

Differential allergen sensitization patterns in chestnut allergy with or without associated latex-fruit syndrome

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Background: Chestnut allergy has been almost exclusively considered in the context of the latex-fruit syndrome. Chestnut allergens not linked to latex hypersensitivity have not been studied.

Objective: We sought to explore whether differences in sensitization patterns between chestnut allergy with or without associated latex-fruit syndrome can be detected.

Methods: Twelve patients sensitized to chestnut but not to latex and 3 control patients with latex-chestnut allergy were analyzed. A major chestnut allergen was purified and characterized. IgE immunoblotting, specific IgE determination, and skin prick tests with 5 isolated allergens involved in food allergy or latex-fruit syndrome were also performed.

Results: A major 9-kd allergen was detected in chestnut extract, isolated, and identified as lipid transfer protein (LTP) Cas s 8. Specific IgE to this allergen was found in 91% (by means of IgE immunoblotting) and 58% (by means of ELISA) of sera from patients with chestnut but not latex allergy. Moreover, 66% of these patients had positive skin prick test responses to Cas s 8. Additionally, allergenic LTPs from peach fruit and *Artemisia vulgaris* pollen were also reactive. In contrast, avocado class I chitinase and latex hevein, allergens associated with the latex-fruit syndrome, showed no reaction. The opposite situation was exhibited by patients with latex-chestnut allergy.

Conclusions: Patients with chestnut allergy with or without associated latex hypersensitivity present different patterns of major allergens (LTPs and class I chitinases, respectively).

Clinical implications: LTPs and class I chitinases can be used as diagnostic tools in patients with chestnut allergy to predict whether an associated latex sensitization and a risk of potential cross-reactivity with other plant foods and pollens exist. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Chestnut, food allergy, latex-fruit syndrome, lipid transfer protein, class I chitinase, hevein, mugwort pollen, peach

Tree nuts are among the most allergenic foods worldwide,¹ and therefore allergens from hazelnut, walnut, cashew, and Brazil nut have been identified.² In contrast, chestnut allergy has been almost exclusively considered in the context of the latex-fruit syndrome, thus focusing on cross-reactive allergens with latex and other fruits, such as avocado and banana, which are closely linked to the syndrome.³⁻⁶ Class I chitinases with a hevein-like domain from chestnut (Cas s 5), avocado (Pers a 1), and banana (Mus a 1), as well as latex hevein (Hev b 6.02), have been identified as the main allergens responsible for these cross-reactions.⁷⁻¹⁰

In addition to Cas s 5,⁷ only 2 potential allergenic proteins have been described in chestnut seeds (nuts). A 24-kd allergen, probably belonging to the legumin family, recognized by more than 50% of sera (n = 9) from Korean patients with positive reactions to chestnut on skin prick tests (SPT) and high specific IgE levels to this nut has been recently described.¹¹ Additionally, a member of the lipid transfer protein (LTP) panallergen family, designated Cas s 8, was purified from chestnut and included in a survey on cross-reactivity among allergenic LTPs from plant foods and *Artemisia* pollen in patients selected on the basis of peach hypersensitivity.^{12,13} However, the role of Cas s 8 in chestnut allergy has not been investigated thus far. Furthermore, neither a clinical study nor the identification of major allergens has been carried out on North American or European patients with chestnut allergy without associated latex sensitization. We present here the results of such a study, which indicate that different main allergens are implicated in patients allergic to chestnut but not latex and patients allergic to latex-chestnut allergy.

METHODS

Selection of patients and control subjects

A prospective study was designed in the Allergy Service of Hospital Dr Negrin (Las Palmas de Gran Canaria, Spain), comprising a detailed clinical history, *in vivo* tests, and blood draws for *in vitro*

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Abbreviations used

LTP: Lipid transfer protein
 MALDI: Matrix-assisted laser desorption/ionization mass spectrometry
 NRL: Natural rubber latex
 SPT: Skin prick test

assays. During a 2-year running period, every patient reporting immediate adverse reactions after chestnut ingestion suggestive of being IgE mediated but with no history of adverse reactions to natural rubber latex (NRL) exposure and showing (1) a positive SPT response to a commercially available chestnut extract (ALK-Abelló, Madrid, Spain) and (2) a negative SPT response to a NRL extract (ALK-Abelló) was recruited.

Three patients previously given diagnoses of latex-chestnut allergy, which is known to be reactive to fruit class I chitinases on the basis of previous studies,¹⁴ and showing specific IgE levels to chestnut of greater than 3.5 kU/L were used as control group. The same *in vivo* and *in vitro* tests were performed in both patients and control subjects. Written informed consent was obtained from all participants, and the study was approved by the Research and Ethics Committees of the Hospital Dr Negrín (Las Palmas de Gran Canaria, Spain).

SPTs were performed according to standard procedures by trained personnel who were unaware of the subjects' clinical histories, with panels of aeroallergens, including dust mites, fungi, pollens, animal danders, and NRL, and food allergens, including milk, egg, fish, nuts, fruits, mustard, kiwi, cereals, seafood, and legumes, in commercial extracts, as detailed elsewhere.¹⁵

Total IgE was determined by using the UniCAP System (Pharmacia Diagnostics, Uppsala, Sweden), according to the manufacturer's instructions. Specific IgE levels to chestnut (code f299), to common aeroallergens, and to other foods implicated in adverse reactions for each patient were also determined by using the CAP method, and values greater than 0.35 kU/L were considered positive.

Isolation and characterization of allergens

Mature chestnuts (*Castanea sativa*) were peeled, freeze-dried, ground, and defatted with cold acetone ($2 \times 1:10$ [wt/vol] for 1 hour at 4°C) and ethanol/ether ($1:3$ [vol/vol], $2 \times 1:10$ [wt/vol] for 1 hour at 4°C). After drying, nuts were extracted with PBS buffer (0.1 mol/L sodium phosphate [pH 7.4] and 0.15 mol/L NaCl, $1 \times 1:5$ [wt/vol] for 1 hour at 4°C). After centrifugation (10,000 rpm for 30 minutes at 4°C), the supernatant was dialyzed (cutoff point, 3.5 kD) against H₂O and freeze-dried. Protein concentration was quantified according to the method of Bradford.¹⁶

The PBS chestnut extract was fractionated by means of gel filtration on a Sephacryl S-200 HR column (2.5×85 cm; Amersham Pharmacia Biotech, Uppsala, Sweden), eluting with 0.1 mol/L ammonium acetate (pH 6.8; 50 mL/hour). Fractions enriched in Cas s 8 were freeze-dried and further separated by means of RP-HPLC on a Vydac C4 column (2.2×25 cm; particle size 10 μ m; Separations Group, Hesperia, Calif). Elution was carried out with a 3-step linear gradient of acetonitrile in 0.1% trifluoroacetic acid (10% to 35% in 140 minutes, 35% to 50% in 100 minutes, and 50% to 85% in 15 minutes; 1 mL/min). Chromatographic fractions containing the chestnut LTP Cas s 8 were located by means of immunodetection after SDS-PAGE with both anti-peach LTP Pru p 3 rabbit polyclonal antibodies and a serum pool from patients with chestnut allergy.

Allergenic LTPs from peach fruit (Pru p 3) and *Artemisia vulgaris* pollen (Art v 3) were isolated as previously described.¹² Avocado class I chitinase allergen Pers a 1 and hevein from *Hevea brasiliensis* latex were also purified according to methods previously reported.^{7,17} All isolated allergens were quantified by using a commercial bicin-chonic acid test (Pierce, Cheshire, United Kingdom).

SDS-PAGE was performed on Bio-Rad Miniprotein II system gels (15% polyacrylamide), according to the method of Laemmli,¹⁸ by using both reducing (10% β -mercaptoethanol in the sample buffer) and nonreducing (replica gels) conditions. Similar results were obtained in both cases regarding the allergens studied (LTPs and class I chitinases). Figs 1 and 2 show results under reducing conditions. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI) analysis and N-terminal amino acid sequencing were carried out by using standard methods with a Biflex III spectrometer (Bruker-Franzen Analytik, Bremen, Germany) and an Applied Biosystems 477A gas phase sequencer (Foster City, Calif), respectively.

Immunodetection assays

Samples (20 μ g of PBS chestnut extract and 2 μ g of purified allergens) were separated by means of SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes. After blocking, membranes were incubated overnight with a serum pool or individual sera from patients with chestnut or latex-fruit allergy (1:6 dilution), washed, and then treated with mouse anti-human IgE mAb HE-2 ascitic fluid.¹⁹ After washing, a rabbit anti-mouse IgG peroxidase-conjugated antibody (1:5000 dilution; DAKO, Glostrup, Denmark) was added, and finally, IgE-binding components were detected by means of enhanced chemiluminescence, according to the manufacturer's instruction (Amersham Biosciences, Little Chalfont, United Kingdom). A pool of sera from patients with dust mite allergy but not plant food or pollen allergy was tested as a negative control.

Immunoblot inhibition assays with Cas s 8 as the inhibitor (5 μ g/mL) were carried out as described above, except that the serum was preincubated with Cas s 8 (3 hours at 25°C) before being used for IgE immunodetection.

Additionally, replica gels were electrotransferred onto polyvinylidene difluoride membranes and immunodetected with rabbit polyclonal antibodies to the peach LTP Pru p 3 (1:200 dilution) and then treated with alkaline phosphatase-conjugated anti-rabbit IgG (1:500 dilution; Sigma, St Louis, Mo), and finally, putative LTP components were detected with a 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium solution (Sigma).

Specific IgE determinations and SPTs with purified allergens

ELISAs to determine specific IgE of individual sera (1:8 dilution) from patients with chestnut allergy but not latex or latex-fruit allergy to purified Cas s 8, peach Pru p 3, or avocado Pers a 1 (3 μ g/mL) or to the PBS chestnut extract (30 μ g/mL) were performed as previously described.²⁰ PBS buffer with 1% BSA and a serum pool from patients with house dust mite allergy but not plant food or pollen allergy were used as negative controls. All tests were performed in triplicate.

SPTs with the purified natural allergenic LTPs Cas s 8, peach fruit Pru p 3, and mugwort pollen Art v 3 (20 μ g/mL in a 1:1 [vol/vol] PBS buffer/glycerol solution), as well as with purified natural avocado Pers a 1 (50 μ g/mL) and latex hevein Hev b 6.02 (10 μ g/mL), were carried out according to standard procedures. Histamine dihydrochloride (10 μ g/mL) and physiologic saline solution served as positive and negative controls, respectively. Duplicates of each sample were assayed. A mean wheal diameter of 3 mm or larger (15 minutes after puncture) compared with that produced by the negative control was considered a positive response.

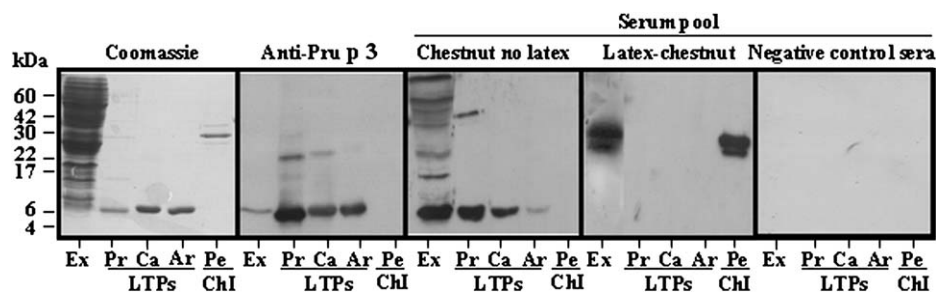


FIG 1. Protein staining with Coomassie blue and immunodetection with anti-Pru p 3 rabbit polyclonal antibodies or IgE of a pool of sera from patients with chestnut but not latex allergy, latex-chestnut allergy, or house dust mite allergy (negative control) of the following SDS-PAGE-separated samples: chestnut PBS extract (*Ex*) and the isolated allergens Pru p 3 (*Pr*), Cas s 8 (*Ca*), Art v 3 (*Ar*), and Pers a 1 (*Pe*).

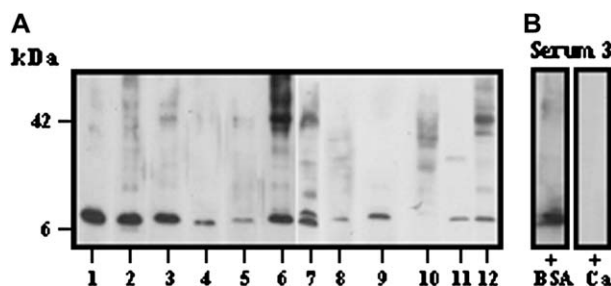


FIG 2. **A,** IgE immunodetection with individual sera (lanes 1-12) from patients with chestnut but not latex allergy (numbers as in Table I) of the SDS-PAGE-separated chestnut PBS extract. **B,** Immunoblot inhibition of serum 3 with BSA (+BSA; negative control) and Cas s 8 (+Ca) as inhibitors. Experimental conditions are as in Fig 2, A, except that the serum was previously incubated with each inhibitor.

RESULTS

Clinical characteristics of patients with chestnut allergy but not latex allergy

Twelve patients with chestnut allergy were included in the study, the general characteristics of whom are summarized in Table I. Mean $\pm$ SD age was 27.2 ± 7.9 years (rang, 17-46 years), with a predominance of female patients (8:4). All but 1 patient (92%) were atopic, as defined by a personal history of respiratory allergy, together with a positive SPT response to several common aeroallergens, which included mugwort pollen in most cases. Of the atopic group, 6 (54%) patients had rhinitis and bronchial asthma, and 5 (45%) had only rhinitis.

Clinical manifestations of chestnut allergy reported by patients consisted of oral allergy syndrome in 5 (42%) patients, oral allergy syndrome with urticaria-angioedema in 3 (25%) patients, isolated urticaria in 1 (8%) patient, and systemic anaphylaxis in 3 (25%) patients (2 of them with chestnut-dependent exercise-induced anaphylaxis). SPT responses to chestnut were positive in all patients, with mean $\pm$ SD wheals of 7.2 ± 3.4 mm. Total IgE levels ranged from 54 to 895 kU/L, with a geometric mean of 194.3 kU/L. Specific IgE to chestnut showed positive results in 11 (92%) of 12 patients, with a geometric mean of 3.4 kU/L (range, <0.35-16.8 kU/L). All our patients had several associated clinically relevant food allergies,

including other nuts in 10 (83%) patients, mustard in 7 (58%) patients, peach in 6 (50%) patients, and avocado in 2 patients (Table I).

In addition to chestnut skin reactivity, most patients also had positive SPT responses to peach (11/12, 92%) and mugwort pollen (10/12, 83%) commercial extracts, but negative responses to NRL (Table II).

Different IgE-binding patterns to chestnut proteins were detected with sera from allergic patients with or without associated latex hypersensitivity

A PBS extract from chestnuts was separated by means of SDS-PAGE and immunodetected with a pool of sera from patients with chestnut but not latex allergy. Up to 8 IgE-binding bands from 80 to 9 kd were detected, with the major one being that at 9 kd (Fig 1). In contrast, only a double band of around 30 kd was recognized by a serum pool from patients with latex-chestnut allergy (Fig 1), which is in accordance with data previously reported.⁷ No band was detected by using the negative control sera.

Identification of the major 9-kd chestnut allergen as Cas s 8

The IgE-binding component of 9 kd was isolated from the chestnut PBS extract by means of a 2-step

TABLE I. Clinical characteristics of patients with chestnut IgE-mediated hypersensitivity but without NRL allergy

Patient no.	Age (y)/sex	Atopy	Total IgE (kU/L)	Chestnut			Aeroallergen sensitizations	Other food allergies
				Reported symptoms	SPT (mm)	Specific IgE (kU/L)		
1	21/M	R	739	A	11	9.9	Pollens, dust mites	Nuts, mustard, seafood
2	27/F	R, BA	895	OAS	11	6.7	Pollen, animal danders, dust mites	Avocado
3	28/M	R	465	EIA	9	1.8	Pollens, dust mites	Nuts, mustard, cereals
4	46/F	R, BA	65	OAS, U	3	0	Pollens	Avocado, peach, nuts, legumes, kiwi, papaya
5	17/F	R	277	OAS	7	3.1	Pollens, dust mites	Peach, nuts
6	23/F	R, BA	212	EIA	6	1.4	Pollens	Nuts, mustard
7	29/F	R, BA	57	OAS	7	13.2	Pollens	Nuts, mustard, peach
8	17/M	R, BA	130	U	9	1.7	Pollens, animal danders	Nuts, mustard
9	32/F	—	54	OAS	4	0.8	Pollens, dust mites	Nuts, mustard, peach
10	32/F	R, BA	221	OAS, AE	3	1.1	Pollens	Nuts, mustard, peach, seafood
11	30/M	R	294	OAS	13	4.6	Pollens	Mustard, fish, chicken
12	24/F	R	95	OAS, U	4	16.8	Pollens, dust mites	Peach, nuts, kiwi

M, Male; F, female; R, rhinitis; A, anaphylaxis; BA, bronchial asthma; OAS, oral allergy syndrome; EIA, exercise-induced anaphylaxis; U, urticaria; AE, angioedema.

TABLE II. Results of SPTs in patients with chestnut but not latex immediate hypersensitivity and control patients with latex-chestnut allergy

Patient no.	Chestnut	Peach		Mugwort pollen		Latex		Avocado
	Cas s 8	CE	Pru p 3	CE	Art v 3	CE	Hev b 6.02	Pers a 1
1	5	7	—	6	5	—	—	—
2	4	7	7	11	—	—	—	—
3	ND	—	ND	8	ND	—	ND	ND
4	—	7	9	—	—	—	—	—
5	3	5	—	4	—	—	—	—
6	—	8	5	8	5	—	—	—
7	4	6	8	5	6	—	—	—
8	ND	10	ND	8	ND	—	ND	ND
9	9	16	7	7	5	—	—	—
10	ND	4	ND	4	ND	—	ND	ND
11	6	3	—	6	3	—	—	—
12	—	5	—	—	4	—	—	—
C1	—	—	—	—	—	8	10	4
C2	5	—	3	—	—	8	7	5
C3	—	—	—	—	—	10	7	3

Values are expressed in millimeters of mean wheal diameter.

CE, Commercial extract; —, negative response; ND, not done; C1-C3, control patients with latex-chestnut allergy.

chromatographic protocol, which included gel-filtration chromatography, followed by RP-HPLC (not shown). The purified protein behaved as a single band of the expected apparent molecular size in SDS-PAGE and was recognized by both anti-Pru p 3 (peach LTP) antibodies and IgE of sera from patients with chestnut but not latex allergy (Fig 1, lines Ca). The isolated 9-kd allergen showed a single peak at 9763 d in MALDI analysis, as well as a single N-terminal amino acid sequence, ITCTQVSKSL, which is identical to that of Cas s 8 allergen, except for the absence of the N-terminal serine previously described.¹² Together, all these data strongly indicated that the purified

9-kd protein and the chestnut LTP Cas s 8 were the same allergen.

LTPs are major allergens linked to chestnut but not latex allergy

The relevance of Cas s 8 in patients with chestnut but not latex allergy was highlighted by 3 different lines of experimental evidence. First, 11 (92%) of 12 individual sera from these patients recognized the SDS-PAGE band corresponding to Cas s 8 in IgE immunodetection assays (Fig 2). Furthermore, this band was the most reactive one with most sera. Second, significant specific IgE

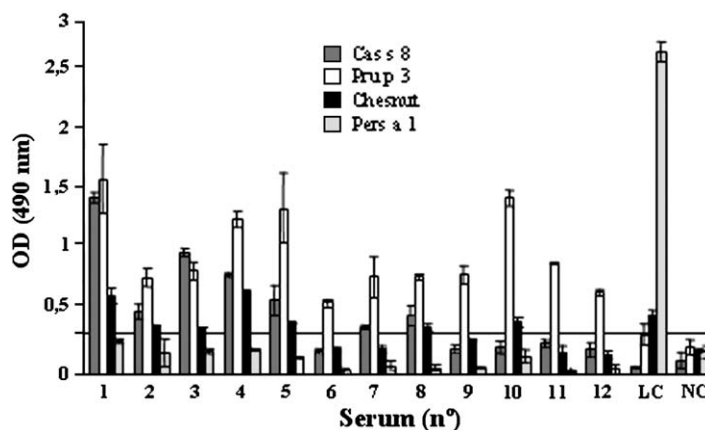


FIG 3. Specific IgE to Cas s 8, Pru p 3, Pers a 1, and PBS chestnut extract of 12 individual sera from patients with chestnut but not latex allergy (lanes 1-12) and a serum pool from patients with latex-chestnut allergy (LC). A pool of sera of subjects with allergy to mites (NC) and BSA in solid phase tested against the different sera ($n = 30$; mean, 0.087; SD, 0.042) was assayed as a negative control. Means ($n = 3$) and SDs (bars) are represented. Specific IgE levels were considered positive when greater than 0.3 OD units.

levels to the purified allergen were found in 7 (58%) of these sera by means of ELISA (Fig 3). Third, 6 (66%) of 9 patients had positive SPT responses to Cas s 8 (Table II).

On the basis of the above-mentioned results, together with the high rate of skin sensitization to peach fruit (92%) and mugwort pollen (83%) of the group of patients studied, the main LTP allergens from these plant sources (Pru p 3 and Art v 3, respectively) were also tested. Both allergens bound IgE of the pool of sera from patients with chestnut but not latex allergy (Fig 1). Moreover, all the individual sera (12/12, 100%) presented specific IgE to Pru p 3 (Fig 3), and more than 50% of the patients tested (5/9 for Pru p 3 and 6/9 for Art v 3) had positive reactions *in vivo* (SPT) to these 2 allergenic LTPs (Table II).

In sharp contrast with their sensitization to Cas s 8, as well as to its food and pollen homologues, patients without associated latex hypersensitivity did not show either specific IgE to class I chitinases, represented by avocado Pers a 1 (Figs 1-3), or an *in vivo* response to this allergen or to latex hevein Hev b 6.02 (negative SPT response in 9/9 cases assayed, Table II).

The opposite scenario was displayed by the population with latex-chestnut allergy. Their sera strongly reacted with Pers a 1 but not with allergenic LTPs in immunodetection assays (Fig 1) and, accordingly, showed very high specific IgE levels to Pers a 1 but negative responses to Pru p 3 and Cas s 8 (Fig 3). Positive SPT responses were obtained with Hev b 6.02 and Pers a 1 in the 3 patients, whereas only one of them was reactive to LTP allergens (Table II).

DISCUSSION

Up to 40% to 50% of patients with latex allergy show hypersensitivity to freshly consumed fruits or nuts, mainly avocado, banana, chestnut, and kiwi, a clinical picture

known as latex-fruit syndrome.⁶ This fact could explain why most studies on allergens from these foods (except kiwi) have been performed in patients also allergic to latex. In addition to other potential cross-reactive components,^{10,21,22} fruit class I chitinases and latex hevein Hev b 6.02 have been identified as the major allergens responsible for the syndrome.^{7-10,14} The *in vitro* and *in vivo* results here obtained with the population with latex-chestnut allergy testing avocado Pers a 1 as a model of fruit class I chitinases fully confirmed this sensitization pattern.

Patients belonging to the same population of subjects with latex-chestnut allergy but selected on the basis of a clinical history of chestnut but not latex sensitization, supported by SPT and specific IgE determination responses, showed a very different pattern of major allergens. Class I chitinases (Pers a 1) do not seem to play an important role, according to their lack of reactivity in immunodetection, ELISAs, and SPT assays (Figs 1-3 and Table II). However, a 9-kd protein was detected as the major IgE-binding chestnut component when searching with the individual sera of patients with chestnut but not latex allergy. The isolation and characterization of this allergen allowed us to identify it as the chestnut LTP, corresponding most probably to the previously described putative allergen Cas s 8.¹² The relevance of Cas s 8 in these patients was confirmed by its positive specific IgE levels and SPT responses (58% of sera and 66% of patients tested, respectively).

All our subjects with chestnut but not latex allergy were also sensitized to mugwort pollen and plant foods, particularly to peach fruit. Accordingly, in addition to Cas s 8, specific IgE and *in vivo* reactions to its peach (Pru p 3) and *Artemisia* pollen (Art v 3) homologues have been found in the studied patients. Interestingly, recent data on a group of patients with cabbage allergy from the same geographic area and with similar clinical characteristics to those here selected (except for chestnut

hypersensitivity) also point to a member of the LTP family (Bra o 3) as a major cabbage cross-reactive allergen.²³ Thus an actual clinical background that includes sets of patients with confirmed IgE-mediated symptoms to different plant foods and *Artemisia* pollen supports the relevance of the potential cross-reactivity previously reported among Pru p 3, Art v 3, and Cas s 8.^{12,13}

A virtually opposite situation to that described above was detected in the population with latex-chestnut allergy. No specific IgE to Cas s 8 or Pru p 3 was found in their sera, and only one of them had a positive SPT response to the LTP allergens tested. Therefore this panallergen family does not seem to play an important role in the latex-fruit syndrome, although cross-sensitization between plant food LTPs and their latex counterpart (Hev b 12)²⁴ cannot be excluded in some cases.

To the best of our knowledge, only a single work on chestnut allergy not linked to latex sensitization has been previously published.¹¹ It concerns patients from Korea, where chestnut accounts for the third greatest prevalence among food allergens. Up to 21 IgE-binding components with apparent molecular sizes of between 13 and 99 kd were detected, with 9 of them being recognized by more than 50% of the sera tested (n = 9). Only a 24-kd protein, presumably belonging to the legumin family, was characterized. These results suggest a very different pattern of allergen sensitization to that found in our group of patients.

In conclusion, chestnut allergy not linked to the latex-fruit syndrome presents Cas s 8 LTP as the major allergen in the Spanish population, whereas the main allergens involved in the syndrome, class I chitinases, do not seem to play a significant role in sensitization to this nut regarding patients not sensitized to NRL. Therefore basic research observations on LTP and chitinase allergens suggest that improved diagnosis and therapy might become possible on the basis of their reported characterization at the biochemical and clinical levels.²⁵

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