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Pru p 7 sensitization is a predominant cause of severe, cypress pollen-associated peach allergy

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Abstract

Background: Peach is a common elicitor of food allergic reactions. Peach-induced immediate reactions may occur as benign pollen-food syndromes, usually due to birch pollen-related PR-10 cross-reactivity in temperate climates, and as potentially severe primary food allergies, predominantly related to nsLTP Pru p 3 in Mediterranean regions. The newly described peach allergen Pru p 7 has gained recent attention as a potential peach allergy severity marker. Sensitization to Pru p 7 and its allergenic homologues of the gibberellin-regulated protein family occurs in areas with high *Cupressaceae* tree pollen exposure.

Objective: We sought to investigate the distribution, clinical characteristics and molecular associations of Pru p 7 sensitization among subjects with suspected peach allergy in different regions of France.

Methods: Subjects with suspected peach allergy (n = 316) were included. Diagnostic work-up was performed according to current guidelines, including open food challenge when required. IgE antibody measurements and competition experiments were performed using the ImmunoCAP assay platform.

Results: Sensitization to Pru p 7 was present in 171 (54%) of all subjects in the study and in 123 of 198 (62%) diagnosed as peach allergic, more than half of whom were sensitized to no other peach allergen. Frequency and magnitude of Pru p 7 sensitization were associated with the presence of peach allergy, the clinical severity of peach-induced allergic reactions and the level of cypress pollen exposure. Cypress pollen extract completely outcompeted IgE binding to Pru p 7. Pru p 7 was extremely potent in basophil activation tests.

Conclusion and Clinical Relevance: A subtype of *Cupressaceae* pollinosis, characterized by Pru p 7 sensitization, can be an underlying cause of severe peach allergy.

KEYWORDS

allergens and epitopes, anaphylaxis, basophil, cypress pollinosis, food allergy, IgE, immunological tests, peamaclein, Pru p 7

1 | INTRODUCTION

Fruit and vegetables account for the most common aetiology of food allergies in teenagers and adults.¹⁻⁴ Severe reactions are frequent, with fruit and vegetables reported as causative in 5% of all cases of food anaphylaxis in children and adolescents in a range of European countries,⁵ and confirmed as culprit in 7% of children seeking emergency care at a major Madrid hospital for anaphylaxis-like

symptoms.⁶ Peach-induced immediate reactions are frequent in pollen-allergic patients and often occur as pollen-food syndromes (PFS).⁷⁻⁹ Birch pollen-related PR-10 sensitization underlies most cases of peach-induced oral allergy syndrome (OAS) occurring in temperate climates, while sensitization to non-specific lipid transfer proteins (nsLTPs) is predominant in Mediterranean regions and may induce OAS as well as severe reactions.^{1,4,9-11} In southern Europe, primary food allergies, including peach allergy (PA), may account for

up to 96% of food allergy cases.¹ Sensitization to nsLTPs, either as primary food allergy or as PFS, accounts for most of PA cases in the Mediterranean region.^{1,12,13} However, some PA patients have been found to lack sensitization to Pru p 1 (PR-10), Pru p 3 (nsLTP) and Pru p 4 (profilin), despite a convincing clinical history and a strongly positive skin test with fresh peach.¹⁴⁻¹⁶ Pru p 7, also referred to as peamaclein, a member of the gibberellin-regulated protein (GRP) family, was described as a novel peach allergen.¹⁴ We have previously reported Pru p 7 as a major allergen associated with severe clinical symptoms and strong cypress pollen (CP) sensitization in PA patients from Marseille, southern France.¹⁶ As France comprises a wide spectrum of geographic and climatic conditions, including continental, oceanic, Mediterranean and alpine regions, a variety of pollen sensitizations may occur across the country, making a fuller understanding of different pollen-related food allergies particularly relevant. In this study, we set out to further characterize the implications of Pru p 7 sensitization in PA in Mediterranean and non-Mediterranean regions of France.

2 | METHODS

2.1 | Patients

Three hundred sixteen outpatients residing in southern France (42nd to 46th latitude north), seeking allergy work-up for a suspicion of peach-induced food allergy symptoms, were included in this study. Clinical data were collected by means of a standardized questionnaire retrospectively completed by the allergists. Continental, oceanic and Mediterranean climates were represented. Five geographic regions were defined based on cypress and birch pollen counts¹⁷ and numbered 1-5 according to ascending order of cypress/birch pollen count ratio: region 1 (Lyon and continental environments), region 2 (Bordeaux and neighbouring Atlantic coastal regions), region 3 (alpine and continental regions including Toulouse and southern Alps), region 4 (Nice and Montpellier regions) and region 5 (Marseille and Var regions, southernmost Mediterranean environments) (Table 1). Each participant's area of residency was categorized as belonging to one of these regions.

2.2 | Diagnostic criteria and procedures

Diagnosis of PA was confirmed or rejected according to ICON and EAACI guidelines for food allergy.¹⁸⁻²⁰ The combination of a convincing clinical history of immediate reaction to peach in the past year, positive skin prick tests (SPT) to peach extract (Stallergènes-Greer, ALK-Abelló) or fresh peach defined as a weal >50% of the positive control, and/or IgE sensitization to peach, formed the basis of a PA diagnosis.²⁰ Convincing clinical history was defined as one or more immediate reaction (s) within 2 hours after peach ingestion, inhalation or direct contact, presenting as acute urticaria or angioedema, contact urticaria, OAS, food-dependent exercise-induced anaphylaxis, laryngeal swelling, immediate vomiting, rhinitis, cough, wheezing, bronchospasm, hypotension or loss of consciousness. The use of peach-specific IgE determination in conjunction with clinical history and peach SPT helped reduce the need

for oral food challenge (OFC) as advised.²¹ Diagnostic OFC was performed according to ICON and EAACI guidelines^{18,20} and previous PA studies^{3,11} if negative SPT and IgE results were discordant with clinical history. Patients who had had a life-threatening reaction to peach during the last 12 months did not undergo OFC, in line with current guidelines.¹⁸ A total of 61 patients were subjected to OFC, of whom 41 failed (PA) and 20 passed (peach tolerant, PT).

OFC followed an open protocol, with peach peel and pulp tested separately. After a negative labial challenge test, doses of fresh peach were administered in sequential 20-minute steps, starting the provocation with 1/128 of one fruit (approximately 1 g), then proceeding with 1/64, 1/32, 1/16, 1/8, 1/4 and 1/2 (highest dose approximately 65 g). The OFC was stopped if objective or severe symptoms appeared: erythema, urticaria, sneezing, wheezing, cough, vomiting, severe abdominal pain, hypotension, neurological symptoms or difficulty to breathe. Patients were kept in day-care units at close proximity of an intensive care unit during the challenge procedure and were monitored for 4 hours after ingestion of the last dose of peach. At the end of the diagnostic procedure, patients were categorized as PA or PT.

The diagnostic investigation for other food allergies followed the same steps as that for PA.

2.3 | Assessment of clinical severity

The severity of clinical reactions was graded following the recently proposed classification of food allergic and anaphylactic reactions^{22,23}: grade 1 comprising local reactions without systemic involvement: redness, swelling and itching; grade 2 mild to moderate systemic reactions involving distant skin locations, upper airways and/or gastrointestinal symptoms, but without cardiovascular or respiratory involvement: urticaria, angioedema, flush and gastrointestinal symptoms; grade 3 severe systemic reactions with cardiovascular and/or bronchial and laryngeal involvement: wheezing, stridor, objective dyspnoea, tachycardia, lowered blood pressure and shock.

2.4 | Ethics statement

Determination of IgE to whole allergen extracts and molecular allergens was part of routine investigations. Patients received written laboratory reports and the study was based on a retrospective review of medical records and laboratory results. In accordance with the Declaration of Helsinki and the French law on research involving humans, ethics committee approval and patient consent were not required for this type of non-interventional study, provided the patients received information and retained the right to oppose to the use of excess serum and anonymized medical data for research purposes.^{24,25}

2.5 | IgE antibody measurements

IgE antibodies directed to natural allergen extracts and molecular allergens were measured with the ImmunoCAP[™] assay platform (Thermo Fisher Scientific, Uppsala, Sweden) during the routine diagnostic procedure.

All sera were assayed for IgE to peach, CP, PR-10 protein rPru p 1, nsLTP rPru p 3, profilin rPru p 4, the major and specific CP allergen pectate lyase nCup a 1, and MUXF3, representing cross-reactive carbohydrate determinants (CCD). IgE assays to additional allergen extracts and components were performed according to each patient's history. IgE to natural and recombinant Pru p 7 was measured on excess serum with experimental ImmunoCAP™ tests. rPru p 1 and rPru p 4 were taken as surrogates for their respective allergen families due to high level of molecular similarity and cross-reactivity.^{2,26} Pru p 3, the immunodominant nsLTP and a possible primary sensitizer for this allergen family,^{12,27} was considered representative of nsLTP sensitization. Specific IgE values exceeding the assay's stated limit of quantitation of 0.10 kU_A/L were considered positive.²⁸ For comparison of the experimental ImmunoCAP assay to an independent method for detection of IgE to nPru p 7, a subset of sera were analysed with the multiplexed FABER® assay (CAAM, Rome, Italy).^{29,30}

2.6 | Preparation of natural and recombinant peach allergens and experimental ImmunoCAP™ tests

Natural Pru p 7 was purified from extract of canned peaches by sequential steps of cation exchange, size exclusion and reversed phase chromatography. Bioaffinity adsorption was then performed to eliminate any residual amount of LTPs. Natural Pru p 3 was purified from peach peel extract by affinity chromatography using a monoclonal anti-Pru p 3 antibody, followed by cation exchange chromatography. Recombinant Pru p 7 and Pru p 3 were expressed in *Pichia pastoris* and purified from culture medium by cation exchange or metal ion affinity chromatography, followed by size exclusion or cation exchange chromatography. Purity, homogeneity and identity of the allergen preparations were verified by SDS-PAGE, analytical

size exclusion chromatography, mass spectrometry and immunological activity using ImmunoCAP™ and serum samples with defined IgE reactivities. All chromatography media and equipment were obtained from GE Healthcare Life Sciences (Uppsala, Sweden). Experimental ImmunoCAP™ tests were prepared as described.³¹

2.7 | IgE competition experiments

Cross-reactivity between Pru p 7 and CP was studied by preincubating serum samples for 2 hours at room temperature either with CP extract (15% w/v final concentration, 42 sera) or with rPru p 7 (100 µg/mL final concentration, 33 sera) prior to measurement of IgE binding to rPru p 7 or CP ImmunoCAP solid phase, respectively. Percent inhibition was calculated as reduction of IgE binding in comparison with serum samples pre-incubated with buffer alone. Dose-dependent inhibition was performed with four samples. Two samples were incubated with 15% (w/v) CP extract and a 4-fold dilution series thereof, followed by measurement of IgE binding to Pru p 7. Two other samples were instead pre-incubated with rPru p 7 in a 4-fold dilution series from 200 to 0.2 µg/mL, followed by measurement of IgE binding to CP extract.

2.8 | Basophil activation tests

Basophil activation tests (BAT) were performed with a commercial CD193 (CCR3)/CD63 kit (Bühlmann Laboratories, Hochbuch, Switzerland) in a subset of patients. Peach extract, Mal d 1 (PR-10), Tri a 12 (profilin), and cow's milk extract (Bühlmann Laboratories) and recombinant and natural Pru p 7 and Pru p 3 were used as allergen reagents. Milk extract served as a negative control for foods regularly consumed and tolerated by each tested patient. Extracts

TABLE 1 Geographic and demographic characteristics of the study population

	All regions	Region 5	Region 4	Region 3	Region 2	Region 1
Pollen counts (per year and m3)						
Birch	639	69	176	226	1077	1648
Cypress	11 408	18 782	17 251	4276	9021	7712
Cypress/birch ratio	18	272	98	19	8	5
All patients	316	205	27	35	28	21
Sex ratio (M/F)	0.7	0.8	0.9	0.5	1.0	0.8
Age (years; median, IQR)	18 (11-33)	17 (11-33)	28 (18-47)	21 (12-32)	16 (10-27)	16 (11-32)
Peach allergic patients						
Sample size (number, %)	198 (63%)	133 (65%)	22 (81%)	20 (57%)	13 (46%)	10 (48%)
Sex ratio (M/F)	0.8	0.8	1.0	0.5	1.2	0.8
Age (years; median, IQR)	21 (13-36)	21 (13-36)	32 (21-47)	19 (14-32)	17 (9-31)	16 (13-34)
Peach tolerant patients						
Sample size (number, %)	118 (37%)	72 (35%)	5 (19%)	15 (43%)	15 (54%)	11 (52%)
Sex ratio (M/F)	0.7	0.8	0.7	0.4	0.9	0.8
Age (years; median, IQR)	15 (10-28)	14 (9-26)	15 (13-24)	23 (10-32)	15 (10-25)	17 (10-31)

Regions 1-5 were defined in ascending order of cypress to birch ratio. Outpatients seeking allergy work-up for a history of peach-induced symptoms were included. Peach allergy was confirmed or rejected according to ICON and EAACI guidelines for food allergy.

and molecular allergens obtained from Bühlmann Laboratories were used at final concentrations of 2.5, 1, 0.5 and 0.05 times the manufacturer's recommended concentration, that is 62.5, 25, 12.5 and 1.25 ng/mL for peach and milk extracts, and 625, 250, 125 and 12.5 ng/mL for Mal d 1 and Tri a 12.

Natural and recombinant Pru p 7 was applied at 11 different concentrations, ranging from 0.25 pg/mL to 2.5 µg/mL. Natural and recombinant Pru p 3 was applied at final concentrations of 5 ng/mL, 50 ng/mL and 2.5 µg/mL. Positive and negative controls (patient background, anti-FcεRI and formyl-methionyl-leucyl-phenylalanine) were assayed in duplicate at the start and end of the experiment for each patient. Analysis was performed with a Canto II flow cytometer and FACS Diva software (BD Biosciences, Franklin Lakes, NJ). Five hundred basophils or more were analysed for each activation condition. Background CD63 expression was lower than 5% in all patients, and no non-responder (anti-FcεRI-induced CD63 expression of 10% or less) was found. BAT was considered positive for an allergen concentration if CD63 expression exceeded twice the highest background result in each patient. EC₅₀, CD-sens and the ratio of maximal activation Pru p 7/anti-FcεRI activation were calculated as described.^{32,33}

2.9 | Statistical data analysis

Median and IQR for IgE were calculated for sensitized subjects in each group. GraphPad Prism (version 7, La Jolla, CA), SPSS (version 25, Chicago, IL) and R (version 3.3.2, R Foundation for Statistical Computing, Vienna, Austria) were used for statistical analyses. Differences with a *P* value < 0.05 were considered statistically significant. Binomial variables were compared using Fisher's exact test and modelled according to multiple binomial logistic regression models. Ordinal variables (reaction grade and region of residency according to pollen counts) were compared using the asymptotic general independence test and modelled according to multiple ordinal regression models. Continuous variables were compared using Mann-Whitney's test and modelled according to multiple linear regression models. Correlation studies were done by calculating Spearman's correlation coefficient. Odds ratios were calculated as previously described.³⁴

3 | RESULTS

3.1 | Demographic and food allergy characteristics of the study population

A total of 316 patients from the southern part of France were retrospectively included in the study, with a median age of 18 years (range 0.13-74) and sex (M/F) ratio 0.7. Subjects were classified according to two diagnostic categories: PA and PT (Table 1). PA was diagnosed in 198 (63%) patients. Allergy to other fruits (mainly *Rosaceae*, *Citrus* and exotic fruits) was found in 157 (79%) of PA patients, while allergy to legumes, tree nuts, cereals and seeds was found in 46 (23%). Among the 118 PT patients, allergy to other

fruits or vegetables was found in 42 (36%) and to legumes, tree nuts, cereals and seeds in 64 (54%). In 33 (28%) of the PT subjects, no plant food allergy was found (Figure S1). Involvement of a cofactor was reported in 24 (12%) of the PA patients, exercise being the most frequent (*n* = 19). Among the symptoms experienced, eyelid oedema was reported by 19 (10%) of the PA patients.

3.2 | Pru p 7 sensitization is present in all regions and is higher and more common in PA than PT patients

Overall, Pru p 7 sensitization was found in 171/316 (54%) patients (Table 2). A gradient of Pru p 7 sensitization from north (region 1, 29% prevalence) to south (region 5, 60% prevalence) was observed, *P* = 0.003 (Figure 1). Pru p 7 sensitization was more frequent in PA (123/198, 62%) than in PT (48/118, 41%) patients, *P* = 0.0002. It was positively and independently related to PA (OR [CI95]: 2.5 [1.4-4.6]; *P* = 0.003). Apparent monosensitization to Pru p 7, that is without detectable IgE to any of the other tested peach allergens (Pru p 1, Pru p 3, Pru p 4), was more frequent in PA (66/123, 54%) than in PT (8/48, 17%) patients, *P* < 0.0001.

The concentrations of IgE to Pru p 7 were higher in PA than PT patients sensitized to this allergen (median [IQR]: 3.4 [0.5-8.3] vs 0.3 [0.2-0.5] kU_A/L; *P* < 0.0001) (Table 2). Data on sensitization to other molecular allergens in PA and PT patients are available in Tables S1 and S2.

For comparative purposes, sera of six PA subjects with IgE to Pru p 7 by ImmunoCAP were also analysed with the FABER[®] assay (CAAM, Rome, Italy) and concordant results were obtained in five of these (83%).

3.3 | Pru p 7 sensitization is associated with the severity of reaction and cofactor involvement

Figure 2 shows the levels of IgE antibody responses to peach, individual peach components, CCD and CP in the PA and PT subjects, illustrating the prominent difference in Pru p 7 sensitization between these groups. Further, Pru p 7 sensitization was more frequent in PA patients who experienced grade 3 reactions (61/78, 78%) than in those who experienced grade 2 (38/59, 64%) or grade 1 (24/61, 39%) reactions, *P* < 0.0001 (Figure 3A). Likewise, the severity of reactions elicited by peach was significantly associated with the concentration of IgE to Pru p 7 (Figure 3B). Odds ratio calculation showed that Pru p 7 sensitization was positively and independently related to a higher prevalence of grade 3 reactions (OR [CI95]: 3.7 [1.6-8.5]; *P* = 0.002), and negatively related to grade 1 (OR [CI95]: 0.2 [0.1-0.4]; *P* < 0.0001). This association remained significant after adjustment for sensitization to other peach allergens and to CP as potential confounding covariates.

Cofactor involvement was significantly more frequent in Pru p 7-sensitized patients (22/171 vs 7/145, *P* = 0.01), but eyelid oedema during the allergic reaction was not (15/171 vs 5/145, *P* = 0.06).

3.4 | Pru p 7 induces basophil activation in PA patients

The ability of Pru p 7 to activate effector cells was assessed with BAT (Figure 4). The test was performed with peach extract, natural and recombinant Pru p 7, available representative molecules for relevant allergen families: nsLTP (Pru p 3), PR-10 (Mal d 1) and profilin (Tri a 12), and a tolerance control allergen, that is cow's milk, in 9 PA patients sensitized to Pru p 7 and in one PT, Pru p 7 non-sensitized patient with plant food-induced anaphylaxis. Comparable results were obtained with the natural and recombinant Pru p 7 and with natural and recombinant Pru p 3. All Pru p 7-sensitized patients displayed basophil activation induced by peach extract and Pru p 7 as monitored by CD63 membrane expression, but no activation with the other allergens tested, nor with cow's milk extract (Table S3). The PT patient's basophils were not activated by any of the extracts or molecular allergens tested (Table S3). Basophil sensitivity to Pru p 7-induced activation was monitored through EC₅₀, CD-sens and the lowest activating concentration. Median EC₅₀ was 0.05 ng/mL (IQR 0.0125-1.25) and median CD-sens was 2000 (IQR 80-8000). EC₅₀ and the lowest activating concentration were lower in PA patients without cofactor involvement (*n* = 6) than in those who required a cofactor for reaction to peach (*n* = 3), with a similar trend for CD-sens (Table 3). The ratio of maximal rPru p 7-induced activation to anti-RFceI-induced activation was close to 1 in both groups.

3.5 | IgE to Pru p 7 is associated with and cross-reactive to IgE to cypress pollen

We have previously described a ubiquitous co-sensitization to CP in Pru p 7 sensitized individuals.¹⁶ Among the 171 Pru p 7 sensitized subjects in the present study, all but one, who had Pru p 7 IgE at the positivity threshold of the assay (0.10 kU_A/L), were also sensitized to CP.

To study more directly a likely cross-reactivity between Pru p 7 and CP, we performed a dose-response IgE competition experiment with sera from four patients sensitized to CP and Pru p 7. Preincubation with different concentrations of CP extract caused a gradually increasing and ultimately almost complete (98-99%) inhibition of IgE binding to Pru p 7 at the final inhibitor concentration of 15% (w/v) (not shown). In contrast, preincubation with Pru p 7 only partly (67-72%) prevented IgE binding to immobilized CP extract even at the highest inhibitor concentration applied (not shown).

We next performed single-point inhibition of IgE binding to Pru p 7 in 42 patients' sera, with a fixed final concentration of 15% (w/v) of CP extract. This resulted in >80% inhibition in 38 of the 42 sera and >60% in the remaining four (Figure 5A). Conversely, single-point inhibition of IgE binding to CP extract by Pru p 7, at a fixed final concentration of 100 µg/mL, was performed in 33 patients' sera and resulted in >40% inhibition in only 9/33 (27%) and less than 20% in 10/33 (30%) of the sera tested (Figure 5B). The ability of Pru p 7 to inhibit IgE binding to CP extract was negatively correlated to the ratio of IgE to Cup a 1/Pru p 7 (Spearman rho -0.7, CI95 -0.9 to

-0.5, *P* < 0.0001), reflecting the proportion of CP-specific IgE directed to Cup a 1, which is structurally unrelated to Pru p 7.

3.6 | Cypress pollen exposure determines regional peach molecular sensitization profiles

Using a multivariate model of CP sensitization including all tested peach allergens as covariates, we found that no peach allergen other than Pru p 7 was significantly associated with CP sensitization (Table S4). The relationship between CP and Pru p 7 sensitization was further assessed by sorting patients by region of residency as described in Methods (Table 1). Ordinal logistic regression models illustrated a strong relationship between the cypress/birch pollen count ratio and the occurrence of sensitization to CP, Pru p 7 or Pru p 1 (Table S5). This relationship was positive for CP and Pru p 7 sensitization (*P* < 0.0001 and *P* = 0.0001, respectively), and inverse for Pru p 1 sensitization, a surrogate of Bet v 1 sensitization (*P* = 0.03). Further multivariate analysis of CP, Pru p 1 and Pru p 7 sensitizations found independent relationships between these covariates and cypress/birch pollen count ratios, consisting of a quantitative effect of CP exposure associated with higher prevalence of Pru p 7 (*P* = 0.03) and lower prevalence of Pru p 1 sensitization (*P* = 0.003) among CP sensitized subjects (Table S5).

4 | DISCUSSION

We report here that Pru p 7 is a major allergen in PA patients from southern France, in this study showing a prevalence of Pru p 7 sensitization exceeding 60%. In 54% of Pru p 7-sensitized PA patients, there was no concomitant sensitization to other available peach allergens. The occurrence and level of Pru p 7 sensitization were higher in severe PA with peach-induced anaphylactic manifestations, that is grade 3 reactions of the EAACI classification,²³ than in PA patients with lower grade reactions. The functional potency of Pru p 7 was confirmed with BAT. Strong basophil activation was induced in Pru p 7-sensitized PA patients, with lowest activating concentrations of Pru p 7 in the range of picograms/millilitre and median EC₅₀ at 0.05 ng/L, while median CD-sens was 2000, indicating a biological potency higher than other food allergens.^{33,35-37}

In all but one borderline case in this study, Pru p 7 sensitization was associated with CP sensitization. The link between CP and Pru p 7 sensitization was further explored with IgE competition experiments. We found that CP extract could inhibit 80-100% of IgE binding to Pru p 7 in 90% of the 42 sera analysed, whereas Pru p 7 only partly inhibited IgE binding to CP extract. Based on these results and the association between Pru p 7 sensitization and the intensity of CP exposure, we believe that CP acts as a primary sensitizer, eliciting in a proportion of individuals an IgE response that cross-reacts with Pru p 7. A partially characterized 14 kDa CP protein called BP14 has been shown to share a stretch of homology and cross-reactivity with snakin-1 from potato, a protein related to Pru p 7 and other members of the GRP protein family.³⁸ In our study, clinical reactions to

TABLE 2 Pru p 7 sensitization by geographic region and peach allergy or tolerance

rPru p 7 IgE	All regions	Region 5	Region 4	Region 3	Region 2	Region 1
Peach allergic patients						
Prevalence (number, %)	123 (62%) ***	88 (66%) **	12 (55%)	13 (65%) ***	7 (54%)	3 (30%)
Median (IQR) (kU _A /L)	3.4 (0.5-8.3)	4.1 (0.7-8.6)	2.8 (1.0-10.4)	3.7 (1.1-7.3)	0.2 (0.1-0.6)	0.3 (0.2-0.6)
Pru p 7 monosensitization	66/123 (54%) ***	49/88 (55%) ***	7/12 (58%)	8/13 (62%)	1/7 (14%)	1/3 (33%)
Peach tolerant patients						
Prevalence (number, %)	48 (41%) ***	35 (49%) **	4 (80%)	2 (13%)	4 (27%)	3 (27%)
Median (IQR) (kU _A /L)	0.3 (0.2-0.5)	0.3 (0.2-0.7)	0.3 (0.2-0.4)	0.2 (0.2; 0.2)	0.1 (0.1-0.2)	0.2 (0.2-0.4)
Pru p 7 monosensitization	8/48 (17%) ***	7/35 (20%) ***	0	0	0	1/3 (33%)
All patients						
Prevalence (number, %)	171 (54%)	123 (60%)	16 (59%)	15 (43%)	11 (39%)	6 (29%)
Median (IQR) (kU _A /L)	1.3 (0.2-6.4)	2.3 (0.3-6.7)	1.2 (0.5-5.1)	3.4 (0.3-6.3)	0.2 (0.1-0.2)	0.2 (0.2-0.5)
Pru p 7 monosensitization	74/171 (43%)	56/123 (46%)	7/16 (44%)	8/15 (53%)	1/11 (9%)	2/6 (33%)

Bold figures with asterisks denote statistically significant differences between PA and PT patients. Median and IQR were calculated for samples with Pru p 7 IgE ≥0.10 kU_A/L. Statistical significance flags for P-value levels: * < 0.05; ** < 0.01; *** < 0.001.

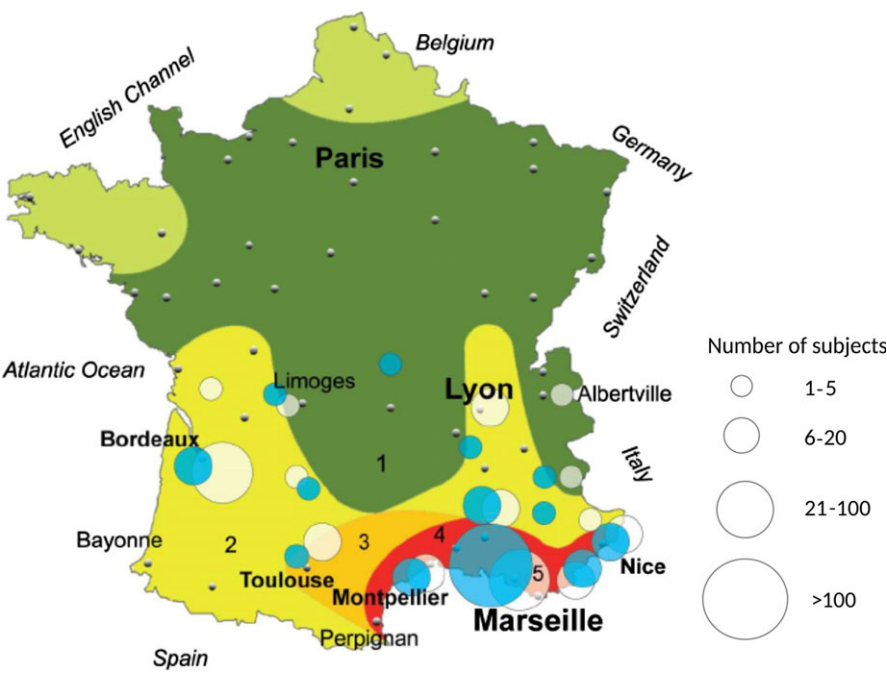


FIGURE 1 Prevalence of Pru p 7 sensitization in southern France in relation to cypress pollen exposure levels. Cypress pollen levels: green, low (exposure region 1); yellow, intermediate (exposure region 2); orange, high (exposure region 3); red, very high (exposure regions 4 and 5). Birch pollen exposure follows an inverse gradient and is virtually absent in the southernmost part defining the region of Marseille (5). Pru p 7 sensitization: blue circles: sensitized subjects; white circles: non-sensitized subjects. The size of each circle indicates the number of subjects it represents. Small black and white symbols indicate sites of pollen counting. Cypress pollen map reproduced with permission¹⁷

potato were reported by only two Pru p 7 sensitized patients, one of which was additionally sensitized to Pru p 4.

Our data show that Pru p 7 sensitization in PA patients from southern France belongs to the PFS through its association with CP. Similar to other examples of PFS, Pru p 7-induced reactions occur mainly in adolescents and young adults. However, food allergy symptoms are more severe in Pru p 7-related PFS than in previously described examples of PFS.

Despite early reports on the association of PA with cypress pollinosis,³⁹ the prevalence of cypress-associated peach sensitization and PA is currently unknown. In a cohort of 1280 cypress-sensitized patients from France, 165 (13%) patients had peach sensitization as defined by positive prick-to-prick tests to peach, and 29 of these 165 (18%) had a history of peach-induced symptoms.⁴⁰ In regard to molecular sensitization relevant to peach allergy in our region,

preliminary data indicate that 15-25% of cypress pollinosis patients may be Pru p 7 sensitized (unpublished observations). Peach IgE sensitization rates across European countries as reported from the Euro-prevall cohort peak at 11.9% in Madrid and 13.4% in Zurich.²

Similarly, little is known about allergen(s) other than Pru p 7 potentially involved in the cypress-peach syndrome.⁴¹ Teams from southern France and Spain reported an association of CP and PA in Pru p 3 sensitized patients.^{42,43} Further, in a series of seven CP and PA patients, all displayed IgE binding to a 45-kDa CP protein, which could be out-competed by peach extract.³⁹ Additionally, certain allergens identified in Japanese cedar (*Cryptomeria japonica*) pollen, such as Cry j 3 (thaumatin-like protein), CJP-6 (isoflavone-reductase) and CPA-9 (subtilisin-like protease), show sequence identity of 40% or higher with homologues in *Rosaceae* fruits and other plant foods, suggesting that cross-reactions may occur through multiple allergen families.⁴⁴

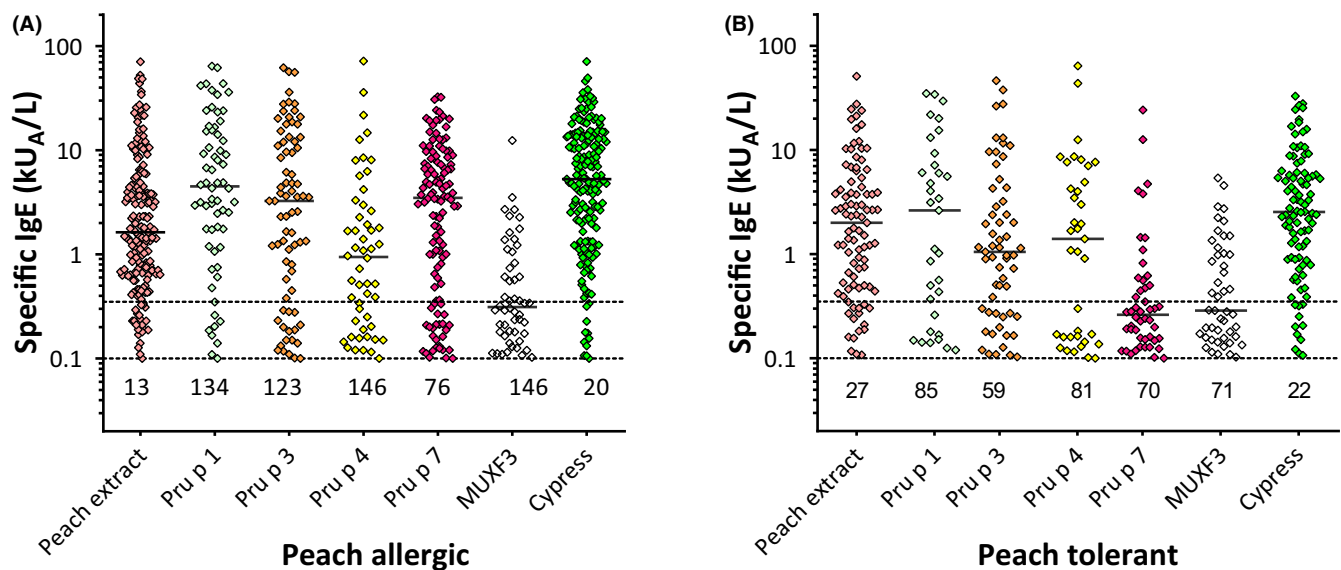


FIGURE 2 IgE antibody levels in peach allergic and peach tolerant patients. Levels of IgE to peach and peach components, MUXF3 and cypress pollen in peach allergic (A) and peach tolerant (B) subjects. Horizontal bars indicate median levels among positive (≥ 0.10 kU_A/L) observations. Dotted horizontal lines indicate the 0.10 and 0.35 kU_A/L levels. Indicated above the x-axis in each graph are the numbers of sera with IgE <0.10 kU_A/L

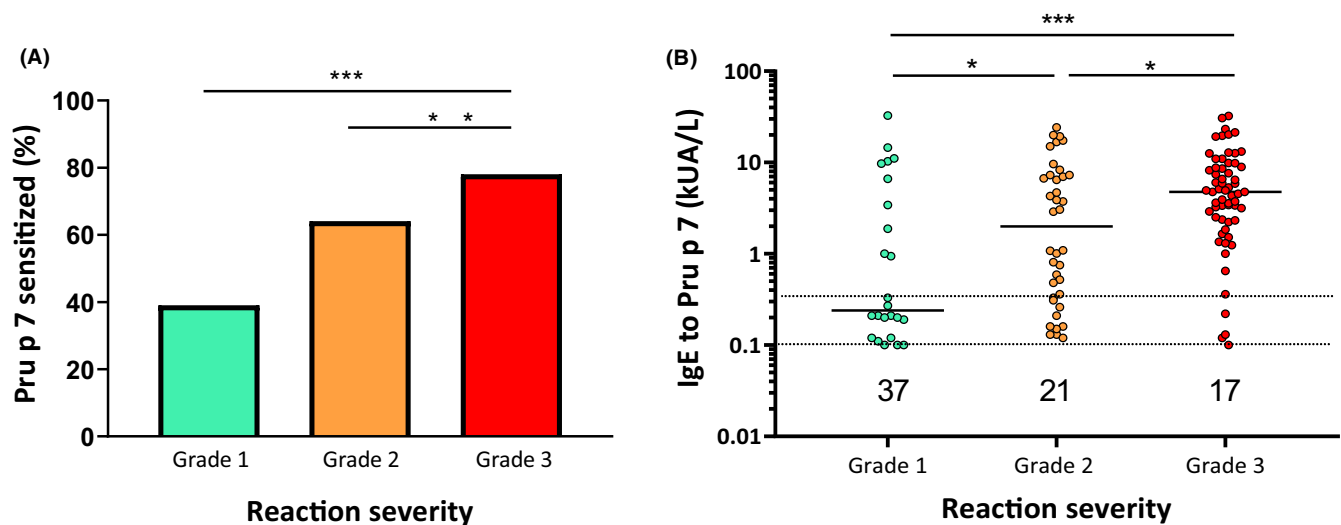


FIGURE 3 Prevalence and magnitude of Pru p 7 sensitization in relation to the severity of reactions to peach. Prevalence (A) and concentration (B) of IgE to Pru p 7 in patients with mild, moderate or severe reactions to peach. Statistical significance flags for *P*-value levels: * < 0.05, ** < 0.01, *** < 0.001. In B, horizontal bars indicate median levels among positive (≥ 0.10 kU_A/L) observations and dotted horizontal lines mark the 0.10 and 0.35 kU_A/L levels. Indicated above the x-axis is the number of sera with IgE <0.10 kU_A/L

Although cypress trees are typical of Mediterranean regions, *Cupressaceae* pollen dispersal also affects non-Mediterranean environments, including Central Europe, North America, Mexico and Japan.^{41,45–48} GRP-related allergy to *Rosaceae* and *Citrus* fruits was reported in conjunction with Japanese cedar pollinosis.^{46,47,49} In south-central United States, mountain cedar (*Juniperus ashei*) pollen is a major cause of allergic rhinoconjunctivitis.⁴⁸ Pollinosis to *Cupressaceae* has been steadily increasing during the past decades, possibly as a result of extended species distribution due to climate change and ability of *Cupressaceae* pollen to travel over hundreds of

kilometres.^{41,44,48} Individuals with *Cupressaceae* pollinosis who display similar sensitization profiles due to the homology of allergens from different *Cupressaceae* species⁵⁰ might become exposed to previously unencountered, cross-reacting foods, when changing residence regions, travelling overseas, or expanding their consumption of fruits and vegetables as a result of healthy living trends.⁵¹ Indeed, we found that Pru p 7 sensitization was associated with reactions to an array of plant foods, including but not limited to *Rosaceae*. Members of the GRP family are widely distributed in the plant kingdom, share high levels of sequence identity^{14,49,52} and have been

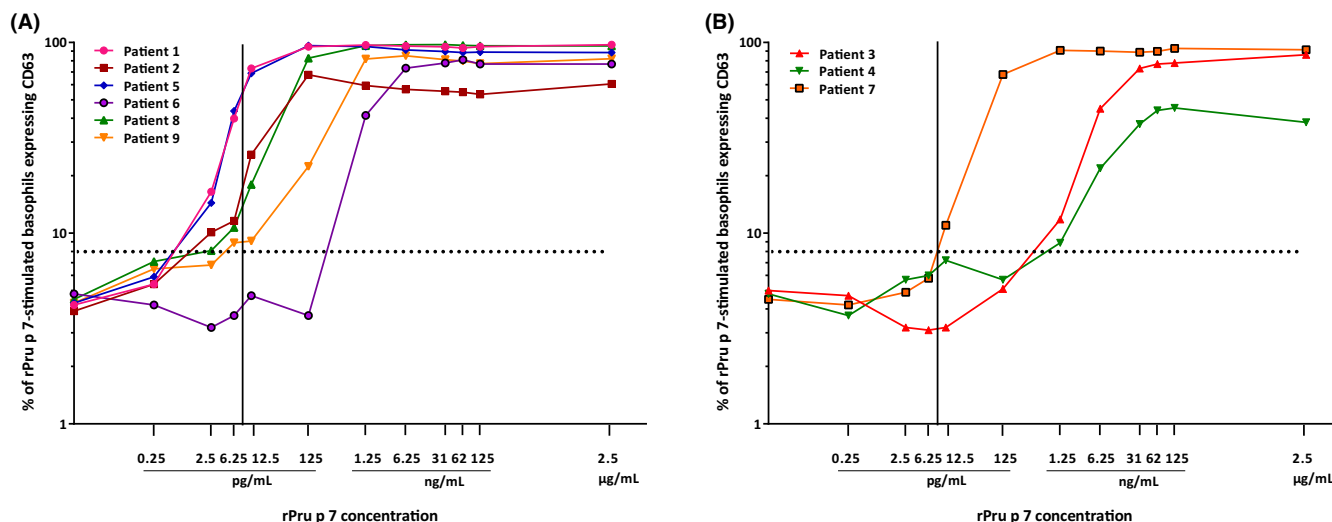


FIGURE 4 Dose-response of basophil activation with rPru p 7. Dose-response BAT with rPru p 7 was performed in nine PA patients monosensitized to Pru p 7. A, BAT in individual PA patients without cofactor requirement. B, BAT in individual PA patients with clinical reactions requiring cofactors. The dotted line represents the positivity threshold. The solid line shows the separation between BAT with lowest activating concentrations below or greater than 6.25 pg/mL of rPru p 7

TABLE 3 Basophil activation test with rPru p 7 in a sample of peach allergic patients

	All	Without cofactor	With cofactor	P
Number of subjects	9	6	3	
EC ₅₀ ng/mL (median, range)	0.05 (0.006-6.3)	0.03 (0.006-1.3)	6.3 (0.05-6.3)	0.03
CD-sens (median, range)	2000 (80-16 000)	5000 (80-16 000)	16 (16-2000)	0.08
AUC	9990	10 579	8987	0.30
Lowest activating concentration ng/mL (median, range)	0.006 (0.006-1.3)	0.004 (0.003-1.3)	1.3 (0.01-1.3)	0.048
Ratio maximal rPru p 7-induced activation/anti-FcεRI (median, range)	1 (0.5-1.8)	1 (0.9-1.8)	0.9 (0.5-1)	0.2

Bold figure denotes statistically significant differences between peach allergic patients experiencing clinical reactions to peach without cofactor intervention and peach allergic patients requiring a cofactor.

associated with particularly severe food allergic reactions in at least three environments: Italy,¹⁴ France¹⁶ and Japan.^{49,53}

In our cohort, increasing CP exposure and associated Pru p 7 sensitization was related to lower prevalence of PR-10 sensitization even in regions with substantial birch pollen exposure. Conversely, a potential protective effect of birch pollen exposure has been suggested with respect to LTP-related food allergies.⁵⁴ The pathophysiological background of such interactions between pollen exposure and the resulting host sensitization may bring further insight into the geographic restriction patterns of food allergy.

In conclusion, we report here that Pru p 7 sensitization is common in patients allergic to peach, often in the absence of sensitization to other peach allergens. Sensitization to Pru p 7 is invariably associated with CP sensitization and appears to be driven by CP exposure, rather than reflecting a primary food sensitization. Biologically, Pru p 7 was found to be extremely potent, causing basophil activation at sub-picomolar concentrations. Because CP is present in continental as well as Mediterranean environments, and given the world-wide distribution of related *Cupressaceae* species, Pru p 7 and homologous food proteins may be an important culprit in severe allergic reactions to peach and other plant foods in many regions of the world.

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CONFLICT OF INTEREST

AEE, JÖ and JL are employees of Thermo Fisher Scientific. RAL is employee of Beckman Coulter. PD has received speaker and consultancy fees from ALK, Circassia, Stallergènes Greer, Allergopharma, Thermo Fisher Scientific, Meda, Menarini and grants from AstraZeneca and from MEDA Pharma through CHRUM administration. LGC has received reimbursement for attending a symposium and a fee for organizing education from Thermo Fisher Scientific. PAA has received reimbursement for attending a congress from Thermo Fisher Scientific and a research grant from the French Society of Allergology. YPMB has received speaker fees from Thermo Fisher Scientific, ALK-Abello, Stallergènes. IAM has received research grants from the European Union. JV has received speaker and consultancy fees from Meda

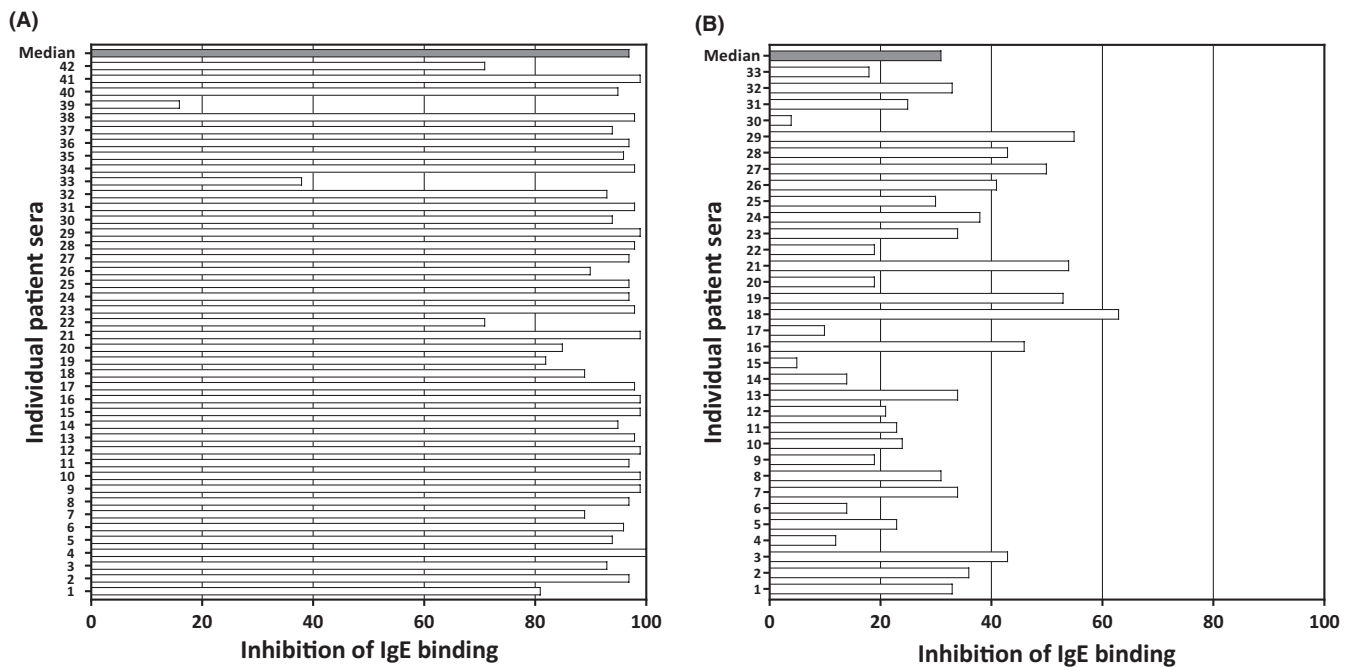


FIGURE 5 Reciprocal IgE inhibition experiments. Sera of peach allergic subjects were pre-incubated with either (A) cypress pollen extract (15% w/v, $n = 42$) or (B) rPru p 7 (100 $\mu\text{g/mL}$, $n = 33$) prior to measurement of IgE binding to rPru p 7 or cypress pollen abstract, respectively, by ImmunoCAPTM. Resulting degrees of inhibition are expressed as percent reduction of IgE concentration values as compared to measurements performed after preincubation with buffer alone, without inhibitor. Median inhibition is represented as a grey bar at the upper end of each graph

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AUTHOR CONTRIBUTIONS

CK, JL and JV conceived, designed and supervised the study. CK, AS, PAA, MV, LG, CS, SM and JV supervised laboratory procedures and collected and analysed clinical and laboratory data. YC and JV performed statistical analysis. RAL and JV performed BAT experiments. AEE and JÖ produced natural and recombinant Pru p 7 and Pru p 3, performed experimental ImmunoCAP IgE measurements and IgE inhibition tests. AP, VL, CA, JB, FP, AMC, DPC, JLB, PD, SG, FB, BL, CP, SL, TB, LGC, MG, AAD, DC, TS, ICB, YPM, BH, NB, MDG, BS, AC, SV, BP, LN, GL, SM performed clinical allergy work-up and collected and analysed clinical data. CK, YC, AEE, AS, AMC, JLB, DC, BS, JL and JV wrote the paper. IAM and JLM revised the paper for important intellectual content. All authors read and approved the final manuscript.

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