients with malignancies, systemic immune diseases or major anatomical abnormalities of the nose were also excluded, as recommended by guidelines (1).

The MPLA-SCIT was given according to the manufacturer's instructions, with four pre-seasonal injections given at weekly intervals, at the doses of 300, 800, 1,000 and 2,000 units. The course was repeated before the season for 3 years. All the subjects received the same standard drug

therapy for the treatment of rhinitis, conjunctivitis and asthma, according to current guidelines (12, 13). All patients, receiving or not MPLA-SCIT, underwent the same diagnostic procedures at baseline, after 3 years (end of SCIT) and after 8 years from baseline (5-year discontinuation). The following evaluations were made:

- clinical assessment of the presence and severity of rhinitis, conjunctivitis and asthma. Rhinitis was classified as mild intermittent or persistent, and moderate/severe intermittent or persistent according to ARIA (12). Asthma was graded in severity according to GINA (13);
- skin prick test with a standard panel of allergens including mites, grasses, parietaria, olive, birch, cypress, ragweed, cat and dog dander. The skin test for Parietaria was performed in duplicate and the mean diameter of the wheal (major diameter plus orthogonal) was recorded;
- pulmonary function test, to record FEV<sub>1</sub> and FVC;
- visual analog scale (VAS). This consisted of a 10 cm line, where the patients had to mark their perceived well-being during the past season, from 0 (very troublesome symptoms) to 10 (total absence of symptoms).

Statistical analysis was performed by a computerized program. Student's t test was used for inter-group and intra-group comparisons. The chi-square test was applied to categorical data.

## Results

Twenty nine patients (15 male, mean age 33.4 years, age range 18-60) were prescribed MPLA-SCIT. Twenty-eight matched patients (13 male, mean age 34.1 years, age range 10-59), who refused SCIT served as control group. The two groups resulted to be homogeneous for the clinical and demographic characteristics at baseline (Tab. 1). In particular, asthma was present in 19% and 20%, and conjunctivitis in 65% and 66% of the active and placebo group, respectively. The rate of monosensitized subjects was slightly greater in the control group (53% vs 41%;  $p = .02$ ). The pattern of skin positivities in the polysensitized subjects is shown in Table 2. There was a significant clinical improvement, as assessed by VAS, only in the active group after 3 years, whereas no change was seen in the control patients. The clinical improvement versus baseline and versus the end of SCIT was maintained at the 5-years assessment (Fig. 1). Also, the distribution of severity of rhinitis was overall decreased at 3 and 8 years in the active group, with no apparent change in the controls (Fig. 2). Of note, the number of patients with conjunctivitis in the

**Table 1** - Baseline characteristics of the two groups

	MPLA-SCIT n=29	CONTROLS n=28	P chi square
Mean age ± SD	33.4±11.8	34.1±13	NS *
Age range	18-60	10-59	-
Male (%)	15 (52)	13 (46)	NS
Conjunctivitis (%)	19 (65)	18 (66)	NS
Asthma (%)	6 (19)	6 (20)	NS
Mild persistent rhinitis (%)	6 (19)	6 (20)	NS
Moderate/sev intermittent rhinitis (%)	0	2 (5)	NS
Moderate/sev persistent rhinitis (%)	23 (62)	20 (75)	NS
Mean FEV1% ± SD	100±.8	96±9	NS *
Mean FVC% ± SD	101±9	100±10	NS *
Parietaria wheal mean diameter ± SD	9.5±2.1	8.9±1.6	NS *
VAS score ± SD	2.9±1.5	2.7±1.1	NS *
Monosensitized	12 (41)	15 (53)	.02

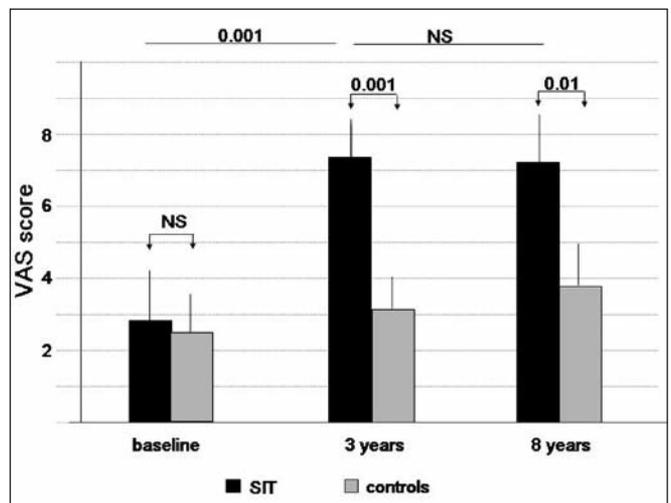
\* Student's t

**Table 2** - Pattern of sensitisation in polysensitized subjects

	MPLA-SLIT n = 17	CONTROLS n = 13
Grass, n (%)	6 (35)	4 (30)
Mite, n (%)	5 (29)	3 (23)
Olive, n (%)	5 (29)	3 (23)
Cypress, n (%)	2 (12)	2 (15)
Hazelnut, n (%)	3 (18)	1 (8)
Cat, n (%)	2 (12)	3 (23)
Mugwort, n (%)	2 (12)	2 (15)

active group decreased from 19 to 6 at the end of the treatment and was of 9 after 5 years of discontinuation. In parallel, there was a decrease in the number of patients with asthma symptoms (from 6 to 2 to 4). In the control group there was no change in the number of patients with conjunctivitis, whereas those with asthma were doubled after 8 years. A significant reduction in the mean wheal diameter of the Parietaria skin test was seen in the active group at the end of the MPLA-SCIT (9.5±2.1 mm VS 6.4±2.6 mm; p=.01), but this reduction was lost at the 5-year follow up (9.5±2.1 mm VS 8.4±2.7mm; p= NS). No change was observed in the control group (8.9±1.6 mm VS 8.8±1.1 mm VS 8.1±1.5 mm). Concerning the spirometric parameters, in the control group there was a slight decline over time in the FEV<sub>1</sub> and FVC, but a significant

**Figure 1** - Visual Analog Scale score (mean and SD) at the three time-points. The 3-year point corresponds in the SCIT group to the end of the treatment. The significant differences are plotted above the bars



difference was detected only for FEV<sub>1</sub> at 8 years versus baseline (Fig. 3). At the end of the follow up only one out of the 12 monosensitized patients in the active group had developed new sensitizations, whereas this happened in 6/15 monosensitized of the control group. The behaviour of the main clinical parameters during follow-up is shown in table 3. The treatment was overall well tolerated. Only

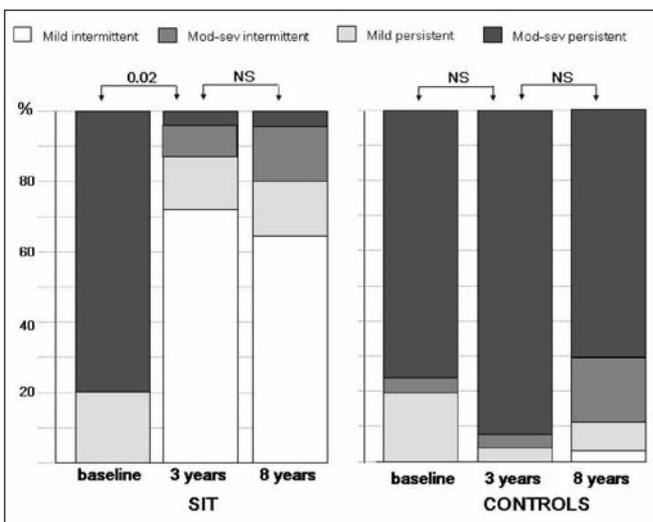
four patients displayed a large local reaction during the first course with the 2,000 U dose. The dose was repeated the next week without further problems.

**Discussion**

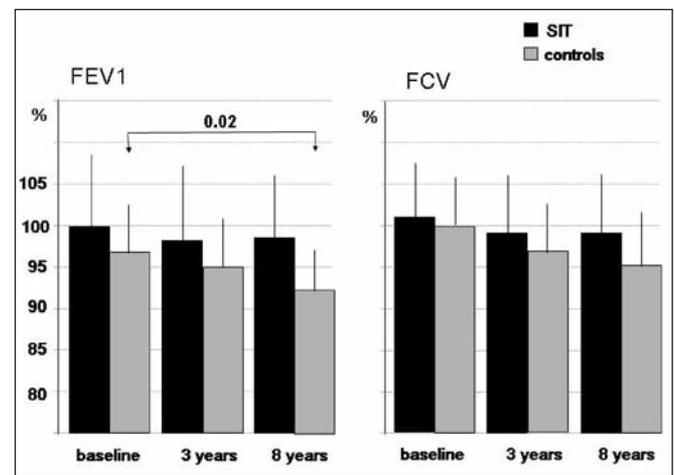
The so-called “additional effects” of immunotherapy, namely the preventive action and the long-term persistence of the clinical effects, make this treatment profoundly different from the standard medications. In particular, the long-lasting effect was clearly demonstrated for the traditional SCIT (for review see 9, 11), although only few randomized double blind trials were specifically

designed to assess this effect (14-17). No datum is so far available for the adjuvanted SCIT, which is largely used in clinical practice since about ten years. Thus, we designed this study to evaluate the persistence of the clinical benefit of MPLA-SCIT for Parietaria. In order to have available all the data in the long-term, the design had to be kept as simple as possible, and adherent to the real-life. For this reason, only those parameters which are usually employed to evaluate the effects of immunotherapy were chosen, namely the clinical assessment of disease’s severity (1) and the patient’s reported perception of the well being. The main limitation of the study stands in the open non randomized design. Nonetheless, in real life, it is not feasible to have a double blind approach maintained for such a long time, and in fact an open design was used in

**Figure 2** - Percentage distribution of the severity of rhinitis (according to ARIA) at the three time-points in the SCIT and control groups. Chi-square p values are reported above the bars



**Figure 3** - Mean (SD) values of FEV1 and FVC measured at the three timepoints. Significant p value differences are reported above the bars



**Table 3** - Summary of the clinical characteristics of the patients in the two groups

	SCIT (N= 29)			Controls (N=28)		
	Baseline	3 yrs	8 yrs	Baseline	3 yrs	8 yrs
Conjunctivitis (%)	19 (65)	6 (20)	9 (31)	18 (66)	21 (75)	21 (75)
Asthma (%)	6 (20)	2 (7)	4 (14)	6 (20)	9	12
Mild intermittent (%)	0	21 (72)	19 (65)	0	0	2 (7)
Mild persistent (%)	6 (20)	4 (14)	4 (14)	6 (20)	1 (3)	3 (10)
Mod/sev intermitt (%)	0	3 (11)	5 (8)	2 (7)	1 (3)	4 (14)
Mod/sev persist (%)	23 (80)	1 (3)	1 (3)	20 (73)	26 (94)	19 (68)
Monosensitized (%)	12 (41)	11 (38)	11 (38)	15 (53)	12 (43)	9 (32)
Wheal mean diameter	9.5±2.1	6.4±2.6	8.4±2.7	8.9±1.6	8.8±1.4	8.1±1.5

other studies to assess the long-term effect of sublingual immunotherapy (18). For the same reasons, it was not feasible to have a detailed recording of the medication intake, although this would have added a relevant and confirmatory information. In addition, the study was not designed to assess the efficacy of the treatment, but the persistence of the effects on the long term. Finally, the absence of a randomization is counterbalanced by the ascertained homogeneity of the two groups at baseline. As far as the clinical parameters are concerned, it was observed that the overall severity of the disease (rhinoconjunctivitis) was decreased after MPLA-SCIT, and that the improvement was maintained at 5 years after discontinuation. The reduction of the skin reactivity at the 3rd year can be interpreted as an indirect marker of the immunological effect. This is also corroborated by the prevention of the onset of new sensitisations in monosensitized patients after discontinuation, which is similar to that described elsewhere (19).

In conclusion also the relatively new immunotherapy with an MPLA adjuvant results to have a long-lasting carry-over effect, which is similar to that of traditional SCIT. This encourages the use of this modality of treatment (20), in order to combine a good safety profile (21) with the achievement of beneficial effects for years after the discontinuation.

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# The prevalence of nasal polyps and the corresponding urinary $LTE_4$ levels in severe compared to mild and moderate asthma

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## KEY WORDS

*Nasal polyps; severe asthma; asa-intolerance; cysteinil leukotrienes*

## SUMMARY

**Background:** Several comorbid conditions may contribute to worsening asthma symptoms, including nasal polyps (NPs). Cysteinyl leukotrienes (Cys-LTs) play a crucial role in asthma pathophysiology, and specific receptors for CysLTs are reported as up-regulated in nasal polyp tissues. The aim of the present study was to assess the prevalence of nasal polyps in severe vs mild and moderate asthma, and to compare the corresponding levels of urinary Leukotriene E<sub>4</sub> ( $LTE_4$ ). **Materials and methods:** A cohort of 386 asthma patients were studied: n=166 with mild, n=146 with moderate and n=74 severe asthma. All patients performed a nasal endoscopy and urine were collected in the morning for the quantitative  $LTE_4$  immunoenzymatic assay (Cayman Chemical, Mi, USA). Intolerance to ASA was also assessed by means of a nasal provocation test with L-ASA. **Results:** The prevalence of NPs was the following: 8 cases (4.8%) in mild; 14 (9.6%) in moderate, and 33 (44.6%) in severe asthma. Mean urinary  $LTE_4$  levels were increasing according to the disease severity. ASA-intolerance was assessed in 1 patient in mild asthma (0.6%), 14 in moderate asthma (9.6%) and 28 in severe asthma (37.8%). **Conclusions:** Nasal polyps represent a comorbid which is highly frequent in severe asthma. Both their prevalence and the corresponding mean  $LTE_4$  levels in urine proved in strict, direct relationship with asthma severity. In severe asthma, nasal polyps represent a condition which is associated with the highest excretion of urinary  $LTE_4$  and ASA intolerance.

## Introduction

GINA guidelines define severe asthma as a condition in which any of the following are manifest: continuous symptoms prior to treatment; frequent exacerbations and nocturnal symptoms; impairment of lung function ( $FEV_1 \leq 60\%$  predicted or  $PEF \leq 60\%$  of personal best, and  $PEF$  variability  $\geq 30\%$ ) (1).

Severe asthma, although present in a relatively small proportion of the whole asthma population, comprises those subjects with the highest morbidity and who are in partic-

ular need of careful evaluation (2). Bronchial asthma is a costly disease and the related social impact is ever increasing, particularly in terms of indirect costs in severe asthma (3).

A number of pathological factors may contribute to poor control of asthma, including nasal polyps (NPs), which usually affect asthma severity in real life (4). Generally speaking, NPs consist in the prolapse of the mucosal lining of the nose and nasal sinuses (in particular from the lining of the ethmoid sinuses), which protrude down into the middle meatus and present as a smooth, round, pale

and translucent swelling, and are characterized by infiltration of the mucosa with eosinophils, lymphocytes and mast cells (5). NPs are not only associated with atopy but also with asthma; aspirin intolerance; cystic fibrosis; allergic fungal sinusitis, and Churg-Strauss syndrome (6).

Cysteinyl leukotrienes (cys-LTs), namely leukotriene C<sub>4</sub> (LTC<sub>4</sub>), LTD<sub>4</sub> and LTE<sub>4</sub>, play an extremely important role in the pathophysiology of asthma. Cys-LTs cause potent bronchoconstriction; mucosal edema; vasodilatation; vascular permeability and increased mucus secretion within the airways of asthmatic patients (7). In particular, LTE<sub>4</sub> has been identified as a major metabolite of LTC<sub>4</sub>, and urinary LTE<sub>4</sub> (u-LTE<sub>4</sub>) is now regarded as the most reliable analytic parameter for monitoring the endogenous synthesis of cys-LTs (8).

Basal urinary levels of LTE<sub>4</sub> excretion are significantly higher in aspirin-intolerant asthma patients (AIA) than in aspirin-tolerant asthma subjects (9). Interaction of CysL-Ts receptors (Cys-LT1 and Cys-LT2) with their ligand LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> play a disease-regulating role also in chronic rhinosinusitis (CHRS) and NPs, particularly in the aspirin intolerance syndrome, which is often related to these conditions (9). Cys-LTs receptors have been described as up-regulated in nasal polyp tissue, and their expression is related with eosinophilic inflammation (10).

CHRS/NPs are involved in cys-LTs overproduction of asthmatic patients and are not strictly associated with aspirin intolerance itself but rather with clinical features (11). Urinary LTE<sub>4</sub> excretion proves directly proportional to the extent of nasal structural changes occurring in ASA-intolerant asthmatics, being subjects with NPs those with the highest LTE<sub>4</sub> values, immediately followed by those with hypertrophic rhinitis (12).

The prevalence of NPs is considered to be around 4% in the general population, but near 30% in patients with NPs and asthma (13); nevertheless, their true prevalence in severe asthma is still unknown.

The aim of the present study was to assess the incidence of NPs in severe vs mild and moderate asthma, and to compare the corresponding levels of urinary Leukotriene E<sub>4</sub> (LTE<sub>4</sub>).

## Materials and methods

A cohort of studied 386 asthma patients was studied and divided in: n=166 with mild (80 males; 18 -76 years; FEV<sub>1</sub>= 85.6 % pred. ± 8.9 sd); n=146 with moderate (72

males; 19 - 73 years; FEV<sub>1</sub>= 69.7% pred. ± 10.1 sd), and n=74 with severe asthma (23 males; 21 - 69 years; FEV<sub>1</sub>= 55.1% pred. ± 7.3 sd), according to GINA guidelines.

All patients performed a nasal endoscopy according to the European Position Paper on Rhinosinusitis and Nasal Polyps (14).

## Urinary LTE<sub>4</sub>

Urinary LTE<sub>4</sub> concentration was measured by enzyme immunoassay (ACETM Competitive Enzyme Immunoassay, Cayman Chemical, Ann Arbor, Mich, USA), as reported by Pradelles et al (15). This assay is based on the competition between LTE<sub>4</sub> and an LTE<sub>4</sub>-acetylcholinesterase (AChE) conjugate (LTE<sub>4</sub> tracer) for a limited amount of LTE<sub>4</sub> antiserum. Because the concentration of the LTE<sub>4</sub> tracer is held constant while the concentration of LTE<sub>4</sub> varies, the amount of LTE<sub>4</sub> tracer that is able to bind to the LTE<sub>4</sub> antiserum will be inversely proportional to the concentration of LTE<sub>4</sub> in the well. This antibody-LTE<sub>4</sub> complex blinds to a mouse monoclonal anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent (which contains the substrate to AChE) is added to the well. This reagent consists of acetylthiocholine and 5,5'-dithio-bis-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by AChE produces thiocholine. The non-enzymatic reaction of thiocholine with 5,5'-dithio-bis-(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm. AChE has several advantages over other enzymes commonly used for enzyme immunoassays. Unlike horseradish peroxidase, AChE does not self-inactivate during turnover. In addition, the enzyme is highly stable under the assay conditions, has a wide pH range (pH 5-10), and is not inhibited by common buffer salts and preservatives. The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412 nm. The intensity of this colour, determined spectrophotometrically, is proportional to the amount of LTE<sub>4</sub> tracer bound to the well, which is inversely proportional to the amount of free LTE<sub>4</sub> present in the well. LTE<sub>4</sub> was measured by enzyme immunoassay on all samples according to the manufacturers' instructions and expressed in pg/mg creatinine (pg/mg). Because treatment with β<sub>2</sub>-agonists or with anti-inflammatory drugs, including oral or inhaled corticosteroids, sodium cromoglycate, or oral leukotriene receptor antagonists themselves, does not affect u-LTE<sub>4</sub> levels (16), these

medications were not withheld at the time of the urine sample collection in this study.

### Nasal Provocation test with L-ASA

Each patient performed the NPT according to the method described by Casadevall and co-workers by means of the acoustic rhinomanometry (12, 17). This method consists in the measurement of acoustic reflections from the nasal cavity of a sound pulse created by a spark in a sound tube connected to the nasal cavity via a nosepiece. Unlike conventional rhinomanometry, acoustic rhinomanometry does not require generation of nasal flow, and therefore its use is less limited by the presence of nasal polyps and nasal obstruction. The response was evaluated by the EccoVision Acoustic Rhinomanometry System (TM Hood Laboratories, USA) with the measurement of (18):

- 1) calculated resistance, based on a tube with the same area and laminar flow (REQ, mmH<sub>2</sub>O/l/min);
- 2) the total volume of the nostrils (VOL, cm<sup>3</sup>) represents the nasal cavity volume in the analysis segment;
- 3) the minimal cross sectional area (cm<sup>2</sup>);
- 4) its distance from the nosepiece (cm).

Rhinomanometric measurements were performed while the subject was in apnoea after a non-forced expiration. The rhinomanometer was calibrated daily with a calibration tube provided by the manufacturer. The analysis of data was performed using the Kwikstat program (TM Texasoft). Baseline nasal function was measured with Acoustic Rhinomanometry, while the subjects were in a sitting position, then 80 µl of L-ASA solution (180 mg/ml L-ASA) was applied locally through nose droplets on the inferior nasal concha in both nostrils. The total deposited dose of L-ASA was equivalent to 25 mg of acetylsalicylic acid. Acoustic Rhinomanometry was then performed bilaterally every 10 minutes for the next 2 hours. L-ASA was prepared freshly each day by dissolving crystalline L-ASA in 0.9% sodium chloride to produce a solution containing 180 mg/ml.

NPT was considered positive when: nasal resistance increased more than 40% in at least one nostril as compared with the corresponding baseline value; when the volume of one nostril decreased more than 10% from baseline; both parameters sustained for at least two consecutive measurements, and were accompanied by clinical symptoms persisting at least 30 minutes. The dose of aspirin, the duration of the observation period, and criteria for positivity of the test were established on the basis of previous experiments (12, 17).

Pulmonary function (FEV<sub>1</sub>; forced expiratory volume in 1 sec) was measured simultaneously by means of a computerized pneumotachograph (Masterlab, Jaeger). The maximal fall in REQ, VOL and FEV<sub>1</sub> observed in the 2 hours following the NPT were calculated.

### Statistical analysis

Mean values ± sd obtained before and after NPT for each variable were compared by t test, and p<0.05 was assumed as the lowest limit for the statistical significance.

### Results

The prevalence of nasal polyps was: 8 cases (4.8%) in mild asthma; 14 (9.6%) in moderate asthma, and 33 (44.6%) in severe asthma (Tab. 1).

According to a semi-quantitative score for classification (14), the endoscopic appearance score was: 7,6 ± 2,3sd in mild asthma; 10,1 ± 1,9sd in moderate asthma, and 12,6 ± 2,2sd in severe asthma, respectively (severe vs moderate p<0.001; moderate vs mild p< 0.05).

Furthermore, in severe asthmatics, nasal polyps were much more frequent and also more clinically relevant, such as characterized by a more frequent bilateral occurrence, by the presence of severe oedema and consistent discharge.

**Table 1** - Prevalence of nasal polyps

	Polyps n.	Polyps %	Prevalence AIA	u-LTE <sub>4</sub> (pg/ml)
Mild asthma	8/166	4.8	0.6%	129.1±74.8
Moderate asthma	14/146	9.6	1.4%	330.7±72.3 °
Severe asthma	33/74	44.6	37.8%	432.3±88.1 * ^

\* p<0.001 Severe vs Mild; ^ p<0.001 Severe vs Moderate; ° p< 0.001 Moderate vs Mild asthma

Urinary LTE<sub>4</sub> levels proved to increase according to asthma severity: 129.1 pg/ml ± 74.8sd in mild asthma; 330.7 pg/ml ± 72.3 sd in moderate (severe vs mild p<0.001), and 432.3 pg/ml ± 88.1 sd in severe asthma (severe vs moderate p<0.001; moderate vs mild p< 0.001).

Moreover, the higher proportion of ASA intolerance (37.8%) was found in severe asthma, vs 1,4% in moderate and 0,6% in mild asthma.

## Discussion

Severe asthma is not a frequent condition in the whole population of asthma patients, even though its burden in terms of health care utilization; hospitalization or access to emergency department, and frequent exacerbations is quite elevated (3).

A disproportionately large amount of the total cost of illness is generated by a relatively small proportion of patients with severe symptoms. The underlying structural changes still are not well understood (in particular their response to inhaled corticosteroids), but probably comorbidities play a crucial role in deteriorating lung function and quality of life. Some patients may continue to require high-intensity asthma treatment because of persistent symptoms owing to particular conditions, such as rhinosinusitis, gastro-oesophageal reflux or psychosocial problems, despite the best available therapeutic strategies for managing these conditions (19).

Results of present study confirmed the high incidence of ASA-intolerance in severe asthma. AIA develops according to a characteristic sequence of symptoms characterized by the presence of: persistent rhinitis (which usually starts at an average age of 30 years and which is followed by asthma); ASA sensitivity, and nasal polyps (9). Rhinorrhea and nasal congestion are usually the earliest symptoms of AIA, which generally persist and become perennial; they are difficult to treat, and frequently combine with recurrent or chronic sinusitis, anosmia, and nasal polyps. Asthma and ASA sensitivity tend to become manifest later (an average of 1 to 5 years after the onset of rhinitis), being AIA-patients with nasal polyps a much more difficult clinical condition to treat when compared to that of subjects with AIA-induced rhinitis only.

Clinical signs of AIA are bronchoconstriction and, in many cases, naso-ocular, gastrointestinal, and/or skin reactions, which occur shortly after ingestion of ASA or of non-steroidal anti-inflammatory compounds. As patients with AIA are intolerant to all drugs that inhibit cyclooxy-

genase, it has been postulated that this sensitivity may stem from the up-regulation of the 5-lipoxygenase pathway of arachidonic acid metabolism with the resultant production of mediators such as the cys-LTs. In particular, genetic predisposition to cys-LTs pathway up-regulation can be related to the overactive expression of the LTC<sub>4</sub>S-444C allele (20). AIA is therefore associated with an elevated urinary excretion of LTE<sub>4</sub>, both in basal conditions and after ingestion, inhalation (9), or nasal instillation (21) of ASA.

Most of the proinflammatory actions of CysLTs are mediated by their binding to the Cys-LT<sub>1</sub> receptors (22-23). CysLT<sub>1</sub>-receptor antagonists attenuate asthma which is elicited by aspirin challenge in aspirin-sensitive subjects (24-25), even though the elevated number of nasal inflammatory leukocytes expressing Cys-LTs receptors in ASA-sensitive patients with chronic rhino-sinusitis is probably also critical in the pathogenesis of aspirin sensitivity (26).

The underlying role of Cys-LTs is confirmed by the positive effect of Montelukast, a selective Cys-LT<sub>1</sub> receptor antagonist, on nasal function, nasal reactivity to L-ASA, and on blood markers of eosinophilic inflammation in mild- to-moderate AIA in the presence of nasal symptoms (27).

Even though the number of eosinophils, neutrophils and plasma cells in NPs is significantly higher than in nasal mucosa, NPs represent a comorbid characterised by an extremely high level of systemic inflammation and for this reason they should be always taken into account in the management of severe asthma. Then, the role of NPs in triggering severe asthma should not be mainly attributed to the trivial occurrence of a mechanical post-nasal drip, but mainly to the occurrence of a sustained local and systemic inflammation. It is easily mirrored by the high levels of urinary LTs and of other plasma eosinophilic mediators measurable in these conditions.

As NPs confirm to represent a true risk factor for increasing asthma severity, this evidence supports the need of a much more careful assessment of asthma patients, with particular attention to their nasal conditions. Actually, in order to define the most convenient therapeutic strategy and to obtain the best control of the disease, the united assessment of airways (such as proximal and distal) should be mandatory in all asthma patients.

In conclusion, nasal polyps represent a comorbid condition which is more highly frequent in severe asthma. For a better disease management it is crucial to investigate the presence of concomitant disorders, such as ASA-intolerance and NPs which can exacerbate asthma. Their preva-

lence, together with their urinary LTE<sub>4</sub> concentrations, increases in strict relationship with the level of asthma severity, even though nasal polyps represent a peculiar condition which proves to be associated with the highest excretion of urinary LTE<sub>4</sub> and with the highest prevalence of ASA intolerance.

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# Cypress pollen does not cross-react to plant-derived foods

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## KEY WORDS

*Nasal polyps; severe asthma; asa-intolerance; cysteinil leukotrienes*

## SUMMARY

**Background and Objective:** Some studies hypothesize the existence of cross-reactivity between allergy to *Cupressus sempervirens* pollen and plant-derived foods. We aimed to assess whether this holds true. **Methods:** 72 patients monosensitized to cypress pollen were investigated for food allergy to peach, apple, tomato and *Juniperus oxycedrus* berry. **Results:** No patient had a history of clinical allergy or showed in-vitro or in-vitro reactivity to peach, apple, and tomato. Two patients scored positive on SPT with *Juniperus oxycedrus* berry but in-vitro tests ruled out cross-reactivity with the corresponding pollen. **Conclusion:** Airborne allergy to Cupressaceae pollen is not associated with allergy to plant-derived foods.

Some pollen allergens, such as the major birch allergen, Bet v 1, and the plant pan-allergen, profilin, are well known to cross-react to homologous allergens in vegetable foods causing the so-called oral allergy syndrome. In contrast, the existence of cross-reactivity between specific cypress pollen allergens and plant-derived foods is poorly defined. In recent years, some authors reported cross-sensitisation to tomato, banana and apple in patients allergic to *Juniperus ashei* or *Criptomeria japonica* pollen (1-5), and a French study observed potential cross-reactivity between Cypress pollen and peach (6). Further, in France the berry of *Juniperus oxycedrus* is present in several dishes, but data about potential cross-reactivity with the corresponding pollen are missing. We assessed whether cross-reactivity between *Cupressus sempervirens* and peach and other fruits or between *Juniperus oxycedrus* pollen and *Juniperus oxycedrus* berry occur.

Seventy-two patients diagnosed as being monosensitized to *Cupressus sempervirens* at the outpatients allergy departments of Marseille, France, (n=39) and Bordighera, Italy, (n=33) were studied. The diagnosis was based on a clinical history of rhino-conjunctivitis with or without asthma from November to the end of March, confirmed both by positive SPT and increased levels of IgE specific for *Cupressus sempervirens*. All patients were thoroughly interviewed about adverse food reactions (including oral allergy syndrome, ur-

ticaria/angioedema, gastrointestinal symptoms immediately after the ingestion of specific foods). All patients underwent skin prick tests with our routine panel of aeroallergens (house dust mites, pellitory, grass, olive, *Cupressus sempervirens*, *Betula alba*, *Alternaria*, Cockroaches, Cat and Dog). Further, *Juniperus oxycedrus* fruit was tested by the prick-prick technique. IgE to peach, tomato and apple were measured by Immuno-CAP (Padia, Uppsala, Sweden) in all subjects. Thirty patients allergic to pollens other than cypress were used as controls. All skin tests were carried out using Lofarma extracts (Milan, Italy; 1/20 w/v), and were performed and read following established methods.

*Juniperus oxycedrus* pollen (Jo) and desiccated Jo berries underwent 5% (w/v) aqueous extraction in 0.125 M NH<sub>4</sub>HCO<sub>3</sub> for 4 h at 4°C under stirring at 4°C. The suspensions were centrifuged at 20,000 g for 1 hour at 20°C and supernatants were extensively dialyzed against distilled water. Protein content of samples was assessed by BioRad assay. Specific IgE to *Juniperus oxycedrus* pollen or berry extracts were measured by ELISA using a pool of sera from patients allergic to *Cupressus sempervirens* pollen. A pool of 5 sera from non-atopic individual was used as control. To this purpose, 5 µg of Jo pollen and berry extracts in 100 µl of buffer (15 mmol/L Na<sub>2</sub>CO<sub>3</sub> and 35 mmol/L NaHCO<sub>3</sub>, pH 9.6) per well of 96-microtitre plates (Maxisorp Nunc, Roskilde, Den-

**Table 1** - ELISA inhibition of Jo pollen extract using pollen or fruit Jo extracts or *J. Oxycedrus* pollen extract as inhibitors

Inhibitor dilution	inhibitor			
	<i>Juniperus oxycedrus</i> pollen extract		Juniperus fruit extract	
	UA	% inhibition	UA	% inhibition
1:1	0.05	100	1.101	0
1:4	0.046	100	1.008	2
1:16	0.128	88	1.034	0
no inhibition	1.024	0	1.024	0

mark) were used in the coating phase. After washings with 0.15 M phosphate-buffered saline, pH 7.4 (PBS) and 0.05% Tween 20 (Sigma, Milan, Italy), wells were saturated with 2% bovine serum albumin (BSA) in PBS (saturation and dilution buffer) for 2 hours at room temperature. Subsequently, after further washing, 100 µl of positive or negative pool diluted 1:2 in dilution buffer were added to the wells and incubated for 2 hours at room temperature. Wells were washed, and bound specific IgE was detected by peroxidase-conjugated anti-human IgE from goat (diluted 1:1500, Biospecific, Emeryville, CA, USA); a colorimetric reaction was induced using tetramethyl-benzidine/H<sub>2</sub>O<sub>2</sub> as substrate. The enzyme reaction was stopped after 20 minutes by the addition of 1 mol/L HCl. Absorbance values were read at 450 nm by spectrophotometer. In inhibition studies, patients' sera positive to both pollen and berry extracts were pooled before pre-absorption for 2 hours at room temperature with different concentrations of Jo pollen or berry extracts (40 µl of sera and 80 µl of inhibiting extract diluted 1:1, 1:4 or 1:16). Subsequently, 100 µl of such solutions were added to Jo-coated wells and ELISA performed as before performing ELISA as described before. IgE levels were expressed as optical density units (OD). Based on the mean + 2SD of IgE levels found in normal controls, values less < 0.100 OD were considered negative.

No patient allergic to *Cupressus sempervirens* reported clinical allergy or showed either in-vivo or in-vitro hypersensitivity to peach. Similarly, none out of 33 patients reported clinical allergy or immunological sensitivity to tomato or apple. Only 2/72 patients scored positive on SPT with Jo berry. The pool of sera used in the study showed strong IgE reactivity to *Juniperus oxycedrus* (O.D.: 1.024 UA) (Tab. 1). As a difference from patients, 25% of 30 control subjects with pollen allergy reported oral allergy syndrome following the ingestion of vegetable foods. SPT with *Juniperus oxycedrus* pollen extract scored positive in 90% of patients, thus confirming the cross-reactivity between *Cupressus* and *Juniperus* pollen. No cross-reactivity between *Juniperus* pollen and berry was ob-

served as shown by the lack of any inhibition of IgE reactivity to pollen pre-adsorption sera with Jo berry extract (Tab. 1). Altogether, our findings clearly rule out an association between *Cupressus sempervirens* pollen allergy and hypersensitivity to plant-derived foods. The two cases of skin reactivity to *Juniperus* berry in French subjects are probably the result of alimentary habits, and we weren't able to find any cross-reactivity between *Juniperus oxycedrus* pollen and berry extracts.

#### Acknowledgements

The pollen of *Juniperus oxycedrus* was a kind gift of Prof. Dr. Paolo Radici - Istituto di Patologia delle Piante, CNR, Sesto Fiorentino (FI).

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G. D'AMATO<sup>1</sup>

## Respiratory allergy induced by exclusive polysensitization to serum albumins of furry animals

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### KEY WORDS

*Allergic rhinitis, allergic sensitization, animal allergy, bronchial asthma, cat, dog, hypersensitivity, respiratory allergy, pet, serum albumin*

### SUMMARY

*In this report we describe an unusual case of exclusive allergic sensitization to furry animals, as a possible study model to speculate about different modalities of sensitization to allergens of common and less common mammalian species. A 27-year-old woman referred in our Allergological Centre for the occurrence of conjunctival and severe respiratory symptoms after contact with several animals such as cats, dogs, rabbits, horses, cows etc. Patient underwent clinical and anamnestic evaluation including a detailed information on the modality of exposure to different furry animals. Skin-prick-test (SPT) was performed with our routine panel of commercial standardized extracts (Lofarma Laboratories, Milan, Italy). Some animal allergenic extracts (rabbit, horse, rat, mouse, cavia, cow and hamster) have been tested by SPT one week after the routine SPT. A blood sample was taken for measurement of total IgE and specific IgE (CAP System, Phadia, Uppsala, Sweden) as well as Immunoblotting procedures. The results of in vivo and in vitro procedures revealed allergic sensitization only to animal – derived allergens. Total IgE were 59,3 kU/L. Immunoblotting showed a specific IgE-mediated sensitization of the patient to cow's, rabbit's and horse's serum albumins (SA). In conclusion, our case report confirms the role of SA as cross-reacting agent in allergic sensitization to furry animals. This finding suggests to perform SPTs to several furry animal allergens in all individuals with high level of allergic sensitization to common pets (cats and / or dogs) in order to identify allergy to other animals and consequently to avoid future exposures at risk.*

### Introduction

Exposure to furry animal – derived materials is a well recognized cause of occupational sensitisation for people who are in contact with animals in labs and other settings such as pet shops, farms, etc. (1).

It is well known that a large percentage of cat or dog-sensitized individuals has never kept these animals in their domestic environments (2). As a consequence, these pets' allergens are now considered ubiquitous being transferred in pet-free environments (private homes, schools, public

transport vehicles etc) through different transfers such as clothes and hair (3, 4).

Another mechanism of allergic sensitization to animal allergens without previous contact includes a cross-reaction through the lipocalins (5) or serum albumin (6).

To the best of our knowledge, no previous report on multiple allergic sensitization to different animal allergens has been published.

We describe an unusual case of exclusive allergic sensitization to several furry animals through different modalities of sensitization (direct/indirect contact and cross-reaction).

## Case report

A 27-year-old woman was referred in our Allergological Centre due to the occurrence of conjunctival and severe respiratory symptoms (rhinitis, cough, wheezing and dyspnea) mainly after contact with common pets such as cats and dogs, but also after occasional exposure to other animals (e.g. rabbits, horses, cows etc.).

Patient reported that no animal was kept steadily in her domestic environment.

Respiratory allergic symptoms of slight/moderate intensity were persistent all year round. Asthmatic symptoms were not controlled by usual long-term anti-asthma therapy (Fluticasone propionat: 500 mcg + Salmeterol xynafoate: 50 mcg – bid/die) plus Montelukast (10 mg/die). The patient denied any cutaneous symptom after contact with animals and/or after the ingestion of meats/milk.

## Methods

### *Personal data*

Detailed information about exposure to different furry animals was sought.

A familiar history of atopy was reported but patient did not have a personal history of previous cutaneous and/or respiratory symptoms.

### *Skin-prick-tests (SPT)*

SPT were performed with commercial extracts (Lofarma Laboratories, Milan, Italy) of house dust mites, *Parietaria* species, grasses, cat and dog dander, olive, birch, *Alternaria alternata*, *Cladosporium herbarum* and mugwort. Further, rabbit, horse, rat, mouse, cavia, cow and hamster were tested as well. Skin tests were performed and read according to accepted guidelines (7).

### *Evaluation of specific IgE antibodies*

Specific IgE to animal allergens were measured by ImmunoCAP (Phadia, Uppsala, Sweden).

### *PolyAcrylamide Gel Electrophoresis (PAGE) and Immunoblotting*

Protein separation was performed by electrophoresis using gels with the following composition:

*Running gel:* 15% acrylamide; 0.14% bis-acrylamide; 0.36 M TRIS-HCl buffer pH 8.8; 35% glycerol; 0.02% ammonium persulfate; and 0.15% N,N,N',N'-tetramethylethylenediamine (TEMED).

*Stacking gel:* 3.5% acrylamide; 0.09% bis-acrylamide; 0.125 M TRIS-HCl buffer pH 6.8; 0.02% ammonium persulfate; and 0.15% TEMED.

*Running buffer:* 25mM TRIS, 0.19M glycine, pH 8,8.

### *Immunoblotting*

After PAGE, proteins were transferred to PVDF membrane (Millipore) by western blotting in a Trans-blot Electrophoretic Transfer Cell (Bio-Rad). The membranes were blocked with 1% gelatin and washed three times with 0.25% gelatin solution (in 150 mM NaCl, 5mM EDTA, 50 mM Tris, 0.05% Triton-X) to prevent non-specific adsorption of the immunological reagents. The membrane was then immersed in 10 mL of 0.25% gelatin solution containing 300 µL of serum from allergic subject. Antigen-IgE complexes were detected using 10 µL of goat anti-human IgE antibodies labeled with alkaline phosphatase (Sigma, Milan, Italy). The developing solution contained 15% bromochloroindolyl phosphate (BCIP) and 30% nitro blue tetrazolium (NBT) (Sigma, Milan, Italy) in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl<sub>2</sub>).

Quantitative evaluation of the immunoreactive bands was performed by a gel scanner (Sharp JX-330, Pharmacia Biothec) and the Image Master™ 1D Software. It allows the quantification of proteins by calculating the average density of pixels across the band length and integrating over the band width.

Classes of positive reactions were defined on the basis of an arbitrary scale of densitometric values and 6 classes of reactivity were identified.

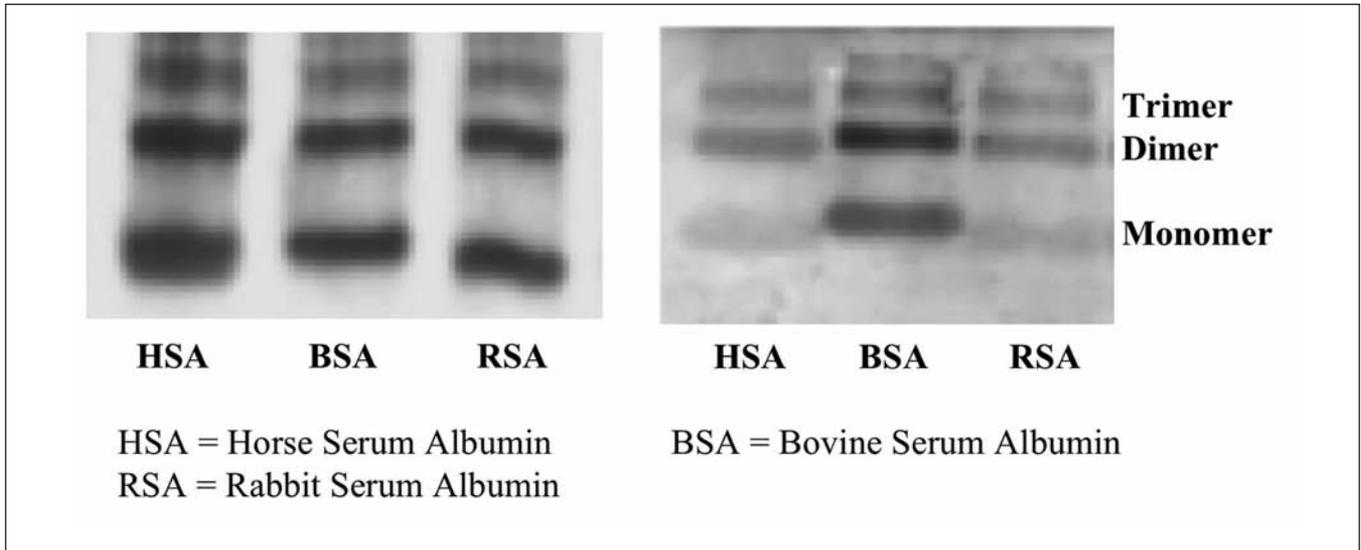
## Results

SPT revealed positivity only to animal – derived allergens (dog, cat, rabbit, horse, rat, mouse, cavia, cow and hamster).

Specific IgE were detected for all tested animal allergens (Tab. 1). Immunoblot analysis revealed IgE-reactivity to cow's, rabbit's and horse's serum albumins, with a good correlation between severity of response in SPT (wheal diameter) and the densitometric class of reaction in immunoblotting. No IgE reactivity against other proteins has been found (Fig. 1).

The strict avoidance of animal contact outdoors along with an intensive cleaning of indoor environments resulted in a progressive significant reduction of respiratory symptoms over the next two months.

**Figure 1** - SDS-PAGE (left) and Immunoblotting (right) of mammal's serum albumin. These procedures revealed IgE-reactivity to cow's, rabbit's and horse's serum albumins



**Table 1** - Cutaneous, laboratory and exposure data of a patient sensitized only to allergens of furry animals

Allergen	SPTs (Wheal diameter)	IgE (KU/L)	IgE (Class)	Animal exposure
Dog epithelia	9 x 9 mm	19.7	4	Direct (outside her home)
Cat dander	10 x 11 mm	24.8	4	Direct (outside her home)
Horse epithelia	7 x 6 mm	1.1	2	No
Rabbit epithelia	9 x 10 mm	1.7	2	Indirect
Hamster epithelia	8 x 9 mm	4.0	3	No
Cow epithelia	10 x 12 mm	0.5	1	No
Mouse epithelia	11 x 11 mm	0.4	1	No
Rat epithelia	7 x 7 mm	2.0	2	No
Guinea pig epithelia	6 x 6 mm	8.8	3	No
Rabbit serum	n.a	4.5	3	Indirect
Rabbit urine	n.a.	2.9	2	Indirect
Horse serum prot	n.a.	2.3	2	No
Rabbit meat (*)	4 x 4 mm	1.7	2	n.a.
Cow milk (*)	5 x 4 mm	0.5	1	n.a.

n.a. = not applicable

(\*) No symptoms after ingestion

## Discussion

To the best of our knowledge this is the first report of a multiple sensitization to furry animal allergens in a subject without professional exposure.

As shown in table 1, allergic sensitization to common pets

can be easily explained by the reported direct and frequent contact with cats/dogs although outside patient's home and the transport of cat/dog allergens indoors through her clothing.

Allergic sensitization to rabbit epithelia as well as rabbit serum and urine is likely to be induced by an indirect

mechanism of exposure because the frequent presence, at patient's home, of a couple of friends owners of four rabbits as pets.

Sensitization to other animal allergens cannot be explained neither by exposure direct (denied by patient) nor indirect considering their low ownership in our geographical area.

A likely explanation of high number of animal sensitization in our patient is the cross-reactivity between serum albumins of different mammals.

Serum albumin (SA), a thermolabile protein of approximately 68 kDa, constitutes an important panallergen involved in milk, meat, and epithelia allergy (8-10). Some important allergens of cat such as Fel d 2 (11) and dog such as Can f 3 (12) are SA and it may explain the high frequency of allergic sensitization to both pets. It has been shown that SA constitutes the cross-reacting allergen between epithelia of cat, dog, horse and pig (13, 14).

Although the primary modality of allergic sensitization to SA is the ingestion of different meats such as beef, pork etc., in some cases an inhalation route has been demonstrated (15).

Recently, it has been shown that first contact with SA was through cow's milk and that sensitization to SA may occur even without direct contact with animals (6). This mechanism could explain why this patient became allergic to uncommon mammalian allergens even in the absence of any contact with such animals.

Our patient showed cutaneous and serological sensitization to cow milk, rabbit meat and relative serum albumins, but the ingestion of these foods did not induce cutaneous/respiratory symptoms. The lack of reactions is likely connected to the heat lability of serum albumin with consequent modification of sensitization mechanisms (16).

In conclusion, our case report confirms the role of SA as cross-reacting agent in allergic sensitization to furry animals. This finding suggests to perform SPTs to several furry animal allergens in all individuals with high level of allergic sensitization to common pets (cats and/or dogs) in order to identify allergy to other animals and consequently to avoid future exposures at risk.

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