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Letter to the Editor

A new allergen family involved in pollen food-associated syndrome: Snakin/gibberellin-regulated proteins

To the Editor:

Around the Mediterranean basin, Cupressaceae pollen is considered as the primary cause of respiratory allergies with symptoms of rhinoconjunctivitis, chronic cough, and asthma.¹ The pollinosis can be severe including infectious complications, partly due to winter pathologies occurring within cypress pollinating period.² In Southern France, a pollen food-associated syndrome (PFAS) was described involving cypress pollen and peach and/or citrus sensitizations inducing mainly oral syndrome but also urticaria and angioedema. Up to now, the cross-reactive allergen at the basis of this syndrome has not yet been unraveled. Besides the 4 groups of allergens already described in the various Cupressaceae species, an as yet unidentified basic allergen of 14 kDa (BP14), overexpressed in *Cupressus sempervirens* pollen and different from a lipid transfer protein, was found to sensitize 37% of cypress pollen allergic patients (CPAPs) in Southern France.³ The BP14 IgE epitopes are not related to cross-reactive carbohydrate determinants and are heat resistant but destroyed under reducing conditions.

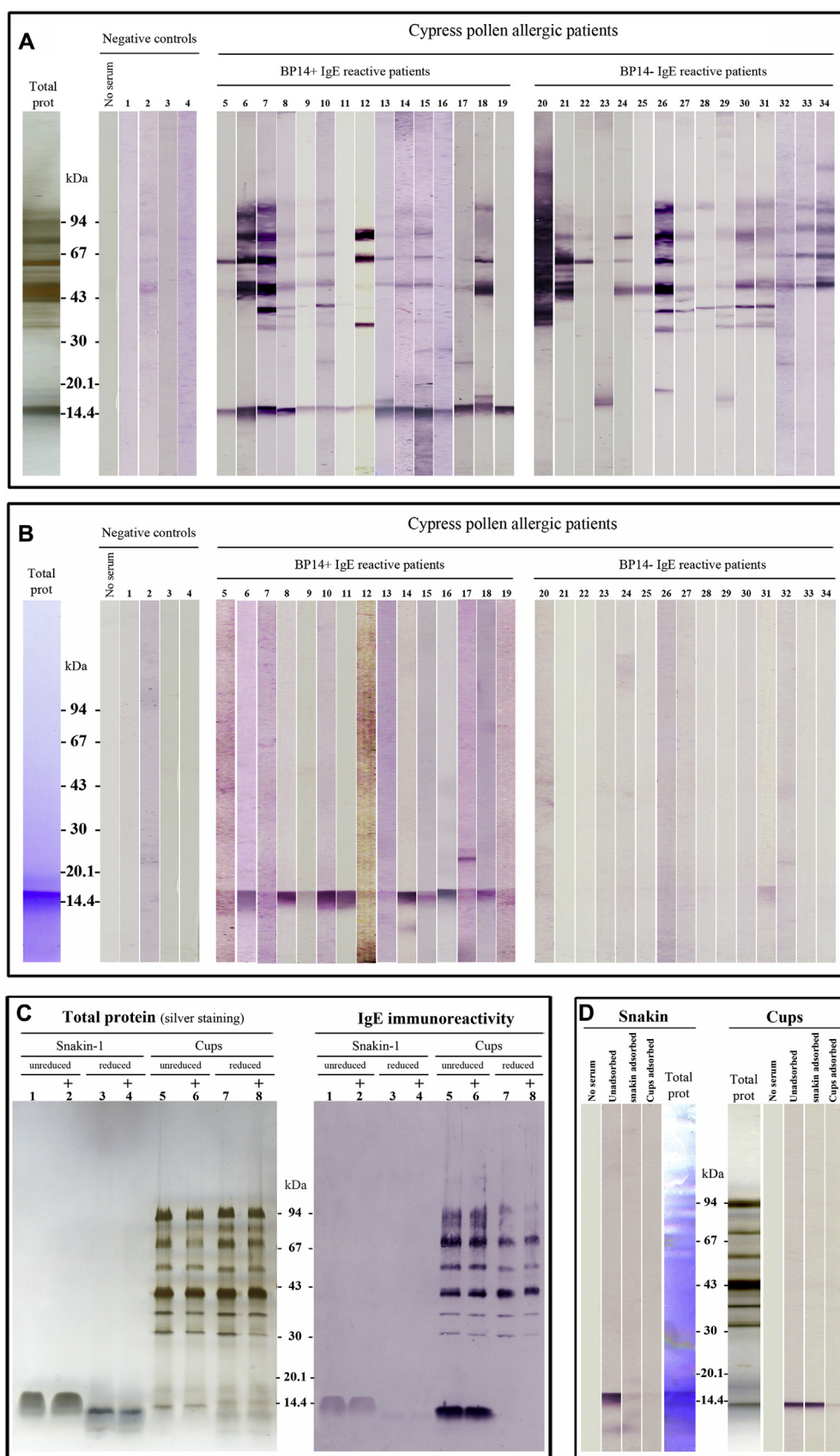
To identify BP14, proteins extracted from *C. sempervirens* pollen were run in SDS-PAGE and the corresponding band was submitted to mass spectrometry analysis (see this article's [Methods](#) section in the Online Repository at [www.jacionline.org](#)). Except a contaminant peptide of polygalacturonase, 2 overlapping peptides of the gibberellin-regulated protein (GRP) peamaclein (Pru p 7 from peach) were found: R.CLKYCGICCEK.C and K.YCGICCEK.C. Furthermore, 1 very similar peptide of the GRP of *Theobroma cacao* was identified:

R.CLKYCGICCK.K (Fig 1, A). Using the Basic Local Alignment Search Tool algorithm, the identified peptides were evidenced in proteins of the snakin/GRP family from various plant species. One of them, the snakin-1 from potato, produced in recombinant form⁴ and sharing 82.5% sequence identity with Pru p 7 and 79.4% with the GRP of *Theobroma cacao* (Fig 1, B), was tested in direct and inhibition western blots for its binding with serum IgE from CPAP. Out of 30 CPAP sera, all those expressing IgE to BP14 exhibited IgE reactivity to snakin-1 (n = 15) (Fig 2, A and B). No IgE binding was observed in 14 of the 15 sera tested from BP14-negative CPAPs. One serum (no. 31), tested negative for BP14, was positive for snakin-1. Available clinical data from patients are given in [Table E1](#) in this article's Online Repository at [www.jacionline.org](#). Similarly to BP14, the reduction of snakin-1 resulted in decreased apparent molecular mass and abolished IgE reactivity (Fig 2, C), pointing out the presence of conformational IgE epitopes, which is in agreement with the highly folded globular structure of GRP including 6 disulfide bridges.⁵ Crossed inhibitions in western blot were carried out using a 2-step "depletion" protocol to prevent unwanted molecular interactions occurring in a 1-step "competitive" approach between the 2 allergenic sources, snakin-1 and cypress pollen extract. Specific IgEs were depleted on a first allergenic source and residual IgEs were then assayed to the blotted proteins from the second allergenic source. Depletion of BP14-reactive IgE by adsorption on nitrocellulose-dotted pollen extract or on migrated-electrotransferred BP14 only was efficient to decrease the IgE reactivity to snakin-1 as well as to BP14 (Fig 2, D). However, depletion of snakin-1-reactive IgE with nitrocellulose-dotted or migrated-electrotransferred snakin-1 was not able to inhibit the binding to BP14. These results suggest that the primary sensitizer might be the cypress pollen grains. The results depicted in Fig 2, D, were obtained with CPAP number 14

A									
PROTEIN					PEPTIDE				
Accession	Function	Species	MW [kDa]	pI	#Peptides	SC [%]	Sequence	Range	Score
Q9FY19	Polygalacturonase	<i>Juniperus ashei</i>	55.7	10.0	1	2.6	K.VDGTIAAYDPDAK.W	121 - 133	65.1
P86888	Peamaclein	<i>Prunus persica</i>	6.9	8.6	2	17.5	K.YCGICCEK.C	25 - 32	27.2
							R.CLKYCGICCEK.C	22 - 32	21.2
A0A061FGF7	Gibberellin-regulated protein	<i>Theobroma cacao</i>	11.4	10.1	1	9.3	R.CLKYCGICCK.K	47 - 56	27.1

B									
Peamaclein:	-----	-----	-----	-----	-----	-----	-----	-----	-----
Snakin-1†:	-----	-----	-----	-----	-----	-----	-----	-----	-----
GRP Theo:	MKLILVTFLL	VSLVLSSFF	EVSMAGSGFC	DSKCKVRCCK	AGAKD	RCLKY	CGICCKKCKC	60	
Peamaclein:	VPSGTYGND	ECPCYRDLN	SKGNPKCP	-----	63				
Snakin-1†:	VPSGTYGND	ECPCYRDLN	SKGKSKCP	-----	63				
GRP Theo:	VPSGTYGND	ECPCYRDMN	SKGQLALAGL	NCTQR	95				

FIG 1. A, Mass spectrometry analysis of in-gel-separated BP14 proteins from *Cupressus sempervirens* pollen extract. The gel was run under reducing condition. For detailed information, see this article's [Methods](#) section. **B**, Sequence alignment of peamaclein from *Prunus persica* (P86888), snakin-1 from *Solanum tuberosum*, and GRP from *Theobroma cacao* (GRP Theo, A0A061FGF7). The enlightened sequences correspond to the peptides identified in mass spectrometry analysis. MW, Molecular weight; pI, isoelectric point. SC (%) percent coverage. *Amino acid identity with peamaclein sequence. †Snakin-1 sequence without signal peptide as described in Kuddus et al.⁴



and similar results were observed with other patients' sera (see Table E2 in this article's Online Repository at www.jacionline.org). The set of IgE epitopes recognized on BP14 might be different from those recognized on snakin-1. This might be related to the existence of a highly variable region in amino-acid composition and sequence length in the snakin/GRPs.⁵

In consequence we showed using mass spectrometry and direct and crossed inhibition binding assays that BP14 allergen from *C. sempervirens* is cross-reactive with a member of the snakin/GRP protein family while also displaying some specific IgE epitopes. Snakin/GRPs are involved in plant development such as pollen maturation, responses to biotic or abiotic stress, hormone crosstalk, and redox homeostasis. They are small cationic polypeptides described in flowers, vegetables, and fruits, with 12 highly conserved cysteins and with antimicrobial activity.⁵ Interestingly, antimicrobial cationic proteins participating in innate immunity such as cathelicidins and defensins also exist in humans.⁶

Four snakin/GRPs from fruits were shown to be allergens, Pru p 7 in peach,⁷ Pun g 7 in pomegranate,⁸ and Pru m 7 and Cit s 7 in Japanese apricot and sweet orange, respectively (www.allergen.org). Because Pru p 7 shares more than 80% sequence identity with snakin-1 and more than 95% with other fruit GRP allergens, BP14 should be considered as the cross-reactive allergen in the 2 documented PFAS involving peach and/or citrus.¹ Preliminary experiments showed that patients allergic to cypress pollen and citrus expressed IgE against cypress pollen BP14 cross-reactive with a cationic 12-kDa allergen from grapefruit (unpublished data). Therefore, snakin/GRP may represent a new diagnostic marker of PFAS in addition to other allergens such as Bet v 1-like proteins involved in the well-studied birch/apple syndrome.⁹ Whether specific allergenic immunotherapy using a BP14-containing cypress pollen extract can reduce the food-related symptoms remains unknown for the moment and would deserve more investigations. With regard to allergy to Cupressaceae pollen, Inuo et al.¹⁰ reported that a specific allergenic immunotherapy performed with a Japanese cedar pollen extract led to a decrease in basophil activation to tomato in a patient with a cedar/tomato syndrome. Snakin/GRP sensitization was reported to be clinically associated with eyelid edema, systemic reaction, or food-dependent exercise-induced anaphylaxis.¹¹

To conclude, we show herein that a snakin/GRP from pollen can be allergenic and is cross-reactive with other members of this protein family from fruits or vegetables. The results delineate the structural basis of the clinically reported PFAS between cypress pollen and peach and/or citrus. PFAS are increasing in prevalence because risk factors combine lifestyles (such as dietary habits) and environmental changes.⁹ The knowledge of the diversity of

allergen content in pollen and food is therefore crucial to discriminate between a cosensitization and/or a cross-reactivity to improve the diagnosis and the design of subsequent allergenic immunotherapy.

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FIG 2. IgE immunoreactivity of BP14+ (n = 15) and BP14- (n = 15) CPAP tested on cypress pollen extract (A) or snakin-1 (B). Total cypress proteins are silver stained (Fig 2, A) and snakin-1 is stained with Coomassie blue (Fig 2, B). Detailed procedures are described in the Methods section. Molecular masses are indicated in kDa. C, IgE immunoreactivity is abolished when snakin-1 or BP14 is reduced. Left part corresponds to silver-stained total proteins, and right part to IgE immunoreactivity using serum number 7 from a BP14+ CPAP. D, Crossed inhibition using a "depletion" protocol. NCA-blotted snakin or NCA-blotted cypress pollen extract (Cups) strips were incubated with either unadsorbed or snakin-adsorbed or cypress pollen extract-adsorbed (Cups) CPAP serum number 14 as indicated and IgE binding was revealed. Total proteins (Total prot) were stained either with Coomassie blue (snakin-1) or with silver nitrate (Cups). *Cups*, *Cupressus sempervirens* pollen extract; +, alkylation with iodoacetamide. Molecular masses are indicated in kDa.

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METHODS

Pollen protein extractions and recombinant snakin-1

Cupressus sempervirens (Cups) pollen was supplied by Allergon AB (Angelholm, Sweden). Pollen grains (10%, w:v) were suspended in PBS, pH 7.4, and grinded using a multidimensional grinder FastPrep 24 (MP-Biomedicals, Illkirch, France) for 40 seconds at 4°C at 6 m/s using matrix C (silice beads of 1-mm diameter).^{E1} After centrifugation (18,000g 4°C), the supernatant was collected and stored as aliquots at -20°C until use.

Recombinant snakin-1 was prepared in *Pichia pastoris* and purified essentially as described in Kuddus et al.^{E2} Briefly, after the induction phase, yeast pellet and supernatant were separated by centrifugation (14,000g, 4°C, 30 minutes). The snakin-1-containing supernatant was collected and purified on a HiTrap SP HP cation exchange column (GE Healthcare) followed by an RP-HPLC on a Cosmosil 5C18-AR300 column (Nacalai Tesque Research, Japan). The purified recombinant snakin-1 was then lyophilized and stored at -30°C.

For some experiments (MS/MS and ) proteins of Cups pollen extract and snakin-1 were reduced for 3 minutes at 100°C with 3% 2-mercaptoethanol and then alkylated with 250 mmol/L iodoacetamide (Sigma-Aldrich, St Louis, Mo) essentially as reported by Shahali et al.^{E3}

Patients and patient' sera

All CPAPs were selected according to their clinical symptoms (rhinitis, conjunctivitis, and asthma) during the cypress pollinating season and skin prick test to cypress pollen extract. Sensitizations were determined with the usual fluorescence enzyme immunoassay ImmunoCAP (ThermoFischer Scientific, Villebon, France and Phadia, Uppsala, Sweden) on the ImmunoCAP 2500 apparatus, according to the manufacturer's recommendations with a detection limit set at 0.10 kU/L. Mean Cups-specific IgE levels are similar in the 2 categories, BP14-positive and BP14-negative CPAP. Sera corresponded to residues from biological analysis laboratories for the diagnosis of allergy (see Table E1) and written informed consent was obtained from patients.

Sera from atopic patients with another type of allergy (food, nettle, or pinus pollen) (see Table E1) and the serum from a healthy individual (nonallergic, nonatopic) were used as negative controls. The sera were stored at -20°C until use. Mean age of CPAP was 26.8 years, with a range of 3 to 55 years distributed in 13 females and 18 males (see Table E1).

Gel electrophoresis analysis

Proteins were separated in SDS-PAGE in a 8% to 18% gradient polyacrylamide gel (ExcelGel, GE Healthcare, Uppsala, Sweden) and run on a flat-bed electrophoretic chamber (Multiphor II, GE Healthcare) at 12°C. Molecular mass markers (GE Healthcare, Little Chalfont, UK) ranging from 14.4 to 94 kDa were used as comparative references. Separated proteins were then either electrotransferred onto a 0.2-μm cyanogen bromide-activated nitrocellulose (NCA) membrane (Optitrans BA-S 83, Schleicher and Schuell, Dassel, Germany) for western blotting assays as described in Poncet et al.^{E4} or stained with Coomassie blue or silver nitrate for detection of the separated proteins.

Double 1-dimensional gel electrophoresis (D1-DE) was performed according to Shahali et al.^{E5} for Cups extract. After first dimension, isoelectric focusing in pH gradient 2 to 11, the whole width of basic proteins in the pH range 8.5 to 9.5 was run in SDS-PAGE as described above. Isoelectric point markers (Serva, Heidelberg, Germany) ranging from 4.45 to 9.6 were used as comparative references in first dimension.

Immunoblotting

Electroblotting of separated proteins was performed onto NCA sheets with a semidry Novablot apparatus (LKB, Uppsala, Sweden), 75 mn at 1 mA/cm². The membranes were then dried and blocked with PBS containing 0.3%

(v/v) Tween 20 (Sigma-Aldrich) (PBS-Tw) for 1 hour at 20°C. For 1-DE screening, each NCA was then cut into 2.5-mm wide strips that were individually incubated with diluted patient or control sera (1:10 or more when indicated) in PBS-Tw 0.1% (18 hours at 20°C). After washing, the membrane was incubated for 2 hours at 20°C with alkaline phosphatase-conjugated goat antihuman IgE (Sigma-Aldrich). The AP activity was detected using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma-Aldrich) in 0.1 mol/L tris acetate buffer, pH 9.5. For each immunoblot analysis, the serum from a healthy individual was used as a negative control.

Crossed inhibitions of IgE reactivity

Serum IgEs were depleted in specific IgE by adsorption on NCA-dotted or NCA-blotted first allergenic source before incubation with an NCA-blotted second allergenic source electrotransferred after separation of proteins by SDS-PAGE in 1-DE or in D1-DE.

Allergenic source-dotted immunosorbent. Virgin NCA strips were incubated for 1 to 2 hours with either Cups pollen extract (1/10 dilution) or snakin-1 (100 μg/mL) in PBS to carry out NCA-dotted immunosorbents. The membranes were then dried and blocked with PBS-Tw 0.3%. Selected sera were added to the NCA-dotted immunosorbents for 1 hour at 20°C under gentle rocking. Adsorbed sera were then incubated with NCA strips (2.5 mm wide) blotted with either an electrophoresis-separated Cups protein extract or snakin-1 and revealed in the same conditions as described in the section "Immunoblotting."

Allergenic source-blotted immunosorbent. Adsorptions were also performed on migrated and electrotransferred proteins (snakin-1 or BP14 from cypress pollen). Pieces of NCA (3 cm × 0.7 cm) containing either blotted snakin-1 or blotted BP14 (separated by D1-DE) were dried and blocked with PBS-Tw 0.3%. Selected sera were added to the NCA-blotted immunosorbents for 1 hour at 20°C under gentle rocking and the experiments proceeded exactly as with NCA-dotted immunosorbents.

Mass spectrometry analysis and database search

The reduction of BP14 resulted in a shift in apparent molecular mass from 14 kDa to 12 kDa as previously reported by Shahali et al.^{E3} Therefore, the Coomassie blue-stained protein band at 12 kDa was manually excised from a gel run in reducing conditions and further cut into 1 mm³ cubes. An in-gel digestion of proteins was carried out according to Hlaváčková et al.^{E6} Briefly, pieces of gel were destained using 100 mM NH₄HCO₃/acetonitrile 1:1 (v/v), disulfide bridges reduced with dithiothreitol (56°C, 45 minutes), and free cysteines alkylated with iodoacetamide (25°C, 30 minutes, in dark). Finally, proteins were digested for 3 hours at 37°C in 50 mM NH₄HCO₃ with trypsin (concentration 12.5 μg/mL, sequencing grade, Pierce, Rockford, Ill). The resulting peptides were extracted with 0.1% trifluoroacetic acid and then desalted and concentrated using ZipTip C-18 (Millipore, Billerica, Mass). Mass spectra were obtained using Maxis Impact mass spectrometer (Bruker Daltonics, Bremen, Germany) connected online with Dionex Ultimate 3000 RSLC UHPLC. For peptide separation, Acclaim PepMap RSLC C18 column was used with linear gradient of acetonitrile (3%-35% over 30 minutes). Flow rate was set to 300 nL/min. Eluted peptides were directed to Captive spray ion source. Precursors for fragmentation were selected in the range of 400 to 1400 m/z; up to 10 precursors could be selected from each MS spectrum. Peaklists were extracted from raw data by Data Analysis 4.1 and uploaded to Proteinscape 3.1 (both Bruker Daltonics, Bremen, Germany) data management system. Mascot server version 2.4.1 (Matrix Science, London, UK) was used for protein identification. Whole Swissprot database (version 2014-01) and Viridiplantae part of NCBI nr database (version 20140107) were searched in succession with following parameters: enzyme trypsin (1 missed cleavage allowed), cysteine carbamidomethylation as fixed and oxidation of methionine as variable modifications, peptide tolerance 10 ppm in the MS mode and 0.05 Da tolerance for peptide fragments in the MS/MS mode.

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TABLE E1. Clinical data of the 35 studied pollen- and/or food-allergic patients

Serum #	Sex	Age	Specific IgE (kU/L)			
			Cups pollen	Peach	Citrus	Other
1	M	67	ND	ND	ND	Negative control, nonatopic, nonallergic
2	M	4	0.35	1.80	ND	Potato: 1.22
3	M	47	<0.10	ND	ND	Nettle pollen: 9.84
4	F	26	<0.10	ND	ND	Pine pollen*
5	M	19	10.2	2.62	1.09	Mustard: 3.29; mite<0.10
6	F	3	26.0	1.80	ND	Grass: 87.5; weed: 28.6; mite: 0.23
7	M	3	6.99	14.7	3.08	Pea: 4.4; lentil: 4.2
8	M	30	11.5	<0.10	0.14	Juniperus: 40.1; dander: 1.8; mite: 0.3
9	M	14	2.27	0.29	<0.10	Juniperus: 13.6; pumpkin: 9.8
10	F	17	4.89	0.6	0.55	Weed: 3.2; olive: 11.4
11	M	21	2.86	<0.10	<0.10	Grass: 1.8; olive: 0.69; mite: 17.0
12	M	44	4.33	0.11	<0.10	Grass: <0.10; weed: 0.16
13	F	31	5.82	ND	3.96	
14	F	17	8.39	0.41	0.43	Grass: 0.26
15	M	13	*	ND	*	
16	F	25	7.50	*+	0.76	
17	F	31	10.9	ND	2.34	
18	F	39	59.8	<0.10	ND	<i>C. arizonica</i> : 36.7; <i>J. sabinoides</i> >100; mite 1.7
19	F	48	4.66	0.98	1.44	Curry: 1.19; coriandre: 0.74; gluten: 0.49
20	M	42	23.1	21.7	15.6	Grass: 26.7; mimosa: 1.44; dander: 5.3
21	M	72	21.6	0.57	0.87	Grass: 2.21; dander: 5.96
22	M	10	8.15	0.18	0.29	Grass: 24.4
23	M	13	11.8	6.41	3.85	Weed: 36.9; oak: 35.5
24	M	19	8.15	<0.10	<0.10	Grass: 0.11
25	F	21	0.88	<0.10	<0.10	Grass: <0.10; dander: <0.10; mite: 12.0
26	F	11	12.1	5.2	4.57	Ara h 8: 9.6; Ara h 9: 4.7; grass: 8.2; olive: 75.5; dander: 3.3; mite 50.7
27	M	5	3.35	<0.10	<0.10	Weed: 0.1; mite: 0.3
28	M	41	1.54	<0.10	<0.10	Mimosa: <0.10
29	M	11	8.8	4.69	8.15	Pea, lentil, chickpea>100; grass: >100; olive: 18.0; dander: 7.7
30	M	55	1.11	0.7	0.72	Grass: 1.4; weed: 4.8; mite: 10.9
31	F	48	0.47	0.35	0.35	Grass: 0.48; mite: 0.11
32	M	Unknown	51.36	<0.10	ND	
33	F	37	3.62	0.12	0.16	Grass: 0.18; weed: 0.16
34	M	21	13.7	0.49	0.45	Grass: 40.3; weed: 0.59
35	F	44	13.1	ND	<0.10	Weed: 0.17; dander: 13.0; mite: <0.10

F, Female; M, male; ND, not done.

*Positivity was only qualitatively assessed.

TABLE E2. Crossed inhibition between snakin-1 and BP14 from cypress pollen studied with a “depletion” protocol using 5 different patient sera in immunoblotting experiments

Patient no.	IgE reactivity on	Immunosorbent for depletion	
		Snakin-1	Cypress pollen extract
5	Snakin-1	+	+
	BP14 in basic fraction	—	+
12	Snakin-1	+	+
	BP14 in basic fraction	—	+
14	Snakin-1	+*	+†
	BP14 in total extract	—	+
16	BP14 in basic fraction	—*	+†
	Snakin-1	ND	ND
19	BP14 in basic fraction	—	+
	Snakin-1	+	+
19	BP14 in basic fraction	—	+

+, IgE inhibition; —, no IgE inhibition; ND, not done.

*Same results were obtained upon depletion on migrated snakin electrotransferred on NCa.

†Same results were obtained upon depletion on D1-DE-migrated BP14 electrotransferred on NCa.