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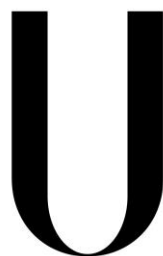
**Characterization of allergens from  
several tree nuts and their role in  
plant food allergy**

*Caracterização de alérgenos de frutos secos e o seu papel na alergia a  
alimentos vegetais*

**Cátia Liliana Morais Pereira**

**Mestrado em Controlo da Qualidade e Toxicologia dos Alimentos**

**2013**



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# **Characterization of allergens from several tree nuts and their role in plant food allergy**

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**Mestrado em Controlo da Qualidade e Toxicologia dos Alimentos**

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## **ABSTRACT**

Food allergies are a common issue in western countries. In the last decade, these diseases has increased significantly, and nowadays it is estimated that affects 2-8% of the population. Within the food allergies, plant food is the most frequent in adult population and the most part of the plant food allergens belong to protein families with defense or storage functions.

Among plant food allergies there is a special interest in tree nut allergy. In the course of history, nuts have been part of the diet around the world. Tree nuts have a high nutritional value and they are very important in the human diet. However, in the developed world, the allergic reactions caused by tree nuts represent one of the first causes of food allergies in children and the first in adults.

Understanding the mechanism by which a harmless protein to the organism is capable of inducing an allergic response is the basis to prevent and treat this type of disease.

Until now, in food allergy, the only possible treatment is avoiding the consumption of the culprit food. Although, the existence of cross-reactivity between allergens and the specific sensitization profiles of each patient, makes it difficult to know which foods are related and which ones the patient should avoid.

In order to develop safe and effective immunotherapy, it is necessary to characterize the allergens involved both at molecular and immunological level.

The major allergens described in tree nuts are 7S vicilins, 11S legumins, 2S albumins, lipid transfer proteins (LTPs) and thaumatin-like proteins (TLPs).

In this thesis, the allergenic molecular basis of these proteins was studied in order to try to understand the possible mechanisms that are mediating sensitization and cross-reactivity and the prevalence of these proteins in a Spanish population, with the use of protein microarrays.

**Key words:** food allergy, seed storage proteins, protein microarrays, tree nuts, LTPs, TLPs, cross-reactivity

## RESUMO

As alergias alimentares são um problema comum nos países ocidentais. Na última década, estas doenças têm aumentado significativamente e actualmente é estimado que afectem 2-8% da população. Nas alergias alimentares, a alergia a alimentos vegetais é a mais frequente na população adulta e a maioria dos alergenos de alimentos vegetais pertencem a famílias de proteínas com funções de defesa e armazenamento.

Entre as alergias a alimentos vegetais, há um interesse especial na alergia a frutos secos. No decurso da história, os frutos secos têm feito parte da dieta em todo o mundo. Os frutos secos têm um elevado valor nutricional e são muito importantes na dieta humana. Contudo, no mundo desenvolvido, as reacções alérgicas causadas pelos frutos secos, representam uma das primeiras causas de alergia alimentar em crianças e a primeira em adultos.

Conhecer o mecanismo pelo qual uma proteína inofensiva ao organismo é capaz de induzir uma resposta alérgica, é a base para prevenir e tratar este tipo de doença.

Até agora, na alergia alimentar, o único tratamento possível é evitar o consumo do alimento culpado pela alergia. Todavia, a existência de reactividade-cruzada entre alergenos e os perfis específicos de sensibilização dos pacientes, torna difícil saber que alimentos estão relacionados e quais os alimentos que o paciente deve evitar.

De modo a desenvolver imunoterapia segura e eficaz é necessário caracterizar os alergenos envolvidos, tanto a nível molecular como a nível imunológico.

Os alergenos maioritários descritos nos frutos secos são vicilinas 7S, leguminas 11S, albuminas 2S, proteínas de transferência de lípidos (LTPs) e proteínas similares a taumatinas (TLPs).

Nesta tese, a base molecular alergénica destas proteínas foi estudada de modo a perceber os possíveis mecanismos que medeiam a sensibilização e a reactividade-cruzada e a prevalência destas proteínas numa população Espanhola, com a utilização de *microarrays* de proteínas

**Palavras-chave:** alergia alimentar, proteínas de armazenamento de sementes, *microarrays* de proteínas, frutos secos, LTPs, TLPs, reactividade cruzada

## INDEX

<b>ACKNOWLEDGEMENTS .....</b>	<b>III</b>
<b>ABSTRACT .....</b>	<b>IV</b>
<b>RESUMO .....</b>	<b>VI</b>
<b>INDEX .....</b>	<b>VIII</b>
<b>FIGURES INDEX .....</b>	<b>XI</b>
<b>LIST OF ABBREVIATIONS.....</b>	<b>XIV</b>
<b>1. INTRODUCTION .....</b>	<b>1</b>
<b>1.1. Allergy mechanisms .....</b>	<b>1</b>
<b>1.2. Sensitization Pathways.....</b>	<b>3</b>
1.2.1. Gastrointestinal sensitization .....	3
1.2.2. Respiratory sensitization .....	5
1.2.3. Skin sensitization.....	6
<b>1.3. Food allergy .....</b>	<b>7</b>
<b>1.4. Allergen cross-reactivity .....</b>	<b>7</b>
<b>1.5. Seed storage proteins.....</b>	<b>8</b>
1.5.1. 2S albumins .....	8
1.5.2. Globulins.....	9
<b>1.6. Pathogenesis related proteins .....</b>	<b>10</b>
1.6.1. Lipid Transfer Proteins .....	10
1.6.2. Thaumatin-like Proteins .....	11
<b>1.7. Tree nut allergy .....</b>	<b>11</b>
1.7.1. Hazelnut .....	12
1.7.2. Walnut.....	13
1.7.3. Chestnut .....	13
1.7.4. Peanut .....	14
1.7.5. Pine nut .....	14
1.7.6. Sunflower seed.....	15

1.7.7.	Cashew nut.....	15
<b>1.8.</b>	<b>Diagnosing allergy .....</b>	<b>15</b>
1.8.1.	Skin prick test (SPT) .....	16
1.8.2.	Double blind placebo controlled food challenge .....	17
1.8.3.	Immunodetection: ELISA and Western-Blot .....	18
1.8.4.	Protein Microarrays .....	19
<b>2.</b>	<b>AIMS .....</b>	<b>21</b>
<b>3.</b>	<b>MATERIAL AND METHODS.....</b>	<b>22</b>
<b>3.1.</b>	<b>Patient's Sera .....</b>	<b>22</b>
<b>3.2.</b>	<b>Methods .....</b>	<b>22</b>
3.2.1.	Protein extraction and protein quantification .....	22
3.2.1.1.	Protein extraction .....	22
3.2.1.2.	Electrophoretic methods .....	23
3.2.2.	Immunochemical methods .....	23
3.2.2.1.	Western-Blot with polyclonal antibodies .....	23
3.2.2.2.	Western-Blot with patient's sera .....	24
3.2.3.	Isolation of allergens .....	25
3.2.3.1.	2S Albumins isolation.....	25
3.2.3.2.	11S isolation.....	25
3.2.3.3.	LTP and TLP isolation .....	25
3.2.4.	Quality of purified proteins.....	26
3.2.5.	Protein microarrays.....	26
3.2.5.1.	Printing.....	26
3.2.5.2.	Hybridization.....	26
3.2.5.3.	Array reading .....	27
3.2.6.	Statistical analysis.....	27
<b>4.</b>	<b>RESULTS .....</b>	<b>29</b>
<b>4.1.</b>	<b>Study of the cross-reactivity among tree nut species .....</b>	<b>29</b>
<b>4.2.</b>	<b>Characterization of allergens in tree nuts and their prevalence in an adult population .....</b>	<b>31</b>
4.2.1.	Purification and characterization of 2S albumins from walnut .....	31
4.2.2.	Purification and characterization of 11S from hazelnut .....	31
4.2.3.	Purification and characterization of LTP from Hazelnut, walnut and chestnut.....	33

4.2.4.	Purification and characterization of TLP from hazelnut and chestnut .....	36
4.2.5.	Study of the prevalence of the most relevant allergens.....	40
<b>5.</b>	<b>DISCUSSION.....</b>	<b>43</b>
<b>6.</b>	<b>CONCLUSIONS .....</b>	<b>52</b>
<b>7.</b>	<b>REFERENCES .....</b>	<b>53</b>

## FIGURES INDEX

<b>Figure 1:</b> Paracelular transport (Ménard, S., <i>et al.</i> , 2010) .....	4
<b>Figure 2:</b> Transcelular transport. (Ménard, S., <i>et al.</i> , 2010).....	5
<b>Figure 3:</b> The dual-allergen exposure hypothesis of food allergy.....	6
<b>Figure 4:</b> Skin prick tests to common allergens in the forearm. Compared with histamine, the positive control, indicating the presence of allergen-specific IgE to different allergens (Tordesillas, L., 2012).....	17
<b>Figure 5:</b> Schematic illustration of an immunoblotting (Western-blot) (Adapted from: Goldsby, R.A., <i>et al.</i> , 2003). .....	19
<b>Figure 6:</b> Schematic description of protein microarray testing of allergen-specific IgE. Allergens are potted onto slides in triplicate. Serum incubation is followed by a second anti-human IgE antibody for detection. The mean fluorescence intensity is determined by two different laser settings (Jahn-Schimid, B., <i>et al.</i> , 2003).....	20
<b>Figure 7:</b> SDS-PAGE from PBS extracts of cashewnut (C) (10µg), hazelnut (H) (5µg), peanut (P) (10µg), chestnut (Ct) (10 µg), walnut (W) (15 µg), pine nut (Pn) (10 µg) and sun seeds (S) (10 µg): <b>A</b> , staining with Blue Coomassie; <b>B</b> , Immunodetection with pool serum from Jimenez Diaz Foundation (FJD); <b>C</b> , Immunodetection with pool serum from Hospital La Princesa University Hospital; <b>D</b> , Immunodetection with pool serum from Basurto Hospital (Bilbao). .....	29
<b>Figure 8:</b> SDS-PAGE from PBS extracts of cashewnut (C) (10µg), hazelnut (H) (5 µg), peanut (P) (10µg), chestnut (Ct) (10µg) walnut (W) (15µg), pine nut (Pn) (10µg), and sun seeds (S) (10 µg); <b>A</b> , Immunodetection against Pru p 2.0201 (TLP) (1:5000); <b>B</b> , Immunodetection against Hela 1 (11S legumin) (1:10000); <b>C</b> , Immunodetection against Pru p 3 (LTP) (1:1000) .....	30
<b>Figure 9:</b> SDS-PAGE from PBS extracts (10 µg) of walnut; <b>A</b> , staining with blue comassie of the extract (E) and of the retained fraction of the molecular exclusion chromatography (ME); <b>B</b> , Immunodetection with polyclonal antibodies against 2S.....	31
<b>Figure 10:</b> SDS-PAGE from PBS extracts (10µg) (E) of hazelnut, the retained fraction obtained by anion-exchange chromatography in Waters cartridge (5µg)	

(A) and in FPLC (5µg) (R) **A**, staining with Blue Coomassie; **B**, Immunodetection with polyclonal antibodies against Hela 1 (11S legumin) (1:10000) ..... 32

**Figure 11:** SDS-PAGE from PBS extracts (10µg) of hazelnut (H), walnut (W) and chestnut (Ct): **A**, staining with Blue Coomassie; **B**, Immunodetection with polyclonal antibodies against Pru p 3 (LTP) (1:1000). ..... 33

**Figure 12:** SDS-PAGE of the retained fraction obtained by cation-exchange chromatography in Waters cartridge (5µg) of hazelnut (H), walnut (W) and chestnut (Ct): **A**, staining with Blue Coomassie; **B**, Immunodetection with polyclonal antibodies against Pru p 3 (LTP) (1:1000). ..... 34

**Figure 13:** Purification of LTPs from hazelnut, walnut and chestnut. A,B and C purified by HPLC reverse-phase. All selected picks contain the purified proteins. .... 34

**Figure 14:** SDS-PAGE from purified proteins (5µg) of hazelnut (H), walnut (W) and chestnut (Ct) . **A**, staining with Blue Coomassie; **B**, Immunodetection with polyclonal antibodies against Pru p 3 (LTP) (1:1000). ..... 35

**Figure 15:** MALDI-spectrum of the purified allergen Jug r 3..... 35

**Figure 16:** MALDI-spectrum of the purified allergen Cor a 8..... 36

**Figure 17:** MALDI-spectrum of the purified allergen Cas s 8. .... 36

**Figure 18:** SDS-PAGE from PBS extracts (10µg) of hazelnut (H) and chestnut (C): **A**, staining with Blue Coomassie; **B**, Immunodetection with polyclonal antibodies against Pru p 2.0201(TLP) (1:5000). ..... 37

**Figure 19:** SDS-PAGE of the retained fraction obtained by cation-exchange chromatography in Waters cartridge (5µg) of hazelnut (H) and chestnut (Ct): **A**, staining with Blue Coomassie; **B**, Immunodetection with polyclonal antibodies against Pru p 2.0201 (TLP) (1:5000). ..... 37

**Figure 20:** Purification of LTPs from hazelnut and chestnut. A and B purified by HPLC reverse-phase. All selected picks contain the purified proteins..... 38

**Figure 21:** SDS-PAGE from purified proteins (5µg) TLP hazelnut and TLP chestnut **A**, staining with Blue Coomassie; **B**, Immunodetection with polyclonal antibodies against Pru p 2.0201 (TLP) (1:5000). ..... 39

**Figure 22:** MALDI-spectrum of the purified allergen TLP hazelnut. .... 39

**Figure 23:** MALDI-spectrum of the purified allergen TLP chestnut. ....40

**Figure 24:** Recognition frequencies of the different allergens by geographical area. Frequencies, shown as percentage of positive response (%), were obtained by incubating the allergens microarray with single sera from allergic patients of Jimenez Diaz Foundation (FJD), La Princesa University Hospital and Basurto Hospital (Bilbao). Percentage positive responses and significant differences ( $p>0,05$ ) are indicated. ....42

**Figure 25:** Frequency of sensitizations obtained by the different allergens microarray using sera from all patients. ....42

## LIST OF ABBREVIATIONS

<b>Ana o 1</b>	Cashew nut allergen (7S)
<b>Ana o 2</b>	Cashew nut allergen (11S)
<b>Ana o 3</b>	Cashew nut allergen (2S)
<b>APC</b>	Antigen presenting cell
<b>Ara h 1</b>	Peanut allergen (7S)
<b>Ara h 2</b>	Peanut allergen (2S)
<b>Ara h 3</b>	Peanut allergen (11S)
<b>Ara h 6</b>	Peanut allergen (2S)
<b>Ara h 9</b>	Peanut allergen (LTP)
<b>BALT</b>	Bronchial-associated lymphoid tissue
<b>Bet v 1</b>	Birch tree allergen
<b>Cas s 5</b>	Chestnut allergen (chitinase)
<b>Cas s 8</b>	Chestnut allergen (LTP)
<b>Cor a 1</b>	Hazelnut allergen (Bet v 1-like)
<b>Cor a 2</b>	Hazelnut allergen (2S)
<b>Cor a 8</b>	Hazelnut allergen (LTP)
<b>Cor a 9</b>	Hazelnut allergen (11S)
<b>Cor a 11</b>	Hazelnut allergen (7S)
<b>Da</b>	Dalton
<b>DBPCFC</b>	Double-blind placebo-controlled food challenge
<b>FJD</b>	Jimenez Diaz Foundation
<b>FPLC</b>	Fast-Performance Liquid Chromatography
<b>GALT</b>	Gut-associated lymphoid tissue

<b>Hel a 1</b>	Sunflower seed allergen (11S)
<b>Hel a 2S</b>	Sunflower seed allergen (2S)
<b>Hel a Oleosins</b>	Sunflower seed allergen (Oleosin)
<b>HPLC</b>	High-Performance Liquid Chromatography
<b>IgE</b>	Immunoglobulin E
<b>IgG</b>	Immunoglobulin G
<b>Jug r 1</b>	Walnut allergen (2S)
<b>Jug r 2</b>	Walnut allergen (7S)
<b>Jug r 3</b>	Walnut allergen (LTP)
<b>Jug r 4</b>	Walnut allergen (11S)
<b>LTPs</b>	Lipid Transfer Proteins
<b>MALDI</b>	Matrix-assisted laser desorption/ionization
<b>MHC</b>	Major Histocompatibility Complex
<b>PAGE</b>	Polyacrilamide electrophoresis gel
<b>PBS</b>	Phosphate buffered saline
<b>PR</b>	Pathogenesis related
<b>Pru p 2.0201</b>	Peach allergen (TLP)
<b>Pru p 3</b>	Peach allergen (LTP)
<b>PVDF</b>	Polyvinylidene difluoride
<b>RP</b>	Reversed phase
<b>SALT</b>	Skin-associated lymphoid tissue
<b>SPT</b>	Skin Prick Test
<b>TCR</b>	T-Cell Receptor
<b>TLPs</b>	Thaumatolike proteins
<b>TOF</b>	Time of Flight mass spectrometry

## **1. INTRODUCTION**

Allergies are immunogenic diseases that nowadays affect more than 30% of the world population and their development depend both on genetic and environmental factors (Kay, A.B., 1997). The most common sources of allergens are dust mites, mold, pollen and plants (Hoffmann-Sommergruber, K., 2000). Allergies can be produced by inhalation, ingestion or contact with allergens that are capable of inducing the synthesis of immunoglobulin E (IgE) (Huby, R.D.J., *et al.*, 2000). In IgE-mediated reactions, clinical symptoms are caused by the rapid and massive release of inflammatory mediators. Moreover, this type of disease involves an important cost for the European health systems, sometimes bigger than that produced by season flu (Holgate, S., *et al.*, 2006).

Food allergies are the most common cause of anaphylaxis evaluated in emergency rooms in all age groups and they occasionally result in fatalities (Nowak-Wegrzyn, A., and Sampson, H.A., 2011).

### **1.1. Allergy mechanisms**

The first attempt to define food allergy came in 1984 from the American Academy of Allergy and Immunology, and the National Institute of Allergy and Infectious Diseases. They classified adverse food reactions in two types, food allergy (hypersensitivity) and food intolerance, depending on the existence or not of an underlying immunological mechanism, respectively (San Miguel Moncin, M.M., *et al.*, 2007).

Food allergy is constituted by a hypersensitivity reaction in which symptoms appear quickly and are caused by exposure to exogenous macromolecules, also known as antigens or allergens. This reaction results in a marked increase in reactivity and responsiveness to the antigen or allergen on ensuing exposure, resulting in adverse health effects and normally mediated by

IgE. Meanwhile, food intolerances are non-immune-mediated reactions whose symptoms can sometimes take days to manifest (Mills, E. N. C., 2003). This way, food allergy can be divided into IgE-mediated and non-IgE mediated reactions, where the food allergens are the antigenic molecules which induce the immunologic response (Fernandéz-Rivas, M. and Miles S., 2004).

The IgE mediated reactions are also called type I Hypersensitive, and involve a complex immunologic process, which is divided in two phases, the sensitization and subsequent reaction to re-exposure to an allergen.

Upon contact with the mucosa, the antigen is taken up by the antigen presenting cells (APC), which will process it in the endocytic pathway. The generated peptides are presented on the cell surface bound to a receptor known as major histocompatibility complex (MHC) type II. Then, these APCs such as mucosal macrophages, progenitor B-cells and dendritic cells migrate to the regional lymph nodes where the antigen presentation will occur to T-cells. The T-cell receptors of CD4+ T helper cells (TCR) recognize the complex of foreign peptide plus MHC molecule on the APC surface. After this interaction the CD4+ T cells are activated and differentiate into Th2 cells (Berin, M.C., and Shreffler, W.G., 2008). Thus, they secrete specific cytokines, as interleukins, responsible for differentiation towards one particular subtype of T-helper lymphocytes and for the proliferation of B lymphocytes that synthesize specific IgE antibodies. The IgE molecules enter into circulation and bind to their high affinity receptors (FcεRI), which are present in mast cells, blood basophils and dendritic cells.

Consecutive exposures to the allergen trigger the second phase, where a huge number of IgE molecules are bound to the surface of the effector cells such as mast cells and basophils. Thus, the cross-linking is produced, triggering the cell degranulation, with release of preformed pharmacological mediators such as histamine, and subsequent newly formed mediators such as cytokines and leukotriene (Bird, J.A., 2010).

## **1.2. Sensitization Pathways**

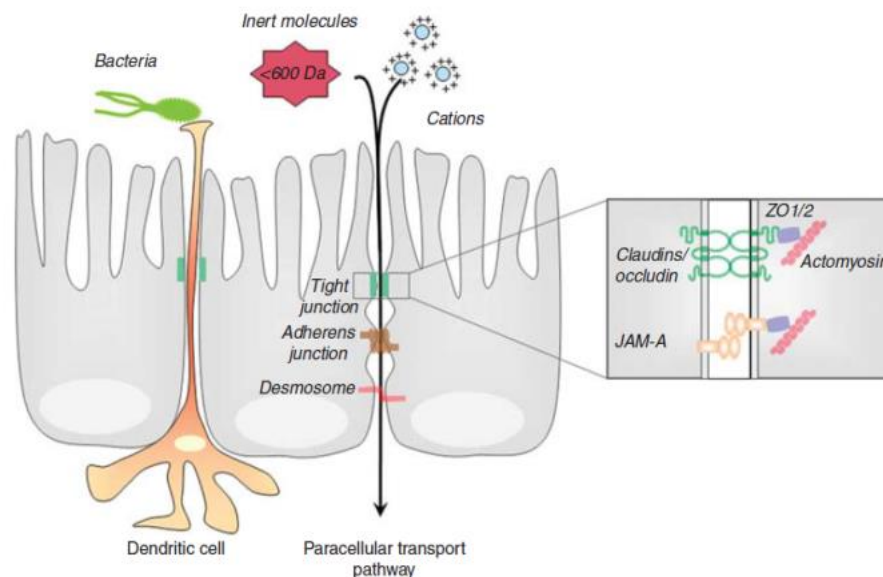
The allergic response needs that the responsible allergen gets in contact with the immune system. This occurs when the antigen crosses through epithelial barriers as the skin, intestines and the respiratory tract. The epithelia represent a physical barrier against the entrance of infectious microorganisms, toxins and harmful antigens, and also biochemical barriers, as the cells that actively participate in development of immune responses (Bartemes, K.R., and Kita, H., 2012). Therefore, epithelia dysfunction causes an increased risk of sensitization (Groschwitz, K.R., and Hogas, S.P., 2009; Perrier, C., and Corthésy, B., 2010).

### **1.2.1. Gastrointestinal sensitization**

After ingestion, digestive enzymes degrade food proteins into small peptides which are absorbed in the intestine. However, some proteins resist the enzymatic activities, reaching the intestinal lumen intact and then they interact with the lymphoid tissue associated to the digest tract, or GALT (gut-associated lymphoid tissue), and may conduct an immune response (Ménard, S., *et al.*, 2010). The ability of molecules to cross the epithelium is dependent on its size, shape, polarity, three-dimensional structure and aggregation status (Perrier, C. and Corthesy, B., 2001).

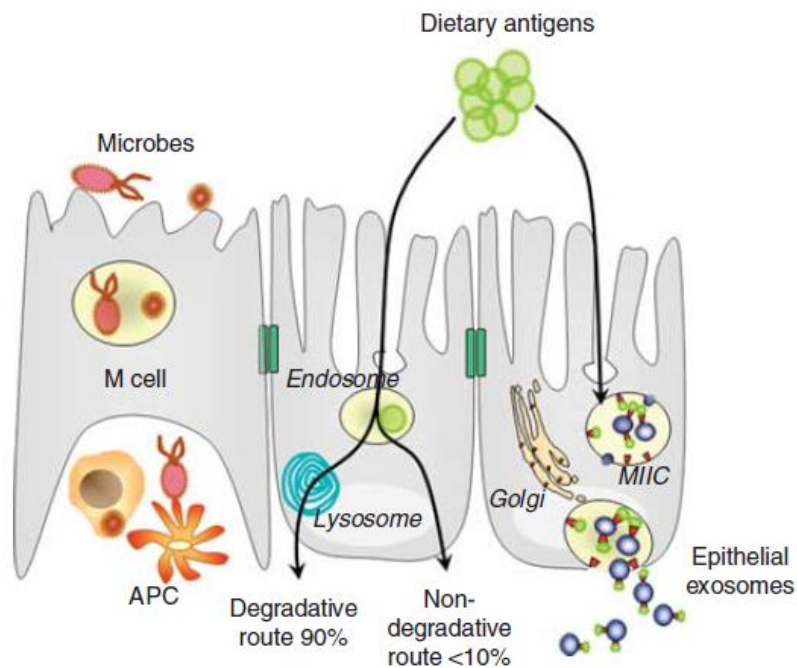
The gut epithelia monolayer is the main element of the epithelial barrier. Its integrity depends on the binding complexes between cells, especially the tight junctions. Below the monolayer, is the lamina propria, which is rich in immune cells (dendritic cells, lymphocytes, macrophages and others), and above is covered by a mucus layer. The monolayer avoids the entry of harmful antigens and infectious organisms, being permeable only to water, electrolytes and essential nutrients (Groschwitz, K.R., and Hogas, S.P., 2009). However, some

components of the human diet can be carried through the monolayer. The intestinal transport of molecules from the intestinal lumen to the lamina propria can occur through two distinct mechanisms: paracellular and transcellular transport. The paracellular transport, occurs through the adjacent epithelial cells, where is allowed the transportation of molecules smaller than 600 Da. The tight junctions situated at the apical area, regulate the paracellular permeability, and, when there are some dysfunction, the risk of sensitization increases once the augmentation in the permeability allows the transportation of non-degraded proteins (figure 1) (Perrier, C., and Chortésy, B., 2010).



**Figure 1:** Paracellular transport (Ménard, S., *et al.*, 2010)

In the transcellular transport, proteins enter into the cell thanks to the formation of endosomes, being degraded in small peptides by the activity of the lysosomes. The resulting peptides are released into the basolateral side, playing an important role in the body's immunity. Early endosomes may emerge with vesicles rich in MHCII molecules which expose protein-fragments even partially degraded. In the end, the vesicle releases its content, in the basolateral side, (figure 2) (Ménard, S., *et al.*, 2010).



**Figure 2:** Transcellular transport. (Ménard, S., *et al.*, 2010)

### 1.2.2. Respiratory sensitization

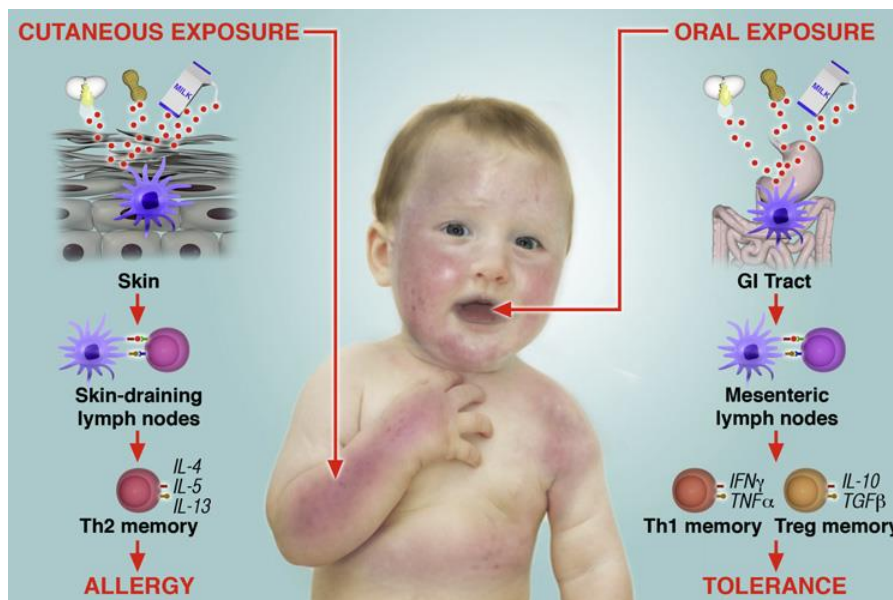
The human lung inhales 10000 liters of air, each day, which causes the inhalation of many allergens (Holgate, S.T., 2007). The respiratory sensitization occurs when those allergens get in touch with the bronchial associated lymphoid tissue (BALT). Therefore, it is only required an environmental exposure for a person to stay sensitized.

The bronchial epithelium is similar to the gut, structurally and functionally. The epithelium cells, tight junctions and the mucus layer form the respiratory physical barrier. This barrier is semi-permeable, allowing the transport of water, nutrients and gases. The mucus layer above the monolayer also works as a barrier, but in this case as a biochemical and immunological barrier, since it contains cytokines and antimicrobial compounds (Vareille, M., *et al.*, 2011). Furthermore, the epithelial cells are also involved in the immune

reaction mediated by the release of the immune response mediators (Bartemes, K.R., and Kita, H., 2012).

### 1.2.3. Skin sensitization

Another sensitization pathway is the skin sensitization which explains the development of food allergies without previous consumption of the food. The dual-allergen exposure hypothesis suggests that the exposition to food allergens by the oral route induces oral tolerance, meanwhile, the exposition to these allergens by cutaneous route induce sensitization (figure 3) (Lack, G., 2008).



**Figure 3:** The dual-allergen exposure hypothesis of food allergy (Lack, G., 2008).

This cutaneous sensitization occurs when the allergens cross through the skin and interact with the skin-associated lymphoid tissue (SALT). Allergens get in touch with Langerhans cells, inducing the activation of T-cells towards an immune response type I.

### **1.3. Food allergy**

Nowadays, the prevalence of food allergies is about 3% in adults, and between 6 and 8% in children (Burks, W., and Ballmer-Weber, B.K., 2006). Food allergens, apparently, do not share specific biochemical characteristics which determine a protein to be an allergen. Why, in the same family, a protein is allergic and other not, is something that is still unknown. Within the food allergies, plant food is the most frequent in adult population. To this day, many plant food allergens have been identified (Breiteneder, H., and Radauer, C., 2004), most of whom belong to only 31 protein families of over 2600 plant protein families described. The most part of the plant food allergens belong to protein families with defense or storage functions (Chapman, M.D., *et al*, 2007). Some families of allergens do not follow this rule, such as profilins (Radauer, C., and Hoffmann-Sommergruber, K., 2004).

Seed storage proteins accumulate mainly in mature seeds and they are mobilized during germination to provide a source of nitrogen for the early stages of development (Higgins, T.J.V., 1984). The families of storage proteins described as allergens are vicilins, legumins, 2S albumins and prolamins. Plant defense proteins are involved in mechanisms of response to pests, pathogens and stressful environment. This group includes enzyme inhibitors, thionins, thaumatins, lipid transfer proteins (LTPs), chitinases, lectins, and more. There are 14 families of defense proteins, eight of which include members that have been described as allergens (Hoffmann-Sommergruber, K., 2002).

### **1.4. Allergen cross-reactivity**

Cross-reactivity is a major problem in allergy and the term is used to describe clearly defined clinical features that show reactivity to a source without previous exposure (Ferreira, F., *et al.*, 2004). The phenomenon where an IgE

antibody raised against one allergen, binds or recognizes a similar protein from another source, it is called allergen cross-reactivity (Ferreira, F., *et al.*, 2004). Its molecular basis is the presence of homologous allergens in different species that are recognized by the same type of specific IgE. The IgE cross-reactions are due to shared features at the level of primary and tertiary structures of proteins (Aalberse, R.C., *et al.*, 2001).

## **1.5. Seed storage proteins**

### **1.5.1. 2S albumins**

Albumins are a group of seed storage proteins soluble in water at low concentrations and with a sedimentation coefficient ( $S_{20.w}$ ) of 2. They are widely distributed in dicot seeds, including cultivated species such as brassicas, legumes, sunflower, cotton, and castor bean (Shewry, P. R., *et al.*, 2004).

The 2S albumins are characterized for being small globular proteins rich in sulfur amino acids as cysteine and methionine (Shewry, P. R. *et al.*, 1995). They are also rich in other amino acids, such as glutamine and arginine which contain 2 and 3 nitrogen atoms, respectively, which have a main role on the storage of nitrogen. Besides the storage of nitrogen, another biological role is the storage of sulfur and carbon germination and seedling growth (Férrandez-Rivas, M. *et al.*, 2004).

All 2S albumins contain 8 conserved cysteine residues and 4 disulfide bonds, having, this way, higher proportions of cysteine than other proteins. Although they vary considerably in their amino acid sequence, they are typically synthesized as a pre-protein which is processed to give small and large subunits, with 4-5kDa and 9-11kDa, respectively, with 4 disulfide bonds, two between the subunits and two within the large subunit (Monsalve, R.I., *et al.*, 2004). This

disulfide bond arrangement confers an unusually high stability, both to thermal denaturation and to digestion by proteolytic enzymes, which allows the allergens to reach the gastrointestinal tract, almost intact, and also favors increased cellular uptake, reduced neutralization by secretory antibodies and decreased degradation in the blood stream (Férrnandez-Rivas, M., *et al.*, 2007).

### **1.5.2. Globulins**

The globulins belong to the cupin superfamily and are present in monocotyledonous and dicotyledonous plants (Shewry, P. R. *et al.*, 2004). Their basic structure is composed by a double stranded  $\beta$  helix domain, which led to the name “cupin” based on the Latin for a small barrel or cask (Mills, E.N.C., *et al.*, 2003). This family is divided in two different groups of proteins, with different sedimentation coefficients, the 7S vicilins, with  $S_{20.w}$  7/8 and the 11S proteins with  $S_{20.w}$  11/12 (Shewry, P.R., *et al.*, 2004). Globulins are also deficient in cysteine and methionine, although 11S usually contain slightly higher levels of these amino acids.

The 11S globulins, also called legumins, are the most widely group of seed storage proteins occurring in most dicotyledonous species, in cereals oats and rice (Shewry, P.R., *et al.*, 1995). Legumins are hexameric proteins with a molecular mass of 300-400 kDa, comprising 6 subunits of 60 kDa. These subunits are processed in a large (acidic) and small (basic) chains with approximately 40 kDa and 20 kDa, respectively, remaining associated by a single disulfide bond (Férrnandez-Rivas, M., *et al.*, 2007).

The 7S are often called vicilins, because of their presence in the *Viciae* group of legumes. They are trimeric proteins with a molecular mass of 150 to 200 kDa. The post translational proteolysis and glycosylation give origin to subunits with 40 to 80 kDa (Breiteneder, H. and Radauer, C., 2004). This group

of proteins lack cysteine residues, hence they cannot form disulfide bonds (Shewry, P.R., *et al.*, 1995).

## **1.6. Pathogenesis related proteins**

### **1.6.1. Lipid Transfer Proteins**

Lipid transfer proteins (LTPs) form a broad family of proteins very widespread in nature. They are constituted by 7 to 9 kDa monomeric proteins that are held together by 4 disulfide bonds to form a hydrophobic tunnel (Breiteneder, H. and Radauer, C., 2004; Salcedo, G., *et al.*, 2007). The disulfide bonds are responsible for their high stability, thus being resistant to proteolysis, harsh pH changes, and thermal treatments and can refold to their native structure on cooling (Marion, D., *et al.*, 2004).

The three-dimensional structure of the members of this family is highly conserved, with four alpha-helices separated by short turns and an unstructured C-terminal coil (Douliez, J.P., *et al.*, 2000; Salcedo, G., *et al.*, 2007).

LTPs have been isolated from fruits leaves, roots and pollen and were identified as major allergen on peach, apple and apricot in Mediterranean populations (Breiteneder, H., and Radauer, C., 2004; Salcedo, G., *et al.*, 2007; Diaz Perales, A., *et al.*, 2000). Usually they accumulate in the outer epidermal layers of plant organs thus explaining the stronger allergenicity of peels compared with pulps of several species, such as peach (Fernández-Rivas, M., and Cuevas, M., 1999).

Initially, investigators thought that the main function of this family was associated with their *in vitro* properties of facilitating inter-membrane nonspecific transfer of lipids, thus the name Lipid Transfer Proteins, but further studies have highlighted their importance *in vivo* action in the defense of plants from different kinds of pathogens and environmental stress (Molina A., *et al.*,

1993; Pastorello, E.A., *et al.*, 2001; Salcedo, G., *et al.*, 2007). Due to this new function, they were classified into the pathogenesis related proteins (PR), with the name of PR-14 (Breiteneder H., and Ebner, C., 2000; Salcedo, G., *et al.*, 2007).

### **1.6.2. Thaumatin-like Proteins**

Thaumatin-like proteins (TLPs) are universal in plants and because their expression is induced by environmental stresses as pathogen invasion drought, wounding and cold hardiness, plant TLPs were assigned to form family 5 of the Pathogen Related proteins (PR5) (Liu, J., *et al.*, 2010).

TLPs are polypeptides of about 200 amino acid residues that share sequences similarity with thaumatin (Breiteneder, H., 2004).

This family of proteins is also called as antifungal proteins, and has a molecular mass ranging from 21 to 26 kDa (Hoffmann-Sommergruber, K., and Mills, E.N.C., 2009). They are formed by 16 conserved cysteins, which form 8 disulfide bridges. These disulfide bridges help to stabilize the molecule and allow the correct folding and high stability under extreme thermal and pH conditions as well as the resistance to protease degradation (Férrandez-Rivas, M., *et al.*, 2007).

### **1.7. Tree nut allergy**

Tree nuts are fruits that have a dry and hard seed-vessel surrounding their seeds. They belong to different botanical families but they are grouped together for their functional analogy. They have a high nutritional value and thus are very important in the human diet. Tree nuts are directly consumed or as part of the bakery and pastry products, ice creams, sauces, etc. The intake of tree nut has increased in the last years especially because consumption of this

fruits has been proven to be a healthy dietary habit (Férrnandez-Rivas, M., *et al.*, 2007). Several studies show that nuts have a beneficial effect on the outcome of coronary disease and cholesterol serum levels (Kris-Etherton, P.M., *et al.*, 2007). This group mainly includes almonds, hazelnuts, cashew nuts, walnuts, chestnuts, pine nuts, pistachios nuts, sun seeds and peanuts.

In the developed world, the allergic reactions caused by tree nuts represent one of the first cause of food allergies in children and the first in adults. The frequencies in which individuals get sensitized rely on age, atopic stage of the patient and on the consumption of these foods in different countries. The presence of pollinosis in the region is another factor that affects the sensitization to these foods (Burks, W., and Ballmer-Weber, B., 2006).

The sensitization to just one tree nut is more common in children, meanwhile adult patients present sensitization to multiple tree nuts.

### **1.7.1. Hazelnut**

Hazelnut (*Corylus avellana*) is a very common allergenic food, often involved in severe allergic reactions, as reported in several studies. This type of tree nut is widely used in pre-packaged foods, especially in the pastry industry and in ice-creams. The vast and varied use of the hazelnut in the food industry represents a significant risk to the individuals that are allergic to this type of tree nut, since the labels do not identify the ingredients in small quantities, or food that can be present through contamination (Ortolani, C., *et al.*, 2000)

The type of allergic reaction and the specific allergens recognized can vary according to the geographic region. The main allergen in hazelnut, was identified as a pathogen protein, with a molecular mass of 17 kDa (Cor a 1), which is homologous to the main allergen of birch tree (Bet v 1), causing the allergy to hazelnut to be particularly prevalent in individuals with respiratory diseases, to birch, pollen, hazelnut and alder (Roux, K.H., *et al.*, 2003).

This tree nut consists of 5 different proteins, in which the major one, Cor a 1, is divided in four isoforms, Cor a 1.01-0.4. Besides Cor a 1, hazelnut is formed by other allergens as a Profilin, Cor a 2 (14kDa), a Lipid Transfer Protein, Cor a 8 (9 kDa) (Shocker, F., *et al.*, 2003), a legumin like protein (11S), Cor a 9 (40 kDa) (Beyer, K., *et al.*, 2002), a 7S Vicilin-like protein, Cor a 11 (48 kDa) (Lauer, I., *et al.*, 2004) and finally, by a thaumatin like protein (25 kDa).

### **1.7.2. Walnut**

The English walnut (*Juglans Regia*) is an important tree nut associated with allergic reactions to food. In the US, more patients are allergic to walnut than any other tree nut (Wallowitz, M., *et al.*, 2006).

The first protein described was a 2S albumin, Jug r 1, with a molecular mass of 15 to 16 kDa (Sordet, C., *et al.*, 2009). After that, a second protein was discovered, and this time it was a 7S globulin, named Jug r 2 (44 kDa) (Teuber, S.S., *et al.*, 1998).

Besides Jug r 1 and Jug r 2, walnut is also formed by an LTP, with 9 kDa, called Jug r 3 and a 11S-legumin like protein, Jug r 4 (Roux, K.H., 2003).

### **1.7.3. Chestnut**

Fresh and boiled chestnuts are commonly ingested around the world and its allergy is widely reported in the latex-fruit syndrome (Lee, S., *et al.*, 2005). Few studies address allergy to chestnuts in patients reacting primarily to this food.

Chestnut reactivity is frequently associated to clinical allergy not only to fruits but also to other tree nuts (Rico, P., *et al.*, 2004).

There have been few studies to identify the IgE binding components within chestnut allergen extracts, and until now only Cas s 5, a chitinase, Cas s 8, an LTP with 9 kDa and a TLP with 24 kDa were isolated (Roux, K.H., *et al.*, 2003).

#### **1.7.4. Peanut**

Allergy to peanut (*Arachis hypogea*) is common and can be a potentially severe form of food allergy. During the last decade there has been an increased concern over the avoidance of mortality associated with peanut allergy (Grundy, J., *et al.*, 2002). The prevalence of self-reported peanut allergy is estimate to be around 0.5 % to 1 % of the unselected population in both adults and children (Sicherer, S.H., 1999).

Consumption of peanut in western countries may also be rising because of its use as a source of protein in health food, the popularity of vegetarianism and increased use of prepared foods (Fleisher, D.M., *et al.*, 2003).

The major allergens from peanut are: Ara h 1, a 7S Vicilin, Ara h 2, a 2S albumin, Ara h 3, a 11S legumin, Ara h 6, 59 % homologous to Ara h 2 but 2-4 kDa smaller and Ara h 9 a lipid transfer protein (Zhuang, Y., and Dreskin, S.C., 2012).

#### **1.7.5. Pine nut**

Pine nuts, seeds from the tree *Pinus pinea*, are frequently used as seasoning in the cuisine of the Mediterranean area of Spain (Marinas, D., *et al.*, 1998). There have been several reports of allergic and anaphylactic responses to pine nut but little is known about its allergenic peptides (Roux, K., *et al.*, 2003).

### **1.7.6. Sunflower seed**

Sunflower seed (*Helianthus annuus*) is extensively used in oil form, in margarine, as a condiment and in bread. However, few clinical reports have indicated that this nut causes anaphylactic reactions in sunflower seed-allergic subjects. The main allergens in this type of nut are: Hel a 1 (11S legumin) (Jimenez, A., *et al.*, 1994), Hel a 2S albumin (Kelly, J.D., *et al.*, 2000), and Hel a Oleosins.

### **1.7.7. Cashew nut**

Cashew nuts (*Anacardium occidentale*) are globally popular and are valued for their sensory qualities, especially the unique flavor and texture (Teuber, S., *et al.*, 2002). This type of nut is associated with IgE mediated anaphylaxis and cashew nut allergy is the second most commonly reported tree nut allergy in the United States (Robotham, J., *et al.*, 2005). Allergy to cashew nuts is becoming more recognized in clinical practice, but there is few information on the clinical characteristics with which informs practice (Clark, A.T., *et al.*, 2007). The major allergens of cashew nut are: Ana o 1, a 7S Vicilin, Ana o 2, a 11S Legumin and Ana o 3, a 2S Albumin.

## **1.8. Diagnosing allergy**

Identification of the culprit allergens and potentially cross-reactive structures is of supreme importance in order to practice appropriate allergen avoidance (Ebo, D.G., *et al.*, 2004) or elimination from the diet followed by a positive reaction to an oral challenge with the food in study. There is no test able to identify all patients with food allergies due to the limited diagnostic value of many tests used (Cocco, R., and Solé, D., 2009).

Diagnostic tests used can be *in vivo* (clinical methods), such as skin prick test, or *in vitro* (laboratorial methods), including immunoassays (Dreborg, S., 2001).

In the case of food allergies, they are suspected more often than found by specific diagnostic methods and only less than 20% are confirmed by oral challenges. Because IgE mediated reactions occur so soon after ingestion, the patient's history is vital for the correct diagnosis (Cianferoni, A., and Spergel, J.M., 2009).

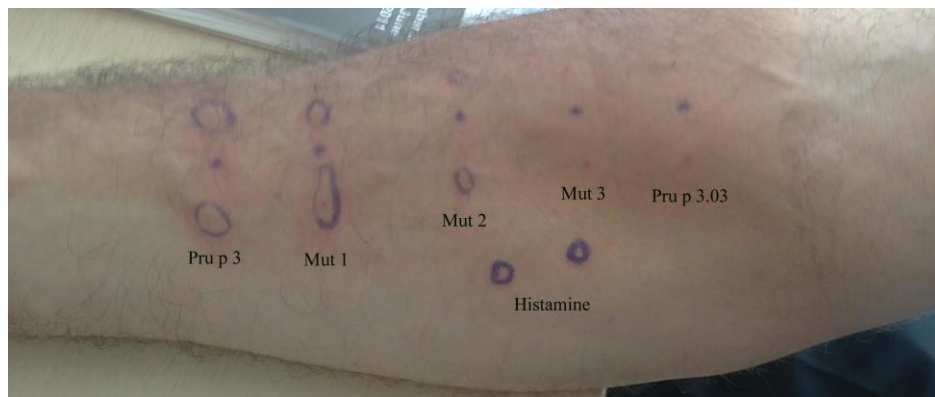
### **1.8.1. Skin prick test (SPT)**

Skin prick test (SPT) is an important diagnostic method of atopy and it comprises the skin reactivity related to the quality, potency and standardization of allergen extracts. This method is frequently used for food-specific IgE and can be easily performed, it is cheap and safe and the results are available in 15 min. The diagnostic accuracy of SPTs depends on the quality of the food allergen extracts used. For many foods, in specially the plant-derived ones, commercial extracts of SPTs show a low sensitivity leading to a high rate of false-negative results. This happens due to the low abundance or the lack of stability of several allergens to endogenous enzymatic process taking place in plant food extracts. When this occurs, skin testing with native foods shows a clearly superior performance (Asero, R., *et al.*, 2007)

This test consists in gently “pricking” a few drops of the purified allergen on the skin surface (usually on the forearm), as well as positive and negative controls (histamine and glycerin, respectively). A positive SPT is characterized by a wheal-and-flare reaction in the injection site, around 15 minutes after the contact with the allergen. This reaction occurs due to the degranulation of mast cells when bound to the allergen-specific IgE. The larger the size of the wheal, the more likely a patient will react to the food but it does not predict the

severity of the reaction. These immediate hypersensitivity skin tests have high negative predictive values (around 95 %) but their positive predictive accuracies are only about 50% (Cianferoni, A., and Spergel, J.M., 2009).

Factors as age, previous exposures and type of food might change the predictive value of the wheal size. As so, negative values are very helpful to exclude food allergies.



**Figure 4:** Skin prick tests to common allergens in the forearm. Compared with histamine, the positive control, indicating the presence of allergen-specific IgE to different allergens (Tordesillas, L., 2012).

### **1.8.2. Double blind placebo controlled food challenge**

Oral food challenges are essential to establish the identity of specific food triggers and are a diagnostic test which provides strong evidence of a food allergy, and allows the clinician to recommend a correct elimination diet (Cianferoni, A., and Spergel, J.M., 2009).

Double-blind placebo-controlled food challenge remains the gold standard for the diagnosis of food allergy, although it is time consuming, costly and might be life-threatening (Cocco, R., and Solé, D., 2009).

The food is given to the patient in fasting conditions, starting with a low dose unlikely to cause symptoms. Incremental amounts of food are given at time intervals, between 15 and 60 minutes, until a positive reaction appears or the patients eat an amount of the food corresponding to a normal serving, and

the severity of the symptoms is compared to a placebo group in order to confirm diagnosis (Asero, R., *et al.*, 2007).

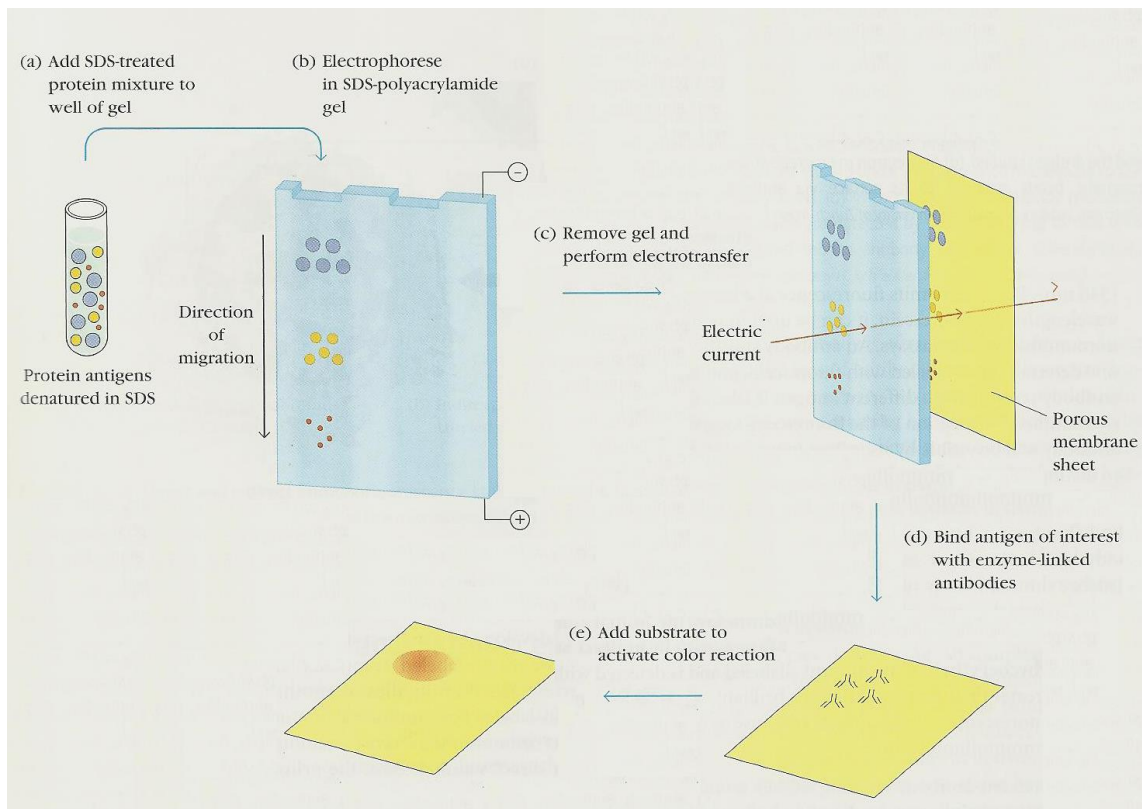
### **1.8.3. Immunodetection: ELISA and Western-Blot**

During the last two decades there has been a great increase in the number and variety of immunodiagnostic tests performed. The cause of this increment has been due to the development of methods which use labeled antigens or antibodies, resulting in tests with very high levels of sensitivity and specificity (Voller, A., *et al.*, 1978).

Enzyme-linked immunosorbent assay (ELISA) is a highly sensitive immunochemical method for detection or quantification of an antibody or antigen by giving a colored soluble reaction product. The reaction occurs due to the use of a ligand (such as an anti-immunoglobulin) conjugated to an enzyme, usually peroxidase or alkaline phosphatase, that leads to the change of the color of the substrate. By keeping the known antigen constant and diluting the serum to be tested, it can be produced a curve of decreasing optical density (OD) readings which will indicate the amount of antibody in a given serum when compared to a standard control (Zabriskie, J.B., 2009). ELISA is a fairly simple assay and is easily completed within a day, allowing the analysis of a large number of samples in a relatively short time. The data are objective as the OD of each well is automatically read and expressed as a numerical value from a continuous scale. Similar to immunoblotting and immunoprecipitation, ELISA data provides information on antigen identity. ELISA assays can be divided in direct and indirect ELISA, depending on the amount of given antigen or antibody we want to measure (Roitt, I.M., and Delves, P.J., 2001; Voller, A., *et al.* 1978).

Immunoblotting, also called Western blot, is used to identify target antigens (Figure 5) (Zabriskie, J.B., 2009). This method is explained in the

chapter 3.2.2.1. Nevertheless, is time-consuming and thus not suitable for routine screening of a large number of serum samples (Rusznack, C., and Davies, R.J., 1998). However, it allows to compare different proteins, toxins and cellular products at the same time and being therefore considered a simple procedure by immunologists.



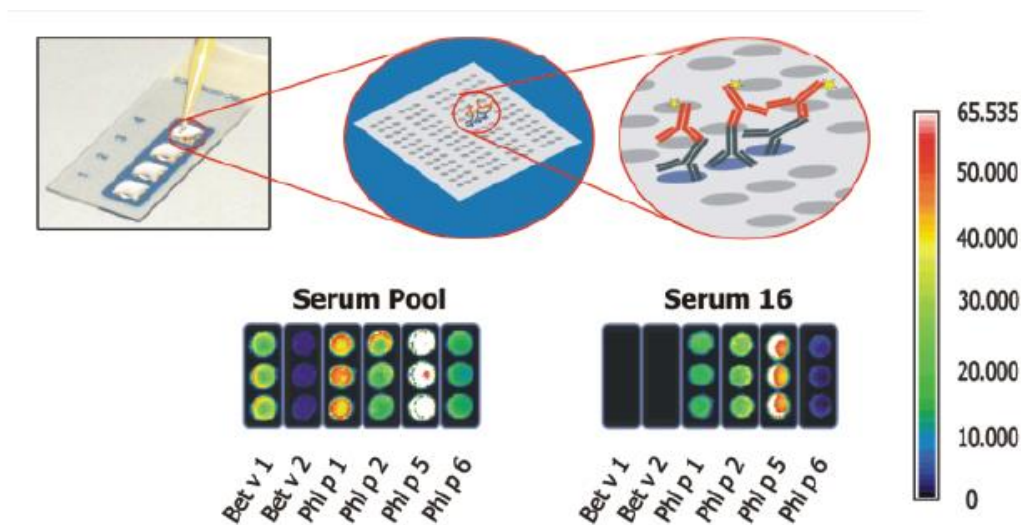
**Figure 5:** Schematic illustration of an immunoblotting (Western-blot) (Adapted from: Goldsby, R.A., *et al.*, 2003).

#### 1.8.4. Protein Microarrays

Protein microarrays, also called protein chips, are a powerful method to detect large numbers of protein interactions in parallel, such as protein-antibody, protein-protein, enzyme-substrate, etc., with high efficiency and sensitivity (Hall, D.A., *et al.*, 2007).

They consist in a solid phase ligand binding assays using proteins immobilized on support surfaces and are able to detect proteins, monitor their expression levels, interactions and functions, making possible a complete analysis by automated means. Besides these advantages, this method requires low amount of sample and gives origin to an abundance of data in a single experiment (Taussing, M., and Stoevesandt, O., 2009).

In allergy, the application of protein microarrays relates to improved diagnosis of IgE profiling, replacement of uncomfortable procedures, multiplexed screening of minimal samples, discovery of novel allergens and development of antigen-specific therapies. As in Western-blot assays, we can detect antigens or antibodies in blood samples which for sure accelerate immunodiagnosics significantly (Hall, D.A., *et al.*, 2007; Templin M.F., *et al.*, 2002). This method is described in more detail in the chapter 3.2.5.



**Figure 6:** Schematic description of protein microarray testing of allergen-specific IgE. Allergens are applied onto slides in triplicate. Serum incubation is followed by a second anti-human IgE antibody for detection. The mean fluorescence intensity is determined by two different laser settings (Jahn-Schimid, B., *et al.*, 2003).

## 2. AIMS

The main objective of this work was to purify and characterize biochemically and immunologically, proteins belonging to the principal allergen families from hazelnut, walnut, chestnut, cashew nut, peanut, pine nut and sunflower seeds.

Specific aims:

- a) The analysis of patterns of IgE binding of sera against extracts of hazelnut, walnut, chestnut, cashew nut, peanut, pine nut and sunflower seeds by immunodetection.
- b) The study of the prevalence of the most relevant purified allergens: hazelnut, walnut, chestnut, peanut and sunflower seed allergens using microarray.

### **3. MATERIAL AND METHODS**

#### **3.1. Patients' Sera**

Patients' sera with a convincing clinical history of allergic reactions to the ingestion of tree nuts, confirmed with double-blind placebo-controlled food challenge (DBPCFC) and with positive skin prick test (SPT), were selected from the allergy services from Basurto Hospital (Bilbao; n=78), Jimenéz Diaz Foundation (FJD) (Madrid; n=23), and La Princesa University Hospital (Madrid; n=30).

#### **3.2. Methods**

##### **3.2.1. Protein extraction and protein quantification**

###### **3.2.1.1. Protein extraction**

Raw nuts (hazelnut, walnut and chestnut) were purchased from a local market. One kilogram of each nut was peeled and chopped with a blender and the flour was dissolved in acetone [1:5 (p/v)], for 1 hour, at 4°C. The acetone supernatant was eliminated with vacuum filtration and the dried ground was suspended in PBS buffer [1:5 (p/v), sodium phosphate 10 mM; pH 7.4; NaCl 1.5 M] for 1 hour, at 4°C in agitation. After that it was centrifuged at 8000 rpm, for 30min at room temperature. The supernatant was dialyzed against distilled water, with a cut-off of 6000-8000 Da membrane for 48 h in agitation, at 4°C.

After dialysis, all extracts were frozen in liquid nitrogen and lyophilized, followed by quantification with the Bradford method (Bradford, M.M., 1976).

### **3.2.1.2. Electrophoretic methods**

Samples were separated by SDS-PAGE on Bio-Rad Miniprotean III System gels (15% polyacrylamide) (Bio-Rad Laboratories, Hercules, CA, USA), following the method of Laemmli (Laemmli, U.K., 1970).

For the separation gel, was used a polyacrylamide solution at 15 % (p/v) in Tris-HCl 0,125 M, pH 8.8, SDS 0,1 % (p/v); and for the stacking gel was used a polyacrylamide solution at 4 % (p/v) in Tris.HCl 0.125 M, pH 6.8, SDS 0.1 % (p/v). The samples were dissolved in Tris-HCl 0.0625M, pH 6.8, SDS 2 % (p/v), urea 8 M; bromophenol blue 0.00 1%, and  $\beta$ -mercaptoethanol 10 %.

The electrophoresis was carried at 20 mA/gel, at a maximum of 200 mV, using as an electrophoresis buffer Tris 0.25M, glycine 1.29M, SDS 1 % (p/v), pH 8.3.

Protein staining was performed with Coomassie Brilliant Blue R-250 [Coomassie R-250 0.25 % in methanol: acetic (50:9) (v/v)].

### **3.2.2. Immunochemical methods**

#### **3.2.2.1. Western-Blot with polyclonal antibodies**

SDS-PAGE-replicas were electrotransferred into polyvinylidene difluoride (PVDF) membranes.

Membranes were previously activated with methanol and washed with distilled water, and then equilibrated in a transfer buffer (Tris 50 mM, Boric 50 mM, pH 8.3). The electroblotting was carried out at 70 V for 1 hour, in ice.

For the immunodetection, membranes were blocked with 5 % milk in PBS for 1 hour in agitation or overnight at 4.°C. After blocking, membranes were incubated with the primary antibody – anti –Hela 1 (11S globulin, dilution 1:10 000), anti- Pru p 3 (LTP, dilution 1:1000), and anti-Pru p 2.0201 (TLP, dilution

1:5000) in PBS-blocking Buffer (10:1 v/v), 1 hour at room temperature and agitation.

After washing 3x 10 minutes in PBS, Tween 0.05%, membranes were incubated with secondary antibodies anti-IgG- alkaline Phosphatase-conjugated in defatted milk-PBS (dilution 1:5000), for 1 hour in agitation. After washing again 3x 10min, BICP/NBT liquid substrate (SIGMA) was added in dark until reactive bands appeared.

### **3.2.2.2. Western-Blot with patients' sera**

For the immunodetection with patient sera, the same protocol as 3.2.2.1 was used with the following modifications.

After blocking, the membranes were incubated with a serum pool diluted 1:3 (in blocking solution 1:10 PBS), overnight at 4°C, in agitation and covered. After washing, anti-human IgE rabbit monoclonal antibody HRP-conjugated (Invitrogen Corporation, De Schelp, the Neederlands) (dilution 1:1000 in PBS-Blocking) was added for 1 hour in agitation, covered at room temperature. Membranes were washed as before, and chemiluminescent substrate (SuperSigma West Pico Chemiluminescent Substrate, Thermo Scientific, Rockford, USA) was added for 5 minutes and chemiluminescence measured in a dark room. The membranes were placed in a support with a chemiluminescence high performance film and left exposing.

### **3.2.3. Isolation of allergens**

#### **3.2.3.1. 2S Albumins isolation**

After lyophilization, the extract was subjected to a molecular exclusion chromatography on HiLood 16/60 Superdex 75 column. Fractions were separated with PBS buffer 35 mM, pH 7.5, NaCl 1 M (0,8mL/min).

#### **3.2.3.2. 11S isolation**

After lyophilization, hazelnut extract was fractioned by an anion-exchange chromatography with a 20 mM ethanolamine, pH 9.0, on a Vac 6cc cartridge (Waters, Ireland). Elution was carried out 1 M NaCl in the same buffer (1 mL/min). The retained fraction of the extract was repurified by FPLC on a mono-Q1 (Biorad) column in the same buffer.

#### **3.2.3.3. LTP and TLP isolation**

Hazelnut, walnut and chestnut extracts, were fractioned by a cation-exchange chromatography with 10 mM formic acid, pH 4.0 on a VacRC (Waters, Ireland) cartridge. The retained material was then eluted with 0.75mM NaCl in the same buffer (1 mL/min). The retained fractions were purified by a RP-HPLC on a Nucleosil 300-C4 column (7x250 mm; particle size 5 µm; Tecknokroma, Barcelona, Spain). Elution was performed with a linear gradient of acetonitrile in 0.1 % trifluoroacetic acid (15 % for 10 min and 15-85 % over 150 min; 0.5mL/min).

### **3.2.4. Quality of purified proteins**

Purified proteins were quantified by the method of bicinchoninic acid test (BCA) (Smith *et al*, 1985) and purity was measured by SDS-PAGE following by confirmation with Western-blot, mass spectrophotometric analysis with a AXIMA CFR MALDI-TOF, and fingerprinting after tryptic digestion, using standard methods.

### **3.2.5. Protein microarrays**

#### **3.2.5.1. Printing**

Purified proteins were printed (0.25 mg/mL and 0.125 mg/mL in 1X Protein Binding Buffer (Whatman, USA) containing 0.02% Tween 20) on epoxy-activated glass slides (TeleChem International, Sunnyvale, CA, USA) with 16 microarrays per slide, using a MicroGrid II TAS microarrayer (BioRobotics, Genomic Solutions, USA). Each condition was printed triplicate by the arrayer.

#### **3.2.5.2. Hybridization**

Each microarray well was blocked with 150  $\mu$ L of commercial Blocking Solution 1X (Sigma) for 1 hour in agitation, in darkness. After blocking, 80 $\mu$ L of serum was added to the slides, and left incubated overnight, at 4  $^{\circ}$ C and covered. After, the slides were washed with 100  $\mu$ L of washing solution (PBS 1X + Tween20 at 0,1%), 3 times, for 5 minutes. Secondary antibody, Anti-IgE\*PE647, was added in a dilution 1:100 in PBS, 70  $\mu$ L to each well, and incubated 1 hour at room temperature, in agitation, and covered. As a blank control, one microarray well per slide was always incubated solely with PBS (Sigma, St. Louis, CO, USA) instead of serum and, after washing incubated with

the fluorescence secondary antibody. Finally, the slide was washed again 3 times for 5 minutes, centrifuged to eliminate the remaining washing solution and scanned at 650 nm.

### **3.2.5.3. Array reading**

After centrifuged, the slide was put in the scanner with the printing downwards and fitted with the flange. To scan the slide, was used the program GenePix™ (Genomics Solutions, US), at a wavelength of 650 nm. After that, the scanning area was selected and the chip was scanned 2 times to avoid the files from being too big. Spots with obvious defects and those replicate spots having a signal-to-noise ratio less than 3, as measured by GenePix™ software, were removed from the analysis. Only those allergen spots in which at least two of the three replicates fulfilled the analytical criteria were considered for quantification. The IgE binding of each allergen spot was calculated as the final fluorescence intensity, obtained by subtracting the local background  $B$  from the observed value, measured by GenePix™ software and then the fluorescence intensity from the blank control by applying the following equation:  $I = (F_{650} - B)_{\text{sample}} - (F_{650} - B)_{\text{blank}}$ . Fluorescence intensity levels higher than 200 units were considered to be positive.

### **3.2.6. Statistical analysis**

Fluorescence levels from each patient's serum were analyzed by contingency tests. Differences in the quantitative variables were analyzed by the non-parametric Kruskal-Wallis test. Differences among regions were analyzed by the Dunn's multiple comparisons test, followed by a two-way ANOVA test,

with Turkey's multiple comparisons test, to analyze the allergens that cause the differences. Values of  $p < 0.05$  were considered significant for all tests.

## 4. RESULTS

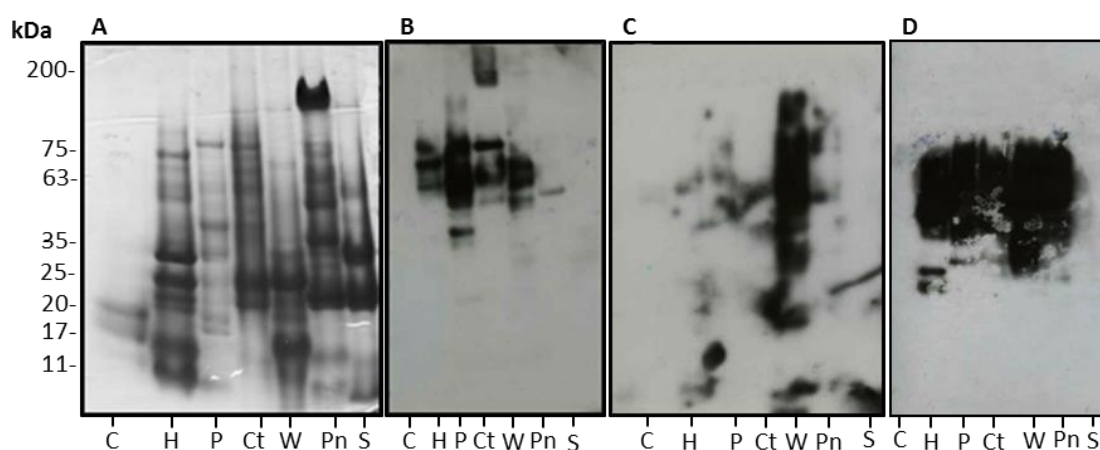
### 4.1. Study of the cross-reactivity among tree nut species

We selected the most relevant allergenic nuts such as cashew nut, hazelnut, peanut, chestnut, walnut, pine nut and sunflower seeds to study their implication in cross-reactivity.

For this, sera from patients with clear clinical history to one of these nuts were obtained from the Allergy Services of La Princesa University Hospital (Madrid), of Jimenez Diaz Foundation (FJD) (Madrid) and Basurto Hospital (Bilbao), from Spain.

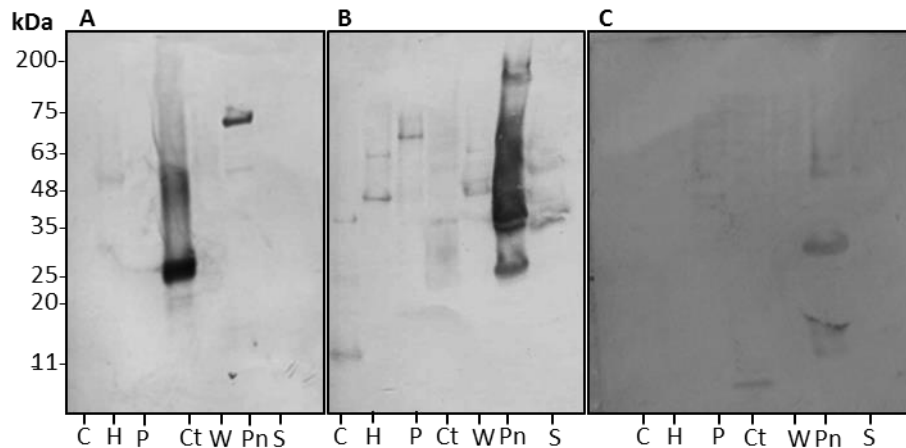
Protein PBS extracts of the selected nuts were fractionated by SDS-PAGE and stained with Coomassie Blue (figure 7 A).

Replicas were electro-transferred and incubated with a serum pool of patients from each hospital (figure 7, B, C and D). We observed different IgE binding profiles in each case, and different IgE binding bands in each nut. Thus, not all extracts were recognized by IgE, therefore showing that not all extracts are implied in cross-reactivity.



**Figure 7:** SDS-PAGE from PBS extracts of cashewnut (C) (10  $\mu$ g), hazelnut (H) (5  $\mu$ g), peanut (P) (10  $\mu$ g), chestnut (Ct) (10  $\mu$ g), walnut (W) (15  $\mu$ g), pine nut (Pn) (10  $\mu$ g) and sun seeds (S) (10  $\mu$ g): **A**, staining with Coomassie Blue; **B**, Immunodetection with pool serum from Jimenez Diaz Foundation (FJD); **C**, Immunodetection with pool serum from La Princesa Hospital University Hospital; **D**, Immunodetection with pool serum from Basurto Hospital (Bilbao).

In order to identify the nature of these IgE binding allergens recognized by the different pools, we incubated SDS-PAGE replicas with specific polyclonal IgG antibodies produced against peach TLP (Pru p 2.0201), peach LTP (Pru p 3) and sunflower seed 11S legumin (Hel a 1) (figure 8).



**Figure 8:** SDS-PAGE from PBS extracts of cashew nut (C) (10  $\mu$ g), hazelnut (H) (5  $\mu$ g), peanut (P) (10  $\mu$ g), chestnut (Ct) (10  $\mu$ g) walnut (W) (15  $\mu$ g), pine nut (Pn) (10  $\mu$ g), and sun seeds (S) (10  $\mu$ g); **A**, Immunodetection against Pru p 2.0201 (TLP) (1:5000); **B**, Immunodetection against Hel a 1 (11S legumin) (1:10000); **C**, Immunodetection against Pru p 3 (LTP) (1:1000)

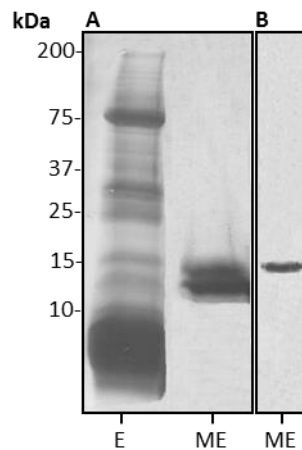
A clear self-recognition was observed when the membrane was incubated with anti-peach TLP antibody, although a reactive band was shown by pine nut extract and a weaker one in the hazelnut. In the case of anti-Hel a 1, we can observe reactive bands in the majority of the nuts such as cashew nut, hazelnut, peanut, chestnut, walnut, pine nut and sunflower seeds. This means there is a high concentration of members of this protein family in the nuts.

By contrast, with the antibodies produced against LTP, only in chestnut extract we could see a 9 kDa band. In the pine nut extract we could also observe higher reactive bands.

## 4.2. Characterization of allergens in tree nuts and their prevalence in an adult population

### 4.2.1. Purification and characterization of 2S albumins from walnut

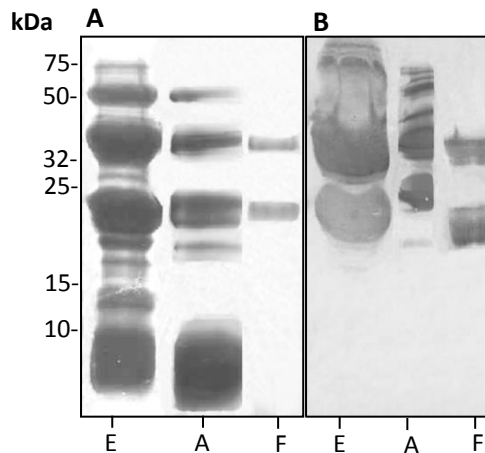
In order to purify a 2S albumin from walnut, PBS extract was fractionated, by electrophoresis in presence of SDS (SDS-PAGE) (figure 9, A). After, the extract was subjected to a molecular exclusion chromatography and the retained fraction showed a band of about 15 kDa (figure 9, A and B).



**Figure 9:** SDS-PAGE from PBS extracts (10  $\mu$ g) of walnut; **A**, staining with Coomassie Blue of the extract (E) and of the retained fraction of the molecular exclusion chromatography (ME); **B**, Immunodetection with polyclonal antibodies against 2S.

### 4.2.2. Purification and characterization of 11S from hazelnut

One of the major allergens in hazelnut belongs to the 11S seed storage protein family, called Cor a 9. The 11S seed storage protein was fractionated and isolated from mature hazelnut extract, based on their solubility in water and salt solution, by electrophoresis in presence of SDS (SDS-PAGE), as shown in figure 10.



**Figure 10:** SDS-PAGE from PBS extracts (10 µg) (E) of hazelnut, the retained fraction obtained by anion-exchange chromatography in Waters cartridge (5 µg) (A) and in FPLC (5 µg) (F) **A**, staining with Coomassie Blue; **B**, Immunodetection with polyclonal antibodies against Hela 1 (11S legumin) (1:10000)

After extraction, unlike the other purified proteins mentioned above, an anion-exchange chromatography was performed in order to purify the 11S from hazelnut (figure10).

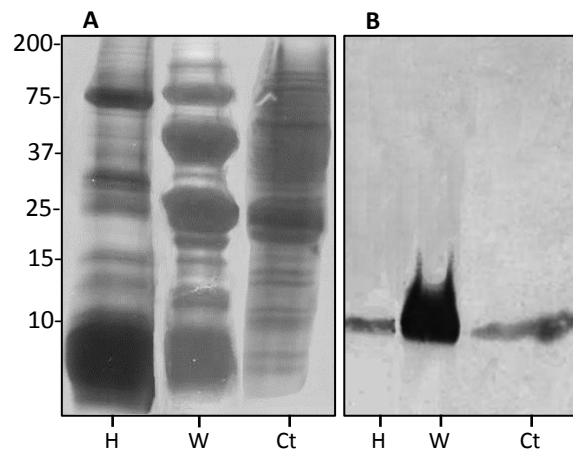
The retained fraction showed the presence of 11S, both in Blue Coomassie and in the immunodetection with polyclonal antibodies against 11S. This method was followed by FPLC with a mono Q column, which revealed two bands corresponding to the acidic and basic fraction of the 11S, as known as Cor a 9.

In order to confirm the purity of Cor a 9 a fingerprint was performed. The results can be found in this website:

[http://www.matrixscience.com/cgi/protein\\_view.pl?file=../data/20130308/FtEmfzStT.dat&hit=13](http://www.matrixscience.com/cgi/protein_view.pl?file=../data/20130308/FtEmfzStT.dat&hit=13)

#### 4.2.3. Purification and characterization of LTP from hazelnut, walnut and chestnut

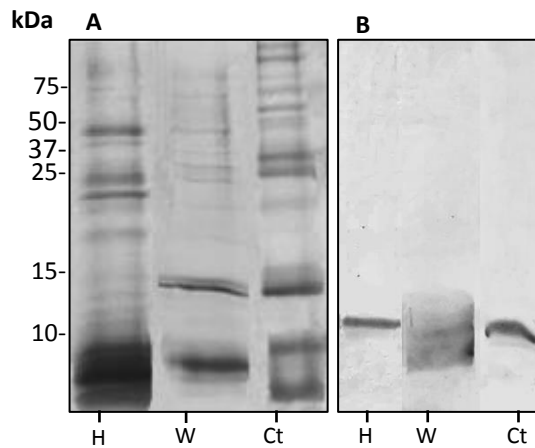
Raw extracts from hazelnut, walnut and chestnut, were fractionated by electrophoresis in presence of SDS (SDS-PAGE), showing, in all of them, a complex pattern of bands between 10 and 200 kDa (figure 11 A).



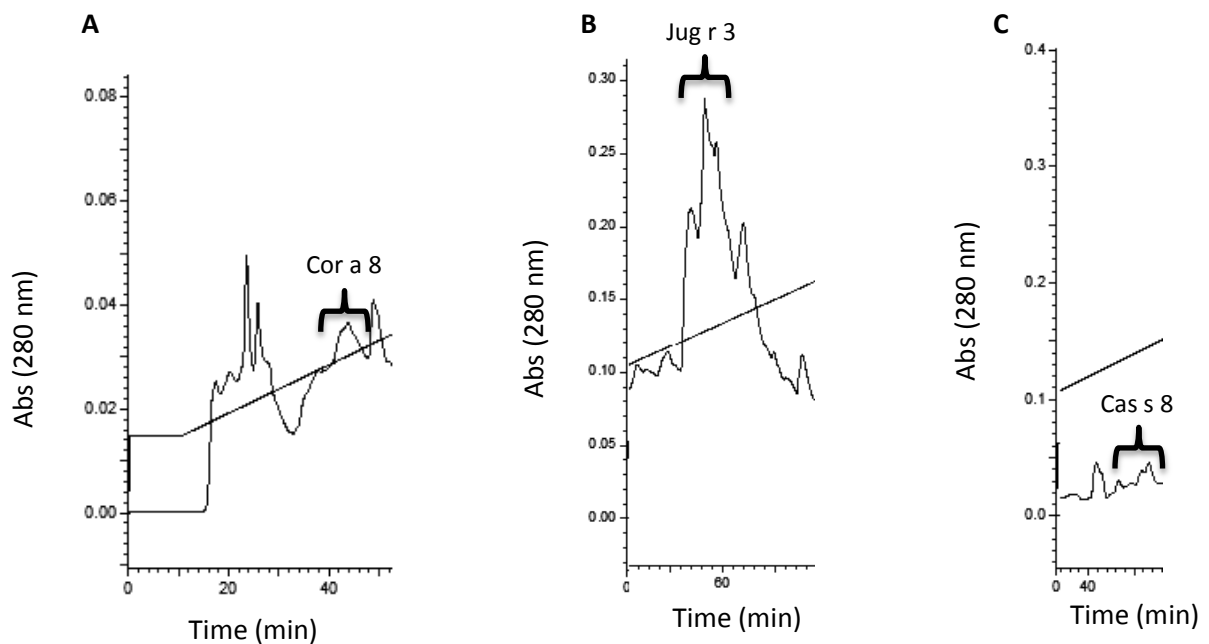
**Figure 11:** SDS-PAGE from PBS extracts (10  $\mu$ g) of hazelnut (H), walnut (W) and chestnut (Ct): **A**, staining with Coomassie Blue; **B**, Immunodetection with polyclonal antibodies against Pru p 3 (LTP) (1:1000).

Among the majority of bands, components with molecular mass ranging between 9 and 10 kDa, along with larger ones, were recognized by polyclonal antibodies, against Pru p 3 (figure 11 B).

In order to purify LTPs recognized by Western-blot, extracts were, fractionated by cation-exchange chromatography (figure 12). The retained fractions with 9 kDa bands were repurified by reversed-phase HPLC (figure 13).

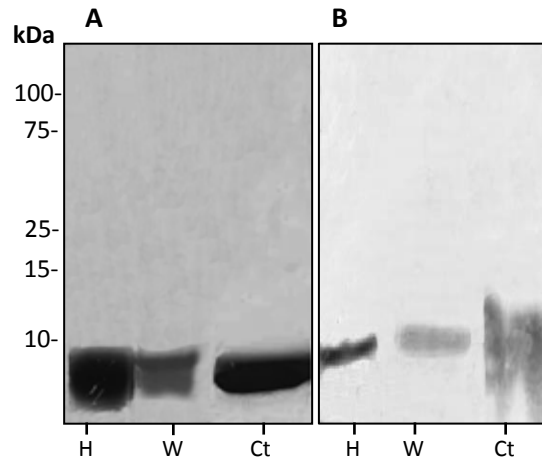


**Figure 12:** SDS-PAGE of the retained fraction obtained by cation-exchange chromatography in Waters cartridge (5  $\mu$ g) of hazelnut (H), walnut (W) and chestnut (Ct): **A**, staining with Coomassie Blue; **B**, Immunodetection with polyclonal antibodies against Pru p 3 (LTP) (1:1000).



**Figure 13:** Purification of LTPs from hazelnut (A), walnut (B) and chestnut (C) by HPLC reverse-phase. All selected picks contain the purified proteins.

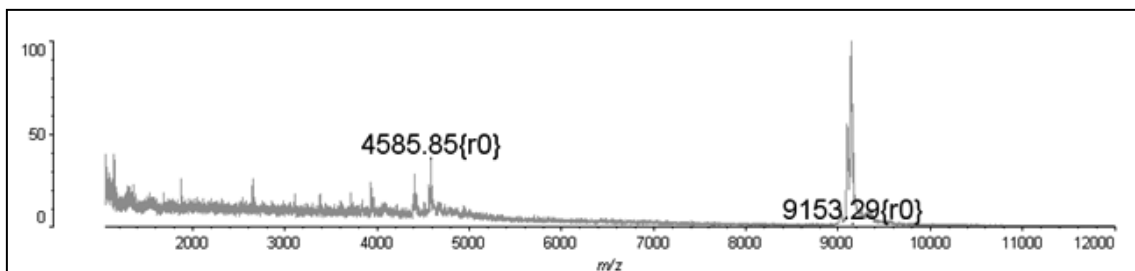
The presence of LTPs was confirmed by SDS-PAGE and recognized by polyclonal antibodies (figure 14).



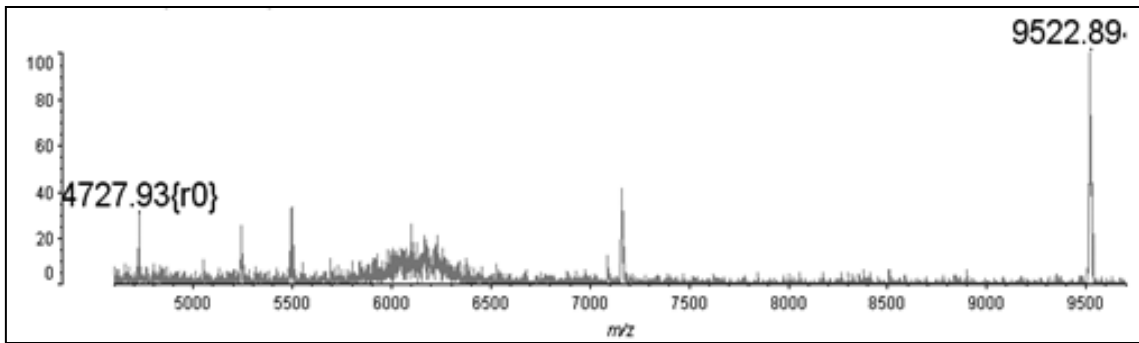
**Figure 14:** SDS-PAGE from purified proteins (5 $\mu$ g) of hazelnut (H), walnut (W) and chestnut (Ct) . **A**, staining with Coomassie Blue; **B**, Immunodetection with polyclonal antibodies against Pru p 3 (LTP) (1:1000).

Even more, the nature of these proteins was confirmed by mass spectrometry using MALDI-TOF.

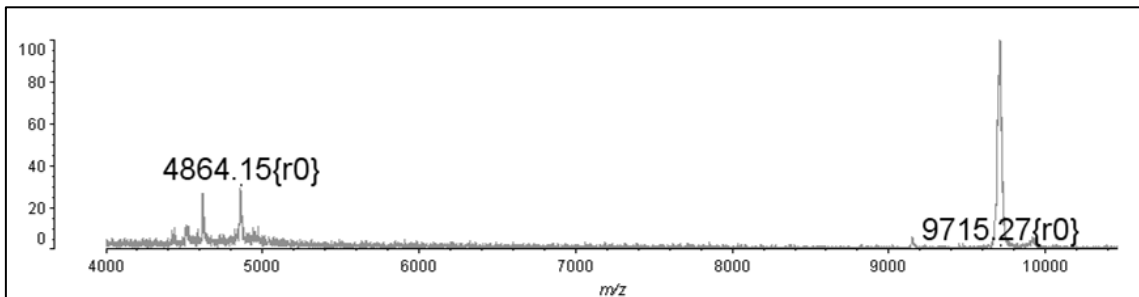
All graphs showed one peak at approximately 9 kDa and their half peak at 4.5 kDa, thus indicating that Jug r 3 (figure 15), Cor a 8 (figure 16) and Cas s 8 (figure 17) were purified.



**Figure 15:** MALDI-spectrum of the purified allergen Jug r 3.



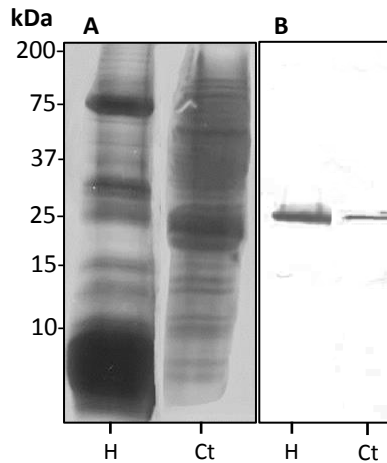
**Figure 16:** MALDI-spectrum of the purified allergen Cor a 8.



**Figure 17:** MALDI-spectrum of the purified allergen Cas s 8.

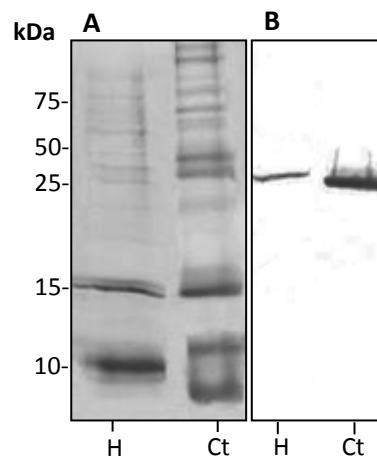
#### **4.2.4. Purification and characterization of TLP from hazelnut and chestnut**

In order to purify TLPs, it was used a similar method as for the purification of LTPs. PBS extracts were fractionated in SDS-PAGE, showing similar electrophoretic patterns. The immunodetection with polyclonal antibodies against TLP revealed one band of about 25 kDa, in each extract (figure 18).



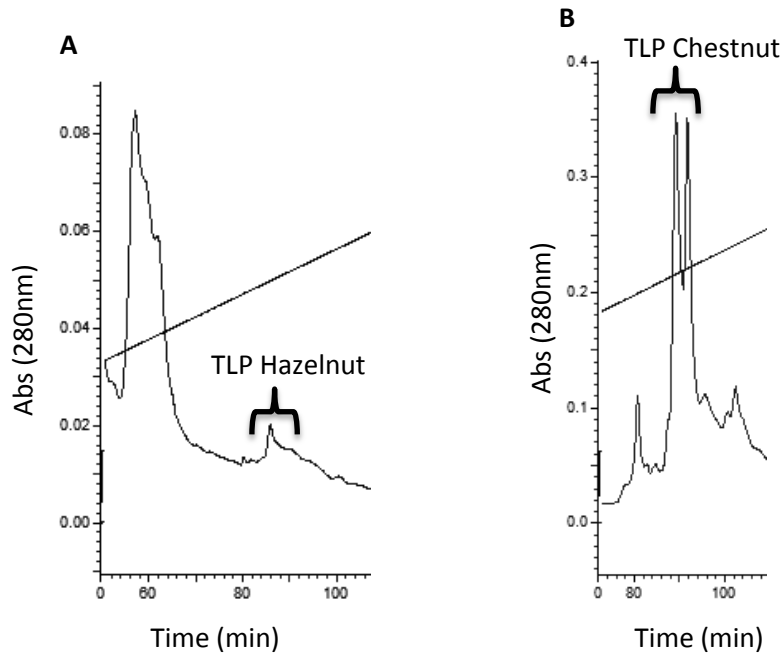
**Figure 18:** SDS-PAGE from PBS extracts (10 µg) of hazelnut (H) and chestnut (C): **A**, staining with Coomassie Blue; **B**, Immunodetection with polyclonal antibodies against Pru p 2.0201(TLP) (1:5000).

Knowing that each extract contains TLPs, they were used to purify TLP following the same protocol as used for the purification of LTPs. First, both extracts were fractionated by cation-exchange chromatography (figure 19 A) and the retained fractions showed the presence of TLPs (figure 19 B).



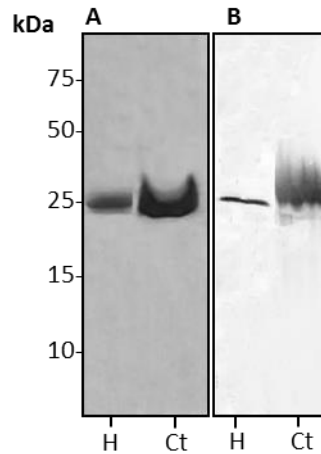
**Figure 19:** SDS-PAGE of the retained fraction obtained by cation-exchange chromatography in Waters cartridge (5 µg) of hazelnut (H) and chestnut (Ct): **A**, staining with Coomassie Blue; **B**, Immunodetection with polyclonal antibodies against Pru p 2.0201 (TLP) (1:5000).

After, the retained fraction of each extract was separated by reversed-phase HPLC, thus obtaining one peak for hazelnut (figure 20 A) and two majority peaks for chestnut (figure 20 B).



**Figure 20:** Purification of LTPs from hazelnut (A) and chestnut (B) by HPLC reverse-phase. All selected picks contain the purified proteins.

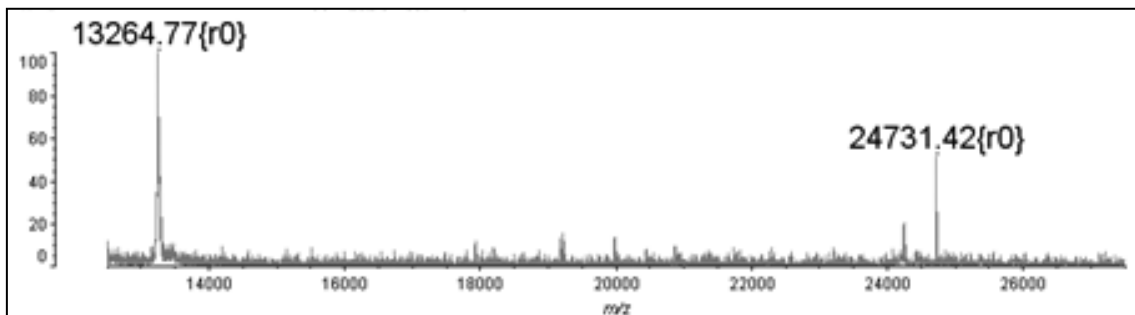
The two major peaks from chestnut showed a single band in SDS-PAGE, with a molecular weight of approximately 25 kDa, and the single peak from hazelnut showed a similar band (figure 21).



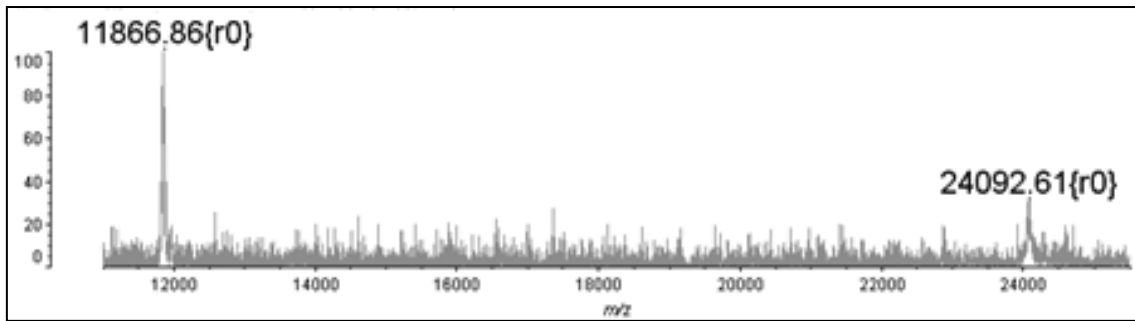
**Figure 21:** SDS-PAGE from purified proteins (5 $\mu$ g) TLP hazelnut and TLP chestnut **A**, staining with Coomassie Blue; **B**, Immunodetection with polyclonal antibodies against Pru p 2.0201 (TLP) (1:5000).

To confirm the purity of each purified protein, mass spectrometry was performed using a MALDI-TOF device.

All graphs showed one peak at approximately 25 kDa and their half peak at 12 kDa, thus indicating that TLP hazelnut (figure 22) and TLP chestnut (figure 23) were purified.



**Figure 22:** MALDI-spectrum of the purified allergen TLP hazelnut.



**Figure 23:** MALDI-spectrum of the purified allergen TLP chestnut.

#### 4.2.5. Study of the prevalence of the most relevant allergens

In order to establish the role of each protein family in tree nut allergy and their putative involvement in cross-reactivities with other foods, purified proteins Cor a 8, Cas s 8, Jug r 3, Cor a 9, TLP hazelnut and TLP chestnut, among proteins from sunflower seeds (2S, 11S and oleosins), and Jug r 1 which were already purified, were used to perform *in vitro* assays by microarrays.

These proteins were printed on a protein microarray. This microarray was tested with the sera from 131 allergic patients from 3 different hospitals.

Basurto Hospital in Bilbao had the higher percentage of sensitized patients. Most of the allergens (12/16), in this region, had a positive response between 20 and 60% (figure 24).

In La Princesa University Hospital, all 16 allergens had a positive response, with percentages between 5 and 30% (figure 24).

Foundation Jimenez Diaz (FJD), showed the lowest positive responses, with values lower than 20% of positive response. In this region 11 from 16 allergens showed a positive response (figure 24).

Ara h 1 (7S), Ara h 2 (2S), Cor a 14 (2S), Cor a 9 (LTP), Hel a 2 (11S) and Jug r 1 (2S), were the allergens that showing a higher positive response in every region (figure 25).

With respect to peanut allergens, only Ara h 3 (11S) and Ara h 9 (LTP) had the lowest positive responses (figure 25). Ara h 3 was not recognized in patients from FJD and Ara h 9 was not recognized by patients from Bilbao (figure 24).

Chestnut showed the lowest percentages, with values below 20% (figure 25), and none of the two allergens (TLP chestnut and Cas s 8) were present in the three regions (figure 24).

Hazelnut showed good percentages in almost every allergen, least Cor a 8 with only 10% of recognition (figure 25) and was only recognized in patients from La Princesa University Hospital (figure 24).

Walnut was the only group of tree nuts in which the two allergens (Jug r 1 and Jug r 3) were recognized in all 3 regions with percentages between 10 and 60% (figure 24).

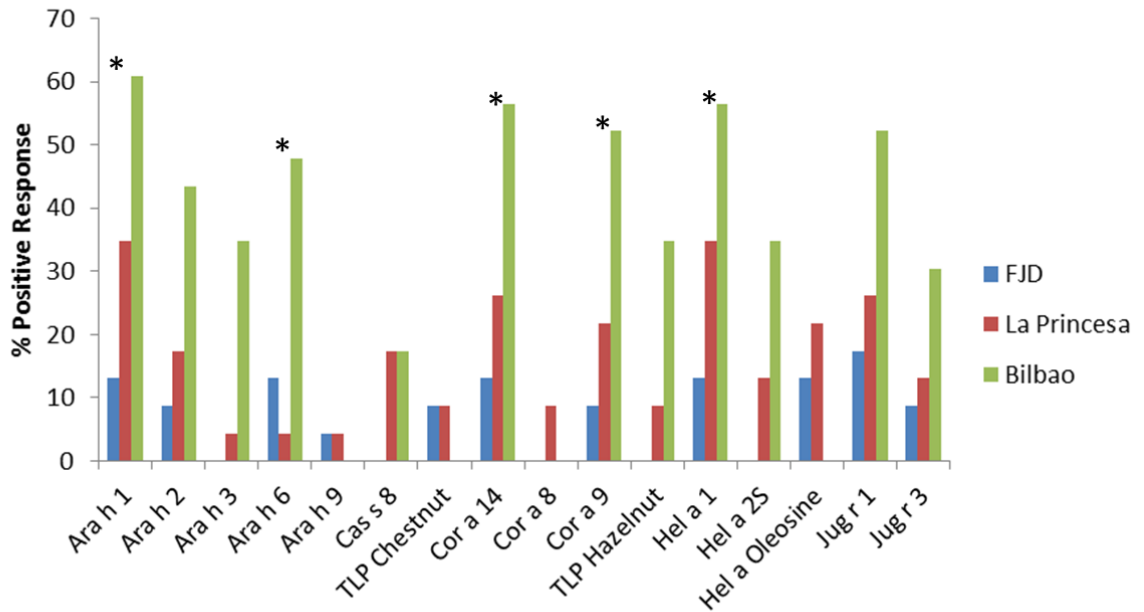
Regarding allergen groups, Ara h 2, Cor a 14, Jug r 1, Hel a 2S and Jug r 1, all 2S albumins, were highly recognized in every region, except Hel a 2S that did not showed recognition in patients from FJD (figure 24).

In the LTP group, Ara h 9, Cor a 8, Cas s 8 and Jug r 3, only Jug r 3 was recognized by patients from the 3 regions (figure 24). Ara h 9 and Cor a 8 showed the lower positive responses (figure 25). Cor a 8 was only recognized by patients from La Princesa University Hospital.

In the legumin group, 11S, Ara h 3, Cor a 9 and Hel a 1 had a high positive response in every region, except Ara h 3 that was not recognized in patients from FJD (figure 24).

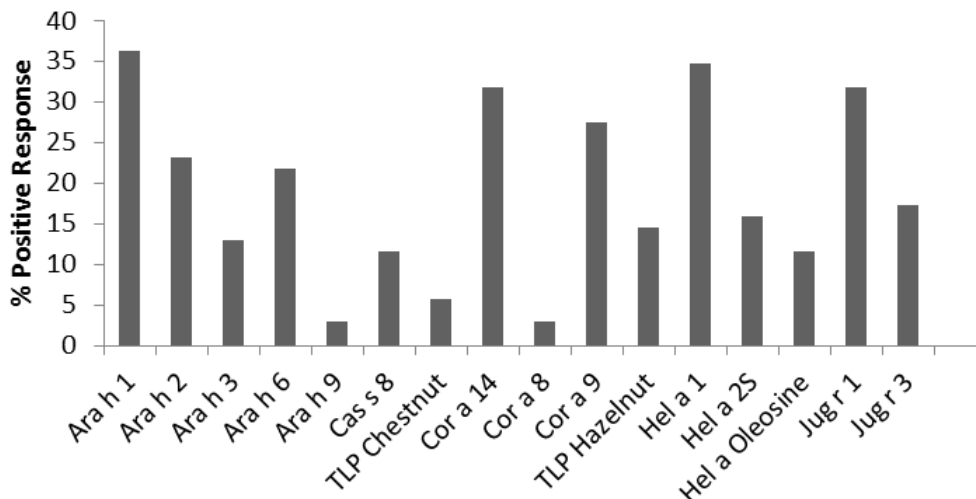
Both TLPs were recognized in less than 15% of the patients, with exception of TLP hazelnut that showed a positive response of 60% in Bilbao. TLP hazelnut and TLP chestnut were not recognized in patients from FJD and Bilbao, respectively (figure 24).

## Nut Allergens



**Figure 24:** Recognition frequencies of the different allergens by geographical area. Frequencies, shown as percentage of positive response (%), were obtained by incubating the allergens microarray with single sera from allergic patients of Jimenez Diaz Foundation (FJD), La Princesa University Hospital and Basurto Hospital (Bilbao). Percentage positive responses and significant differences ( $p < 0,05$ ) are indicated.

## Global Prevalence



**Figure 25:** Frequency of sensitizations obtained by the different allergens microarray using sera from all patients.

## 5. DISCUSSION

Food allergy is a topical issue in western countries. As happens with other allergic diseases prevalence appears to be increasing with special interest in tree nut allergy. The prevalence of food allergy is suffering an increase in recent years in both adults and children. This pathology, usually, begins in the first years of life to decrease progressively during childhood to adulthood (Moncín, M.M.S.M., *et al.*, 2007).

In the course of history, nuts have been part of the diet around the world. Tree nuts have a high nutritional value, meaning that, they are really important in human diet, and their consumption has intensified in the past few years due to investigations that show that tree nuts have a beneficial effect on human health, especially on the outcome of coronary disease and cholesterol serum levels (King, J.C., *et al.*, 2008), although their beneficial effects, the truth is that in the developed world, the allergic reactions caused by tree nuts represent one of the first cause of food allergies in children and the first in adults (Sampson, H.A., 1999).

When a patient is diagnosed as positive to one tree nut species, the treatment prescribed, is the strict avoidance of the offending food (Crespo, J.F., *et al.*, 2006). Despite, most of the professionals recommend the avoidance of all nuts and tree nuts, usually, the patient is only sensitized to one or two species, and not to all the 20 different tree nut species. Not all species cross-react, but what we need to know is which species are related. Thus, it is important to characterize the allergens responsible for the allergy to tree nuts, and understand their cross-reactivity. By these mean, it will be possible to improve the lifestyle of these allergic patients.

In this study, extracts from cashew nut, hazelnut, peanut, chestnut, walnut, pine nut and sunflower seeds were used to observe their implication in cross-reactivity among nuts. Patients from different regions and with tree nut

allergy presented a complex profile of bands by immunodetection against the tree nut extracts showing different IgE binding profiles in each case.

The results suggest that not all nuts show cross-reactivity. The relationship between the chemical and functional properties of a protein and its allergenic ability remains unknown, thus these relationships should be defined to improve the recommendations to patients.

In this study, only chestnut nut, hazelnut and peanut showed a clear relationship, as the IgE band profile is very similar in the three extracts. In order to clarify and demonstrate these relationships, inhibition assays would be necessary.

The structural characteristics of proteins are major determinant factors of cross-reactivity. As mentioned before, IgE cross-reactions occur because of shared features at the level of primary and tertiary structure of proteins and cross-reactivity seems to require more than 70% sequence identity (Ferreira, F., *et al.*, 2004). IgE cross-reactivity between the different tree nuts has been demonstrated by several authors. Sutherland *et al.*, demonstrated partial cross-reactivity between hazelnut and macadamia nut (*Macadamia integrifolia*) (Sutherland, M.F., *et al.*, 1998). Hazelnut has also been shown to own multiple cross-reacting proteins with sesame seed and poppy seed (Vochs, E., *et al.*, 1993). Walnut and hazelnut seems to cross-react with the 2S albumin from almond (Poltronieri, P., *et al.*, 2002). Pistachio nut appears to cross-react with cashew nut (Fernandez, C., *et al.*, 1995). The 11S legumin of cashew nut, walnut and hazelnut appears to share the same IgE epitopes (Wallowitz, M.L., *et al.*, 2004). Cross-reactivity was demonstrated, at the T cell level, among cashew nut, walnut and peanut (Kulis, M., *et al.*, 2009). And, some studies have suggested that there may be common allergens between pistachio, peanut, walnut, chestnut, almond and cashew nuts, as well as pine nut and almond (Marinas, D., *et al.*, 1998; Parra, F.M., *et al.*, 1993).

According to these previous studies, the responsible for the cross-reaction is the protein and not the extract. For this reason, it is necessary to identify the nature of the allergens implicated in this type of sensitization. Thus polyclonal antibodies against Pru p 2.0102 (anti-TLP), Hel a 1 (11s) and Pru p 3 (LTPs), were used for analysing the tree nut extracts.

The presence of specific antibodies against Hela 1, in almost every extract, showed that there is a higher concentration of legumins (11S) in tree nuts, which, from literature, is the most widely group of seed storage proteins in almost every dicotyledonous species. The fact that the double-stranded  $\beta$ -helix comprises the cupin fold, leads to a remarkably stable structural motif, resisting to thermal denaturation and proteolysis. This high stability may play a major role in allowing sufficient immunologically active fragments to pass down the gastrointestinal tract, and is responsible, in part, for the thermostable nature of the allergenic activity of these proteins. This, attached to the abundance of storage globulins in the diet, must contribute to their ability to act as potent allergens (Mills, E.N.C., *et al.*, 2002). In spite of all this powerful properties, there is little evidence of IgE cross-reactivity between allergens from this family (Breiteneder, H., and Mills, E.N.C., 2005).

Regarding LTPs, only chestnut and pine nut showed a 9kDa band, but that does not mean that walnut or hazelnut are not abundant in LTPs. In fact walnut is very abundant in Jug r 3 and the same happens to Cor a 8 in hazelnut, the problem is the affinity of these proteins to the antibody.

In the past few years a number of allergens have been identified in a variety of food and plants. Not only has this information well-marked the proteins that are directly responsible for food allergies but it has also revealed structural relationships between allergens (Willison, L.N., *et al.*, 2008).

The characterization of allergens and the study of their expression can, in consequence, prevent unwanted contacts in patients allergic to plant food (Sicherer, S.H., and Sampson, H.A., 2006). Thus based on the IgE reactive bands

against the different extracts, majority allergens from hazelnut, walnut and chestnut were isolated (allergens from the other extracts were already isolated and stored in the lab.)

In this study, we isolated LTPs from hazelnut, walnut and chestnut. From literature, and as mentioned above, LTPs have a molecular mass of 9 kDa and because they have an isoelectric point of about 9.0, to purify the proteins we had to use a cation-exchange chromatography. In this method the column is coated by a negatively charged resin that binds all the substances positively charged. The retained fraction was repurified by reversed-phase HPLC and confirmed by MALDI-TOF. Finally, we isolated Cor a 8 from hazelnut, Jug r 3 from walnut and Cas s 8 from chestnut.

Members of the lipid transfer protein family are known to be important allergens associated with plant food allergy, particularly in the Mediterranean region. The importance of LTPs as allergens may lie in their high resistance to heat treatments and degradation by digestive enzymes (Asero, R., *et al.*, 2000), which enables them to maintain their integrity in the digestive tract, where they can induce an immune response associated with the gastrointestinal epithelium (Marion, D., *et al.*, 2004). This demeanor has led to them being reckoned as true food allergens. Due to their high stability, LTPs are associated with severe systemic symptoms, with or without Oral Allergy Syndrome (OAS) (Fernandez-Rivas, M., *et al.*, 2003). Cross-reactivity has been described between LTP from botanically unrelated fruits, vegetables and pollen (Asero, R., *et al.*, 2002; Diaz-Perales, A., *et al.*, 2000). Cross-reactivity can vary between geographical areas, since they depend on the local flora that produces pollen and the dietary patterns of the population.

In order to purify TLPs, from hazelnut and chestnut, the same method as for LTPs was used. These proteins have an isoelectric point of 8.5, thus cation-exchange chromatography was used. The repurified proteins were eluted, by RP-HPLC, some minutes after LTPs, because of their molecular mass of about 24

kDa. Mass spectrometry was performed to confirm the purity of TLP hazelnut and TLP chestnut.

Thaumatin-like proteins are panallergens and may be responsible for cross-reactivity among foods and pollen (Breiteneder, H., 2004). TLPs have been identified in several food allergies such as apple, cherry, kiwi, grape and some pollen (Palacín, A., *et al.*, 2010). They have a compact three-dimensional structure maintained by eight disulfide bridges, which helps their resistance to the process of digestion in the gut and heat treatments at acidic pHs (Breiteneder, H., 2004; Perri, F., *et al.*, 2008). This features could favour the permanence of this type of allergens in foodstuff (after cooked or industrial processing), and therefore their allergenic potential (Marzban, G., *et al.*, 2009).

In order to purify 2S albumins, molecular exclusion chromatography was performed. This technique was chosen based on the protein profile of walnuts. This type of nut has a lot of proteins and we only wanted the 30-70 kDa fraction.

The 2S albumins are defined on the basis of their sedimentation coefficient and are widely distributed in both mono- and di-cotyledonous plants (Ferreira, F., *et al.*, 2004). They are heterodimeric proteins with the two subunits linked by disulphide bonds and they are rich in arginine, glutamine, asparagine and often cysteine. These proteins are subjected to modification after their synthesis (Breiteneder, H., and Ebner, C., 2000). This family is thought to sensitize via the gastrointestinal tract, meaning that these proteins can resist and survive to harsh conditions as acid pH, denaturing effects of surfactants and proteolytic activities of digestive enzymes (Moreno, F.J., and Clemente, A., 2008). Thereafter, these proteins could be absorbed facilitating the exposition of these allergens to the immune system in order to elicit an allergic response (Moreno, F.J., *et al.*, 2005; Murtagh, G.J., *et al.*, 2003). Thus, the stability to food processing is an important feature in the assessment of the intrinsic allergenicity of 2S proteins (Mills, E.N.C., *et al.*, 2009). Regardless 2S albumins

high structural homology, cross-reactivity between members of this family seems to be uncommon (Moreno, F.J., and Clemente, A., 2008).

The 11S legumins, with an isoelectric point of 6.0, were purified by anion-exchange chromatography. In this method, the column is coated by a positively charged resin that binds all the substances negatively charged, followed by a re-purification by FPLC. The 11 S have shown in an electrophoretic pattern with 2 bands, corresponding to the acidic and the basic subunits, with 40 and 20 kDa, respectively. Performing a peptide mass fingerprint we conclude that we had purified Cor a 9.

The analysis of the IgE binding of different allergenic families using data from large numbers of patients could clarify recognition patterns and help in the prediction the prevalence of each allergen in food allergy (Hiller, R., *et al.*, 2002). For this purpose, a representative panel of LTP, TLP, 11S, 2S and oleosins from hazelnut, peanut, chestnut, walnut and sunflower seeds, was used, based on the most frequent sensitization associated with tree nut allergy in three different populations in Spain.

A microarray approach was chosen as the best *in vitro* high-throughput immunological assay to test a large number of proteins and sera, based on the small quantities of protein and sera required and which can help us in the improvement the diagnosis and treatment of allergy (Beyer, K., and Teuber, S.S., 2005; Sanz, M.I., *et al.*, 2011; Shreffler, W.G., 2011). Other advantages are the lower costs and the more fast processing of the samples (Salcedo, G., and Díaz-Perales, A., 2010).

In this study LTPs showed little recognition in every region, comparing to all allergens. Only in patients from La Princesa Hospital, every LTP was recognized by patients, unlike in Bilbao, that from four LTP, only Jug r 3 and Cas s 8 were recognized. This LTP was also the only protein recognized by patients from the three different regions. Thus we can say that patients from Madrid,

especially in La Princesa, are more sensitized to LTPs than in Bilbao and Jug r 3, the LTP from walnut, is the allergen that has the higher prevalence in all regions.

Lipid transfer proteins have been a widely studied family regarding plant food allergenic reactions and their role in food and/or pollen cross-reactivity (Salcedo, G., *et al.*, 2007). As said before, allergens from this family have been found in most vegetable sources, and the model member of LTPs is the peach LTP, Pru p 3. This allergen is recognized by 75% of patients who suffer from peach allergy, the most frequent plant food allergy in Spain and has been involved in food cross-reactivity, especially those involving fruits and nuts (Salcedo, G., *et al.*, 2004)..

In a previous study (Palacín, A., *et al.*, 2012), using microarrays as well, fruit allergic patients from Bilbao and Madrid showed an over 70 % of positive response to Pru p 3. Nevertheless, in our study, the prevalence of LTPs has never been higher than 30%, meaning that tree nut LTPs were not principal allergens, although all of them showed cross-reactivity with Pru p 3.

With respect to TLPs, they did not obtain a high positive response (up to 15%) in the studied regions. Between the two TLPs, TLP hazelnut showed a higher recognition in patients from Bilbao (30%) thus meaning, that TLPs may be important allergens in the north of Spain.

As mentioned above, members of the thaumatin-like protein (TLP) family have been identified as important allergens in different vegetable sources, but as LTPs family, TLPs are very present in peach fruit (Pru p 2.0201) (Breiteneder, H., 2004). This family is thought to be responsible for cross-reactivity between pollen and fruit, however at present this is not supported by enough experimental data. A previous study about TLPs and microarrays (Palacín, A., *et al.*, 2012) showed that Pru p 2.0201 had a prevalence of more than 50% both in Bilbao and in Madrid.

Comparing to our study, none of the two TLPs have recognition higher than 30%. This means that, as happens with LTPs, the first sensitizer is not the TLPs from tree nuts, but rather Pru p 2.0201, that cross-reacts with other TLPs.

However, it seems that seed storage proteins (2S albumins and 11S legumins) show a relevant role in tree nut allergies. All 2S showed high recognition in almost every region. Jug r 1 and Cor a 14 were the allergens with more recognition. The 2S albumins are unique among seed proteins in terms of the higher sulfur content in some species compared with other storage proteins (Teuber, S., *et al.*, 2008) and they are present in almost edible seed.

The 11S legumin were highly recognized by all patients, being Hel a 1, the 11S from sunflower seed, the allergen with highest recognition in every region.

Ara h 1, was the only vicilin (7S) studied, and showed a high prevalence in the different regions. Vicilins represent one of the most abundant proteins found in vegetables used for human diet (Shin, D.S., *et al.*, 1998). The enzymatic activity, of this class of proteins, is unknown, but it is thought to interact with each other to form unique higher order oligomeric structures that may help in packaging these proteins into seeds (Lawrence, M.C., *et al.*, 1994). Ara h 1 was the first major allergen identified in peanut and more than 90% of patients with positive challenges to peanut have specific IgE to Ara h 1 (Mills, E.N.C., *et al.*, 2004). Peanuts are often subjected to roasting, a thermal treatment performed at low levels of hydration, unlike walnuts, that are often eaten raw. It has been reported that, Ara h 1, has a dramatic increase in allergen activity of peanut extracts, from roasted compared with raw nuts (Mills, E.N.C., *et al.*, 2004). Due to the fact that peanut is one of the tree nuts most consumed all over the world and because Ara h 1 showed to be an abundant protein that survives intact in most food processing methods and is stable to digestion, the prevalence to this allergen is really high all over Spain (Shin, D.S., *et al.*, 1998). Because of the low prevalence of peanut allergy in Spain, it must be assumed that this positive

response to Ara h 1, occurs due to the cross-reactivity with vicilins from other tree nuts, as walnut and hazelnut.

## 6. CONCLUSIONS

- Protein extracts from chestnut, peanut, walnut and hazelnut are recognized specifically by patients' sera according to their origin. No cross-reactivity was observed among the studied extracts.
- Through microarray approaches, we analyzed the IgE binding of different allergenic patients. Patients from Madrid (center of Spain) showed higher sensitization to LTPs than patients from Bilbao (north of Spain).
- LTP family is a relevant allergen in the studied nuts, Jug r 3, the LTP from walnut, was the allergen with higher prevalence in all regions.
- TLPs from tree nuts are not the first sensitizers but rather peach TLP (Pru p 2.0201).
- The 2S albumins and 11S legumins showed a relevant role in tree nut allergies.

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