

Role of Art v 3 in pollinosis of patients allergic to Pru p 3

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Background: Food allergy caused by lipid transfer protein (LTP) from peach (Pru p 3) is frequently associated with sensitization to mugwort LTP (Art v 3). Although *in vitro* cross-reactivity is already well known, it has yet to be elucidated whether a pollen LTP can induce rhinitis.

Objective: The aim of this study was to investigate whether mugwort LTP could elicit respiratory symptoms and whether a primary food LTP allergy could lead to a respiratory allergy.

Methods: Patients with confirmed Pru p 3 allergy and control subjects were selected. Immediate responses to nasal allergen provocation tests (NAPT) with Art v 3, Pru p 3, and mugwort were assessed by using the visual analog scale score, total nasal symptom score, and acoustic rhinometry. Tryptase and cysteinyl leukotriene (cysLT) levels were measured in nasal lavage fluid. Immunoblotting, ELISAs, and ELISA inhibition assays were also performed.

Results: Fifteen patients and 9 control subjects were selected. NAPT results with Art v 3 and Pru p 3 showed significant changes in acoustic rhinometry, visual analog scale scores, total nasal symptom scores, and cysLT levels ($P < .001$). Tryptase levels were only increased in NAPT with Pru p 3. NAPT with mugwort were used in those patients who were only sensitized to Art v 3, with similar results ($P < .05$). No significant changes were detected in control subjects.

Conclusion: The results demonstrated that a pollen LTP can elicit rhinitis in sensitized patients. Findings also suggest that a primary sensitization to Pru p 3 can lead to a respiratory allergy through cross-reactivity. (*J Allergy Clin Immunol* 2013;■■■:■■■-■■■.)

Key words: Lipid transfer protein, food allergy, pollinosis, nasal allergen provocation test, Pru p 3, Art v 3

Abbreviations used

AR:	Acoustic rhinometry
HEP:	Histamine equivalent prick units
IQR:	Interquartile range
LTP:	Lipid transfer protein
NAPT:	Nasal allergen provocation test
sIgE:	Specific IgE
SPT:	Skin prick test
TNSS:	Total nasal symptom score
VAS:	Visual analog scale
VOL 2-5 cm:	Volume of nasal cavity encompassing the lower turbinate

A considerable number of patients with pollen allergy have allergy to plant foods.¹⁻³ The core of this association is the existence of cross-reactive IgE antibodies against proteins from related and unrelated allergenic sources, which are widely distributed in the vegetable kingdom, the so-called “panallergens.”⁴

In some cases the association between pollen and plant foods is determined by a well-studied sensitization profile, such as cross-reactivity to Bet v 1–like allergens. In fact, in central and northern Europe almost 70% of patients with birch pollen allergy also have plant food allergy.^{5,6} A major birch allergen, Bet v 1 (a protein from the pathogenesis related 10 family), is the main cause of cross-reactivity with plant foods. Sensitization to Bet v 1 homologues appears to result from a previous sensitization to birch pollen, and therefore “birch-free” regions are not at risk of cross-reactivity between birch pollen and food allergens.

In the Mediterranean area the lipid transfer protein (LTP) family is involved in severe allergic reactions related to plant food. Patients allergic to LTP usually show multiple plant food sensitizations,^{7,8} the so-called LTP syndrome.⁹ Food LTPs have also been described as inducers of respiratory symptoms, mainly occupational asthma.¹⁰⁻¹⁴ However, it has never been directly demonstrated that an LTP could be the protein responsible for these symptoms.

The association between LTP-mediated food allergy and pollinosis is currently a matter of debate. LTPs have been considered true food allergens, with the ability to act as primary sensitizers independent of pollen LTPs.¹⁵ There are studies reporting cross-reactivity between peach LTP (Pru p 3) and both plane tree LTP (Pla a 3) and mugwort LTP (Art v 3),¹⁶⁻¹⁸ but although both proteins have been suggested as primary sensitizers, depending on the population studied,¹⁸⁻²¹ it has yet to be elucidated whether these pollen LTPs have a role in pollinosis.

We designed the present study to clarify whether a pollen LTP alone is capable of inducing respiratory symptoms and whether sensitization to peach LTP can lead to respiratory allergy. We used an experimental model of the nasal allergen provocation test (NAPT) with Pru p 3 and Art v 3. Two hypotheses have been

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established. First, mugwort LTP (Art v 3) alone can induce respiratory allergy in sensitized patients, and second, patients with peach allergy who are primarily sensitized to Pru p 3 can have respiratory symptoms when exposed to mugwort pollen because of cross-reactivity with Art v 3.

METHODS

Study subjects

Fifteen adults with a documented history of anaphylaxis, a generalized cutaneous reaction with involvement of at least another organ,²² to peach and a positive skin prick test (SPT) response, serum specific IgE (sIgE) level, or both to Pru p 3 were recruited. Nine subjects (5 nonatopic and 4 atopic) sensitized to neither mugwort nor any other food allergen tested were recruited as the control group. Other possible plant food allergies were established on the basis of a clinical history of symptoms compatible with food allergy (urticaria, oral allergy syndrome, or gastrointestinal symptoms, including functional dyspepsia, crampy abdominal pain, nausea, vomiting and/or diarrhea and/or anaphylaxis) and a positive SPT response to the culprit/suspected food. Respiratory allergy caused by pollen was also assessed according to the clinical history and SPT responses based on a panel of common commercial pollen allergen extracts.

The study was approved by the Hospital Clinic's ethics committee, and written informed consent was obtained from all patients.

SPTs

SPTs were performed with commercial extracts of the most prevalent aeroallergens in our area, including biologically standardized allergenic extracts (unpublished data) of house dust mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), molds (*Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarum*, and *Penicillium notatum*), pollens (*Lolium perenne*, *Parietaria judaica*, *Artemisia vulgaris*, *Olea europea*, *Platanus acerifolia*, and *Cupressus arizonica*), dog and cat dander (Laboratorios LETI S.L., Madrid, Spain), and purified Art v 3 (100 µg/mL). The plant food allergens tested were as follows: hazelnut, peanut, kiwifruit, peach, maize, wheat, chickpea, mustard, apple, melon, green bean (Laboratorios LETI, Barcelona, Spain), palm profilin (Pho d 2, 50 µg/mL; ALK-Abelló, Madrid, Spain), and Pru p 3 (100 µg/mL; Bial-Aristegui, Bilbao, Spain). A complete list of extract concentrations is provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org. Suspected allergens not included in the standard panel were also tested with a commercial extract. In case of a negative result with the suspected allergen, a prick-by-prick test with the fresh food was carried out. SPTs were performed on the volar surface of the forearm with a 1-mm single peak lancet (ALK-Abelló) according to the European Academy of Allergology and Clinical Immunology recommendations.²³ Histamine hydrochloride (10 mg/mL) and saline solution were used as positive and negative controls, respectively. The SPT response was considered positive if the longest diameter was greater than 3 mm compared with that elicited by the negative control.

Total IgE and sIgE levels in serum

Individual sera from all the recruited patients were tested with an allergen microarray immunoassay, including 103 allergenic components (ImmunoCAP ISAC; Thermo Fisher Scientific, Uppsala, Sweden), according to the manufacturer's protocol. Total IgE and sIgE levels to plane tree, mugwort, peach, and rPru p 3 were determined by means of immunoassay (ImmunoCAP, ThermoFisher Scientific). The cutoff value for sIgE was 0.35 kU/L for the ImmunoCAP and 0.3 ISAC Standardized Unit (ISU) for the ImmunoCAP ISAC.

NAPT

By using a micropipette, 100 µL of saline solution was instilled on the inferior turbinate of each nostril to exclude nasal hyperreactivity. Fifteen minutes later, NAPT was performed with 50 µL of purified

nArt v 3 (5 µg per nostril), 50 µL of freshly reconstituted mugwort (10 BU/mL) extract (ALK-Abelló), and 100 µL of nPru p 3 (2.5 µg per nostril) on different days separated by at least a 2-week interval.

Nasal symptoms. Symptoms of nasal obstruction, rhinorrhea, itching, and sneezing were each recorded by placing a vertical mark on a horizontal visual analog scale (VAS) of 100 mm and using a 4-point Likert scale from 0 to 3 (0, no symptoms; 1, mild symptoms; 2, moderate symptoms; and 3, severe symptoms). The total VAS score was the sum of 4 VAS scores (total range, 0-400). The total nasal symptom score (TNSS) was obtained from the sum of all 4 individual symptom scores, with a total possible score ranging from 0 (no symptoms) to 12 (maximum symptom intensity).

Acoustic rhinometry. Nasal obstruction was assessed by means of acoustic rhinometry (AR) with the use of an SRE 2000 rhinometer (Rhinometrics, Lyngø, Denmark) according to the guidelines of the Standardization Committee on Acoustic Rhinometry.²⁴ The volume of nasal cavity encompassing the lower turbinate (VOL 2-5 cm) was analyzed in each nostril. Volumes were transformed in percentages; the postdiluent value was considered 100%, and therefore the other time-point percentages are with respect to that value.

NAPT monitoring. Patients were evaluated at baseline, after diluent, and at 15 and 30 minutes and 1, 2, 4, and 5 hours after the challenge test through examination of nasal symptoms and AR. Physical examination and forced spirometry were also carried out to exclude systemic symptoms, bronchial obstruction, or both.

Nasal inflammatory mediators. Nasal irrigation was performed at baseline and 15, 60, and 300 minutes after NAPT. Five milliliters of saline solution was instilled into the nasal cavity by using a nasal device that permitted the retention of saline solution for 30 seconds and its subsequent collection.

Samples were centrifuged at 3000g for 10 minutes at 4°C, and supernatant was stored at -80°C and then lyophilized. Tryptase was measured by means of immunoassay (ImmunoCAP), and cysteinyl leukotriene (cysLT) levels were measured with the Cysteinyl Leukotriene EIA kit (Cayman Chemical, Ann Arbor, Mich), according to the manufacturer's instructions. The cutoff value for cysLT detection was 34 pg/mL, and that for tryptase was 1 ng/mL.

Immunoblotting, allergen isolation, ELISA, and ELISA inhibition assay techniques are described in the [Methods](#) section in this article's Online Repository.

Statistical analysis

SPSS software (version 18.0; SPSS, Chicago, Ill) was used for statistical analysis. Data were expressed as the median and interquartile range (IQR). The clinical, demographic, and NAPT data were compared between groups by using the Mann-Whitney *U* test. The Wilcoxon signed-rank test was used to identify significant differences in tryptase levels, cysLT levels, TNSSs, VAS symptoms, and VOL 2-5 cm in patients by using AR within groups. *P* values of less than .05 were considered statistically significant.

RESULTS

Subjects

One male and 14 female subjects were included in the patient group (median age, 37 years; IQR, 21-46 years), and 3 male and 6 female subjects were included in the control group (median age, 30 years; IQR, 21-60 years). The demographic data and sensitization profiles are presented in [Table I](#); nonatopic control subjects have been omitted from the table as long as they had no sensitizations. Mugwort and plane tree pollen were the most prevalent pollens by far, with sensitization in 14 of 15 and 15 of 15 patients, respectively, in the allergic group, followed by wall pellitory pollen, with sensitization in 4 of 15 patients. All patients (15/15) had allergic rhinitis caused by any pollen, with 3 of 15 cases associated with asthma. All patients had allergic reactions to multiple plant foods (median, 6 foods;

TABLE I. Demographic data and sensitization profile

Subject no.	Pollinosis		Aeroallergen sensitization (SPT)	Culprit food/symptomatology	Plant food sensitization (SPT)	Other sensitizations (SPT)
	Rhinitis	Asthma				
Patient group						
1	+	–	hdm, wp, mw, pl	<i>pe, to</i> (An), mz (GIS), hz , pe, wn (U)	hz, pe, mz, wh, mu	–
2	+	–	mw, pl	pe (An), hz, pn, che (U), le, to, cb (GIS)	hz, pe, mz, gb, pn	che, as, cb, le, to
3	+	–	hdm, mw, pl	pe, al (An), ba, ml, wm, pr, gb (GIS)	pe, mu, ml	ml, ba
4	+	+	wp, mw, pl	pe, to (An), wn, pn, hz (U)	hz, kw, pe, mz, mu, ml, gb	–
5	+	–	hdm, gr, mw, pl , cd, dd	pe, le, gb, cb (An), H z (U)	hz, kw, pe, mz, gb	–
6	+	–	hdm, mw	pe (An), che (U), le, ap (GIS)	hz, pe, mz, wh, mu	le, che
7	+	–	hdm, mw , cla, cd	pe (An), wn, pn (U), zu , ba (OAS), pp (GIS)	hz, kw, pe, mz, wh, pn, mu, gb	pp, wn, zu, ba
8	+	–	hdm, gr, pl , cd, dd	pe, wn (An), le, pn (OAS), gb (CU)	kw, pe, gb, pn	le
9	+	–	wp, mw, pl	pe, wn, pp, mz, le, ba, che (An)	hz, pe, mz, pn, mu, ml	wn, sf, che, ba, pp, le
10	+	–	hdm, pl	pe, le (An), ak, gb, che (GIS)	kw, pe, mz, mu, gb	le, che
11	+	–	hdm, mw , ol, alt	pe (An), le, gb (GIS), wn, hz (U)	hz, pe, mz, pn, mu, gb	gp, wn, le
12	+	–	hdm, mw, pl	pe (An), wm (OAS), le, gb (GIS), kw, hz, pn (U)	hz, kw, pe, mz, pn, mu	le
13	+	+	hdm, gr, wp, mw, ol, pl, cy , cd, dd	pe (An), le, gb (GIS)	hz, kw, pe, mz, mu, gb	le
14	+	–	hdm, mw, pl	pe, hz (An), le, to (GIS), ml, wm (OAS), pi (U)	hz, kw, pe, mz, wh, mu, gb	le, to, ml
15	+	+	hdm, mw , ol	pe (An), ap, kw, gp, mz, ml, to, as (OAS), le (GIS)	hz, kw, pe, mz, pn, mu	ba, le, gp
Atopic group						
21	+	–	hdm, pl , cd, dd	NA	–	–
22	+	–	gr, alt, cd	NA	–	–
23	+	+	hdm	NA	–	–
24	+	–	cd	NA	–	–

Pollen sensitizations with an LTP analog and LTP-containing foods are marked in boldface.

F, Female; M, male; NA, not applicable.

Aeroallergens: *alt*, *Alternaria* species; *cla*, *Cladosporium* species; *cd*, cat dander; *cy*, cypress; *dd*, dog dander; *gr*, grass; *hdm*, house dust mite; *mw*, mugwort; *ol*, olive; *pl*, plane tree; *wp*, wall pellitory.

Plant foods: *ak*, Artichoke; *ap*, apple; *as*, asparagus; *ba*, banana; *cb*, cabbage; *che*, cherry; *gb*, green bean; *gp*, grape; *hz*, hazelnut; *kw*, kiwi; *le*, lettuce; *ml*, melon; *mu*, mustard; *mz*, maize; *pe*, peach; *pi*, pineapple; *pn*, peanut; *pp*, pepper; *pr*, pear; *sf*, sunflower seed; *to*, tomato; *wh*, wheat; *wm*, watermelon; *wn*, walnut; *zu*, zucchini.

Symptoms: *An*, Anaphylaxis; *CU*, contact urticaria; *GIS*, gastrointestinal symptoms; *OAS*, oral allergy syndrome; *U*, urticaria.

IQR, 5–7 foods) and a great variety of manifestations from oral allergy syndrome to anaphylaxis. All of them had at least 1 episode of anaphylaxis with peach, and 8 of 15 had anaphylaxis with at least 1 other food. Gastrointestinal symptoms were the second most frequent clinical manifestation, occurring in 11 of 15 patients. In Table II we show the patient group results on SPT responses and sIgE measurements for peach, mugwort, and plane tree, as well as the available components included in the array that were considered relevant for the study (Pru p 3, Art v 1, and Art v 3). Complete ISAC results are available in Fig E1 in this article's Online Repository at www.jacionline.org. Art v 3 sensitization was detected in 13 of 14 patients sensitized to mugwort pollen. Detection of sIgE to Pru p 3 and Art v 3 by means of both ISAC and ELISA showed that sIgE levels to Pru p 3 were higher than those to Art v 3 in all cases (Table II).

NAPTs with Art v 3

The 13 patients sensitized to Art v 3 (positive SPT response, ISAC result, or both) were challenged with purified nArt v 3. Significant differences were detected between patients and control subjects at different time points in AR volumes, total VAS scores, TNSSs, and cysLT levels (Fig 1). The median VOL 2–5 cm significantly decreased from 100% (postdiluent value) to 68% at 15 minutes (Fig 1, A), with the maximum reduction in nasal patency ($P < .001$) at this time point. Patients presented with significant increases in total VAS scores and TNSSs at 15 minutes (Fig 1, B), reaching normal values at 2 hours after the challenge test. CysLT levels were significantly increased at 15 minutes after NAPT ($P < .001$; Fig 1, C). Tryptase levels were increased in 5 patients at 15 minutes after NAPT, coinciding with those who reacted most on NAPT with Art v 3, although no statistical significance was reached (data not shown).

TABLE II. SPT responses and sIgE profiles in the patient group

Subject	SPT			Total IgE (kU/L)	sIgE ImmunoCAP (kU/L)			ISAC (ISU)			ELISA (OD 492 nm)	
	Pru p 3	Mugwort	Art v 3		Peach	Pru p 3	Mugwort	Pru p 3	Art v 1	Art v 3	Pru p 3	Art v 3
1	+	+	+	95.4	4.96	6.04	1.72	2.35	<0.3	1.33	2	0.91
2	+	+	+	1311	18.20	21.30	2.04	8.10	<0.3	2.00	3.04	1.20
3	+	+	—	751	NA	14.60	2.91	6.60	<0.3	1.80	3.04	1.83
4	+	+	+	51.2	4.47	5.11	0.78	4.63	<0.3	1.83	0.82	0.24
5	+	+	+	153	4.89	5.17	1.23	5.00	<0.3	0.70	NA	NA
6	+	+	+	130	33.70	38.60	5.93	5.30	<0.3	2.00	2.60	1.02
7	+	+	+	306	27.60	29.60	7.29	7.80	<0.3	4.40	2.26	1.03
8	+	—	—	55.9	<0.35	<0.35	<0.35	0.80	<0.3	<0.3	<0.21	<0.21
9	+	+	+	136	47.00	61.00	22.30	9.80	<0.3	6.90	3.40	3.09
10	+	—	—	81.7	1.53	1.60	0.53	0.60	<0.3	<0.3	0.42	0.30
11	+	+	+	42.1	4.64	1.23	ND	5.60	<0.3	3.60	1.55	1.16
12	+	+	+	NA	4.54	5.57	1.22	1.10	<0.3	<0.3	0.60	0.22
13	+	+	+	102	NA	10.30	1.24	3.20	<0.3	0.80	0.82	0.27
14	+	+	+	254	27.30	37.10	8.29	30.00	<0.3	16.00	1.80	1.20
15	+	+	+	57.4	13.00	16.00	2.91	2.30	<0.3	0.70	1.80	<0.21

ELISA results are considered positive if ODs are greater than 0.21 units.

NA, Not available; ND, not determined.

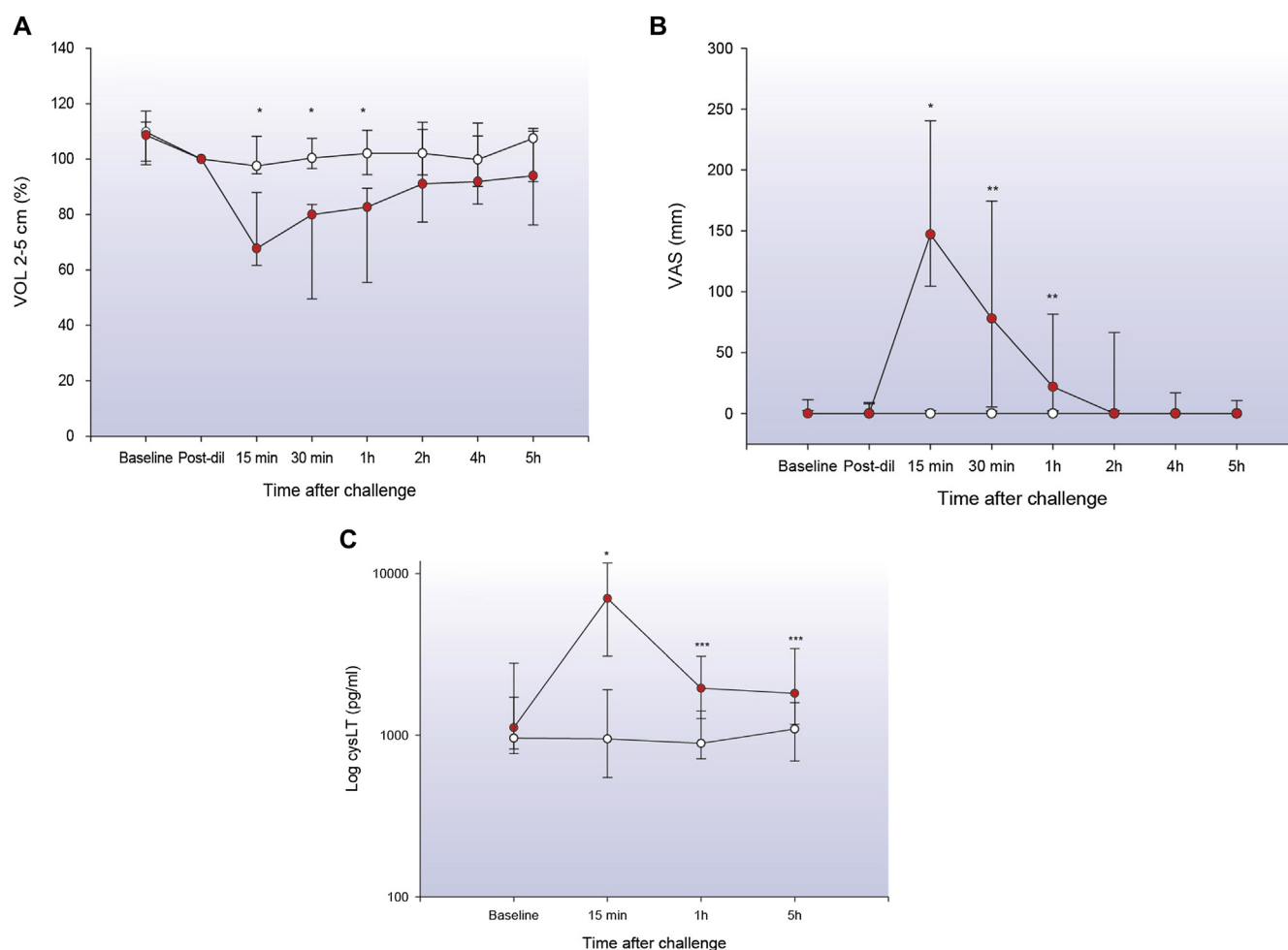


FIG 1. NAPT with Art v 3. Changes in AR (A), total VAS scores (B), and nasal cysLT levels (C) in patients (red dots) and control subjects (white dots) after NAPT with Art v 3. Data are expressed as medians and ranges. Significant differences between groups are indicated as follows: * $P < .001$, ** $P < .01$, and *** $P < .05$.

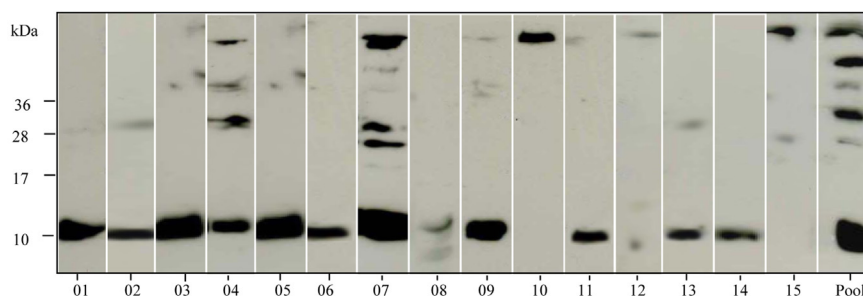


FIG 2. IgE immunoblotting of mugwort extract with the sera of the 15 patients with Pru p 3 allergy. Patient numbers correspond to those shown in [Tables I and II](#).

No significant changes were detected within the control group at any point. No adverse reaction was seen in any subject.

NAPTs with mugwort

Sensitization to the major allergen of mugwort, Art v 1, was ruled out in all patients by using the ImmunoCAP ISAC. Immunoblotting was carried out to assess sensitization to other mugwort proteins, if any. Thirteen of 15 had a band at 10 to 12 kDa corresponding to Art v 3 ([Fig 2](#)), with this being the only detectable band in 8 of these subjects. The 8 patients monosensitized to Art v 3 were challenged against a complete extract of mugwort pollen to assess whether regular exposure to mugwort pollen would elicit respiratory symptoms. The median VOL 2-5 cm significantly decreased from 100% (postdiluent value) to 78% at 15 minutes after NAPT ($P < .05$); TNSSs and VAS scores significantly increased at the same time point after NAPT ($P < .05$; [Fig 3](#)). No adverse reaction was seen in any subject.

NAPTs with Pru p 3

Significant differences were detected between patients and control subjects at different time points in AR volumes, total VAS scores, TNSSs, and tryptase and cysLT levels ([Fig 4](#)). The median VOL 2-5 cm of the 15 patients significantly decreased from 100% (postdiluent value) to 70% ($P < .001$) at 15 minutes after the nasal challenge, coinciding with a significant increase in total VAS scores and TNSSs ($P < .001$), but the maximum reduction in nasal patency was detected at 30 minutes (58%, $P < .001$). Normal values were recovered for 2 hours after the NAPT ([Fig 4, A and B](#)). Significant increases in tryptase levels were detected at 15 minutes ($P < .01$); tryptase levels were undetectable in 6 patients at 15 minutes, and no tryptase was detected at any other time point. CysLT levels were significantly higher at 15 and 60 minutes ([Fig 4, C and D](#)). No significant changes were detected within the control group. No adverse reaction was seen in any subject.

ELISA inhibition

ELISA inhibition experiments were carried out with the purified allergens Pru p 3 and Art v 3 and with serum pools. IgE binding to Art v 3 was completely inhibited by both Art v 3 and Pru p 3. In the case of Pru p 3 on the solid phase, IgE binding was completely inhibited by itself, but inhibition with Art v 3 was less, reaching a maximum of 91% ([Fig 5](#)). Single-sera inhibition experiments with Pru p 3 on the solid phase showed how Art v 3 inhibition with the concentrations used was only fully achieved in 3 cases, whereas in 7 other sera the inhibition was less than 40%;

on the other hand, 7 of 11 sera were fully inhibited by Pru p 3. Full individual ELISA inhibition results are shown in [Table E1](#) in this article's Online Repository at www.jacionline.org.

DISCUSSION

Cross-reactivity between pollen and food LTPs has been a matter of debate in the last few years. To date, pollen LTPs have been described as allergens in 6 different species: ragweed,²⁵ mugwort,¹⁶ Japanese cedar,²⁶ olive,²⁷ plane tree,¹⁸ and wall pellitory,²⁸ with different identities with plant food LTPs. Salcedo et al¹⁵ proposed classifying pollen LTPs into 2 groups: the first group includes plane tree Pla a 3 and mugwort Art v 3, which shares 40% of its determined N-terminal sequence with Pru p 3, and both are clearly involved in plant food–pollen cosensitization.^{16,18,19} The second group includes the LTPs from ragweed Amb a 6 and olive Ole e 7 as minor allergens and wall pellitory Par j 1 and Par j 2 as major allergens, with less than 35% sequence identity with Pru p 3, which appears not to be involved in pollen–plant food cross-reactivity.^{25,27,28}

Although there are indirect results relating Pla a 3 and Art v 3 to pollinosis,^{18,19} there is currently no direct evidence to demonstrate that these LTPs can be responsible for respiratory symptoms.

To prove the hypothesis that pollen LTPs can induce respiratory symptoms, we performed the nasal challenge with Art v 3. It was carried out in patients sensitized to this protein, revealing significant changes in nasal patency, respiratory symptoms, and release of cysLT in nasal lavage fluid. To demonstrate that these results were not artificially generated and that regular exposure to mugwort pollen could precipitate rhinitis in these patients, NAPTs with mugwort were carried out in those patients monosensitized to Art v 3, with similar results. These findings appear to support our first hypothesis, which establishes that mugwort LTP alone can induce rhinitis when sensitized patients are exposed to this pollen. To our knowledge, this is the first time that an *in vivo* study proves that an LTP from pollen can elicit respiratory symptoms.

It is very difficult to demonstrate that sensitization to food allergens can lead to respiratory allergic symptoms through cross-reactivity between pollen and food allergens. LTPs can sensitize patients through both the oral and inhalational routes,¹⁰⁻¹⁴ and therefore cross-sectional studies can hardly help clarify which comes first. ELISA inhibition is a widely accepted method for differentiating whether an allergen is a primary sensitizer.^{19,20} In most cases Pru p 3 is considered the primary sensitizer, but Art v 3 has also been reported as a primary sensitizer in some cases of allergy to mugwort.¹⁹ In our study

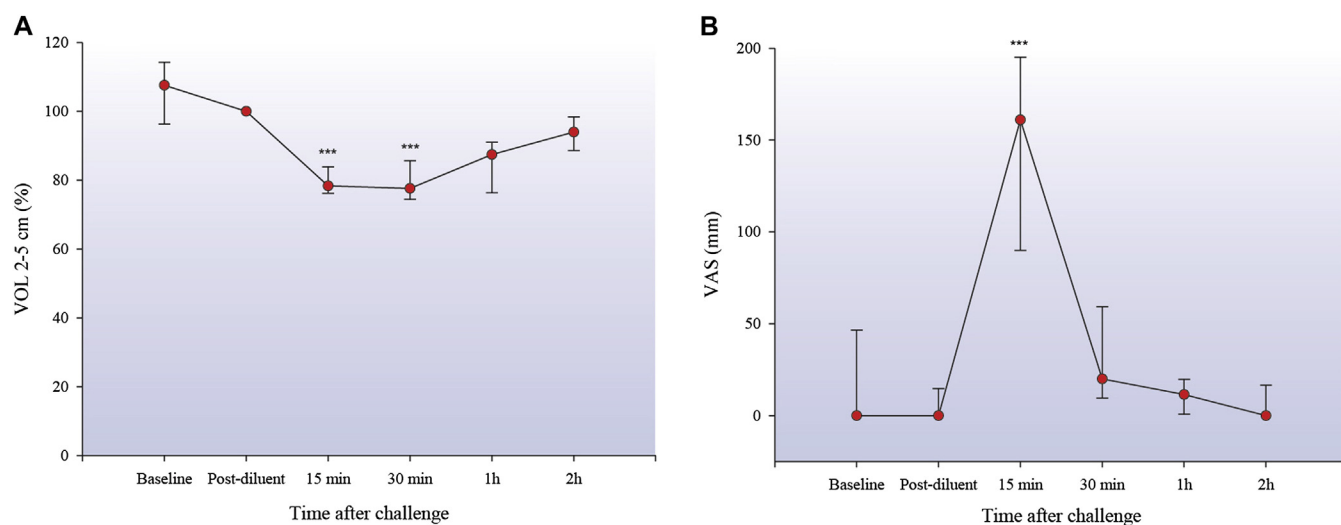


FIG 3. NAPT with mugwort pollen extract. Changes in AR (**A**) and total VAS scores (**B**) after NAPT with mugwort. Data are expressed as medians and ranges. Significant differences between postdiluent values and values 15 and 30 minutes after challenge are indicated as follows: *** $P < .05$.

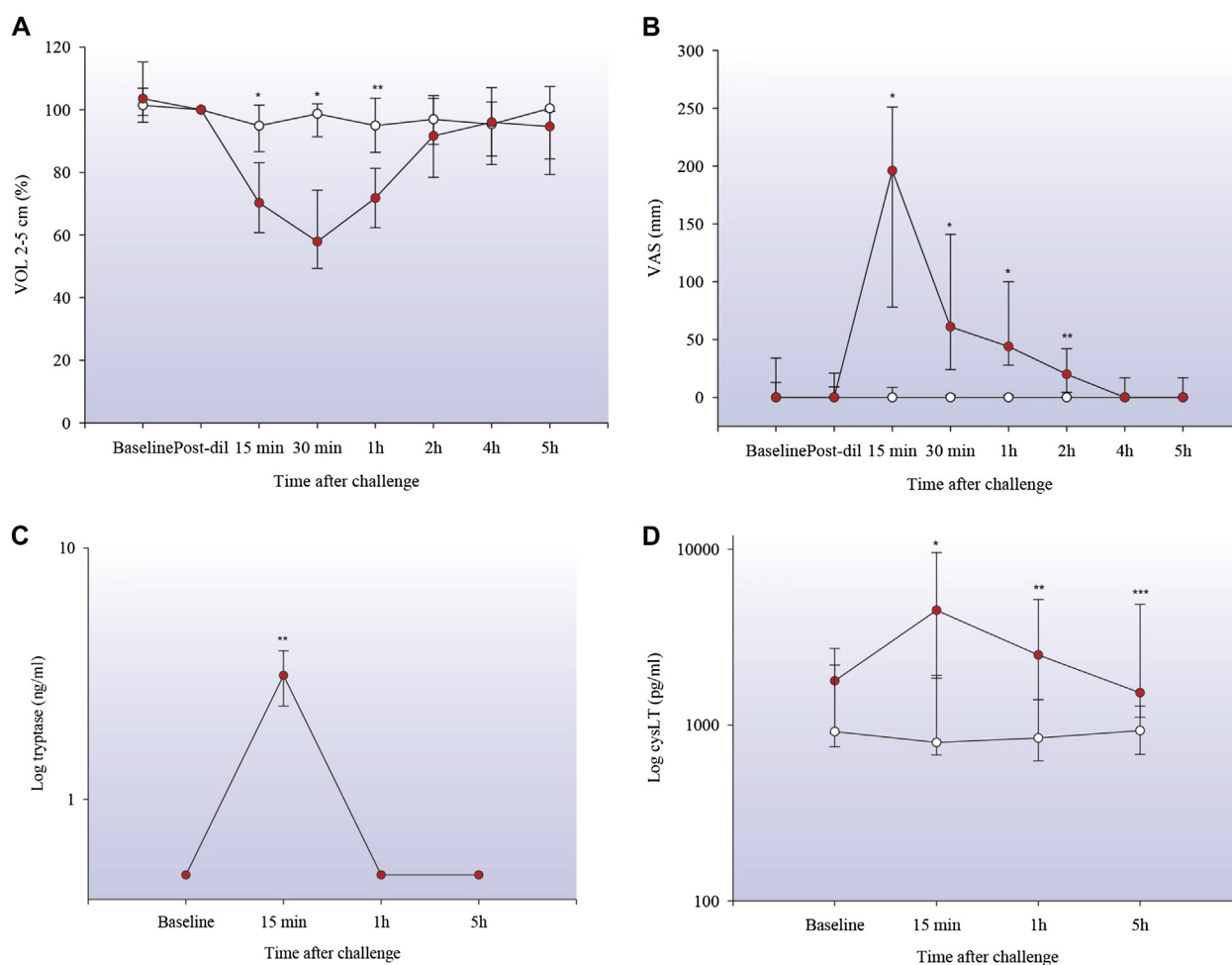


FIG 4. NAPT with Pru p 3. Changes in AR (**A**), total VAS scores (**B**), nasal tryptase levels (**C**), and nasal cysLT levels (**D**) of patients (red dots) and control subjects (white dots) after NAPT with Pru p 3. Data are expressed as medians and ranges. Significant differences between groups are indicated as follows: * $P < .001$, ** $P < .01$, and *** $P < .05$.

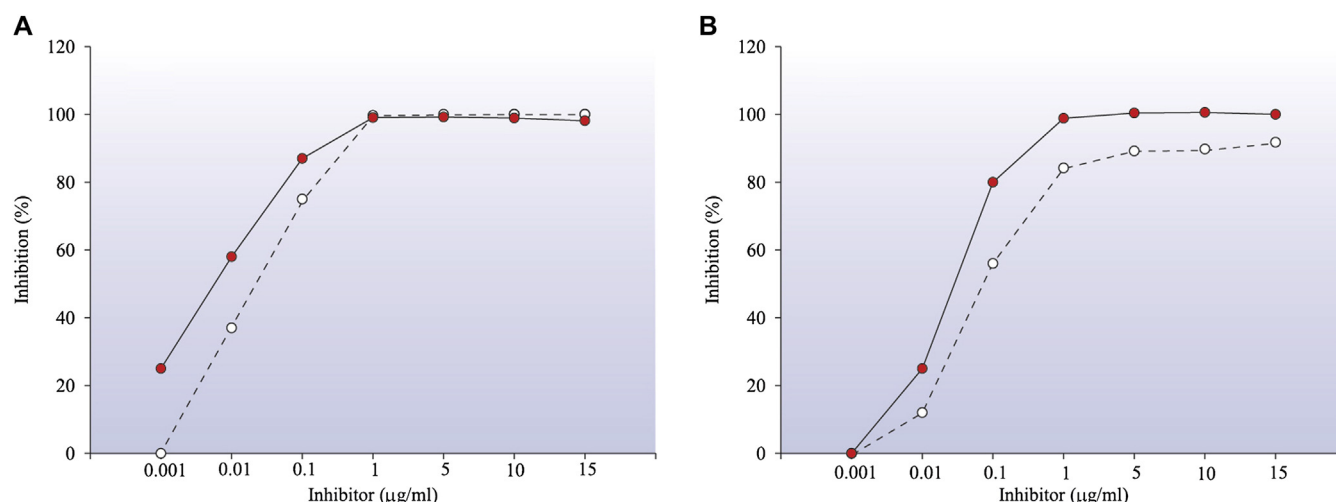


FIG 5. ELISA inhibition with Art v 3 (**A**) and Pru p 3 (**B**) in the solid phase. A serum pool was preincubated with the corresponding inhibitor (Art v 3, white dots; Pru p 3, red dots) at different concentrations (0.001, 0.01, 0.1, 1, 5, 10, and 15 µg/mL).

patients were selected according to a confirmed Pru p 3 allergy, and although 93% of patients were sensitized to mugwort and more than 50% were monosensitized to Art v 3, there was a lack of respiratory symptoms during the mugwort pollen season. In this regard it is worth mentioning the low *Artemisia vulgaris* pollen counts, usually a mean of 5 grains/m³,²⁹ during the pollen season in our area (Barcelona, Spain), which represents a very low probability of having a genuine sensitization to mugwort. Thus we reasoned that the primary sensitization should be dependent on Pru p 3. Moreover, the ELISA inhibition experiments performed support this notion. Considering both the ELISA results and the demonstration that Art v 3 can elicit symptoms *per se*, there is strong support for the hypothesis that a food allergen like Pru p 3 can lead to a respiratory allergy.

Another challenge was to demonstrate whether a protein that can sensitize through both the gastrointestinal and respiratory mucosae could induce symptoms not only in the digestive tract but also in the nasal mucosa. Thus NAPT with Pru p 3 were carried out, eliciting nasal symptoms (itching, rhinorrhea, sneezing, and nasal obstruction) in all patients, as demonstrated by VAS scores, TNSSs, AR, and detection of released inflammatory mediators (cysLT and tryptase) in nasal lavage fluid. Interestingly, the nasal reaction caused by Pru p 3 was stronger than that after NAPT with Art v 3, even when using a lower protein concentration. This finding provides additional support to the hypothesis that Pru p 3 was the primary allergen in these patients.

One innovative aspect of the present study was the use of purified allergenic proteins in the NAPT. NAPT are usually performed with complete extract of the suspected allergen, and this procedure has proved very useful for both characterizing the responsible allergens and assessing the efficacy of therapies. The use of purified proteins will further help us examine the individualized role of the various allergens that usually participate in allergic responses.^{30,31}

One potential limitation of our study is the presence of plane tree sensitization in all the patients. Plane tree pollen is one of the most frequent types of pollinosis in our geographic area,³² and Pla a 3 is considered the major allergen in patients with coexisting

plane tree pollen and Pru p 3 allergy.¹⁸ The Art v 3 sensitizations observed in our patients could therefore also result from cross-reactivity with Pla a 3. Unfortunately, Pla a 3 was unavailable in our component-resolved diagnostic evaluation when the study was carried out. However, sensitization to plane tree pollen in terms of sIgE was lower than that to peach in most cases; moreover, 5 of 13 patients sensitized to Art v 3 were sensitized to plane tree but not to Pla a 1 or Pla a 2, suggesting that sensitization to this pollen could be due to Pla a 3 recognition secondary to a cross-reactivity with Pru p 3, as proposed for Art v 3.

A second limitation of our study is the use of Art v 3 in the NAPT to prove our hypothesis. Our population is not naturally exposed to mugwort pollen, and therefore although the model worked well during the provocation tests, we did not prove that the mechanism is also present in real life.

In summary, for the first time, this study demonstrates that a pollen LTP, Art v 3, can elicit respiratory symptoms. Equally important, our findings suggest that there is a link between food and respiratory allergy in patients with LTP syndrome: a primary sensitization to a food LTP can lead to respiratory symptoms caused by a pollen LTP through a cross-reactivity mechanism.

Clinical implications: In patients with LTP-dependent food allergy, pollen LTPs can contribute to symptoms during the pollen season.

REFERENCES

1. Cuesta-Herranz J, Barber D, Blanco C, Cistero-Bahíma A, Crespo JF, Fernández-Rivas M, et al. Differences among pollen-allergic patients with and without plant food allergy. *Int Arch Allergy Immunol* 2010;153:182-92.
2. Caballero T, Martín-Esteban M. Association between pollen hypersensitivity and edible vegetable allergy: a review. *J Invest Allergol Clin Immunol* 1998;8:6-16.
3. Bircher AJ, Van Melle G, Haller E, Curty B, Frei PC. IgE to food allergens are highly prevalent in patients allergic to pollens, with and without symptoms of food allergy. *Clin Exp Allergy* 1994;24:367-74.
4. Hauser M, Roulias A, Ferreira F, Egger M. Panallergens and their impact on the allergic patient. *Allergy Asthma Clin Immunol* 2010;6:1.
5. Juhlin-Dannfelt C. About the occurrence of various forms of pollen allergy in Sweden. *Acta Med Scand* 1948;131:563-77.

6. Geroldinger-Simic M, Zelniker T, Aberer W, Ebner C, Egger C, Greiderer A, et al. Birch pollen-related food allergy: clinical aspects and the role of allergen-specific IgE and IgG4 antibodies. *J Allergy Clin Immunol* 2011;127:616-22.
7. Pascal M, Muñoz-Cano R, Reina Z, Palacín A, Vilella R, Picado C, et al. Lipid transfer protein syndrome: clinical pattern, cofactor effect and profile of molecular sensitization to plant-foods and pollens. *Clin Exp Allergy* 2012;42:1529-39.
8. Asero R, Mistrello G, Roncarolo D, Amato S, Caldironi G, Barocci F, et al. Immunological cross-reactivity between lipid transfer proteins from botanically unrelated plant-derived foods: a clinical study. *Allergy* 2002;57:900-6.
9. Pastorello EA, Robino AM. Clinical role of lipid transfer proteins in food allergy. *Mol Nutr food Res* 2004;48:356-62.
10. Palacín A, Quirce S, Armentia A, Fernández-Nieto M, Pacios LF, Asensio T, et al. Wheat lipid transfer protein is a major allergen associated with baker's asthma. *J Allergy Clin Immunol* 2007;120:1132-8.
11. Enrique E, Ahrazem O, Bartra J, Latorre MD, Castelló JV, de Mateo JA, et al. Lipid transfer protein is involved in rhinoconjunctivitis and asthma produced by rice inhalation. *J Allergy Clin Immunol* 2005;116:926-8.
12. Tabar AI, Alvarez-Puebla MJ, Gomez B, Sanchez-Monge R, García BE, Echechipia S, et al. Diversity of asparagus allergy: clinical and immunological features. *Clin Exp Allergy* 2004;34:131-6.
13. Borghesan F, Mistrello G, Roncarolo D, Amato S, Plebani M, Asero R. Respiratory allergy to lipid transfer protein. *Int Arch Allergy Immunol* 2008;147:161-5.
14. García BE, Lombardero M, Echechipia S, Olaguibel JM, Díaz-Perales A, Sánchez-Monge R, et al. Respiratory allergy to peach leaves and lipid-transfer proteins. *Clin Exp Allergy* 2004;34:291-5.
15. Salcedo G, Sánchez-Monge R, Barber D, Díaz-Perales A. Plant non-specific lipid transfer proteins: an interface between plant defence and human allergy. *Biochim Biophys Acta* 2007;1771:781-91.
16. Díaz-Perales A, Lombardero M, Sánchez-Monge R, García-Selles FJ, Pernas M, Fernández-Rivas M, et al. Lipid-transfer proteins as potential plant panallergens: cross-reactivity among proteins of *Artemisia* pollen, *Castanea* nut and *Rosaceae* fruits, with different IgE-binding capacities. *Clin Exp Allergy* 2000;30:1403-10.
17. García-Selles FJ, Díaz-Perales A, Sánchez-Monge R, Alcántara M, Lombardero M, Barber D, et al. Patterns of reactivity to lipid transfer proteins of plant foods and *Artemisia* pollen: an in vivo study. *Int Arch Allergy Immunol* 2002;128:115-22.
18. Lauer I, Miguel-Moncin MS, Abel T, Foetisch K, Hartz C, Fortunato D, et al. Identification of a plane pollen lipid transfer protein (Pla a 3) and its immunological relation to the peach lipid-transfer protein, Pru p 3. *Clin Exp Allergy* 2007;37:261-9.
19. Lombardero M, García-Selles FJ, Polo F, Jimeno L, Chamorro MJ, García-Casado G, et al. Prevalence of sensitization to *Artemisia* allergens Art v 1, Art v 3 and Art v 60 kDa. Cross-reactivity among Art v 3 and other relevant lipid transfer protein allergens. *Clin Exp Allergy* 2004;34:1415-21.
20. Gao ZS, Yang ZW, Wu SD, Wang HY, Liu ML, Mao WL, et al. Peach allergy in China: A dominant role for mugwort pollen lipid transfer protein as a primary sensitizer. *J Allergy Clin Immunol* 2013;131:224-6.
21. Pastorello EA, Pravettoni V, Farioli L, Rivolta F, Conti A, Ispano M, et al. Hypersensitivity to mugwort (*Artemisia vulgaris*) in patients with peach allergy is due to common lipid transfer protein allergen and is often without clinical expression. *J Allergy Clin Immunol* 2002;110:310-7.
22. Sampson HA, Muñoz-Furlong A, Campbell RL, Adkinson NF, Bock SA, Branum A, et al. Second symposium on the definition and management of anaphylaxis: summary report—Second National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network symposium. *J Allergy Clin Immunol* 2006;117:391-7.
23. Dreborg S, Backman A, Basomba A, Bousquet J, Dieges P, Malling HJ. Skin tests used in type I allergy testing Position paper. Sub-Committee on Skin Tests of the European Academy of Allergology and Clinical Immunology. *Allergy* 1989;44(Suppl 10):1-59.
24. Hilberg O, Pedersen OF. Acoustic rhinometry: recommendations for technical specifications and standard operating procedures. *Rhinology* 2000;16:3-17.
25. Hiller KM, Lubahn BC, Klapper DG. Cloning and expression of ragweed allergen Amb a 6. *Scand J Immunol* 1998;48:26-36.
26. Ibrahim AR, Kawamoto S, Nishimura M, Pak S, Aki T, Díaz-Perales A, et al. A new lipid transfer protein homolog identified as an IgE-Binding antigen from Japanese Cedar Pollen. *Biosci Biotechnol Biochem* 2010;74:504-9.
27. Tejera ML, Villalba M, Batanero E, Rodríguez R. Identification, isolation, and characterization of Ole e 7, a new allergen of olive tree pollen. *J Allergy Clin Immunol* 1999;104:797-802.
28. Ayuso R, Carreira J, Lombardero M, Duffort O, Peris A, Basomba A, et al. Isolation by mAb based affinity chromatography of two Par j I isoallergens. Comparison of their physicochemical, immunochemical and allergenic properties. *Mol Immunol* 1993;30:1347-54.
29. Historical pollens and spores data of Barcelona—Point of Information on Aerobiology (PIA). Available at: <http://lap.uab.cat/aerobiologia/en/historical/barcelona>. Accessed January 31, 2013.
30. Godnic-Cvar J, Susani M, Breiteneder H, Berger A, Havelec L, Waldhör T, et al. Recombinant Bet v 1, the major birch pollen allergen, induces hypersensitivity reactions equals to those induced by natural Bet v 1 in the airways of patients allergic to tree pollen. *J Allergy Clin Immunol* 1997;99:354-9.
31. Ruiz-García M, García del Potro M, Fernández-Nieto M, Barber D, Jimeno-Nogales L, Sastre J. Profilin a relevant aeroallergen? *J Allergy Clin Immunol* 2011;128:416-8.
32. Navarro Pulido A. Rinitis. In: *Alergológica 2005. Factores Epidemiológicos, clínicos y socioeconómicos de las enfermedades alérgicas en España en 2005*. Madrid: Luzan 5, S.A. de ediciones; 2006. p. 107-31.

METHODS

SPTs

Aeroallergens were tested at the following concentrations: house dust mite (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), 100 histamine equivalent prick units (HEP)/mL; *Alternaria alternata*, 30 HEP/mL; *Aspergillus fumigatus*, *Cladosporium herbarum*, and *Penicillium notatum*, 5 mg/mL; pollens (*Lolium perenne*, *Parietaria judaica*, *Artemisia vulgaris*, *Olea europea*, *Platanus acerifolia*, and *Cupressus arizonica*) and dog and cat dander, 30 HEP/mL.

Food allergens were tested at the following concentrations: hazelnut, peanut, kiwifruit, peach, apple, and melon at 5 mg/mL and maize, wheat, chickpea, mustard, and green bean at 10 mg/mL.

Immunoblotting

Mugwort pollen protein extract (10 mmol/L PBS [pH 7.4] and 150 mmol/L ClNa; 20 µg) was separated by using SDS-PAGE, and replica gels were electrotransferred onto polyvinylidene difluoride membranes. After blocking with blocking buffer (Sigma-Aldrich, St Louis, Mo) for 1 hour at room temperature, the membranes were incubated overnight with single sera from patients (1:5 dilution in PBS) washed 3 times with PBS–Tween 20 0.05% for 10 minutes and then incubated with goat anti-human IgE-peroxidase conjugate (1:5000 dilution in PBS; Invitrogen, Carlsbad, Calif). IgE-binding components were detected by means of chemiluminescence (Amersham Biosciences, Little Chalfont, United Kingdom).

Allergen isolation

LTP from mugwort pollen (nArt v 3) was purified from a Tris-HCl extract of defatted samples, as previously described.^{E1} After centrifugation, dialysis against H₂O, and freeze-drying, the extract was fractioned by means of reversed phase HPLC on a Vydac-C4 column (22 × 250 mm; particle size, 10 µm; The Separations Group, Hesperia, Calif). Elution was performed with a 2-step linear gradient of 2-propanol in 0.1% trifluoroacetic acid (0% to 30% in 120 minutes, 30% to 50% in 30 minutes, and 80 minutes at

50%; 1 mL/min). Fractions containing the LTP were pooled, freeze-dried, and further rechromatographed on a semipreparative reversed phase HPLC Nucleosil 300-C4 column (8 × 250 mm; particle size, 5 µm; Sugelabor, Madrid, Spain), eluting with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (0% to 80% in 150 minutes, 0.5 mL/min).

The protein quality was determined by means of wide-range mass spectrometry using standard peptide fingerprint methods. The absence of LPS in the samples was checked by using anti-LPS antibodies (rabbit anti-*Escherichia coli* LPS; AbD Serotec, Kidlington, United Kingdom) and THP1-XBlue cells (InvivoGen, Toulouse, France).

ELISA and ELISA inhibition assay

sIgE levels to Pru p 3 and Art v 3 were determined by means of direct ELISA, as previously reported,^{E1} with 14 single sera. Plates were covered with nPru p 3 or nArt v 3 at 3 µg/mL. Dilution of sera was 1:2, and goat anti-human IgE-peroxidase conjugate antibody was used at 1:3000 dilution (Biosource, Camarillo, Calif). Blocking solution (Sigma) without a solid phase was used as a negative control, and absorbance (OD) values of greater than 0.208 units (n = 9; mean + 3 × SD = 0.094 + 3 × 0.038 OD units) were considered positive. Serum dilution was determined by using titration curves. For ELISA inhibition assays, plates were coated as indicated above, and a serum pool was preincubated (3 hours at 25°C) with the corresponding inhibitor (nArt v 3 or nPru p 3) at different concentrations. Single-sera inhibition experiments were also performed by using nPru p 3 on the solid phase; unfortunately, there was not enough nArt v 3 protein available to perform individual experiments with this protein on the solid phase. All experiments were made in triplicates, and the results are the means of these experiments.

REFERENCE

- E1. Díaz-Perales A, Lombardero M, Sánchez-Monge R, García-Selles FJ, Pernas M, Fernández-Rivas M, et al. Lipid-transfer proteins as potential plant panallergens: cross-reactivity among proteins of *Artemisia* pollen, *Castanea* nut and *Rosaceae* fruits, with different IgE-binding capacities. *Clin Exp Allergy* 2000;30:1403-10.

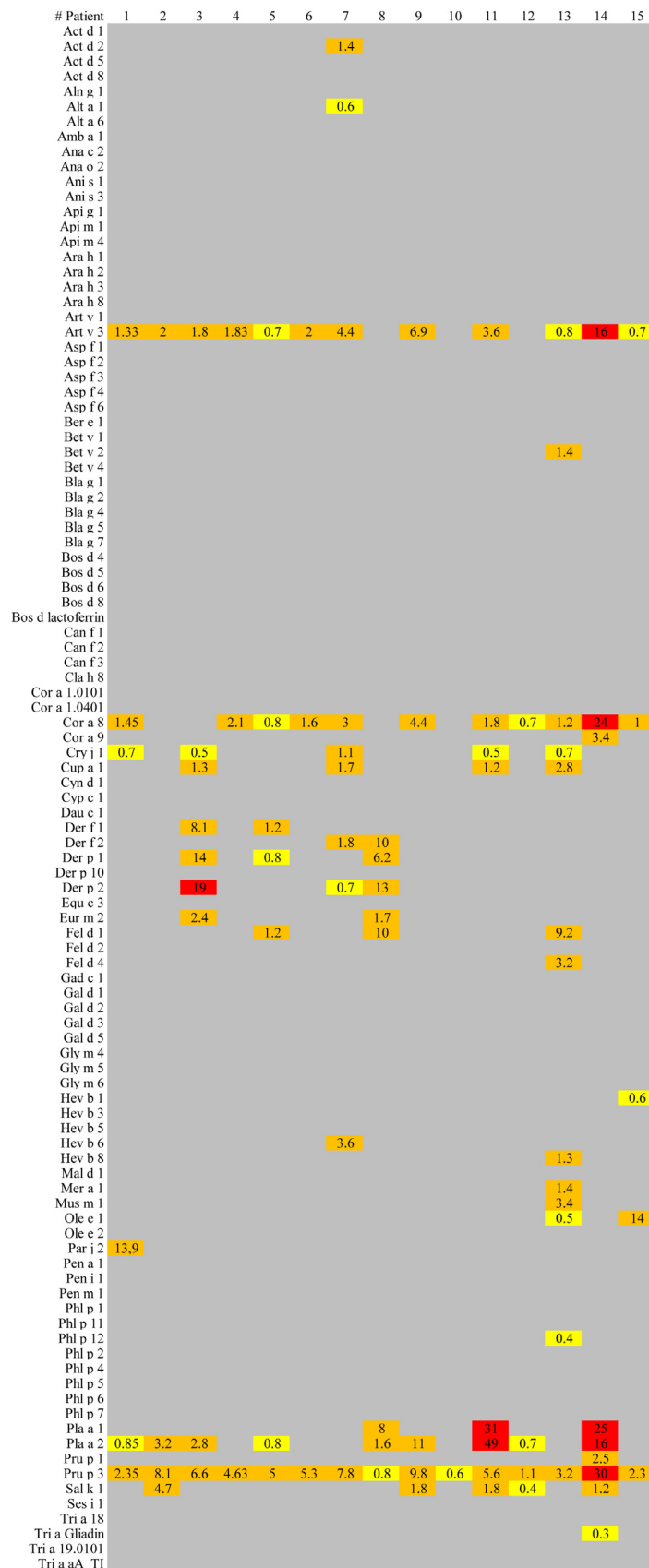


FIG E1. Schematic representation of sIgE detection by using a microarray. *Columns* represent each subject, and *rows* represent each allergenic component included in the array. Levels are expressed as ISU units. Color code: *gray*, less than the cutoff of 0.3 ISU units; *yellow*, 0.3 to 0.9 ISU units; *orange*, 1 to 14.9 ISU units; and *red*, greater than 15 ISU units.

TABLE E1. Individual ELISA inhibition results (percentage inhibition IgE-binding) with purified allergens

Pru p 3 on solid phase		
Patient	Inhibitor	
	Pru p 3	Art v 3
1	100	39
2	78	11
3	100	10
4	100	100
6	96	0
7	100	78
9	100	5
11	100	100
12	100	100
14	82	25
15	83	34

Plates were coated with Pru p 3 at 3 µg/mL. The corresponding inhibitor (Art v 3 or Pru p 3) concentration was 5 µg/mL, as determined from preliminary dose-response experiments until reaching a plateau level.