The study about the role of LTP and TLP families in the development of the treenut allergy.

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Summary

Background: Food allergy caused by the ingestion of treenuts is mediated by lipid transfer protein (LTP) and thaumatins (TLPs) described as food allergens.

Objective The main objective of this study was to establish the role of LTPs and TLP families in the development of the allergy to chestnut, hazelnut and walnut.

Methods: For the study of sensitization to allergenic protein of nuts, we used sera from 13 patients with symptoms after ingestion, and positive skin prick test. These sera were provided by the Hospital de Basurto (Bilbao).

Immunodetection assays of specific IgE were made with protein extracts and purified allergens by HPLC. The purity of proteins was characterized by the N-terminal amino acid sequencing and MALDI molecular size analysis.

Results: This study identified and characterized potential allergen members of the family LTPs and TLPs in nuts. Most patients were sensitized to LTPs, showing a positive response to Cor a 8 and Jug r 3 both allergen constituting as major allergens in patients with allergy symptoms nuts.

Patients showed a positive response to 62% TLP-chestnut and hazelnut TLP 85%, showing a high response in our patient population constituting it as the second family of allergens causing allergy to nuts.

Conclusion: The LTP and TLP families were identified in nuts as principal and major allergens, having an important role in food allergy in the population studied. Severe symptoms after nuts ingestion are associated with high levels to Cor a 8, Jug r 3, Cas s 8, TLP-chestnut and TLP-hazelnut promising data for future use thereof in diagnostic assays in vivo of allergy to nuts.
1 Introduction

Allergies are nowadays diseases affecting more than 30% of the population in western countries. They are caused by inhalation, ingestion or contact with allergens, antigens responsible for the inducing synthesis of specific immuglobulin E (IgE) that mediate allergic reactions under determine conditions [1].

In the case of food allergies, the current incidence is estimated about 3% in the adult population and 6–8% in the pediatric [2]. Food allergens are mostly soluble proteins with a molecular weight of between 10-70 kDa [3]. Allergy to nuts is one of the most common food allergies, the tree nuts are among the most allergic foods worldwide [4], and therefore hazelnut, walnut, cashew, peanut, chestnut and Brazil nut have been identified [5]. (Table 1). The characterization of nut allergens, has revealed that the majority belong to storage protein families such as vicilins (7S globulins composed of subunits of about 50 kDa), legumins (11-13S globulins composed of subunits acid peptide of 30-40 kDa and 15-20 kDa basic) and 2S albumin (15 kDa).

### Table 1. Treenuts allergens

<table>
<thead>
<tr>
<th>Fruit</th>
<th>Allergen</th>
<th>Function/type</th>
<th>Molecular weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cashew</td>
<td>Ana o 1</td>
<td>Vicilins (7S)</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Ana o 2</td>
<td>Legumins (11S)</td>
<td></td>
</tr>
<tr>
<td>Hazelnut</td>
<td>Cor a 1</td>
<td>PR-10 (Bet v 1-like)</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Cor a 2</td>
<td>Profilins</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Cor a 8</td>
<td>PR-14 (LTP)</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Cor a 9</td>
<td>Globulins (11S)</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Cor a 11</td>
<td>Vicilins (7S)</td>
<td>48</td>
</tr>
<tr>
<td>Peanut</td>
<td>Ara h 1</td>
<td>Vicilins</td>
<td>63.5</td>
</tr>
<tr>
<td></td>
<td>Ara h 2</td>
<td>Conglutins</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Ara h 3</td>
<td>Glycins</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Ara h 4</td>
<td>Glycins</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Ara h 5</td>
<td>Profilins</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Ara h 6</td>
<td>Conglutins</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Ara h 7</td>
<td>Conglutins</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Ara h 8</td>
<td>PR-10</td>
<td>17</td>
</tr>
<tr>
<td>Chestnut</td>
<td>Cas s 5</td>
<td>Chitinases Ib</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cas s 8</td>
<td>PR-14 (LTP)</td>
<td>9.7</td>
</tr>
<tr>
<td>Brazil nuts</td>
<td>Ver e 1</td>
<td>Albumins (25)</td>
<td>9</td>
</tr>
</tbody>
</table>
A previous study demonstrated that chestnuts were accounted for the third greatest prevalence among the food allergens and it is the most prevalent tree nut allergen for both adult and pediatric allergy patients in Korea [6]. Chestnut food allergy has also been reported in those patients presenting with latex-fruit or oral allergy syndrome [7].

Hazelnut is a common cause of food-induced allergic reactions. In Europe the prevalence of hazelnut allergy is estimated to be between 0.1% and 0.5% [8]. A multicenter study confirmed allergy to hazelnut by means of double-blind, placebo-controlled food challenge [9]. Often specific IgE antibodies to several nuts (sensitization or immunological reactivity) have been found. In this sense, 142 peanut-allergic patients were tested by skin prick test (SPT) using different nut protein extracts. This study rendered that over 50% of patients showed a positive response against almonds, 40% cashew, 30% pistachio, 26% to Brazil nuts and hazelnuts 21% [10].

The homology between potential allergens as 2S albumins, vicilins, legumins, profilins or LTP could theoretically justify a broad immunological cross-reactivity. However, we must consider that a change in a single amino acid can alter the ability to bind IgE antibodies or binding avidity, so some highly homologous proteins have no immunological cross-reactivity. The family of proteins called LTPs (non-specific lipid transfer proteins) have been identified as major allergens plant taking its name from its ability to bind lipids in vitro and transferred between different membrane systems [11]. They are widely distributed in the plant kingdom, with about 100 potential members in more than 50 different species.

A large number of allergens of this family have been identified in plant foods especially fruits of Rosaceae family and other fruits, vegetables, nuts and grains, and latex being the peach (Prunus persica) Pru p 3, the model for the study of food allergy.

Moreover, the presence of allergic LTPs has been described in some pollens, suggesting that LTPs may have an additional role as aeroallergens [12], [13], [14]. The LTPs found in pollen of Artemisia vulgaris (Art v 3) [11] and Platanus acerifolia (Pla a 3) [11], have a sequence identity of 40% with Pru p 3, can be responsible for the cross-sensitization between pollen and plant foods [15], [16], [17].

The high resistance of LTPs to both heat treatment and digestive proteolytic attack has been related to the capability of these allergens to induce severe systemic symptoms in many patients [18], [19], [20]. LTPs are probably sensitizers through the oral route, but preliminary data suggest an alternative role as inhalant allergens, linked in some cases to plant food and pollen cross-reactions. [18], [20]

The lipid transfer proteins (LTPs) of 9 kDa (90-95 amino acid residues) basic polypeptides [15], [16]. Despite the large heterogeneity in the conservation of their primary structure, they have eight cysteine residues conserved, forming 4 disulfide bridges responsible of your high stability [6]. Similarly, the three-dimensional structure of the members of this family is highly
conserved, forming a compact domain consisting of four α-helices separated by short turns, and a random C-terminal tail [21]. However, being compatible with some of these possible functions, the strongest evidence points to involvement of LTPs in plant defense mechanisms against pathogens [22], [23], [12]. This has led to classify as PR proteins, including them in the family PR-14 [24].

The family of thaumatin-like proteins (TLPs) are also panallergens and may be responsible for cross-reactivity among foods and pollen [25]. These allergens have been identified in several food allergies such as those of apple [26], cherry [27], pepper [28], olive [29], grape [30] and kiwi [31]. In pollen, TLPs have proved to be important sensitized in cedar [32], cypress [33] and some species of juniper [34].

TLPs are proteins with a molecular weight of 20-30 kDa, and a very compact three-dimensional (3D) structure maintained by eight disulphide bridges [35]. This highly conserved 3D folding helps their resistance to the process of digestion in the gut and heat treatments at acidic pHs [36], [37]. This protein family is comprised of plant defense proteins (pathogenesis-related protein; PR5) (especially against fungal attacks). There are two types of TLPs differing with respect to their cellular location: acidic, which are directly driven to the apoplastic space; and basic, which are transported to the vacuole and release from the cell under some stress conditions [35]. Mal d 2 was revealed to be a major allergen with a 75% positive response in apple-allergic patients [38], Act d 2, the kiwi thaumatin, was recognized by 64% of Spanish patients [39], cherry (Pru av 2) [40] and Tri a-TLP in wheat [41].

The main objective of this study was to establish the role of LTPs and TLPs in the development of allergy to chestnut, hazelnut and walnut.

2 METHODS

2.1 Patients and sera:

Sera from 13 patients with allergy to nuts were selected by the Hospital de Basurto (Bilbao). All patients had a convincing clinical history of immediate allergic reactions after nuts ingestion (urticaria/angioedema or anaphylactic symptoms); and showing positive skin prick test (SPT) to fresh nuts. The clinical date are summarized in Table 2. The written informed consent was obtained from all patients and the ethics committees of the corresponding hospitals approved the study (Hospital de Basurto, Bilbao, Spain). A pool of sera from these patients was used for IgE immunodetection assays, and individual sera were tested in specific IgE determination by ELISA assays.
Table 2. Clinical data of the nut allergic patients, used in this study selected in the Hospital de Basurto of Bilbao, Spain.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Clinic history</th>
<th>Symptoms</th>
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<tbody>
<tr>
<td></td>
<td>Food</td>
<td>Pollens</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
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<tr>
<td>2</td>
<td>+</td>
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<td>3</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>13</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

2.2 Nuts extracts

Chestnut (Castanea sativa), hazelnut (Corylus avellana) and walnut (Juglans regia) were peeled, cut into small pieces, were defatted with cold acetone [2 x 1:5 (w/v) for 1 hour at 4°C] and ethanol: ether [1:3 (v/v), 3 x 1:5 (w/v) for 1 hour at 4°C]. After drying, extracted with phosphate-buffered saline (PBS) buffer (0.1 M sodium phosphate, pH 7.0 and 0.15 M NaCl; 1 x 1:5 (w/v), 1 h, 4°C). After centrifugation (12000 x g, 30 min, 4°C), the supernatant was dialyzed against H$_2$O (cut-off point, 3.5 kDa) and freeze-dried. Protein concentration was quantified according to the method of Bradford [42].
2.3 Isolation and characterization of allergens

The nuts extracts were fractionated by means of cation-exchange chromatography on an Acell Plus CM Waters SepPak cartridge (Waters, Milford, Mass) equilibrated with 20 mM formic acid, pH 4 and eluted with 75 mM NaCl in the same buffer. The fractions were dialysed (cut-off point 3.3 kDa) against H$_2$O and freeze dried. The retained fractions containing the different nuts allergens were identified by SDS-PAGE fractionation and immunodetection with the serum pool from nuts-sensitized patients or using polyclonal antibodies produced against Pru p 3 (anti-LTP), produced against Pru p 2.0201 (anti-TLP), produced against Cash 5, (class I chitinase of chestnut and major allergen; anti-chitinase), or produced against Bet v 1 (PR10 and major allergen of birch pollen), respectively.

The retained material was further separated by means of reverse-phase HPLC on a Nucleosil 300-C4 column (8 x 250 mm; particle size, 5 µm; Scharlau Science, Barcelona, Spain), eluting with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (0% to 10% for 15 minutes and 10% to 100% in 150 minutes; at a flow rate of 1 mL/min).

Putative lipid transfer proteins (LTPs) or thaumatin-like protein (TLPs) were immunodetected with both anti-LTP and anti-TLP antibodies. The isolated allergens were quantified using a commercial bicinchoninic acid test (BCA; Pierce, Cheshire, UK) [43].

SDS-PAGE was performed on Bio-Rad Miniprotean III System (Bio-Rad Laboratories, Hercules, CA, USA) gels (15% polyacrylamide) under reducing conditions, according to Laemmli [44].

N-terminal amino acid sequencing was performed with an Applied Biosystems 477A gas-phase sequencer (Foster City, Calif) and matrix-assisted laser desorption ionization (MALDI) mass spectrometry analysis with a Bi-flex III Spectrometer (Bruker-Franzen Analytik, Bremen, Germany), according to standard methods [45].

2.4 Immunodetection assays

Samples (10 µg of protein crude extract from nuts and 5 µg of purified allergens) were fractionated by SDS-PAGE and then electrotransferred (transfer buffer: 50 mM Tris, 50 mM boric acid, pH 8.3) onto polyvinylidene difluoride membranes (pore size 0.45 µm, Immobilon, Millipore Corporation) [46]. After blocking with 1X commercial solution (Sigma, St. Louis, USA), membranes were incubated overnight with a pool of sera from 13 nut-sensitive patients [1:2 dilution (v/v)] in PBS-blocking 1:10 overnight at 4°C, washed and then treated with anti-IgE-SP secondary antibody diluted in PBS-1:4 (dilution 1:3000, 1h). After washing membranes were incubated with the chemiluminescent HRP substrate for 5 minutes, and revealed by autoradiography (Amersham Hyperfilm-ECL).
Additionally, blocked membranes were immunodetected with different polyclonal antibodies: anti-LTP (dilution 1:1000), anti-TLP (1:10000), anti-chitinase (1:5000) or anti-Betv1 (1:50). The membranes were blocked with 5% skim milk (w/v) in PBS buffer. Then were treated with alkaline phosphatase-conjugate anti-rabbit IgG, except Betv1 that was incubated with alkaline phosphatase-conjugate anti-mouse IgG (1:5000 dilution; Sigma, St Louis, MO, USA) and revealed with a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution.

2.5 Specific IgE determination by ELISA assays

Specific IgE of 13 individual sera (1:4) dilution to extract (30 µg/mL as solid phase) or to the purified allergens Cas s 8, chestnut TLP, Cor a 8, hazelnut TLP and Jug r 3 (3 µg/mL as solid phase) was measured by ELISA following a previously described method [47]. Blocking solution (Sigma) was used as a negative control and specific IgE levels >0.22 OD units [mean (OD) + 3 SD of the negative control value to extract] and specific IgE levels >0.11 units [mean (OD) + 3 SD of the negative control value to purified allergens] were considered positive. All test were performed in triplicate.

3 RESULTS / DEVELOPMENT

3.1 General characteristics

Thirteen nuts-sensitive patients were included in the study, whose clinical characteristics are summarized in Table 2. Clinical manifestations of nuts allergy reported by patients consisted of oral allergy syndrome (OAS) in 2 cases (15.4%), systemic symptoms in 10 cases (76.9%). Most of our patients showed SIS with urticaria/angioedema in 6 cases (46%) and systemic anaphylaxis in 4 (31%).

3.2 Analysis of the presence of potential allergens in hazelnut, chestnut and walnut.

With the objective to analyze the presence of potential allergens in hazelnut, chestnut and walnut, nuts extracts in PBS buffer (0.5 M NaCl) were performed. These extracts were separated by SDS-PAGE, showing components from 9 kDa to 200 kDa (Fig. 1). For to analyze the presence of specific IgE in those nuts extracts, a replica was transferred to PVDF membrane and immunodetection was performed using the serum pool from 13 nuts-
sensitive patients. The sera used reacted with proteins in all 3 extracts, recognizing bands around 10-95 kDa (Fig. 1). Protein bands of approximately 10, 17 and 24 kDa were recognized by polyclonal and monoclonal antibodies: anti-Pru p 3, anti-chestnut TLP, anti-chi and anti-Bet v 1 (Fig. 1).

![Fig 1. The chestnut, hazelnut and walnut extracts](15 µg) SDS-PAGE and stained with Coomassie Blue. The replica gels were electrotransferred to PVDF membranes and incubated with a pool of sera from nut-sensitive patients or with antibodies to Pru p 3 (anti-LTP, 1:500), chestnut TLP (anti-TLP; 1:10000), chestnut chitinase (anti-Chi, 1:5000) and Bet v 1 (anti-Bet v 1, 1:50).

### 3.3 Purification and characterization of allergens in hazelnut and chestnut.

The recognition by sera of patients allergic to extracts (Fig. 1) suggested that some proteins in hazelnut, chestnut and walnut may be important allergens in these species, and based on these data, the 10 and 24 kDa putative allergens were isolated. The purity of the isolated allergens was checked by protein staining after SDS-PAGE, further confirmed by immunodetection with anti-Pru p 3 and anti-TLP antibodies (Fig. 2). To support this notion, the retained were re-purified by High Pressure Liquid Chromatography method (HPLC).
The isolated allergen were collected individually them was checked by protein staining after SDS-PAGE and IgG-binding capacity was confirmed by immunodetection with antibodies anti-Pru p 3 and anti-TLP, protein bands of approximately 10 and 28 kDa were recognized, could be potential hazelnut allergens (Cor a 8 and hazelnut TLP) and chestnut (Cas s 8 and chestnut TLP) (Fig. 3).

The isolated allergen, Cas s 8, behaved as a single band of the expected apparent molecular weight in SDS-PAGE, which was recognized by anti-Pru p 3 antibodies (Fig. 3a).
3.4 Allergen characterization

The purity of Cas s 8 was confirmed by N-terminal amino acids sequence: SITXTQVSKLMPXL, as well as by the single and sharp peak at 9718.6 d detected in MALDI analysis. Together, all these data strongly indicated that the purified 9 kDa protein and the chestnut LTP Cas s 8 were the same allergen.

3.5 Determination of specific IgE by ELISA assays in sera from nuts-sensitized patients

ELISA assays using nuts extracts and different purified allergens as a solid phase led to determine the specific IgE levels to extracts and purified allergens in the individual sera. The results are summarized in Fig. 4. In the group of patients (n=13), positive IgE levels to extracts were found in around 77% hazelnut (n=10), 100% chestnut (n=13) and 85% walnut (n=11), and the positive recognition were observed in 100% for Cor a 8 (n=13), 85% for hazelnut TLP (n=11), 46% for Cas s 8 (n=6), 62% for chestnut TLP (n=8) and 100% for Jug r 3 (n=13).

The positive responses to Cor a 8 and Jug r 3 (LTPs) were observed in 100% of patients tested, these results confirmed the relevance of LTPs family as a major allergen involved in nuts allergen was supported by results obtained (Table 3).
The role of LTPs and TLPs as major allergens to fruits (peach, apple, cherry, and grape), vegetable (asparagus and lettuce), and nut (hazelnut and chestnut) in the Mediterranean population frequently are associated with severe systemic symptoms, is well established [7], [9], [11], [12]. A substantial proportion (46%) of the selected population in this study developed symptoms as angioedema and urticaria after nuts ingestion, additionally (31%) of patients developed severe symptoms (systemic anaphylaxis) and short group (8%) another symptom associated a nuts allergy.

Table 3: Recognition of specific IgE (%), with sera from nuts allergic patients in extracts and purified proteins:

<table>
<thead>
<tr>
<th>Table 3: Recognition of specific IgE (%)</th>
<th>with sera from nuts allergic patients in extracts and purified proteins:</th>
<th>Cor a 8, hazelnut TLP, Cas s 8, chestnut TLP and Jug r 3.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hazelnut</td>
</tr>
<tr>
<td>Patients n =</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Recognition %</td>
<td>77</td>
<td>100</td>
</tr>
</tbody>
</table>

4 Discussion

The role of LTPs and TLPs as major allergens to fruits (peach, apple, cherry, and grape), vegetable (asparagus and lettuce), and nut (hazelnut and chestnut) in the Mediterranean population frequently are associated with severe systemic symptoms, is well established [7], [9], [11], [12]. A substantial proportion (46%) of the selected population in this study developed symptoms as angioedema and urticaria after nuts ingestion, additionally (31%) of patients developed severe symptoms (systemic anaphylaxis) and short group (8%) another symptom associated a nuts allergy.
Profile analysis of the recognition of specific IgE in extracts showed a recognition pattern of proteins with around molecular weights ranging from 10 to 95 kDa. Demonstrated in previous studies, the involvement of LTPs as a family of panallergens involved in IgE-mediated reactions, both in food and pollen [48], [49], [50], [51] and the importance of TLPs in relation to nuts allergy [25], and great stability of the members of this family to protease digestion and heat treatment at acid pHs could favor their permanence in foodstuff (after cooked or industrial processing), and thereby their allergic potential, although these proteins have not been found in processed foods such as jam or juice [36].

The isolation of Cas s 8 has allowed us to characterize as lipid transfer protein allergen (LTP), abundant in the chestnut extract, displays an N-terminal amino acid sequence typical of the LTP family, a deduced molecular size 9718.6 d.

The three extracts were recognized by individual sera analyzed by ELISA, the 100% of patients reacted with chestnut extract, the high proportion of recognized may be considered as the nuts with major sensitive patients in the population studied (Table 3).

The allergenicity of the purified proteins were confirmed by ELISA assays. Cor a 8 and Jug r 3 were recognized by 100%, hazelnut TLP by 85%, chestnut TLP by 62% and Cas s 8 with significantly low 46% patients (Table 3). This low response IgE-binding from patients with Cas s 8 may be related to the sensibilization from patients to TLP-chestnut, demonstrating the importance today of TLPs in the context of nuts allergy in Spain [52].

Cor a 8 and Jug r 3, the nuts LTPs used as models of this family displayed 100% positive recognition of nuts-allergic patients with ELISA assays, this result could be explained for the cross-reactivity between hazelnut and walnut observed also with clinical symptoms. Furthermore, the group of patients with anaphylaxis showed a significantly higher level of IgE to Cor a 8 and Jug r 3, suggesting potential clinical relevance of LTPs. Other authors have also emphasized the high recognition prevalence of Cas s 8 allergen in nuts-sensitized patients [28], but this scenario seems to be different, where Cas s 8 is not recognized by most 50% of 13 sera from nuts-sensitized patients, thus not is considerate as the major allergen in this population studied.

The second allergen family with proteins around of 28 kDa, also abundant in the nuts extracts corresponds to the Thaumatin-like allergen, the sensitized patients to TLPs showed a low response against to LTPs, the specific IgE detected were 62% chestnut TLP and 85% hazelnut TLP, of individual sera by ELISA, constituting it as the second family of allergens causative of nuts allergy in this population.

Chestnut TLP and hazelnut TLP, was used as a model of this family represents a minor treenut allergens base on its low specific IgE prevalence (62% and 85% respectively) in sera from treenut-sensitized patients. The potential clinical relevance of TLPs are further
supported by the statistical correlation between IgE levels to this allergen and anaphylactic symptoms.

Furthermore, the group of patients with anaphylaxis showed a significantly higher level of IgE, as well as sensitization rates, to hazelnut TLP than the patients with other symptoms, thus suggesting its potential clinical relevance. Other authors have also emphasized the high recognition prevalence of this allergens in TLP-sensitized patients from different European areas[49],[52],[53], but this scenario seems the different in the United Kingdom, where TLPs are not recognized by any of 13 sera from treenut-sensitized patients, thus not being a major allergen in this population.

The close identity with other food TLPs described as allergens, such as Act d 2, Mal d 2 or Pru av 2, suggests cross-reactivity among these, although more detailed studies are required. Likewise, further work is required to evaluate the role this family as panallergens and their importance as mediators of cross-reactions [36], [37]. On the other hand, the presence of members of this family described as pollen allergens [25], could explain the high prevalence of polinosis (50%) in the patients studied. This results should be included in routine test for allergy diagnosis.

Together, all these suggest a potential role of LTPs and TLPs families as major nuts allergens in population studied, as well as the need for further research in diagnostic assays in vivo of nuts allergy.

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