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***Quercus ilex* pollen allergen, Que i 1, responsible for pollen food allergy syndrome caused by fruits in Spanish allergic patients**

Running title: Que i 1, the new member of the Bet v 1 family, is responsible for PFAS in the Spanish population

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27 **Authors' contributions:** MP: selection of patients, study design, statistical analysis, VMGS: Holm oak
28 transcriptome database search; NCB: western blotting, production and purification of Que i 1; DLA:
29 selection of patients; MÁC: Proteomic studies; SQ: selection of patients; JVJN: proteomic studies; RRP:
30 study design, coordination, writing the manuscript.

ABSTRACT

Background: Pollen food allergy syndrome (PFAS) related to PR10 from vegetables is common in northern Europe, whereas in Mediterranean countries PFAS has been preferentially associated with profilins. However, there are pollen-allergic patients reactive to Bet v 1 in birch-free regions. Since it cannot be the primary sensitizer, there has to be another culprit. *Quercus ilex* is a good candidate as it belongs to the order *Fagales*. This order includes trees with highly sensitizing pollen such as alder, hazel, hornbeam, oak and chestnut because of the presence of PR10 allergens. PR10 allergens have indeed been described in other *Quercus* species.

Objective: Our goals were to determine the rate of sensitization to *Q. ilex* in central Spain and the associated frequency of PFAS; secondly to identify and clone the *Q. ilex* allergen PR10.

Methods: We included 224 allergic patients with respiratory symptoms to estimate the rate of sensitization. A skin prick test (SPT) and ImmunoCAP were performed. A total of 38 *Q. ilex*-sensitized patients were tested using western blotting to determine the rate of Que i 1. Peptides from Que i 1 were analysed by MALDI-TOF/TOF and Orbitrap LC-MSMS. The Que i 1 sequence was first obtained from the Holm oak transcriptome then cloned and expressed in bacteria.

Results: 59.8% of pollen-allergic children were sensitized to *Q. ilex*. We described and cloned the *Q. ilex* PR10, Que i 1, which has a sensitization rate of 60.5% and was recognized by 65.4% patients reporting PFAS.

Conclusion and clinical relevance: Sensitization to *Q. ilex* pollen has increased significantly since 1995. This sensitization could be important, as the presence of PFAS in this population is higher than in patients not sensitized to *Q. ilex*. The first *Q. ilex* allergen has been described and is related to PFAS in Spanish patients sensitized to PR10 but not exposed to birch pollen.

Keywords: oral allergy syndrome, PR10, pollen food allergy syndrome, *Quercus ilex*, Que i 1.

Abbreviations: PFAS, pollen food allergy syndrome; LTP, lipid transporting protein; SPT, skin prick test.

1 INTRODUCTION

Spain contains the world's largest area of *Quercus ilex* (Holm oak) woodland. These trees release significant amounts of pollen. In fact, this source of pollen contributes up to 30% of the total pollen count recorded in Madrid from May to June¹. Madrid is located in the centre of Spain, in the Mediterranean area where spring pollinosis is caused mainly by the *Poaceae* family¹. However, in two Spanish populations of patients with spring hay fever, 3.5% in one region and 14% in another tested positive in skin tests for Holm oak pollen^{1,2}. As no Holm oak-monosensitized patients were found and all oak-sensitized patients were also sensitized to *Poaceae*, Holm oak pollen has not been considered to be clinically relevant from an allergological point of view for 25 years. There are no recent reports, nor specific studies, addressing the role of Holm oak in pollinosis.

Holm oak belongs to the order *Fagales* and family *Fagaceae*. This order includes species such as birch, alder, hazel and hornbeam, the pollens of which are potent triggers of spring pollinosis in central Europe. The presence of panallergens, mainly PR10 and profilins, in these pollens has been demonstrated in great detail³. Likewise, the capacity of *Fagales* species other than birch to sensitize atopic individuals has been described⁴.

Madrid, located in the centre of Spain, is a region that is virtually birch-free. However, there are pollen-allergic patients reactive to Bet v 1 and other PR10 allergens as determined by commercial methods such as ImmunoCAP and the ImmunoCAP-ISAC platform (Thermo Fisher Scientific, Uppsala, Sweden). This observation has also been reported for other birch-free Mediterranean populations such as those in central and southern Italy⁵. Since Bet v 1 from birch cannot be the primary sensitizer in these populations, there has to be another culprit. Other *Fagales* trees such as beech, hazelnut, alder, hornbeam and chestnut are present in the north of Spain, but they are uncommon in the centre and south of the country (<http://www.euforgen.org/species/>). Holm oak is a good candidate as a source of PR10 in Spain, as it belongs to the *Fagales* order and because PR10 allergens, namely Que a 1⁶ and Que m 1, have been reported to be present in other *Quercus* species⁷. Furthermore, *Quercus spp.* has been associated with pollen food allergy syndrome in the Korean adult population⁸ and in Mexican children⁹.

Pollen food allergy syndrome (PFAS) is clinically characterized most frequently by isolated oral and pharyngeal symptoms with immediate onset following food intake, and is also called oral allergy syndrome (OAS)¹⁰. The number of foods that trigger PFAS continues to grow, with differences among the various populations studied, depending on the prevalence of the various species of plant in different countries as well as food-consuming habits¹¹. In northern European countries, many patients with birch tree pollen sensitization develop PFAS induced mainly by fruits of the Rosaceae family and are reactive to Bet v 1 homologues¹². Conversely, in southern European countries, the allergens involved in PFAS are diverse, including one or more panallergens such as PR10, profilin, LTP and other less common allergens¹³. This is related to the fact that Mediterranean patients with PFAS are sensitized to various pollens, and the vegetables involved are more diverse than in northern European populations¹⁴.

Here we present the rate of allergy to Holm oak (*Q. ilex*) pollen in a Spanish population from the centre of Spain and the frequency of PFAS associated with the first Holm oak allergen to be described, Que i 1, which belongs to the PR10 family. Que i 1 was also cloned and expressed in bacteria.

2 MATERIAL AND METHODS

2.1 Patients and ethics

A total of 224 Spanish patients, from Spanish parents, living in Madrid region, complaining of respiratory symptoms suggestive of pollinosis prospectively underwent skin prick tests (SPT) for aeroallergens (*C. arizonica*, *P. acerifolia*, ALK-Abelló, Madrid, Spain; *O. europaea*, *Q. ilex*, *P. pratensis*, Roxall-Medicina, Zamudio, Spain), LTP (Pru p 3, ALK-Abelló, Madrid, Spain) and profilin (Pho o 2, Roxall-Medicina) between May and October 2019. The SPT was performed according to European guidelines¹⁵, using commercial extracts from *Q. ilex* pollen (Roxall). In a subset of patients (n=49) SPT with oak (*Q. robur*) and birch (*B. verrucosa*) was also performed. The SPT was considered to be positive if the diameter of the wheal was 3 mm greater than that induced by the negative control. The presence of PFAS was specifically recorded in this population. Diagnosis of PFAS was made according to routine clinical practice, which includes SPT with commercial extract of involved foods, prick-prick testing with fresh foods, sIgE determination to involved foods and/or panallergens. Oral food challenges were performed when diagnosis was not clear.

Clinical assessment included examination of the patient's history, SPT and determination of specific IgEs (sIgE), which were measured by ImmunoCAP 100 (Thermo Fisher, Uppsala, Sweden) according to the manufacturer's instructions.

Patients allergic to Holm oak pollen were selected based on their case histories and positive SPT. PFAS was diagnosed following our routine clinical practice protocols, namely SPT with commercial extracts, sIgE determination and skin-prick testing with fresh food plant when necessary. In a few cases, when the diagnosis was not clear, oral food challenge (OFC) was performed. Sera (n=38) were obtained from the Paediatric Allergy Unit at La Paz Hospital in Madrid, Spain.

The local ethics committee (protocol number PI-2243) approved the study. Written informed consent was obtained from all patients' parents or their legal representatives.

2.2 Pollen extract

Commercial *Q. ilex* SPT extract (Roxall) was concentrated through 10 kDa-pore size Amicon Ultra-15 (Merck Millipore, MA, USA) and used for SDS-PAGE, mass spectrometry analysis (MALDI-TOF/TOF and Orbitrap LC-MSMS) and western blotting.

2.3 Mass spectrometry (MS) sample preparation

Q. ilex pollen extract (20 µg) was separated by 4-20% precast mini-protean TGX gels (Bio-Rad) and stained with Coomassie Brilliant Blue (CBB, Bio-Rad). Protein bands were excised from the gel and digested as described¹⁶. Briefly, gel plugs were destained with 200 mM ammonium bicarbonate (AmBic)/40% acetonitrile (AcN) at 37°C. After three dehydration and rehydration cycles, the samples were digested with trypsin and incubated in 10% TFA to extract the peptides. The peptide fragments were

purified (microcolumn C18 Millipore) and crystallized with α -cyano-4-hydroxycinnamic acid, and then underwent mass spectrometry using a MALDI LIFT-TOF/TOF mass spectrometer (Ultraflextreme, Bruker) in the m/z range 700–3500 Da, with an accelerating voltage of 20 kV. The eight most abundant peptide ions underwent MSMS fragmentation analysis to determine the peptide sequence.

2.4 MALDI-TOF/TOF MSMS analysis

A combined peptide mass fingerprinting (PMF) tandem mass spectrometry 'LIFT' (MS+MSMS) search was performed using ProteinScape 4.0 (Bruker) software (Applied Biosystems) over the species-specific "DB qiproteome V3_parsed.fasta" database¹⁷ employing the MASCOT search engine (Matrix Science, London). The parameters allowed were a minimum of four peptide matches, a maximum of one-miscleavage and peptide modifications by carbamidomethylcysteine and methionine oxidation permitted. The maximum tolerance for peptide mass-matching was limited to 50 ppm.

2.5 Shotgun LC-MSMS analysis

Q. ilex extract (40 μ g) underwent 12% SDS-PAGE electrophoresis to obtain a unique band. The gel was then stained with CBB and the resulting band excised to be digested as described above. Generated peptides were separated by nano-LC in a Dionex Ultimate 3000 nano UPLC system (Thermo Scientific, San Jose, CA) with a C18 75 μ m x 50 cm Acclaim PepMap column (Thermo Scientific). Peptides were concentrated and cleaned up on a 300 μ m x 5mm Acclaim PepMap cartridge (Thermo Scientific) as described¹⁸. The peptides were ionized by a nano-electrospray ionization source and analysed with a trihybrid Thermo Orbitrap Fusion mass spectrometer (Thermo Scientific) operating in positive mode. Method settings were as described¹⁸.

The raw data were processed using Proteome Discoverer (version 2.1.0.81, Thermo Scientific). MS2 spectra were searched with the SEQUEST engine against the species-specific "DB qiproteome V3_parsed.fasta" database. *In-silico* peptide lists were generated by theoretical tryptic digestion allowing up to one missed cleavage, carbamidomethylation of cysteines as a fixed modification, and oxidation of methionine as a variable modification. Peptides were grouped into proteins according to the law of parsimony and filtered to 1% FDR and a minimum XCorr of 2.

2.6 Cloning of Que i 1

A synthetic Open reading frame (ORF) of Que i 1 was produced and cloned into the pUC57 ampicillin resistance vector. Subsequently, the Que i 1 sequence was sub-cloned into the pHTP1 expression vector (kanamycin resistance). This plasmid produces a protein with an N-terminal hexa-histidine tag and TEV cleavage site (NZYtech; Lisbon, Portugal). Sequence similarity was analysed with an NCBI BlastP program using the All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF and excluding environmental samples from WGS projects databases¹⁹. Sequence alignments were carried out using the Clustal Omega program.

2.7 Protein expression and purification

The Que i 1 expression construct was expressed from the pHTP1 construct, transformed into *E. coli* KRX cells (Promega, Madison WI, USA) and grown in LB medium supplemented with kanamycin (30 µg/ml). Protein expression was induced by the addition of 0.1% rhamnose with an OD600 of 0.8 and growth was continued at 22°C overnight. Harvested cells were resuspended in 1/50 culture volume of BugBuster master mix reagent (Merck, Darmstadt, Germany), incubated in a rotating platform for 20 minutes at room temperature and then centrifuged at 16000 x g for 15 min at 4°C. Inclusion bodies containing the recombinant protein were purified from the pellet. It was resuspended in 1/10 diluted BugBuster, incubated for 5 minutes at room temperature and centrifuged for 15 min at 16000 x g, at 4°C; this step was repeated twice. Purified inclusion bodies containing Que i 1 were solubilized in 20 mM Tris-HCl, pH 8, 500 mM NaCl, 5 mM imidazole, 8 M urea.

Recombinant Que i 1, expressed as a 6-His-tagged protein, was purified from the solubilized inclusion body fraction using the His Spin Trap kit and His Buffer kit (GE Healthcare Bio-Sciences AB, Uppsala Sweden) under denaturing conditions, following the manufacturer's specifications. After purification, recombinant allergen was dialyzed against 20 mM Tris-HCl, pH 8, 200 mM NaCl with decreasing urea concentrations to refold the protein. Purified protein was analysed by 16% SDS-PAGE electrophoresis. Protein concentration was assessed by the Bradford method (Bio-Rad).

2.8 Gel SDS-PAGE and western blotting

Q. ilex pollen extract, *E. coli* lysates and recombinant purified proteins were separated using 16% SDS-PAGE gels for western blotting experiments. After electrophoresis, proteins were stained with CBB or electro-transferred onto nitrocellulose sheets as described²⁰. Subsequently, membranes were incubated with the sera (diluted 1:20). Bound IgE was detected using a monoclonal anti-IgE antiserum (Ingenasa, Madrid, Spain) and an alkaline phosphatase-labelled goat anti-mouse antiserum (Biosource Int., Camarillo, CA). Finally, the signal was visualized with the alkaline phosphatase 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/4-nitroblue tetrazolium (NBT) system (Amresco, Solon, Ohio). Western blotting inhibition was performed as described above, except that serum from *Quercus*-allergic patients was preincubated overnight at 4°C and for 2 hours at room temperature with 10 µg of recombinant Que i 1²⁰.

3 RESULTS

3.1 Frequency of Holm oak sensitization

A total of 224 patients allergic to pollen (134, 59.8% males, mean age 10.99 ± 3.10 years) were included in the study. Two hundred and two (90.2%) presented rhinoconjunctivitis and 124 (55.4%) had asthma. One hundred (44.6%) patients presented both asthma and rhinoconjunctivitis. SPT sensitization to *P. pratensis* was detected in 89.3%, to *O. europaea* in 88.8%, to *P. acerifolia* in 68.6%, and to *C. arizonica* in 73.4%. SPT showed LTP to be recognized in 23.2% of patients and profilin in 22.8%. Interestingly, 134 (59.8%) showed sensitization to *Q. ilex*, although only two patients (0.9%) were monosensitized. Sensitization to *Q. robur* and *B. verrucosa* was found in 57.1% and 51% of patients respectively.

Holm oak pollen-sensitized patients showed a similar frequency of recognition of LTP than those not sensitized (26.8% vs. 18.3%, $p=0.174$). Sensitization to profilin in Holm oak-sensitized patients was higher than in those not sensitized (31.2%, vs. 11.1%, $p=0.001$). The mean wheal diameter in patients sensitized to Holm oak ($n=134$) was 5.12 ± 1.99 mm for *Q. ilex*, 1.74 ± 3.07 mm for LTP and 2.08 ± 3.38 for profilin.

3.2 Frequency of PFAS in Holm oak-sensitized patients

The rate of PFAS in our population of paediatric patients allergic to pollen was 19.2% (mean age 10.99 ± 3.10 years). PFAS was more frequently reported by *Q. ilex*-sensitized patients (24% vs. 13.5%, $p=0.059$), although this difference did not reach statistical significance. In the 29 *Q. ilex*-sensitized patients with PFAS, it was not associated with sensitization to LTP ($p=1.000$) or profilin ($p=0.154$). Moreover, fourteen of them (48.3%) showed no sensitization to profilin or LTP. Thus, we decided to investigate the presence of other panallergens in *Q. ilex* pollen related to PFAS, namely PR10. *Q. ilex* is a good candidate source of PR10 allergens as it belongs to the order *Fagales*, in which PR10 allergens have been described as major allergens^{6,7}.

Thirty-eight sera (26 males, 68.4%; mean age 11.24 ± 2.64 years) from *Q. ilex*-sensitized patients were selected. The characteristics of these patients are summarized in Table 1. Thirty-six of the 38 Holm oak pollen-sensitized patients (94.76%) were polysensitized. There is no commercial sIgE assay for Holm oak available; the closest sIgE test available, *Q. alba*, was performed when sera were available (25 patients). *Q. alba* sIgE was positive in 23 patients (92%). The results of sIgE assays and skin prick testing are shown in Table 2.

Twenty-six of the 38 *Q. ilex* selected patients (68.4%) presented with PFAS when any food plants were eaten. PFAS symptoms were most frequently reported with banana (18/36), melon (12/36), peeled apple (9/38), watermelon (9/34), walnut (9/27), peeled pear (6/36), kiwi (6/28), lentil (6/37) and peeled peach (5/28). Sensitization to PR-10 and profilin of these patients can be seen in Table 3.

Data on profilin and PR10 sensitization were available in 34 out of 38 patients. Of these, 19 (55.9%) were sensitized only to PR10, 4 (11.8%) only to profilin, and 7 (20.6%) were sensitized to both profilin and PR10 allergens.

3.3 IgE binding of Holm oak pollen extract

Sera from patients sensitized to Holm oak recognized many putative allergens in *Q. ilex* pollen extract by western blotting (data not shown). The most conspicuous one had a molecular weight of around 17kDa, consistent with a PR10 protein; this band was flanked above and below by two weaker bands (Fig. 1B). This “triplet” band was almost fully inhibited by preincubation with purified recombinant Que i 1 (Fig. 1C).

3.4 Identification of PR10 oak allergen

Holm oak pollen extract was separated by gradient SDS-PAGE and bands in the range of 17-20 kDa were excised and analysed by MALDI-TOF/TOF (Fig 2A). The band corresponding to approximately 17 KDa matched the protein PR10 (contig-4069000012) of the *Q. ilex* transcriptome database¹⁷, with high confidence parameters (score value of 116 and 41.9% of sequence coverage, Fig. 2B). This sequence corresponded to the PR10 major allergen Pru ar-like with high identity, at both transcript level (95%, appendices 1) and protein level (89%, appendices 2).

Shotgun analysis of the pollen extract proteome showed 895 identified proteins with high confidence (FDR≤0.01), 557 of which fulfilled the following requirements: score value ≥2 and two or more identified peptides per protein. The PR10 major allergen Pru ar-like protein was identified with high confidence parameters (55.3% coverage, 22 peptides and 236.6 score). The PR10 peptides identified by both proteomic strategies (MALDI-TOF/TOF and LC-MSMS), covered altogether 65% of the predicted protein sequence (Fig. 2B). This combined strategy (Peptide Mass Fingerprint+LC-MSMS) allowed identification of 11 peptides belonging to this protein by PMF, and the identification was confirmed by MSMS fragmentation of KGDFQLKEEQIQATIEKI (m/z 1876.9157) peptide with a high score (113.1) (Fig. 2B).

3.5 Molecular cloning of Que i 1

The cloned sequence coded for 159 amino acids with a calculated mass of 17384.79 kDa and an isoelectric point of 5.93 (Fig. 2B). The translated sequence showed highest homology with Cas s 1 (90% identity), followed by Que a 1 (89% identity) and Que r 1 (86% identity) (Fig. 3). Furthermore, Que i 1 shares 62% identity with Bet v 1 and 60% with Mal d 1. This sequence was deposited into the GenBank database under accession No. MN563736, and was designated as Que i 1.0101 by the WHO/IUIS Allergen Nomenclature Committee.

3.6 Production of recombinant Que i 1

Recombinant protein was produced with 26 additional amino acids including 6 histidine residues (MGSSHHHHHSSGPQQGLRENLYFQG) at the N-terminus. The protein yield was 14 mg/L of bacterial culture in insoluble form. After denaturation with urea and refolding by dialysis, the recombinant protein

showed an apparent molecular weight of 22 kDa by SDS-PAGE. As described for other PR10 allergens, a dimer of around 45 kDa was found⁴. The approximate percentage purity of rQue i 1 was 99% (Fig. 4A).

3.7 Frequency of recognition of Que i 1 in allergic patients

The IgE reactivity of the recombinant Que i 1 was measured by western blotting (Table 1, Fig. 4B, C). Thirty-eight sera samples from Holm oak-allergic patients were tested. sIgE to Que i 1 was detected in 23 out of 38 (60.5%, Fig 4B) sera tested whereas the remaining 15 negative patients (39.5%, Fig 4C) recognized profilin (Bet v 2, 33%) or LTP (Pru p 3, 62.5%, Table 2) and some patients recognized allergens not identified by western blotting with *Quercus* pollen extract (data not shown). Interestingly, no differences between SPT and sIgE were detected between Que i 1-sensitized patients and non-sensitized patients, but differences were detected between Que i 1-sensitized patients and sIgE-PR10 allergens (Table 2). The Kappa index for agreement between Que i 1 recognition and other commercially available PR10 allergens was high (Bet v 1: 0.933, $p < 0.0001$; Cor a 1: 0.906, $p < 0.0001$; Gly m 4 0.798, $p = 0.001$; Pru p 1 0.733, $p = 0.001$; Mal d 1 0.685, $p = 0.002$; Ara h 8 0.526, $p = 0.006$) (Table 2).

4 DISCUSSION

We analysed sensitization to Holm oak pollen and the presence of PFAS in a cohort of unselected children with pollinosis in a birch-free Mediterranean area. We found the rate of Holm oak sensitization to be 59.8%, with 0.9% monosensitized patients. Twenty-five years ago, this sensitization was 14% in the same region¹. Although the patients included in that study were adults and ours were children, it seems that Holm oak sensitization has been increasing during that time. One possible explanation is the rising amount of pollen in the environment and the increase in the length of the pollen season due to global warming and higher levels of CO₂ in the atmosphere²¹. Furthermore, the diagnostic tools used for the diagnosis of pollen sensitization have improved significantly in the last 25 years.

The variety and amount of pollen in Mediterranean countries in spring is vast and diverse, with many species coexisting at the same time. As these patients have symptoms during the months of Holm oak pollination (from mid-April to mid-June, with the peak in mid-May), and as they recognize allergens from Holm oak pollen extract, it seems clear that this pollen is contributing to the symptoms. As has been proposed, the whole spectra of pollen sensitizations should be taken into account in order to prescribe the correct immunotherapy and to assess whether its outcome is successful²².

Some Bet v 1 homologues found in the pollen of *Fagales* trees have been extensively described (e.g. Cor a 1, Aln g 1, Car b 1, Que a 1)^{4,23} and identified as major allergens causing spring pollinosis, especially in patients living in birch-free areas. These allergens should therefore be taken into account in order to improve the diagnosis and allergen-specific immunotherapy of *Fagales* tree pollen allergy in different geographical areas¹³, as has been already suggested for Car b 1 and Que a 1²³. This could be applicable to Que i 1 and other Bet v 1 homologues from other *Fagales* tree pollens in Mediterranean countries.

The sequence identity of Que i 1 and Bet v 1 is 62%; when amino acids with similar characteristics are included, this figure rises to 73%. However, identity in areas where epitopes of Bet v 1 have been defined is much higher. For the critical amino acids of the first defined epitope (amino acids 49 to 58), the identity is 9/10; and for the second and third epitopes, that span from 73-103, the identity is 14/30, and for similar amino acids 20/30²⁴. This explains why 95.5% of the Que i 1 patients cross-react with Bet v 1 and explains why 0% of the Que i 1-negative patients did not recognize Bet v 1. Likewise, 85.7% of Que i 1-negative patients recognize *B. verrucosa* pollen extract in a SPT, which can be explained by the presence of other panallergens such as profilin (33.3% of Que i 1-negative patients recognize Bet v 2) and by recognition of other allergens not determined here. Interpreting the reactivity of pollen extracts is more difficult than with individualized allergens because there are many factors involved, e.g. the quality of the extracts, which affects the amount and diversity of allergens extracted; the sensitization pattern of the patients who are usually polysensitized.

Recently, Scala et al⁵ published the IgE reactivity to a wide panel of PR10s in patients from an Italian region that was birch-free, but rich in other *Fagales* trees. They found that the most frequently recognized allergen, besides Bet v 1, was Cor a 1.0101 (71%), but 26% of the patients did not recognize Bet v 1. Interestingly, the combination Bet v 1, Cor a 1.0101 and Aln g 1 was associated with respiratory symptoms,

whereas the combination Ara h 8, Cor a 1.0401, Gly m 4, Mal d 1 and Pru p 1 was linked to PFAS⁵. It is clear that birch is not the main sensitizer in that population; the authors suggested hornbeam, alder and oak as possible primary sensitizers. According to our results, *Q. ilex* (Que i 1) is a good candidate as it is abundant in all the coastal areas of Italy and in northern Italy close to Lake Garda, which has a "Mediterranean-like" climate²⁵.

We found that 19.2% of our patients had PFAS with food plants consumption. This rate of PFAS is similar to the rate of 22.2% found in central Italy, a population with similarities in terms of climate, latitude, and pollen exposure pattern complexity¹⁴. Mastroianni et al¹⁴ found that 13.1% of Italian children with PFAS were monosensitized to PR10 and 15.3% to profilin, and that 23.3% were multi-panallergen sensitized (profilin, PR10 and/or LTP). Their data are concordant with the Spanish population with PFAS studied here, since 55.9% of our patients were PR10-only positive, 11.8% profilin-only positive and 20.6% recognized both (LTP data were not available for all our patients and were not taken into consideration). In fact, our population fits in the clusters 2a and 2d defined by Mastroianni et al¹⁴. Cluster 2a consisted of patients living in central Italy and sensitized to profilin, *Fagales*, plantain, plane and olive tree with oral symptoms triggered by banana, *Cucurbitaceae*, peach and kiwi fruit. The cluster 2d includes patients living in the same region but sensitized to PR10 whose oral symptoms are triggered by apple, peach, and kiwi fruit¹⁴. The authors proposed that the PR10 in the cluster 2d patients can be attributable to *Quercus spp*, *Juglans spp*, *Carpinus spp* and other *Fagales* species. In our population, PFAS was more common among *Q. ilex*-sensitized patients than those who were not (24% vs. 13.5%). Considering our results, their results and the similarities between both populations, *Q. ilex* is a good candidate to be involved in PR10 syndrome in patients in central Italy, as we have found in patients in central Spain. This is the first report to link a tree from the genus *Quercus* and its PR10 allergen with PFAS in the south of Europe.

We describe Que i 1 as a major Holm oak allergen that belongs to the PR10 protein family. The low yield of natural Que i 1 observed in the pollen extract led us to obtain the recombinant allergen in order to study the rate of IgE recognition among our patients. To obtain the recombinant allergen, we started from mass spectrometry results, searched on a Holm oak transcriptome database^{17,26} and designed a synthetic gene. The recombinant allergen obtained was solubilized with urea, as has been described for other PR10 allergens^{27,28}. After refolding, the recombinant protein tended to form dimers (Fig. 4A), which also seem to be present in the natural form, considering the 35 kDa band that disappears in western blotting inhibition (Fig. 1). The tendency to form dimers has been described for other PR10 allergens although not all of them²⁸. Nevertheless, 60.5% of the patients recognized the recombinant allergen, meaning that it retains its IgE binding capacity. Further studies are necessary to establish the relationships with other PR10 allergens, either from tree pollen or from vegetables, with respect to cross-reactivity and epitopes.

In summary, our results demonstrate that Holm oak is a relevant source of sensitization in central Spain. We have described the first Holm oak allergen, Que i 1, as a major allergen. Likewise, this allergen is responsible for PFAS associated with PR10 allergens in a birch-free area.

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CONFLICTS OF INTEREST

The authors declare that they have no relevant conflicts of interest.

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TABLES

Table 1. Characteristics of *Q. ilex*-sensitized patients. *Comparisons are made using Chi square test for qualitative variables and T test for continuous variables (age). Percentages are in brackets.

	Whole population (n=38) (%)	Que i 1 + (n=23) (%)	Que i 1 – (n=15) (%)	p*
Age (years± SD)	11.2 ± 2.6	11.6 ± 2.8	10.6 ± 2.4	0.251
Gender (male)	26 (68.4)	15 (65.2)	11 (73.3)	0.728
FH pollinosis	18 (47.4)	7 (35)	11 (73.3)	0.041
FH Food allergy	6 (15.8)	4 (20)	2 (13.3)	0.680
Previous AD	11 (28.9)	1 (5)	3 (20)	0.292
Current AD	5 (10.5)	7 (35)	4 (26.7)	0.721
RC	36 (94.7)	22 (95.7)	14 (93.3)	1.000
BA	28 (68.4)	17 (73.9)	9 (60)	0.481
PFAS	26 (68.4)	17 (73.9)	9 (60)	0.481

AD: atopic dermatitis; BA: bronchial asthma; FH: family history; RC: rhinoconjunctivitis; PFAS: pollen food allergy syndrome.

Table 2. Data on skin prick testing (SPT) and specific IgE (sIgE) determined by ImmunoCAP expressed as percentages, Que i 1 determined by western blotting. *Comparisons are made using Chi-square, statistically significant results are shown in bold.

	Q. ilex sensitized (n=38) (%)	Que i 1 + (n=23) (%)	Que i 1 – (n=15) (%)	p
Skin prick test				
<i>Q. ilex</i>	100	100	100	-
<i>C. arizonica</i>	88.2	85.7	92.3	1.000
<i>P. acerifolia</i>	94.1	100	84.6	0.139
<i>O. europaea</i>	88.2	90.5	84.6	0.627
<i>P. pratensis</i>	90	84.2	100	0.279
<i>Q. robur</i>	84.4	94.7	71.4	0.128
<i>B. verrucosa</i>	76.5	85	64.3	0.228
LTP (Pru p 3)	45.9	43.5	50	0.745
Profilin (Pho d 2)	34.2	30.4	40	0.728
sIgE				
<i>Q. alba</i>	95.8	100	88.9	0.375
<i>B. verrucosa</i>	93.8	100	85.7	0.438
Bet v 2	37.5	40	33.3	1.000
Pru p 3	63.6	64.3	62.5	1.000
Bet v 1	63.6	95.5	0	<0.0001
Pru p 1	64	88.2	12.5	0.001
Mal d 1	78.3	100	37.5	0.002
Cor a 1	73.1	100	12.5	<0.0001
Ara h 8	41.7	62.5	0	0.006
Gly m 4	61.9	92.3	12.5	0.001
Que i 1	60.5	100	0	-

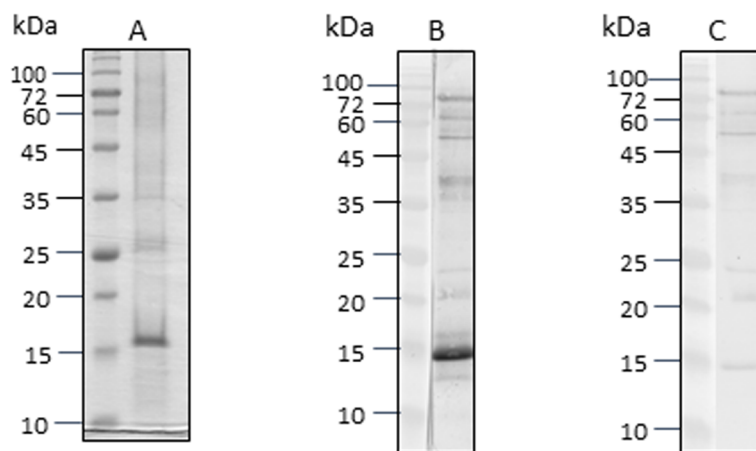
Table 3. Sensitization to PR-10 and profilin in patients presenting with PFAS according to offending food. Percentages are in brackets.

	PR-10	Profilin	Profilin and PR10	None
Banana (n=18)	7 (38.9)	4 (22.2)	5 (27.8)	2 (11.1)
Melon (n=12)	5 (41.7)	3 (25.0)	2 (16.7)	2 (16.7)
Peeled apple (n=12)	5 (41.7)	2 (16.7)	2 (16.7)	0 (0.0)
Watermelon (n=9)	6 (66.7)	2 (22.2)	0 (0.0)	1 (11.1)
Walnut (n=9)	5 (55.6)	0 (0.0)	2 (22.2)	2 (22.2)
Peeled pear (n=6)	4 (66.7)	1 (16.7)	1 (16.7)	0 (0.0)
Kiwi (n=6)	3 (50.0)	1 (16.7)	1 (16.7)	1 (16.7)
Lentil (n=6)	3 (50.0)	0 (0.0)	2 (33.3)	1 (16.7)
Peeled peach (n=5)	1 (20.0)	1 (20.0)	3 (60.0)	0 (0.0)

FIGURE LEGENDS

1. Analysis of *Q. ilex* pollen extract (10µg) by SDS-PAGE and western blot. **A**, Coomassie-stained gel of *Q. ilex* pollen extract under reducing conditions. **B**, western blotting with *Q. ilex* allergic patient serum recognizing Que i 1. **C** western blotting inhibition with 10µg of rQue i 1.
2. **A**, Analysis of *Q. ilex* pollen extract by gradient SDS-PAGE. Coomassie-stained gel: lane M molecular mass markers, lane 1 *Q. ilex* pollen extract, arrow indicates the excised band containing the identified PR10 major allergen Pru ar-like protein analysed by MALDI-TOF/TOF and LC-MSMS. **B**, The nucleotide and amino acid sequence of Que i 1 from *Q. ilex*. The full-length sequence coded for 159 amino acids. Peptides identified by MALDI-TOF/TOF (41.90% coverage) are in cursive and by LC-MSMS (55.28% coverage) are in bold, when identified by both techniques are in cursive and bold. Underlined the peptide MSMS fragmented on the combined (PMF+MSMS) MALDI-TOF/TOF strategy.
3. Amino acid sequence alignment with allergenic PR10. Que i 1 (*Q. ilex*, Holm oak), Cas s 1 (*Castanea sativa*, Chestnut, ACJ23861.1), Que a 1 (*Q. alba*, White oak, ABZ81045.1), Que r 1 (*Q. rubra*, Northern red oak, AFF59686.1), Que m 1 (*Q. mongolica*, Mongolian oak, AUH28179.1), Fag s 1 (*Fagus silvatica*, European beech, ACJ23865.1), Pru ar 1 (*Prunus armeniaca*, Apricot, O50001.1), Cor a 1 (*Corylus avellana*, European hazelnut, CAA96549.1), Pru du 1 (*P. dulcis*, Almond, ACE80941.1), Pru p 1 (*P. persica*, Peach, AJE61291.1), Pru av 1 (*P. avium*, Sweet cherry, O24248.1), Bet v 1 (*Betula verrucosa*, Silver birch, CAA96540.1), Mal d 1 (*Malus domestica*, Apple, AAS00052.1). *) positions which have a single, fully conserved residue; :) conservation between groups of strongly similar properties; .) conservation between groups of weakly similar properties. % identity value measures the number of identical residues ("matches") in relation to the length of the alignment (*); % similarity value also considered the residues with strongly similar properties ("*" plus ".").
4. **A**, Purification of Que i 1 using His Spin trap kit analysed by SDS-PAGE and Coomassie staining. Lane 1, purified inclusion bodies containing Que i 1. Lane 2, non-retained fraction. Lane 3, wash step, lane 4, eluted Que i 1 with 1 M imidazole. **B**, Western blotting analyses of 23 sera from *Q. ilex*-sensitized patients with positive detection of recombinant Que i 1. **C**, Western blotting analyses of 15 sera from *Q. ilex*-sensitized patients with negative detection of recombinant Que i 1; a and b sera from no *Q. ilex* sensitized patients.

FIG 1



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FIG 2



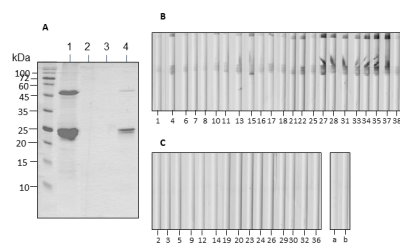
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Fig. 3

Que i	1	MGVSTHESQETSVIPPARLFKAFVLDSNLIQKVPQAIAKSIERIEGNGGPGTIKKITFGEASKFKYSKHRIDALDPENCTYSFSVIEGDA-LTD-MVSV	100
Cas s	1	MGVFTHESQETSVIPPARLFKAFVLDSNLIQKVPQAIAKSTIEIEGNGGPGTIKKITFGEASKYKYSKHRIDALDPENCTYSFSVIEGDV-LTD-IENV	100
Que a	1	MGVFTHESQETSVIAPARLFKALFLDSNLIQKVPQAIAKSTIEIEGNGGPGTIKKITFGEASKFYAKHRIDALDPENCTYSFSVIEGDA-LTV-MESV	100
Que r	1	MGVFTHESSEETSIAIPPARLFKALVLDSNLIQKVPQAIAKSTIEIEGNGGPGTIKKITFGEASKYKAKHRIDAPDPENCTYSFSVIEGDA-LTD-IASV	100
Que m	1	MGVVAYESEDASVIPPARLFKAFVLDSNLIQKVPVQALKSTIEIEGNGGPGTIKKITFGEAGSHLKAKHRIDVIDPENFTFSFSVIEGDA-LFDKLENV	100
Fag s	1	MGVFTYSESTTVIPARLFKAFVLDSNLIQKVPQAIAKSEIEIEGSGGPGTIKKITFGEAGSQFNMYKHRIDEIDNANFTYATLIEGDA-ISETLEKI	100
Pru ar	1	MGVFTYETEFTSVIPPEKLFKAFILDADNLIQKVPVAVKGTIEIEGDDGGVTIKKVTFGEAGSQYAYVKHRVDGIDKDNLSYSYTLIEGDA-LSDVIENI	100
Cor a	1	MGVFNYETETTSVIPPARLFKRFVLDSNLIQKVPAPKAIAKSIEIEIEGNGGPGTIKKICFDEGSPFNYYIKQKVEEIDQANFSYRYSVIEGDA-LSDKLEKI	100
Pru du	1	MGVFTYETEFTSVIPPEKLFKAFILDADNLIQKVPVAVKGTIEIEGDDGGVTIKKVTFGEAGSQYGYVKHRIDGIDKDNLTYSYTLIEGDV-LSDVIEKI	100
Pru p	1	MGVFTYETEFTSVIPPEKLFKAFILDADNLIQKVPVAVKGTIEIEGDDGGVTIKKVTFGEAGSQYGYVKHRIDGIDKDNLTYSYTLIEGDA-LSDVIEKI	100
Pru av	1	MGVFTYSEFTSEIPPARLFKAFVLDSNLIQKVPQAIAKSIEIEGDDGGPGTIKKITFGEAGSQYGYVKHRIDSDIKENYSYTYLIEGDA-LGDTLEKI	100
Bet v	1	MGVFNYETETTSVIPPARLFKAFILDADNLIQKVPQAIASSVENIEGNGGPGTIKKITFPEGSPFYVKKERVDEVDHANFYKYSYSMIEGGA-LGDTLEKI	100
Mal d	1	MGVFTYETEFISVIPPPRLFKAFILDADNLIQKVPVAVKGTIEIEGDDGGVTIKKVTFGEAGSQLGFVKHRIDGIDKDNFVYSYTLIEGDGLSDKIEKV	100

			aa	% Identity	% similarity
Que i	1	STEIKFVASPDGGSIKKSTTKYQTKGDFQLKEEQIQATIEKITGLSKAVEAYLLAHPDLYT	159	100	100
Cas s	1	STETKFVASPDGGTIMKSTTKYQTKGDFQLKEEQVQA A IEKATGLFKAVEAYLLANPDLYK	159	90	93
Que a	1	STEIKCVASPDGGSIMKSTTKYQTKGDFQLKEEQIQA A IEKAAGLLKAVEAYLVAHPDLYK	159	89	90
Que r	1	STEMKFVASPDGGSIMKSNTKYQTKGDFQLKEEQVQATIDKAAGHFKAVEAYLLAHPDPYN	159	86	89
Que m	1	SETKIVASPDGGSIVKSTCKYQTKGDFQLTDELIRGGKEKASGVFKAVEAYLVAHPDGYY	160	74	84
Fag s	1	AYEIKLVASPDGGSILKSTSKYHTKG DHEIKEDEQIKAGKEEASGIFKAVEAYLLANPAAYH	160	67	83
Pru ar	1	AYDIKLVASPDGGSIVKTTSHYHTKG DVEIKEEQVKAGKEKAAGLFLK LVEAYLLANPDAYN	160	61	81
Cor a	1	NYEIKIVASPHGGSILKSISKYHTIG DHELKDEQIKAGKEKASGLFKAVEGYLLAHSDAYN	160	65	76
Pru du	1	AYDIKLLASPNGGSIVKTTSHYHTKG DVEIKEEQVKAGKEKAAGLFLK LVEGYLLANPDAYN	160	59	79
Pru p	1	VYDIKLVASPNGGSIVKTI SHYHTKG DVEIKEEQVKAGKEKAAGLFLK LVEGYLLANPDAYN	160	59	78
Pru av	1	SYETKLVASPSGGSIIKSTSHYHTKGNVEIKEEHVKAGKEKASNLFKLIETY LKGHPDAYN	160	61	76
Bet v	1	CNEIKLVATPDGGSILKISNKYHTKG DHEMKA EHMKA I KEKAEALLRAVESYLLAHSDAYN	160	62	73
Mal d	1	AYETKLVASPDGGSIVKSTSHYHAKGDVEIKEEQVKAGQEASGLFLKL VESYLLANPDAYN	161	60	77
		: * : * * * * * : * : *			

FIG 4



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