Allergenic protein and epitope recognition in food allergy: a new perspective for the molecular and clinical characterization of shellfish and lipid transfer protein allergy.

Reconeixement de proteïnes i epítops al·lergènics en al·lèrgia alimentària: una nova perspectiva per la caracterització clínica i molecular de l'al·lèrgia al marisc i a les proteïnes de transferència de lípids.

Mariona Pascal i Capdevila

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Doctoral Thesis in Biomedicine
Universitat de Barcelona, 2011

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Als meus pares i a en Xavi.

" La nostra recompensa es troba en l'esforç i no en el resultat. Un esforç total és una victòria completa."

Mahatma Gandhi
(1869-1948)

AGRAÏMENTS

Ara que arriba el moment de posar punt i final a aquesta Tesi, voldria fer arribar el meu agraïment a totes les persones que, d'una manera o altre, m'heu ajudat en aquest camí. S'acaba una etapa que ha estat molt important i positiva per a mi, i que ha resultat una experiència enriquidora en molts sentits... I tot això ha estat possible perquè he estat envoltada de gent que m'ha recolzat i animat en tot moment. Miro enrere i m'adono que totes les pedres que m'he anat trobant al llarg d'aquest camí s'han fet petites i insignificants, mentre que només queden els bons records i la satisfacció de l'esforç que he compartit amb tots vosaltres.

En primer lloc, vull fer constar el meu especial agraïment als directors de la Tesi, la Dra. Rosalia Ayuso i el Dr. Manel Juan, per haver-me donat l'oportunitat de dur a terme la meva Tesi i per la seva dedicació en el desenvolupament d'aquest projecte i en tota la meva formació. Molt especialment vull agrair al Dr. Jordi Yagüe el seu suport incondicional, per creure que podria tirar endavant el meu projecte, pel seu ajut i paciència en els moments més difícils, però sobretot per ensenyar-me uns valors que van més enllà d'aquesta Tesi, que m'han fet créixer com a persona i que segur que m'ajudaran al llarg de tota la meva vida professional.

A tots els membres del Servei d'Immunologia de l'Hospital Clínic, per tots els moments que hem compartit junts des de que vaig entrar de R1, per tot el que amb paciència i sempre de bona gana m'heu ensenyat, pels esmorzars compartits, les nits de guàrdia i mil anècdotes més... Al Dr. Ramon Vilella, a en Jordi Milà i a la Luchy vull fer una menció especial, perquè amb ells vaig començar aquesta aventura de l'al·lèrgia... A la Mercè li he d'agrair la seva incondicional disposició a donar-me un cop de mà, sense ella, la "paperassa" hauria estat impossible.

Aquest projecte no hauria estat possible sense els meus companys del "zulo", als quals vull agrair la confiança dipositada en mi, tot el que m'han ensenyat, les sessions que hem compartit i sempre obrir-me les portes als vostres projectes. He agrair molt especialment al Dr. Joan Bartra haver estat el meu "al·lergòleg de capçalera" durant tot aquest camí. Joan, és molt llarga la llista de totes les coses que t'he d'agrair, però per mi el més important ha estat la teva il·lusió per tirar endavant, sense deixar-me mai sola en aquesta aventura i sempre al peu del canó quan t'he necessitat, dedicant-me un temps que de ben segur no tenies...

A la Dra. Maria Luisa Sanz, per la meva mini-estada al seu laboratori de la Clínica Universitària de Navarra, que em va permetre iniciar aquesta aventura i fer créixer el meu interès per l'al·lèrgia.

Al Dr. Hugh Sampson he d'agrair molt especialment l'oportunitat d'entrar a formar part del seu grup d'investigació i desenvolupar bona part d'aquesta Tesi al seu laboratori, pel seu suport i la seva reflexió sobre molts aspectes de la vida professional. A tots els membres del laboratori del Mount Sinai, però en especial a la Dra. Mirna Chehade i a la Galina Grishina, per la seva inoblidable acollida i companyia durant la meva estada a Nova York i per tot el que m'han ensenyat a nivell personal i professional. Encara que sembli estrany vull agrair també a la ciutat de Nova York tots els racons que tenen quelcom especial que t'ajuden a créixer com a persona i a tirar endavant dia a dia, pas a pas.

A les meves amigues, Maria R., Laura E. Maria T. i Núria C., per fer-me costat durant tot aquest camí, però especialment per recolzar-me en les decisions i els moments més difícils, quan tot semblava una bogeria...

A tu Xavi, t'ho he d'agrair tot. Ets la persona que més ha "patit" aquesta Tesi i et vull donar les gràcies per tot l'esforç que ens ha suposat arribar fins aquí, per la teva comprensió, per sempre donar prioritat als meus interessos per davant dels teus, per la teva paciència i recolzament en els moments més difícils, per haver-te robat tantes hores i per haver omplert la meva vida de bons moments i fer que aquests anys hagin estat tant especials per a nosaltres. I perquè sé que passi el que passi sempre ets al meu costat.

Als meus pares, perquè ho sou tot i us ho dec tot. Per donar-me l'oportunitat de formar-me i poder arribar fins aquí, però molt especialment per haver-me ensenyat des de petita el valor de la responsabilitat, l'esforç i la dedicació com a claus per aconseguir allò que vols a la vida. Gràcies per "aguantar" tant bé les meves absències d'aquests últims anys, per tots els bons i mals moments que no hem pogut compartir i que ens han unit més que mai.

I a la resta de la meva família, pel seu suport incondicional. A la Marta, la Rosalia i l'Enric, per acompanyar-me en tot aquest viatge i per ser valents quan les coses no han sigut del tot fàcils. A les meves Àvies, per sempre fer-me costat, de manera senzilla, alegrant-se del meus avanços i desitjant el millor per mi, encara que la meva feina sovint no és fàcil d'entendre... A en Raül i a la Irene per preocupar-se per mi i animar-me durant tot aquest temps.

A tots aquells que d'una manera o altra m'han ajudat, encara que el seu nom no figuri de forma explícita en aquestes línies, sense ells tampoc hauria estat possible. A tots, el meu més sincer agraïment.

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3. MATERIAL AND METHODS

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LIST OF ABBREVIATIONS

AK	Arginine kinase
APC	Antigen presenting cell
BAT	Basophil activation test
BcR	B cell receptor
BSA	Bovine serum albumin
CD40L	CD40 ligand
CRD	Component-Resolved Diagnosis
CRIT	Component-Resolved Immunotherapy
DBPCFC	Double blind placebo controlled food challenge
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DP	*Dermatophagoides pteronyssinus*
EDTA	Ethylenediamine tetra-acetic acid
Eff.	Efficiency
e.g.	exempli gratia = for example
ELISA	Enzyme linked immuno-sorbent assay
FABP	Fatty-acid binding protein
FAP	Facilitated antigen presentation
FcεRI	High affinity IgE receptor
FcεRII	Low affinity IgE receptor, also known as CD23
FDR	False discovery rate
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FN	False negative
FP	False positive
GI	Gastrointestinal
GID	Gastrointestinal disorders
HDM	House dust mite
Hemo	Hemocyanin
HRF	Histamine-releasing factor
HAS	Human serum albumin
i.e.	id est = that is, which means…
Ig	Immunoglobulin (E, G4…)
IL	Interleukin (-3,-4,-5,-13…)

IPTG	Isopropyl-beta-D-thiogalactopyranoside
ISU	ISAC standardized unit
IT	Immunotherapy
ITAM	Immunoreceptor tyrosine-based activation motif
kDa	KiloDalton
L-ASA	L-acetyl salicylic acid
LB	Lysogeny or Luria Broth
LT	Leukotrien (-C4, -D4, -E4…)
LTP	Lipid transfer protein
MLC	Myosin Light Chain
NGS	Normal Goat Serum
NPV	Negative predictive value
NSAID	Non-steroidal anti-inflammatory drug
nsLTP	Non-specific lipid transfer proteins
OAS	Oral allergy syndrome
OD	Optical density
OFC	Oral food challenge
OIT	Oral immunotherapy
O/N	Overnight
OU	Optical units
PAF	Platelet activating factor
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline containing Tween 20
PCR	Polymerase chain reaction
PG	Prostaglandin
PPV	Positive predictive value
RAST	Radioimmunoassay test
SCP	Sarcoplasmic calcium-binding protein
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Sensitivity
SP	Specificity
SPT	Skin prick test
Th0	Naïve CD4+ T cell
Th1 / Th2	T helper type 1 / type 2

TM	Tropomyosin
TN	True negatives
TP	True positives
TpC	Troponin C
TSLP	Thymic stromal lymphopoietin

CHAPTER 1. INTRODUCTION

INTRODUCTION

1.1. Allergy

1.1.1. Definitions

Clemens Von Pirquet introduced the term *allergy* in 1906 as “an altered capacity of the body to react to a foreign substance” [1]. That was an extremely broad definition that included in fact all immunological reactions. Nowadays the definition is restricted to “disease following a response by the immune system to an otherwise innocuous antigen” [2].

In 1920, Prausnitz and Küstner demonstrated the involvement of immunoglobulins in the allergic response by injecting Küstner’s (fish allergic) sera into Prausnitz’s (not allergic) forearm and with that transferring him the sensitivity to the fish extract. However, it was not until 1967 that the responsible molecule was characterized and termed Immunoglobulin E (IgE) by Ishizaka (confirmed with experiments of Johannson and Bennich) [3].

Allergy is one of a class of harmful immune system responses that are termed *hypersensitivity reactions* that produce tissue injury and can cause serious disease. In 1963, Gell and Coombs classified them in four groups (Types I-IV) based on the immune reactant, the antigen and the effector mechanism involved [2,4]. Allergy is often equated with type I hypersensitivity (immediate-type hypersensitivity reactions mediated by IgE that lead to mast cell activation) (Figure 1.1).

The World Health Organization (WHO) has classified the common allergic diseases, which include asthma induced by aeroallergens, allergic rhinitis, atopic dermatitis, drug allergy and food allergy, as one of the six more frequent pathologies that affect the world population. Prevalence of these diseases in Europe is estimated around 25-30% of the population. Apparently their prevalence is increasing and they are a frequent cause of disability in the developed world [5].

Atopy is the term used to describe an exaggerated tendency to mount IgE responses to a wide variety of common environmental allergens. It is considered that 20% of the worldwide population is atopic. This state has a strong familial basis and is influenced by several genetic loci. Atopic individuals have higher total levels of IgE and

eosinophils in the circulation than their normal counterparts. They are more susceptible to allergic diseases such as hay fever and asthma [2].

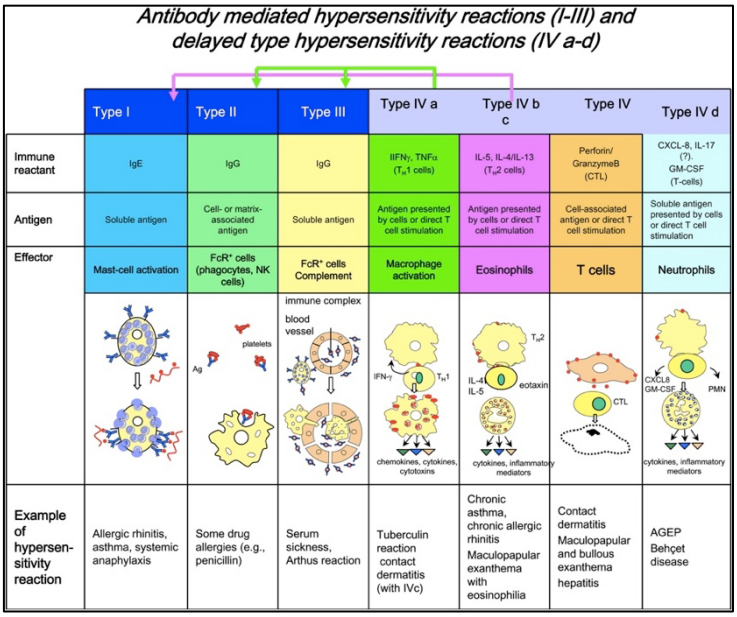

Figure 1.1. Classification of hypersensitivity reactions. Schematic representation of the main characteristics of each type of hypersensitivity reaction. From: [4].

1.1.2. Effector cells: mast cells and basophils

Mast cells and basophils are considered critical components of the allergic response. They express the high-affinity IgE receptor (FcεRI) and, in response to aggregation of this receptor by antigens acting through bound IgE, secrete inflammatory mediators (contained in basophilic plasma granules) known to be responsible for the symptoms and pathology of allergic diseases [6]. The state when allergen specific IgE antibodies arm tissue mast cells and blood basophils by binding to its receptors is called *sensitization*.

Moreover, both cell types are known to express other receptors that also induce secretion of mediators responsible for allergic symptoms. Some of these receptors are now considered part of the innate immune response and mast cells have been implicated in mediating several non-allergic diseases (e.g., inflammatory diseases, neurological diseases and functional diseases) thanks to their responsiveness to these other types of stimulation (e.g., adenosine, complement component 3a, chemokines, cytokines, pathogen-associated molecular patterns (PAMPs), sphingosine 1-phosphate

and stem-cell factor/KIT ligand). Nevertheless, secretion from mast cells and basophils and their participation in diseases is not restricted to IgE-mediated reactions [6].

Human mast cells have been traditionally described as the tissue equivalents of basophil granulocytes, which have been seen for long as a surrogate with which to study the mast cell [5, 6]. This view, however, may no longer be valid, since there is substantial developmental and physiological evidence that these two cell types differ from one another more than they are alike [6].

1.1.2.1. Mast cells

Mast cells (mastocytes) are potent tissular effector cells of hematopoietic origin, closely related to monocytes and macrophages. Development of these cells involves the release of intermediate precursors from the bone marrow that are capable of targeting specific tissue sites for maturation. Mast cell survival on specific tissue sites has been estimated on the order of months. They are particularly abundant in a perivascular distribution in connective tissues and at mucosal surfaces, at sites in the body that are exposed to the external environment, such as the skin. In these locations, they are found in close proximity to blood vessels, where they can regulate vascular permeability and effector-cell recruitment. Although not having direct cell–cell contact with local populations of antigen-presenting cells (e.g., Langerhans cells in the skin), they can modulate the behavior of these and other neighboring effector cells through the release of mediators. In addition to their role in allergy, they regulate many tissue functions and they have a central role in innate immunity to bacterial and parasitic infections [7].

1.1.2.2. Basophils

Basophils develop from pluripotent stem cell precursors found in the bone marrow and share properties mainly with eosinophils. While the exact factors important in their differentiation remain unknown, the cytokine interleukin (IL) -3, likely plays a critical role, as well as in the survival and activation of mature basophils.

Upon release from the bone marrow as mature cells, basophils have very little capacity for further development and are thought to survive only for days in the blood circulation.

It was first thought that their function was restricted to histamine and leukotriene (LT) C4 secretion, but now, there is firm evidence that basophils are the predominant cellular source of IL-4 and IL-13, perhaps the two most important cytokines having a role in the pathogenesis of allergic disease. This fact, together with the evidence that these cells infiltrate allergic lesions and are capable of responding to variety of stimuli, has launched a renewed interest in them and in their role in allergic inflammation and disease [8,9].

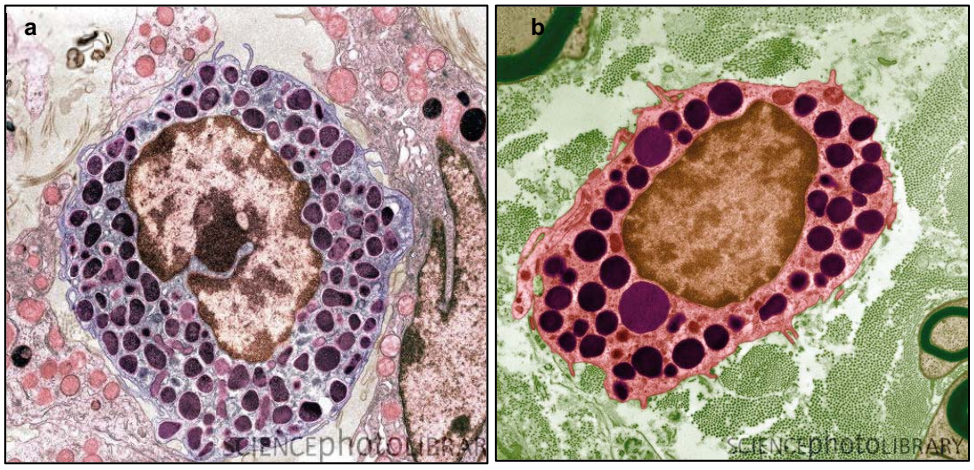

Figure 1.2. Mast cell and basophil. a) Mast cells are a type of leukocyte found in connective tissue. The large oval (pink and brown) is the cell's nucleus and within the cell's cytoplasm (purple) there are granules (dark purple) containing chemical mediators. b) Basophils are the least common of the white blood cells. The nucleus is shown in brown and granules in the cell cytoplasm in purple. Cell sections, colored transmission electron micrograph (TEM). Magnification: x5000 when printed at 10 centimeters wide. From Science Photo Library.

1.1.3. Antigens

Allergen is the specific term used to describe the antigen that has the capacity to stimulate the production of IgE by selectively triggering a T helper type 2 (Th2) response in a genetically disposed individual and to develop an allergic reaction in the individual that has previously been sensitized to it (i.e., induce allergic symptoms in an individual to whom IgE production has been already induced due to a prior exposure to the allergen). If the antigen is only able to induce the allergic reaction, but not to sensitize is known as an incomplete allergen, whereas antigens with full capacity to sensitize and induce symptoms are known as complete allergens [5].

There is currently not known structure or feature that makes a substance an allergen or not. However, size, solubility, molecular compactness and stability seem to be traits that influence notably in allergenicity. Most allergens are common, innocuous small proteins or glycoproteins with a molecular weight of 5-100 kilo Dalton (kDa). Major allergen sources include: herbaceous dicotyledons, tree and grass pollens, fungi, animal dander, house-dust mites, cockroaches and foods such as fresh fruits, vegetables, nuts, fish and shellfish. Noteworthy, several of the clinically relevant allergens possess biochemical properties that may facilitate the actual sensitization process (e.g., the protease activity can enhance epithelial permeability or stimulate proinflammatory cytokine release) [5].

Allergen exposure may occur within and outside home as well as in the workplace. Allergens enter the body at very low doses via a number of routes such as the respiratory (the most clinically important) and gastrointestinal tracts, but they may also be injected (e.g., venoms, drugs).

IgE is usually produced against the protein part. Although some data suggest that the glycan part of a glycoprotein may be also allergenic (e.g., that associated with *Cupressus arizonica* pollen allergens [10]). IgE production to allergen glycan moieties may contribute to cross-reactivity (i.e., phenomenon that occurs when the antibody reacts not only with the original allergen that has caused sensitization, but also with a similar allergen) [5, 10].

The number of proteins from any given allergenic source that may be allergenic vary and sensitized patients producing IgE to a source usually recognize more than one allergenic protein. Those allergens in a source recognized by more than 50% of allergic individuals are usually termed ‘major’ but some of those considered ‘minor’ on a population basis may, of course, be of clinical significance at an individual level [5].

The impact of molecular biology and genomics on understanding of allergen structure and function over the past 25 years has been enormous. The majority of clinically significant allergens have now been sequenced and their endogenous biochemical activities determined. These advances have facilitated the determination of the three-dimensional structures of a significant number of allergens, the determination of allergen T cell- and B cell-reactive epitopes and the construction of hypoallergenic variants for use in the treatment of allergic disease (protein or DNA vaccines). However, questions regarding what is unusual about the proteins that are common allergens, as well as why only some of the people who are exposed make IgE antibodies against them, are not clearly answered yet [5].

1.1.3.1. Allergen Nomenclature

Before the arrival of detailed sequence information, the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee introduced guidelines to facilitate the consistent naming of purified allergens from complex sources [6].

The naming procedure is based on using the first three letters (although four are sometimes used to avoid confusion e.g., 'Cand' and 'Can' for *Candida* and *Canis*, respectively) of the genus source (e.g., *Dermatophagoides*) and combining it with the first one or two letters of the species name (e.g., *pteronyssinus*) followed by an Arabic numeral reflecting either the order in which the allergen was isolated or its clinical importance, or both.

Allergens from different species within a genus or across phylogenetically related genera but similar on the basis of sequence identity use the same numbering arrangement. For example, the related house-dust mite cysteine protease allergens from mites such as *Dermatophagoides pteronyssinus, D. farinae, Euroglyphus maynei* and *Blomia tropicalis* are individually referred to as Der p 1, Der f 1, Eur m 1 and Blo t 1 respectively, or collectively as the Group 1 mite allergens.

Isoallergens from the same species (>67% sequence identity) are given a suffix (00–99) (e.g., Amb a 1.01, Amb a 1.02). For allergens from the same species that differ by only a few residues, an additional two digits are used to differentiate them (e.g., Amb a 1.0101) [6].

1.1.4. Development of the allergic reaction

The allergic reaction involves two important phases, a first encounter with the allergen that leads to asymptomatic production of allergen-specific IgE (sensitization); and a second phase that occurs on re-exposure to the allergen that causes the allergic reaction and the clinical symptoms.

1.1.4.1. Priming Th2 cells that drive IgE responses

The destiny of a naïve CD4+ T (Th0) cell responding to a peptide presented by a dendritic cell (DC) is determined by: the cytokines that it is exposed to (before and during this response) and the intrinsic properties of the antigen (dose and the route of

presentation). The DCs at these sites of entry take up the antigen, efficiently process it (at the same time that they become activated) and migrate to regional lymph nodes, where differentiated into professional antigen-presenting cells (APCs) with co-stimulatory activity, they interact with Th0 cells driving them to become effector Th2 cells, the T cell type that allows IgE class switching. It is not fully understood how DCs induce the differentiation Th2 versus Th1, Th17 or even iTreg response (accessory signals and cytokines (including some chemokines) produced by activated accessory immune cells, like monocytes, that avoid the activation of some key transcription factors T-bet (Th1), RORγt (Th17) or Foxp3 (iTreg) seem to be key elements in this polarization) [2,9, 11].

Exposure to IL-4 induces the development of Th2 cells that express the transcription factor GATA-3 and produce IL-4, IL-5, IL-6 IL-9, IL-10, IL-13 and GM-CSF. These cells express cell surface receptors, which target their trafficking to allergic sites and trigger activation in settings of allergic inflammation, including the chemokine receptors CCR3, CCR4, CRTH2, CCR8 and the IL-33 receptor, T1/ST2 [9,11]. Transmucosal antigen presentation at very low doses seems also to be a particularly efficient way of inducing Th2-driven IgE responses.

Apart from their role in allergic diseases, IgE antibodies are important in host defense against infection with multicellular parasites. Therefore, this defense system is anatomically distributed mainly at the sites of potential entry of such parasites (i.e., under the skin, under the epithelial surfaces of the airways (the mucosal-associated lymphoid tissues) and in the submucosal of the gut (the gut-associated lymphoid tissues)). At these sites, cells of the innate and adaptive immune systems are specialized to secrete predominantly cytokines that drive Th2 responses and IgE production, and also amplify the polarization of the response [9, 12, 13].

1.1.4.2. Class switching to IgE

There are two main components of the immune response leading to IgE production. The first consists of signals that favor the differentiation of Th0 cells to a Th2 phenotype. The second comprises the action of cytokines and co-stimulatory signals from Th2 cells that stimulate B cells to switch to producing IgE antibodies (Figure 1.3) [6].

IL-4 and IL-13 activate the kinases Jak1 and Jak3 leading to the phosphorylation of the transcriptional regulator Stat6 present in T- and B-lymphocytes.

Mice lacking functional either IL-4, IL-13 or Stat6 have impaired Th2 responses and impaired IgE switching, therefore their role is crucial in signaling for the heavy chain class switching from IgM to IgE. The other key signal for IgE production consists on the co-stimulatory interaction between CD40 ligand (CD40L or CD154) on T-cell surface and CD40 on the B cell surface, which is actually required for all antibody class switching.

Mast cells and basophils can also amplify the already initiated IgE response. Once activated by antigen cross-linking of their FcεRI-bound IgE, these cells express CD40L on their surface and secrete IL-4 and IL-13. All of them bind to their receptors on activated B cells. Thus, they can provide both required signals for IgE synthesis. Importantly, this interaction can occur at the site of the allergic reaction, since B cells are observed to form germinal centers at inflammatory foci [6, 11, 13].

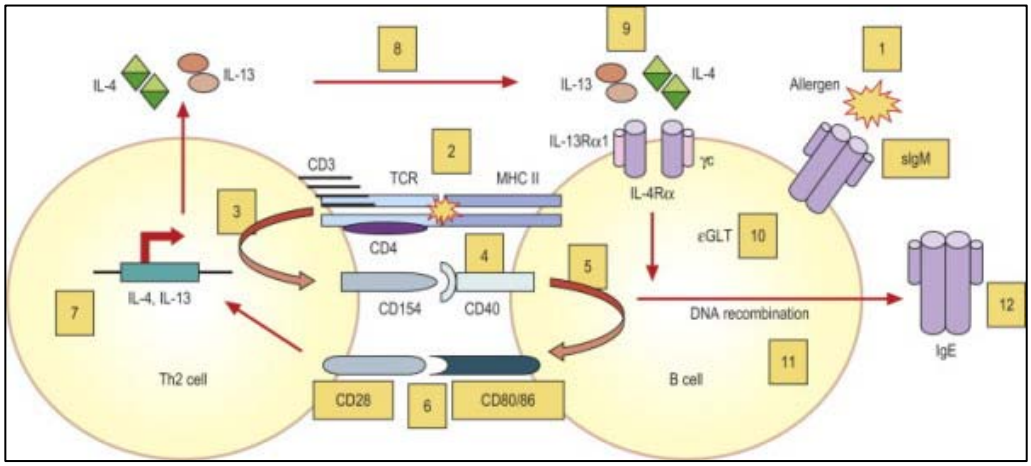

Figure 1.3. T/B cell interactions leading to IgE isotype switching. The signals required for isotype switching are provided to the B cell through a complex series of interactions with an allergen-specific T cell. A B cell that expresses IgM specific for the allergen **(1)** binds the allergen via surface immunoglobulins (sIgM), processes it and presents it to an allergen-specific Th2-like T cell **(2).** Engagement of the T cell receptor/CD3 complex by MHC class II molecules results in the rapid expression of CD154 (CD40L) **(3),** which engages CD40 on B cells **(4).** T/B cell interactions mediated via CD40/CD154 are amplified by interactions between the co-stimulatory molecules (CD28/CD80-CD86). Engagement of CD40 upregulates CD80-CD86 expression on B cells **(5).** CD80-CD86 engage CD28 **(6)** inducing high-rate transcription **(7)** and secretion **(8)** of IL-4 and/or IL-13 that will bind their heterodimeric receptors **(9).** At this stage, the B cell is receiving both signals required for IgE switching: IL-4 triggers ε germline transcription **(10),** thereby targeting the ε switch region for recombination. Cross-linking of CD40 by CD40L activates DNA recombination to the targeted ε S region **(11),** leading to IgE isotype switching and IgE secretion. From [6].

1.1.4.3. IgE receptors and allergen presentation

After class switching, IgE secreted by plasma cells binds to its receptors on cell surfaces (state termed *sensitization*, condition sine qua non for an allergic reaction to occur on reexposure to the allergen). Two receptors have been described for IgE: a high-affinity receptor (FcεRI) present on mast cells, basophils and APCs (these are Langerhans cells and interdigitating epithelial dendritic cells); and a low-affinity receptor (FcεRII or CD23) on lymphocytes, Langerhans cells, follicular dendritic cells, macrophages, monocytes, eosinophils and platelets (Figure 1.4) [6,11].

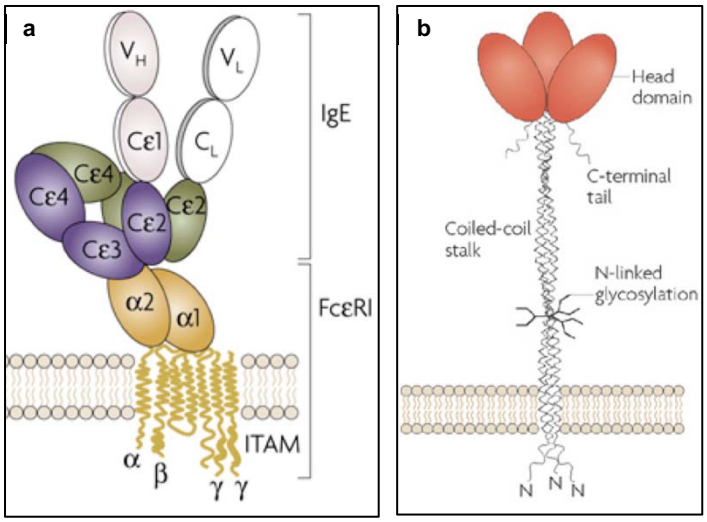

Figure 1.4. IgE receptors. a) Schematic representation of the entire IgE molecule bound to the extracellular domains of the FcεRI α-chain, according to the structural information from the FcεRI complex and the bent IgE-Fc structure. The β- and γ-chains of FcεRI, with their immunoreceptor tyrosine-based activation motifs (ITAMs), are also shown. b) A schematic representation of membrane-bound CD23 showing the extracellular trimeric α-helical coiled-coil 'stalk', the three C-type lectin domain 'heads' and the C-terminal 'tails'. N-linked glycosylation sites near the base of the stalk are also shown. Adapted from [11].

On re-exposure, allergen binding to the IgE of FcεRI on the surface of APCs leads to the presentation of allergenic peptides to Th2 cells, either after migrating to local lymph nodes or on site.

Similarly in the mucosa, allergen binds IgE bound to CD23 that is expressed by allergen-activated B cells. This process facilitates allergen presentation to T cells (termed Facilitated Antigen Presentation (FAP)). The interaction between CD23 and HLA-DR in the cell membrane is involved in the trafficking of the allergen–IgE–CD23

complex to endosomes. In the endosomes, allergens are processed and derived peptides are loaded onto HLA-DR molecules for B cell presentation. Antigen presentation through the membrane bound B-cell receptor (BcR, a surface Ig) involves the interaction of cognate B cells with Th cells (thus the number of cognate B cells is a limitation). However, FAP may overcome this limitation, as all antigen-activated B cells (that is, CD23-expressing) are able to present a variety of peptides, even from totally unrelated allergens, to cognate T cells, regardless of the specificity of the own BcR.

This is important for the phenomenon of 'epitope spreading', not only within a single allergen, but also to unrelated allergens. Thus, an antigen activated B cell expressing CD23 can in effect behave as a 'professional' APC (i.e., dendritic cell), which can simultaneously process unrelated antigens through FcγRs and cause epitope spreading to other antigens. Actually, CD23-mediated FAP is known to be as efficient as FcγR-mediated antigen presentation by dendritic cells, orders of magnitude more efficient than B-cell internalization via BcR. IL-4, IL-13 and CD40L also participate in the process by stimulating the expression of CD23 and its release [11].

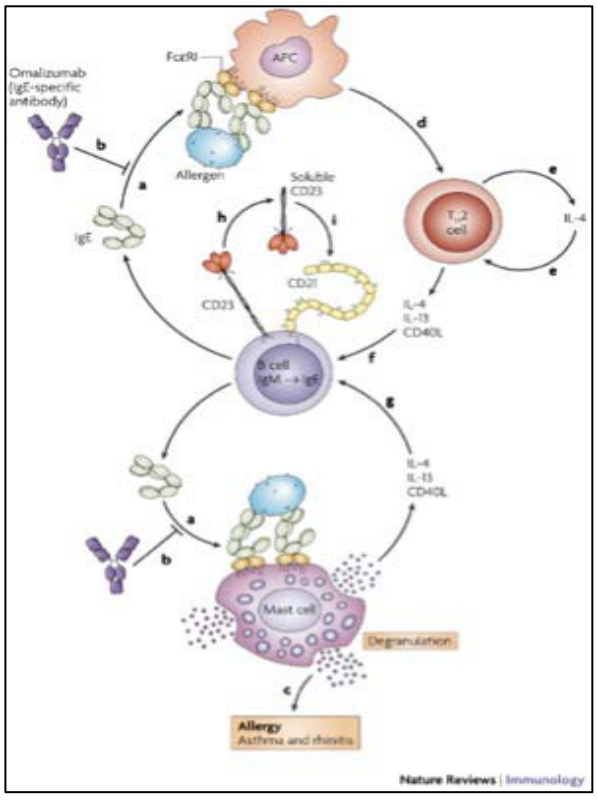

Figure 1.5. Allergen acts in pump priming of the allergic response. IgE is synthesized and secreted by B cells, binds to FcεRI on mast cells and APCs (**a**) and sensitizes these cells to allergens. Omalizumab inhibits this binding (**b**). Allergen binding to IgE triggers mast-cell

degranulation (**c**). Allergen binding to the APC leads to the presentation of allergenic peptides to Th2 cells (**d**), which secrete IL-4 (**e**) to maintain the Th2 cell lineage and recruit more Th cells into this lineage (**e**). Also they secrete IL-13 and express CD40L, which together with IL-4 stimulates class switching to IgE (**f**). Allergen-activated mast cells contribute to the production of IL-4 and IL-13 (and express CD40L), which may also stimulate class switching to IgE (**g**). IL-4, IL-13 and CD40L also stimulate the expression of CD23 and the release of soluble CD23 (**h**). In humans, soluble trimeric CD23 upregulates IgE synthesis and secretion through CD21 (**i**). From [11].

The allergen binding to IgE of FcεRI on the surface of mast cells causes cross-linking of the receptors leading to cell activation that through the corresponding signaling pathways ends up with degranulation and mediators release. Moreover, upon activation by allergens, mast cells secrete IL-4, IL-13 and CD40L that maintain Th2 cell lineage and recruit more Th cells into this lineage [12,13]. Also with IL-4 the IgE class switching is favored. Thus, the actual allergen acts in pump priming of the allergic response (Figure 1.5) [11].

1.1.4.4. Mast-cell activation pathways

Despite the fact that mast cells and basophils only share some attributes, signaling transduction pathways following FcεRI aggregation or other forms of stimulation are frequently discussed as if the mechanisms were the same for both cell types and are mainly referred to mast cells.

The manifestations of allergic reactions are considered to be mainly a consequence of the release of pro-inflammatory mediators following antigen-induced aggregation of IgE-bound to FcεRIs expressed at the mast-cell surface (Figure 1.6). However, there is increasing evidence that receptors for other ligands might markedly influence mast-cell activation in a physiological context [6].

1.1.4.4.1 FcεRI-mediated mast-cell activation

Antigen-dependent mast-cell activation is regulated by a complex series of intracellular signaling processes that are initiated following FcεRI aggregation. FcεRI is a multimeric receptor that comprises an α-chain (responsible for IgE binding), a β-chain and a disulphide-linked γ-chain homo dimer, which perform critical signal transduction functions and their intracellular domains contain immunoreceptor tyrosine-based activation motifs (ITAMs) that act as docking sites for SH2 domain containing signaling proteins.

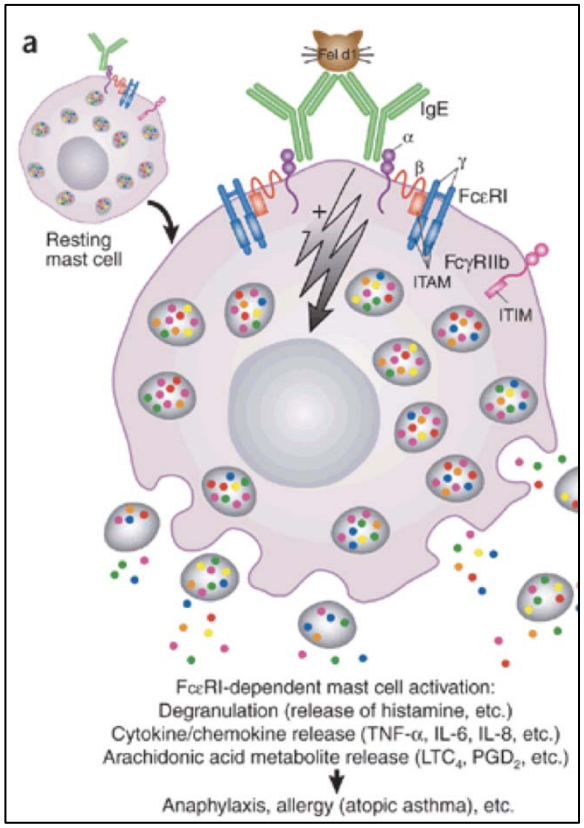

Figure 1.6. FcεRI-mediated mast-cell activation. Subjects sensitized to specific allergens, such as the cat allergen Fel d1, produce IgE antibodies specific to these allergens, which bind to high-affinity receptors (FcεRI) on the surface of mast cells and basophils. Aggregation of FcεRI, typically by the binding of di- or multivalent allergen recognized by the IgE, induces mast cell and basophil activation. Adapted from [14].

In mast cells, the main SRC family kinase that is involved in initial stages is LYN, which mainly resides in lipid rafts. The association of aggregated FcεRI, typically caused by the binding of di- or multivalent allergen recognized by the IgE, with activated LYN might be sufficient to shift the equilibrium of FcεRI from a nonphosphorylated state to a phosphorylated state, thereby initiating FcεRI-mediated degranulation.

The tyrosine residues that are phosphorylated by LYN are present in the ITAMs of the FcεRI β- and γ-chains. When phosphorylated, they provide high-affinity docking sites for the SH2 domains of LYN and for the SH2 domains of the ZAP70 (ζ-chain-associated protein kinase of 70 kDa)-related tyrosine kinase SYK (spleen tyrosine kinase), respectively. The subsequent SYK- and/or LYN-mediated tyrosine phosphorylation of the transmembrane adaptor molecule LAT (linker for activation of T cells) is crucial for coordination of the downstream signaling pathways.

Phosphorylation of LAT results in the recruitment of several types of molecules: cytosolic adaptor molecules, such as GRB2 (growth-factor-receptor-bound protein 2), GADS (GRB2-related adaptor protein), SHC (SH2-domain-containing transforming protein C) and SLP76 (SH2-domain-containing leukocyte protein of 65 kDa); guanine-nucleotide-exchange factors and adaptor molecules, such as SOS (son of sevenless homologue) and VAV; and signaling enzymes, such as phospholipase Cγ1 (PLCγ1). These interactions with LAT result in the formation of a macromolecular signaling complex. The four terminal tyrosine residues in LAT (Y132, Y171, Y191 and Y226) are crucial and sufficient for the ability of LAT to regulate signaling in mast cells and ultimately their degranulation.

The main signaling enzyme regulated by both direct and indirect interactions with these tyrosine residues is PLCγ. When activated, it catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) in the plasma membrane. The resulting products, inositol-1,4,5-trisphosphate (InsP3) and diacylglycerol (DAG), induce mobilization of cytosolic calcium and activation of protein kinase C (PKC), respectively. Since the calcium signal induced by InsP3 is transient, it is the calcium sequestered from extracellular stores by capacitive entry, as a consequence of depletion of intracellular stores, which allows the signal to be sustained. The PLCγ-dependent increases in both cytosolic free calcium and PKC activation are essential signals for degranulation to proceed.

The sequence of events that leads from LAT to cytokine production has not been as clearly defined as the sequence that leads to degranulation. However, the pathways that lead to cytokine-gene expression require the guanine-nucleotide-exchange factors VAV and SOS to activate RAS. After it has been activated, RAS positively regulates the RAF-dependent pathway that leads to phosphorylation and, in part, activation of the mitogen-activated protein kinases (MAPKs) extracellular-signal-regulated kinase 1 (ERK1) and ERK2.

The MAPKs JUN amino terminal kinase (JNK) and p38 are similarly activated in a LAT-dependent manner in mast cells, but the mechanism(s) that regulates these responses is less well-defined. These molecules (ERK1, ERK2, p38 and JNK), in turn, activate transcription factors (including the activator protein 1 (AP1) components (FOS and JUN), nuclear factor of activated T cells (NFAT) and nuclear factor-κB (NF-κB)) leading to cytokine generation (Figure 1.7) [6, 14-17].

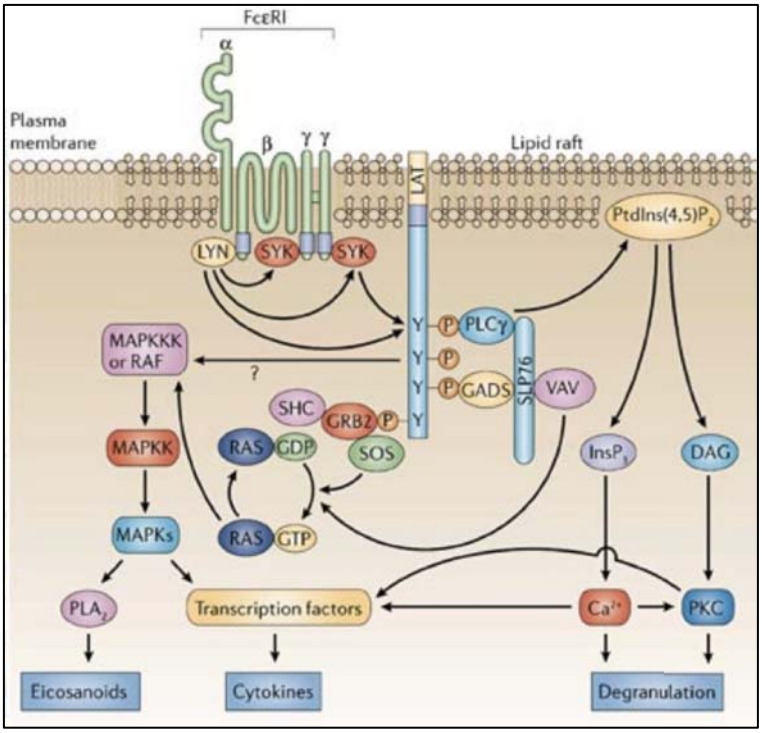

Figure 1.7. Signaling cascade in activated mast cells. For clarity, only one high-affinity receptor for IgE (FcεRI) is shown. DAG, diacylglycerol; $InsP_3$, inositol-1,4,5-trisphospha-te; PLA_2, phospholipase A2; PtdIns(4,5)P_2, phosphatidylinosi-tol 4,5-bisphosphate. From [16].

1.1.4.5. Inflammatory responses after mast cell activation

Classically, mediator secretion is considered the measured outcome of cell activation and leads to inflammatory reactions.

For mast cells, it is possible to define three types of secretion, each one with characteristic mediators: (1) rapid from pre-formed pools stored in the secretory granules (e.g. histamine, neutral proteases, preformed cytokines and proteoglycans), (2) rapid but newly synthesized lipid mediators that are the products of endogenous arachidonic acid metabolism (e.g. PGD2, LT-B4 and -C4, the parent molecule of the cysteinyl-LTs) and (3) slow but newly synthesized proteins (e.g., proinflammatory cytokines, chemokines and growth factors) [6].

In basophils, at least two major non-cytotoxic degranulation patterns have been described depending on the type of stimulus used: 1) anaphylactic degranulation, a very rapid and explosive event that is characterized by a regulated granule extrusion by exocytosis that can ultimately result in an intact cell that is completely degranulated. As expected, this pattern is common with IgE-mediated degranulation resulting from

the binding of specific antigen or anti-IgE antibody; 2) piecemeal degranulation, initially used to describe the pattern of degranulation observed in basophils found in certain cell-mediated pathological conditions, including contact dermatitis, skin graft rejection, Crohn's disease, ulcerative colitis and irritable bowel syndrome. It is characterized by an induced vesicular transport of granular content that does not involve direct granule extrusion. Various cytokines and chemokines that modulate basophil activity are believed to induce this type of degranulation [18].

The net result of the mediators release process includes: first, an immediate (starting within seconds) allergic reaction, mainly due to the activity of histamine, PGs and other preformed or rapidly synthesized mediators that cause the rapid increase in vascular permeability (plasma extravasation, tissue edema) and the contraction of smooth muscle (bronchoconstriction). Second, after 8-12 hours, a more sustained inflammation process, known as the late phase response, that takes place and is caused by the induced synthesis and release of mediators including PGs, LTs, chemokines and cytokines from the activated mast cells.

This late response involves the recruitment of other effector cells, notably Th2 lymphocytes, eosinophils and basophils, which contribute significantly to the immunopathology of the allergic response. Moreover, a second phase of smooth muscle contraction mediated by T cells occurs, with persistent inflammation, sustained edema and tissue remodeling. The late-phase reaction and its long-term sequel, chronic allergic inflammation, contribute to a much serious long-term illness, as, for example, chronic asthma (Figure 1.8) [6].

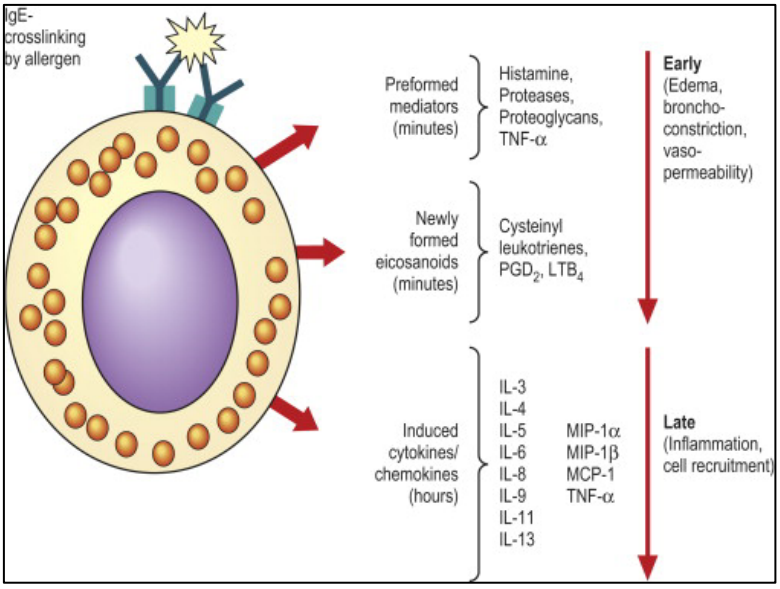

Figure 1.8. Mediators released from mast cells upon IgE-mediated activation. Upon cross-linking of FcεRI-IgE by allergen, mast cells immediately release preformed mediators from storage in secretory granules via exocytosis. Concomitantly, leukotrienes and PGD2 are generated from arachidonic acid, and cytokine and chemokine production is induced. From [6].

1.1.5. Clinical manifestations of the allergic response

The consequences of the IgE mediated mast-cell activation depend on the dose of antigen and its route of entry.

There are two main anatomical distributions of mast cells, those associated with vascularized connective tissues (connective tissue mast cells) and those found in submucosal layers of the gut and the respiratory tract (mucosal mast cells). The overall response to an allergen depends on which mast cells are activated. Allergen in the bloodstream activates connective tissue mast cells throughout the body, resulting in the systemic release of histamine and other mediators. Whereas, subcutaneous administration of allergen activates only local connective tissue mast cells, leading to a local inflammatory reaction. Inhaled allergen, penetrating across epithelia, activates mainly mucosal mast cells, causing smooth muscle contraction in the lower airways, leading to bronchoconstriction and difficulty in expelling inhaled air. Similarly, ingested allergen penetrates across gut epithelia, causing vomiting due to intestinal smooth muscle contraction and diarrhea due to outflow of fluid across the gut epithelium. Food allergens can also be disseminated in the bloodstream, causing urticaria (hives) when the food allergen reaches the skin or generalized systemic reactions (anaphylaxis) [6].

1.2. Food Allergy

1.2.1. Definitions

A *food allergy* is defined as "an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food" [19]. In 2001, the European Academy of Allergy and Clinical Immunology (EAACI) Nomenclature Committee revised the definitions of adverse reactions to food and proposed a new nomenclature that was reassured by the World Allergy Organization (WAO) Nomenclature Committee in 2003. Briefly, they propose that any adverse reaction to food should be defined as *food hypersensitivity*; among these, the reactions mediated by immune mechanisms are defined as *food allergy*, whereas the ones without an immune mechanism are considered together as *non-allergic food hypersensitivity* (previously known as *intolerance*). Food allergy is further classified as IgE- or non-IgE mediated (Figure 1.9). The IgE-mediated are the most frequent and account for the majority of well-characterized food allergic disorders, although a number of non-IgE-mediated immune reactions, especially in the gastrointestinal tract, have been delineated [5].

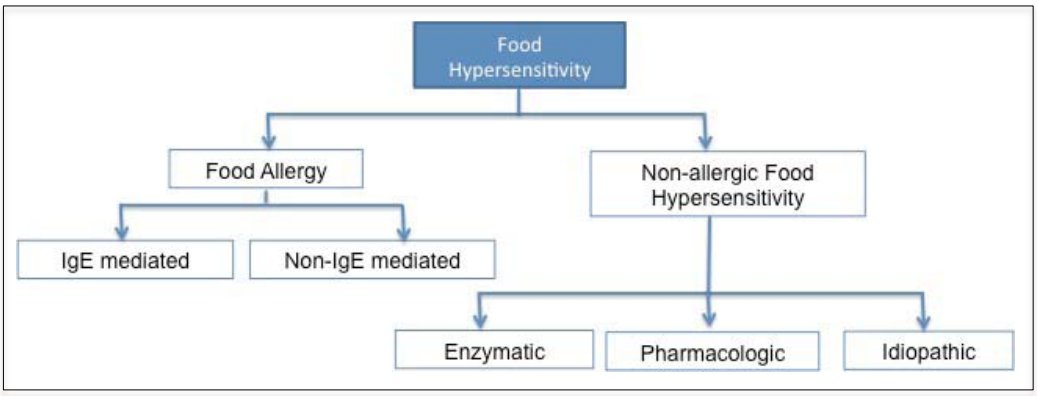

Figure 1.9. Classification of food hypersensitivity reactions. Adapted from [5].

Importantly, food allergy must be distinguished from a variety of adverse reactions to foods that do not have an immune basis but may resemble it in clinical manifestations. They comprise the majority of adverse reactions to foods and may be due to factors inherent in food ingested, such as toxic contaminants, pharmacologic properties of the food, metabolic disorders and/or idiosyncratic responses of the host. Food aversions may mimic adverse food reactions, but typically cannot be reproduced

when the patient ingests the food in a blinded fashion. Examples of non-allergic adverse reactions to food are: lactose intolerance, pancreatic insufficiency, food poisoning, caffeine and panic disorder among others.

1.2.2. Prevalence and epidemiology

Food allergy is common and might be increasing in prevalence representing an important public health problem. Food allergy significantly affects quality of life, especially due to the difficulties associated with food avoidance, which is the primary treatment of food allergy [20, 21]. Epidemiologic studies based on food challenges indicate that 1 to 10.8% of the general population have immune-mediated nontoxic food hypersensitivity [22]. However, the actual prevalence is difficult to determine, mainly due to a lack of accurate controlled population-based studies using the gold standard procedure for food allergy diagnosis, the double blind placebo controlled food challenge (DBPCFCs), but also because of innumerable factors such as misclassification, biased participation, lack of simple diagnostic tests, rapid evolution of disease, large numbers of potential triggers and varied clinical phenotypes [20].

Studies to address the reasons for the increased prevalence and persistence of food allergies have included the hygiene hypothesis, changes in the components of the diet, the use of antiacids (resulting in exposure to more intact protein), food processing and/or extensive delay of oral exposure [23].

1.2.3. Natural history of food allergy

Natural history of food allergy refers to the evolution of this disease from the very beginning with the allergen sensitization to the potential lost of this sensitization. It needs to be taken into account not only the development or not of tolerance to the allergenic source causing the first allergy, but also the potential development of new sensitizations to food allergens and/or aeroallergens [5].

The prevalence of food hypersensitivity is greatest in the first few years of life. Food allergy in early infancy is generally transient, however, several studies have shown that is also one of the first manifestations of the process known as “atopic progression”, in which the first manifestation would be the atopic dermatitis and, after a food allergy, the respiratory allergy would be developed [5]. Most young children allergic to cow’s milk and egg ‘outgrow’ their food allergy (become tolerant) within a few

years, except in the majority of cases of peanut, tree nut and seafood allergy that persist into adulthood. Eighty-five percent of milk allergic children and 66% of egg allergic children become food tolerant by age 5 years. In contrast, only approximately 20% of all children with peanut allergy become peanut tolerant. It appears that the natural history of allergy to seeds, fish and shellfish are similar to peanut [24]. Although younger children are more likely to 'outgrow' their food allergies, it is apparent that older children and adults also may lose their reactivity if the responsible food allergen is identified and eliminated from the diet. Approximately one-third of children and adults will lose their clinical reactivity after 1-2 years of allergen avoidance [25]. Puncture skin test and serum specific IgE results typically remain positive and do not predict which patients will lose their clinical reactivity.

Most non-IgE-mediated gastrointestinal food allergies occur in infants and are outgrown in the first 2–3 years of life. However, allergic eosinophilic gastroenteritis is frequently seen in adults and the number of cases developing in young children and adolescents appears to be increasing. Long-term studies have not been completed, so the prognosis of this disorder remains to be determined. No formal studies on the natural history of non-IgE-mediated cutaneous or respiratory disorders have been undertaken, but these sensitivities are believed to be long lasting.

Food allergy in young children may be viewed as a marker of an atopic predisposition. In many children, food allergy coexists with other atopic conditions, such as atopic dermatitis, asthma and allergic rhinitis. Sensitization to egg white in children with atopic dermatitis and a family history of atopy is associated with a 70% risk for respiratory allergic disease (asthma or allergic rhinitis) at 5 years of age [26]. Therefore, subjects with past and current food allergy should be considered at high risk for asthma and environmental allergy.

1.2.4. Routes of sensitization

Sensitization to specific proteins within food allergens may occur in the gastrointestinal tract (oral route) or as a consequence of a primary allergic sensitization to inhalant allergens. The immunologic mechanisms responsible for the development of allergic sensitization rather than tolerance to foods are not well understood, although there have been a number of recent advances in the understanding of why some foods are inherently allergenic [5].

In 2000, Breiteneder and Ebner suggested a classification of food allergy based on the immunological mechanisms involved, the sensitization route and the pattern of allergens implicated [27]. Class I food allergy (also viewed as “traditional”) is the one that occurs due to sensitization through the oral route and is due to class I allergens, which are hidrosoluble glycoproteins of 10-70kDa, very stable and resistant to heat, acid and proteases (gastric digestion). Typical allergens of this class are: egg proteins, milk proteins, fish parvalbumin, shellfish tropomyosin, lipid transfer proteins in plant-foods, etc. Class II food allergy occurs as a consequence of a primary allergic sensitization to inhalant allergens, given the existence of common epitopes between the food allergen and the aeroallergen. Thus, the food allergen is able to cause an allergic reaction but not to induce sensitization (i.e., incomplete allergens). Most class II allergens are thermolabile proteins, susceptible to enzymatic degradation. Examples are: raw carrot, apple, melon secondary to birch or ragweed pollen sensitivity, respectively [5].

1.2.4.1. Oral tolerance

Despite the large extent of dietary antigenic exposure, only a small percentage of individuals have food allergy. This is due to development of oral tolerance to dietary proteins, which refers to a state of active inhibition of immune responses to an antigen by means of prior exposure to that antigen through the oral route [28]. However, in a susceptible host, a failure to develop or a breakdown in the process of oral tolerance may result in hypersensitivity responses to ingested food antigens. Thus, once food enters the gastrointestinal tract, three distinct immune responses may occur: a) systemic tolerance for cellular and humoral immunity, which constitutes the normal response; b) local immune reaction at the mucosa with IgA production; or c) systemic activation that can affect both arms of immunity, humoral and cellular (food hypersensitivity). Several factors affect the induction of oral tolerance to a dietary antigen. Some are antigen related, namely the dose and nature of the antigen. Other factors are inherent to the host, including age, genetics and intestinal flora [5].

Briefly, oral tolerance can be divided into two forms, each with a distinct mechanism. One due to exposure of high doses of oral antigen (in which T-cell receptor cross-linking can occur in the absence of costimulation or in the presence of inhibitory ligands (CD95 and CD95 ligand), leading to anergy or deletion, respectively) and the other one due to exposure to low doses of oral antigen leading to the activation

of regulatory T cells, which suppress immune responses through soluble or cell surface–associated suppressive cytokines (IL-10 and TGF-β) (Figure 1.10). In the latter mechanism, the antigen/allergen (after suffering modifications in the gut lumen) contacts with the APC, mainly DCs residing in the gastrointestinal lamina propria, that capture the antigen, migrate and initiate oral tolerance in the draining lymph node by activation of antigen-specific T cells with a regulatory phenotype (there is a lack of consensus about the phenotype of these regulatory T cells: CD4+CD25+, T_H3, T_R1, CD8+ and/or Natural Killer T cells). Then, regulatory T cells migrate back to the lamina propria, resulting in a suppression of the immune response [5, 28].

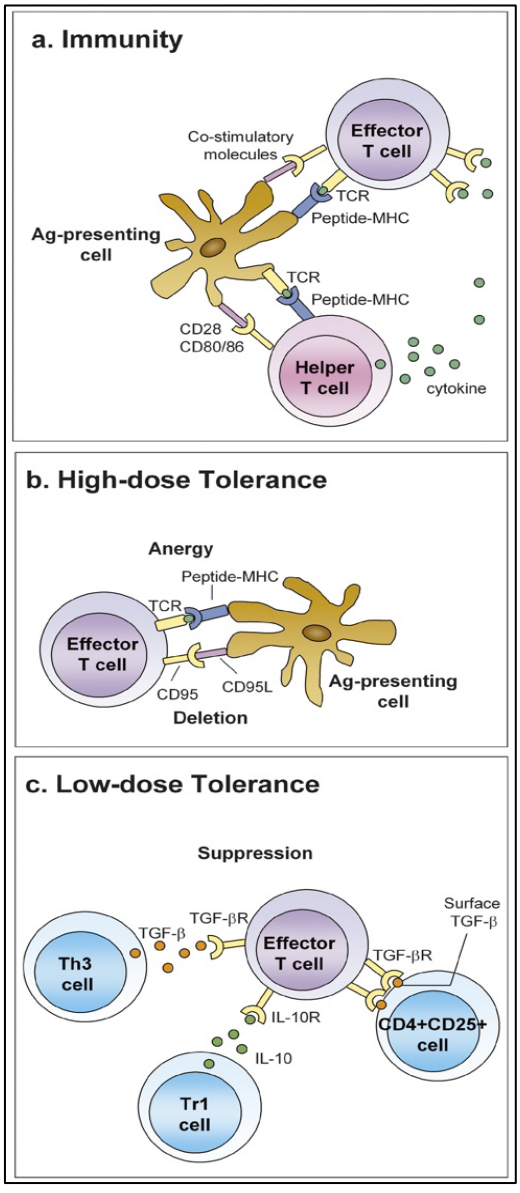

Figure 1.10. Mechanisms of oral tolerance. a) Generation of an immune response, which requires ligation of the T-cell receptor with peptide-MHC complexes in the presence of appropriate costimulatory molecules and cytokines. b) Effect of high doses of oral antigen. c) Effect of low doses of oral antigen. From [28].

1.2.4.2. Potential role of non-inherently tolerogenic routes

Emerging data suggest that allergic sensitization may occur if the primary route of exposure is not the naturally tolerogenic oral route. The observation that it is possible to sensitize mice to food allergens through the skin without an adjuvant suggests that the skin may be an important route of sensitization. In humans, household exposure to peanut has been shown to be associated with allergic sensitization to peanut in children, independent of maternal ingestion. However, the assumption that the skin is inherently allergenic cannot be made, since tolerance can also be induced via skin exposure [29, 30].

1.2.4.3. Food allergens activate the innate immunity

Several food allergens have been shown to directly activate various components of the innate immunity that may influence the adaptive immune response to food allergens, promoting or suppressing allergic sensitization. One example of such effect are soy isoflavones, which directly suppress gastrointestinal DCs [31]. This fact has been proposed as an explanation for soy being a weaker food allergen than peanut allergens despite the homology between them (Figure 1.11).

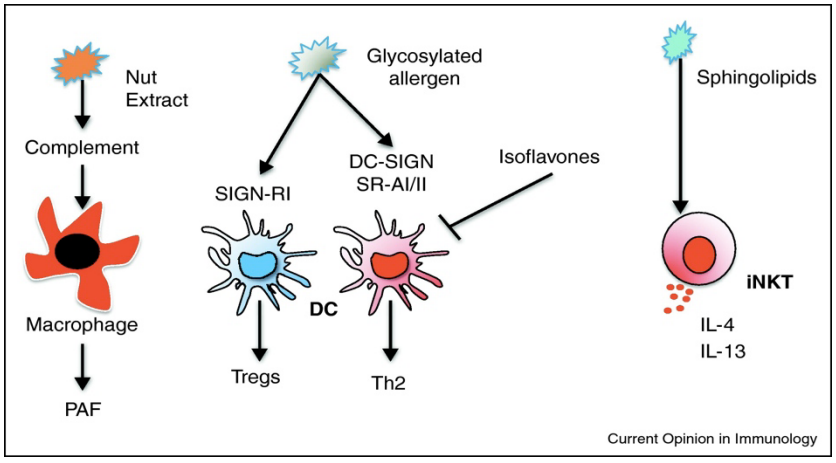

Figure. 1.11. Food allergens activate the innate immunity. Nut extracts activate complement, leading to macrophage activation and release of platelet activating factor (PAF). Allergens with different glycosylation patterns can bind to innate receptors: SIGNR1 on dendritic cells (DCs) promotes the generation of regulatory T cells and DC-SIGN or the scavenger receptor-alpha type I or II (SR-AI/II) alters the phenotype of the DC to promote the generation of Th2 cells. Isoflavones from soy suppress DC activation. Sphingolipids found in milk can directly act on

invariant NKT (iNKT) cells, leading to preferential release of the Th2 cytokines (IL-4 and IL-13). From [29].

Innate activity of allergens does not explain why only some individuals become sensitized to foods. Gastrointestinal epithelial cells at the interface between the gastrointestinal contents and the mucosal immune system are host factors that likely determine the immune response to foods. Supporting evidence for this hypothesis in humans is that epithelial cells from food allergic subjects express higher levels of galectin-9 that can act on DCs to promote allergic sensitization. In mice, the epithelial cytokine thymic stromal lymphopoietin (TSLP) has been described critical for gastrointestinal but not systemic manifestations of food allergy. Indeed, mutations upregulating TSLP expression are associated with eosinophilic esophagitis but the relationship to IgE-mediated food allergy has not yet been addressed in humans [29].

1.2.5. Food allergens

Food allergens are defined as “those specific components of food or ingredients within food (typically proteins, but sometimes also chemical haptens) that are recognized by allergen specific immune cells and elicit specific immunologic reactions, resulting in characteristic symptoms” [19]. Although an allergy can be triggered by virtually any food, major allergens sources responsible for most significant reactions include milk, egg, peanut, tree nuts, fresh fruits, shellfish, fish, wheat and soy. Allergy to additives and preservatives is generally uncommon [23] (Figure 1.12).

Figure 1.12. Major food allergen sources. From: http://freeblogspot.org/blog/food-allergy-and-intolerances/

In food allergy, cross-reactivity occurs when a food allergen shares structural or sequence similarity with a different food allergen or aeroallergen, which may then trigger an adverse reaction similar to that triggered by the original food allergen. This phenomenon rarely occurs between allergens that have an identity below 50% and in most cases it requires an identity above 70%. It is common, for example, between plant-foods and pollens (Bet v 1 homologues), among different shellfish and among different tree nuts [5].

Some allergens (most often from fruits and vegetables) cause allergic reactions primarily if eaten when raw. However, most food allergens can still cause reactions even after they have been cooked or have undergone digestion in the stomach and intestines. It has been shown that allergens that are stable to heat and digestion are more likely to cause a severe clinical reaction, whereas heat and digestion labile allergens are more likely to be tolerated or only cause milder/local symptoms. Food preparation can also affect allergenicity. For instance, the high temperature of roasting (180ºC) peanuts leads to a Maillard reaction or the emulsification procedure to obtain (peanut butter) appears to increase stability and allergenicity [23].

Additional characteristics of the manner in which foods are ingested might be relevant. For example, recent studies suggest that 70% to 80% of young children allergic to milk or eggs can tolerate baked (heat-denatured) forms of the protein but not the unbaked form. It is suggested that these children make IgE antibodies primarily to conformational epitopes on the food proteins and represent the children who will naturally outgrow their food allergies [32].

1.2.6. Pathophysiologic mechanisms

Generally, food allergic disorders involve more than one of the classic mechanisms of hypersensitivity reactions described by Gell and Coombs. Therefore, it is conceptually and diagnostically helpful to categorize food-induced allergic disorders based on immunopathology among those that are mediated or not mediated by IgE antibodies [23].

1.2.6.1. IgE-mediated food hypersensitivity

The best characterized food allergic reactions involve IgE-mediated responses. After a first phase of sensitization that implies IgE production, an effector phase is

going to happen on reexposure to the culprit allergen. The effector phase is characterized by an acute phase (with an immediate onset after ingestion) a potential late phase and a chronic phase.

Briefly, when food allergens penetrate mucosal barriers and reach IgE antibodies bound to mast cells or basophils, mediators are released within seconds to minutes and vasodilatation, smooth muscle contraction and mucus secretion is induced, resulting in symptoms of immediate hypersensitivity (acute phase). The activated mast cells also may release a variety of cytokines, which may contribute to the IgE-mediated late-phase response. During the initial 4-8 hours, primarily neutrophils and eosinophils are recruited to the site of response. These first infiltrating cells are activated and release a variety of mediators including PAF, peroxidases, eosinophil major basic protein and eosinophil cationic protein. In the subsequent 24-48 hours, lymphocytes and monocytes infiltrate the area and an inflammation is established. Successive repetitions of late-phase responses contribute to the establishment of a chronic allergic inflammation (chronic phase). Moreover, repeated ingestion of a food allergen stimulates mononuclear cells to secrete histamine-releasing factor (HRF), a cytokine that interacts with IgE molecules bound to the surface of basophils (and perhaps mast cells) and increases their releasability. The 'spontaneous' generation of HRF by activated mononuclear cells *in vitro* has been associated with increased bronchial hyperreactivity in patients with asthma and increased cutaneous irritability in children with atopic dermatitis [6].

1.2.6.2. Non-IgE mediated food hypersensitivity

Type II antigen-antibody dependent cytotoxic reactions occur when specific antibody binds to a surface tissue antigen or hapten associated with a cell and induces a direct effect (such as complement activation, where complement activation products promote the generation of various inflammatory mediators that lead to subsequent tissue damage). This effect is defined by the specificity of the recognition of the antibody. A few reports have implicated an antibody-dependent thrombocytopenia secondary to the ingestion of milk. However, little evidence supports any significant role for type II hypersensitivity in food allergic disorders [6].

Type III antigen-antibody complex-mediated hypersensitivity has been implicated in patients with a variety of complaints and elevated serum food antigen-antibody complexes. However, food antigen-antibody complexes have been

demonstrated in the sera of normal individuals as well as patients with suspected food hypersensitivity. For instance, the complexes formed by the interaction of IgG, IgA or IgM antibodies to β-lactoglobulin are found 1–3 hours after ingesting milk in normal children and adults [6].

Type IV and extended type IV cell-mediated hypersensitivity has been implicated in food allergic disorders with a delayed onset of clinical symptoms (several hours after ingestion). Ingestion of the sensitizing antigen may provoke mucosal lesions. In humans, a few investigators have found increased lymphocyte proliferation to food antigens in food allergic individuals, but increased proliferation is found in many asymptomatic subjects as well. Cell-mediated hypersensitivity reactions contribute to a number of gastrointestinal disorders, such as allergic eosinophilic esophagitis and gastroenteritis, atopic dermatitis and celiac disease [6].

1.2.7. Clinical manifestations of food allergy

Unfortunately, there is no pathognomonic symptom of food allergy, since the patient may present a wide range of allergic symptoms, from very mild (not even perceptible as pathologic) to death [5].

A variety of symptoms have been associated with IgE-mediated allergic reactions involving the skin, the gastrointestinal (GI) and/or respiratory tracts, and/or the cardiovascular system. Reactions can be either generalized (urticaria, angioedema, hypotension, shock, anaphylaxis or food-dependent exercise-induced anaphylaxis); or restricted to the skin (flushing, pruritus, rash, acute contact urticaria), to the respiratory tract (ocular pruritus and tearing, nasal congestion, pharyngeal edema and wheezing, rhinoconjunctivitis and acute bronchospasm) and/or to the GI tract (lip, tongue, and palatal pruritus and swelling, laryngeal edema, vomiting and diarrhea). It is not known why foods provoke different constellations of symptoms in different individuals. Atopic dermatitis and chronic airway hyperreactivity (asthma) involve activation of other cell types through IgE-mediated mechanisms [5, 6].

1.2.7.1. Local manifestations

Local gastrointestinal food allergic disorders mainly include: Immediate GI allergic reactions (acute GI hypersensitivity) and Pollen-Food Allergy Syndrome (or Oral Allergy Syndrome, OAS).

In acute GI hypersensitivity, symptoms generally develop within minutes to two hours of consuming the responsible food allergen and consist of nausea, abdominal pain, cramps, emesis and/or diarrhea (typically in conjunction with cutaneous and/or respiratory symptoms). It is shown at any age and the prognosis is variable depending on the allergen. Local vasodilation, edema, mucus secretion and petechial hemorrhaging have been observed by endoscopy. After adverse food reactions leading to diarrhea, increased stool and serum PGE2 and PGF2 have been detected.

The OAS is elicited by a variety of plant-food allergens cross-reacting with their homolog proteins in pollens (aeroallergens). Symptoms are limited almost exclusively to the oropharynx (pruritus, tingling, erythema and/or angioedema), rarely involving other target organs, after ingestion of raw fresh fruits and vegetables (usually cooked forms are well-tolerated since the allergens causing these reactions are labile). Any age group can suffer from OAS, although it is most common in young adults. It is estimated that it affects up to 50-70% of adults suffering from pollen allergy (especially, birch, ragweed and mugwort pollens) with exacerbated symptomatology during the pollen season.

Short of breathiness due to glottis edema, bronchospasm or both, are infrequent as isolated presentation of food allergy and are usually associated to severe generalized reaction; however they may occur. Moreover, food can also induce respiratory symptoms by inhalation of volatile allergens (e.g., cooking steam, pulverization, etc.) at home, at work (occupational food allergy) or due to environmental contamination. Thus, respiratory symptoms need to be also considered in food allergy [5,6].

1.2.7.2. Generalized manifestations

Anaphylaxis is currently defined as “a serious allergic reaction that is rapid in onset and might cause death” [33].

Food-induced anaphylaxis is the leading single cause of anaphylaxis treated in emergency departments in the USA, especially in childhood [34,35]. It usually manifests within one hour of exposure, but the onset of symptoms may also occur a few hours afterwards, possibly related to a less severe reaction or delayed absorption of the food. Symptoms are most commonly seen in the skin (urticaria, angioedema, pruritus, flushing) and respiratory tract (cough, difficulty breathing, wheezing), whereas the cardiovascular system is less often affected compared to anaphylaxis of other

causes, especially in children. Importantly, the clinical presentation (onset of symptoms, clinical severity and sequence of symptom progression) can differ between individuals and between reactions in the same individual and is likely dependent on concomitant factors, such as the amount of food ingested, consumption to an empty versus full stomach, concurrent illness, exercise, alcohol, medications, menstruation, among others. Asthmatics, adolescents and those with a prior reaction are at increased risk for more severe reactions. However, also patients without a known allergy may be at risk for food-induced anaphylaxis. Most of the anaphylactic reactions occur to ingested food allergens; however, anaphylaxis to inhaled food allergens have also been reported including fish, shellfish, seeds, soybeans, cereal grains, egg, milk, and other foods in the form of allergen flour in the air and vapors during cooking or roasting.

Currently, total tryptase level is the most commonly measured marker to establish a diagnosis of anaphylaxis. Tryptase levels increase immediately, peak at 1–2 hours and return to baseline 24 hours after complete resolution of symptoms. Levels are ideally obtained within 3 hours of onset of symptoms and serial measurements may help establish the diagnosis. However, lack of tryptase elevation is also commonly seen probably due to a slow onset of reactions or because mucosal mast cells and basophils, the major players in food-induced anaphylaxis, contain less to no tryptase as compared with skin mast cells.

The mainstay of treatment of any anaphylactic reaction is the timely intramuscular administration of epinephrine into the lateral thigh. Adjunctive therapies include H1-antihistamine, which may relieve skin symptoms and rhinorrhea, H2 blockers, oxygen, bronchodilators, and corticosteroids, given with the goal of preventing or ameliorating a late phase reaction, although their role here has not been proven [36].

The skin is one of the more frequent targets in food allergy. Acute urticaria is characterized by transient pruritus and erythema lesions distributed to any part of the skin with less than 24 hours of evolution. Sometimes, urticaria can be presented with localized edemas, generally in the face, eyes, lips and limbs, cursing as angioedema. Both are frequently observed in food allergy, but they are unspecific symptoms and with multiple potential triggers [5].

1.2.8. Role of cofactors in food allergy

The severity with which the food allergy is expressed, or even more, its clinical manifestation in a sensitized patient after exposure to the culprit allergen, depends on several factors either related to the food or to other concomitant ones, such as exercise or the previous intake of a non-steroidal anti-inflammatory drug (NSAID).

Both have been described as risk factors for developing a severe episode of food-induced anaphylaxis. Recently, it has been published that NSAIDs intake is present in 22% of the food allergy induced anaphylactic shocks, representing a risk factor with an odds ratio >11 [37]. Thus, if the drug and the food intake temporarily coincide, the drug can exacerbate the allergic response towards the food, leading to an anaphylactic episode or, even more, the drug may be a required cofactor for such clinical manifestation of the food allergy. This clinical event is known as anaphylaxis in the context of NSAIDs-induced food allergy or Food-Dependent Aspirin-Induced Anaphylaxis, FDAIA). However, this role as a cofactor is not universal in all food allergy patients, since a severe anaphylactic reaction can be induced without a previous intake of NSAID, as well as, these drugs not always exacerbate a mild symptomatology manifested by the food allergic patient.

Limited studies in the literature demonstrate this synergistic effect and even less go into detail on the physiopathogenesis. Currently, two main hypotheses are intended to explain the reason of this clinical event. Briefly, on one hand it is stated that the NSAID increases the permeability of the intestinal membrane causing a major absorption of the culprit allergen [38]. On the other hand, there is the hypothesis that NSAIDs have a direct effect on the mast cell and the basophil amplifying its degranulation response [39]. A recent study supports the latter, showing in mice that NSAIDs are capable of increasing the IgE-mediated degranulation of mast cells after a specific IgE stimulus [40]. Also, an increase of the membrane permeability could not be caused only by the effect of NSAIDs, but also by the degranulation of mast cells and basophils [41], which would lead to a “mix-hypothesis”. Therefore, the ones raised are not contrary hypothesis, allowing the mast cell and the basophil to be the common denominator in both of them.

1.2.9. Food allergy diagnosis

1.2.9.1. Clinical history and physical examination

The evaluation requires a thorough history and physical examination to consider a broad differential diagnosis, to ascertain possible trigger foods, and to determine a likely general pathophysiologic basis, specifically whether the food-induced allergic disorder is likely IgE-mediated or not, which will guide testing.

The clinical history should determine the possible causal food(s), quantity ingested, time course of reaction, concomitant factors (exercise, drugs and alcohol) and reaction consistency. Moreover, it also focuses on details that might contribute to estimating the prior probability of an allergic reaction to a specific food. For example, reasoning dictates that a food ingested infrequently is more likely responsible for an acute reaction than one previously tolerated; that contamination of a meal by a previously diagnosed allergen should be considered ahead of a less likely explanation, such as development of a new allergy to a previously tolerated food; and that major allergens are inherently more likely to be triggers than other foods.

To arrive at a diagnosis, the clinician should consider the epidemiologic aspects of the disease (i.e., common triggers and common associations) and the details of the specific history. Afterwards, and in the context of these prior observations, the appropriate testing has to be considered [23, 42].

1.2.9.2. Detection of food specific IgE

1.2.9.2.1. Skin prick test

The skin prick test (SPT) introduces a tiny amount of allergen into the skin to detect presence or absence of allergen-specific IgE in the surface of mast cells. Therefore it only applies for the diagnosis of IgE mediated allergic reactions. The procedure is quick, inexpensive and considered generally safe, although anaphylactic reactions due to testing have occasionally been reported. The test can be carried out on all age groups including babies, although the response will be considerably smaller than in an adult.

Allergens tested are selected in accordance with the patient's clinical history. A drop of the allergen (extract) solution is placed in the inner forearm and the skin is then

pricked through the drop using the tip of a lancet (Figure 1.13). Puncture allows penetration of the extract on the superficial layers of the skin. With a positive test to an allergen (defined as a mean wheal diameter 3 mm above the negative control [43]), the skin becomes itchy within a few minutes and then becomes red and swollen with a "wheal" in the center. Importantly, the size of the wheal does not indicate the severity of symptoms. Negative SPT responses essentially confirm the absence of IgE-mediated allergic reactivity (negative predictive accuracy, >90%). However, a positive test response does not necessarily prove that the food is causal (specificity, <100%) and merely shows sensitization.

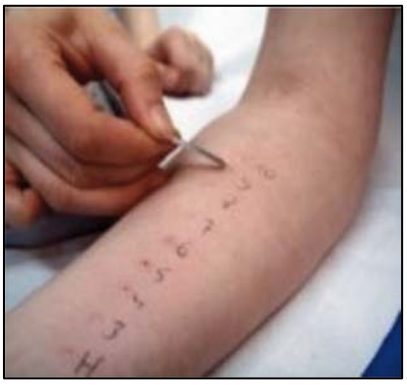

Figure 1.13. Skin Prick Test. A drop of the allergen (extract) solution is placed in the inner forearm and the skin is then pricked through the drop using the tip of a lancet. From: Sciencephoto.com.

The negative control is a saline (salt-water) solution to which a response is not expected. If a patient reacts to the negative control, then this will indicate that the skin is, for whatever reason, extremely sensitive and that the results need to be interpreted accordingly. The positive control solution contains histamine, to which everyone is expected to react. Failure to do so could mean that medicines the patient is taking might be blocking the response. It is recommended to avoid taking some antihistamines about 6 weeks prior to the test.

When evaluating allergy to many fruits and vegetables, the fact that commercially prepared crude extracts are often inadequate because of the lability of the responsible allergen needs to be considered when the SPT result is negative and the clinical history strongly suggests a particular sensitization. In these cases, the fresh food might be used directly for testing (prick by prick test).

1.2.9.2.2. Serum specific IgE

Serum immunoassays to determine food-specific IgE antibodies in serum provide another modality to evaluate IgE-mediated food allergy. The first *in vitro* test to detect specific IgE antibodies to suspected or known allergen was a radioimmunoassay test (RAST) in which the amount of radioactivity was proportional to the serum IgE for the allergen. However, this method is no longer used and the extensively validated ImmunoCAP system (or similar non-radioactive immunoassays) is the one now used by primary care physicians and specialists in the routine evaluation of food allergies due to the test ease, good performance and availability [6].

Similar to SPTs, many published studies have attempted to correlate serum-specific IgE levels with results of food challenges and therefore to provide the clinician with IgE levels that can predict the likelihood that a patient will react on ingestion of the food [44]. Unfortunately, data from these studies are not always consistent and have not been able to assign one specific cutoff value that provides optimum specificity and sensitivity for any single food across all populations. However, in all studies, a direct correlation has been reported between increasing concentrations of food-specific serum IgE and the probability that an individual will react to an ingested food. Importantly, undetectable serum food-specific IgE might be associated with clinical reactions for 10% to 25%. Consequently, if there is a suspicion of possible allergic reactivity, a negative SPT response and/or negative physician-supervised food challenge result, are necessary to confirm the absence of clinical allergy [42].

Whole extracts from different allergenic sources (e.g., foods, pollens) have been traditionally used for serum specific IgE detection. These extracts contain mixtures of allergenic and non-allergenic components and are difficult to standardize for their major allergens. Among the allergenic proteins, there are unique allergen molecules, which are markers for a genuine sensitization for an allergen source, and cross-reactive molecules (allergens that are present in many different allergen sources and induce cross-reactivity between allergen extracts). Thus, the use of whole extracts for diagnosis implies that a positive test does not clarify if there is a genuine sensitization to a particular allergen source or it is only a cross-reactivity phenomenon [45, 46].

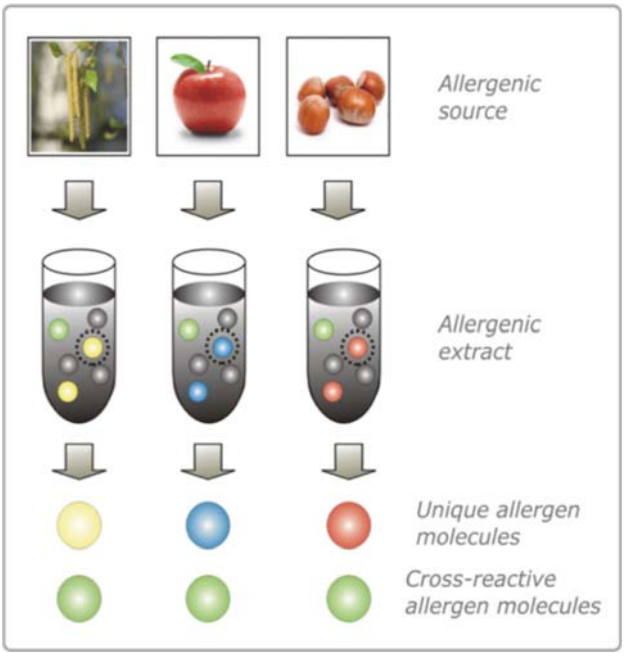

Figure 1.14. Molecular content of extracts. Extracts made of biological allergen sources contain allergenic as well as non-allergenic compounds and are hard to standardize. *Pan-allergens* are molecules present in many different allergen sources and capable of inducing cross-reactivity between allergen extracts of different allergenic sources [47]. Unique allergens are markers for a genuine sensitization for an allergen source. From: Phadia website.

To overcome this problem, detection of specific IgE to individual components of the allergenic source has recently been introduced in the clinical practice, allowing the identification of the "real" culprit causing the allergic reaction. Not only a more accurate diagnosis is achieved but also it has permitted to associate IgE responses to specific proteins in foods with particular clinical outcomes. This novel approach is termed *Component-Resolved Diagnosis* (CRD) [45] and is further discussed below.

1.2.9.3. Oral food challenges

Accurate diagnosis of food allergy and appropriate treatment options depend on the verification of clinically relevant allergen specific IgE antibodies, as well as on the identification of the responsible allergenic molecule(s). Importantly, a positive test (i.e., SPT, serum specific IgE) result merely identifies sensitization to a particular allergen and does not permit definitive differentiation between clinically relevant and non-relevant IgE reactivity.

The oral food challenge (OFC) consists on a gradual feeding of a possible allergen under medical supervision to determine tolerance or clinical reactivity [42]. Since severe reactions could be elicited, the procedure has to be undertaken by properly trained personnel with medications and equipment to treat anaphylaxis on hand. Feeding is generally stopped when objective or persistent subjective symptoms are elicited. For chronic disorders, in which an ingested food is currently a part of the diet, diagnosis typically includes a period of elimination of the possible trigger food(s) to determine whether symptoms resolve before an OFC. Caution is advised because acute severe reactions are sometimes noted after reintroduction of a potential allergen after prolonged dietary elimination.

Open or single blind OFCs are often used to screen for reactions. However, the Double-Blind Placebo-Controlled Food Challenge (DBPCFC) is considered the *gold standard* for diagnosis because bias is minimized. If the blinded challenge result is negative, it must be confirmed by means of an open supervised feeding of a typical serving of the food in its natural form to rule out a false-negative challenge result (approximately 1% to 3%) [42].

1.2.9.4. Other tests

The basophil activation test (BAT) is an *in vitro* assessment of allergic response that requires only a small amount of whole blood and allows for a measurement of a functional response beyond just the presence of IgE. The assay currently uses flow cytometry to detect upregulation of certain cell surface markers (e.g., CD63) after antigen stimulation in order to identify activated basophils. To date, some studies have validated this method using mainly drug allergens, but its role in food allergy diagnosis has yet to be defined in the clinical setting [42].

Food-specific IgG and IgG4 antibody levels do not have a role in the diagnosis of food allergy. They are both likely to be positive in food allergic individuals as well as healthy controls simply due to exposure [48].

Although not commercially available, determination of specific IgE-binding epitopes on an allergen might provide increased diagnostic utility. The specific profiles of epitopes bound might reflect distinctions in binding to areas of an allergen that are dependent on protein folding (conformational epitopes) and are a feature of mild/transient allergy versus areas that represent linear binding regions (sequential epitopes) that are stable, reflecting a severe persistent allergy.

1.2.10. A therapy for food allergy

Despite food allergy is an increasingly prevalent problem in westernized countries, there is currently no effective treatment than strict avoidance of the causal food(s) [49]. While symptoms of the reaction can be reduced by pharmacological treatment, allergen-specific immunotherapy (IT), based on the administration of increasing doses of allergens to induce a state of allergen-specific non-responsiveness [50], probably represents the only causative allergy treatment.

Several therapeutic strategies, both foods allergen-specific and nonspecific, are under investigation targeting foods that most frequently provoke severe IgE-mediated anaphylactic reactions (peanut, tree nuts and shellfish) or are most common in children, such as cow's milk and hen's egg.

Allergen-specific approaches include oral (OIT), sublingual (SLIT) and epicutaneous (EPIT) immunotherapy (desensitization) with native food allergens and mutated recombinant proteins of decreased IgE-binding activity coadministered within heat-killed *Escherichia coli* (HKE) to generate the maximum immune response. Diets containing extensively heated (baked) milk and egg represent an alternative approach to food OIT and are already changing the paradigm of strict dietary avoidance for patients with food allergy [32, 51, 52].

Nonspecific approaches include monoclonal anti-IgE antibodies (e.g., Omalizumab), which might increase the threshold dose for food allergen in patients with food allergy, and a Chinese herbal formulation (FAHF-2), which prevented peanut-induced anaphylaxis in a murine model [53] and is currently being investigated in clinical trials [49].

1.3. Molecular Diagnosis of Food Allergy

1.3.1. Component-Resolved Diagnosis

Diagnosis and specific IT have been traditionally performed with extracts obtained from natural allergen sources. Attempts to isolate and purify the disease-eliciting allergens from the natural allergen sources have been found too difficult and expensive for routine application on diagnosis and therapeutic purposes. However, advances in biochemistry and molecular biology in the last two decades allowed the

introduction of recombinant DNA technology in the field of allergen characterization, providing an increasing number of recombinant allergens that in most cases have immunological properties comparable with their natural counterparts. Actually, molecular biology was initially applied in the field of allergy to study the primary structure and molecular identity of allergens rather than to produce recombinant allergens for diagnostic and therapeutic purposes. Nevertheless, the idea to produce panels of recombinant allergens for clinical applications gained support after several observations [45, 46].

First, sequence analysis of allergens indicated that structurally related allergens exist in taxonomically related and unrelated species and that these cross-reactive molecules are often not strictly discriminated by the patient's immune system [54]. Second, immunological and biological investigations have shown that many, not all [55], recombinant allergens behave similar to their natural counterparts [56]. Third, it is possible to produce recombinant allergens which display most of the IgE epitopes present in natural allergen extracts; and finally, the observation that diagnosis of most patients with tree and grass pollen allergy was possible using only a limited number of recombinant allergens [57, 58].

For the concept of using defined allergens (molecules) as opposed to allergen extracts for diagnosis of Type I allergy, the term Component-Resolved Diagnosis (CRD) was proposed [45]. Thus, CRD uses single allergenic molecules (*components*), either recombinant or naturally purified, to precisely determine the individual patient's humoral reactivity profile (mainly IgE) with the aim of identifying the disease eliciting molecule(s), i.e., the real triggers of the allergic reaction.

CRD generates valuable information relating to the clinical significance of particular IgE reactivity profiles to allergens, eventually leading to the design of diagnostic tests for more precise clinical questions or with enhanced diagnostic or prognostic value. For instance, a positive reaction to an allergenic component with cross-reactive potential may predict allergic reactions to all those allergen sources, which contain immunologically related molecules. The remarkable increase in knowledge of allergen components of a wide range of allergenic sources and their applicability in allergy diagnosis and food allergy in particular has launched to their acceptation in routine allergy diagnosis as a useful tool [46].

Moreover, the application of the microarray technology on CRD enables sIgE testing in a multiplex format and allows for the simultaneous measurement of many IgE clones with different specificities only using minute amounts of sera and antigens [59]. Component-resolved diagnostics using a commercially available microarray format

(ImmunoCAP ISAC™, Phadia ThermoFisher Scientific ®, Sweden) has recently been introduced into the clinical allergy practice.

Although, microarrays with large numbers of allergenic components can be very helpful in epidemiological and basic research studies, the clinical practice probably requires a simplified diagnostic tool with limited set of well-validated sensitization markers that can facilitate the diagnosis and predict potential cross-reactivity and clinical symptoms, which actually aims to solve a diagnostic problem. Thus, a careful selection of the allergens included based on their diagnostic value and not on their availability is needed [60].

1.3.2. Component-Resolved Immunotherapy

Although a great progress has been achieved on the standardization of allergen extracts by using monoclonal antibodies, current forms of extract-based treatment still cannot be individualized to include relevant allergens only. Rather, all patients showing allergic reactions to proteins present in a particular allergen source are treated with the same complex extract regardless of their individual sensitization profiles.

The allergen extract is commonly administered with an adjuvant and may therefore induce immune reactions against extract components not recognized before treatment. Such immunotherapy-induced sensitization has been reported to grass pollen allergens [61], for instance.

CRD is very important not only because it allows a more reliable diagnosis of the allergic patient, but also because it helps in the identification of better candidates for therapeutic purposes. Components identified as real triggers of the allergic response to a particular allergen can be genetically engineered or serve as templates for the design of synthetic hypoallergenic allergen derivatives with reduced anaphylactic activity for use in patient-tailored treatment. This concept is known as Component-Resolved Immunotherapy (CRIT) [45] and it opens new perspectives for food allergy therapy [62] (Figure 1.15).

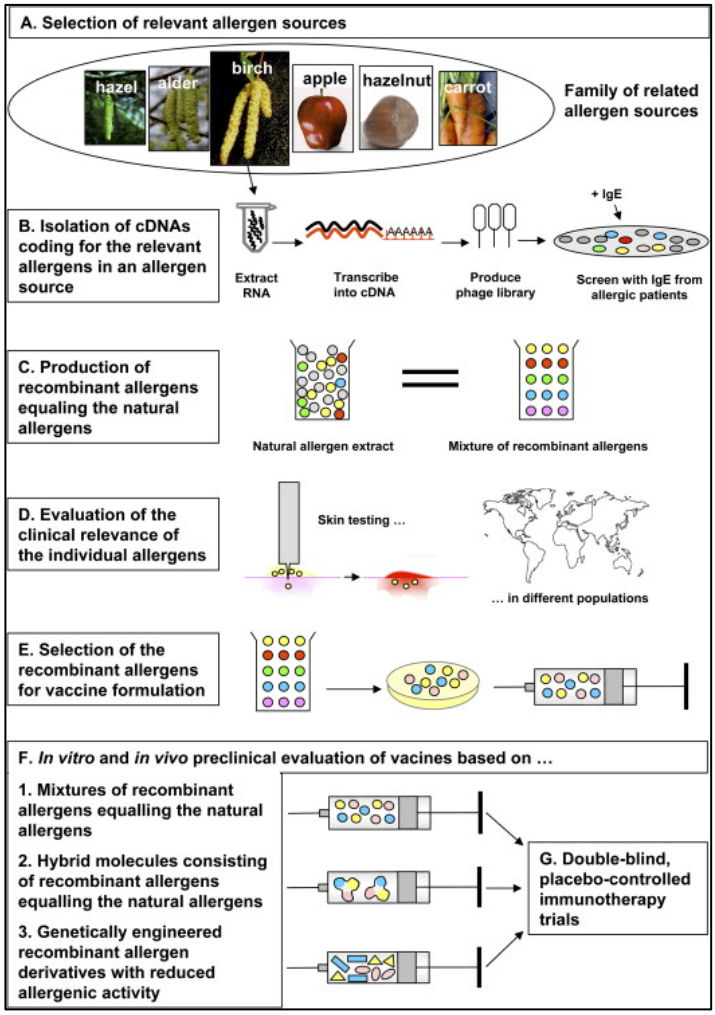

Figure 1.15. Production of recombinant allergens and selection for vaccine formulation. From [62].

1.3.3. Epitope mapping

The group of amino acids within allergenic proteins that is bound by IgE antibodies is called *IgE binding epitope* or *B-cell epitope* [63]. Each allergen source contains many different allergenic components and each of them has several epitopes. Importantly, every species contain species-specific allergen epitopes and antibodies formed to these structures bind only to the allergen epitopes in this particular species. However, since proteins with similar structures are often also present in biologically related species, epitopes can be shared between them, leading to cross-reactivity phenomena.

There are two types of IgE-binding epitopes in allergens, sequential and conformational [64]. A sequential (or linear) epitope is a sequence of contiguous amino

acids in the primary structure of the protein, whereas a conformational epitope comprises amino acids that line up because of the tertiary structure of the protein. Sequential epitopes have been suggested to be more important in food allergens, which are usually consumed once cooked and go through digestion in the gastrointestinal tract (heat and acid conditions cause denaturation and alteration in tertiary structure before reacting with the immune system). In contrast, conformational epitopes are described more relevant in respiratory allergens and patients with oral allergy syndrome (due to cross-reactivity between conformational epitopes of food allergens and homologous pollen-related allergens).

However, the relevance of IgE binding to short peptides has also been challenged [65]. Recently, IgE binding to short peptides of Pen a 1 (shrimp tropomyosin) and Ara h 2 (peanut seed storage protein) was found to contribute little to the IgE binding particularly in fluid phase antibody/target interaction, suggesting that conformational epitopes are more important.

Methodology to determine IgE sequential epitopes has developed over the last few years. The conventional method, the fragmentation approach, involved partial protease digestion or chemical cleavage at sites of specific amino acids of the food allergen, followed by western blotting using patients' sera and amino acid sequencing of IgE-binding fragments. This method was restricted by the position of the cleavage sites of the allergens. Next a more flexible system, the SPOT membrane-based immunoassay technology, was introduced. On that, overlapping peptides covering the primary amino acid sequence were synthesized *in situ* on a nitrocellulose membrane, incubated with the patient's sera and peptides bound by IgE antibodies were detected using antihuman-IgE antibody labeled with peroxidase, followed by the addition of a chemoiluminescent substrate and exposure to radiographic film. Several disadvantages of the SPOT-membrane technique (e.g., large volumes of patient sera required, time-consuming peptide synthesis, expensive and error prone) called for the development of a novel immunoassay that utilizes the high throughput microarray platform and commercially synthesized peptides [66]. The microarray allows the screening of thousands of target peptides in parallel using microliter quantities of serum. The peptide spots bound by human IgE antibodies are detected using fluorescent labeled secondary antibodies followed by scanning with a microarray scanner, which is much more convenient than radiographic film development, providing quantitative data rather than semi-quantitative or qualitative results such as the previous methods. Moreover, its redundancy allows more robust replication and statistical approaches [67].

IgE epitope-mapping with peptide microarrays have been shown to provide an additional tool for allergy diagnosis and prognosis [63]. Informative IgE-binding epitopes have been identified as biomarkers of clinical severity, persistence or both in patients' with some food allergies. For peanut allergy, clinical sensitivity determined by means of DBPCFC has been positively related to broader epitope recognition [68]. Also, patients with persistent milk allergy showed increased epitope diversity and stronger IgE affinity binding compared to patients with transient milk allergy or tolerance to extensively heated milk [69].

Studies of conformational epitopes are far behind those of sequential epitopes due to the limitation of available three-dimensional structures of food allergens and the difficulty of maintaining protein stability. The recently developed mimotope mapping technique is another option for mapping conformational epitopes [70]. Briefly, using random peptide phage-display libraries, peptides are generated and screened with purified allergen specific IgE. The selected peptides that mimic the binding sites of the allergen but do not correspond to its sequential sequence are known as mimotopes. The resulting mimotope can be mapped onto the three dimensional structure of the allergen using computational biological techniques. Using this technique, IgE conformational epitopes of important major allergens, such as fish parvalbumin [71] and the peach LTP (Pru p 3) [72], have been identified.

For all described so far, IgE epitope mapping of allergens is considered a further step in developing molecular allergy diagnosis with attractive perspectives.

1.4. Shellfish Allergy

1.4.1. Prevalence and clinical characteristics

Shellfish allergy is common among children and adults in western countries such as Europe, United States and Australia, but seems to be even more prevalent in Asian countries where allergic reactions to seafood and particularly shellfish are very common, probably due to their dietary habits [73]. In fact, the actual prevalence is difficult to determine due to a lack of accurate controlled population-based studies incorporating DBPCFCs. Reported rates of shellfish allergy vary among countries: Canada, 1.42%; USA, 2%; and Singapore, 5.2% (14-16 years-olds) [20]. In a prospective Spanish study, Alergológica 2005, shellfish was the offending food in 22%

of the subjects diagnosed with food allergy, being the third most common food allergy culprit, after fruits and tree nuts [74].

The pattern of allergic symptoms after ingestion of shellfish appears similar to the symptoms experienced due to other allergenic foods. Reactions are usually immediate (reported mostly within 2 hours), but late-phase reactions have also been reported up to 8 hours after ingestion. Respiratory reactions are often seen. In particular, crustacean allergic subjects often experience OAS, within minutes after ingestion they suffer itching and angioedema of the lips, mouth and pharynx. Patients may have a single symptom but often there is multi-organ involvement and reactions are severe, with frequent anaphylaxis. Noteworthy, one of the first clinical reports highlighting the existence of exercise-induced anaphylaxis due to food was presented by Maulitz et al. [75] after the ingestion of oysters. Similar findings have subsequently been reported for other shellfish species [73]. According to the National Electronic Injury Surveillance System (NEISS) in the United States, shellfish may account for up to 24% of the emergency department visits for food-allergic events [76, 77]. Recent studies estimated the frequency of United Stated emergency department visits/year at 203,000 for food-related acute allergic reactions and 90,000 for those with anaphylaxis [78].

Allergic symptoms occur not only from ingestion, but can also be triggered by inhaling cooking vapors and handling shellfish in the domestic and/or occupational environment. In this case, symptoms manifest mainly as upper and lower airway respiratory symptoms and dermatitis, while systemic anaphylaxis is rarely seen with this type of exposure [73].

Despite the high prevalence and severity of these food-allergic reactions, there is still no cure and accurate diagnosis and avoidance are the only recommended options [79]. Shellfish allergic patients usually avoid all types of shellfish for life after diagnosis [80].

1.4.2. Shellfish allergens and cross-reactivity

Shellfish includes both crustaceans and mollusks. However, most species provoking allergic reactions belong to the class Crustacea, which includes shrimp, by far the most frequently involved. It is noteworthy that crustacean and mollusk allergens do not cross-react with fish allergens and no reactivity between known allergens or homologous proteins has currently been demonstrated [73].

Tropomyosin (TM) is considered to be responsible for most of the allergenic activity of shrimp [73, 81], but other allergens have been identified and characterized in crustaceans: Arginine kinase (AK) [82, 83], Myosin Light Chain (MLC) [84], Sarcoplasmic Calcium-binding Protein (SCP) [85], as well as, three new allergenic shrimp proteins that have been identified, although not fully characterized (hemocyanin, fatty-acid binding protein and troponin C) [86].

In shrimp, tropomyosin represents 20% of soluble proteins and it is really abundant in the cooking broth. Its thermostability permits its vehiculation on cooking steam and therefore sensitization or allergic reactions can occur through inhalation (respiratory exposure). Over 85% of shrimp allergic patients are sensitized to tropomyosin and the IgE response towards this allergen represents the 80% of the IgE response to the whole extract of shrimp [5].

Tropomyosins represent a family of highly conserved proteins, present in muscle and non-muscle cells of vertebrates and invertebrates, but only the ones of invertebrates are allergenic. Tropomyosin is the main panallergen in the animal world and sensitization to it accounts for the cross-reactivity between shellfish and other invertebrates, such as arthropods (dust mites (Der p 10, Der f 10) and cockroach (Per a 7, Bla g 7), nematodes parasites and mollusks [73, 79 and 87].

To date, there is limited information about the clinical relevance of sensitization to particular shrimp allergens as well as the role of allergens other than tropomyosin in cross-reactivity phenomenon, despite the improvement on knowledge of shellfish allergens in the last years, when important allergens have been cloned and characterized.

1.4.3. Diagnosis of shellfish allergy

Shellfish allergy diagnosis is aided by clinical history, SPT and quantification of sera specific IgE. However, positive test results, alike other food allergies, are not necessarily proof of clinical reactivity, basically due to the high cross-reactivity that exists with other arthropods [79].

Therefore, DBPCFC is still the most reliable method to confirm the clinical relevance and to identify putative species. However, as mentioned earlier on this chapter, they are not entirely practical considering time requirements and the risk of inducing potential severe reactions. Thus, research is focusing on the development of

diagnostic methodology that may eliminate the need for oral food challenges. However, a limited number of studies are published trying to address this issue.

The relevance of sensitization to tropomyosin has recently been studied in dust mite-allergic subjects sensitized to shrimp. Tropomyosin-specific IgE (Pen a 1, Phadia) was found to have greater diagnostic efficiency compared to whole shrimp-specific IgE or SPT (88.5%, 74.2% and 65.7% respectively) [88]. A panel of six allergens (tropomyosin, arginine-kinase, sarcoplasmic calcium-binding protein, a novel myosin light chain, troponin C and triosephosphate-isomerase) of the North Sea shrimp (*Crangon crangon*) has been very recently suggested to develop a more sophisticated and clinically adequate methods of diagnosis and treatment of shrimp allergy [89].

Ayuso R et al. identified the IgE-binding epitopes of four shrimp allergens (Lit v 1-4) in 2010 [90]. However, no attempt was then made to correlate epitope or allergen recognition with different clinical profiles. Just recently, Ayuso R et al. identified some of these epitopes as clinically relevant in a pilot study [91].

1.5. Plant-Food Allergy

1.5.1. Main culprit foods and allergen families

Plant food allergy is the most common food allergy among older children and adults [92-95]. Peanut, followed by tree nuts, are the plant foods most commonly involved in allergic reactions in the United States and United Kingdom. However, in Central and Northern Europe (especially in birch trees rich areas), and in the Mediterranean, the prevalence of peanut allergy decreases but tree nuts (mainly hazelnut, followed by walnut) and fruits – especially those belonging to the Rosaceae family (including apple, peach and pear) and kiwi – emerge as important food allergies [96]. A meta-analyses on published studies including food challenge tests for diagnosis showed prevalence ranging from 0.1% to 4.3% each for fruits and tree nuts, 0.1% to 1.4% for vegetables, and <1% each for wheat, soy and sesame [97]. Fresh fruits, mainly *Rosaceae*, were found responsible for 33.3% of the diagnosed food allergies (7.4% of 4991 patients) in Spain [74].

Remarkably, only 4 protein families contain nearly 60% of all plant-food allergens. This indicates that conserved structures and biological activities play a role in determining or promoting allergenic properties. These families are the prolamin

superfamily (includes 2S-albumin seed storage proteins and the non-specific lipid transfer proteins among others), the cupin superfamily (contains the vicilin and the legumin-type seed storage globulins), the profilins and proteins related to the major birch pollen allergen Bet v 1 [98-100]. Figure 1.16 shows a classification of these plant food proteins based on their function in plants [5].

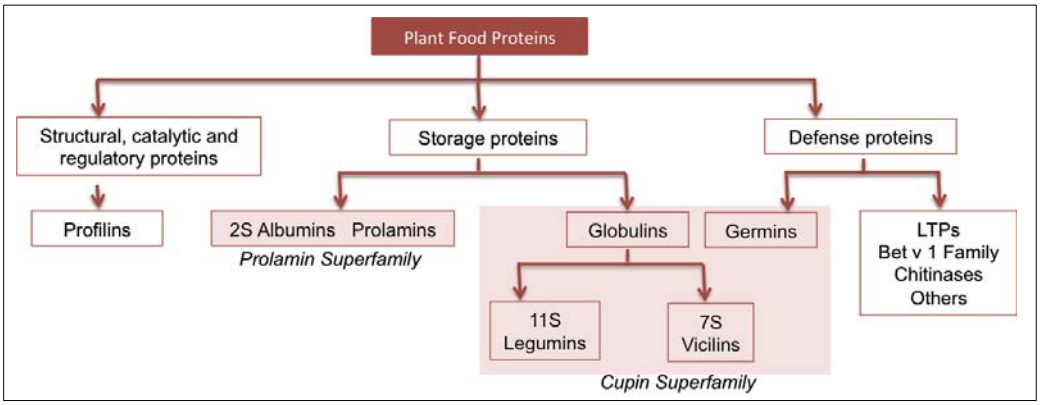

Figure 1.16. Plant-food proteins described as allergens. Adapted from [5].

Fruit allergy has a remarkable different picture across Europe [101-104]. In the Northern and Center, it represents an important cross-reactivity phenomenon involving homologous allergens in both pollens and plant-foods, mainly Bet v 1 homologues [105] and profilins [106], usually expressed as OAS. In contrast, in the South, both fruit allergy with and without (>20%) [107, 108] pollen sensitization is observed. Non-specific lipid transfer proteins (nsLTPs) are mainly involved and, despite OAS is also present, higher frequency of allergic systemic reactions are reported [109]. Apple is the most frequent culprit in Northern- and Central-Europe, whereas peach is definitely the one in the South, where clinically relevant peach allergy is mainly related to Pru p 3 (peach lipid transfer protein, LTP), its major allergen [108].

1.5.2. Non-specific lipid transfer proteins

NsLTPs, usually referred as LTPs (Lipid transfer proteins) only, are important allergens in fruits, vegetables, nuts, pollen and latex. Despite their wide distribution throughout the plant kingdom, their clinical relevance is largely confined to the Mediterranean area, but the reasons for this fact are not clearly understood yet [110].

They belong to the prolamin superfamily, which includes a range of important plant allergens. Their small structure is characterized by a highly conserved pattern of

six to eight cysteines forming three to four disulfide bridges. They have low molecular mass (around 9 kDa), basic character and they are highly expressed in the epidermal or peripheral layers of cutinized organs. Importantly, they are stable at low pH and resistant to proteolysis and temperature [111]. Thanks to these peculiar molecular characteristics, nsLTPs have been proposed as a model allergen for true food allergy (food allergy class I). They reach the intestinal immune system in an immunogenic way allowing them a direct sensitization and in the following expositions to induce severe systemic reactions [112].

1.5.3. The LTP-syndrome

Sensitization to LTP can be clinically presented through a range of mild to severe, sometimes life-threatening, symptoms. In most cases, the ingestion of plant food LTPs causes symptoms in the oral cavity. However, it became evident from very early reports on these allergens that they are also capable of eliciting severe generalized reactions. Unlike other allergen groups (e.g., Bet v 1-related food allergens or profilins), LTPs can be responsible for severe reactions upon the ingestion of fresh as well as processed foods [109].

Clinically relevant cross-reactivity among LTPs from taxonomically related and unrelated plants has been demonstrated, especially when the amino acid sequence identity is quite high [110]. The widely documented extensive cross-reactivity, *in vivo* and *in vitro*, is probably due to the ubiquitous distribution of LTPs in the plant kingdom, which makes sensitization to multiple foods very common in these patients, commonly referred to *LTP Syndrome* [109].

Although sensitization to Pru p 3 can be found with and without associated pollinosis, the majority of patients allergic to peach present an associated pollen allergy as well as other food allergies, mainly to other *Rosaceae*, but also to other non-*Rosaceae* vegetable foods. Actually in 2007, Zuidmeer and Van Ree [113], reported that there is possibly more than one *LTP-Syndrome*: (1) primary sensitization to food LTP without concomitant pollen allergy; (2) primary sensitization to a food allergen against a background of existing pollen allergy, (3) primary sensitization to a pollen allergen.

Given all this peculiar characteristics of LTPs, the clinician encounters with a clinically complex patient with sensitization to multiple plant-food allergens and with multiple clinical manifestations of variable severity that in some occasions can in addition be exacerbated by cofactors. Variability of severity of symptoms may

represent distinct patterns of allergen sensitization. The large number of allergenic members of plant food allergens described, brings up the need of accurate diagnostic tools to determine the allergen sensitization profile of these patients.

Despite substantial advances have been achieved in the last years regarding allergy characterization, there is still a need to further study the elements involved in the allergic response, not only with the aim to improve diagnosis and treatment, but to clarify the underlying mechanisms involved that lead to the diversity of clinical and molecular profiles observed in allergy, and food allergy in particular.

CHAPTER 2. HYPOTHESIS AND OBJECTIVES

HYPOTHESIS AND OBJECTIVES

2.1. Hypothesis

All described above encourages the use of panels of allergenic molecules (components) as well as synthetic sequential peptides for an elaborate molecular analysis of sensitization patterns in patients with food allergy. The hypothesis of this thesis was that CRD and epitope mapping improve the clinical and molecular characterization of shellfish allergy and LTP-syndrome.

2.2. Objectives

Thus, the whole aim of this thesis is to characterize shellfish allergy and LTP-syndrome at a clinical and molecular level by CRD and epitope mapping.

Specific objectives:

Part 1. Component-Resolved Diagnostics and epitope recognition in shellfish allergy

1. To determine profiles of shellfish allergen sensitization resulting into clinical reactivity.

Part 2. Lipid Transfer Protein syndrome: clinical pattern and molecular sensitization profile to plant-foods and pollens

1. To clinically characterize LTP-syndrome in patients from our area.

2. To determine the utility of CRD in a microarray format as a tool in the diagnostic work-up of patients with multiple plant-food allergies who present a broad diversity of clinical symptoms that do not reveal a particular pattern of sensitization.

CHAPTER 3. MATERIAL AND METHODS

MATERIAL AND METHODS

3.1. Patient selection

3.1.1. Shrimp sensitized subjects

This was a multicenter study with participation of the Allergy Clinic of Mount Sinai Medical Center, New York, USA; the Division of Clinical Immunology and Allergy, University of São Paulo School of Medicine, São Paulo, Brazil; and the Allergy Clinic of Hospital Infantil Universitario Niño Jesús, Madrid, Spain. Subjects were recruited from 2008 to 2011. Inclusion criterion was positive shrimp SPT and/or shrimp-specific serum IgE.

Subjects were divided into two groups based on whether histories of immediate allergic reactions following shrimp ingestion were reported. DBPCFC was performed to all the individuals, except those reporting an anaphylaxis episode within the previous 3 months. When DBPCFC was negative, an oral open challenge with shrimp was performed to reassure tolerance.

Two group controls were included, one of subjects sensitized to house-dust mite (HDM) and/or cockroach with no sensitization to shrimp and no reported histories of immediate allergic reactions after ingestion, and one of non-atopic individuals.

3.1.2. Multiple plant-food allergic subjects

Patients diagnosed of multiple plant-food allergy with at least two of the different plant-foods involved (peach and an other plant-food not taxonomically related to Rosaceae), sensitized to LTP and other inhalant and food allergens were selected from the Allergy Unit – Department of Pneumology and Allergy, Hospital Clínic, Barcelona, Spain, for further analysis. Diagnosis on these patients had been established on the basis of symptoms compatible with IgE-mediated food allergy: urticaria, oral allergy syndrome (OAS), gastrointestinal disorders (GID), anaphylaxis; positive skin prick test (SPT) and specific IgE determination. All subjects recruited were inhabitants of the Barcelona area (Catalonia, Spain). Written informed consent was obtained and the Local Ethics Committee approved the study.

For both groups (shrimp and plant-food allergic subjects), clinical history of each patient was reviewed looking for symptoms, culprit foods, cofactors and other epidemiological relevant data. The local ethical committees approved the studies and informed consent was obtained from all patients.

3.2. Skin Prick Test

As described above (section 1.2.9.2.1), SPTs with commercial extracts are used to detect the presence of allergen specific IgE on the surface of human mast cells (state termed sensitization). Prick-by-prick tests are performed by direct contact with the allergenic source.

For detection of sensitization to shrimp prick-by-prick tests with raw shrimp obtained from a local market were performed.

For the evaluation of the sensitization to plant-food and pollen allergenic sources SPTs were performed using whole extracts commercially available (ALK-Abelló, Madrid, Spain). The same standard panels of common allergens and pollens tested in the Allergy Unit of Department of Pneumology and Allergy, Hospital Clínic, Barcelona for routine diagnosis were used for the present study.

The plant-food allergens panel includes: peach peel, maize, wheat, hazelnut, mustard, lettuce, kiwi; and the aeroallergens panel consists of: grass, mugwort (*Artemisia vulgaris*), wall pellitory (*Parietaria judaica*), olive (*Olea europaea*), cypress (*Cupressus sempervivens*), plane tree (*Platanus acerifolia*), dust mites, alternaria, aspergillus, cat- and dog-dander.

The tests were performed following standard procedures for SPTs [43] to all subjects, except for three that rejected being tested. Briefly, on the forearm area comprised between 5 cm above the wrist and 3 cm below the elbow, the spots where each extract would be applied were conveniently labeled with a pen and separated by at least 2 cm. On each spot, a drop of the corresponding extract was applied and the center was punctured with a lancet (single use) with a 90º angle to the skin. Read-out of the reaction was done usually after 15-20 minutes of test performance that coincides with the maximal reaction point of extracts, although them maximal response to histamine is usually achieved after 10-15 minutes. The wheal produced by the allergen was measured and compared to the negative control. Histamine dihydrochloride (10 mg/mL) and PBS buffer/glycerol solution were positive and negative controls,

respectively. A mean wheal diameter of ≥ 3mm compared to negative control was considered positive.

3.3. Oral food challenge

All the subjects and controls recruited for the shellfish allergy characterization study were submitted to oral food challenge (DBPCFC) to evaluate their clinical reactivity, except those with a moderate – severe anaphylactic reaction following shrimp ingestion within the previous 3 months. A cold pudding was made of chocolate and vanilla ice cream, vanilla aroma and cacao powder. The active meal contained also grinded boiled shrimp obtained from a local market. The maximum amount of shrimp in a given blinded challenge was 24 grams (12 peeled shrimp tails). Five investigators confirmed that preparation was adequately blinded. None of the subjects were lactose intolerant or allergic to any compound in the pudding. In case of a negative DBPCFC, the subject received an open challenge with shrimp.

Subjects diagnosed of multiple plant-food allergy were not challenged after recruitment for the present study.

3.4. Specific IgE detection by ImmunoCAP System

The ImmunoCAP System (Phadia Thermo Fisher Scientific, Uppsala, Sweden) is an automated system based on the fluorescent enzyme immunoassay (FEIA) that measures allergen-specific IgE in serum or plasma samples. Over 650 different allergens (extracts) and 70 allergen components are commercially available to date (Figure 3.1). The ImmunoCAP solid phase consists of a cellulose derivative enclosed in a capsule to which the allergen is covalently attached after being activated with cyanogen bromide. The hydrophilic, highly branched polymer provides an ideal microenvironment for allergens, binding them irreversibly while maintaining their native structure. The test is designed as a sandwich immunoassay. This technology ensures binding of all relevant antibodies, regardless of antibody affinity, still giving low non-specific binding.

ImmunoCAP specific IgE detects IgE antibodies in the range 0 to 100 kU_A/L, where A represents allergen-specific antibodies. The result is reported quantitatively. In clinical practice, 0.35 kU_A/L has commonly been used as a cut-off, although 0.1 kU_A/L

has been also suggested. A large number of studies have evaluated the clinical performance of this test in allergy diagnosis. Thus, sensitivity and specificity have been reported from multi-center studies including several hundred patients tested for a range of different allergens and they have been estimated of 84-95% and 85-94%, respectively. Moreover, the system includes standard reagents for a calibration curve as well as the corresponding quality controls for the procedure.

Briefly, first the serum is incubated with the CAP System containing the allergen and following a washing step is performed to clear the non-specific IgE present in the sample. Afterwards, incubation with a secondary antibody (IgG monoclonal anti-IgE antibody) labeled with an enzyme (beta-galactosidase) is performed. If allergen specific IgE is present on the patient serum, an immunocomplex is formed (allergen > serum allergen-specific IgE > anti-IgE-enzyme). Incubation is followed by another washing step to clear not bound secondary antibody. After, a developing solution (4-metil-umbeliferil-beta-D-galactoside) is added to the system that acts as a substrate for the enzyme, resulting in a fluorescent product. Finally the fluorescent reaction is interrupted with a stop solution (calcium carbonate). Fluorescence is measured with a fluorometer, which extrapolates the values obtained for the patient sample to a calibration curve set with the standard reagents and the controls. The change in fluorescence of the sample is directly proportional to the IgE content of the serum.

Using the ImmunoCAP system it is also possible to determine the total content of IgE in a sample. However, in this case a monoclonal anti-IgE is attached to the solid phase instead of a specific allergen [114].

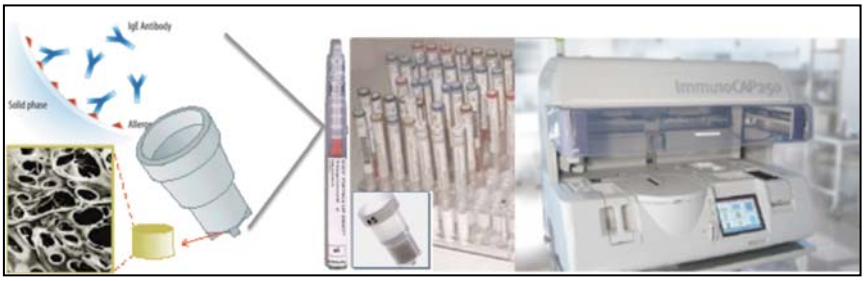

Figure 3.1. Main components of the ImmunoCAP System. The cellulose discs coated with the allergen are placed inside capsules. Capsules are presented inside cylinders (resembling a pen) containing usually 10-12 units. Auto analyzers are able to identify both the sample and the allergen through barcode or sample identification. There are several auto analyzers available depending on the amount of samples that can be processed in one run (e.g., ImmunoCAP 100, 250, 1000, etc.). Adapted from [114].

The ImmunoCAP System with the auto analyzer ImmunoCAP 250 (Phadia Thermo Fisher Scientific®, Uppsala, Sweden) was used to detect total IgE and specific IgE to commercially available extracts and specific components (Phadia Thermo Fisher Scientific®, Uppsala, Sweden).

In multiple plant-food allergic subjects, available data on total and specific IgE to peach (f95, *Prunus persica*), mugwort (w6, *Artemisia vulgaris*), plane tree (t11, *Platanus acerifolia*), and the components Pru p 3, Pru p 1, Pru p 4 (f420, peach LTP; f419, PR-10/Bet v 1 homolog and f421, profilin; respectively) determined by ImmunoCAP (Phadia, Thermo Fisher Scientific®), was collected from patients' clinical history. Specific IgE antibodies > 0.35 kU_A/L (ImmunoCAP) were considered positive.

In the shellfish allergic group and the corresponding controls of the study, specific IgE to shrimp (f24, which is a mixture of 4 different species of shrimp: *Pandalus borealis, Penaeus monodon*, *Metapenaeopsis barbata* and *Metapenaeus joyneri*), dust mite (d1, *Dermatophagoides pteronyssinus)* and cockroach (i6, *Blatella germanica*) extracts were measured.

Specific IgE antibodies > 0.35 kU_A/L were considered positive for all of them.

3. 5. Specific IgE detection by microarray

ImmunoCAP ISAC (Phadia Thermo Fisher Scientific®, Uppsala, Sweden) is an advanced *in vitro* diagnostic test including biochip technology that allows simultaneous measurement of specific IgE antibodies to a broad spectrum of allergen components in a single test, using a minute amount of sample.

The panel of allergen components includes genuine markers of sensitization (species specific) as well as cross-reactive ones. The version used for this study includes 103 components of over 50 different allergenic sources (Table 3.1). Allergen components are immobilized on the biochip in triplicates. The system is available for IgE and IgG4 antibodies detection.

Its use is especially indicated for complex cases such as those with inconsistent case history, unsatisfactory response to treatment or polysensitized patients.

Briefly, 20µL of serum are added to the microarray and incubated for two hours at room temperature. Successive washes are done prior to one-hour incubation with an anti-human IgE secondary antibody-fluorescence-labeled at room temperature protected from light. After washes and drying, fluorescence signal is detected using a laser scanner (Perkin Elmer). Analysis of corresponding digitized microarray image

data is performed with the Phadia MIA software to obtain numerical data according to calibration sera of known IgE content (Figure 3.2). ImmunoCAP ISAC is a semi-quantitative test and results are reported in ISAC Standardized Units for specific IgE (ISU) [114].

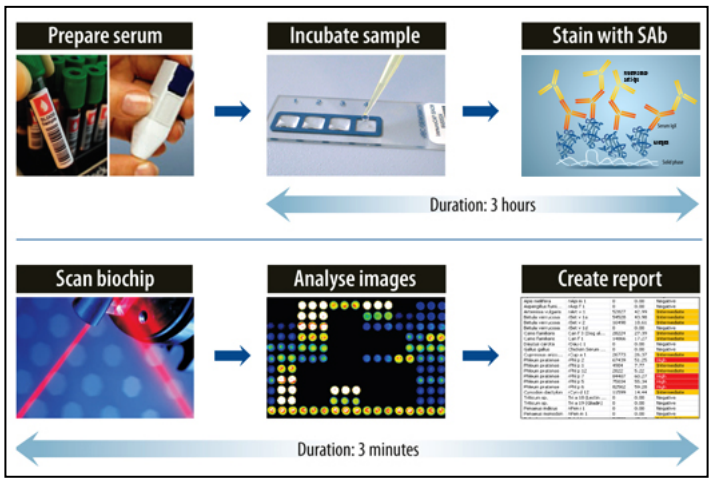

Figure 3.2. ImmunoCAP ISAC test principle. Schematic representation of ImmunoCAP ISAC test performance. From [114].

The ImmunoCAP ISAC was used following the manufacturer's protocol briefly described above to evaluate the presence and absence of specific IgE towards the components present in the array in the cohort of patients diagnosed of multiple plant-food allergy.

Specific IgE antibodies > 0.3 ISU were considered positive for all of them.

ALLERGEN COMPONENT	ALLERGEN SOURCE		PANALLERGEN
Plants, Specific Markers			
Act d 1	Kiwi	*Actinidia deliciosa*	
Act d 2	Kiwi	*Actinidia deliciosa*	
Act d 5	Kiwi	*Actinidia deliciosa*	
Act d 8	Kiwi	*Actinidia deliciosa*	PR-10
Aln g 1	Alder	*Alnus glutinosa*	PR-10
Amb a 1	Ragweed	*Ambrosia artemisiifolia*	
Ana o 2	Cashew nut	*Anacardium occidentale*	
Api g 1	Celery	*Apium graveolens*	PR-10
Ara h 1	Peanut	*Arachis hypogaea*	
Ara h 2	Peanut	*Arachis hypogaea*	
Ara h 3	Peanut	*Arachis hypogaea*	
Ara h 8	Peanut	*Arachis hypogaea*	PR-10
Art v 1	Mugwort	*Artemisia vulgaris*	
Art v 3	Mugwort	*Artemisia vulgaris*	LTP
Ber e 1	Brazil nut	*Bertholletia excelsa*	
Bet v 1	Birch	*Betula verrucosa*	PR-10
Cor a 1.0101	Hazel pollen	*Corylus avellana*	PR-10
Cor a 1.0401	Hazelnut	*Corylus avellana*	PR-10
Cor a 8	Hazelnut	*Corylus avellana*	LTP

Cor a 9	Hazelnut	*Corylus avellana*	
Cry j 1	Japanese cedar	*Cryptomeria japonica*	
Cup a 1	Cypress	*Cupressus arizonica*	
Cyn d 1	Bermuda grass	*Cynodon dactylon*	
ALLERGEN COMPONENT	**ALLERGEN SOURCE**		**PANALLERGEN**
Dau c 1	Carrot	*Daucus carota*	PR-10
Gly m 4	Soybean	*Glycine max*	PR-10
Gly m b-conglycinin	Soybean	*Glycine max*	
Gly m glycin	Soybean	*Glycine max*	
Hev b 1	Latex	*Hevea brasiliensis*	
Hev b 3	Latex	*Hevea brasiliensis*	
Hev b 5	Latex	*Hevea brasiliensis*	
Hev b 6	Latex	*Hevea brasiliensis*	
Mal d 1	Apple	*Malus domestica*	PR-10
Ole e 1	Olive	*Olea europaea*	
Par j 2	Wall pellitory	*Parietaria judaica*	LTP
Pla a 1	Plane tree	*Platanus acerifolia*	
Pla a 2	Plane tree	*Platanus acerifolia*	
Phl p 1	Timothy	*Phleum pratense*	
Phl p 2	Timothy	*Phleum pratense*	
Phl p 4	Timothy	*Phleum pratense*	
Phl p 5	Timothy	*Phleum pratense*	
Phl p 6	Timothy	*Phleum pratense*	
Phl p 11	Timothy	*Phleum pratense*	
Pru p 1	Peach	*Prunus persica*	PR-10
Pru p 3	Peach	*Prunus persica*	LTP
Sal k 1	Saltwort	*Salsola kali*	
Ses i 1	Sesame seed	*Sesamum indicum*	
Tri a 18	Wheat	*Triticum aestivum*	
Tri a 19 crude gliadin	Wheat	*Triticum aestivum*	
Tri a a 19 Omega 5 Gliadin	Wheat	*Triticum aestivum*	
Tri a aA_TI	Wheat	*Triticum aestivum*	
Plants, Cross Reactive Markers			
Ana c 2	Bromelin	*Ananas comosus*	CCD marker
Bet v 2	Birch	*Betula verrucosa*	Profilin
Bet v 4	Birch	*Betula verrucosa*	CBP
Hev b 8	Latex	*Hevea brasiliensis*	Profilin
Mer a 1	Annual mercury	*Mercurialis annua*	Profilin
Ole e 2	Olive	*Olea europaea*	Profilin
Phl p 7	Timothy	*Phleum pratense*	CBP
Phl p 12	Timothy	*Phleum pratense*	Profilin

Non-Plants, Specific Markers			
Alt a 1	Alternaria	*Alternaria alternata*	
Alt a 6	Alternaria	*Alternaria alternata*	
Ani s 1	Anisakis	*Anisakis simplex*	
Api m 1	Honey bee venom	*Apis mellifera*	
Api m 4	Honey bee venom	*Apis mellifera*	
Asp f 1	Aspergillus	*Aspergillus fumigatus*	
Asp f 2	Aspergillus	*Aspergillus fumigatus*	
Asp f 3	Aspergillus	*Aspergillus fumigatus*	
Asp f 4	Aspergillus	*Aspergillus fumigatus*	
Asp f 6	Aspergillus	*Aspergillus fumigatus*	
Bla g 1	Cockroach	*Blattella germanica*	
Bla g 2	Cockroach	*Blattella germanica*	
Bla g 4	Cockroach	*Blattella germanica*	
Bla g 5	Cockroach	*Blattella germanica*	
Bos d 4	Milk, α-lactalbumin	*Bos domesticus*	
Bos d 5	Milk,β-lactoglobulin	*Bos domesticus*	
Bos d 6	BSA	*Bos domesticus*	
Bos d 8	Milk, Caseins	*Bos domesticus*	
Bos d lactoferrin	Milk, Lactoferrin	*Bos domesticus*	
Can f 1	Dog	*Canis familiaris*	
Can f 2	Dog	*Canis familiaris*	
Can f 3	Dog	*Canis familiaris*	
Cla h 8	Cladosporium	*Cladosporium herbarum*	

Cyp c 1	Carp	*Cyprinus carpio*	
Der f 1	House dust mite	*Dermatophagoides farinae*	
Der f 2	House dust mite	*Dermatophagoides farinae*	
Der p 1	House dust mite	*Dermatophagoides pteronyssinus*	
Der p 2	House dust mite	*Dermatophagoides pteronyssinus*	
Equ c 3	Horse	*Equus caballus*	
Eur m 2	Storage mite	*Euroglyphus maynei*	
Fel d 1	Cat	*Felis domesticus*	
Fel d 2	Cat	*Felis domesticus*	
Fel d 4	Cat	*Felis domesticus*	
Gad c 1	Cod	*Gadus callarias*	
Gal d 1	Egg, Ovomucoid	*Gallus domesticus*	
Gal d 2	Egg, Ovalbumin	*Gallus domesticus*	
Gal d 3	Egg, Conalbumin	*Gallus domesticus*	
Gal d 5	CSA (Livetin)	*Gallus domesticus*	
Mus m 1	Mouse	*Mus musculus*	
Non-Plants, Cross-reactive Markers			
Ani s 3	Anisakis	*Anisakis simplex*	Tropomyosin
Bla g 7	Cockroach	*Blattella germanica*	Tropomyosin
Der p 10	House dust mite	*Dermatophagoides pteronyssinus*	Tropomyosin
Pen a 1	Shrimp	*Penaeus aztecus*	Tropomyosin
Pen i 1	Shrimp	*Penaeus indicus*	Tropomyosin
Pen m 1	Shrimp	*Penaeus monodon*	Tropomyosin

Table. 3.1. Panel of allergenic components included in the microarray. Components listed in alphabetical order classified as: plants and non-plants, and subdivided into specific markers and cross-reactive markers. PR-10: pathogenesis-related group 10; LTP: Lipid Transfer Protein; CBP: Calcium-binding protein; CCD: Carbohydrate Cross-reactive Determinants.

3.6. Enzyme Linked Immuno-Sorbent Assay (ELISA)

The Enzyme Linked Immuno-Sorbent Assay (ELISA) is a powerful technique for detection and quantitation of biological substances such as proteins, peptides, antibodies and hormones. By combining the specificity of antibodies with the sensitivity of simple enzyme assay, the ELISA can provide a quick and useful measurement of the concentration of an unknown antigen or antibody. Currently, there are three major types of ELISA assays commonly used. They are: indirect ELISA (typically used for antibody screening), sandwich ELISA (or antigen capture, for analysis of antigen present) and competitive ELISA (for detection of antigen specificity).

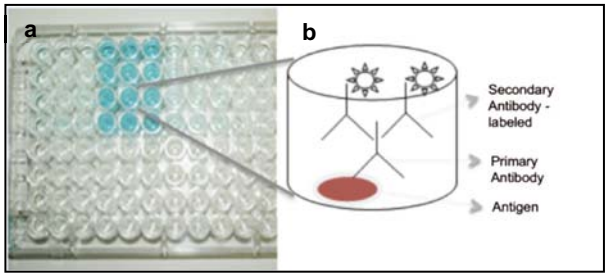

Figure 3.3. ELISA assay. a) Typical ELISA output. Darker wells indicate higher levels of analyte in the original sample. b) Schematic representation of an indirect ELISA in one well. Adapted from [115].

Briefly in the indirect ELISA, a buffered solution of the antigen to be tested is adhered to each well of a microtiter plate. A solution of non-reacting protein, such as bovine serum albumin (BSA), is added to block any plastic surface in the well that remains uncoated by the antigen. Next, the primary antibody is added, which binds specifically to the test antigen that is coating the well. The primary antibody in this case would be in the serum of a patient to be tested for reactivity towards the antigen (allergen). Afterwards, a secondary antibody is added, which binds the primary antibody. This secondary antibody is labeled with an enzyme attached that does not effect on the binding properties of the antibody. A substrate for this enzyme is then added and changes color upon reaction with the enzyme. The color change shows that secondary antibody has bound to primary antibody, which strongly implies that the patient's serum has had an immune reaction to antigen tested. The higher the concentration of the primary antibody that was in the serum, the stronger the color change. Often a spectrometer is used to give quantitative values for color strength and results are expressed as optical density (OD) units (Figure 3.3) [115].

An indirect ELISA was used to test individual sera from 21 patients of the multiple plant-food allergy group for specific IgE to wheat LTP (rTri a 14).

1. Coat Polystyrene 96-well microtiter plates (Costar 3590, Corning Life Sciences) with 50 µL of purified protein at 2 µg/mL in phosphate buffer saline (PBS) (0.1M sodium phosphate [pH 7.0] and 0.15 mol/L NaCl) for 1 hour at 37°C.

2. Block the coated wells (5 µg/ml solid phase) with 1% BSA in PBS buffer.

3. Incubate with 50 µL of a single dilution of the individual serum (1:3) for 14 hours at 25°C.

4. Wash with 0.1% Tween-20 in PBS buffer (PBS-T 0.1%) and incubate with a peroxidase-labeled antihuman IgE (Dako A/S, Glostrup, Denmark) for 1 hour at 25°C.

5. Wash again and then develop with 50 µL of peroxidase substrate buffer (code S2045, Dako).

6. After 30 minutes, stop the reaction with 50 µL of 4N H_2SO_4 and measure OD at 492 nm.

PBS buffer with 1% BSA was tested as a negative control for each solid phase. Additionally, blocking solution without solid phase was used as negative control. Tests were performed in triplicate. A read out (λ 492 nm) above 0.11 OD units was considered positive [116].

3.7. Boiled and raw shrimp western blotting

3.7.1. Preparation of boiled and raw shrimp extracts

Extracts from boiled and raw pacific white shrimp (*Litopenaeus vannamei*) were obtained from the Mount Sinai Food Allergen Repository. Extracts had been prepared from raw shrimp tail muscles manually homogenized in a mortar to achieve a smooth paste. Protein extraction had been made by agitation in PBS with a protease inhibitor cocktail, without ethylenediamine tetra-acetic acid (EDTA, Roche, Indianapolis, Ind), and sodium azide (NaN_2) 1:400) as preservative and incubated overnight at 4ºC. The mixture was centrifuged at 3000 rpm for 10 minutes at 4ºC and then at 15000 rpm for 5 minutes at 4ºC. The boiled extract was prepared the same way but previously boiling the peeled tails for 5 minutes in distilled water [84].

3.7.2. Protein separation and western blotting

First, Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) was used to separate proteins contained in whole extracts. The SDS-PAGE uses a highly cross-linked gel of polyacrylamide as the inert matrix through which the proteins migrate. The proteins themselves are not in a single aqueous solution but in one that includes a powerful negatively charged detergent, sodium dodecyl sulfate (SDS) (Figure 3.4). This detergent binds to hydrophobic regions of the protein molecules and with the treatment of strong reducing agents to remove secondary and tertiary structure (e.g. disulfide bonds [S-S] to sulfhydryl groups [SH and SH]), the protein is unfolded into extended polypeptide chains (denaturation).

Proteins of the sample become covered in the negatively charged SDS and move to the positively charged electrode through the mesh of the gel that is packed on cassettes. Smaller proteins migrate faster, thus proteins are separated according to size (usually measured in kDa). The concentration of acrylamide determines the resolution of the gel. The greater the acrylamide concentration, the better the resolution of lower molecular weight proteins. The lower the acrylamide concentration the better the resolution of higher molecular weight proteins.

Samples are loaded into wells in the gel. One lane is usually reserved for a marker or ladder, a commercially available mixture of proteins having defined molecular weights, typically stained so as to form visible, colored bands (e.g., SeeBlue®Plus 2 Molecular Weight, Invitrogen). When voltage is applied along the gel, proteins migrate into it at different speeds. These different rates of advancement (known as different electrophoretic mobility) separate into bands within each lane [117].

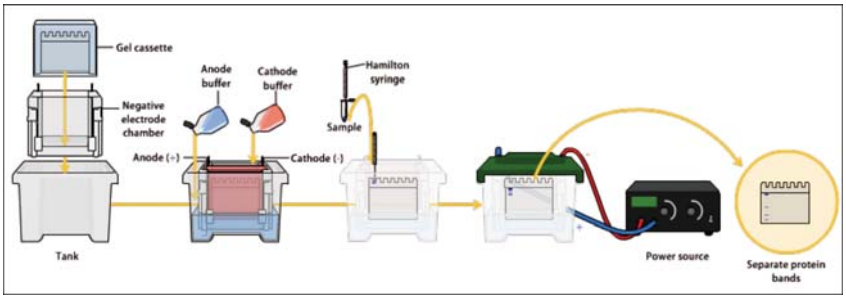

Figure 3.4. Protein electrophoresis. By far the most common type of gel electrophoresis employs polyacrylamide gels and buffers loaded with sodium dodecyl sulfate. Source: [118].

Second, western blotting was used to test IgE reactivity of patient's sera to the proteins contained in the shrimp extract.

Western blotting is an extension of protein electrophoresis. Sometimes called Immunoblotting, it is a reliable method for investigating any sample (tissue or cells that are homogenized in a buffer) for the presence of a specific antigen. After protein separation with SDS-PAGE, proteins are transferred to a membrane (typically nitrocellulose or polyvinylidene fluoride (PVDF)) for detection, based on molecular weight, with primary and secondary antibodies (Figure 3.5).

In the particular case of allergy, proteins from allergenic sources are immobilized on membranes and are detected with antibodies in a two-step process:

1. First, the primary antibody, which corresponds to the IgE directed against the allergenic protein in question that is present in the sera of the subject.

2. Then, a secondary antibody (an antihuman IgE antibody). Two approaches are common: a secondary antibody conjugated with the enzyme horseradish peroxidase (HRP) that catalyzes a chemiluminescent reaction (oxidation of luminol to 3-aminophthalate accompanied by emission of low-intensity light that can be enhanced in the presence of certain chemicals (modified phenols) increasing the sensitivity of the reaction (enhanced chemiluminescence, ECL), or a secondary antibody labeled with iodine 125 isotope. Both reactions are visualized through exposure of X-ray film, but the radioactive one is more sensitive and shows less background on proceeding [118].

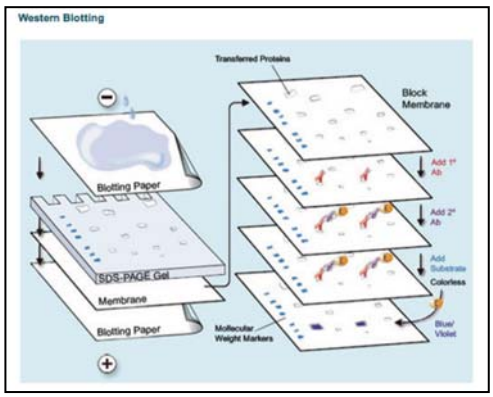

Figure 3.5. Western blotting procedure. Source: http://www.leinco.com/general_wb

Hereby, for SDS-PAGE analyses, samples were heated at 70ºC for 10 minutes with Nupage sample buffer® (Invitrogen, Carlsbad, CA) in the presence of 0.05M dithiothreitol (DTT). The proteins of the extract were then separated by means of SDS-PAGE using Nupage 4-12% Zoom Gels® (Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions and as described earlier on this chapter.

After gel separation, proteins were transferred onto Immobilon-P membrane® (Millipore®, Bedford, Mass) for immunoblot detection of IgE binding (western blot), according to manufacturer instructions. Membranes were cut on strips of 4mm wide with a razor blade to test individual sera from all patients and controls recruited for the shellfish study. Sera dilutions ranged from 1:5 to 1:20 (in PBS-T 0.05%, 1% BSA and 10% normal goat serum (NGS)) depending on the shrimp specific IgE levels (f24, Phadia, Sweden) of the individual. The membrane was incubated with diluted sera for 1 hour. After rinsing with PBS (four quick rinses), the membranes were incubated for 1 hour at room temperature with a secondary antibody (iodine 125–labeled goat anti-human IgE, DiaMed, Windham, Me) diluted as per the manufacturer's instructions, washed (four quick rinses with PBS) and exposed to Kodak Imaging Film (Carestream

Health Inc.®, Rochester, NY) for a short period (2-4 days) and a long one (12-15 days). As a negative control, serum from a nonatopic subject was used.

3.8. Purification of recombinant proteins

3.8.1. Overview

Recombinant DNA technology consists of isolating a target DNA sequence and transferring it to a cloning vector that has the ability to self-reproduce. The DNA of the cloning vector interacts with the target DNA and produces a new blueprint of gene information called recombinant DNA that is transferred to RNA, which in turn produces a *recombinant protein* [119].

Thanks to molecular biology, recombinant allergens can be produced as defined molecules in consistent quality and unlimited amounts according to the corresponding DNA template that exactly mimic the properties of the natural allergens (i.e., recombinant wild-type allergens), but also as modified variants with advantageous properties for therapeutic purposes (i.e., reduced allergenic activity or increased immunogenicity) [62].

The most widely used expression systems are those using *Escherichia coli* (*E. coli*) as the host cell. However, there are also several eukaryotic cell–based expression systems available: insect cells infected with recombinant baculovirus, yeast (e.g., *Saccharomyces cerevisiae* and *Pichia pastoris*) and mammalian cells. The choice of protein expression system depends on the intended use and characteristics of the protein. If a protein can be expressed in *E. coli* in a soluble state either directly into the cytoplasm or by secretion into the periplasm, then it may be possible to isolate large amounts of correctly folded and active protein. On the other hand, if the protein's full biological activity requires posttranslational modification (i.e., glycosylation or phosphorylation), then an eukaryotic expression system must be used.

The basic approach used to express foreign genes in *E. coli* begins with insertion of the gene into an expression vector, usually a plasmid. The next step involves transforming a suitable *E. coli* host strain with the construct, for example, by electroporation or thermic shock. Transformed cells are then subjected to evaluation of plasmid stability and foreign protein expression after induction. Once small-scale shaker flask experiments have identified successful expression systems of the

controllable transcriptional promoter present in the expression vector, the transformed *E. coli* strain can be used in large-scale growth systems. Production is followed by protein purification and characterization (Figure 3.6) [120].

An essential component of typical *E. coli* expression vectors is the promoter, which when induced, can direct the production of large amounts of mRNA from the cloned gene. One of the most common routinely used is the *lac* promoter system, which utilizes the transcriptional control elements from the *E. coli* β-galactosidase gene. The Lac repressor controls this promoter by binding to an operator sequence in the promoter region. Induction of the promoter is generally accomplished by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG), a lactose analog that binds to the lac repressor and prohibits its binding to the lac operator. Moreover, a translation initiation sequence (Shine and Dalgarno sequence or ribosome binding site (RBS)) is required in close proximity to an initiator methionine for the initiation of translation on mRNAs [120].

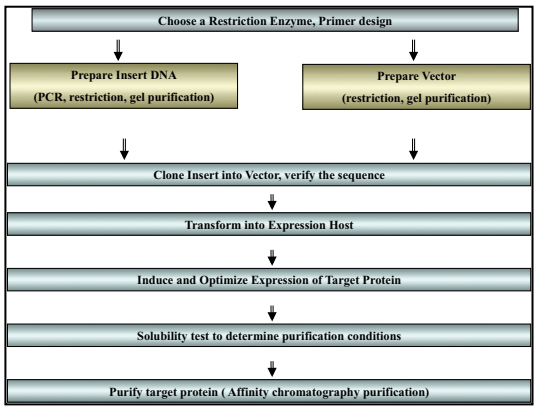

Figure 3.6. Overview of the production of recombinant proteins. Source: Galina Grishina, Mount Sinai School of Medicine, New York.

Recombinant proteins can be expressed in soluble or insoluble (as inclusion bodies) forms in the periplasmic space or in the cytoplasm of the bacteria. Inclusion bodies are composed of densely packed denatured protein molecules in the form of particles that require additional steps of solubilization and refolding [120].

Therefore, strategies for protein purification depend on the localization of the protein and its solubility. Probably, the most significant advancements for protein purification have been the development of fusion proteins and the fusion tag expression and purification techniques, resulting in a more consistent production of

soluble and active protein, and allowing for simple and efficient purification and/or detection of the proteins from bacterial lysates [120].

3.8.2. Crustacean recombinant allergens

Ten recombinant (r) crustacean proteins were considered for this study: Tropomyosin (TM, Lit v 1), Arginine Kinase (AK, Lit v 2), Myosin Light Chain (MLC, Lit v 3), Sarcoplasmic-Calcium-Binding protein (Lit v 4, SCP isoform alpha), Hemocyanin (Hemo), Fatty-Acid Binding Protein (FABP) (all from pacific white shrimp, *Litopenaeus vannamei*), lobster-Sarcoplasmic-Calcium-Binding protein (SCP, isoform beta), and 3 different Troponin C proteins [TpC, 2 from lobster (*Homarus americanus*) GenBank Acc. FE535729 (=$TpCB_5$) and FD699849 (=$TpCC_{10}$) (FE and FD amino acid sequences: 42.2% identity; 55% similarity), 1 from green shore crab (*Carcinus maenas*) GenBank Acc. DN634859, =$TpCA_2$) (FD and DN: 44% identity/ 58.4% similarity; FE535729 and GC: 75% identity/ 84% similarity. $TpCA_2$, $TpCB_5$ and $TpCC_{10}$ are arbitrary names derived from the process of cloning (A, B, C: name of the Lysogeny or Luria Broth (LB) agar plate used and the number corresponds to the colony peaked and grown).

3.8.3. Expression of recombinant protein

Clones had already been constructed and were obtained from the Mount Sinai Food Allergen Repository, except for Troponin C clones that were kindly provided by Mount Desert Island Biological Laboratory (MDIBL, Salsbury Cove, ME, USA).

Briefly, the cDNAs of interest were amplified by means of polymerase chain reaction (PCR) and ligated into pET24b vector using the Rapid Ligation Kit (Roche®) and introduced into XL1-Blue *E.coli* strain (Agilent Technologies®) following manufacturer's protocol. Next, the size of the cloned DNA inserts and their orientation in the vector were confirmed by PCR of individual colonies off a transformation plate. Plasmid purification from the positive colonies was performed using the miniprep plasmid extraction kit (Qiagen®). The nucleotide sequence of the cDNA insert and the adjacent vector fragments was verified by direct sequencing. For protein production each construct was introduced into expression *E. coli* strains - BL21 (Agilent Technologies® or Rosetta strains (Novagen®), if experiencing difficulties for protein expression.

Protein expression was performed as follows. Transformed cells had been grown overnight (O/N) at 37ºC on LB agar plates containing 30µg/µl kanamycin. The next day, a new O/N culture (in 3mL LB 30µg/µl kanamycin) was inoculated with a single colony and grown at 37ºC with mild shaker agitation overnight. Next morning, the culture optical density was checked at 600nm (1:10 dilution in LB) and a new culture was started by inoculating 6 mL LB medium with volume of the overnight culture containing ~0.12 optical units (O.U) at 600nm, total. The culture was grown at either 37ºC or 30ºC until it reached 0.4-1 O.U. at 600nm. Then, protein expression was induced by adding IPTG at 1mM final concentration. The optimal induction time was determined by Western blot analysis of the expressed protein at different time points – typically at 2, 4, 6 and 16 hours post-induction. At each time point, culture optical density was measured as above and cells from the culture aliquot (~0.2 O.U. at 600nm) were collected for analysis In order to optimize the purification conditions the aggregate state of the expressed protein was determined by solubility test employing SDS-PAGE/Western blot analysis.

For this project, all recombinant proteins were expressed after induction of bacterial cultures (45 mL LB-kanamycin) with IPTG at the chosen conditions (summarized in Table 3.2). After induction, cell pellets were obtained from cultures by centrifugation at 5,000 g for 15 minutes using pre-weighed centrifuge tubes and stored at -80ºC until purification.

Protein	Expression host	Induction Conditions	Expression form
TM	BL21	37ºC, 16h	Native
SCP-alpha	BL21	37ºC, 16h	Native
SCP-beta	Rosetta	37ºC, 2h	Native
MLC	BL21	30ºC, 16h	Native
AK	BL21	30ºC, 16h	Denatured
Hemo	BL21	37ºC, 16h	Denatured
FABP	BL21	37ºC, 16h	Native
TpC A2	Rosetta	37ºC, 2h	Native
TpC B5	Rosetta	37ºC, 2h	Native
TpC C10	Rosetta	37ºC, 4h	Native

Table 3.2. Summary of the conditions for recombinant allergens expression.

3.8.4. Purification of recombinant protein

Affinity purification is based on the specific interaction of a target molecule with an immobilized ligand. For recombinant proteins, the addition of fusion tags using appropriate expression vectors enables affinity purification by a number of strategies.

Briefly, fusion tags are small stretches of amino acids added to the N-terminal or C-terminal end of a protein. Not usually helping to increase expression levels or protein solubility, they can be advantageous in protein purification and detection. The tags are generally chosen either because they encode an epitope that can be later detected and purified using an antibody that binds to it, or because the tag amino acids provide a physical characteristic that can be exploited for easy and specific protein purification. As an example is the polyhistidine tag (His Tag), usually a stretch of six consecutive histidine residues added to either the N- or C-terminus of a protein that provides specific binding to metal chelate resins. There are a number of polyhistidine vectors on the market.

Several commercially available products have been specifically designed for the purification or detection of fusion proteins tag sequences. These products are optimized for purification of proteins expressed in bacterial, yeast, insect, or mammalian systems. For maximal recovery of intact target proteins from cell cultures, a first step in purification should be an efficient, gentle extraction**.**

For this project, all recombinant allergens were purified under native conditions using the His·60 Ni Buffer Set (Clontech®, Cat. No. 635665), except for the recombinant Hemocyanin and AK that were purified under denatured conditions using the BugBuster Ni-NTA His•Bind Purification Kit (Novagen®).

The main difference between working under denatured or native conditions resides on the sample preparation to obtain the recombinant protein (i.e., cultured cells preparation using different buffers). Both methods are based on affinity purification using nickel (Ni) charged resin columns, to which the His-tag of the recombinant protein binds. Later on, this binding is disrupted and the protein of interest is eluted.

3.8.4.1. His·60 Ni Superflow™ resin and gravity columns

The His·60 Ni Superflow™ Resin (Clontech®) is a high-capacity Ni-IDA resin that has been optimized for the efficient purification of expressed his-tagged proteins from bacterial, mammalian and baculovirus-infected cells. His-tagged proteins are

purified from total soluble protein extract utilizing a high capacity His60 Ni resin charged with Ni immobilized onto Superflow™ agarose beads. The combination of the high density of Ni (2+) ion and high flow rates allow the efficient capture of target his-tagged proteins (Figure 3.7) [121]. The manufacturer's protocol was followed, briefly described hereby:

A. Sample Preparation

1. Add 2 mL of His60 Ni x Tractor Buffer per 100 mg of cell pellet. Gently pipet up and down until the cell pellet is fully resuspended.

2. To the resuspended pellet, add 1 µL of Benzonase® for every 2 mL of extract (i.e., every 100 mg of cell pellet) and mix gently.

3. Incubate on ice, with intermittent mixing, for 15 min. Centrifuge for 20 min at 10,000 g at 4°C.

4. Carefully collect the clear supernatant, which is the starting sample.

B. Gravity-Flow Column Purification (1 mL pre-packed resin)

1. Equilibrate the column and all buffers to room temperature.

2. Wash the column with 5-10 column volumes of His·60 Ni Equilibration Buffer. Put the bottom stopper on the outlet of the column.

3. Add the clarified sample (supernatant) to the column and carefully connect the top stopper to the top of the column. Allow target protein to bind by slowly inverting the column for 1 hour (preferably at 4°C).

5. Install the column in a vertical position and let the resin settle at the bottom of the column.

6. Put a stand containing clean empty tubes under the outlet of the column.

7. Carefully remove the top stopper. Remove the bottom stopper and start collecting 1 mL fractions.

8. Wash the column with 10 column volumes of His·60 Ni Equilibration Buffer followed by 10 column volumes of His·60 Ni Wash Buffer (prepared by mixing 710 µL of His·60 Ni Elution Buffer with 9.29 mL of His·60 Ni Equilibration Buffer)

9. Elute the target protein with approximately 10 column volumes of Elution Buffer and collect 1 mL fractions.

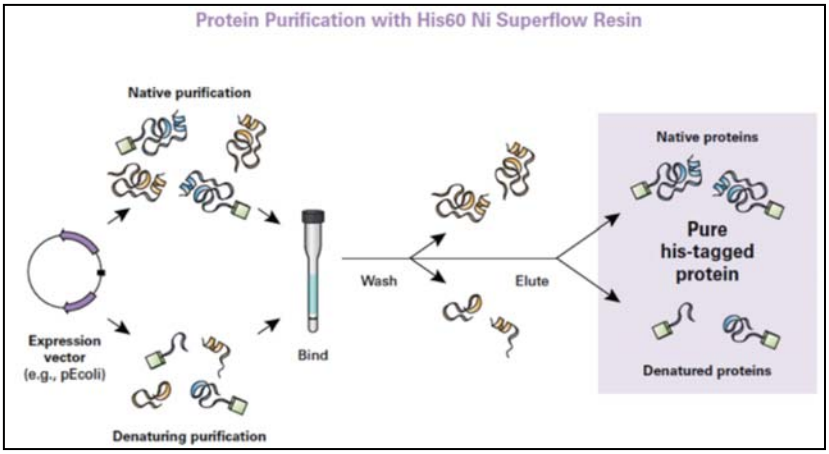

Figure 3.7 Overview of protein purification with His·60 Ni Superflow™ resin. Source: [121].

Collect all washes and elutes into separate tubes for the analysis on the gel (SDS-PAGE) or performing a Bradford protein assay to identify the elution fraction that has a major content of the protein of interest.

The His60 Ni Gravity Column can be quickly regenerated by adding 20 mL of His60 Ni Equilibration Buffer or by washing with 10 column volumes of 20 mM MES, 0.3 M NaCl; pH 5.0 buffer. Regeneration allows the column to be reused to purify the same protein multiple times without significant loss of binding capacity. For extended storage (over 1 week), wash the column with five column volumes of water after each use and store in 20% ethanol. Attach supplied bottom stopper, followed by the top stopper. Store the column at 4°C.

3.8.4.2. BugBuster Ni-NTA His·Bind purification kit

The BugBuster Ni-NTA His·Bind Purification Kit (Cat. No. 70751, Novagen, Merck4Biosciences®) combines the Ni-NTA His·Bind Resin and BugBuster Protein Extraction Reagent for convenient preparation of soluble cell extracts and affinity purification of His·Tag fusion proteins. The BugBuster Protein Extraction Reagent is a ready to use solution formulated for the gentle disruption of the cell wall of *E. coli* resulting in the liberation of soluble protein. The kit includes Benzonase® Nuclease for viscosity reduction and removal of nucleic acids from protein preparations. Cells are harvested by centrifugation as usual, followed by suspension in BugBuster reagent. During a brief incubation, soluble proteins are released without denaturation. The

extract is clarified by centrifugation, which removes cell debris and insoluble proteins. The clarified extract is ready to apply to the Ni-NTA His·Bind Resin.

Ni-NTA His·Bind Resin is used for rapid one-step purification of proteins containing a His·Tag® sequence by metal chelation chromatography. The His·Tag sequence binds to Ni^{2+} cations, which are immobilized on the Ni-NTA His·Bind Resin. After unbound proteins are washed away, the target protein is recovered by elution with imidazole. The versatile system allows proteins to be purified under gentle, non-denaturing conditions or in the presence of either 6 M guanidine or urea.

With the His·Tag/His·Bind technology, purification is based on the affinity between the 6-10 neighboring histidines of the His·Tag sequence and an immobilized metal ion (usually Ni^{2+}). The metal is held by chelation with reactive groups covalently attached to a solid support. The Ni-NTA His·Bind Resins use nitriloacetic acid (NTA) as the chelator, which has four sites available for interaction with metal ions [122].

A. Sample preparation

1. Completely resuspend the cell pellet in room temperature the BugBuster reagent by pipetting or gentle vortexing (using 5 mL reagent per gram of wet cell paste (1.2mL)).

2. Add 1 µL (25 units) of Benzonase per mL of BugBuster reagent used for resuspension.

3. Incubate the cell suspension on a shaking platform or rotating mixer at a slow setting for 30 min at room temperature.

4. Remove insoluble cell debris by centrifugation at 16,000 g for 20 min at 4°C.

5. Transfer the supernatant to a fresh tube. Save the pellet for inclusion body purification.

Inclusion body purification

1. Resuspend the pellet in the same volume of BugBuster reagent used to suspend the original cell pellet. Pipette up and down and vortex to obtain an even suspension.

2. Add 3 µL of rlysozyme™ (1:10 diluted from the stock). Mix by vortex and incubate at room temperature for 5 min.

3. Add 6 volumes of 1:10 diluted BugBuster reagent (in deionized water) to the suspension and vortex for 1 min.

4. Centrifuge the suspension at 16,000 g for 15 min at 4°C to collect the inclusion bodies.

5. Remove the supernatant with a pipette. Resuspend the inclusion bodies in one half the original culture volume of 1:10 diluted BugBuster, mix by vortexing, and centrifuge again at 16,000 g for 15 min at 4°C. Repeat this wash step 2 more times.

6. Resuspend the final pellet of purified inclusion bodies in 1 mL Binding Buffer "B".

7. Rotate the tube for 25 min at room temperature.

8. Centrifuge 5 min at 10,000g and load the supernatant on the already prepared column.

Column preparation

1. Use 4 mL/column of 50% Ni-NTA His-Bind slurry (1V= 2mL resin column)
2. Wash column with 5V (10mL) H_2O
3. Equilibrate column with 3V of Binding buffer (B)

B. Column purification

1. Once sample is loaded, wash two times with Binding buffer "B" (2V= 4mL).
2. Wash three times with Wash Buffer "C" (2V= 4mL).
3. Elute two times with Elution buffer "D" (1V = 2mL).
4. Elute four times with Elution buffer "E" (1V = 2 mL).

Collect all washes and elutes into separate tubes for the analysis on the gel (SDS-PAGE) or protein concentration determination. The fraction that contains the desired protein needs to be dialyzed to reduce the concentration of urea to 2M before protein storage or blotting use.

Buffers for purification under denaturing conditions

Denaturing binding buffer

Buffer B: 8 M urea; 0.1 M NaH_2PO_4; 0.01 Tris-Cl, pH 8.0

Denaturing washing buffer

Buffer C: 8 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris-Cl pH 6.9

Denaturing elution buffers

Buffer D: 8 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris-Cl pH 5.9

Buffer E: 8 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris-Cl pH 4.5

Note: Due to the dissociation of urea, the pH of Buffers B, C, D and E should be adjusted immediately prior to use. Do not autoclave these buffers.

3.8.5. Protein analysis

Purified proteins were analyzed on SDS-PAGE, as described above, and stained with Simply Blue SafeStain® reagent (Invitrogen®).

The Simply Blue SafeStain® reagent is a fast and easy staining protocol for visualizing protein bands on polyacrylamide gels. The gel is rinsed with three 5-minute washes with distilled water and then the already prepared reagent is added (approximately 20 mL per gel) to the gel in a cuvette, which is kept in a stirring plate until bands develop.

Bands begin to develop immediately in the stain (1 µg of protein can be detected after just 5 minutes in the stain). Destaining with distilled water is not required, but it is recommended to achieve maximum sensitivity and minimal background (Figure 3.8) [123].

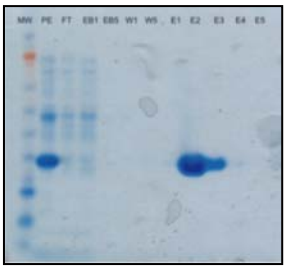

Figure 3.8. SDS-PAGE analysis of elution fractions. Example: purification of SCP using Clontech purification columns. Each lane represents an elution fraction. E2 and E3 contain the protein. PE represents pre-elution protein and MW is the molecular weight.

3.8.6. Protein dialysis

Dialysis consists on the removal of buffer salts (desalting) and small contaminants from proteins and other macromolecules. It can also be used for sample concentration. Slide-A-Lyzer Dialysis Cassettes® (Pierce Protein Research®, Thermo Scientific) facilitate this process compared to the traditional dialysis tubing, since they provide faster buffer exchange and better sample recovery. There are three different cassette sizes to accommodate sample volumes between 0.1 and 12 mL.

Briefly, cassettes are constructed from two sheets of low binding, regenerated-cellulose dialysis membrane that are hermetically sealed on either side of an inert plastic frame. The membrane and cassette materials are compatible with most common laboratory chemicals and buffers. Liquid samples are easily added and removed by penetrating the self-sealing gasket with a hypodermic needle attached to a syringe. No knots, clips or caps are needed to seal the units and prevent leaking and sample-loss during the dialysis procedure (Figure 3.9) [124].

Figure 3.9. Protein dialysis using the Slide-A-Lyzer Dialysis Cassettes. Source: [124].

Native proteins were dialyzed against PBS and denatured proteins were dialyzed against a buffer containing urea 4M and 2M for Hemo and AK, respectively.

3.8.7. Determination of protein concentration

Protein concentration was determined by Coomassie Plus (Bradford) Protein Assay® (Pierce Protein Research®, Thermo Scientific, Rockford, Ill).

The Pierce Coomassie Plus Assay Reagent® is a single, ready-to-use solution for measuring protein concentration in a colorimetric assay at room temperature. Simply, the reagent is added to equal volumes of samples and standards, mixed by gentle pipetting and the absorbance is measured straightforward on a standard spectrophotometer or plate reader at 595 nm. The assay can be performed in either test tubes or in a microplate. Protein detection range is 1 to 1500 µg/mL.

Briefly, in the acidic environment of the reagent, protein binds to the Coomassie dye. This results in a spectral shift from the reddish/brown form of the dye (absorbance maximum at 465 nm) to the blue form of the dye (absorbance maximum at 610 nm). The difference between the two forms of the dye is greatest at 595 nm, so that is the optimal wavelength to measure. Development of color has been associated with the presence of certain basic amino acids (primarily arginine, lysine and histidine). The

reagent is compatible with most salts, solvents, buffers, thiols, reducing substances and metal chelating agents encountered in protein samples [124].

3.9. Dot blot printing and immunolabelling

3.9.1. Dot blotting

The Minifold I manifold® (Schleicher & Schuell, Inc. ®, Keene, NH, USA) is a 96-well filtration/incubation unit for processing dot-blot assays of nucleic acids or proteins. The unique design of the device eliminates cross-lateral flow of reagents across the solid phase and forms discrete, uniform test dots for easy isolation and quantitation of results.

The system includes a sample well plate with metal clamping plate and silicone O-rings, a filter support plate and a vacuum plenum. The Minifold is easily adjusted for use with many different types of transfer matrices for optimal sample retention and biological activity. The manifold is available in either a standard acrylic unit or in a chemically resistant model (Delrin®), recommended for sterile procedure as it withstands autoclaving [125].

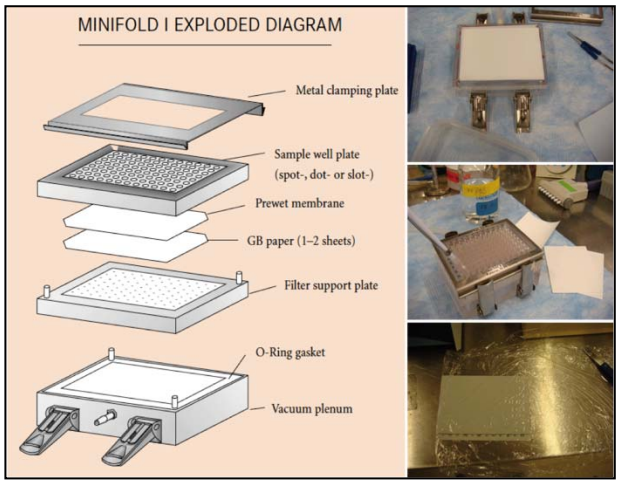

Figure 3.10. The Minifold Dot Blotter. a) Diagram of Minifold I system assembly. Source: [125]; b) Pictures of the Minifold I system used for this project.

Briefly, set up Minifold I system to vacuum source. Wet 2 filter sheets and one membrane (e.g., nitrocellulose) in PBS and place them on the filter support plate on top

of the vacuum plenum, previously aligned with the registration pins on either corner. Place the sample well plate, with O-rings facing down, on the top of the filter. Be sure each O-ring is fully seated to prevent lateral flow. Again, registration pins ensure proper alignment. Place the clamping plate on top of the sample well plate. Clamp the "sandwich" together with the adjustable stainless steel latches. The clamping plate ensures that even pressure is applied to all sides of the plate. Apply sample material, generally in a 200 µL volume, carefully not to puncture the membrane with the tip (for this project, proteins were disposed as depicted in Figure 3.11). Turn on vacuum source and make sure all sample has pulled through well. Wash each well with 200 µL of PBS under vacuum. Tap the device on bench top to release bubbles. Shut vacuum off after all wash buffer has gone through. Release clamps and carefully remove the membrane with the dotted samples. Wash the membrane with PBS-T 0.05% for five minutes in a stirring plate, avoid drying until use (wrap the membrane with plastic wrap) (Figure 3.10).

IgE-reactivity to all crustacean recombinant allergens was tested by dot blotting immunoanalysis using individual sera from all subjects recruited for the shellfish allergy study. One microgram of protein was dotted on nitrocellulose membrane (Trans-Blot® Transfer medium, Bio-Rad Laboratories) using The Minifold I Dot Blotter® described above. Proteins were displayed as it is shown in Figure 3.11. The manufacturer's protocol was followed [125]. After printing, the membrane was kept frozen at -20ºC until next day.

3.9.2. Immunolabelling

Before immunolabelling the membrane was defrosted at room temperature and cut into strips, using a razor blade, in a way that two strips contained all ten recombinant proteins (2 strips/ individual) (Figure 3.11).

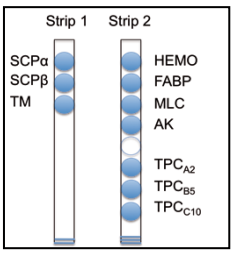

Figure 3.11. Nitrocellulose strips with printed recombinant allergens

The membrane was blocked with 1% BSA, 10% NGS in PBS-T 0.05% and afterwards incubated with diluted sera for one hour at room temperature. Iodine 125-labeled goat anti-human IgE (DiaMed, Windham, Me) was used as secondary antibody, also for one hour at room temperature. Membranes were exposed to Kodak Imaging Film (Carestream Health Inc, Rochester, NY) for both a short (2-4 days) and a long exposure (16-20 days). Three investigators qualitatively evaluated outcome results independently (positive/negative IgE binding compared to the background). Read outs were pooled together for agreement and analysis of frequencies of IgE recognition.

3.10. Epitope mapping

3.10.1. Synthetic overlapping peptides and printing conditions

Overlapping peptides (15-mers, overlapping by 10 amino acids) corresponding to the primary sequence of the 5 shrimp allergens: Lit v 1 (TM), Lit v 2 (AK), Lit v 3 (MLC), and Lit v 4 (SCP)) and TpC were commercially synthesized (JPT Peptide Technologies, Berlin, Germany).

Peptides were re-suspended in Dimethyl Sulfoxide (DMSO) - 99.7% Extra Dry Over Molecular Sieve, AcroSeal™ (Acros Organics®, Geel, Belgium) at 4 mg/mL, diluted 1:2 in dH_2O to a final concentration of 2 mg/mL. Using a NanoPrint60 (ArrayIt Corporation®, Sunnyvale California), diluted peptides were printed on epoxy-derivatized glass slides (3D Expoxy; JPT Peptide Technologies GmbH®) in 2 sets of triplicates to improve precision and to determine intra-assay variation. Each printed array also included spots from a 50:50 mixture of DMSO / dH_2O, used as solvent control and for background normalization, and fluorochrome-labeled BSA, for the purpose of grid alignment during analysis.

ARGININE KINASE		TROPOMYOSIN		SARCOPLASMIC		MYOSIN LIGHT CHAIN		TROPONIN C	
Pp #	Sequence	Pp #	Sequence	Pp #	Sequence	Pp #	Sequence	Pp #	Sequence
AK-01	MADAAVIEKLEAGFK	TM-01	MDAIKKKMQAMKLEK	SCP-01	MAYSWDNRVKYVVRY	MLC-01	MSRKSGSRSSSKRSK	TpC-01	MDSLDEEQIETLRKA
AK-02	VIEKLEAGFKKLEAA	TM-02	KKMQAMKLEKDNAMD	SCP-02	DNRVKYVVRYMYDID	MLC-02	GSRSSSKRSKKSGGG	TpC-02	EEQIETLRKAFDSFD
AK-03	EAGFKKLEAATDCKS	TM-03	MKLEKDNAMDRADTL	SCP-03	YVVRYMYDIDNNGFL	MLC-03	SKRSKKSGGGSNVFD	TpC-03	TLRKAFDSFDTEKTG
AK-04	KLEAATDCKSLLKKY	TM-04	DNAMDRADTLEQQNK	SCP-04	MYDIDNNGFLDKNDF	MLC-04	KSGGGSNVFDMFTQR	TpC-04	FDSFDTEKTGSITAE
AK-05	TDCKSLLKKYLTKEV	TM-05	RADTLEQQNKEANNR	SCP-05	NNGFLDKNDFECLAV	MLC-05	SNVFDMFTQRQVAEF	TpC-05	TEKTGSITAETIATI
AK-06	LLKKYLTKEVFDKLK	TM-06	EQQNKEANNRAEKSE	SCP-06	DKNDFECLAVRNTLI	MLC-06	MFTQRQVAEFKEGFQ	TpC-06	SITAETIATIMRMMG
AK-07	LTKEVFDKLKDKKTS	TM-07	EANNRAEKSEEEVHN	SCP-07	ECLAVRNTLIEGRGE	MLC-07	QVAEFKEGFQLMDRD	TpC-07	TIATIMRMMGVKISE
AK-08	FDKLKDKKTSLGATL	TM-08	AEKSEEEVHNLQKRM	SCP-08	RNTLIEGRGEFSADA	MLC-08	KEGFQLMDRDKDGVI	TpC-08	MRMMGVKISEKNLQE
AK-09	DKKTSLGATLLDVIQ	TM-09	EEVHNLQKRMQQLEN	SCP-09	EGRGEFSADAYANNQ	MLC-09	LMDRDKDGVIGKTDL	TpC-09	VKISEKNLQEAIAET
AK-10	LGATLLDVIQSGVEN	TM-10	LQKRMQQLENDLDQV	SCP-10	FSADAYANNQKIMRN	MLC-10	KDGVIGKTDLRGTFD	TpC-10	KNLQEAIAETDEDGS
AK-11	LDVIQSGVENLDSGV	TM-11	QQLENDLDQVQESLL	SCP-11	YANNQKIMRNLWNEI	MLC-11	GKTDLRGTFDEIGRI	TpC-11	AIAETDEDGSGLLEF
AK-12	SGVENLDSGVGIYAP	TM-12	DLDQVQESLLKANIQ	SCP-12	KIMRNLWNEIAELAD	MLC-12	RGTFDEIGRIATDQE	TpC-12	DEDGSGLLEFEEFVE
AK-13	LDSGVGIYAPDAEAY	TM-13	QESLLKANIQLVEKD	SCP-13	LWNEIAELADFNKDG	MLC-13	EIGRIATDQELDEML	TpC-13	GLLEFEEFVELSAKF
AK-14	GIYAPDAEAYTLFAP	TM-14	KANIQLVEKDKALSN	SCP-14	AELADFNKDGEVTVD	MLC-14	ATDQELDEMLADAPA	TpC-14	EEFVELSAKFLIEED
AK-15	DAEAYTLFAPLFDPI	TM-15	LVEKDKALSNAEGEV	SCP-15	FNKDGEVTVDEFKQA	MLC-15	LDEMLADAPAPINFT	TpC-15	LSAKFLIEEDEEALK
AK-16	TLFAPLFDPIIEDYH	TM-16	KALSNAEGEVAALNR	SCP-16	EVTVDEFKQAVQKHC	MLC-16	ADAPAPINFTMLLNM	TpC-16	LIEEDEEALKAELRE
AK-17	LFDPIIEDYHVGFKQ	TM-17	AEGEVAALNRRIQLL	SCP-17	EFKQAVQKHCQGKKY	MLC-17	PINFTMLLNMFAERQ	TpC-17	EEALKAELREAFRIY
AK-18	IEDYHVGFKQTDKHP	TM-18	AALNRRIQLLEEDLE	SCP-18	VQKHCQGKKYGDFPG	MLC-18	MLLNMFAERQTGESD	TpC-18	AELREAFRIYDKEGN
AK-19	VGFKQTDKHPNKDFG	TM-19	RIQLLEEDLERSEER	SCP-19	QGKKYGDFPGAFKVF	MLC-19	FAERQTGESDDDDVV	TpC-19	AFRIYDKEGNGFITT
AK-20	TDKHPNKDFGDVNSF	TM-20	EEDLERSEERLNTAT	SCP-20	GDFPGAFKVFIANQF	MLC-20	TGESDDDDVVAKAFL	TpC-20	DKEGNGFITTDVLKE
AK-21	NKDFGDVNSFVNVDP	TM-21	RSEERLNTATTKLAE	SCP-21	AFKVFIANQFKAIDV	MLC-21	DDDVVAKAFLAFADE	TpC-21	GFITTDVLKEILAEL
AK-22	DVNSFVNVDPEGKFV	TM-22	LNTATTKLAEASQAA	SCP-22	IANQFKAIDVNGDGK	MLC-22	AKAFLAFADEEGNID	TpC-22	DVLKEILAELDPRLT
AK-23	VNVDPEGKFVISTRV	TM-23	TKLAEASQAADESER	SCP-23	KAIDVNGDGKVGLDE	MLC-23	AFADEEGNIDCDTFR	TpC-23	ILAELDPRLTPADLE
AK-24	EGKFVISTRVRCGRS	TM-24	ASQAADESERMRKVL	SCP-24	NGDGKVGLDEYRLDC	MLC-24	EGNIDCDTFRHALMT	TpC-24	DPRLTPADLENIIEE
AK-25	ISTRVRCGRSMQGYP	TM-25	DESERMRKVLENRSL	SCP-25	VGLDEYRLDCITRSA	MLC-25	CDTFRHALMTWGDKF	TpC-25	PADLENIIEEVDEDG
AK-26	RCGRSMQGYPFNPCL	TM-26	MRKVLENRSLSDEER	SCP-26	YRLDCITRSAFAEVK	MLC-26	HALMTWGDKFSSQEA	TpC-26	NIIEEVDEDGSGTLD
AK-27	MQGYPFNPCLTESQY	TM-27	ENRSLSDEERMDALE	SCP-27	ITRSAFAEVKEIDDA	MLC-27	WGDKFSSQEADDALD	TpC-27	VDEDGSGTLDFDEFM
AK-28	FNPCLTESQYKEMEA	TM-28	SDEERMDALENQLKE	SCP-28	FAEVKEIDDAYNKLT	MLC-28	SSQEADDALDQMDID	TpC-28	SGTLDFDEFMEMMNG
AK-29	TESQYKEMEAKVSST	TM-29	MDALENQLKEARFLA	SCP-29	EIDDAYNKLTTEDDR	MLC-29	DDALDQMDIDDGGKI		
AK-30	KEMEAKVSSTLSSLE	TM-30	NQLKEARFLAEEADR	SCP-30	YNKLTTEDDRKAGGL	MLC-30	QMDIDDGGKIDVQGV		
AK-31	KVSSTLSSLEGELKG	TM-31	ARFLAEEADRKYDEV	SCP-31	TEDDRKAGGLTLERY	MLC-31	DGGKIDVQGVIQMLT		
AK-32	LSSLEGELKGTYYPL	TM-32	EEADRKYDEVARKLA	SCP-32	KAGGLTLERYQDLYA	MLC-32	DVQGVIQMLTAGGGD		
AK-33	GELKGTYYPLTGMSK	TM-33	KYDEVARKLAMVEAD	SCP-33	TLERYQDLYAQFISN	MLC-33	IQMLTAGGGDDAAAE		
AK-34	TYYPLTGMSKEVQQK	TM-34	ARKLAMVEADLERAE	SCP-34	QDLYAQFISNPDESC	MLC-34	AGGGDDAAAEEA		
AK-35	TGMSKEVQQKLIDDH	TM-35	MVEADLERAEERAET	SCP-35	QFISNPDESCSACYL				
AK-36	EVQQKLIDDHFLFKE	TM-36	LERAEERAETGESKI	SCP-36	PDESCSACYLFGPLK				
AK-37	LIDDHFLFKEGDRFL	TM-37	ERAETGESKIVELEE	SCP-37	SACYLFGPLKVVQ				
AK-38	FLFKEGDRFLQAANA	TM-38	GESKIVELEEELRVV						
AK-39	GDRFLQAANACRYWP	TM-39	VELEEELRVVGNNLK						
AK-40	QAANACRYWPAGRGI	TM-40	ELRVVGNNLKSLEVS						
AK-41	CRYWPAGRGIYHNDN	TM-41	GNNLKSLEVSEEKAN						
AK-42	AGRGIYHNDNKTFLV	TM-42	SLEVSEEKANQREEA						
AK-43	YHNDNKTFLVWVNEE	TM-43	EEKANQREEAYKEQI						
AK-44	KTFLVWVNEEDHLRI	TM-44	QREEAYKEQIKTLTN						
AK-45	WVNEEDHLRIISMQM	TM-45	YKEQIKTLTNKLKAA						
AK-46	DHLRIISMQMGGDLG	TM-46	KTLTNKLKAAEARAE						
AK-47	ISMQMGGDLGQVFRR	TM-47	KLKAAEARAEFAERS						
AK-48	GGDLGQVFRRLTSAV	TM-48	EARAEFAERSVQKLQ						
AK-49	QVFRRLTSAVNEIEK	TM-49	FAERSVQKLQKEVDR						
AK-50	LTSAVNEIEKRIPFS	TM-50	VQKLQKEVDRLEDEL						
AK-51	NEIEKRIPFSHHDRL	TM-51	KEVDRLEDELVNEKE						
AK-52	RIPFSHHDRLGFLTF	TM-52	LEDELVNEKEKYKSI						
AK-53	HHDRLGFLTFCPTNL	TM-53	VNEKEKYKSITDELD						
AK-54	GFLTFCPTNLGTTVR	TM-54	KYKSITDELDQTFSE						
AK-55	CPTNLGTTVRASVHI	TM-55	TDELDQTFSELSGY						
AK-56	GTTVRASVHIKLPKL								
AK-57	ASVHIKLPKLAANRE								
AK-58	KLPKLAANREKLEEV								
AK-59	AANREKLEEVAGKYN								
AK-60	KLEEVAGKYNLQVRG								
AK-61	AGKYNLQVRGTRGEH								
AK-62	LQVRGTRGEHTEAEG								
AK-63	TRGEHTEAEGGIYDI								
AK-64	TEAEGGIYDISNKRR								
AK-65	GIYDISNKRRMGLTE								
AK-66	SNKRRMGLTEFQAVK								
AK-67	MGLTEFQAVKEMQDG								
AK-68	FQAVKEMQDGILELI								
AK-69	EMQDGILELIKIEKE								

Table 3.3. Sequences of the synthetic overlapping peptides tested in the microarray.

3.10.2. Immunolabelling

The slides were blocked with 400 µL of 1% human serum albumin (HSA) in PBS-T for 60 minutes at room temperature, followed by incubation with 250 µL of patient serum diluted 1:5 in PBS-T/HSA for 24 hours at 4ºC. Slides were then washed with PBS-T and incubated for 24 hours at 4ºC with a cocktail of 3 biotinylated

monoclonal anti-human IgE antibodies, one from Invitrogen® (Carlsbad, CA) diluted 1:250, one from BD Biosciences Pharmingen® (San Jose, CA) diluted 1:250, and one from Phadia® (Uppsala, Sweden) diluted 1:1000, and one monoclonal anti-human IgG4-fluorescein isothiocyanate (SouthernBiotech®, Birmingham, AL) diluted 1:1000 in PBS-T/HSA.

After incubation with secondary antibodies, slides were incubated for 3 hours at 31ºC with a cocktail of Anti-Biotin Dendrimer Oyster 550 (350; Genisphere®, Hatfield, MN) and Anti-Fluorescein Isothiocyanate Dendrimer Oyster 650 (350; Genisphere®) in Dendrimer Buffer (Genisphere®), both at 0.6 µg/mL, with the addition of 0.02 mg/mL salmon sperm DNA (Invitrogen®). Afterwards, several successive washing steps were performed with PBS-T, 15 mmol/L Tris, 0.1X PBS, and 0.05X PBS. Slides were centrifuge dried and scanned with a ScanArrayGx (PerkinElmer®, Waltham, MA). Images were saved in TIFF format (Figure 3.12).

3.10.3. Microarray data analysis

Fluorescence signal of each spot was digitized with the program Scan Array Express (Perkin Elmer®) and data were exported as comma-delimited text files and analyzed with R programming language. Briefly, the read out for each spot, including all replicates and the spots for background control, is the median fluorescent signal of the spot divided by the local background and $\log_2$ transformed. Thus, a z score value is calculated for each spot using background control values within the same array. The total z score value for each peptide is the median of the z-scores of the six replicated spots [63, 67]. An individual peptide sample was considered positive if its z score exceeded the value of 3, meaning that the signal was significantly above the background ($P < 0.003$).

TileMap, a tool for tiling array analysis, based on a hierarchical empirical Bayes model with moving average method [126], was applied to identify IgE and IgG4 binding sites with statistical differences between groups. As hundreds of peptides were analyzed simultaneous, the false discovery rate (FDR) was calculated to adjust for multiple comparisons. A p value of less than 0.01 and FDR of less than 0.05 were considered as significant.

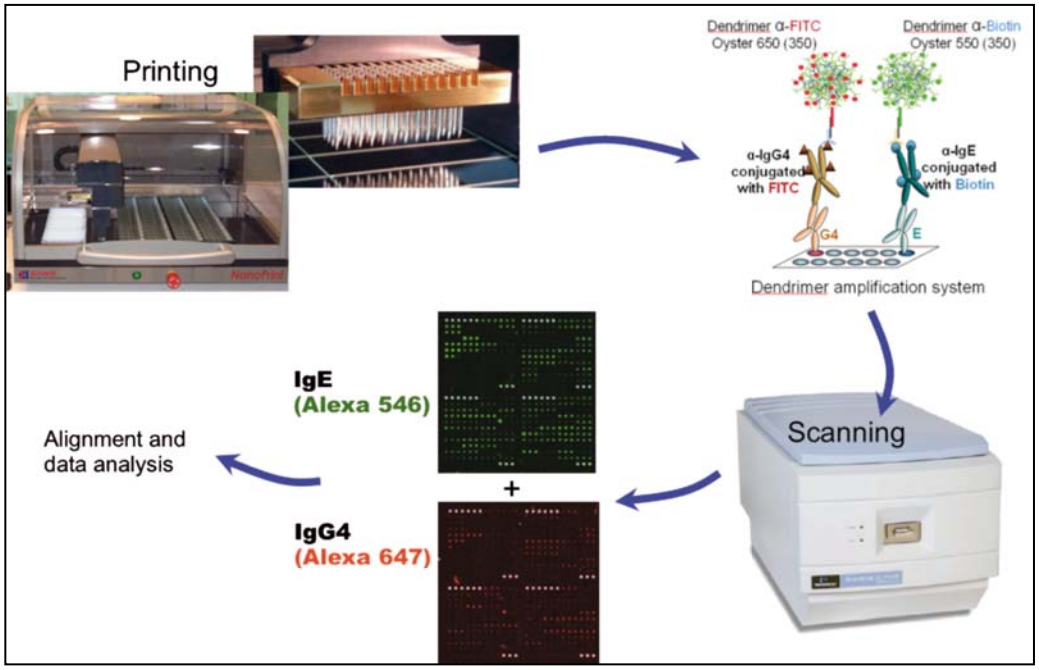

Figure 3.12. Overview of the general procedure of peptide microarray. Source: The Jaffe Food Allergy Institute, Mount Sinai School of Medicine, NY.

3.11. Basophil activation test

The basophil activation test (BAT), as briefly described in chapter 1, is an *in vitro* assessment of the allergic response that requires only a small amount of whole blood and allows for a measurement of a functional response of basophils beyond just the presence of IgE antibody bound to its receptor on the surface of such cells.

Allergen stimulation causes changes in the basophil cell membrane that can be detected by flow cytometry using the BAT. Thus, this assay uses flow cytometry to detect the upregulation of certain cell surface markers that in resting basophils are either anchored to intracellular basophilic granules (CD63) or are present in smaller quantities on the basophil cell membrane (CD203c) (Figure 3.13).

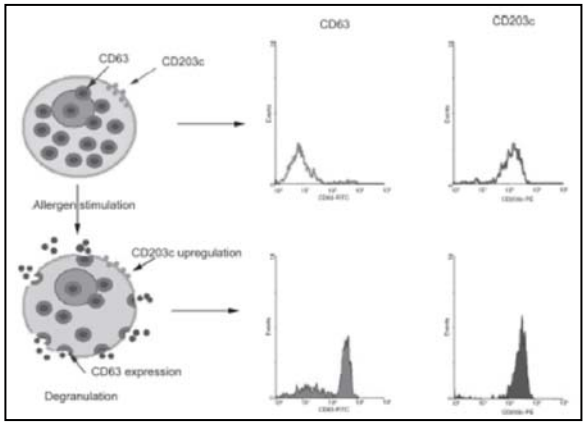

Figure 3.13. Basophil activation test. Basophils upon encounter with specific allergen recognized by surface receptor FcεRI-bound IgE, not only secrete and generate quantifiable bioactive mediators but also up-regulate the expression of different markers (CD45, CD63, CD69, CD203c) which can be detected by multicolor flow cytometry using specific monoclonal antibodies. Source: [18].

Twenty-four patients diagnosed of peach allergy, according to clinical history, positive SPT and serum specific IgE to Pru p 3 (the peach LTP) > 0.75 kU_A/L, presenting clinical exacerbation when NSAIDs intake were selected (Group A). All of them were NSAIDs tolerant. A cohort of 7 peach allergic patients, positive SPT and serum specific IgE to Pru p 3 > 0.75 kU_A/L, not clinically exacerbated by NSAIDs (Group B) and a cohort of 5 healthy subjects (Group C) were also included.

The basophil activation protocol using CD63 and CCR3 (Flow^{2}CAST^{TM} kit, Bühlmann®, Schönenbuch, Switzerland) was the cellular test of choice to develop an *in vitro* model for the cofactor effect of NSAIDs on plant-food allergy. The manufacturer's protocol was carefully followed. Briefly, the fresh within 1 hour of blood sampling in 4 mL EDTA tubes (BD Vacutainer^{TM}) blood samples were gently homogenized by inverting several times. For each patient polystyrene tubes were prepared with 50µL of allergen at the required concentrations diluted in stimulation buffer. As positive controls, both a monoclonal anti-FcεRI antibody and N-formyl-methionyl-leucyl-phenylalanine (fMLP) (2 mM) were used. In order to evaluate basal degranulation values without stimulation, 50 µL of stimulation buffer were applied to separate tubes (negative control).

Stimulation with allergens was performed in 2 separate phases, with the shortest delay between them and using the same blood sample:

- Phase 1: Pru p 3 alone at 2, 1, 0.5, 0.250, 0.125 and 0.0625 ng/mL.

- Phase 2: Pru p 3 (single concentration) in combination with different concentrations of L-acetyl salicylic acid (L-ASA, 3mM, 1mM, 0.3mM and 0.1mM). The concentration of Pru p 3 used for this phase was selected based on the following criterion: the concentration inducing a % of CD63+ cells equivalent to half of the ones achieved when stimulating with the monoclonal anti-FcεRI antibody (positive control) in the first phase. For example, if in the first phase for the positive control 68% of CD63+ cells were observed, the concentration of Pru p 3 inducing around 34% of CD63+ cells was selected. This criterion was strictly followed in all the patients and the rationale was to use a concentration of Pru p 3 that did not induce a maximal degranulation so we can observe if the L-ASA is increasing or not the number of CD63+ cells.

To each tube containing the appropriate allergen/s, 100 µL of stimulation buffer (containing calcium, heparin and IL-3 (2 ng/mL)), 50 µL of patients blood and 20 µL staining reagent containing a mix of anti-CD63-FITC and anti-CCR3-PE monoclonal antibodies were added. The tubes were covered with an adhesive plastic sheet and incubated at 37ºC for 15 min in a water bath. The stimulation was stopped by addition of 2 mL of lysing buffer and the tubes were left at room temperature for 5 min. After centrifugation for 5 min at 1600 rpm, the supernatant was decanted and 300 µL of washing buffer was added to each tube. The cells were resuspended by gently vortex before flow cytometric analysis.

Flow cytometric analysis of the cells was performed using a FACS-Canto™ flow cytometer (Becton-Dickinson Immunocytometry System®, Heidelberg, Germany), using FACS-Diva™ software. Basophilic cells were selected out of the lymphocyte population using anti-CCR3. At least 300 basophils were assessed in each assay to consider it valid. Basophil response was evaluated through the expression of the basophil activation marker CD63. The upregulation of the CD63 activation marker was calculated as the percentage of the CD63-positive cells within the total identified basophils. A high percentage of CD63+ cells in the negative control tubes (>5%, following the manufacturer's recommendations) or a lack of stimulation when using the monoclonal anti-FcεRI antibody and the fMLP as stimuli where considered a criteria for excluding the sample from the study.

3.12. Statistical analysis

3.12.1. CRD and epitope recognition in shellfish allergy

Quantitative variables were described by median, range, interquartile range and qualitative variables with absolute frequencies and percentages using GraphPad Prism™ version 4.0c for Macintosh (GraphPad Software, Inc. ®, La Jolla, CA, USA). The analysis of differences in IgE levels between groups was assessed using the non-parametrical Kruskal-Wallis test.

The Fisher exact probability test was applied to assess statistically significant differences between the frequencies of IgE reactivity of each group to individual recombinant proteins.

Properties of recombinant allergens and shrimp extract as diagnostic tests to discriminate between shrimp allergic (DBPCFC positive: group 1, n=58) and shrimp tolerant (DBPCFC negative: groups 2a, 2b and C1, n=40) individuals were evaluated. Sensitivity (SE, defined as the proportion of true-positive results/tests among all shrimp allergic subjects) and specificity (SP, defined as the proportion of true-negative results/tests detected among all shrimp tolerant subjects) of each recombinant allergen were calculated according to the method of Goldman [127] with the following formulas: SE = TP/(TP + FN) and SP = TN/(TN + FP), where TP is the number of true positives (patients with positive DBPCFC and positive recombinant allergen). FP is the number of false positives (patients with negative DBPCFC and positive recombinant allergen). TN is the number of true negatives (patients with negative DBPCFC and negative recombinant allergen) and FN is the number of false negatives (patients with positive DBPCFC and negative recombinant allergen).

Furthermore, the positive predictive value (PPV), negative predictive value (NPV) and efficiency of each recombinant allergen were calculated using two-by-two contingency tables. PPV describes the proportion of positive challenge subjects among those having a positive test result (recognition of a recombinant allergen; PPV= TP/TP+FP), whereas NPV describes the proportion of the negative challenge subjects among those having a negative test result (NPV= TN/TN+FN). Efficiency (Eff., also known as Accuracy) was defined as the proportion of true positive and true negative results detected among the total number of tests (Eff. = TP+TN/TP+TN+FP+FN).

The Mann Whitney t-test non-parametric was appropriately used for comparison of number of peptides bound by IgE, IgG4 and both. All tests were two sided.

3.12.2. Lipid transfer protein syndrome: clinical pattern and molecular sensitization profile to plant-foods and pollens

Quantitative variables were described by median, range, interquartile range and qualitative variables with absolute frequencies and percentages using GraphPad Prism^{TM} version 4.0c for Macintosh (GraphPad Software, Inc. ®, La Jolla, CA, USA).

Estimation of proportion of positive tests to plane tree, mugwort, wall-pellitory, cypress, grass pollen or olea in a subject was analyzed using logistic regression models utilizing the Generalized Estimating Equations methodology with an unstructured matrix to account for intra-subject correlations. A Type I Error: 0.05 was considered.

For the basophil activation test *in vitro* model, differences upon the number of CD63+ cells induced by each stimulus were analyzed with the Wilcoxon Matched Pairs test.

CHAPTER 4. RESULTS

RESULTS

4.1. CRD and epitope recognition in shellfish allergy

4.1.1. Study profile: patient characterization

A total of 86 individuals with a positive shrimp SPT and/or shrimp-specific serum IgE were recruited. Seventy-four (86%) reported history of immediate allergic reactions following shrimp ingestion and 12 (14%) did not. Of these 74, 58 (78%) had a positive Double-Blind Placebo-Controlled Food Challenge (DBPCFC) to shrimp (**Group 1**) and 16 (22%) a negative one (**Group 2a**). The 12 (14%) subjects with positive shrimp test but not reported history of shrimp immediate allergic reactions were also challenged, with negative result in all cases (100%) (**Group 2b**). All negative DBPCFC were confirmed with an oral open challenge with shrimp. Twelve control subjects sensitized to HDM and/or cockroach allergic but not sensitized to shrimp were also recruited. These subjects had no history of reactions to shrimp and a shrimp challenge was negative in all cases (**Group Control 1**). Five non-atopic individuals were included (**Group Control 2**). The study profile is shown in Figure 4.1.

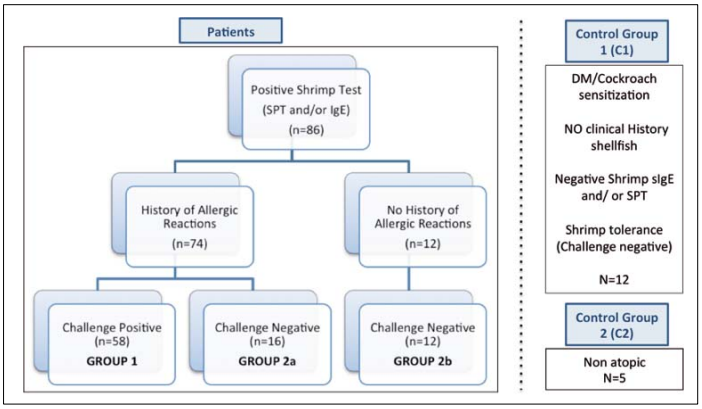

Figure 4.1. Study profile: subjects recruited. sIgE: specific IgE, SPT: Skin prick test, DM: Dust mite.

Gender, age, frequency of sensitization to shrimp, dust mite (*Dermatophagoides pteronyssinus*, DP) and cockroach, specific IgE levels to these allergens, as well as history of allergic reactions to shrimp and DBPCFC outcome for

each group are summarized in Table 4.1. A detailed description of the subjects recruited for each group is presented in Table 4.3.

	Group 1	Group 2a	Group 2b	Group Control 1
N	58	16	12	12
Gender Male, % (no.)	50% (29)	50% (8)	50% (6)	17% (2)
Age median[range]	17[3-52]	25[5-45]	29[13-47]	32.5[16-60]
Sensitization - Shrimp - Dust Mite (DP) - Cockroach	 100% (58/58) 94.6% (53/56) 78.8% (41/52)	 100% (16/16) 93.8% (15/16) 40% (6/15)	 100% (12/12) 100% (12/12) 63.6% (7/11)	 0% (0/12) 100% (12/12) 16.7% (2/12)
Specific IgE kU_A/L median[range] - Shrimp - Dust Mite (DP) - Cockroach	 27.10[<0.35->100] 14.0[<0.35->100] 2.7[<0.35->100]	 0.52[<0.35-16.5] 2.24[<0.35-60.2] 0.79[<0.35-3.6]	 1.53[<0.35-5.7] 54.1[1.89->100] <0.35[<0.35-1.29]	 <0.35 8.35[<0.35->100] <0.35[<0.35-0.53]
History Shrimp Reaction	+	+	-	-
DBPCFC	+	-	-	-

Table 4.1. Characteristics of the subjects recruited for the study. DP: *Dermatophagoides pteronyssinus*; DBPCFC: Double Blind Placebo Controlled Food Challenge; "+": positive, "-": negative.

	sIgE Shrimp	sIgE Dust Mite	sIgE Cockroach
KW Test p value	<0.0001	0.012	<0.0001
Dunn's test p value - 1 vs 2a - 1 vs 2b - 1 vs C1 - 2a vs 2b - 2a vs C1 - 2b vs C1	 <0.001 <0.01 <0.001 ns ns ns	 ns ns ns <0.05 ns ns	 ns <0.01 <0.001 ns ns ns

Table 4.2. Comparison of shrimp-, dust mite- and cockroach-specific IgE levels. KW: Kruskal Wallis test for statistical comparison of IgE levels. "ns": not significant, p value >0.05.

Comparison of medians of shrimp-, DP- and cockroach-specific IgE levels showed statistically significant differences among groups, summarized in Table 4.2. Regarding shrimp-specific IgE levels, group 1 had higher levels than all other groups (statistically significant), whereas there were no statistically significant differences between groups with negative DBPCFC. Frequency of DP sensitization was high in all groups (93.8-100%), with similar DP-specific IgE levels (only statistically significant differences between 2a and 2b (n=16 and n=12, respectively)). Frequency of cockroach sensitization varied among groups and cockroach-specific IgE levels of group 1 were statistically significantly higher than those of 2b and C1.

	Subject	GeNAer	Age	Symptoms	DBPCFC	Shrimp	DP	Cockroach
Group 1	1	M	10	U, A	+	49.5	9.5	12
	2	M	17	AE,A, C	+	52.1	9.7	12
	3	M	13	Asth, AE	+	82.4	22.4	32
	4	F	12	C,Asth	+	74.2	12.1	0.4
	5	F	3	U A	+	100	>100	74
	6	M	8	GI,RC, U	+	100	23	30
	7	F	8	AE, U, A	+	30	7	42
	8	F	38	AE, U	+	>100	>100	17
	9	M	34	AE, U, RC	+	>100	>100	<0.35
	10	M	16	A	+	>100	>100	8.9
	11	M	33	A	+	65	40.3	22.6
	12	F	22	AE, RC	+	47.83	59.4	12.1
	13	F	40	AE, A, RC	+	14	8.96	3.2
	14	F	34	A	+	1.07	1.15	2.4
	15	M	30	AE, U	+	15	24	35
	16	M	40	AE, U, A	+	30.3	NA	NA
	17	M	6	A	+	>100	9.3	NA
	18	M	19	U, AE, A, GI	+	8	0.5	0.5
	19	M	5	AE	+	>100	27	33
	20	M	22	OAS, GI	+	78	>100	37
	21	M	12	U	+	>100	>100	NA
	22	F	34	AE,C,A	+	21	3.8	1.7
	23	F	10	U,C,A	+	>100	51	NA
	24	F	16	AE,U,A	+	21	7	3
	25	M	15	A	+	13	<0.35	<0.35
	26	M	9	A	+	>100	25	NA
	27	F	9	U, AE, GI	+	96.8	24	NA
	28	F	33	A, Asth	+	100	46	46.2
	29	M	8	A	+	100	14.1	25.3
	30	F	7	A	+	100	11.1	18.1
	31	F	15	U, RC	+	96.7	18.7	30.3
	32	M	5	U	+	50.4	10.3	14.8
	33	M	14	U, A	+	24.2	10.5	10.2
	34	M	3	A	+	13.7	0.86	1.44
	35	M	9	U, AE	+	11.7	5.75	5.73
	36	F	29	A, Asth	+	7.03	NA	NA
	37	M	33	A	+	6.15	2.27	1.88
	38	F	27	A	+	5.6	<0.35	<0.35
	39	M	9	U, A, GI	+	5.5	2.15	1.02
	40	F	16	GI	+	5.24	26.7	2.27
	41	M	33	OAS, GI	+	2.72	13.9	1.35
	42	M	48	OAS, GI	+	2.47	0.82	2.3
	43	F	12	U,Asth	+	5.9	1.56	0.99
	44	M	8	AE	+	14.4	1.94	3.04
	45	F	27	U, AE	+	58.6	22.6	0.48
	46	F	37	AE, C,Asth	+	52.4	NA(SPT+)	NA(SPT-)
	47	F	16	U	+	0.6	NA(SPT+)	NA(SPT-)
	48	M	39	U	+	0.81	NA(SPT-)	NA(SPT-)
	49	M	17	A	+	> 100	34.5	<0.35
	50	F	25	A	+	2.02	3.79	<0.35
	51	F	17	AE, U	+	0.92	NA(SPT+)	NA(SPT-)
	52	F	12	A	+	4.66	67.8	0.82
	53	F	18	A	+	41.8	24.6	<0.35
	54	F	39	U, GI	+	0.74	73.9	<0.35
	55	M	18	OAS, AE	+	5.53	8.32	<0.35(SPT+)
	56	F	28	AE, U	+	< 0,35(SPT+)	27.2	<0.35
	57	F	50	AE, C	+	15.1	8.97	<0.35
	58	F	52	U	+	0.75	44.3	NA(SPT+)
Group 2a	1	M	10	U	-	0.37	14.1	NA(SPT-)
	2	F	20	U	-	< 0.35(SPT+)	NA(SPT+)	NA(SPT-)
	3	F	34	OAS, C	-	< 0.35(SPT+)	2.24	<0.35
	4	F	28	U	-	< 0.35(SPT+)	NA(SPT+)	NA(SPT-)
	5	F	45	OAS	-	0.44	4.05	<0.35
	6	M	15	AE	-	< 0.35(SPT+)	NA(SPT+)	NA(SPT-)
	7	F	29	U	-	0.44	0.75	<0.35
	8	M	31	U,AE,A	-	0.62	0.46	2.76
	9	F	18	OAS	-	0.55	1.26	<0.35
	10	F	41	RC	-	0.48	9.75	<0.35
	11	M	22	OAS	-	2.32	4.13	2.37
	12	M	31	U	-	5.58	12.2	3.19
	13	F	29	U	-	1.45	60.2	1.24
	14	M	7	GI	-	0.6	<0.35	NA
	15	M	7	RC	-	16.5	2	3.56
	16	M	5	U,GI	-	0.81	0.68	0.79
Group 2b	1	F	32	-	-	<0.35(SPT+)	80.5	<0.35(SPT+)
	2	F	21	-	-	1.54	57.7	<0.35(SPT+)
	3	M	13	-	-	1.19	50.4	<0.35(SPT+)
	4	M	19	-	-	1.53	3.26	0.95
	5	F	47	-	-	2.45	22.9	0.88
	6	M	40	-	-	<0.35(SPT+)	>100	<0.35
	7	F	31	-	-	1.09	>100	<0.35
	8	F	47	-	-	<0.35(SPT+)	1.89	<0.35
	9	M	25	-	-	1.98	>100	<0.35
	10	M	27	-	-	5.7	NA(SPT+)	0.47
	11	M	15	-	-	2.7	8.56	1.29
	12	F	45	-	-	NA(SPT+)	NA(SPT+)	NA
Control group 1 (C1)	1	F	28	-	-	<0.35(SPT-)	2.36	<0.35
	2	F	52	-	-	<0.35(SPT-)	0.41	<0.35
	3	F	60	-	-	<0.35(SPT-)	4.3	<0.35
	4	M	42	-	-	<0.35(SPT-)	<0.35 (SPT+	<0.35
	5	F	25	-	-	<0.35(SPT-)	>100	0.53
	6	F	17	-	-	<0.35(SPT-)	2.22	<0.35
	7	F	39	-	-	<0.35(SPT-)	12.4	<0.35
	8	F	37	-	-	<0.35(SPT-)	2.71	<0.35
	9	F	17	-	-	<0.35(SPT-)	13.8	<0.35
	10	M	37	-	-	<0.35(SPT-)	>100	<0.35(SPT+)
	11	F	16	-	-	<0.35(SPT-)	21.9	<0.35
	12	F	25	-	-	<0.35(SPT-)	52.6	<0.35

Table 4.3. Detailed description of the subjects recruited. Gender: M: male, F: female; Age in years; Symptoms of allergic reactions to shrimp: AE: angioedema, A: anaphylaxis, Asth: asthma, C: cough, GI: gastrointestinal disorders, OAS: oral allergy syndrome, RC: rhinoconjunctivitis, U: urticaria; DBPCFC: Double Blind Placebo Controlled Food Challenge: "+": positive, "-": negative; Shrimp, *Dermatophagoides pteronyssinus* (DP) and cockroach specific IgE levels expressed as kU_A/L; NA: not available; SPT: Skin Prick Test.

Noteworthy, there were no statistically significant differences between groups 2a and 2b and C1 in terms of DP- and cockroach-specific IgE levels (Table 4.2.).

4.1.2. IgE recognition of recombinant allergens by the different groups

Frequencies of IgE reactivity to each recombinant allergen per group are shown in Figure 4.2.

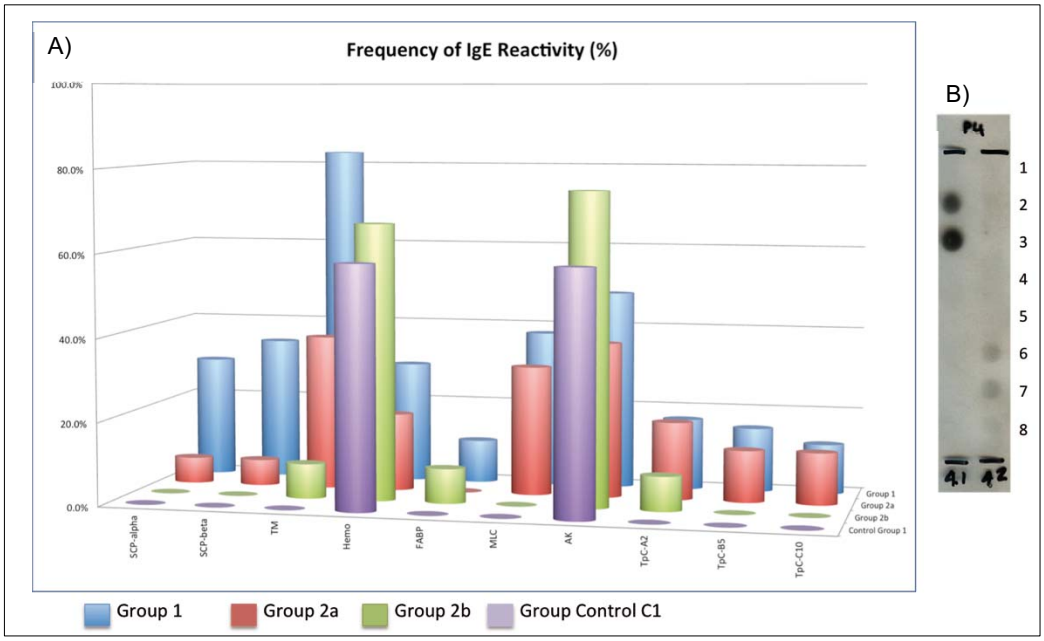

Figure 4.2. IgE recognition of recombinant allergens. A) Histogram showing frequency of IgE recognition per protein and per group, expressed as percentages (%). B) Example of dot blot immunolabelling for one subject. SCP: Sarcoplasmic calcium-binding protein, -alpha and –beta; TM: Tropomyosin; Hemo: Hemocyanin; FABP: Fatty acid binding protein; MLC: Myosin Light Chain; AK: Arginine-kinase; TpC: Troponin C (-A2, -B5, -C10).

Different profiles of IgE recognition were observed between groups. Subjects with a positive challenge (group 1) showed more diversity of allergen recognition (i.e., total number of recombinant allergens recognized by each subject) than the negative challenge groups. The median [range] of allergens recognized by each group and p-values corresponding to the comparison between the median of group 1 and the median of other groups were as follows: group 1, 3[0-9]; group 2a, 1.5 [0-5], $p<0.05$; group 2b, 2[0-3], not significant; group control 1: 1.5 [0-2], $p<0.01$.

Recombinant proteins recognized by each group of patients as well as statistically significant differences of frequencies of IgE recognition are presented in Table 4.4 (Fisher's exact probability test).

Among patients with positive challenge (group 1), eight subjects (8/58, 13.8%) recognized a single protein, which was TM for seven subjects and SCP (both isoforms) for one subject. In-group 1, 18 (31%) subjects recognized simultaneously TM and SCP-alpha and/or -beta, while only one subject from those with challenge negative (groups 2a, 2b and C1, 1/40 (2.5%)) recognized both proteins. Of note, in general both SCPs were recognized simultaneously except for 3 patients in group 1 that recognized SCP beta, but not alpha. In-group 2a, TM, MLC and AK were the allergens more frequently recognized (31-37.5%), whereas in-group 2b, AK and Hemo were the most frequent ones (66.7% and 75%, respectively). No subject of group 2b recognized MLC or SCPs, and only one recognized TM. Group control 1 recognized only Hemo and AK.

In each group, certain patients did not show IgE binding to any recombinant protein by dot blot (3/58 (5.2%) in group 1; 5/16 (31.3%) in group 2a; 2/12 (16.7%) in group 2b and 4/12 (33.3%) in group control 1). However when we tested their IgE reactivity on boiled and/or raw shrimp extracts by immunoblotting, we observed that all patients of group 1 showed IgE binding, whereas only 3 of the 5 of group 2a, 1 of the 2 of group 2b and 2 of the 4 of group control C1 did so. The non-atopic individuals (group control 2) tested did not recognize any recombinant protein by dot blot, nor any protein in boiled and raw shrimp extracts by Western blot.

Allergen	Group 1 n=58	Group 2a n=16	Group 2b n=12	Control 1 n=12
SCP-alpha	17 (29.3%)	1 (6.3%)	-	-
SCP-beta	20 (34.5%)	1 (6.3%)	-	-
TM	48 (82.8%)	6 (37.5%)	1 (8.3%)	-
Hemo	17 (29.3%)	3 (18.8%)	8 (66.7%)	7 (58.3%)
FABP	6 (10.3%)	-	1 (8.3%)	-
MLC	22 (37.9%)	5 (31.3%)	-	-
AK	28 (48.3%)	6 (37.5%)	9 (75.0%)	7 (58.3%)
Tpc A2	10 (17.2%)	3 (18.8%)	1 (8.3%)	-
TpC B5	9 (15.5%)	2 (12.5%)	-	-
TpC C10	7 (12.1%)	2 (12.5%)	-	-

Comparisons	Proteins with Statistically Significant Difference	p value Fischer's test
1 vs 2a	SCP-beta	0.0298
	TM	0.008
1 vs 2b	SCP-alpha	0.031
	SCP-beta	0.0145
	TM	<0.0001
	Hemo	0.0209
	MLC	0.0133
1 vs C1	SCP-alpha	0.031
	SCP-beta	0.0145
	TM	<0.0001
	MLC	0.0133
1 vs (2a+2b)	SCP-alpha	0.0049
	SCP-beta	0.0012
	TM	<0.0001
1 vs (2a+2b+C1)	SCP-alpha	0.0005
	SCP-beta	<0.0001
	TM	<0.0001
	MLC	0.006
2a vs 2b	Hemo	0.0189
(2a+2b) vs C1	-	-

Table 4.4. Frequency of IgE recognition and statistically significant differences between groups. The Fisher exact probability test was applied to assess statistically significant differences between the frequencies of IgE reactivity of each group to individual recombinant proteins. SCP: Sarcoplasmic calcium-binding protein, -alpha and –beta; TM: Tropomyosin; Hemo: Hemocyanin; FABP: Fatty acid binding protein; MLC: Myosin Light Chain; AK: Arginine-kinase; TpC: Troponin C (-A2, -B5, -C10).

4.1.3. Component-resolved diagnosis

4.1.3.1. Allergens that can help differentiate challenge outcome

IgE binding to SCP-alpha, SCP-beta and TM was statistically significantly more frequent in patients with a positive shrimp challenge (group 1) than in subjects sensitized to shrimp but tolerant (negative DBPCFC, 2a and 2b). When comparing

group 1 with all the negative shrimp challenge/ arthropod positive groups (2a, 2b and C1), MLC was also statistically significant different.

If groups 2a and 2b were considered separately, and compared to group 1, also statistically significant differences were observed in terms of recognition of certain proteins. For 1 versus 2a, only two allergens, SCP-beta and TM. For 1 versus 2b, more proteins, both SCPs, TM (the most significant), Hemo and MLC. Group 2a and 2b only differed in binding to Hemo (Table 4.4).

4.1.3.2. Arginine kinase and hemocyanin as cross-reactive allergens

Shrimp proteins recognized by group control 1 were exclusively Hemo and/or AK (6/8 (75%) both proteins). Together, groups 2a and 2b did not show any statistically significant difference in protein recognition compared to the group control 1. Considered separately, group 2b showed no statistically significant differences with this control group, whereas 2a showed statistically significant differences for TM (p=0.0237) and a trend for Hemo (p=0.0497). The comparison of group 1 with control 1 showed the same proteins with statistically significant differences as the comparison 1 versus 2a, 2b and C1, but with higher p values (Table 4.4).

4.1.3.3. No major differences between children and adults

Within group 1, the only statistically significant difference observed in the IgE reactivity profile between children (n=33) and adults (n=25) was a higher frequency of SCP-beta in children (p=0.0128). The same analysis among all challenge negative subjects (groups 2a, 2b and C1, 10 children/ 30 adults) showed no statistically significant differences.

4.1.4. Properties of recombinant allergens as diagnostic tests

Properties of recombinant allergens and shrimp extract as diagnostic tests were described using sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency. Results are shown in Table 4.5. Only those tests that showed statistically significant differences between positive and negative challenge subjects (p value Fisher's test < 0.05: SCP-alpha, -beta, TM, MLC and

shrimp extract) could be considered candidates for a diagnostic test to differentiate challenge outcome.

Allergen	P value Fischer's test	SE (%)	SP (%)	PPV (%)	NPV (%)	Eff. (%)
SCP alpha	**0.0005**	29.3	97.5	94.4	48.8	57.1
SCP beta	**<0.0001**	34.5	97.5	95.2	50.7	60.2
TM	**<0.0001**	82.8	82.5	87.3	76.7	86.6
Hemo	0.1355	29.3	55.0	48.6	34.9	39.8
FABP	0.2348	10.3	97.5	85.7	42.9	45.9
MLC	**0.006**	37.9	87.5	81.5	49.3	58.2
AK	0.5434	48.3	45.0	56.0	37.5	46.9
TpC A2	0.3879	17.2	90.0	71.4	42.9	46.9
TpC B5	0.1911	15.5	95.0	81.8	43.7	48
TpC C10	0.3024	12.1	95.0	77.8	42.7	45.9
Shrimp Extract	**<0.0001**	98.3	48.7	74.0	95.0	77.6

Table 4.5. Properties of recombinant allergens as diagnostic tests. SE: sensitivity, SP: specificity, PPV: positive predictive value, NPV: negative predictive value, Eff.: efficiency. All expressed as percentages (%). SCP: Sarcoplasmic calcium-binding protein, -alpha and -beta; TM: Tropomyosin; Hemo: Hemocyanin; FABP: Fatty acid binding protein; MLC: Myosin Light Chain; AK: Arginine-kinase; TpC: Troponin C (-A2, -B5, -C10). Shrimp extract (f94, ImmunoCAP, Phadia). Statistically significant values shown in bold.

Tropomyosin showed the highest values for all 5 parameters, whereas SCPs showed very high values for specificity (~ 95%) and PPV (97.5%), but low ones for sensitivity (29-35%) and NPV (~ 50%). Similarly, MLC showed high values for specificity (87.5%) and PPV (81.5%), but low ones for sensitivity (38%) and NPV (49%). The shrimp extract was the most sensitive (98%) and with the highest NPV (95%), however it had the lowest specificity (49%) and PPV (74%). Efficiency for SCPs and MLC was around 60%, lower than shrimp extract (f94, ImmunoCAP) (78%) and TM (87%, the highest).

4.1.5. Epitope mapping: IgE and IgG4 binding sites

A subset of subjects recruited for the study (group 1, n=21; group 2a, n=16, group 2b, n=12) were analyzed for IgE and IgG4 binding to synthetic overlapping peptides spanning the whole sequence of five shrimp allergens: TM, SCP, AK, MLC and TpC. IgE and IgG4 binding to the different peptides from all 5 proteins by each patient are shown as heat maps in figures 4.3 and 4.4, respectively. All peptides from the 5 allergens are represented in rows. The recognition of peptides by each patient is

shown in columns. The intensity of binding is presented with average z scores in a grading scale ranging from less than 3 (black) to 5 or greater (yellow for IgE and red for IgG4).

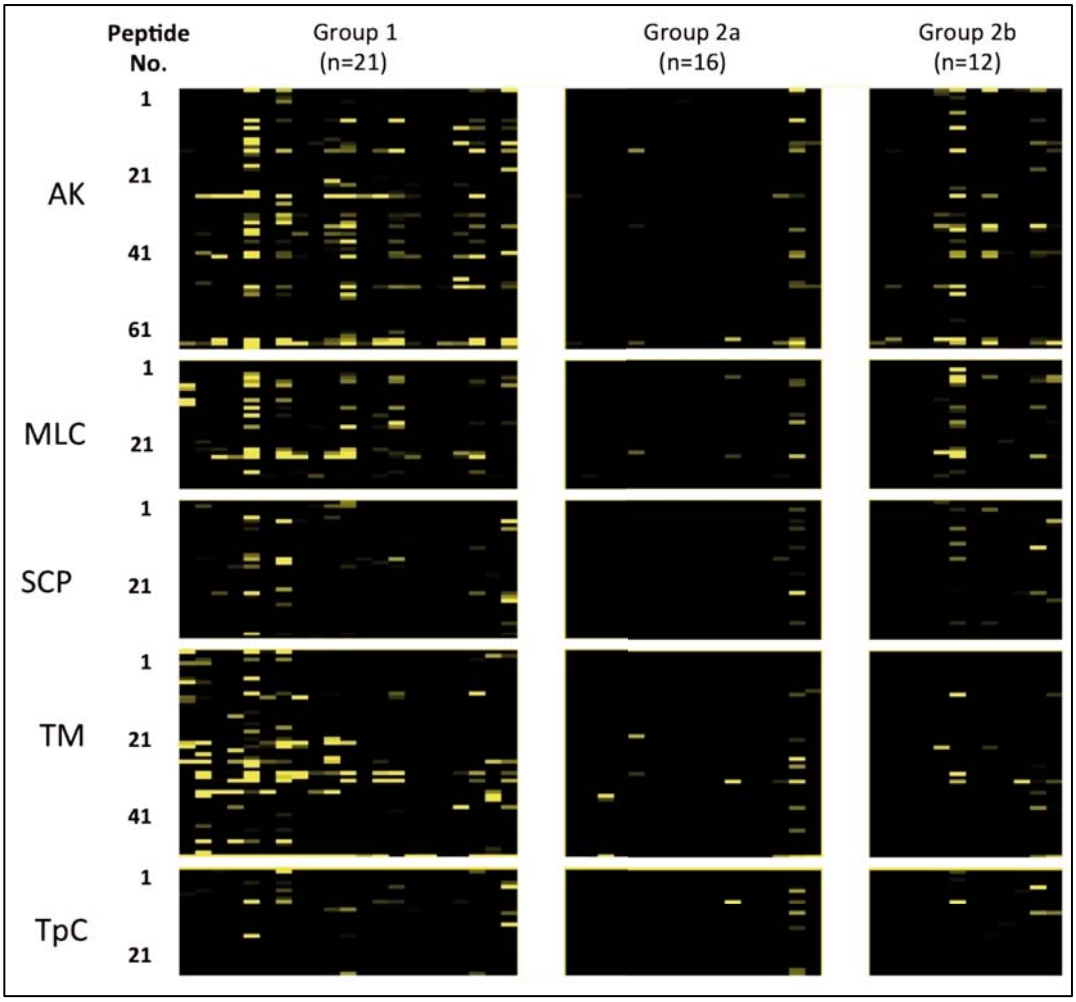

Figure 4.3. Heat map representing IgE binding in the peptide microarray. All the peptides from the 5 shrimp allergens are represented in rows. The recognition of peptides by each patient is shown in columns. The intensity of binding was represented with average z scores in a grading scale ranging from less than 3 (black) to 5 or greater (yellow). SCP: Sarcoplasmic calcium-binding protein; TM: Tropomyosin; MLC: Myosin Light Chain; AK: Arginine-kinase; TpC: Troponin C.

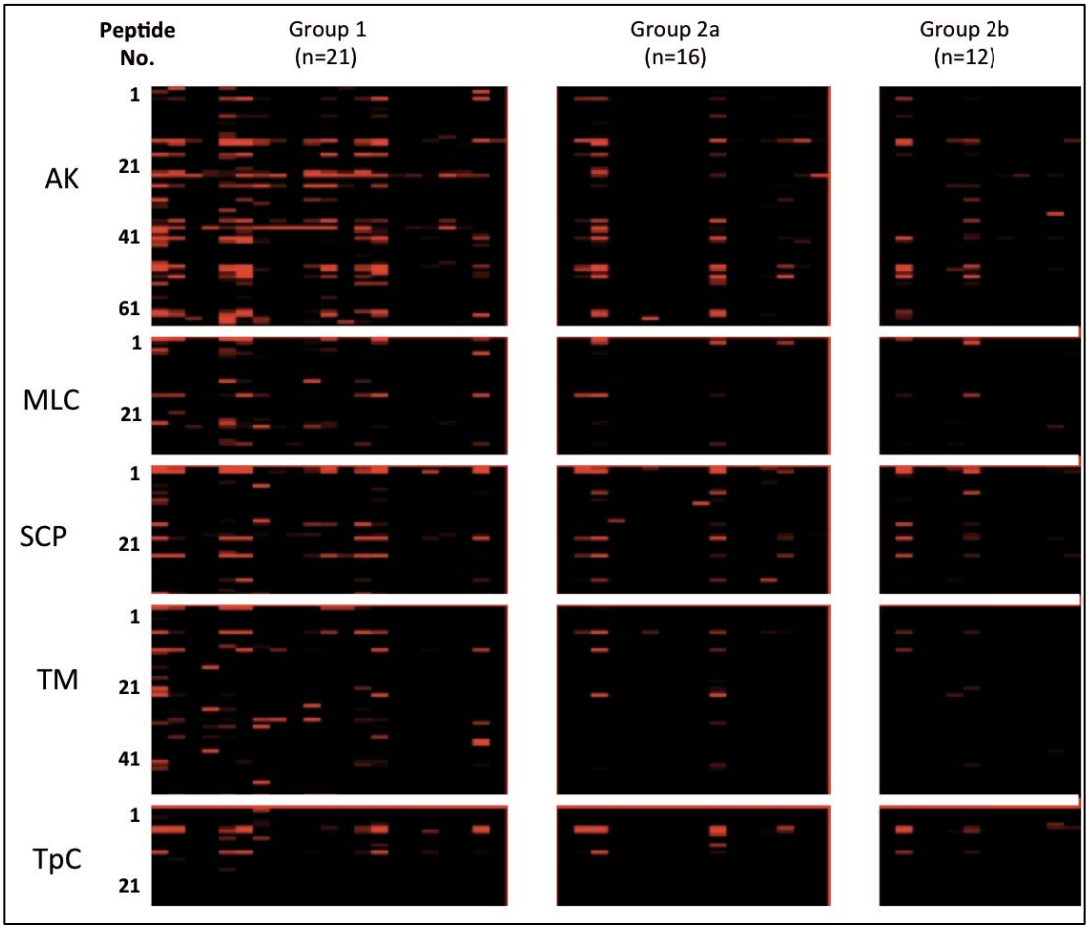

Figure 4.4. Heat map representing IgG4 binding in the peptide microarray. All the peptides from the 5 shrimp allergens are represented in rows. The recognition of peptides by each patient is shown in columns. The intensity of binding was represented with average z scores in a grading scale ranging from less than 3 (black) to 5 or greater (red). SCP: Sarcoplasmic calcium-binding protein; TM: Tropomyosin; MLC: Myosin Light Chain; AK: Arginine-kinase; TpC: Troponin C.

4.1.6. Differential binding sites between groups

TileMap, a tool for tiling array analysis, was applied to identify IgE and IgG4 binding sites with statistical differences between groups (1 versus 2(2a+2b), 1 versus 2a, 1 versus 2b, and 2b versus 2a). As hundreds of peptides were analyzed simultaneously, the false discovery rate (FDR) was calculated to adjust for multiple comparisons. A p value of less than 0.01 and FDR of less than 0.05 were considered as significant. Figure 4.5 and Table 4.6 summarize the binding sites showing statistically significant differences between the groups compared.

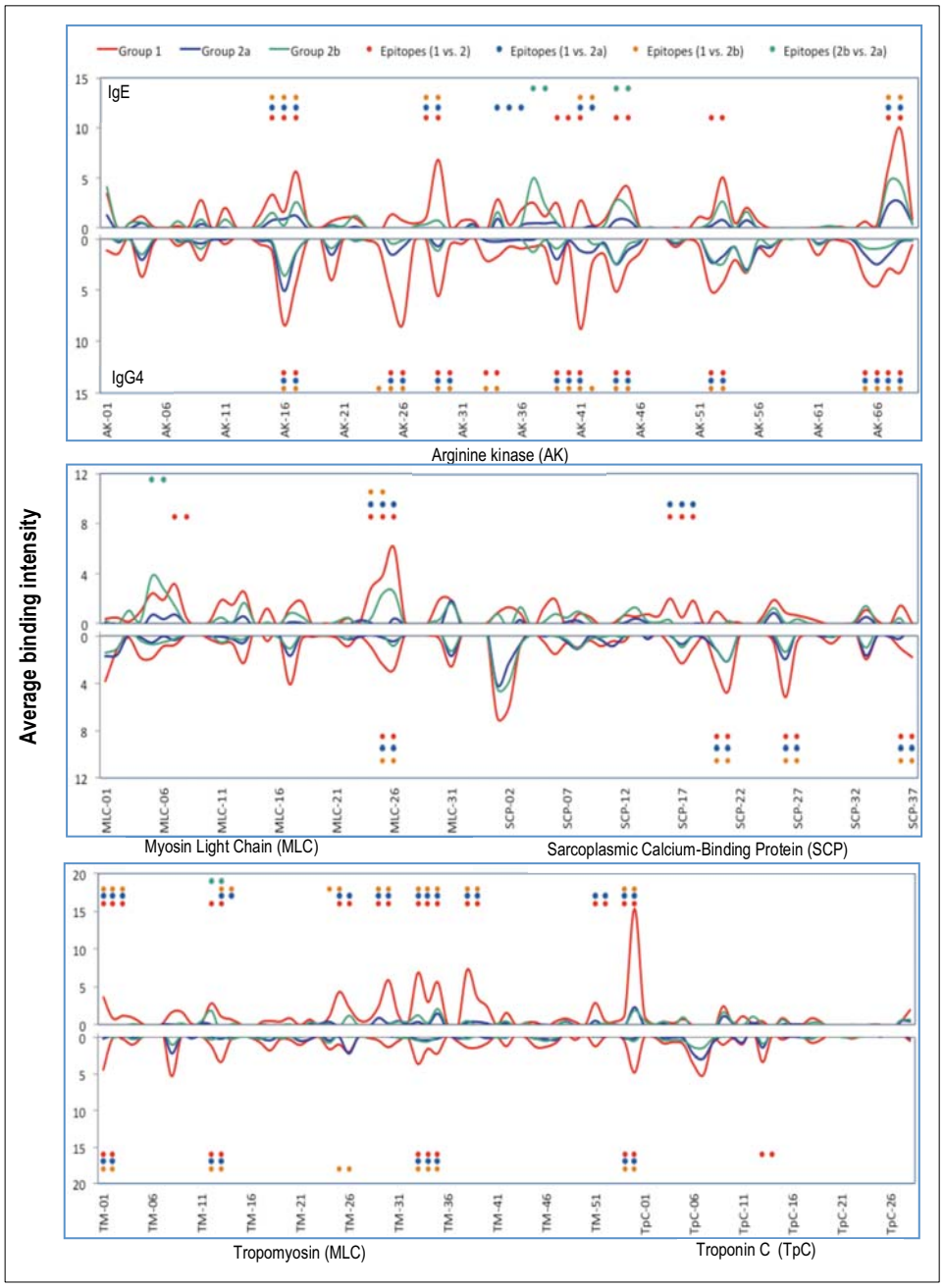

Figure 4.5. TileMap analysis. Lines are showing average binding intensity per group (1, red; 2a, blue; 2b, green). Dots represent binding sites with statistically significant differences between the groups compared (1 vs 2 (2a+2b), red; 1 vs 2a, blue; 1 vs 2b, yellow; 2b vs 2a, green). On the x-axis, the protein and the peptide number is shown (AK: arginine kinase, MLC: myosin light chain, SCP: sarcoplasmic calcium-binding protein, TM: tropomyosin and TpC: troponin C). Significant binding sites have $p<0.01$, FDR<0.05. The top half of each graph corresponds to IgE binding, whereas the other half corresponds to IgG4.

IgE and IgG4 binding sites coincided largely. Minor differences in terms of peptide recognition were observed between groups 2b and 2a. Therefore,

challenge negative subjects were pooled (group 2 =2a+2b) for comparison with group 1 and for further analysis. Regarding IgE binding, tropomyosin was the protein with the largest number of differential binding sites between groups, whereas for IgG4, that was for AK. For SCP, differential binding sites were mainly observed for IgG4 rather IgE. Almost no difference was observed for TpC.

Allergen	1 vs 2		1 vs 2a		1 vs 2b		2b vs 2a	
	IgE	IgG4	IgE	IgG4	IgE	IgG4	IgE	IgG4
AK	15-17 28-29 39-41 44-45 52-53 67-68	16-17 25-26 29-30 33-34 39-41 44-45 52-53 65-68	15-17 28-29 34-36 41-42 67-68	16-17 25-26 29-30 39-41 44-45 52-53 65-68	15-17 28-29 41-42 67-68	16-17 24-26 29-30 33-34 39-42 44-45 52-53 65-68	37-38 44-45	44-45
MLC	7-8 24-26	25-26	24-26	25-26	24-25	25-26	5-6	
SCP	16-18	20-21 26-27 36-37	16-18	20-21 26-27 36-37		20-21 26-27 36-37		
TM	1-3 12-13 25-26 29-30 33-35 38-39 51-52 54-55	1-2 12-13 33-35 54-55	1-3 13-14 25-26 29-30 33-35 38-39 51-52 54-55	1-2 12-13 33-35 54-55	1-3 12-13 24-25 29-30 33-35 38-39 54-55	1-2 12-13 25-26 33-35 54-55	12-13	
TpC		13-14						

Table 4.6. Summary of the differential binding sites identified between groups. "2" refers to the pool of subjects from 2a and 2b. Numbers in the table refer to the number of the peptide for each allergen (peptide ID). SCP: Sarcoplasmic calcium-binding protein; TM: Tropomyosin; MLC: Myosin Light Chain; AK: Arginine-kinase; TpC: Troponin C.

4.1.7. Diversity of peptides bound by IgE and IgG4

For each allergen, the number of positive peptides (i.e., z score above 3) bound by IgE, IgG4 and both in each subject was analyzed. Medians of number of positive peptides bound by each immunoglobulin were compared between allergic (challenge positive, group 1) and tolerant (challenge negative, group 2=2a+2b) individuals (non-parametric t-test: Mann Whitney, p value <0.05 considered significant; Table 4.7).

Subjects of group 1 (shrimp allergic individuals) had more peptides recognized by IgE than group 2 (shrimp tolerant individuals) for all 5 allergens; the difference between the medians was more statistically significant for TM, MLC and AK. Similarly, subjects of group 1 also had more peptides bound by both IgE and IgG4 than subjects of group 2 for all allergens, being the difference between the medians more statistically

significant for TM and AK. Regarding the number of peptides bound by IgG4, the difference between the median number of peptides bound by allergic and tolerant individuals was only statistically significant for 3 allergens, TM, MLC and AK.

	AK		MLC		SCP		TM		TpC	
	1	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
No. peptides bound by IgE										
Median	8	3	4	1	3	2	6	0.5	2	1
Range	1-30	0-20	1-14	0-14	2-11	1-12	0-20	0-9	0-7	0-8
IQ range (25% to 75%)	4-15.5	1-7	1.5-6.5	1-3	2-6	2-3	2.5-9	0-2	1-4	0-2
Mann Whitney p value	0.0021		0.0036		0.0148		<0.0001		0.0145	
No. peptides bound by IgG4										
Median	9	2	2	1	6	2.5	4	0	2	0
Range	2-31	0-26	0-13	0-6	2-14	0-12	0-16	0-9	0-6	0-7
IQ range (25% to 75%)	5.5-22	1-8.5	1-7.5	0-2	2-7.5	2-6.5	0-8	0-2	0-4	0-3
Mann Whitney p value	0.0006		0.0021		0.1111		0.0048		0.2056	
No. peptides bound by IgE and IgG4										
Median	3	1	1	1	3	2	2	0	1	0
Range	1-25	0-6	0-11	0-2	2-7	0-7	0-10	0-2	0-5	0-2
IQ range (25% to 75%)	2-8	1	1-3.5	0-1	2-4	2	0-4.5	0	0-2	0
Mann Whitney p value	<0.0001		0.0088		0.0113		0.0006		0.0269	

Table 4.7. Number of peptides bound by IgE, IgG4 and both per allergen. A peptide is positive if z score is greater than 3. T test non-parametric analysis: Mann Whitney. Comparison of 2 groups: challenge positive 1 versus challenge negative (2=2a+2b). IQ: interquartile range, ns: not significant. SCP: Sarcoplasmic calcium-binding protein; TM: Tropomyosin; MLC: Myosin Light Chain; AK: Arginine-kinase; TpC: Troponin C.

Furthermore, the existence of any correlation between IgE and IgG4 binding was evaluated with scatter plots (Figure 4.6), but no correlation could be found between these two variables neither in shrimp allergic individuals or tolerant ones.

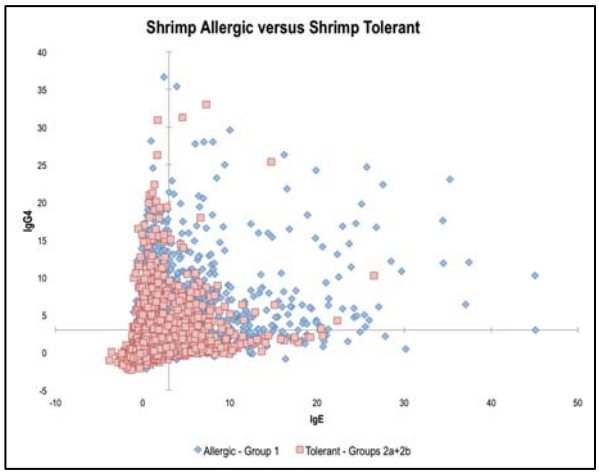

Figure 4.6. Correlation between IgE and IgG4 binding. Scatter plot showing the correlation of IgE and IgG4 binding (expressed as z score values, a peptide is positive if z score is greater than 3) in shrimp allergic (blue squares) and tolerant (red squares) individuals.

4.1.8. Comparison of current results with previous studies

IgE binding sites identified in the current study were compared with the shrimp epitopes defined earlier [90] and with the epitopes found as clinically relevant in a recent pilot study by our group [91] (Table 4.8). IgG4 binding was not assessed in former studies. Subjects included in the present study were recruited from a population similar to previous studies, but now the sample size provided is larger (especially the challenge negative group) and includes subjects from different countries.

Allergen	Peptide #	Aa #	Described Epitope (Aa #)
AK	15-17	71-95	3 (64-96)
	28-29	136-155	4a (121-141)
			4b (142-159)
	39-41	191-215	-
	44-45	216-235	-
	52-53	256-275	-
	67-68	331-350	7 (319-342)*
MLC	7-8	24-26	2 (22-48) *
	24-26		5 (118-141) *
SCP	16-18	76-100	-
TM	1-3	1-25	1 (1-36) *
	12-13	56-75	3 (61-81)
	25-26	121-140	5a (115-150) *
	29-30	141-160	5b (142-162) *
	33-35	161-185	5c (157-183) *
	38-39	186-205	6 (190-210)
	51-52	251-270	7 (246-284) *
	54-55	266-285	
TpC	-		

Table 4.8. Comparison of results with previous studies. Epitopes described by Ayuso R et al. JACI 2010 [90]. "Peptide #" refers to the peptide number/ID in the current study, "Aa #" refers to the corresponding amino acid sequence, and the "Described Epitope (Aa #)" shows the ID of the epitope previously identified and in brackets the corresponding amino acid sequence for that epitope. "*": Epitope also found statistically significant different between allergic and tolerant individuals in Ayuso R et al. CEA 2011 [91]. SCP: Sarcoplasmic calcium-binding protein; TM: Tropomyosin; MLC: Myosin Light Chain; AK: Arginine-kinase; TpC: Troponin C.

Almost all the binding sites identified in the current study corresponded to previously described epitopes for that allergen, only for AK and SCP certain binding sites did not correspond to a previously described epitope.

A recent pilot study by our group [91] identified some epitopes as clinically relevant (for AK epitopes 6 and 7; for MLC, epitopes 1, 2, 4a, 4b, 5; for SCP, epitope 1; for TM, epitopes 1, 2, 5a, 5b, 5c and 7), that is with statistically significant differences between patients with challenge positive (n=15) and challenge negative (n=11) (also by TileMap analysis, FDR <0.05 and p<0.01).

In the present study, including 21 patients with challenge positive (group 1) and 28 with challenge negative (group 2=2a+2b), almost the same epitopes were identified for TM (5/6), with two more epitopes: 3 and 6, but this was not the case for the other allergens. For MLC, only two of the five epitopes described as clinically relevant in the pilot study are found in the present study (epitopes 2 and 5). For SCP, a binding site along amino acids 76-100 (from now on, n) was found in the present study and it had not been described in the pilot study. For AK, only epitope 7 coincided with the epitopes previously stated as clinically relevant (6 and 7) [91], however additional statistically significant differences between groups not detected in the pilot study were found in three other epitopes of this allergen (3, 4a and 4b). Epitopes found as statistically significant different between allergic and tolerant individuals by both the current study and Ayuso R et al. CEA 2011 [91] are shown as asterisks in Table 4.8.

4.1.9. Properties of epitopes as diagnostic tests

Frequencies of IgE recognition for each epitope per group and properties of epitopes as diagnostic tests (described by sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency) are shown in Table 4.9. A statistical analysis of the frequencies of IgE reactivity of each group to individual epitopes was performed. Challenge positive individuals recognized more frequently all epitopes than challenge negative subjects, except for epitope 2 of MLC (p=0.146). Thus, this epitope cannot be considered for the analysis of its properties as a diagnostic test.

Allergen	Epitope ID	Freq. Ch+ (%)	Freq. Ch- (%)	P value Fisher's test	SE (%)	SP (%)	PPV (%)	NPV (%)	Eff.
AK	3	71.4	32.1	0.0096	71.4	67.9	62.5	76.0	69
	4a/4b	61.9	28.6	0.0399	61.9	71.4	61.9	71.4	67.3
	7	81.0	42.9	0.0093	81.0	57.1	58.6	80.0	67.3
MLC	2	28.6	10.7	0.146	28.6	89.3	66.7	62.5	63.3
	5	71.4	32.1	0.0096	71.4	67.9	62.5	76.0	69
SCP	n	33.3	7.1	0.0277	33.3	92.9	77.8	65.0	67.3
TM	1	42.9	0.0	0.0001	42.9	100.0	100.0	70.0	75.5
	3	61.9	14.3	0.0008	61.9	85.7	76.5	75.0	75.5
	5a	38.1	7.1	0.012	38.1	92.9	80.0	66.7	69
	5b	47.6	3.6	0.0004	47.6	96.4	90.9	71.1	75.5
	5c	71.4	32.1	0.0096	71.4	67.9	62.5	76.0	69
	6	38.1	7.1	0.012	38.1	92.9	80.0	66.7	69
	7	85.7	21.4	<0.0001	85.7	78.6	75.0	88.0	81.6

Table 4.9. Properties of epitopes as diagnostic tests. Frequencies (expressed as percentages (%)) of epitope recognition per group (Ch+: positive challenge, group 1; Ch-: negative challenge, group 2=2a+2b). The Fisher exact probability test was applied to assess statistically significant differences between the frequencies of IgE reactivity of each group to individual epitopes. SE: sensitivity, SP: specificity, PPV: positive predictive value, NPV: negative predictive value, Eff.: efficiency. All expressed as percentages (%). SCP: Sarcoplasmic calcium-binding protein; TM: Tropomyosin; MLC: Myosin Light Chain; AK: Arginine-kinase; TpC: Troponin C.

Tropomyosin epitopes showed very high (>90%, epitopes 1, 5a, 5b and 6) or high (>75%, epitopes 3 and 7) specificity. Epitope n of SCP allergen also showed very high specificity, but for the other epitopes this parameter ranged from 57 to 71%. Similarly, the highest values of PPV were observed for TM and SCP (>90%, epitopes 1 and 5b of TM; >75%, epitopes 3, 5a, 5b, 6 and 7 for TM and epitope n for SCP). Sensitivity varied largely for TM epitopes, while epitope 7 showed the highest value of all epitopes analyzed, the others had values from 38 to 71%. Similarly, sensitivity for epitope 7 of AK was 81%, but the other 2 epitopes of the same allergen had lower values. Epitope n of SCP showed the lowest sensitivity (33%). In parallel, the highest NPV were observed for certain epitopes of TM (76-88%) and epitope 7 of AK (80%). Tropomyosin epitopes showed higher efficiency values (76-82%) than the epitopes of the other allergens (63-69%). Epitope 7 of tropomyosin was the only one with high values for all 5 parameters.

4.2. Lipid transfer protein syndrome: clinical pattern and molecular sensitization profile to plant-foods and pollens

4.2.1. Demographics and culprit plant-foods involved

The 45 patients recruited were mainly adults (median [range]: 33 [14-47] years-old), 28 females (62.2%), all previously diagnosed with multiple-plant food allergy due to LTP-sensitization. Culprit foods involved in reactions (recorded in clinical history and with confirmed sensitization) were taxonomically related and unrelated fruits, vegetables and nuts (number of plant-foods involved, median [range]: 4 [2-18]). In some cases, the same plant-food was reported to cause symptoms of different severity in the same individual in different episodes (only the most severe episodes are depicted in Table 4.10).

4.2.2. Clinical symptoms

A heterogeneous pattern of symptoms was observed: OAS (34/45, 75.6%), urticaria (30/45, 66.7%), contact urticaria (5/45, 11.1%), GID (25/45, 55.6%) and anaphylaxis (rapid onset of two or more symptoms affecting: skin, gastrointestinal tract, respiratory system and cardiovascular system) (34/45, 75.6%). Most subjects reported multiple symptoms, depending on the food involved. Isolated episodes of OAS were not observed in our cohort. Only one patient had repeated isolated episodes of anaphylaxis with several plant-foods involved (Table 4.11). Some patients reported episodes of acute urticaria, GID or anaphylaxis suggestive of food allergy but they were unable to identify any potential culprit food (Table 4.10). In patients reporting GID, the existence of a digestive disease potentially responsible for the reported symptoms was ruled-out.

4.2.3. Cofactor effect

Eighteen patients (40%) reported the involvement of cofactors: non-steroidal anti-inflammatory drugs (NSAIDs) (15 patients), exercise (2) or both (1) (Table 4.10). Of the 34 (75.6%) that suffered anaphylaxis, in 11 (32.4%) the presence of the cofactor was mandatory. In cofactor-dependent anaphylaxis, it was very difficult to identify the culprit food by means of the clinical history (Table 4.10).

Subject #	Age/Sex	Pollinosis		Aeroallergens Sensitization (SPT)	Culprit Food / Symptomatology	Cofactor involved and clinical effect	Plant food sensitization (SPT)	Other sensitizations (SPT)
		Rhinitis	Asthma					
1	21/M	+	+	dm, pl, pa	le (An); to, wn (U); wh (GID); fr, le, un, wh (GID); sf (OAS), pe (CU)	Exercise, NSAIDs + unkn = An	hz, kw, pe, mz, mu, le, wh	wn, to, sf
2	45/F	+	-	dm, gr, pa, pl	ap (An); pe, unkn (U)		hz, pe, mz, mu	ap
3	41/M	-	-	dm, ar, ol, pl, cu, dd	unkn (An); pn, hz (OAS); le (GID); mu (U)		hz, kw, pe, mz, mu, le	pn
4	31/F	+	-	dm, pa, ol, pl, cd, dd	unkn (GID), pe (U)	NSAIDs + unkn = An	pe	nd
5	21/F	+	-	cd, dd, ol, pl, ar, gr	pe, hz (An); ap (U), le (GID)		hz, pe, kw, wh, le, mz	ap, wn
6	19/F	-	-	nd	le (An); unkn (GID)		pe, mu, le	nd
7	24/M	+	-	dm, ar, pl, dd, cd	pe, wn, hz, pn (OAS)	NSAIDs + unkn = An	hz, kw, pe, mz, mu, wh	wn, pn
8	28/F	+	-	pl, ar, pa	pn (An); cc (OAS); to (CU)	NSAIDs + unkn = An	hz, kw, le, mu, wh, mz	pn, to, cc
9	27/M	+	-	pa, ar, pl, pnc, cd	wn (An); pe, ba (U); le, ml (OAS)		hz, kw, pe, le	pn, ml, gb, wn, pi, che, sb, cha, cb, ba, cf
10	31/F	+	-	dm, ar, pl, cu	al, wn, hz, pe, che, plm (U); gb (An); cb (CU); unkn (GID)		hz, kw, pe, wh, mu, le	wn, al, che, plm, cb, gb, esc, as, sp, pea, bb
11	42/F	+	+	cd, dd, pl	le (U); fig (OAS); gb (GID)	NSAIDs + unkn = An	pe, mz, le	fig, gb
12	43/F	-	-	dm, ar, pl	to, ml, ba, gb (OAS); wn (An); pe (CU)		hz, kw, pe, mz, le, mu	gb, ml, wn
13	37/F	+	+	pl, pa, ar	wn (OAS); unkn (An)		hz, kw, pe, mz, le	pn, wn, sf, che, cn
14	41/F	-	+	pl, ar, gr	wh, sp (U); ap, wn (OAS); le, ba (GID)		kw, pe, mz, mu, le, wh	ba
15	37/F	+	-	dm, gr, ar, pl, alt, dd, cu, ol	le, wn, hz, gp, pe, fig, ap, gb (OAS); unkn (GID)	NSAIDs + le= An	hz, pe, mz, mu, le	pn, wn, gb, gp, fig, ap
16	47/F	+	-	dm, gr, pa, ar, ol, pl, cd, dd	pe (U); wn, hz, plm, pn, sf, mz, gp, ml, gb, zu, ak, mu, lu, to, che, ml, cb (OAS)		hz, kw, pe, mz, mu, le	wn, pn, plm, lu, sf, ml, gb, to, ak, gp, che, zu, cb
17	34/F	+	-	dm, gr, ar, pl, cd, dd	gb, le (OAS); be (An); plm, cb, sp (U)	NSAIDs + unkn = An	hz, kw, pe, mz, mu, le	gb, sp, cb, plm, be
18	40/M	-	-	na	gb,pn (An); cn (GID); pe (OAS)		na	na
19	33/F	+	+	ar, pl, cd	al, hz, pn, pi, pe, nc, gp (An); fig, ri (OAS)		hz, pe, mz, le	al, pi, pn, fig, ri, gp
20	34/F	-	-	ar, pl	pn, le (An); to (U); unkn (GID)		hz, pe, mz, mu, le	pn, to
21	28/F	+	+	dm, gr, pa, ar, pl, cd, dd	wn, hz, pn, le, pe, gb, gp, ml, fig (OAS)	NSAIDs + unkn = An	hz, kw, pe, mz, mu, le	wn, pn, cb, gb, gp, fig, ml, plm, sb, md
22	23/M	-	-	dm, pl	pe, pa (OAS); unkn (GID)	NSAIDs + unkn = An	kw, pe, mu, le	gb
23	14/M	-	-	dm, gr, pa, ar, ol, pl, cu, cd, dd	sf, cw (An); ap (OAS)		hz, kw, pe, mz, mu, wh	sf, cw, ap
24	20/F	+	-	dm, ar, pl, dd	pe (OAS); le (GID); unkn (U)		pe, mu, le	pn
25	44/F	+	+	dm, ol, pl, alt	le (An); pe (U); ml (OAS); unkn (GID)		hz, pe, mz, le	ml
26	23/M	-	-	dm, ar, pl	to, le, ln (OAS); wh, wn, ro, wh (An)		hz, kw, pe, mz, le, wh	pn, wn, ln, to
27	35/M	+	-	dm, pl	pr, ap, on, le, ca, wn, pe (An)		hz, pe, mz, mu, le	pr, ap, on, wn, pn, ca
28	38/F	+	+	dm, ar, pl	wn, eg (An); mz, pn, to (OAS); pe, unkn (U); wh, le, unkn (GID)		hz, kw, pe, mz, mu, le, wh	pn, wn, to
29	43/F	+	+	dm, pa, ar, ol, pl, asp, pnc	pe (U); unkn (GID)	NSAIDs + le = An	hz, kw, pe, mz, mu, le	char, ce, sp, to, cb, gb
30	40/M	+	-	nd	wh (U); hz (OAS)		pe	nd
31	34/F	+	-	pa, ar, ol	pe (U); le (GID)		pe, mz, mu, le	nd
32	34/F	+	+	dm, gr	wn, hz, le, sb (OAS); pe (U); gb (GID)		hz, pe, mz, le	wn, gb, sb
33	37/M	-	-	na	ap, pe, wn (U); le (GID)	NSAIDs + unkn = An	na	na
34	38/M	+	-	nd	pe, ap, pr, mu, al, hz (OAS); pe (CU); plm, wh (U)		hz, pe, mz, mu, wh	ap, plm, pr, al
35	28/M	+	-	dm, gr, pa, ar, ol, pl, cu	pn, pe (U); le (GID)		hz, pe, mz, mu, le, wh	pn, gb
36	26/M	+	+	dm, gr, ol, alt, cla	ap (An); pe (U); hz (OAS)	NSAIDs + unkn = An	hz, pe, mz	ap
37	28/F	+	-	na	hz, wn, pn, al (U); le, pe, fig, kw (OAS)		na	na
38	28/M	+	-	dm, gr, ar, pl, alt, cu, cd, dd	hz (U); pe (OAS); unkn (GID)	Exercise + unkn = U	hz, pe, mz, mu, le	nd
39	33/M	+	-	dm, gr, pa, dd	pn, gb, ri (OAS); gb, che, ba (GID); hz (An)		hz, pe, mz, le	pn, ba, ri, gb, che
40	37/F	+	-	pl, cu, ar	wn, hz, pn, pe (U); le (OAS)	NSAIDs + le = An	hz, pe, mz, mu, le	pn, wn
41	46/F	+	+	dm, cd, dd, ol, pl, pa	wn, hz, pn, pe, gb, le (OAS)	NSAIDs + le = An	hz, pe, mz, mu, le	pn, wn, gb
42	23/M	+	+	dm, gr, cu, pa, ar, pl	al (OAS); unkn (GID)	NSAIDs + unkn = An	hz, pe, mz, mu	nd
43	24/F	+	+	dm, pl	wn (U); pe (OAS)		pe, mz, mu	wn
44	25/F	+	+	dm, gr, ar, pl	pe (U); lu (An); wn, pn (OAS); ri, ml, wm (GID)	NSAIDs + unkn = An	hz, pe, mz, mu, wh	pn, wn, ml, lu, wm, ri
45	16/F	-	-	dm, ar, pl	pn (An); wn, gb (OAS); pe (U); le (GID)	Exercise + unkn = An	hz, pe, mz, le	pn, wn, gb

Table 4.10. Demographics, clinical characteristics and sensitization. Aeroallergens: alt: alternaria, ar: artemisia, asp: aspergillus, cla: cladosporium, cd: cat dander, cu: cupressus, dd: dog dander, dm: dust mites, gr: grass pollen, ol: olive, pa: parietaria, pl: plane tree, pnc: penicillium; Plant-foods: ak: artichoke, al: almond, ap: apple, as: asparagus, ba: banana, bb: broad bean, be: beer, ca: carrot, cb: cabbage, cc: cucumber, ce: celery, cf: cauliflower, cha: chard, che: cherry, cn: chestnut, cu: cumin, fr: fruits, eg: eggplant, esc: escarole, gb: green bean, gp: grape, hz: hazelnut, kw: kiwi, le: lettuce, ln: lentil, lu: lupine, ml: melon, mu: mustard, mz: maize, nc: nectarine, nm: nutmeg, on: onion, pa: pineapple, pe: peach peel, pn: peanut, pp:

pepper, pi: pistachio, plu: plum, pr: pear, ri: rice, ro: rosaceae, sb: strawberry, sf: sunflower seed, sp: spinach, to: tomato, wm: watermelon, wh: wheat, wn: walnut, zu: zucchini; Symptoms: An: Anaphylaxis, CU: contact urticaria, GID: gastrointestinal disorder, OAS: oral allergy syndrome, U: urticaria, NSAIDs: Non-Steroidal Anti-inflammatory Drugs; na: not available, nd: not detected.

Subject #	OAS	Urticaria	Contact Urticaria	GID	Anaphylaxis	Anaphylaxis only with cofactor
1	+	+	+	+	+	
2	-	+	-	-	+	
3	+	+	-	+	+	
4	-	+	-	+	+	+
5	-	+	-	+	+	
6	-	-	-	+	+	
7	+	-	-	-	+	+
8	+	-	+	-	+	
9	+	+	-	-	+	
10	-	+	+	+	+	
11	+	+	-	+	+	+
12	+	-	+	-	+	
13	+	-	-	-	+	
14	+	+	-	+	-	
15	+	-	-	+	+	+
16	+	+	-	-	-	
17	+	+	-	-	+	
18	+	+	-	+	+	
19	+	-	-	-	+	
20	-	+	-	+	+	
21	+	-	-	-	+	+
22	+	-	-	+	+	+
23	+	-	-	-	+	
24	+	+	-	+	-	
25	+	+	-	+	+	
26	+	-	-	-	+	
27	-	-	-	-	+	
28	+	+	-	+	+	
29	-	+	-	+	+	+
30	+	+	-	-	-	
31	-	+	-	+	-	
32	+	+	-	+	-	
33	-	+	-	+	+	+
34	+	+	+	-	-	
35	-	+	-	+	-	
36	+	+	-	-	+	
37	+	+	-	-	-	
38	+	+	-	+	-	
39	+	-	-	+	+	
40	+	+	-	-	+	+
41	+	-	-	-	+	+
42	+	-	-	+	+	+
43	+	+	-	-	-	
44	+	+	-	+	+	
45	+	+	-	+	+	

Table 4.11. Clinical symptoms. OAS: Oral Allergy Syndrome; GID: Gastrointestinal Disorders; Cofactor refers to NSAIDs and/ or exercise; "+": positive; "-": negative symptoms

4.2.4. Plant-foods sensitization

Sensitization to almost all plant-food allergenic sources included in the SPT standard battery was observed in most patients (median [range]: 5 [1-7] positive extracts). Further sensitizations were detected when SPTs were expanded to include other plant-foods reported by individual patients, to confirm sensitization. All patients that underwent SPT (42/45, 93.3%) had a positive test to peach peel, except one (41/42, 97.6%); 35 (83.3%) were positive to maize, 33 (78.6%) to hazelnut, 32 (76.2%) to lettuce, 29 (69.1%) to mustard, 18 (42.9%) to kiwi, and 12 (28.6%) to wheat (Table 4.10).

All patients with available sera were positive to whole peach extract (40/40, 100%; median [range] kU_A/L: 3.49 [0.38-43.1]), 44/45 (97.8%) to Pru p 3 (5.03 [0.11-36.7]), and none to Pru p 1 (0 [0-0.11]) or Pru p 4 (0 [0-0.07]), by ImmunoCAP™. Specific IgE to rTri a 14 by ELISA was also positive for all patients tested (21/21, 100%) (Table 4.12).

Results from the microarray are shown in Figure 4.7. Sensitization to all the LTPs included in the panel (Pru p 3, Art v 3, Cor a 8 and Par j 2) was observed; the distribution of frequencies is shown in Table 4.13. No sensitization to any other plant-food allergen included in the microarray panel (profilin, Bet-v1 homologues, taumatin, Calcium-binding protein, vicilin, 2s-albumin, 11-s albumin, gliadin, papain-like cistein protease and expansine) was detected.

Peach CRD and Tri a 14						
# Subject	Total IgE	Extract	LTP	PR10	Profilin	Tri a 14
1	132	13.3	13.2	0	0.02	0.30
2	85.5	2.25	5.03	0	0.02	0.27
3	123	4.83	6.58	0	0.01	na
4	265	0.74	0.11	0	0	0.29
5	148	23.4	25.6	0	0	0.29
6	41.4	0.93	1.28	0	0	na
7	205	14.9	20.9	0	0.01	0.23
8	49.6	na	19.6	0	0.02	0.21
9	35.6	na	1.76	0	0.02	0.18
10	34.9	3.2	4.64	0	0	0.18
11	39.3	0.84	1.51	0	0	na
12	na	4.54	5.57	0	0	0.17
13	51.2	4.47	6.35	0	0	na
14	706	1.14	0.89	0	0.02	0.20
15	196	4.64	1.23	0	0.03	0.28
16	50.4	2.18	3.05	0	0	na
17	153	4.89	6.66	0	0.03	na
18	23.5	5.05	7.44	0	0	na
19	103	2.02	2.84	0	0	na
20	367	16.2	21.3	0.11	0.07	na
21	64.8	3.23	3.63	0	0	na
22	83.9	1.77	2.55	0	0	na
23	306	14.4	6.11	0	0	0.34
24	236	6.22	10.2	0	0	na
25	231	0.39	1.65	0	0.07	na
26	72	43.1	35.4	0	0.01	0.27
27	na	na	5.22	0	0	na
28	39.5	1.56	1.64	0	0	0.25
29	111	na	10.8	0	0	0.23
30	196	2.89	3.24	0	0	0.19
31	220	0.89	0.82	0	0	na
32	49.2	0.65	0.89	0	0	na
33	202	4.55	5.63	0	0	na
34	14	0.95	1.27	0	0	0.20
35	138	3.75	5.24	0	0	0.19
36	472	2.35	4.05	0	0	na
37	131	9.51	9.52	0	0	na
38	174	12.3	31.9	0	0	0.20
39	121	2.43	3.35	0	0	na
40	192	31.1	36.7	0	0	0.19
41	55.9	0.38	0.42	0	0	na
42	150	na	14.7	0	0	na
43	78.7	3.85	4.98	0	0	na
44	729	16.7	18.8	0	0	0.18
45	42.2	0.62	0.43	0	0	na

Table 4.12. Sensitization to peach components and Tri a 14. Total and specific IgE levels determined by ImmunoCAP™ (Phadia®), expressed as kU_A/L units, cutoff >0.35; Tri a 14 specific IgE by ELISA (OD 492 nm), cutoff >0.11. "na": not available.

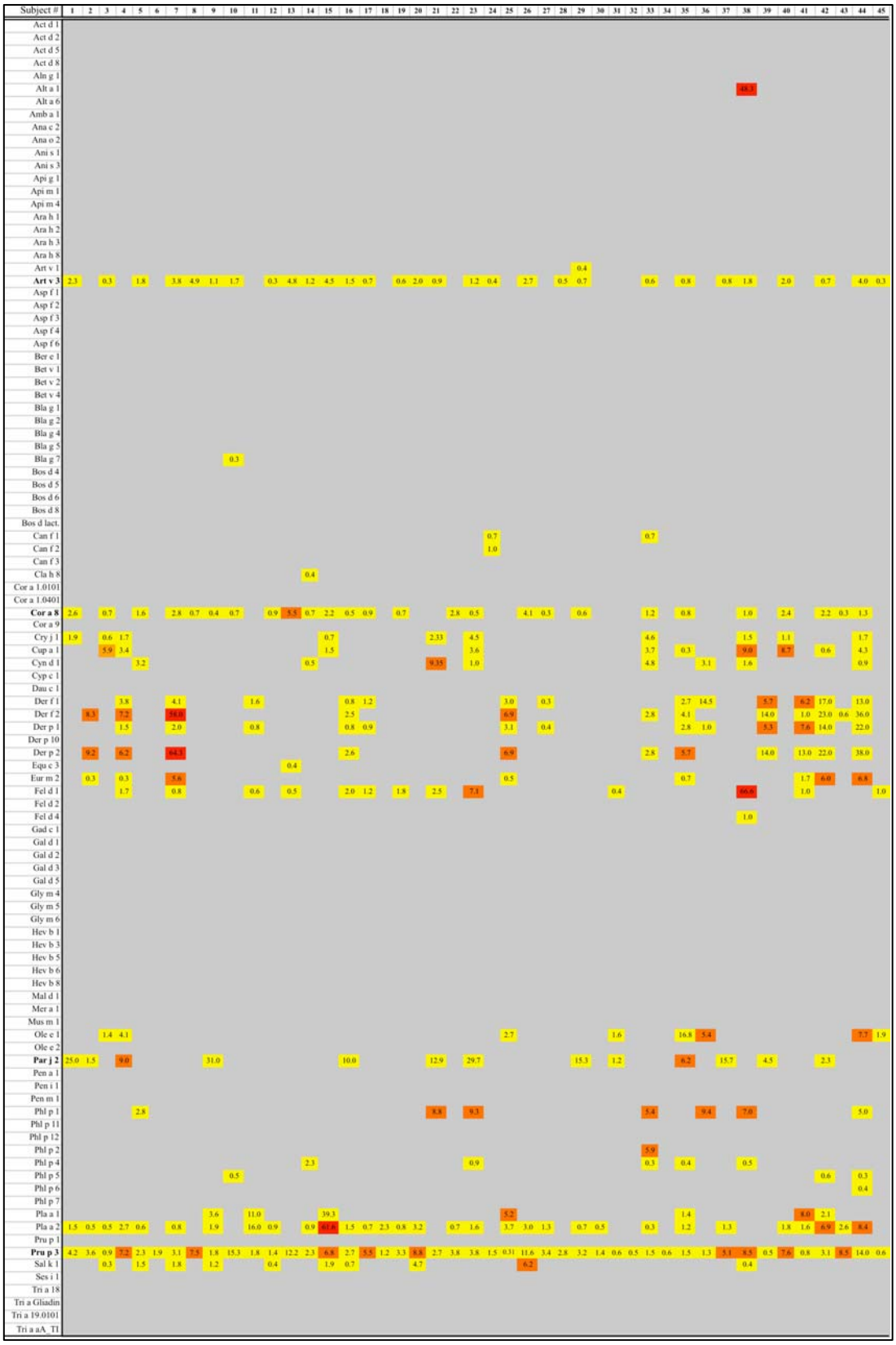

Figure 4.7. Schematic representation of component specific-IgE detection by microarray. A column represents a subject and rows each allergenic component. Levels expressed as ISU

units. Color code: grey, levels below the cutoff 0.3 ISU units; yellow (0.3-5), orange (5-20) and red >20 ISU. In bold the LTPs included in the panel.

4.2.5. Pollen sensitization

Thirty-four (75.6%) subjects were also diagnosed with pollinosis, while all of them had rhinitis, in 14 cases (31.1%) associated with asthma (Table 4.10).

Frequencies of pollen sensitization detected by each method are summarized in Table 4.13. A detailed description of each subject is provided in Table 4.14. By SPT and/or specific IgE, sensitization to some of the pollens tested with a known LTP (mugwort, wall-pellitory, plane tree, cypress and olea) occurred in 43 (95.6%) subjects. Sensitization to plane tree was detected in 42 (93.3%) patients, to mugwort in 33 (73.3%), to wall-pellitory in 16 (35.6%), to olea in 14 (31.1%) and to cypress in 12 (26.7%).

Sensitization	**N (%)**	**Specific IgE** median [range]
Peach Peel SPT	41/42 (97.6%)	-
- Peach Extract ImmunoCAP	45 (100%)	3.49 [0.38-43.1]
- Pru p 3 ImmunoCAP	45 (100%)	5.03 [0.11-36.7]
- Pru p 3 ISAC	45 (100%)	3 [0.3-15.3]
Cor a 8 ISAC	26 (57.8%)	0.5 [0.0-5.5]
Plane Tree SPT	35/42 (83.3%)	-
- Plane tree ImmunoCAP	36/42 (85.7%)	1.41 [0-61]
- Pla a 1 ISAC	7 (15.6%)	0 [0-39]
- Pla a 2 ISAC	31 (68.9%)	0.8 [0-61.6]
Mugwort SPT	27/42 (64.3%)	-
- Mugwort ImmunoCAP	27/43 (63.0%)	0.6 [0-7.0]
- Art v 1 ISAC	1 (2.2%)	-
- Art v 3 ISAC	29 (64.4%)	0.6 [0-4.9]
Wall-Pellitory SPT	15/42 (35.7%)	-
- Par j 2 ISAC	13 (28.9%)	0 [0-81]
Olea SPT	12 (28.6%)	-
- Ole e 1 ISAC	8 (17.8%)	0 [0-16.8]
- Ole e 2 ISAC	0	-
Cypress SPT	9 (21.4%)	
- Cup a 1 ISAC	10 (22.2%)	0 [0-30.4]
Grass pollen SPT	12/42 (28.6%)	-
-Phl p 1 ISAC	7 (15.6%)	0 [0-9.4]
-Phl p 2 ISAC	1 (2.2%)	-
-Phl p 4 ISAC	5 (11.1%)	0 [0-2.3]
-Phl p 5 ISAC	3 (6.7%)	0 [0-0.6]
-Phl p 6 ISAC	1 (2.2%)	-
-Phl p 7 ISAC	-	-
-Phl p 11 ISAC	-	-
-Phl p 12 ISAC	-	-

Table 4.13. Summary of pollen sensitization. Frequencies refer to all subjects of the cohort (n=45) unless specified. Specific IgE median [range] expressed as kU_A/L for ImmunoCAP™ (Phadia) test and ISU units for ImmunoCAP ISAC™ (Phadia®). SPT: Skin Prick Test.

POLLEN SENSITIZATION																								
Subject #	Art v 1	Art v 3	Mugwort ImmunoCAP	Mugwort SPT	Cup a 1	Cypress	Ole e 1	Ole e 2	Olive pollen SPT	Par j 2	Parietaria SPT	Phl p 1	Phl p 11	Phl p 12	Phl p 2	Phl p 4	Phl p 5	Phl p 6	Phl p 7	Grass SPT	Pla a 1	Pla a 2	Plane Tree ImmunoCAP	Plane Tree SPT
1	0.0	2.3	3.61	-	0	-	0	0	-	24.8	+	0	0	0	0	0	0	0	0	-	0	1.5	6.04	+
2	0.0	0	0.06	-	0	-	0	0	-	1.5	+	0	0	0	0	0	0	0	0	-	0	0.5	0.11	+
3	0.0	0.3	0.72	+	5.9	+	1.4	0	+	0	-	0	0	0	0	0	0	0	0	-	0	0.5	0.67	+
4	0.0	0	0	-	30.4	-	4.1	0	+	81	+	0	0	0	0	0	0	0	0	-	0	2.7	1.97	+
5	0.0	1.8	6.97	+	0	-	0	0	+	0	-	2.8	0	0	0	0	0	0	0	-	0	0.6	7.34	+
6	0.0	0	0.44	-	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	-	0	0	0.53	-
7	0.0	3.8	6.04	+	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	-	0	0.8	8.68	+
8	0.0	4.9	2.85	+	0	-	0	0	-	0	+	0	0	0	0	0	0	0	0	-	0	0	4.85	+
9	0.0	1.1	0.2	+	0	-	0	0	-	31	+	0	0	0	0	0	0	0	0	-	3.6	1.9	1.88	+
10	0.0	1.7	0.22	+	0	+	0	0	-	0	-	0	0	0	0	0	0.5	0	0	-	0	0	1.41	+
11	0.0	0	0.04	-	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	-	11	16	21.2	+
12	0.0	0.3	1.22	+	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	-	0	0.9	0.84	+
13	0.0	4.8	0.73	+	0	-	0	0	-	0	+	0	0	0	0	0	0	0	0	-	0	0	0.27	+
14	0.0	1.2	0.6	+	0	-	0	0	-	0	-	0	0	0	0	2.3	0	0	0	+	0	0.9	0.99	+
15	0.0	4.5	2.69	+	1.5	+	0	0	+	0	-	0	0	0	0	0	0	0	0	-	39	61.6	61	+
16	0.0	1.5	0.48	+	0	-	0	0	+	10	+	0	0	0	0	0	0	0	0	+	0	1.5	0.46	+
17	0.0	0.7	1.16	+	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	+	0	0.7	2.3	+
18	0.0	0	0.4	na	0	na	0	0	na	0	na	0	0	0	0	0	0	0	0	na	0	2.3	0.22	na
19	0.0	0.6	0.45	+	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	-	0	0.8	0.81	+
20	0.0	2	2.04	+	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	-	0	3.2	7.13	+
21	0.0	0.9	0	+	0	+	0	0	-	12.1	+	8.8	0	0	0	0	0	0	0	+	0	0	0	+
22	0.0	0	0.02	-	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	-	0	0.7	0.39	+
23	0.0	1.2	1.14	+	3.6	+	0	0	+	29.1	+	9.3	0	0	0	0.9	0	0	0	+	0	1.6	16.7	+
24	0.0	0.4	1.27	+	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	-	0	0	1.28	+
25	0.0	0	0.18	-	0	-	2.7	0	+	0	-	0	0	0	0	0	0	0	0	-	5	3.7	26.6	+
26	0.0	2.7	2.3	+	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	-	0	3	1.9	+
27	0.0	0	0.64	-	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	-	0	1.3	1.99	+
28	0.0	0.5	0.46	+	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	-	0	0	0.35	+
29	0.4	0.7	3.46	+	0	-	0	0	+	15	+	0	0	0	0	0	0	0	0	-	0	0.7	6.6	+
30	0.0	0	0.11	-	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	-	0	0.5	0.06	-
31	0.0	0	0.04	+	0	-	1.6	0	+	10.2	+	0	0	0	0	0	0	0	0	-	0	0	0.04	-
32	0.0	0	0	-	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	+	0	0	0	-
33	0.0	0.6	1.16	na	3.7	na	0	0	na	0	na	5.4	0	0	5.9	0.3	0	0	0	na	0	0.3	1.46	na
34	0.0	0	0	-	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	-	0	0	0.03	-
35	0.0	0.8	1.12	+	0.3	+	16.8	0	+	6.2	+	0	0	0	0	0.4	0	0	0	+	1.4	1.2	4.49	+
36	0.0	0	0.04	-	0	-	5	0	+	0	-	9.4	0	0	0	0	0	0	0	+	0	0	0.65	-
37	0.0	0.8	0.19	na	0	na	0	0	na	15.7	na	0	0	0	0	0	0	0	0	na	0	1.3	0.55	na
38	0.0	1.8	3.09	+	8.9	+	0	0	-	0	-	7	0	0	0	0.5	0	0	0	+	0	0	1.3	+
39	0.0	0	-	-	0	-	0	0	-	4.5	+	0	0	0	0	0	0	0	0	+	0	0	0.35	-
40	0.0	2	5.82	+	8.7	+	0	0	-	0	-	0	0	0	0	0	0	0	0	-	0	1.8	5.16	+
41	0.0	0	0.07	-	0	-	0	0	+	0	+	0	0	0	0	0	0	0	0	-	8	1.6	6.36	+
42	0.0	0.7	3.07	+	0.6	+	0	0	-	2.3	+	0	0	0	0	0	0.6	0	0	+	2.1	6.9	10.3	+
43	0.0	0	-	-	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	-	0	2.6	2.19	+
44	0.0	4	3.42	+	4.3	-	7.7	0	-	0	-	5	0	0	0	0	0.3	0.4	0	+	0	8.4	4.75	+
45	0.0	0.3	0.21	+	0	-	1.9	0	-	0	-	0	0	0	0	0	0	0	0	-	0	0	0.13	+

Table 4.14. Detail of pollen sensitization detected by each diagnostic test. Specific IgE to allergen components by microarray in ISU units, cutoff > 0.3; Specific IgE to whole extracts by

ImmunoCAP™ (Phadia®) in kU_A/L, cutoff >0.35; SPT results: positive (+), negative (-) or not available (na).

The proportion of patients positive to plane tree (0.91, 95% CI [0.83-0.99]) and mugwort (0.69, 95% CI [0.55-0.82]) was higher than that of other tested pollens (cypress, olea, wall-pellitory and grass) and this difference was statistically significant (Figure 4.8).

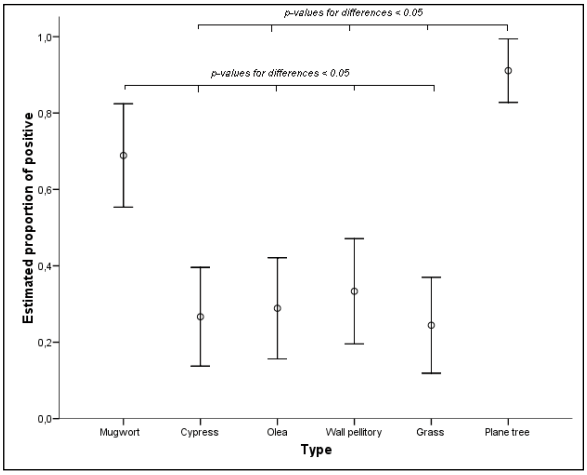

Figure 4.8. Estimation of proportion of positive to pollens. Estimation of proportion of positive tests to plane tree, mugwort, wall-pellitory, cypress, grass pollen or olea in a subject was analyzed using logistic regression models.

4.2.6. NSAID cofactor effect on basophil degranulation *in vitro*

To investigate the effect of NSAID as a cofactor we wanted to establish a model *in vitro* to reproduce the clinical observations. Thus, we designed a basophil activation test based on two stimulation phases, a first phase with only the food allergen (Pru p 3) and a second one with the combination of the drug and the food allergen (L-ASA + Pru p 3). The test was performed to all individuals recruited for this pilot study (Group A: 24 peach allergic patients, presenting clinical exacerbation when NSAIDs intake; Group B: 7 peach allergic patients, not clinically exacerbated by NSAIDs; and Group C: 5 healthy non-atopic subjects) under the same conditions. Tolerance to L-ASA was confirmed in all patients by negative results on basophil activation tests performed at several concentrations of L-ASA (3 mM, 1 mM, 0.3 mM and 0.1 mM).

With this *in vitro* model, a statistically significant increase in basophil activation (measured as expression of CD63) when stimulation was performed with Pru p 3 together with L-ASA at different concentrations (3 mM, 0.3 mM and 0.1 mM) compared to the activation induced by Pru p 3 alone (Wilcoxon Matched Pairs test: at 3mM, $p<0.0001$; at 0.3 mM, $p=0.0117$ and at 0.1 mM $p=0.0085$) could be observed. The L-ASA 1 mM did not show such effect. The percentage of CD63+ cells was very similar to stimulation with L-ASA alone (negative test).

The increase on degranulation upon stimulation with both Pru p 3 and L-ASA was not observed for group B (Figure 4.9). Group C showed no degranulation with any of the stimuli.

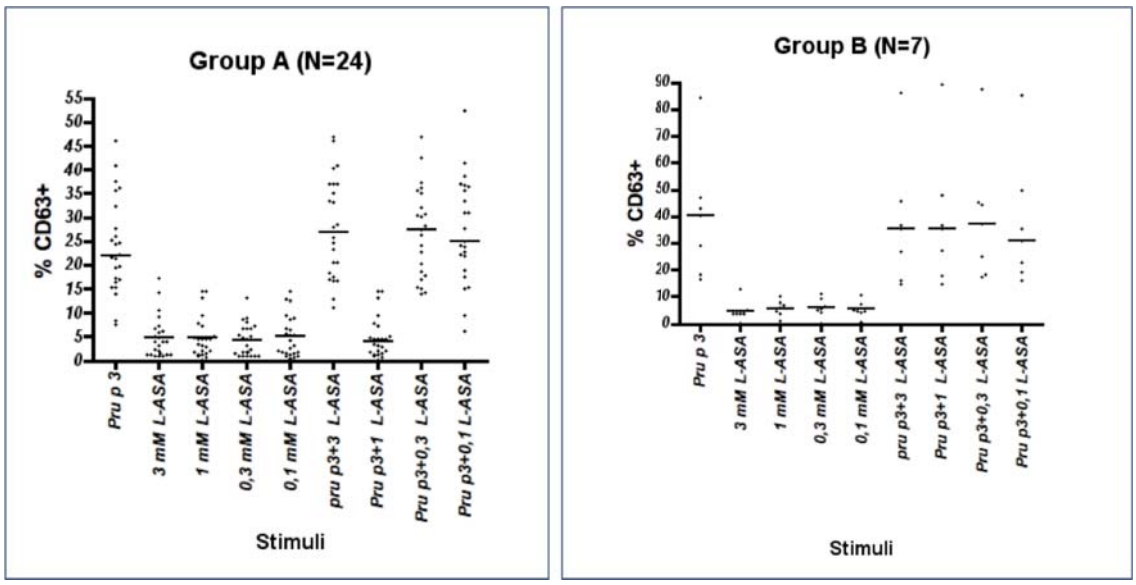

Figure 4.9. Basophil activation test with Pru p 3 and L-ASA. Percentage of CD63+ cells induced by each stimulus in groups A and B.

CHAPTER 5. DISCUSSION

DISCUSSION

5.1. CRD and epitope recognition in shellfish allergy

Despite the high prevalence of shellfish allergy, its diagnosis is still too often a challenge for clinicians, particularly in patients with mild subjective symptoms, inconsistent reactions, or no prior history of reaction on shrimp ingestion but with a positive test result to shrimp (skin prick test and/or specific IgE in serum) obtained during routine workup for allergies. Frequently sensitization to allergens occurs but the clinical repercussion of it is not clear, especially when several potential culprit foods are involved and there is sensitization to allergenic components of all of them. Thus, the double-blind placebo controlled food challenge still remains the gold standard for shellfish allergy diagnosis, despite its limitations considering time requirements and the potential risk of inducing severe reactions.

Component resolved diagnostics, with recombinant or purified allergens, and epitope mapping have been applied to a wide range of food and environmental allergies, allowing the elucidation of distinct sensitization profiles for different clinical pictures [46]. For instance, specific IgE to Ara h 2 is more accurate in predicting clinical peanut allergy than other peanut allergens [128]. In addition, sensitization to particular epitopes of milk [129], egg [130], or peanut [68] has been used as biomarkers for severity and persistence of disease. Furthermore, these new diagnostic tools have allowed the development of new types of immunotherapy, some of which have shown efficacy in human trials [131].

Several shrimp allergens have been cloned and characterized during the last decades [79], as well as their IgE epitopes identified [90]. However, despite the improvement on knowledge of shellfish allergens in the last years, there is limited information about the clinical relevance of sensitization to particular shrimp allergens. Two recent studies aimed to improve the diagnostic approach to shellfish allergy. Yang A et al. [88] studied the relevance of sensitization to tropomyosin in dust mite-allergic subjects sensitized to shrimp. Tropomyosin-specific IgE was found to have greater diagnostic efficiency compared to whole shrimp-specific IgE or SPT (88.5%, 74.2%, and 65.7% respectively). Bauermeister K et al. [89] suggested a panel of six allergens (tropomyosin, arginine-kinase, sarcoplasmic calcium-binding protein, a novel myosin light chain, troponin C and triosephosphate-isomerase) of the North Sea shrimp

(*Crangon crangon*) to develop a more sophisticated and clinically adequate method of shrimp allergy diagnosis and treatment.

The present study aimed to identify profiles of allergen sensitization and significant epitopes in shrimp allergens resulting in clinical reactivity to shrimp. To our knowledge, this is the largest study providing a molecular diagnosis of shrimp allergy, with 86 subjects sensitized to shrimp recruited and challenged, a control group of subjects sensitized to dust-mite and/or cockroach but not to shrimp and including all described crustacean allergens described so far (except triosephosphate-isomerase). Furthermore, IgG4 reactivity at epitope level with five shrimp allergens is assessed for the first time.

The initial recruitment criteria was a positive test with shrimp extract (SPT and/or serum specific IgE), then subjects were subdivided according to their history of allergic reactions with shrimp. A DBPCFC was performed in all cases, except those reporting an episode of anaphylaxis with shrimp within the previous 3 months. An extensive cross-reactivity has been described among crustaceans and dust mite and/or cockroach [79, 132]. Fernandes J et al. reported that house dust mite and cockroach tropomyosins could account for the presence of detectable IgE to shrimp, even in unexposed subjects [133]. Thus, an additional control group allergic to dust-mites and/or cockroach but not sensitized to shrimp was also included. Frequency of sensitization to dust mite (*D. pteronyssinus*, DP) was very high in all groups (94-100%) and no statistically significant differences in the levels of DP-specific IgE were found. Sensitization to cockroach was lower, probably because it is less prevalent in some of the areas where the subjects were recruited. Dust mite and/or cockroach sensitization in groups 1, 2a and 2b could be not only due to a primary sensitization, but also secondary to a cross-reactivity phenomenon with homologue proteins of shrimp and dust mite or cockroach. Thus, both the grade of homology between the same proteins in different species and the frequency of exposure to dust mite, cockroach or both could influence sensitization. Wang et al. suggested recently that shrimp IgE levels might need to be interpreted in the context of environmental exposures after finding that shrimp IgE levels were correlated with cockroach IgE levels and exposure to cockroach allergen at home, but not to dust mite exposure [134].

In terms of recombinant allergen recognition, we observed that shrimp allergic individuals showed more diversity of allergen recognition (i.e., recognized more allergens) than shrimp tolerant subjects; dust mite and/or cockroach allergic subjects not sensitized to shrimp (control group C1) only recognized arginine kinase and

hemocyanin; and arthropod-allergic subjects sensitized to shrimp but tolerant that had been separated depending on their history of allergic reactions on shrimp ingestion (groups 2a and 2b) showed no differences in allergen recognition among them (except for hemocyanin being more frequently recognized 2a, p=0.0189). Thus, the clinical relevance of the differences between these two groups (2a and 2b) is not entirely clear, maybe they are not really different and we do not know to what they may have reacted in the past, especially the group 2a that once had allergic symptoms after shrimp ingestion. Subjects sensitized to shrimp extract (2a and 2b) may recognize other allergens than arginine kinase and hemocyanin, including tropomyosin and others, however no statistically significant differences in frequencies of allergen recognition were found between these two groups (2a+2b) and control group 1. Therefore, all three, regardless of the shrimp sensitization, were considered together as shrimp tolerant for comparative analysis with shrimp allergic subjects.

Statistically significant differences in the frequency of IgE recognition of tropomyosin, sarcoplasmic calcium-binding protein- alpha and/or -beta and myosin light chain were found between shrimp allergic and tolerant subjects. Tropomyosin had the strongest statistically significant difference ($p < 0.0001$). Thus, sensitization to these components, and to tropomyosin in particular, seems to be useful to differentiate clinical reactivity to shrimp (i.e., predictive/associated with positive challenge). Moreover, the analysis of their properties as diagnostic tests showed that tropomyosin had the highest values for all 5 parameters (sensitivity, specificity, PPV, NPV and efficiency). Whereas SCPs and MLC showed high values for specificity (~ 95% and 87.5%, respectively) as well as PPV (97.5% and 81.5%, respectively), but low ones for sensitivity (29-35% and 38%, respectively) and NPV (~ 50%). Efficiency for SCPs and MLC was around 60%, lower than shrimp extract tested by ImmunoCAP (78%) and TM (87%). The shrimp extract was the most sensitive (98%) and with the highest NPV (95%), however it had the lowest specificity (49%) and PPV (74%).

IgE reactivity to whole food extracts on skin prick test and/or ImmunoCAP without clinical correlation has been attributed to sensitization to cross-reacting allergens in other foods or as a result of primary exposure to allergens on sites different from the gastrointestinal tract [101]. In this line, the whole shrimp extract is very sensitive but poorly specific, with a high number of positive tests without clinical relevance. Thus, it could be hypothesized that a great number of positive tests not clinically relevant are due to cross-reactive responses to tropomyosin (described as an invertebrate panallergen [87]), arginine kinase or hemocyanin. Such sensitization could

have been acquired through exposure to these allergens present in foods other than shrimp, parasites, or mites and cockroach through the inhalant route [135]. Yang et al. [88] described that in their group of patients with mite allergy and a high frequency of sensitization to shrimp (SPT and ImmunoCAP) without clinical correlation, sensitization to shrimp tropomyosin was found only in a small proportion of them. In our study, we have also found that only a few subjects with this profile (groups 2a and 2b) recognize tropomyosin. In addition we have observed that they mainly recognize arginine kinase and hemocyanin (allergens not tested by Yang et al. [88]) alike the control group C1. This observation should be confirmed with larger groups of subjects. Thus, from the present study, we identify not only clinically relevant components (SCPs, TM and MLC) in shrimp allergy, but also arginine kinase and hemocyanin as likely markers of cross-reactivity in arthropods, a role that has mainly been attributed to tropomyosin [79, 87, 133]. Only a few subjects in this study, with no statistically significant differences between groups, recognize troponin C allergen.

Many recombinant allergens, such as tropomyosin, have been shown to behave similar to their native counterparts [56, 136]. However this is not universal. This might explain why some subjects were found not to recognize any recombinant protein on dot-blot immunolabelling but then they showed IgE binding to proteins of boiled and raw shrimp extracts by Western blot. Similarly, the low sensitivity observed by the recombinant allergens may be due to post-transcriptional modifications of the native allergens that may differ from the recombinant counterparts.

Although sarcoplasmic calcium-binding protein and myosin light chain were reported to be particularly important in children in prior publications [84, 85, 90], no strong trend on that point was observed in this study. No major differences were found between children and adults IgE recognition within each group. Stronger differences observed in previous studies might be due to the fact that subjects were not challenged and more adults with negative challenges might have been included in the study.

Ayuso R et al. previously identified the IgE-binding epitopes of four shrimp allergens (shrimp TM, AK, MLC and SCP, Lit v 1 to 4, respectively) [90]. In the present study we include another shrimp allergen, Troponin C, as well as IgG4 binding data. The fact that IgE and IgG4 binding areas coincide largely has also been described in peanut and milk microarray studies [68, 69, 137]. TileMap analysis was intended to identify IgE and IgG4 binding sites with statistical differences between groups (FDR <0.05, $p<0.01$). Groups 2a and 2b showed minor differences in binding sites, therefore for the analysis they were considered together (2=2a+2b) as tolerant to compare with

the positive challenge individuals (group 1). Several informative epitopes/binding areas were identified through multiple comparisons between groups that might be useful in predicting clinical reactivity. Interestingly, more differential binding sites for IgE were observed for tropomyosin, a protein associated with positive challenge outcome; whereas for IgG4 more different binding sites were observed for arginine kinase, an allergen that from the part of this study dealing with whole recombinant allergens (dot blot) seems to be more involved in cross-reactivity phenomena. However, we have to keep in mind that for arginine kinase we have also identified some epitopes that differ between challenge positive subjects and challenge negative.

The IgE antigenic areas recognized in the present study for the 4 allergens corresponded well to previously identified epitopes [90]. Moreover, if we compare the results of the current study with the results of a recent pilot study of our group (with smaller sample size) that also aimed to identify clinically relevant epitopes [91], we observe that almost the same epitopes were identified for TM, with two additional ones (epitopes 3 and 6). This was not the case for the other allergens. For MLC, only two epitopes of the five described before as clinically relevant [91] were also found in the current study (epitopes 2 and 5). For SCP, a different binding site not previously reported was identified and the epitope described as clinically relevant by the pilot study could not be reproduced. For AK, only epitope 7 coincided with the results of the pilot study, however statistically significant differences between allergic and tolerant individuals were found in three additional epitopes (3, 4a and 4b) already described by Ayuso et al. [90] but not identified as clinically relevant in the pilot study.

Challenge positive individuals recognized more frequently all epitopes than challenge negative subjects, except for epitope 2 of MLC. Tropomyosin and SCP epitopes showed high specificity (>90%, epitopes 1, 5a, 5b and 6 of TM and n of SCP; >75%, epitopes 3 and 7 of TM) and PPV (>90%, epitopes 1 and 5b of TM; >75%, epitopes 3, 5a, 5b, 6 and 7 for TM and epitope n for SCP), alike TM and SCP complete recombinant allergens. However, the epitopes of the other allergens had lower values for these parameters. Sensitivity varied largely between allergens, as well as, for epitopes of the same allergen. For instance, while epitope 7 of TM showed the highest specificity of all epitopes analyzed, the others had values from 38 to 71%. Epitope n of SCP showed the lowest sensitivity (33%). In parallel, the highest NPV were observed for certain epitopes of TM (76-88%) and epitope 7 of AK (80%). Tropomyosin epitopes showed in general higher efficiency values (76-82%) than the epitopes of the other allergens (63-69%). Epitope 7 of tropomyosin was the only one with high values for all

5 parameters and may be a good candidate to be included in a panel of epitopes to predict clinical reactivity.

In peanut allergy, clinical sensitivity, determined by means of DBPCFC, seemed to be positively related to IgE broader epitope recognition [68]. For milk allergy, peptide microarray studies showed that patients with persistent milk allergy had increased epitope diversity and stronger IgE affinity binding compared to patients with transient milk allergy or tolerance to extensively heated milk [69]. In our study, the number of peptides bound by IgE and both IgE and IgG4 (i.e. epitope diversity) in shrimp allergic subjects was larger for all five allergens than in tolerant subjects. Similarly to peanut and milk allergy, clinical reactivity would be associated to IgE broader epitope recognition of shrimp allergens. Interestingly, for TM, MLC and AK we observed IgG4 broader epitope recognition in allergic individuals (group 1) than tolerant ones (group 2). The role of IgG4 in these differences is hard to address, since this antibody has been related to chronic antigen exposure and it has been shown in other contexts to inhibit the activity of IgE by competing for binding sites becoming somehow protective [138-140]. So one would have expected that the diversity of IgG4 binding would be superior for shrimp tolerant individuals than for allergic ones. However, for milk allergy, it was reported that subjects who were heated-milk tolerant had IgE-binding patterns similar to those of subjects who had outgrown their milk allergy but IgG4-binding patterns that were more similar to those of the allergic group [69]. Then, it was speculated that since strict avoidance is currently the mainstay of treatment of milk allergy, it could be possible that IgG4 binding might increase as the heated milk tolerant group incorporates certain heated milk products into their diets. Alternatively they proposed that as tolerance to milk develops, subjects might lose IgG4 to some epitopes and develop IgG4 to other areas of the milk proteins [69]. In our case, the presence of IgG4 even in shrimp allergic individuals could be due to exposure to other shellfish that they might tolerate, or to chronic exposure to environmental allergens (i.e., dust-mites and/or cockroach) that could cross-react with shrimp allergens having similar sequential epitopes (although conformational epitopes seem to be more relevant than sequential epitopes for environmental allergens [63,67]). An alternative hypothesis maybe that shrimp allergic individuals produce both IgE and IgG4 antibodies and clinical reactivity depends on antibody levels, antibody affinity or other factors. In the present study, affinity of binding was not analyzed, however this would be an interesting point to considerate for future studies intended to identify more differences between subjects with and without clinical reactivity to shrimp, or to identify

sequential epitopes associated with clinical severity and persistence of shrimp allergy. The relevance of these findings of IgG4 in shrimp allergy needs to be further investigated.

In conclusion, sensitization to tropomyosin, sarcoplasmic calcium-binding protein and myosin light chain is associated with clinical reactivity to shrimp. Arginine kinase and hemocyanin are important cross-reactivity markers in arthropods, but still some arginine kinase epitopes may be clinically significant. Detection of specific IgE of these allergenic components should be included in the routine workup of shellfish allergy diagnosis. In addition, the identification of differential binding sites/epitopes with good properties as diagnostic tests to discriminate between allergic and tolerant individuals (e.g., epitope 7 of tropomyosin) opens new perspectives for diagnosis and treatment of shellfish allergy.

5.2. Lipid Transfer Protein syndrome: clinical pattern and molecular sensitization profile to plant-foods and pollens

As expected in a Mediterranean country, our cohort was sensitized to LTPs present in plant-food allergenic sources. More importantly, no sensitization to any other plant-food panallergen was detected by using the microarrayed-CRD system, which allowed us to clinically characterize patients from our area with LTP-syndrome.

In our hands, CRD is a valuable tool for unmasking the diagnosis of LTP-syndrome in patients with a complex clinical pattern and multiple sensitizations to plant-foods and pollens. Specifically, it permits the identification of the components triggering the allergic responses that may help patients avoid severe episodes of anaphylaxis**.** Furthermore, we have found that CRD is useful for identifying those patients who present only mild IgE-mediated symptoms resulting from isolated LTP sensitization. This study shows that mild symptoms, which are usually associated with other plant-food allergens such as profilins or Bet v 1-like proteins, can also be induced by LTPs**.**

Since other allergenic components could be responsible for the symptoms described by the subjects in this study, it is necessary to perform a complete protein screening to thoroughly evaluate other sensitizations. The simultaneous testing of multiple allergenic components from different allergenic protein families with the biochip provides valuable information about the overall picture of each patient's

positive and negative sensitizations. Thus, the greater the number and diversity of validated allergen components in the array panel, the more accurate the diagnosis.

In our group of patients, as previously described in Southern Europe [74, 108, 141, 142], peach is also the main *Rosaceae* fruit involved, and Pru p 3 the major allergen (100% positive). Sensitization to other peach allergens was not detected. Thirty-four (75.6%) patients reported peach allergy, while the remaining eleven were individuals who spontaneously avoided eating peach peel and luckily had never been accidentally exposed to it. Clinical practice reveals that many food-allergic patients avoid eating a particular food while apparently unaware that they are sensitized to it.

Our patients suffered immediate allergic reactions to an extremely diverse panel of plant-foods related with LTP-allergy, including *Rosaceae* and non-*Rosaceae* fruits, such as tree nuts, peanut, green bean, wheat and lettuce. Some patients also reacted to foods previously described as generally safe for LTP-sensitized patients (e.g., banana and melon [143]. In some reactions, the culprit food could not be identified and although this is often observed in food allergy, this phenomenon appears to be more frequent in LTP-sensitized patients. This broad diversity of offending foods is due to the wide distribution of LTPs and their high cross-reactivity demonstrated *in vivo* and *in vitro*, especially when sequence identity is high [110].

A broad spectrum of clinical symptoms has been observed in our study. The high frequency of severe and systemic reactions, in parallel with OAS observed in LTP-sensitized subjects, is consistent with the first description of the clinical picture of LTP-sensitization [144, 145]. In our patients, OAS was found to be very frequent (75%), but almost never as an isolated clinical presentation of LTP allergy. We report that GID were frequently detected (55.6%). As patients with food allergy often do not spontaneously relate abdominal discomfort with foods intake, this observation highlights the need to carefully check digestive symptoms in these patients. In addition, we need to take into account that GID and/or OAS were present in 32/34 (94%) of the patients affected by anaphylaxis in other non-anaphylaxis food allergic episodes and in 12/34 (35%) were the unique other symptoms related with food allergy.

It is well established that in some cases exposure to a food allergen and a concomitant or sequential exposure to physical exercise and/or medication are needed to provoke the reaction. In our group of patients, cofactors were frequently involved (40%) in the clinical expression of allergic symptoms and also influenced their severity, i.e., some subjects tolerated a plant-food and only experienced the allergic reaction with the cofactor, or they suffered a marked worsening of symptoms when the food and

cofactor temporarily coincided. The contribution of a cofactor to the precipitation of episodes of anaphylaxis was mandatory in 11 of the 34 (32.4%) patients. This data emphasizes the need to carefully investigate the presence of more than one trigger in episodes of anaphylaxis, particularly in its most severe forms [37]. Interestingly enough, 55% our patients with anaphylaxis associated with cofactors suffered GID and/or OAS as unique symptoms when exposed only to food allergens. Both clinicians and patients may consider these episodes of food allergy a mild and almost irrelevant process, when in fact it may represent a serious risk of developing severe or even fatal anaphylactic episodes, if cofactors are not detected and the temporary association of food and cofactors is not prevented.

The pathogenic mechanism of cofactors in food allergy remains to be clarified. One theory suggests that changes in mucosal permeability induced by NSAIDs, exercise or a combination of both can enhance allergen absorption, resulting in a more intense exposure of mast cells to allergens [38, 146]. This could partially explain the lack of correlation between LTP-specific IgE levels and severity of symptoms [147]. Although anaphylaxis induced by food allergen associated with cofactor has mainly been described in patients allergic to omega-5 gliadin, our study shows, for the first time, that cofactor-dependent anaphylaxis is also frequent in the LTP-syndrome.

With the basophil activation test developed for an *in vitro* model of the cofactor effect in LTP-syndrome, we are by now able to report that the clinical effect seen *in vivo* were also observed *in vitro*. NSAIDs affected somehow on basophil IgE-mediated responses and this might explain the exacerbation of the clinical symptoms. However, the concentration of L-ASA 1 mM did not show such effect and the percentage of CD63+ cells was very similar to stimulation with L-ASA alone, as if Pru p 3 was somehow blocked to induce degranulation. Further investigations are required to clarify the mechanism of the cofactor effect on basophils and the role of cofactor concentration on basophils behaviour. Moreover, it cannot be ruled out that the effect of L-ASA is caused directly on basophils and not indirectly through other cells present in the sample tested (i.e., monocytes, neutrophils, etc.). Future perspectives for this study include the analysis of the behavior of other cell populations upon stimulation.

Traditionally, LTP-allergy has not been described in association with pollinosis, but this paradigm has changed after recent reports of high rates of co-sensitization to LTPs from pollens from the Mediterranean basin and plant-foods. The link between the two means of sensitization and the potential influence of pollen-LTPs on LTP-food allergy are still subject to debate. Although the IgE cross-reactivity between mugwort

pollen and plant-food LTPs and *Rosaceae* LTPs is only partial [148], peach-allergic patients highly exposed to mugwort pollen showed a significant pattern of sensitization to both Pru p 3 and Art v 3 by SPT, in contrast to areas with less pollen exposure [149]. Art v 3 sensitization has been described not only as a potential epiphenomenon of Pru p 3 sensitization without clinical relevance [150], but also as a major mugwort allergen with the ability to act as a primary sensitizer [151]. The finding that all Spanish hazelnut-allergic patients, with Cor a 8 (LTP) as major allergen [102], are also allergic to *Platanus acerifolia* pollen [152], together with a previous description of the clinical association between plane tree pollinosis and plant-derived food allergy (specially hazelnut) [153], highlighted the potential role of plane tree pollen LTP as a primary sensitizer through the inhalation route and a subsequent cross-reaction with Cor a 8. Subsequently, the plane tree LTP was identified as Pla a 3 and defined as a major allergen in patients with both plane-pollen and peach allergy [154].

In our group of patients, pollinosis was diagnosed in 75.6% of patients. Interestingly, plane tree and mugwort were the most frequent pollens involved and both were found associated with Pru p 3 sensitization, especially in the case of plane tree. The low sequence identity of Pru p 3 with Par j 1 (28%) and Ole e 7 (19%) could explain why sensitization to these pollens was lower in our cohort [110, 155]. With the microarray, it was found that 69% of patients sensitized to plane tree were sensitized to Pla a 2 and 15.6% to Pla a 1, confirming that there is a primary sensitization to plane-tree pollen. This finding is consistent with a geographical area with high levels of this pollen [153]. It is possible that plane-tree pollen may have a dual role in our cohort: as a primary sensitizer or as a co-sensitizer that modulates the spectrum of LTP-sensitization. Accordingly, it is interesting to note that all patients sensitized to Pla a 1 and/or Pla a 2 were also sensitized to Cor a 8. Given that in our geographical area, mugwort pollen levels are not particularly relevant [156], and only 1 patient was positive for Art v 1, considered a major mugwort allergen, we assume that mugwort sensitization resulted from the cross-reactivity with other LTP-sensitizations rather than being a primary sensitization or co-sensitization.

In conclusion, multiple plant-foods not taxonomically related besides peach are involved in LTP-syndrome in our area, as whole allergenic plant-food extract and molecular sensitization profile shows. Local symptoms (OAS, GID) should be evaluated as a risk factor for anaphylaxis because they are frequently associated, especially with cofactor-dependent anaphylaxis. Its association with pollinosis, especially with plane tree, could be part of this syndrome in our area.

CHAPTER 6. CONCLUSIONS

CONCLUSIONS

From the first part of this thesis (paper in progress) we have been able to derive four main conclusions:

1.- Tropomyosin (in particular), sarcoplasmic calcium-binding protein and myosin light chain seem to be the relevant allergens associated with clinical reactivity to shrimp, that could be good candidates to diagnostic tests for shrimp allergy, since they can help predicting challenge outcome.

2.- Arginine kinase and hemocyanin seem to be important cross-reactive allergens between shrimp, dust-mite and cockroach, although some arginine kinase epitopes have been identified as clinically significant.

3.- Several IgE and IgG4 binding sites, have been identified between challenge positive and negative subjects. These epitopes, especially epitope 7 of tropomyosin, could be good candidates to be included in a panel of epitopes to predict clinical reactivity.

4.- Shrimp allergic subjects show more diversity of peptide binding (IgE and IgG4) than shrimp tolerant subjects.

From the second part of this project (paper submitted) we have been able to derive six main conclusions about LTP-syndrome:

1.- A broad diversity of plant-foods are involved besides peach, including plant-foods of the *Rosaceae* family and others taxonomically non-related.

2.- Clinical symptoms are largely diverse. Remarkably, there is a high prevalence of gastrointestinal disorders, the oral allergy syndrome as an isolated presentation is absent and a high frequency of cofactor-dependent anaphylaxis exists.

3.- Anaphylaxis as a single clinical manifestation of the LTP-syndrome is rare. Usually, it is preceded by local symptoms that must be taken into consideration as risk factors to develop anaphylaxis, especially if a cofactor becomes involved.

4.- Association with pollens exists, with a broad spectrum of pollen and pollen-allergen sensitization, especially that of the plane tree

5.- Component-resolved diagnostics, especially in the microarray format, is a valuable tool for unmasking the diagnosis of LTP-syndrome in patients with multiple sensitizations to plant-foods and/or pollens and a complex clinical pattern, with diverse symptoms that resemble those triggered by plant-food allergens other than LTP.

6.- The clinical observation that NSAIDs precipitate or exacerbate allergic symptoms in certain patients with LTP allergy has been reproduced *in vitro* with a basophil activation test model. L-ASA seems to affect somehow the basophil IgE-mediated response. An increase of basophil degranulation is observed when stimulation is performed with L-ASA and Pru p 3 compared to Pru p 3 alone in these patients.

CHAPTER 7. REFERENCES

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CHAPTER 8. SUMMARY IN CATALAN

Reconeixement de proteïnes i epítops al·lergènics en al·lèrgia alimentària: una nova perspectiva per la caracterització clínica i molecular de l'al·lèrgia al marisc i a les proteïnes de transferència de lípids.

1. INTRODUCCIÓ

La resposta al·lèrgica s'origina arrel del reconeixement d'un element innocu com a estrany i/o nociu per part del sistema immunitari de l'individu. Les reaccions al·lèrgiques es defineixen com a reaccions d'hipersensibilitat i en funció del mecanisme implicat es classifiquen en tipus I-IV d'acord amb la classificació de Gell i Coombs (Figura 1.1). L'al·lèrgia mitjançada per IgE és la més freqüent, i en conseqüència la més estudiada, i es classifica com a reacció d'hipersensibilitat de tipus I o immediata [2]. Els antígens en la resposta al·lèrgica reben el nom d'al·lèrgens i es caracteritzen per desencadenar específicament respostes de tipus Th 2 que portaran a la producció d' IgE. Es desconeix quines són les característiques concretes que confereixen a una molècula la seva capacitat al·lergènica [5]. Els al·lèrgens són captats i processats per les cèl·lules dendrítiques i es produeix una diferenciació de les cèl·lules Th0 a cèl·lules Th2, que gràcies al perfil de citocines secretat i altres factors concomitants desencadenaran un canvi d'isotip en el limfòcit B, que començarà a produir i secretar IgE (Figura 1.3) [2]. Aquesta immunoglobulina s'uneix principalment al seu receptor d'alta afinitat (FcεRI, estat de sensibilització) que es troba a la superfície dels mastòcits i els basòfils, cèl·lules claus en la resposta al·lèrgica (Figures 1.2 i 1.4). Quan l'organisme entra de nou en contacte amb l'al·lergen, aquest és reconegut i captat per les IgE de la superfície dels mastòcits i basòfils. La unió de la IgE al corresponent al·lergen suposa l'activació del receptor, que a través d'una complexa cascada de senyalització provocarà l'activació de les cèl·lules (Figures 1.6 i 1.7). L'activació dels mastòcits suposa l'alliberació ràpida de mediadors pro-inflamatoris preformats (e.g., histamina, leucotriens i prostaglandines) que es troben continguts en grànuls dins del seu citoplasma, així com la producció de nous mediadors en les hores que segueixen a l'activació cel·lular. Aquests mediadors són

els responsables de l'aparició immediata de les manifestacions clíniques de l'al·lèrgia (Figura 1.8). Breument, el resultat net del procés d'alliberació de mediadors inclou: en primer lloc, una reacció al·lèrgica immediata (s'inicia en qüestió de segons), principalment deguda a l'activitat de la histamina, prostaglandines i altres mediadors preformats o sintetitzats ràpidament que causen un ràpid increment de la permeabilitat vascular (extravasació plasmàtica, edema tissular) i la contracció del teixit muscular llis. En segon lloc, després de 8-12 hores, es dóna un procés inflamatori més sostingut, conegut com a resposta de fase tardana, que es produeix per la síntesi induïda i posterior alliberació de més mediadors pro inflamatoris: prostaglandines, leucotriens, quimiocines i citocines per part dels mastòcits activats. Aquesta fase tardana implica el reclutament d'altres cèl·lules efectores, principalment limfòcits Th2, eosinòfils i basòfils que contribueixen de forma significativa en la immunopatologia de la resposta al·lèrgica. A més, es dóna una segona fase de contracció del múscul llis per part de les cèl·lules T, amb inflamació persistent, edema sostingut i remodelació tissular. La reacció de fase tardana i la seva seqüela a llarg termini, la inflamació al·lèrgica crònica, contribueixen a una patologia crònica més seriosa, com per exemple, l'asma crònic [6].

La simptomatologia de la reacció al·lèrgica és molt variada i depèn del tipus d'al·lergen i de la seva via d'entrada a l'organisme, la qual condicionarà quins són els mastòcits que s'activaran (mastòcits de teixit connectiu vascularitzat o mastòcits de mucosa). Així doncs, l'activació dels mastòcits de forma generalitzada, amb una alliberació sistèmica d'histamina i altres mediadors, o bé a nivell local, com per exemple a nivell de mucosa respiratòria o digestiva, marcarà la simptomatologia que presenti l'individu (e.g., mucosa digestiva: diarrea, vòmits, etc.; mucosa respiratòria: broncoconstricció i dificultat respiratòria, rinitis, etc.; reaccions generalitzades: urticària amb o sense angioedema i anafilaxis) [5,6].

Les proves diagnòstiques es basen en la detecció de IgE específica per un determinat al·lergen (orientat en funció de la història clínica) a la superfície dels mastòcits (proves cutànies o prick test), lliure en el sèrum (immunoassaig automatitzat ImmunoCAP) o en la superfície dels basòfils (test d'activació dels basòfils). No obstant, aquestes proves només ens indiquen sensibilització del pacient vers a un determinat al·lergen però no ens informen de la seva reactivitat clínica. Així doncs, les proves de provocació, especialment les realitzades a doble-cec i controlades per placebo, són d'elecció per confirmar el diagnòstic de l'al·lèrgia, malgrat el seus

inconvenients logístics i el risc de desencadenar reaccions severes en el pacient [6, 42].

Tradicionalment el diagnòstic de l'al·lèrgia i la immunoteràpia s'ha realitzat utilitzant extractes complets d'aliments difícils d'estandarditzar i que contenen múltiples molècules al·lergèniques i no al·lergèniques. Els extractes ens permeten identificar de forma "grollera" una sensibilització vers a un determinat al·lergen que ens pot explicar la simptomatologia clínica del pacient, però que no ens donen informació de la naturalesa del principal agent responsable, ni ens permeten la previsió de reactivitats creuades amb altres fonts al·lergèniques [5].

L'aplicació dels avenços de les últimes dècades en bioquímica i biologia molecular al camp de l'al·lèrgia, han permès anar més enllà i poder caracteritzar a nivell molecular les proteïnes responsables de la resposta al·lèrgica. Múltiples estudis han permès adoptar el concepte que una font al·lergènica (per exemple en al·lèrgia alimentària, l'aliment) ha de ser interpretada com una mescla de diferents "components" al·lergènics (proteïnes) que a nivell individual poden tenir més o menys rellevància clínica. Així el terme "component" es defineix com l'autèntic agent responsable de la sensibilització i és la base del Diagnòstic Molecular o Diagnòstic Basat en Components (*Component-Resolved Diagnosis*, CRD) que pretén detectar i quantificar els nivells d'anticossos IgE específics per a un determinat component i no per l'extracte complet, proporcionant no només un diagnòstic molt més acurat del pacient al·lèrgic sinó també la possibilitat d'identificar els millors candidats per propòsits terapèutics (disseny d'immunoteràpia al·lergen-específica) [45, 46]. Addicionalment, gràcies a l'aplicació de noves tecnologies com el microarray, s'ha desenvolupat una nova tècnica pel mapatge d'epítops en els al·lèrgens a partir de pèptids seqüencials sintètics que representen tota la seqüència de la proteïna al·lergènica. L'estudi dels epítops B sembla obre noves perspectives pel diagnòstic i tractament de l'al·lèrgia [63, 67].

L'al·lèrgia alimentària es defineix com a un "efecte de salut advers que es desencadena arrel d'una resposta immunològica específica que es dóna de forma reproduïble en l'exposició a un aliment" [19]. La nomenclatura actual proposa que qualsevol reacció adversa a un aliment es defineixi com a hipersensibilitat alimentària, si en el mecanisme intervé un procés immunològic parlem d'al·lèrgia alimentària, la qual al seu torn pot ser classificada com a mitjançada per IgE (més freqüent i millor caracteritzada) o no mitjançada per IgE (especialment descrites a nivell

gastrointestinal) (Figura 1.9) [5]. Essencialment qualsevol aliment pot ser un al·lergen, però els més freqüents són l'ou, la llet, el peix, el marisc i els aliments vegetals (els fruits secs, fruites fresques i verdures) [23]. La sensibilització a proteïnes específiques dels al·lèrgens alimentaris es pot donar a través de la via gastrointestinal (via oral) o com a conseqüència d'una sensibilització al·lèrgica primària a un al·lergen inhalat. Així doncs, es distingeixen dues classes d'al·lèrgia alimentària, en funció del mecanisme immunològic implicat, la via de sensibilització i el patró d'al·lèrgens implicat [27]. En la classe I, la sensibilització es produeix directament a través de la ingesta de l'aliment i els al·lèrgens es caracteritzen per ser glicoproteïnes hidrosolubles molt estables, resistents a la temperatura i a la digestió gàstrica amb capacitat de provocar reaccions al·lèrgiques severes (e.g., proteïnes de la llet, l'ou, la parvalbúmina del peix, la tropomiosina del marisc i les proteïnes de transferència de lípids dels aliments vegetals, etc.). En canvi, en la classe II, la sensibilització es produeix secundària a una sensibilització a un al·lergen inhalat (sensibilització primària) per reconeixement d'epítops comuns entre al·lèrgens. Són al·lèrgens termolàbils i susceptibles a la digestió gàstrica, es caracteritzen per provocar símptomes al·lèrgics restringits a la cavitat oral (e.g., pastanaga, meló o poma crua i el bedoll) [27].

Tot i la gran exposició a proteïnes de la dieta, només un petit percentatge dels individus desenvolupa una al·lèrgia alimentària. Això és gràcies a la tolerància oral que desenvolupa el sistema immunitari vers a les proteïnes de la dieta (estat actiu d'inhibició de les respostes immunològiques a determinats antígens a través de la prèvia exposició a aquest mateix antigen per la via oral). No obstant això, en individus susceptibles, el mecanisme de desenvolupament de la tolerància oral fallaria desencadenant una resposta d'hipersensibilitat als antígens dels aliments ingerits [28].

Desafortunadament, no hi ha cap símptoma patognomònic de l'al·lèrgia alimentària, ja que el pacient pot presentar una àmplia varietat de símptomes al·lèrgica de severitat molt variable, des de molt lleus (o fins i tot imperceptibles com a patològics per part del pacient) fins a la mort [5]. Breument, els símptomes que més s'han associat a les reaccions al·lèrgiques mitjançades per IgE inclouen la pell, els tractes respiratori i/o gastrointestinal, i/o el sistema cardiovascular. Les reaccions poden ser generalitzades (urticària, angioedema, hipotensió, anafilaxis) o restringides a la pell (vermellor, picor, urticaria aguda per contacte), al tracte respiratori (pruïja ocular i llagrimeig, congestió nasal, edema de laringe, sibilàncies, rinoconjuntivitis i broncospasme agut) i/o al tracte gastrointestinal (pruïja labial, lingual i palatal, inflor, edema de laringe, vòmit, dolor abdominal, distensió abdominal i diarrea). Es desconeix

perquè els aliments provoquen diferents símptomes en els individus i perquè la seva severitat pot variar entre episodis [5, 6]. L'anafilaxis és una reacció sistèmica greu d'inici ràpid, que pot comportar la mort, amb una incidència i prevalença en augment. L'únic tractament disponible actualment és l'administració ràpida d'adrenalina a nivell intramuscular [36]. S'han descrit factors concomitants o cofactors, com la presa d'AINEs o l'exercici, que poden modular la severitat de la reacció al·lèrgica a un aliment o fins i tot la poden desencadenar [37]. És a dir, en alguns individus els al·lèrgens alimentaris o la ingesta d'un AINE per si sols no són capaços d'induir una resposta al·lèrgica, però si l'exposició simultània a ambdós. Se sospita que l'AINE "desbloqueja" algun mecanisme de control de la degranulació dels mastòcits i basòfils, però són escassos els estudis que demostrin aquest efecte [38-41].

El diagnòstic de l'al·lèrgia alimentària es basa en una detallada història clínica per identificar els aliments responsables, la severitat dels símptomes, la presència de cofactors, etc.; la detecció de IgE específica (sensibilització) a través de les proves cutànies o lliure en sèrum; i la prova de provocació oral, especialment realitzada a doble cec i controlada per placebo, com a eina d'elecció per a confirmar la reactivitat clínica del pacient [42]. Actualment, no hi ha tractament per l'al·lèrgia alimentària i la dieta d'exclusió, amb totes les seves dificultats i limitacions, és la única intervenció possible [49]. El diagnòstic molecular (tan amb el CRD com el mapatge d'epítops) suposa un notable avenç com a eines pel diagnòstic i desenvolupament d'una teràpia per l'al·lèrgia alimentària [42].

L'al·lèrgia al marisc és l'al·lèrgia alimentària més comuna en adults a Espanya després dels aliments vegetals [74]. El patró de símptomes és molt variat, des de lleus (Síndrome d'Al·lèrgia Oral (SAO) i urticaria), fins a més greus i amb compromís vital (anafilaxis). La majoria d'espècies de marisc que provoquen reaccions al·lèrgiques són crustacis, essent la gamba la més freqüent [73]. La tropomiosina es considera la principal proteïna al·lergènica de la gamba [81], no obstant això en els darrers anys altres al·lèrgens han estat caracteritzats (arginina-quinasa [82-83], cadena lleugera de miosina [84] i proteïna sarcoplàsmica [85]) i altres s'han apuntat com a potencialment rellevants en el diagnòstic i terapèutica de l'al·lèrgia alimentària al marisc (hemocianina, proteïna d'unió a àcids grassos i troponina C [86]). La tropomiosina és el principal panal·lergen del món animal i la sensibilització a aquesta proteïna és la responsable dels fenòmens de reactivitat creuada entre marisc i altres artròpodes (àcars), paràsits nematodes (anisakis) i mol·luscs [79]. Pocs estudis s'han dedicat a

estudiar la rellevància clínica de la sensibilització als diversos al·lèrgens del marisc [88, 89]. L'ús d'al·lèrgens recombinants es considera en aquests moments fonamental pel desenvolupament de noves aproximacions diagnòstiques i estratègies pel disseny d'una immunoteràpia al·lergen específica [79].

Les reaccions al·lèrgiques a aliments vegetals són l'al·lèrgia alimentària més freqüent en adults a Espanya i a tota a l'àrea mediterrània, amb especial rellevància del préssec [74, 108]. El component al·lergènic majoritari d'aquest fruit és el Pru p 3, una proteïna de transferència de lípids, de l'anglès *Lipid Transfer Protein, LTP* [144]. Les LTPs s'han detectat com a proteïnes al·lergèniques en un elevat nombre d'aliments vegetals, particularment de la família *Rosaceae*, però també en altres famílies no taxonòmicament relacionades, pòl·lens i làtex. La distribució ubiqua de les LTP en el regne vegetal és probablement la responsable de l'elevat nombre de reactivitats creuades descrites entre diferents aliments vegetals i pòl·lens [110]. La sensibilització a múltiples aliments és un tret molt comú en els pacients al·lèrgics a les LTP, els quals poden desenvolupar una àmplia diversitat de símptomes amb múltiples aliments vegetals de famílies no necessàriament relacionades, de forma que generalment se'ls diagnostica amb l'anomenat "Síndrome LTP" [113]. Els pacients poden patir símptomes lleus a nivell d'orofaringe, típics del Síndrome d'Al·lèrgia Oral (SAO) generalment causat per una sensibilització a al·lèrgens com les profil·lines o les proteïnes PR-10, o bé reaccions sistèmiques severes, com l'anafilaxis, que fins i tot poden comprometre la vida del pacient, tradicionalment associades a la sensibilització a LTPs o a taumatines entre altres [109]. El fet que la sensibilització a les LTPs pugui presentar-se clínicament d'una forma tant diversa que podria ser associada a diferents al·lèrgens fa que siguin necessàries eines diagnòstiques acurades per determinar exactament a nivell molecular la proteïna responsable de la sensibilització.

2. HIPÒTESI I OBJECTIUS

2.1. Hipòtesi

Tot el que s'ha descrit anteriorment anima a utilitzar panels de molècules al·lergèniques (components) i pèptids sintètics seqüencials per realitzar un anàlisi molecular elaborat dels patrons de sensibilització dels pacients amb al·lèrgia alimentària. La hipòtesi d'aquesta tesi és que el diagnòstic basat en components i el mapatge d'epítops poden millorar la caracterització clínica i molecular de l'al·lèrgia al marisc i del síndrome LTP.

2.2. Objectius

Per tant, l'objectiu global d'aquesta tesi és la caracterització clínica i molecular de l'al·lèrgia al marisc i del síndrome LTP aplicant el diagnòstic basat en components i el mapatge d'epítops.

Objectius específics:

Part 1. Diagnòstic basat en components i reconeixement d'epítops en l'al·lèrgia al marisc

1. Determinar perfils de sensibilització a al·lèrgens del marisc que resultin en reactivitat clínica.

Part 2. Síndrome LTP: patró clínic i perfil de sensibilització molecular d'aliments vegetals i pòl·lens

1. Caracteritzar clínicament el síndrome LTP en els pacients de la nostra àrea.

2. Determinar la utilitat del diagnòstic basat en components en el format de microarray com a eina pel diagnòstic de pacients amb al·lèrgia a múltiples aliments vegetals que presenten una àmplia diversitat de símptomes clínics que no revelen un patró particular de sensibilització

3. MATERIAL I MÈTODES

3.1. Diagnòstic basat en components i reconeixement d'epítops en l'al·lèrgia al marisc

Un total de 86 pacients van ser inclosos en el present estudi. El criteri d'inclusió dels pacients va ser un test positiu a extracte de gamba (IgE específica per ImmunoCAP (Phadia) >0.35 kUA/L i/o prova cutània positiva (Skin Prick Test, SPT)). Els pacients van ser dividits en dos grups en funció de si referien història de reacció al·lèrgica posterior a la ingesta de gamba, i a continuació varen ser sotmesos a una provocació a doble-cec amb gamba (sigles en anglès: DBPCFC) per tal d'avaluar la seva reactivitat clínica. En funció del resultat de la provocació (positiva o negativa) varen ser classificats (Grup 1, 2a, 2b; Figura 4.1.). Una elevada proporció dels pacients sensibilitzats a la gamba, ho estan també als àcars i/o l'escarabat, per tant es va incloure també un grup control d'individus al·lèrgics als àcars i/o escarabat però no sensibilitzats a la gamba (Grup Control 1, n=12). També es va incloure un grup d'individus sans no atòpics com a controls negatius (Grup Control 2, n=5).

Deu proteïnes al·lergèniques recombinants de crustacis varen ser purificades a partir de clons de cDNA expressats en *E. coli* (tropomiosina (TM), cadena lleugera de miosina (*Myosin Light Chain*, MLC), arginina-quinasa (AK), proteïna sarcoplàsmica d'unió de calci (Sarcoplasmic calcium-binding protein, SCP)-alfa i -beta, troponina C (TpC, 3 isoformes diferents: A2, B5 i C10), hemocianina (Hemo) i proteïna d'unió a àcids grassos (*Fatty-Acid Binding Protein*, FABP). Per tal d'avaluar la reactivitat IgE dels individus inclosos en l'estudi per cada un dels al·lèrgens recombinants, aquests es van imprimir sobre un suport de nitrocel·lulosa utilitzant la tècnica de Dot-Blot (Figures 3.10 i 3.11). Addicionalment, sobre un subgrup dels pacients de l'estudi, es va fer un estudi dels llocs d'unió/epítops IgE i IgG4 utilitzant un microarray amb pèptids sintètics solapats de 5 dels al·lèrgens de la gamba estudiats (TM, SCP, MLC, AK i TpC (Figura 3.12).

Les variables quantitatives es van descriure amb medianes, rang i rang interquartil, i les variables qualitatives com a freqüències absolutes en percentatge, utilitzant el software estadístic GraphPad Prism™ version 4.0c for Macintosh (GraphPad Software, Inc. ®, La Jolla, CA, USA). L'anàlisi de les diferències en els nivells IgE entre grups es van analitzar amb el test no paramètric de Kruskal-Wallis. El test de probabilitat de Fisher es va aplicar per analitzar les diferències de

reconeixement IgE dels al·lèrgens recombinants i els epítops. L'anàlisi de les dades del microarray de pèptids es va fer utilitzant l'eina TileMap. Les propietats de les proteïnes recombinants i els epítops com a tests diagnòstics es van avaluar amb els paràmetres de sensibilitat, especificitat, valor predictiu positiu, valor predictiu negatiu i l'eficiència. Els test t no paramètric de Mann Whitney es va utilitzar per la comparació del nombre de pèptids units per IgE, IgG4 i ambdós. Tots els tests aplicats eren de doble cua.

3.2. Síndrome LTP: patró clínic i perfil de sensibilització molecular d'aliments vegetals i pòl·lens

Quaranta-cinc pacients diagnosticats d'al·lèrgia alimentària a múltiples aliments vegetals (involucrant una diversitat significativa de fruites, fruits secs i altres vegetals taxonòmicament relacionats i no relacionats) van ser inclosos en el present estudi per la Unitat d'Al·lèrgia del Servei de Pneumologia i Al·lèrgia de l'Hospital Clínic, després d'obtenir el seu consentiment informat. Tots ells van ser sotmesos a SPT amb extractes comercials dels aliments d'origen vegetal comuns (blat de moro, pell de préssec, avellana, mostassa, enciam, kiwi i llentia) i dels pòl·lens al·lergènics comuns (gramínies, artemísia, parietària, olivera, xiprer i plataner), que són testats de forma rutinària en l'estudi de l'al·lèrgia alimentària a la unitat. A partir de la història clínica, es van recollir els nivells sèrics de IgE total i específica a préssec (extracte complet), i als components: Pru p 3, Pru p 1 i Pru p 4 (LTP, PR-10 i profil·lina del préssec, respectivament) realitzats per ImmunoCAP™ (Phadia®). A tots els individus es va realitzar una detecció de IgE específica front a un panell de 103 components al·lergènics, utilitzant l'immunoassaig comercial en suport de microarray, ImmunoCAP ISAC™ (Phadia®).

Vint-i-quatre pacients amb diagnòstic d'al·lèrgia alimentària al préssec (segons història clínica i SPT i/o IgE específica a Pru p 3 en sèrum positius) que presentaven exacerbació clínica amb la ingesta d'AINE van ser seleccionats per aquest estudi (Grup A). Set pacients també al·lèrgics a préssec, però sense exacerbació clínica amb AINE (Grup B) i 5 individus sans no al·lèrgics (Grup C) també varen ser inclosos. Tots els pacients reclutats eren tolerants als AINEs. A tots ells se'ls va realitzar un Test d'Activació de Basòfils (TAB) a partir de sang fresca utilitzant el kit Flow2CAST™ kit, seguint el protocol del fabricant (Bühlmann®, Schönenbuch, Switzerland). La resposta basofílica va ser avaluada a través de l'expressió del marcador d'activació de basòfils

CD63 per citometria de flux. L'estimulació es va realitzar amb Pru p 3 i L-ASA a diferents concentracions (3mM,1mM, 0,3mM i 0,1mM).

Les variables quantitatives es van descriure amb medianes, rang, rang interquartil, utilitzant el software estadístic GraphPad Prism™ version 4.0c for Macintosh (GraphPad Software, Inc. ®, La Jolla, CA, USA). L'estimació de la proporció de tests positius a pòl·lens en un individu es va analitzar utilitzant models de regressió logística. Es va considerar un Error de Tipus I de 0.05. Pel test d'activació de basòfils, les dades es van analitzar amb el test de Wilcoxon per dades aparellades.

4. RESULTATS

4.1. Diagnòstic basat en components i reconeixement d'epítops en l'al·lèrgia al marisc

4.1.1. Perfil de l'estudi: caracterització dels pacients

Un total de 86 individus amb test positiu a la gamba van ser inclosos a l'estudi. Setanta-quatre (86%) van referir història de reaccions al·lèrgiques immediates al marisc després de la ingesta de la gamba i 12 (14%) no ho van fer. D'aquests 74, 58 (78%) van tenir una reacció en ser provocats amb la gamba a doble cec (**Grup 1**) i 16 (22%) no van reaccionar (**Grup 2a**). Els 12 (14%) individus amb un test a la gamba positiu però sense història de reaccions al·lèrgiques amb la gamba, també varen ser provocats i en tots els casos el resultat va ser negatiu (**Grup 2b**). Tots els resultats negatius a la provocació a doble cec varen ser confirmats amb una provocació oral oberta, que també va ser negativa. Dotze individus sensibilitzats als àcars i/o l'escarabat sense sensibilització a gamba ni història de reaccions al·lèrgiques a la gamba també van ser seleccionats per l'estudi i provocats, amb resultat negatiu en tots els casos (**Grup Control 1**). Es van incloure cinc individus no-atòpics (**Grup Control 2**). El perfil de l'estudi es mostra a la Figura 4.1.

Gènere, edat, freqüència de sensibilització a l'àcar (*Dermatophagoides pteronyssinus*, DP) i l'escarabat, nivells de IgE específica d'aquests al·lèrgens, així com la història de reaccions al·lèrgiques a la gamba i el resultat de la provocació es resumeixen a la Taula 4.1. A la Taula 4.3 es troba una descripció detallada dels pacients.

Amb la comparació de les medianes dels nivells específics de IgE per a cada al·lergen es van mostrar diferencies estadísticament significatives (Taula 4.2). Pel que fa als nivells de IgE específica de la gamba, el grup 1 va presentar nivells més elevats que la resta de grups, mentre que les diferències entre els grups amb provació negativa no van ser significatives. La freqüència de sensibilització a DP fou elevada en tots els grups (93,8-100%), i els nivells de IgE específica similars. La freqüència de sensibilització a l'escarabat fou variable entre els grups i els nivells de IgE específica i les diferències entre grup 1, el 2b i el C1 foren estadísticament significatives. Remarcablement, 2a i 2b no van diferir de C1 en termes de nivells específics de IgE.

4.1.2. Reconeixement IgE dels al·lèrgens recombinants

Les freqüències de la reactivitat IgE per cada al·lergen recombinant per grup es mostren a la Figura 4.2 i Taula 4.4. Es va observar un perfil de reconeixement diferent entre els grups. Els individus del grup 1 mostraren una major diversitat d'al·lèrgens reconeguts (mediana [rang] pel nombre d'al·lèrgens reconeguts per cada grup i els valors de p corresponen a la comparació de la mediana del grup 1 amb cada un dels grups restants; grup 1, 3[0-9]; grup 2a, 1,5 [0-5], $p<0,05$; grup 2b, 2[0-3], no significatiu; grup control 1: 1.5 [0-2], $p<0,01$). Les diferències estadísticament significatives entre grups es mostren a la Taula 4.4.

Alguns individus no van reconèixer cap de les proteïnes recombinants per dot blot (3/58 (5.2%) grup 1; 5/16 (31,3%) grup 2a; 2/12 (16,7%) grup 2b i 4/12 (33,3%) grup control 1). No obstant, alguns d'ells van mostrar reconeixement de proteïnes en extractes complets de gamba crua i bullida per la tècnica de Western blot. Cap dels individus no atòpics (Grup Control 2) va reconèixer cap proteïna recombinant ni cap proteïna dels extractes complets.

4.1.3. Diagnòstic basat en components

4.1.3.1. Al·lèrgens que poden ajudar a diferenciar el resultat de la provocació oral

El reconeixement IgE dels al·lèrgens recombinants SCP-alfa, SCP-beta i TM va ser més freqüent en el grup 1 que en els individus sensibilitzats a la gamba però amb prova de provocació negativa (2a i 2b) (diferència estadísticament significativa). Si es

consideren junts els grups 2a, 2b i C1 per la seva condició de tolerància a la gamba, s'observa que la diferència en freqüència de reconeixement IgE de la proteïna MLC també és estadísticament significativa (Taula 4.4).

Si els grups 2a i 2b es consideren per separat i es comparen amb el grup 1 també s'observen diferències estadísticament significatives. Els grups 2a i 2b només difereixen de forma estadísticament significativa en el reconeixement de l'Hemo (Taula 4.4).

4.1.3.2. L'arginina quinasa i l'hemocianina com a al·lèrgens de reactivitat creuada

En el cas que els individus del grup control 1 reconeguessin algun al·lergen/s recombinant, aquests van ser exclusivament l'Hemo i/o l'AK (6/8 (75%) ambdues proteïnes). Tots els pacients amb provocació negativa junts (2a i 2b) no van mostrar cap diferència estadísticament significativa amb el grup control 1 pel que fa al reconeixement IgE dels al·lèrgens. Considerats per separat, el grup 2b no va presentar cap diferència estadísticament significativa amb el grup control, mentre que en cas de 2a es van observar diferències amb la TM (p=0,0237) i una tendència per l'Hemo (p=0,0497) (Taula 4.4).

4.1.3.3. Diferències menors entre població pediàtrica i adulta

En el grup 1, l'única diferència estadísticament significativa entre el perfil de reconeixement IgE dels nens (n=33) i els adults (n=25) va ser una major freqüència de reconeixement de la SCP-beta en nens (p=0,0128). El mateix anàlisis sobre tots els individus amb provocació negativa (grups 2a, 2b i C1, 10 nens/ 30 adults) no va mostrar cap diferència estadísticament significativa.

4.1.4. Propietats dels al·lèrgens recombinants com a tests diagnòstics

Les propietats dels al·lèrgens recombinants i del l'extracte complet de gamba com a tests diagnòstics es van descriure utilitzant sensibilitat, especificitat, valor predictiu positiu (PPV), valor predictiu negatiu (NPV) i eficiència. Els resultats es mostren a la Taula 4.5. Només els al·lèrgens que mostren diferències de

reconeixement IgE estadísticament significatives entre individus de provocació positiva i negativa (valor p test de Fisher < 0,05: SCP-alfa, -beta, TM, MLC i extracte complet de gamba) poden ser considerats com a candidats per un test diagnòstic capaç de diferenciar el resultat d'una provocació.

La TM va mostrar valors elevats pels 5 paràmetres, mentre que les SCPs van mostrar valors molt elevats per especificitat (~ 95%) i PPV (97.5%), però baixos per sensibilitat (29-35%) i NPV (~ 50%). De forma similar, per la MLC es van observar valors alts de especificitat (87.5%) i PPV (81.5%), però baixos de sensibilitat (38%) i NPV (49%). L'extracte complet de gamba va ser el més sensible (98%) i amb el valor de NPV més alt (95%), però amb la especificitat més baixa (49%) i PPV (74%). L'eficiència del les SCPs i la MLC fou entorn del 60%, menor que l'extracte de gamba (78%) i la TM (87%).

4.1.5. Mapatge d'epítops: llocs d'unió IgE i IgG4

El reconeixement IgE i IgG4 d'un subgrup dels individus inclosos a l'estudi (grup 1, n=21; grup 2a, n=16, grup 2b, n=12) es va analitzar sobre un panel de pèptids sintètics solapats que representen tota la seqüència de cinc al·lèrgens de la gamba (TM, SCP, AK, MLC i TpC) per la tècnica de microarray (Figures 4.3 i 4.4)

4.1.6. Llocs d'unió diferencials entre grups

L'eina d'anàlisi TileMap es va utilitzar per identificar els llocs d'unió IgE i IgG4 amb diferències estadísticament significatives entre grups (1 versus 2(2a+2b), 1 versus 2a, 1 versus 2b, i 2b versus 2a). La Figura 4.5 i la Taula 4.6 resumeixen els resultats de la comparació.

Breument, s'observa que els llocs d'unió IgE i IgG4 coincideixen de forma notable al llarg de la seqüència de les proteïnes. Es van observar diferències menors entre 2b i 2a; per aquest motiu, els individus amb provocació negativa es van agrupar (grup 2 =2a+2b) per comparar els resultats amb el grup 1 i per posteriors anàlisis. Per IgE, el nombre més elevat de llocs d'unió diferencials per IgE va ser en el cas de la TM, mentre que per IgG4, això mateix es va observar per AK. En el cas de la SCP, les diferències es van observar bàsicament per la IgG4. Pràcticament no es va observar cap diferència per la TpC.

4.1.7. Diversitat de pèptids units per IgE i IgG4

El nombre de pèptids units per IgE, IgG4 o ambdós per cada al·lergen fou comparat entre grups (al·lèrgics (1) versus tolerants (2=2a+2b)) (Taula 4.7). La IgE en el grup 1 va unir més pèptids que el grup 2 per tots 5 al·lèrgens. El mateix es va observar pels pèptids units per IgE i IgG4. Per IgG4, la diferència en diversitat d'unió només va ser estadísticament significativa per la TM, MLC i AK.

En cap dels grups no es va observar cap correlació entre el reconeixement IgE i IgG4 (Figura 4.6).

4.1.8. Comparació dels resultats actuals amb estudis previs

El llocs d'unió IgE identificats en el present estudi es van comparar amb els epítops descrits anteriorment [90] i amb els epítops definits com a clínicament rellevants en una publicació recent del nostre grup [91] (Taula 4.8). L'estudi de la IgG4 no s'havia realitzat anteriorment.

Pràcticament tots els llocs d'unió identificats en el present estudi es corresponen amb epítops descrits anteriorment, només per l'AK i la SCP, alguns llocs identificats no corresponen a cap epítop ja descrit.

Un estudi recent del nostre grup va identificar alguns epítops com a clínicament rellevants (per AK, epítops 6 7; per MLC, epítops 1, 2, 4a, 4b, 5; per SCP, epítop 1; per TM, epítops 1, 2, 5a, 5b, 5c i 7), realitzant el mateix tipus d'anàlisi TileMap però amb una mostra més reduïda de pacients, sobretot el grup de provocació negativa o tolerants (al·lèrgics, n=15; tolerants, n=11). Els epítops/ llocs d'unió amb diferències estadísticament significatives entre grups en el present estudi es mostren a la Taula 4.8. Les coincidències amb la publicació pilot anterior del nostre grup (Ayuso R et al. CEA 2011 [91]) es marquen amb asteriscs a la taula.

4.1.9. Propietats dels epítops com a tests diagnòstics

Les freqüències de reconeixement IgE per cada epítop/lloc d'unió per grup i les propietats dels epítops com a tests diagnòstics (sensibilitat, especificitat, valor predictiu positiu (PPV), valor predictiu negatiu (NPV) i eficiència) es mostren a la Taula 4.9. L'anàlisi estadístic de les freqüències de reconeixement IgE de cada grup per als epítops individuals va mostrar que els individus amb provocació positiva reconeixen de

forma més freqüent tots els epítops que els de provocació negativa, a excepció de l'epítop 2 de la MLC (p=0,146) (Taula 4.9). Per tant, aquest epítop va ser exclòs de l'anàlisi de les propietats dels epítops com a tests diagnòstics.

Els epítops de TM van mostrar una alta especificitat (>90%, epítops 1, 5a, 5b i 6, >75%, epítops 3 i 7). L'epítop n de la SCP també va mostrar una especificitat molt alta, però per la resta dels epítops el valor d'aquest paràmetre va oscil·lar entre el 57 i el 71%. De forma similar, els valors més elevats de PPV es van trobar per la TM i la SCP (>90%, epítops 1 i 5b de TM; >75%, epítops 3, 5a, 5b, 6 i 7 per TM i epítop n per SCP). La sensibilitat fou molt variable entre els epítops de la TM, mentre que l'epítop 7 va mostrar el valor més elevat de tots, la resta presentaren valors entre 38 i 71%. De forma similar, la sensibilitat per l'epítop 7 de l'AK va ser del 81%, però pels altres 2 epítops del mateix al·lergen, els valors foren inferiors. L'epítop n de la SCP va mostrar el valor més baix de sensibilitat (33%). En paral·lel, el valor més elevat de NPV es va observar per alguns epítops de TM (76-88%) i l'epítop 7 de l'AK (80%). Es va observar que els epítops de TM tenen valors d'eficiència més elevats (76-82%) que els epítops de la resta d'al·lèrgens (63-69%). L'epítop 7 de la TM fou l'únic amb valors elevats pels 5 paràmetres.

4.2. Síndrome LTP: patró clínic i perfil de sensibilització molecular d'aliments vegetals i pòl·lens

4.2.1. Demografia i aliments vegetals responsables

Els 45 pacients seleccionats per a l'estudi eren majoritàriament adults (mediana [rang]: 33 [14-47] anys), 28 dones (62,2%), tots prèviament diagnosticats d'al·lèrgia alimentària a múltiples aliments vegetals per sensibilització a LTP. Els aliments responsables de les reaccions (recollits en la història clínica i amb sensibilització confirmada) eren fruites fresques, vegetals i fruits secs, relacionats o no taxonòmicament (nombre d'aliments vegetals causants de reacció en cada individu, mediana [rang]: 4 [2-18]). En alguns casos, un mateix aliment va ser referit com a responsable de reaccions de variable severitat en diferents episodis (només les reaccions més severes es descriuen a la Taula 4.10).

4.2.2. Símptomes clínics

La revisió de les històries clíniques dels pacients va mostrar un patró de simptomatologia molt heterogeni (Síndrome d'al·lèrgia oral (SAO) (34/45, 75,6%), urticaria (30/45, 66,7%), urticària de contacte (5/45, 11.1%), trastorns gastrointestinals (25/45, 55,6%) i anafilaxis (34/45, 75,6%). La majoria dels pacients van reportar múltiples símptomes en funció de l'aliment implicat. En el nostre grup de pacients no es van detectar episodis aïllats de SAO. Només un pacient va patir episodis repetits d'anafilaxi amb diferents elements vegetals implicats. Alguns pacients van referir episodis aguts d'urticària, trastorns gastrointestinals o anafilaxis suggestiva d'al·lèrgia alimentària però no van ser capaços d'identificar l'aliment responsable (Taules 4.10 i 4.11).

4.2.3. Efecte cofactor

Dels 34 pacients amb anafilaxis, 17 (50%) no varen descriure un cofactor (AINE i/o exercici) implicat en la reacció, 6 (17,7%) varen patir l'anafilaxis amb i sense cofactor, i 11 (32,4%) només en presència del cofactor. En aquests pacients, la presència del cofactor va representar una exacerbació dels símptomes, des de SAO i/o trastorns gastrointestinals a anafilaxis (Taula 4.11). En l'anafilaxi depenent de cofactor fou molt difícil identificar l'aliment responsable a través de la història clínica (Taula 4.10).

4.2.4. Sensibilització a aliments vegetals

Els pacients van mostrar sensibilització a pràcticament tots els aliments vegetals inclosos en els SPT (mediana [rang]: 5 [1-7] extractes positius). Addicionalment es van detectar altres sensibilitzacions quan en les proves cutànies es van incloure altres aliments vegetals que els pacients referien de forma individual, per tal de confirmar la sensibilització. A tots els pacients que se'ls va realitzar el SPT (42/45, 93,3%) van tenir una reacció positiva a la pell de préssec, excepte un (41/42, 97,6%); 35 (83,3%) foren positius per blat de moro, 33 (78,6%) per avellana, 32 (76,2%) per enciam, 29 (69,1%) per mostassa, 18 (42,9%) per kiwi, i 12 (28,6%) per blat (Taula 4.10).

La sensibilització detectada per IgE específica en sèrum va ser: 40/40, 100%; mediana [rang] kU_A/L: 3,49 [0,38-43,1] per l'extracte complet de préssec, 44/45 (97,8%) pel Pru p 3 (5,03 [0,11-36,7]), 0 [0-0,11] per Pru p 1 i 0 [0-0,07] per Pru p 4 (ImmunoCAP Phadia). La detecció de IgE específica a rTri a 14 per ELISA també va ser positiva per tots els pacients analitzats (21/21, 100%) (Taula 4.12). Els resultats del microarray es mostren a la Figura 4.7. Es va observar sensibilització a totes les LTPs incloses en el microarray (Pru p 3, Art v 3, Cor a 8 i Par j 2); la distribució de freqüències es mostra a la Taula 4.13. No es va detectar sensibilització a cap altra panal·lergen vegetal inclòs en el microarray.

4.2.5. Sensibilització a pòl·lens

Trenta-quatre (75,6%) individus també foren diagnosticats de pol·linosi, mentre que tots presentaven rinitis, en 14 casos (31,1%) associada amb asma (Taula 4.10). Les freqüències de sensibilització a pol·len detectades amb cada mètode es resumeixen a la Taula 4.13. Una descripció detallada de cada individu es mostra a la Taula 4.14. Per SPT i/o IgE específica en sèrum, la sensibilització a alguns dels pòl·lens testats que contenen una LTP coneguda (artemisa, parietària, plàtan d'ombra, xiprer i olivera) es va detectar en 43 (95,6%) individus. La sensibilització al plàtan d'ombra es va detectar en 42 (93,3%) pacients, a l'artemísia en 33 (73,3%), a la parietària en 16 (35,6%), a l'olivera en 14 (31,1%) i al xiprer en 12 (26,7%).

La proporció de pacients positius al plàtan d'ombra (0,91, 95% CI [0,83-0,99]) i artemísia (0,69, 95% CI [0,55-0,82]) fou superior a la de la resta de pòl·lens analitzats (xiprer, olivera, parietària i gramínies) i aquesta diferència fou estadísticament significativa (Figura 4.8).

4.2.6. Efecte dels AINEs en la degranulació del basòfil *in vitro*

En el grup A s'observa un increment estadísticament significatiu de l'activació dels basòfils quan l'estimulació es realitza conjuntament amb Pru p 3 i L-ASA a tres de les concentracions testades (3 mM, 0,3 mM i 0,1 mM) comparat amb l'activació induïda per Pru p 3 sol (Test de Wilcoxon Dades Aparellades: a 3 mM, $p<0,0001$; a 0,3 mM, $p=0,0117$ i a 0,1mM $p=0,0085$). Aquests efectes no van ser observats en el grup B ni en el control (grup C). Curiosament en els pacients del grup A la combinació de Pru p 3 i L-ASA a una concentració 1mM no dóna aquest efecte sinèrgic (Figura 4.9).

5. DISCUSSIÓ

5.1. Diagnòstic basat en components i reconeixement d'epítops en l'al·lèrgia al marisc

Actualment els mètodes diagnòstics per a l'al·lèrgia al marisc no són capaços de predir la reactivitat clínica dels pacients sensibilitzats [42, 79]. Pocs estudis s'han dedicat a analitzar el perfil de sensibilització dels pacients amb reactivitat clínica (al·lèrgics) i els que no la tenen (tolerants) [88, 89]. En el present estudi hem pogut observar que el reconeixement IgE dels al·lèrgens recombinants tropomiosina (en especial), proteïna sarcoplàsmica i cadena lleugera de miosina és més freqüent en els individus al·lèrgics que en els tolerants. Així doncs, podrien ser utilitzats per identificar els pacients realment al·lèrgics a la gamba entre tots els que tenen una prova diagnòstica positiva (test cutani i/o IgE específica). A més, semblaria que els al·lèrgens, arginina quinasa i hemocianina, tindrien un paper important com a molècules de reactivitat creuada entre els artròpodes estudiats (gamba-àcars/escarabat), un rol que tradicionalment ha estat atribuït a l'al·lergen tropomiosina [133]. Addicionalment, la identificació de llocs d'unió o epítops diferencials per IgE i IgG4 (en especial l'epítop 7 de la tropomiosina), així com la detecció d'una major diversitat de reconeixement d'epítops per part dels individus al·lèrgics respecte els tolerants, que coincideixen notablement amb els identificats en un estudi pilot del nostre grup [91], obre noves vies al desenvolupament de nous candidats per a tests diagnòstics i estratègies terapèutiques.

Així doncs, la incorporació d'aquests components al·lergènics i alguns dels seus epítops en les proves diagnòstiques de rutina, actualment només disponible l'extracte complet de la gamba o la tropomiosina, podria minimitzar la necessitat de realitzar una provocació oral del pacient per confirmar la seva reactivitat clínica i evitar els problemes associats a aquesta.

5.2. Síndrome LTP: patró clínic i perfil de sensibilització molecular d'aliments vegetals i pòl·len

En el nostre grup de pacients s'han detectat múltiples aliments vegetals responsables de les reaccions a part del préssec, alguns pertanyents a la família *Rosaceae* i d'altres no relacionats taxonòmicament amb aquesta família. Sembla que

la distribució ubiqua de les LTP en el regne vegetal és probablement la responsable de l'elevat nombre de reactivitats creuades descrites entre diferents aliments vegetals i pòl·lens [110].

Les manifestacions clíniques presentades pels nostres pacients han sigut molt variades i de severitat variable en funció de l'episodi o bé de l'aliment implicat. Remarcablement, la incidència de trastorns gastrointestinals ha sigut elevada. No s'han pogut observar episodis aïllats de Síndrome d'Al·lèrgia Oral (SAO) amb símptomes lleus restringits a nivell d'orofaringe, sinó que generalment aquests s'han acompanyat d'altres manifestacions. Rarament l'anafilaxis s'ha observat com a manifestació aïllada i habitualment aquesta és precedida per símptomes locals, els quals s'han de considerar com a factor de risc pel desenvolupament d'un episodi d'anafilaxis, especialment en presència d'un cofactor. El percentatge elevat de casos en què els pacients pateixen un episodi d'anafilaxis només en presència del cofactor (antiinflamatoris no esteroides (AINE) i/o exercici) posa de manifest que aquest fenomen àmpliament descrit amb altres al·lèrgens com la gliadina, al·lergen del blat, també s'observa en el síndrome LTP [37]. El model del test d'activació de basòfils descrit en aquest projecte podria ser utilitzat per demostrar *in vitro* l'efecte clínic observat *in vivo* i poder estudiar els mecanismes implicats que permetrien que l'AINE moduli la resposta basofílica IgE-mitjançada i explicar l'exacerbació clínica dels pacients.

El patró complex de manifestacions clíniques i la diversitat d'aliments vegetals implicats dificulta en un primer moment el diagnòstic del síndrome LTP. Per exemple, els símptomes a nivell de mucosa oral podrien ser causats per altres al·lèrgens vegetals de distribució ubiqua en el regne vegetal com les profil·lines o les proteïnes PR-10; o bé reaccions sistèmiques severes que fins i tot poden comprometre la vida del pacient, podrien ser donades per al·lèrgens com les taumatines o components específics dels aliments vegetals [111-113]. Aquest fet fa que siguin necessàries eines diagnòstiques acurades per determinar exactament a nivell molecular la proteïna responsable de la sensibilització. El Diagnòstic Molecular o Diagnòstic Basat en Components (*Component-Resolved Diagnostics*, CRD) permet identificar l'autèntic agent responsable de la reacció al·lèrgica (component) i per tant, permet un diagnòstic molt més precís del pacient [46]. A més, la incorporació de la tecnologia del microarray en aquest camp, suposa un gran avenç per la detecció en paral·lel de diferents especificitats IgE [59]. Ens ofereix un panorama de les sensibilitzacions positives i negatives de l'individu, possibilitant un diagnòstic més acurat que utilitzant les

tècniques diagnòstiques convencionals (SPT, ImmunoCAP), sempre que els components estiguin adequadament validats [60]. En el cas particular del Síndrome-LTP, el CRD en suport de microarray és especialment útil per identificar aquells pacients que malgrat presentar només una simptomatologia lleu, típica de la sensibilització a altres panal·lergens dels aliments d'origen vegetal com les profil·lines i les proteïnes PR-10, estan en realitat sensibilitzats a les LTP i per tant es troben en risc de que patir una reacció sistèmica severa, com l'anafilàxia, després de la ingesta d'un aliment vegetal, i en major grau si la ingesta es combina amb un cofactor.

6. CONCLUSIONS

De la primera part d'aquesta tesi (publicació en procés) s'han pogut derivar quatre conclusions principals:

1.- La tropomiosina (en particular), la proteïna sarcoplàsmica i la cadena lleugera de miosina són al·lèrgens associats a la reactivitat clínica, per tant poden ser bons candidats a tests diagnòstics de l'al·lèrgia alimentària a la gamba.

2.- L'arginina quinasa i l'hemocianina són importants al·lèrgens de reactivitat creuada entre la gamba, els àcars i l'escarabat. Tot i això, per a l'arginina quinasa s'han detectat epítops clínicament rellevants.

3.- S'observen diferències en els llocs d'unió/ epítops IgE i IgG4 entre els individus al·lèrgics i tolerants a la gamba. Aquests epítops, especialment l'epítop 7 de la tropomiosina, podrien ser bons candidats per a un panel d'epítops pel diagnòstic de l'al·lèrgia alimentària a la gamba.

4.- Els individus al·lèrgics a la gamba mostren una major diversitat de reconeixement IgE i Ig4 de pèptids seqüencials que els individus tolerants.

De la segona part d'aquesta tesi (paper en procés de revisió) hem pogut obtenir sis conclusions sobre el síndrome LTP:

1.- Una gran diversitat d'aliments vegetals estan implicats a part del préssec, alguns de la mateixa família *Rosaceae* i altres no taxonòmicament relacionats.

2.- Els símptomes clínics són molt diversos. Remarcablement, hi ha una elevada prevalença de trastorns gastrointestinals, el síndrome d'al·lèrgia oral no es presenta de forma aïllada i s'observa una elevada freqüència d'anafilàxia depenent de cofactor.

3.- L'anafilàxia com a manifestació clínica aïllada és molt poc freqüent. Normalment aquesta va precedida de símptomes locals que s'han d'interpretar com a factors de risc pel seu desenvolupament, especialment en presència d'un cofactor.

4.- L'associació amb pòl·lens existeix, amb un ampli espectre de pòl·lens implicats, especialment el plàtan d'ombra.

5.- El diagnòstic basat en components, especialment en el suport de microarray, és una eina útil per desemmascarar el síndrome LTP en pacients amb múltiples sensibilitzacions a aliments vegetals i/o pòl·lens i un patró complex de símptomes que coincideixen amb aquells típicament causats per altres al·lèrgens que no són les LTP.

6.- L'observació clínica que els AINEs precipiten o exacerben els símptomes al·lèrgics en alguns pacients amb al·lèrgia a les LTP s'ha pogut reproduir *in vitro* en un model de test d'activació de basòfils. L-ASA afectaria d'alguna forma la resposta IgE mitjançada, ja que s'observa un increment de la degranulació del basòfil quan l'estimulació es realitza amb L-ASA i Pru p 3 comparat amb Pru p 3 sol en aquests pacients.

APPENDIX A. PUBLICATIONS

APPENDIX A. PUBLICATIONS

The paper reporting the first part of this work showing the recombinant allergens and epitopes that are associated with clinical reactivity is currently in progress.

The second part of this work about the characterization of the clinical pattern and molecular sensitization profile to plant-foods and pollens in the LTP syndrome is currently under peer-review by Clinical Experimental Allergy journal.

Lipid Transfer Protein syndrome: clinical pattern and molecular sensitization profile to plant-foods and pollens

Short Title: LTP syndrome: Clinical pattern and sensitization profile

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