UNIVERSITAT DE BARCELONA

New aproaches for *in vitro* diagnosis of LTP Syndrome

Noves aproximacions en el diagnòstic *in vitro* de la Síndrome LTP

Sara Balsells Vives

New approaches for *in vitro* diagnosis of LTP Syndrome

Noves aproximacions en el diagnòstic *in vitro* de la Síndrome LTP

Memòria de tesi doctoral presentada per **Sara Balsells Vives** per optar al grau de doctor/a per la Universitat de Barcelona

DIRIGIDA PER:

Dra. Mariona Pascal Capdevila
Servei d'Immunologia, Centre de Diagnòstic Biomèdic (CDB), Hospital Clínic de Barcelona; Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS); Departament de Medicina, Facultat de Medicina i Ciències de la Salut, Campus Clínic, Universitat de Barcelona.

Dr. Joan Bartra Tomàs
Servei d'Al·lergologia, Institut Clínic Respiratori (ICR), Hospital Clínic de Barcelona; Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS); Departament de Medicina, Facultat de Medicina i Ciències de la Salut, Campus Clínic, Universitat de Barcelona.

Programa de Doctorat en Medicina i Recerca Translacional
Facultat de Medicina i Ciències de la Salut. Universitat de Barcelona
SETEMBRE 2023

Agraïments

En primer lloc, voldria donar les gràcies a la Dra. Mariona Pascal i al Dr. Joan Bartra, per donar-me aquesta oportunitat. Especialment a la Mariona per confiar en mi quan ens vam conèixer. Per tenir paciència en els meus moments personals més difícils. Gràcies també per exigir-me sempre la perfecció i ensenyar-me tantes coses al llarg d'aquests anys. Durant aquests anys he après molt: m'ha fet fet més forta i més bona professional i persona.

Moltes gràcies a totes les persones que formen el Servei d'immunologia de l'Hospital Clínic de Barcelona. Als tècnics, facultatius i administratius. A la Maria i a la Fina, per ensenyar-me les tècniques d'experimentació que he hagut de desenvolupar i també per ajudar-me i treure'm feina quan m'ha fet falta (tot i que no us en faltava). I als residents i doctorands, per compartir aquests anys. A l'Albert per aportar sempre alegria, humor i ajudar-me en temes informàtics. A l'Àlex per les estones de descans i desconnexió. A la Clara, per compartir projectes. A l'Ana Esteve i a la Raquel, per ser per mi un model a seguir en aquest camí. A l'Anna, la Marta, l'Iñaki i la Natàlia, per la seva alegria, bondat i simpatia. A la Montse Riego per escoltar-me sempre tant. També a tot l'equip del Servei d'Al·lergologia de l'Hospital Clínic i companys del grup de Recerca d'Immunoal·lèrgia Respiratòria Clínica i Experimental de l'IDIBAPS. A la Dra. Rosa Muñoz, per fer-ho tot més fàcil. A la Giovanna A., a la Rocío i a la Sílvia Q. per tantes i tantes hores juntes, citant pacients i fent tests cutanis. A la Gemma López, per ser la meva infermera i ajudar-me en l'extracció de mostres.

També vull donar les gràcies als companys del Servei de Farmàcia. Companys que han esdevingut amics, i fan que vagi a treballar cada dia amb il·lusió. A la Dolors Soy per incorporar-me a l'equip i tenir-me confiança plena. A la Bego per ser una "jefa" amb molt de cor. A la Patri, que viu en primera persona la vida d'un "LTPero". A la Marta, la Maria, la Cris, la Montse, el Kevin, per ser el millor equip de tardes amb qui podria treballar. A la Sandra i a la Judith, les farmacèutiques veteranes, a l'Eduardo i als tècnics (Yolanda, David, Eli, Marta) de qui he pogut aprendre, especialment quan vaig començar.

Diu un proverbi que "Si vols anar de pressa, ves sol; però si vols anar lluny, ves acompanyat". Per sort, tinc un exèrcit de persones estimades que m'ha acompanyat:

Edu, amor, et mereixes tota la felicitat del món. Sense el teu suport (emocional, econòmic i incondicional) jo no hauria arribat fins el final. Gràcies. Aquesta tesis és també la teva tesis. Al Guim i la Clara, la meva felicitat. El vostre somriure m'alegra els dies. Als meus pares, per intentar aconsellar-me i guiar-me sempre. Gràcies mamà per escoltar-me hores i hores al telèfon, la teva bondat et fa ser un model a seguir. Gràcies papà, per exigir-me sempre una mica més, i treure el millor de mi. Als meus germans: al Bernat i la Maria, per aconsellar-me i aportar-me noves idees; al Marc, per compartir la passió per la ciència i interessar-se en cada detall del projecte; al Jordi, per estar sempre pendent de mi i per les teves abraçades. Gràcies a la meva família i també als meus cosins i tiets: *la meva tribu*. Pel seu suport, pel seu interès i les seves bones intencions. Per ajudar-me a veure les coses d'una manera més càlida i aportar sempre un toc d'humor a les situacions complicades. Molt especialment a tu, Victor. Sense tu no hauria estudiat Farmàcia i probablement, no hauria començat el doctorat. Gràcies per orientar-me, aconsellar-me i donar-me la teva opinió. Per obligar-me a fer tonteries i a mirar la vida amb un punt d'ironia. Gràcies per ajudar-me encara ara, des d'allà on siguis. T'estimo i et trobo molt a faltar.

Gràcies a les meves amigues, la meva segona família. Diuen que qui té un amic, té un tresor, i jo soc molt afortunada. A les de la universitat: Estela, Helena, Emma, Cris, Guillem, Alba, Ona, Silvia, Alèxia, Isa, Miguel i Anna. Especialment gràcies a l'Helena per ajudar-me amb la teva experiència en el doctorat i per compartir amb mi estones de filosofia i salut. I a tu, Estela, per ser-hi absolutament sempre. Emma, Ona, Cris: gràcies per cuidar-me des de la distància. A les del Montclar. Sobretot a la Cris, per ajudar-me amb les teves idees de disseny gràfic i infografia. Gràcies també, per ser-hi com a amiga. A la Sònia; a l'Alba, per no deixar mai de fer de psicòloga i molt especialment a la Maria i les llargues hores debatent sobre ciència i el món en el que vivim.

A tots, moltes gràcies.

List of Contents

Agraïments I

List of Contents III

List of Acronyms and Abbreviations V

List of Figures VIII

List of Tables X

Published content XII

Noves aproximacions en el diagnòstic *in vitro* de la Síndrome LTP XIII

Chapter I: Introduction 1

1.1 Allergy 2

1.1.1 Hypersensitivity disorders 2

1.1.2 Antigen 3

1.1.3 Physiopathology 4

1.2 Food allergy 6

1.2.1 Epidemiology 6

1.2.2 Sensitization routes 7

1.2.3 Clinical Manifestations 8

1.2.4 Food Allergens 10

1.2.5 Treatment 14

1.2.6 Diagnostic 16

1.2.7 Molecular Allergy 24

1.3 LTP Syndrome 33

1.3.1 Location and function 33

1.3.2 Structural characteristics 33

1.3.3 LTPs Allergenicity and LTP Syndrome 34

1.3.4 Geographical distribution and prevalence 36

1.3.5 Pollen influence on LTP sensitization 37

1.3.6 Clinical manifestations 38

Chapter II: Hypothesis 43

Chapter III: Objectives 45

Chapter IV: Material and methods 47

4.1 Patient selection 48

4.2 Clinical characterization 49

4.3 Skin prick tests 57

4.4 Serum allergen sIgE immunoassays 59

4.5 Basophil activation test (BAT) 63

4.6 Statistical analysis 65

Chapter V: Results 67

5.1 Clinical relevance of low Pru p 3 sIgE in patients with peach allergy 68

5.2 Molecular sensitization profiles detection on LTP sensitized patients 77

5.3 Improving *in vitro* detection of sensitization to Lipid Transfer Proteins: LTP Strip, a new molecular multiplex IgE assay 90

5.4 Characterization of a LTP syndrome cohort with the LTP-strip 114

Chapter VI: Discussion 121

Chapter VII: Conclusions 132

Bibliography 135

Annexes 164

List of Acronyms and Abbreviations

AIT	Allergen-specific immunotherapy
ALEX	Allergy Explorer® (Macroarray Diagnostics)
AN	Anaphylaxis
AP	Alkaline phosphatase
APCs	Antigen presenting cells
BR	Basophil reactivity
BS	Basophil sensitivity
B reg	Regulatory B cell
CCDs	Cross-reactive carbohydrate determinants
CCL3	Chemokine C-C motif ligand 3
CEFA	Cofactor-enhanced food allergy
CLA	Cutaneous lymphocyte-associated antigen
CMOS	Complementary metal oxide semiconductor
CRD	Component-resolved diagnosis
CSBP	Cytokinin-specific binding proteins
CU	Contact Urticaria
DBPCFC	Double-blind placebo-controlled food challenge
DC	Dendritic cell
EAACI	European Academy of Allergy and Clinical Immunology
EAST	Enzyme-Allergo-Sorbent Test
EMA	European Medicines Agency
EoE	Eosinophilic esophagitis
EPIT	Epicutaneous immunotherapy
FA	Food allergy
FAIT	Food allergen-specific immunotherapy
Fc ε RI	High-affinity receptor for the Fc region of IgE
FDA	Food and Drug Administration
FDEIA	Food-dependent exercise-induced anaphylaxis
FDNIA	Food-dependent NSAID-induced anaphylaxis
fMLP	N-formilmetionil-leucil-fenilalanina
FPIES	Food-Protein-induced enterocolitis syndrome
GERD	Gastroesophageal reflux disease
GI	Gastrointestinal symptoms

GRP	Gibberellin-regulated protein
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
IBS	Irritable bowel syndrome
ICAM-1	Intercellular adhesion molecule 1
IgE	Immunoglobulin E
IgG4	Immunoglobulin G subclass 4
ILC	Innate lymphoid cells
IL	Interleukin
iNKT	Invariant natural killer T
ISAC	ImmunoSorbent Allergen Chip® (Thermofisher Scientific)
ISU	ISAC standardized units
ITAMs	Immunoreceptor tyrosine-based activation motif
IUIS	International Union of Immunological Societies
LoB	Limit of Blank
LoD	Limit of Detection
LoQ	Limit of Quantitation
LR	Likelihood ratios
LTCCs	Dihydropyridine-sensitive L-type Ca2+ channels
LT	Leukotrienes
MAST	Multiple allergen simultaneous tests
MAT	Mast-cell activation test
MC	Mast cell
MHC	Major histocompatibility complex
MIA	Microarray Image Analysis
MLP	Major latex proteins
NK	Natural killer cells
NMR	Nuclear Magnetic Resonance
NPV	Negative predictive value
NSAIDs	Non-steroidal anti-inflammatory drugs
nsLTP	Non-specific lipid transfer proteins
OAS	Oral Allergy Syndrome
OFC	Oral Food Challenge
OIT	Oral immunotherapy
PAF	Platelet-activating factor
PBMCs	Peripheral blood mononuclear cells
PGD2	Prostaglandin D2

PPV	Positive predictive value
PR	Pathogenesis-related proteins
RAST	Radio-allergo-sorbent test
ROC	Receiver operating characteristics
RRP	Ripening-related proteins
RT	Room temperature
SCIT	Subcutaneous immunotherapy
SH	Shock (Anaphylactic)
sIgE	Specific IgE
SLIT	Sublingual immunotherapy
SPT	Skin prick test
TFH	Follicular helper T cells
TGF-β	Transforming growth factor beta
T_H0	Naïve T helper cells
T_H2	Type 2 helper T cell
TLP	Thaumatin-like proteins
TNF	Tumour necrosis factor
T reg	Regulatory T cells
TSLP	Thymic stromal lymphopoietin
U/AE	Generalized Urticaria and/or Angioedema
WAO	World Allergy Organization
WDEIA	Wheat-dependent exercise-induced anaphylaxis
WHO	World Health Organization

List of Figures

CHAPTER I: INTRODUCTION

Figure 1. Allergic response physiopathology. 5

Figure 2. Diagnostic work-out for IgE mediated/type I hypersensitivity reactions. 17

Figure 3. General scheme of immunoassays used for the measurement of IgE in serum. 20

Figure 4. Euroline® multiparameter assay principles and strip representation. 28

Figure 5. Universal arguments for the methodological use of components. 31

Figure 6. Crystal structure of Pru p 3, the LTP from peach. 34

Figure 7. Effects of Pru p 3 sensitization on mast cell activation. 39

CHAPTER VI: MATERIAL AND METHODS

Figure 8. Anamnestic data questionnaire for the cluster identification study. 50

Figure 9. Anamnestic data questionnaire used for the LTP-strip validation study 51

Figure 10. Fresh food cut and distributed in cryotubes before frosting. 57

Figure 11. Prick by Prick performance. 57

Figure 12. Guide tree based on amino acid sequence data. 60

Figure 13. EUROIMMUN-LTP strips performance 62

Figure 14. Evaluation protocol sheet. 63

Figure 15. BAT performance in our laboratory 65

CHAPTER V: RESULTS

5.1 . Clinical relevance of low Pru p 3 sIgE in patients with peach allergy

Figure 16. GrB distribution among EAST classification. 69

Figure 17. Pru p 3 sIgE values distribution among symptoms. 72

Figure 18. Co-sensitization to other LTPs. 74

Figure 19. Basophil activation test under Pru p 3 *stimuli* in grLOW 75

5.2 . Molecular sensitization profiles detection on LTP sensitized patients

Figure 20. Prevalence of sensitized patients for each of the analysed LTPs. 78

Figure 21. LTP sensitization distribution. 79

Figure 22. Frequency of symptoms on sensitized patients. 81

Figure 23. Frequency of symptoms on sensitized patients. 82

Figure 24. sIgE distribution against clusters. 87

5.3. Improving in vitro detection of sensitization to Lipid Transfer Proteins: LTP Strip, a new molecular multiplex IgE assay

Figure 25. Heatmap of sIgE reactivities against nsLTPs of the LTP strip. 100

Figure 26. nsLTP immunoblot assay performance in comparison to PbP results. 103

Figure 27. BAT results for selected nsLTPs with a stimulation concentration of 1 µg/mL. 107

Figure 28. BAT results for a selected set of allergens and patients (Part 1). 108

Figure 29. BAT results for a selected set of allergens and patients (Part 2). 109

Figure 30. CD-sens values distribution. 110

Figure 31. BAT results on negative controls (Part 1). 111

Figure 32. BAT results on negative controls (Part 2). 1112

5.4. Characterization of a LTP syndrome cohort with the LTP-strip

Figure 33. LTP strips heat map. 115

Figure 34. Diversity on family recognition. 117

List of Tables

CHAPTER I: INTRODUCTION

Table 1. Hypersensitivity disorders classification. 2

Table 2. Main plant food allergen families' characteristics. 11

Table 3. Advantages and limitations of singleplex and multiplex assay for sIgE testing. 29

CHAPTER VI: MATERIAL AND METHODS

Table 4. Recombinant nsLTP proteins included on the LTP-strip immunoassay. 58

Table 5. Sequence identity percentages from the proteins included on the strips. 61

CHAPTER V: RESULTS

5.1. Clinical relevance of low Pru p 3 sIgE in patients with peach allergy

Table 6. Clinical relevance frequencies among studied patients. 70

Table 7. Pru p 3 sIgE values distribution among groups. 71

Table 8. Pru p 3/Peach sIgE and Pru p 3/Total IgE ratio median and IQR values 73

Table 9. Sensitization to LTPs from other allergenic sources. 74

Table 10. Characteristics and BAT results of grLOW patients under a Pru p 3 stimulation. 76

5.2. Molecular sensitization profiles detection on LTP sensitized patients

Table 11. Clinical characterization against LTPs. 80

Table 12. Comparison between sensitized and non-sensitized for each of the proteins when classified by local symptoms. 83

Table 13. Comparison between sensitized and non-sensitized for each of the proteins when classified by systemic symptoms. 84

Table 14. Bivariate protein association. 85

Table 15. Protein Combination Clusters obtained from the different reactivity groups. 86

Table 16. Symptoms frequency on multiple reactivity groups. 88

5.3. Improving in vitro detection of sensitization to Lipid Transfer Proteins: LTP Strip, a new molecular multiplex IgE assay

Table 17. Anamnestic Data from the included patients (part 1) 92

Table 18. Anamnestic Data from the patients tested (part 2) 94

Table 19. Anamnestic Data from the patients tested (part 3). 97

Table 20. Plant-food sensitization detected by prick-by-prick test. 99

Table 21. Prick by Prick outcome and anamnestic data. 101

Table 22. LTP-strip outcome and anamnestic data. 102

Table 23. sIgE reactivity of the immunoblot assay compared to the PbP outcome. 104

Table 24. sIgE reactivities in classes against nsLTPs measured by LTP-strips in sera of 28 healthy donors. 106

Table 25. Correlation between ImmunoCAP and Euroline data. 113

5.4. Characterization of a LTP syndrome cohort with the LTP-strip

Table 26. Frequency of LTP plant-food sensitization by the LTP strips. 116

Table 27. Distribution of sensitization on taxonomic families 118

Table 28. Distribution of symptoms on foods over sensitized. 119

Table 29. Culprit foods and cofactor involvement. 120

Published content

Doctoral Thesis in **classical format** with one main objective and 2 specific objectives, and 2 annexed articles.

PAPER 1 – about Objective 1

Balsells-Vives S, San Bartolomé C, Casas-Saucedo R, Ruano-Zaragoza M, Rius J, Torradeflot M, Bartra J, Munoz-Cano R and Pascal M (2022). *Low Levels Matter: Clinical Relevance of Low Pru p 3 sIgE in Patients With Peach Allergy*. Front. Allergy 3:868267. doi: 10.3389/falgy.2022.868267

Journal information: **Frontiers in Allergy**

Recently created journal, not impact factor yet.

PAPER 2 – about Objective 2

Balsells-Vives S, Flügge U, Brix B, Weimann Y, Peralta T, San Bartolomé C, Araujo-Sánchez G, Casas-Saucedo R, Torradeflot M, Lara R, Munoz-Cano R, Bartra J, Suer W, Pascal M. *Improving In Vitro Detection of Sensitization to Lipid Transfer Proteins: A New Molecular Multiplex IgE Assay*. Mol Nutr Food Res. 2023 May 17: e2200906. doi: 10.1002/mnfr.202200906. PMID: 37195823.

Journal information: **Molecular Nutrition & Food Research**

Impact factor (2022):5.2.

Journal Citation Reports (Clarivate, 2023): 34/142 (Food Science & Technology (Science)).

Noves aproximacions en el diagnòstic *in vitro* de la Síndrome LTP

I. INTRODUCCIÓ

Les LTPs (proteïnes de transferència de lípids) són al·lèrgens rellevants principalment a la conca mediterrània, d'àmplia distribució en aliments vegetals, pol·lens i làtex (panal·lergens). La reactivitat creuada entre LTPs de fonts al·lergèniques diferents és molt elevada de manera que és freqüent que els pacients presentin perfils de sensibilització complexes a LTPs de múltiples fonts diverses, l'expressió clínica de la qual pot ser molt variable (Síndrome LTP). El tractament es basa fonamentalment en la dieta d'exclusió. Pel diagnòstic *in vitro* s'avalua l'IgE-específica sèrica (sIgE) mitjançant diversos immunoassaigs. El diagnòstic basat en components permet obtenir un perfil de sensibilització més precís del pacient, així com una millor caracterització clínica, obrint la possibilitat d'una intervenció terapèutica més individualitzada.

II. HIPÒTESIS I OBJECTIUS

Hipòtesis:

Les eines de diagnòstic basades en components són útils per establir perfils moleculars en la Síndrome LTP. Un coneixement més profund i l'optimització dels immunoassajos *in vitro* permetria la identificació de perfils fenotípics diversos en la Síndrome LTP oferint un diagnòstic i intervenció terapèutica personalitzada.

Objectius:

L'objectiu principal és millorar la utilitat de les eines *in vitro* utilitzades pel diagnòstic de l'al·lèrgia a la LTP així com el maneig clínic dels pacients.

1. Millorar la utilitat de les eines *in vitro* actuals pel diagnòstic i maneig clínic dels pacients amb Síndrome LTP.

2. Desenvolupar noves eines *in vitro* basades en diagnòstic molecular per millorar el diagnòstic i maneig terapèutic dels pacients amb Síndrome LTP.

III. MATERIAL I MÈTODES

Estudi de cohort retrospectiu nacional unicèntric (Hospital Clínic Barcelona) amb pacients adults del Servei d'Al·lèrgia (2012-2019), sensibilitzats a almenys una LTP analitzats amb tests cutanis i/o *in vitro* segons pràctica habitual al Servei d'Immunologia. L'estudi ha estat aprovat pel Comitè Ètic (HCB/2016/0361, HCB/2020/0373). Les dades clíniques es van obtenir de l'anamnesi i a través de qüestionaris específics. Es van realitzar proves cutànies amb extractes comercialitzats (SPT) i aliments naturals (Prick-by-Prick). La sIgE sèrica es va mesurar per ImmunoCAP® (ThermoFisherScientific). Un nou immunoblot (Euroimmun,AG) es va desenvolupar per la detecció de sIgE a 28 LTPs de 18 fonts al·lergèniques diferents. Es van realitzar Tests d'activació de basòfils (TAB) a LTPs (Flow2CAST™, Bühlmann Laboratories AG).

IV. RESULTATS

4.1 Rellevància de nivells baixos d'IgE específica a Pru p 3

Es van registrar sIgE Pru p 3 de 496 pacients, 114 (23,0%) amb valors 0,1-0,34 kU_A/L (grLOW), la resta ≥0,35 kU_A/L (grB). El 44,7% del grLOW i el 59,9% del grB eren al·lèrgics. La urticària va ser estadísticament més freqüent al grLOW. En aquest grup els nivells sIgE Pru p 3 van ser significativament més alts en pacients amb símptomes locals versus sistèmics. El TAB amb nPru p 3 es va realitzar a 12 pacients/grup, amb reactivitat similar entre grups. Al grLOW, la reactivitat va ser superior en al·lèrgics i en símptomes sistèmics (p=0,0286).

4.2 Detecció de perfils de sensibilització molecular en pacients sensibilitzats a LTP

Es van incloure 307 pacients amb sIgE a totes les LTPs disponibles comercialment (Pru p 3, Mal d 3, Ara h 9, Jug r 3, Tri a 14, Cor a 8). Reconeixien 4,8 LTP/persona de mitjana. Tots els multireactius eren positius a Pru p 3/ Mal d 3/Jug r 3. Tri a 14 mostra una associació feble amb la resta de LTPs. Els símptomes sistèmics estaven associats a sensibilització a fruits secs (p<0,05). Es van detectar 17 clústers de sensibilització diferents: 46,2% amb 6 LTPs+ (majoritari). Pru p 3 apareix en 14/17 clústers obtinguts. Els símptomes locals eren superiors en el grup <4 LTP mentre que els sistèmics en grups > 4 LTP (p=0,0253).

4.3 Millora de la detecció *in vitro* de la sensibilització a les proteïnes de transferència de lípids: un nou assaig d'IgE multiplex molecular

38 pacients amb síndrome LTP es van utilitzar per validar el rendiment diagnòstic de les noves tires-LTP (versus Prick-by-prick). La concordança era >70% per a la majoria de LTPs. El TAB va demostrar la funcionalitat i la rellevància al·lergènica de nou LTPs recombinants (Pru p 3; Pru du 3; Pru du 3.0101; Pha v 3.0101; Pha v 3.0201; Lac s 1-1; Lac s 1-2; Cuc m LTP; Act d 10).

4.4 Caracterització d'una cohort amb Síndrome LTP amb les tires LTP

Es van analitzar 202 pacients amb les tires-LTP, reconeixent una mediana de 8,5[1-11] famílies taxonòmiques. La sensibilització a les rosàcies estava associada a nivells de sIgE elevats ($p<0,0001$) i era la majoritària: només 2,5% no estaven sensibilitzats a cap d'elles. Dotze pacients (6%) van ser Pru p 3- .Es va observar polisensibilització en 98,5% dels casos, dels quals 92,0% van reconèixer ≥5 LTP. Hi va haver 3 pacients positius a totes 28 LTPs incloses a les tires. Mitjana de reconeixement: 16,6 LTPs. Tres pacients monosensibilitzats.

CONCLUSIONS

Les eines *in vitro* disponibles actualment per al diagnòstic de la Síndrome LTP es poden millorar. Els nivells baixos d'sIgE a Pru p 3 (i.e., per sota del punt de tall tradicionalment considerat) poden ser clínicament rellevants, amb fins a un 20% de reaccions sistèmiques, aspecte rellevant per la pràctica clínica habitual. La meitat dels pacients analitzats estan sensibilitzats a totes les LTPs disponibles comercialment per ImmunoCAP®. El segon perfil de sensibilització més freqüent és sense Tri a 14. La gravetat dels símptomes augmenta en concordança amb la immunoreactivitat. L'absència de sensibilització a Pru p 3 és rara en la nostra població d'estudi (1,9%), en aquests casos els pacients estan sensibilitzats a Tri a 14. Així doncs, la sensibilització a aquest últim s'ha d'estudiar independentment de la sensibilització a Pru p 3 en l'estudi al·lergològic de rutina. El nou immunoassaig desenvolupat que conté 28 LTPs de 18 fonts al·lergèniques variades (LTP-strip) es pot utilitzar amb èxit per a l'estudi al·lergològic *in vitro* dels pacients amb sospita d'al·lèrgia a LTP de la nostra àrea, ampliant l'espectre de proteïnes per les quals es pot analitzar la sensibilització permetent no només un millor diagnòstic del pacient, sinó també un millor maneig terapèutic del mateix.

Chapter I
Introduction

1.1 Allergy

1.1.1 Hypersensitivity disorders

The immune system plays an essential role in host defence against pathogenic agents and also providing protection for future contacts (1,2). When these reactions are not properly controlled, the immunity response stops being a benefit and illness appears, known as Hypersensitivity Disorders (3,4). Classically, they are classified according to the type of immune response and the effector mechanisms that produce tissue and cell damage (Gell & Coombs, 1963), **Table 1**.

Table 1. Hypersensitivity disorders classification.

Hypersensitivity type	Pathologic immunity mechanisms	Tissue injury and disease mechanisms
Type I: Immediate hypersensitivity	T_H2 cells, IgE antibody, mast cells, eosinophils	Mast cells, eosinophils and their mediators (vasoactive amines, lipid mediators, cytokines)
Type II: Antibody-mediated diseases	IgG and IgM antibodies against cell surface or extracellular matrix antigens	▪ Cell opsonization and phagocytosis ▪ Complement and receptor mediated recruitment for Fc and activation of leukocytes (neutrophils, macrophages) ▪ Alterations in cell functions (hormone receptor signals, blockade of receptors for neurotransmitters)
Type III: Immune complex-mediated diseases	Immune complexes of circulating antigens and IgM or IgG antibodies deposited in vascular basement membrane	Complement and Fc-receptor mediated recruitment and leukocyte activation
Type IV: T-cell mediated diseases	CD4+ T cells (T_H1 and T_H17 Lymphocytes)	1. Cytokine - mediated inflammation and macrophage activation
	CD8+ CTL (T-cell mediated cytolysis)	2. Direct target cell death, cytokine-mediated inflammation

Adapted from Abbas *et al.* (1).

Immediate hypersensitivity reactions (Type 1) start a few minutes after antigen provocation on sensitized patients and are known as *Allergy* (2). The EAACI (European Academy of Allergy and Clinical Immunology) defines Allergy as an "immunological hypersensitivity that can lead to a variety of different diseases via different pathomechanisms. It is not a disease itself, but a mechanism leading to disease. In clinical

practice, allergy manifests in form of various different conditions such as anaphylaxis, urticaria, angioedema, allergic rhino conjunctivitis, allergic asthma, serum sickness, allergic vasculitis, hypersensitivity pneumonitis, atopic dermatitis (eczema), contact dermatitis and granulomatous reactions, as well as the colourful spectrum of food- or drug– induced hypersensitivity reactions". Allergy reactions can appear in almost all organs but especially skin and mucosal membranes (5).

1.1.2 Antigen

An antigen (antibody generator) is a substance with the ability of binding specifically to an antibody or a T cell receptor. When these molecules can stimulate an immune response are called immunogenic (1). The antigens triggering immediate hypersensitivity reactions are called *allergens*. Allergens have different sources including foods, drugs, animals, pollens, dust mite products and fungal spores.

These molecules are proteins or substances binding proteins with the ability of activating CD4+ Cells which produce IL-4 (Interleukin-4) and IgE (1,2). Intrinsic and extrinsic factors have been described to induce allergenicity, converting non-allergenic molecules to allergens, such as glycosylation, lipidic bindings and conjugate glycans and also protease proteins (2,6,7). Nevertheless, some non-protein allergens such as drugs (penicillin, chlorhexidine) (8) and galactose-α-1,3-galactose, have also been reported to be responsible of allergic reactions (9).

An "initiator" allergen with the capacity of binding a specific IgE (sIgE) will become a primary sensitizer (10). The term "major" allergen is referred to that allergen recognised by more than 50% of the patients sensitized to the particular allergenic source. A major allergen is not necessarily associated to a more severe clinical reaction. Not all major allergens are initiators (8).

An epitope or antigenic determinant is the part of the antigenic molecule recognised by the immune system and to which an antibody binds. In some cases, epitopes depend on their proteinic primary structure (adjacent aminoacids), known as *lineal epitopes*. In some others, the tertiary structure is necessary to bind the antibody and are called *conformational epitopes*. Food processing affects allergenicity by altering the structure and physicochemical properties of antigens/allergens, e.g., heat can produce the destruction of conformational epitopes as well as gastroduodenal digestion may alter specific conformation arrangements of epitope aminoacids influencing on their sIgE bindings (2,11,12).

The World Health Organization/International Union of Immunological Societies (WHO/ IUIS) Allergen Nomenclature Database stablishes an official, standard nomenclature system for allergens. It is based on the Linnaean binominal classification, typifying the genus and species of the allergenic source. The names of the allergens contain the first 3 or 4 letters of the genus, a space, the first 1 or 2 of the specie, a space and a number that reflects the order of identification in the source. In some cases, also includes a dot and a 4-digit number to identify isoallergens (when more than 67% sequence identity, referred as the percentage of identical residues, occurs) and variants (when among isoallergens, sequence identity is more than 90%) (13–15). For example, the LTP allergen from Peach (*Prunus persica*) is Pru p 3 and Pru p 3.0101, Pru p 3.0102 are its isoallergens (14,15). In September 2023, the WHO/IUIS allergen Nomenclature Database included a total of 1094 allergens (16). Allergen data banks contain information of identified allergens available for the scientific community, i.e., www.allergen.org, www.allergome.org.

1.1.3 Physiopathology

In normal conditions on a healthy subject, when allergen exposition occurs, antigen presenting cells (APCs), mostly dendritic cells (DC), recognise and internalise the antigen migrating it to a lymphatic nodule (4,17). Antigens are presented to naïve T helper cells (T_H0) promoting their differentiation into antigen specific Foxp3+ regulatory T cells (Treg) (18). Treg cells, as well as regulatory B cells (Breg), produce immunoregulatory cytokines (mostly IL-10, TGF-β) resulting on tolerance induction (19–22). Moreover, production of allergen specific Immunoglobulin G subclass 4 (IgG4) is activated (23,24). Allergy seems to be a loose of this tolerance or a failure into tolerance induction. Recovering the immunity tolerance has become one of the main objectives in allergic disease investigation, specially a long-term tolerance (18,21).

Briefly, in atopic individuals, allergens (see **Figure 1**) are captured and processed by DCs (25,26). The entrance of the antigen/allergen activates the innate immunity system by IL-25, IL-31, IL-33 and TSLP (Thymic stromal lymphopoietin) secretion and innate lymphoid cells (ILC, ILC-2 in particular) response (13,16,22,23). ILC-2 will generate an innate response producing T type 2 cytokines (as IL-5, IL-9 and IL-13) which can activate eosinophils, mast cells, B and T cells as well as mucus production, smooth muscle contraction and alveolar macrophage activation (18,21,25–30). TSLP also stimulates immature DC to proinflammatory DCs that can bring the allergens to lymph nodes, process them and present to CD4+ naïve T cells. These, in presence of IL-4 differentiate to CD4+ T_H2 initiating an adaptative immunity response (18,21,27,28). T_H2 produce

cytokines IL-4, IL-5 and IL-13 (2,31,32). These cytokines produced by T follicular helper (TFH) as well as CD40 binding, induce in B cells differentiated to plasmatic cells the change on the immunoglobulin heavy chain class (also known as *class switch*, mediated by IL-4 mostly and IL-13) to ε isotype and the synthesis of IgE.

IgE in human bodies is approximately 50% found as free in vascular bed (with a few days half-life), and 50% bound to effector cells receptors, specially the high affinity FcεRI (100.000 - 250.000 FcεRI/basophil) during about 2 months.

As said, IgE (bivalent) will bind on high-affinity receptor for the Fc region of IgE (FcεRI) on the surface of major effector cells, mast cells and basophils, status known as *allergen sensitization* (32–35). On new encounter with the allergen, these cells can be immediately activated upon cross-linking IgE-allergen. sIgE are cross-linked in pairs or larger aggregates, and about 2000 cross-linkings are necessary to induce a half-maximal response on effector cells (36). Cell activation leads to mediator release leading to allergic symptoms (effector phase of the allergic reaction). A wide range of mediators are newly released and synthesized, as in example histamine, lipidic mediators or cytokines (1,2,32–35,37).

Figure 1. Allergic response physiopathology.

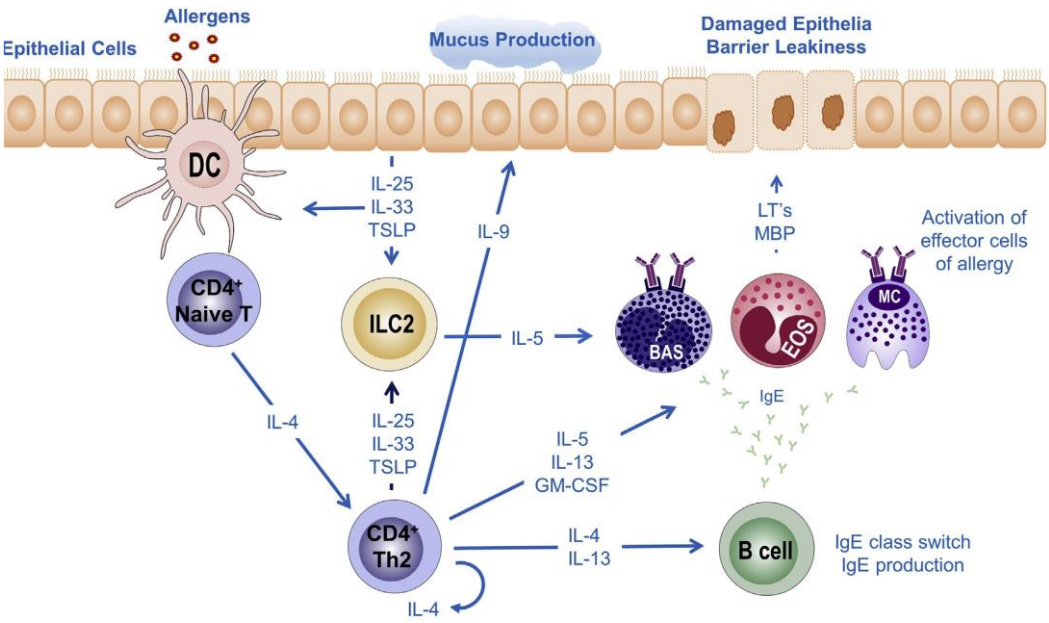


Figure extracted from Kucuksezer *et al.* (21).

1.2 Food allergy

Food allergy (FA) was defined in 2003 (38) by the World Allergy Organisation (WAO) as an adverse health effect to food in which have been demonstrated immunologic mechanisms, including both Ig-E and non-IgE mediated FA. Thus, FA is classified according to the immunological mechanisms: IgE-mediated (immediate) and non-IgE-mediated. Hereby this thesis is focused on IgE-mediated type of FA, which occur within minutes to a few hours after the exposure (39–41).

Frequently, other diseases with similar gastrointestinal symptoms which improve with food avoidance are confused with FA (42). Nevertheless, while other food-related illnesses have localized symptoms in gastrointestinal tract, allergic reactions to food may affect multiple organs which could produce life-threatening reactions (section 1.2.3) (39–41).

1.2.1 Epidemiology

The "Allergic Epidemy" concept has appeared over the last decades with a worldwide increase of allergic rhinitis, asthma and atopic dermatitis prevalence. However, also Food Allergies are becoming more prevalent on population groups with an occidental lifestyle, related with an increase of fatal anaphylaxis over child and adolescents (5,43,44). The frequency of allergic reactions to foods is higher in infants compared to adults (43,45). Over infantile population, allergic diseases are more frequent in boys than girls while in adults, allergy in women tends to be more frequent. This fact could be explained by genetic, endocrinologic and ambiental factors (46).

The *EuroPrevall* project, developed in Europe during the last decade including various countries in which standardized diagnostic protocols were used, aimed at obtaining food allergies prevalence and distribution among children, adolescents and adults in Europe obtained under uniform conditions (47,48). In adults, FA reaches a 6% prevalence in some places in Europe (48).

In 2018, an actualization of the Spanish multicentric study *Alergólogica 2015* on prevalence allergy was published (49), reporting an increase on food allergy prevalence from 3.6% in 1992 to 7.4% in 2005 and 11.4% in 2015. The most frequent offending foods in adults were fruits (44.7%, specially *Rosaceae*), tree nuts (28.4%) and shellfish (14.8%, mainly crustaceans).

Finally, in our region, Catalonia, the *Cibus project* was created (50) to register the most prevalent allergenic foods sources as well as their clinical manifestations. Fruits and tree

nuts were more frequent on adults: peach was the most prevalent food producing allergic reaction (10%). In the Mediterranean area, sensitization to Pru p 3, the peach Lipid Transfer Protein (LTP), is the most important cause of plant food allergy (51).

Prevalence of FA may vary against different geographical locations and may be influenced by multiple factors as dietary habits (52), the cooking method used (11), the age of food weaning (18,27,53), genetic factors (54,55) or the main aeroallergens on each area for which appear diverse molecular sensitization patterns from the same food sources (52,56). Also, societies tending to adopt occidental lifestyles seem to develop more allergic diseases supporting the contribution of environmental risk factors (18,27,42). Besides, antibiotic use altering microbiome, breastfeeding, nutritional factors (vitamin D and ω-3 polyunsaturated fatty acids deficiency) or allergen exposure during lactation and pregnancy seem to have a role on the development of allergy (18,27).

1.2.2 Sensitization routes

The first contact with food allergens is usually oral, although other sensitization ways are possible. Three main sensitization routes have been proposed as relevant exposition sites: gastrointestinal tract, respiratory tract, and skin (epicutaneous). Distinguishing the origin of the allergic sensitization may predict how is the clinical relevance going to be (5).

When primary sensitization is produced in digestive tract (gastrointestinal sensitization route), it is known as **Class I Food Allergy**. In this case, antigens in food (for example, egg, milk, fish, and LTPs (57)) are presented to cells in GI tract and depend on multiple intrinsic factors which could alter the gastrointestinal mucosal barrier epithelium: permeability of the epithelial barrier (tight junctions), stability and ingested quantity of allergen, alteration of gastric pH or microbiome, or developmental immaturity of immune system and GI barrier during infancy (Class I FA is more frequent in paediatric population) (41,58,59).

Primary sensitization can also occur in respiratory tract. It is known as **Class II Food Allergy**, produced by food allergen aerosol inhalation but also for inhaled pollen allergens which will cross-react with food allergens. This phenomenon is called "*pollen food syndrome*". Symptoms can appear years after primary sensitization and are characterised for being mild and restricted in the oral cavity (OAS). Systemic reactions are less frequently seen (41,57) and more likely developed during adulthood (60).

Recently, skin barrier has been proposed as a third sensitization route. A loss of integrity of the skin barrier (i.e., in atopic dermatitis) might participate in food sensitization (61),

known as the "dual allergen exposure hypothesis" proposed by Lack (62). It is evidenced that skin T_H2 inflammation down-regulates filaggrin gene expression leading to a loss-of-function of the epidermal barrier (63) and it is known that a mutation or a lack of its expression produces pro-inflammatory cytokines presence in skin (64). Moreover, it has been reported the predominance of Cutaneous lymphocyte-associated antigen (CLA) plus skin-homing response to peanut in peanut-allergic patients but not in gut-homing (integrin β7+)(65).

1.2.3 Clinical Manifestations

The GI mucosa has the role of host defence against harmful microbes, toxins or damaging nutrients. For this reason, the digestive barrier is arranged with the innate immune system and complementary defence systems as gastric acid, bicarbonate secretion, mucus, digestive enzymes, tight junctions forming an intact epithelium, antimicrobial peptides, peristaltic movements, and phagocytes (2). Sometimes, food proteins are improperly recognised as harmful antigens by the immune system leading to gastrointestinal food allergy symptoms. Waltzer *et al.* (66) proved in 1928 that food antigens could cross over the GI barrier and distribute through the body. Consequently, food allergens could produce an immune response in different tissues generating extra-gastrointestinal symptoms (2). Allergy symptoms are classified as local or systemic symptoms.

1.2.3.1 Local symptoms

FA symptoms exposed on GI are non-specific and can vary depending on the place of mucosal reaction. Are classified as clinical manifestation on the oral mucosa or in the remaining digestive tube mucosa. Oral allergy syndrome (OAS) is produced by lip and oral pruritus, lip and tongue swelling but also pharyngeal pruritus and oedema (sometimes itchy ears are reported). Gastrointestinal tract symptoms (GI) can be localised in the stomach (Reflux disease, Nausea, Vomiting); in the small intestine (Crampy abdominal pain, Malabsorption, Vitamin deficiency) or in the large intestine (Diarrhoea, Constipation, Faecal blood loss). Generally, early reactions appearing few minutes after ingestion, involve oral cavity and stomach while late reactions appearing hours to days later involve small or large intestine (2). In some cases, Contact urticaria (CU) can also occur, a wheal which appears within minutes at the place of contact with the offending food and resolves after 24 hours (67).

1.2.3.2 Systemic symptoms

URTICARIA AND ANGIOEDEMA (U and AE)

The EAACI/GA²LEN (global asthma and allergy European network)/EDF (European dermatology forum)/WAO Guidelines for urticaria (68,69), define Urticaria as "a condition characterized by the development of wheals (hives), angioedema or both". Acute urticaria is one of the most frequent food-induced clinical manifestations. In a subset of allergic patients, cofactors (non-steroidal anti-inflammatory drugs -NSAIDs-, exercise or alcohol) presence combined with food ingestion are necessary to produce urticaria (2). Angioedema in urticaria consists of an abrupt erythematous or rouged skin swelling into lower dermis and subcutis but also in mucous membranes. Can be painful and resolves slower compared to wheals, in some cases about 72 hours (68).

ANAPHYLAXIS (AN)

The EAACI describes Anaphylaxis as "a life- threatening reaction characterized by acute onset of symptoms involving different organ systems and requiring immediate medical intervention"(70). The WAO Anaphylaxis committee proposed a definition for anaphylaxis: "Anaphylaxis is a serious systemic hypersensitivity reaction that is usually rapid in onset and may cause death. Severe anaphylaxis is characterized by potentially life-threatening compromise in airway, breathing and/or the circulation, and may occur without typical skin features or circulatory shock being present" (71). **Anaphylactic shock** (SH) will implicate shock or circulatory collapse.

Co-factors may be symptom aggravating factors in some patients with food allergy, common reported ones are exercise, stress, infection, non-steroidal anti-inflammatory drugs (NSAIDs), menstrual cycle, fasting and alcohol (72–74). This concept is known as co-factor-enhanced food allergy (CEFA)(74).

Exercise induced anaphylaxis consists of a severe- potentially fatal anaphylaxis which converges with physical activity (Food dependent exercise induced anaphylaxis, FDEIA). Requires the presence of exercise before or after food allergen ingestion (75). Wheat-dependent exercise-induced anaphylaxis (WDEIA) is the most characterized of them. Physiopathology mechanisms on FDEIA are not well understood. It is suggested the theory that acidic state promoted by muscular works could induce mast cell degranulation (76). Also, a genetical background could be influent on this type of reactions (75). Alcoholic beverages and non-steroidal anti-inflammatory drugs might influence on the severity of the clinical reactions by accelerating the intestinal allergen absorption (74). On Food-dependent NSAID-induced anaphylaxis (FDNIA) it has been suggested that NSAIDs could rise the intestinal barrier permeability altering the

absorption but also mast cells could play a complementary role (77). Moreover, it has been reported that NSAIDs could have an effect directly on basophils and mast cells response amplification. Aspirin modulates cysteinyl leukotrienes LTC4 in mast cells, by dihydropyridine-sensitive L-type Ca^{2+} channels (LTCCs) (78).

Recently, a mutation in the KARS gene, implicated in antigen dependent–FcεRI activation from mast cells, has been characterised. This mutation would alter important mast cells functions as granules biogenesis and their mediators synthesis conducing to severe anaphylaxis (79).

1.2.4 Food Allergens

Any antigen could potentially produce an allergic response and an increasing number of proteins have been detected as potential allergens, although a restricted number of allergens and proteins families are responsible of most of the FA reactions. Allergic reactions to foods are based on the allergen characteristics which on their turn characterise the alternative sensitization routes. For this reason, many investigation groups are focused on the identification and molecular characterisation of the most important allergens as well as comparing sensitization profiles against different population groups (5,80,81).

- TYPE 1 FOOD ALLERGENS

  Are water-soluble glycoproteins from 10 to 70 kDa, characterized for being resistant to heat, digestion and degradation conditions as gastric acid pH, proteolytic enzymes and bile salts. Its sensitization is produced by the way of gastrointestinal tract inducing Class I Food Allergy. Known as "Complete Antigens" and related with severe clinical manifestations. Seed storage proteins and LTPs belong to this group (5,41,80,81).

- TYPE 2 FOOD ALLERGENS

  Most of them are conformational epitopes and so are thermolabile, which can be degraded by enzymes and digestion. Their sensitization is produced through respiratory tract by cross-reactions with homologous allergens from pollens and reactions appear after food ingestion. This would explain that many patients only experience symptoms on the pharyngeal mucosa and oral cavity. Generally, induce mild symptoms on the oral cavity. Are known as "Incomplete allergens" and induce Class II Food Allergy (5,41,80,81). These allergens are difficult to extract (from their sources) and be isolated. For this reason, using diagnostic equipment results unsatisfactory when natural extracts are used *in vivo* or *in vitro* (58).

Table 2. Main plant food allergen families' characteristics.

Families	Sub-families	Structural characteristics	Location	Symptoms
THAUMATIN LIKE PROTEINS (TLP)		Resistance to high temperatures, pH changes or protease degradation (82)	Growing fruits, pollens, vegetables, nuts and cereals (83)	Mild to severe symptoms, associated to systemic (82)
PROFILINS		Moderate-low stability against heat (41,81)	Vegetal foods, latex, pollens (84,85)	Cross-reactivity: pollen sensitization, pollen-food syndromes, OAS (84–86)
CUPINS	Vicilins	Resistance to protease degradation. Certain thermal stability (87)	Legumes (88,89)	Cutaneous reactions, systemic symptoms in 20% of the patients (90)
	Legumins			
Bet v 1 LIKE PROTEINS SUPERFAMILIY	Ripening-Related Proteins (RRP)/Major Latex Proteins (MLP) Subfamily	Labile under high temperatures and extreme pH conditions (8)	Green kiwi (*Actinia deliciosa*) (91)	Cross-reactivity indicator (60,92) plant-food allergy (8,60,92,93). Mild symptoms (OAS) (94)
	Cytokinin-Specific Binding Proteins (CSBP) Subfamily		Mung bean (*Vigna radiata*) (95)	
	Pathogenesis-Related Protein Subfamily 10 (PR-10)		Fagales plants, fruits, vegetables, nuts and legumes (8)	
PROLAMINS	2S-Albumins Seed Storage Protein	Resistant to heat, gastric pH and proteolytic enzymes (96)	Nuts, legumes, oil seeds, cereals and spices (96)	Severe reactions. Mild reactions over raw and processed foods (96)
	Cereal Prolamin Seed Storage Proteins: Gliadins and Glutenins	High heat stability (8)	Grass cereal grains (8,97,98)	Mild and systemic symptoms, WDEIA (8,97,98)
	α-amylase and protease bifunctional inhibitors	High heat stability (8)	Cereal grains (8,99,100)	Baker's asthma (8,99,100).
	Non-specific lipid transfer proteins (nsLTP) (41,81,101)	Resistance to high temperatures, low pH changes or protease degradation (8,102)	Grains, nuts, vegetables, pollens or natural rubber latex (60)	Mild to severe symptoms (8,102)

The main plant-food allergen families are summarized in **Table 2**, which includes Prolamins and, in concrete, Lipid Transfer Proteins, the allergen family to which this thesis is focused.

1.2.4.1 Cross-reactivity

Not all the allergens have the property of inducing allergen specific IgE antibodies (sensitization), some are *non-sensitizing* (known as secondary or cross-reactive) and will only produce allergic symptoms if another related allergen has caused a previous sensitization (8). Cross-reactivity in allergy refers to the capacity of a secondary allergen to induce an immuno-allergic response (clinically relevant or not) after exposure in an organism previously sensitized to a primary allergen, with which shares 1 or more epitopes (known as cross-reactive epitopes) (103). Allergenic molecules are distributed among different protein families which share common epitopes (81), conserved aminoacid sequences to which the antibodies can bind, either linear epitopes or conformational epitopes (derived from the three-dimensional folding of the protein) (81). Cross-reactivity most likely occurs in allergens sharing >70% aminoacid sequence identity (104) although it has been described that IgE relevant epitopes frequently share less than 20 aminoacids in length (105). Extreme conditions over labile molecules could alter the three-dimensional structure reducing the cross-reactivity reaction by loss of conformational epitopes (104).

IgE (B-cell) cross-reactivity. A primary allergen induces sensitization via Th2 response, activating the synthesis of sIgE antibodies towards a few conformational and/or linear epitopes of its sequence/structure (106,107). Afterwards, when patients are exposed to allergenic-food sources containing homologous allergens, the primary-allergen sIgE antibodies will recognise the secondary allergens through cross-reactive epitopes leading to an immune response (cross-linking, mast cell activation and mediator release). Secondary allergens can be *complete* or *incomplete* when they are not able of producing primary allergic sensitization alone (co-sensitization) (106–109).

The affinity between secondary allergens and sIgE can be weaker compared to primary ones. Multiple factors as the number of cross-reactive epitopes, the binding affinity, physicochemical stability and the homology of aminoacid sequence and/or structure play a role on the relevance of the cross-reactivity (103).

T-cell cross reactivity. It consists on the response of T cells to one or more peptide-MHC ligands of allergens (110). After primary allergen exposure, the allergen is presented by dendritic cells through MHC to naïve T cells, leading to T_H2 cells expansion and differentiation. After the exposure of secondary allergens containing homologous T-cell epitopes, cross-reactivity is produced and primary allergen specific T cells induced and also secondary allergen sIgE is generated (108). Usually, it is produced by sequence homology between cross-reactive sequence residues and related allergen sources. A

cross-reactive T-cell epitope is able to stimulate memory T cells and induce subsequent IgE production (111).

1.2.4.2 Panallergens

Panallergens are proteins which intervene on key biological processes from organisms which contain conserved structures and sequences (84,112). They are constituted by different protein families from animal and plant sources of phylogenetic related or unrelated allergens, which widely cross-react with allergens from other allergenic sources. Thus, cross-reactions (i.e., cross reactivity) can occur between multiple pollen sources (113), between plant foods and pollens (114) or between animal foods and invertebrate inhalants, among others (115). The most relevant panallergens from vegetal sources are profilins, polcalcins, non-specific lipid transfer proteins and pathogenesis-related type 10 proteins (PR-10) (41,81).

In daily medical practice, a significant number of patients are sensitized to multiple foods, pollens or sea-foods, not necessary being part of the same taxonomic family. Panallegen sensitization has epidemiologic particularities (112) and yields complexity on the allergenic profile of the patients (116). Cross-reactivity to panallergens can produce variate sIgE responses to different allergenic sources leading to positive allergological tests (either *in vitro* tests or skin prick tests) without clinical relevance (117). Furthermore, it has been reported that a high degree of panallergen sensitization is associated with the severity of allergic symptoms (118).

1.2.4.3 Pathogenesis Related Proteins (PR)

Many proteins that are produced by the plant as a defence response against pathogens or environmental stress are allergens (41,81) and they are expressed in multiple organs and tissues (60). In 1994 their nomenclatures were unified based on biological/enzymatic activity, serological relationship or aminoacid residues sequences (119) and grouped into 17 families (Pathogenesis Related Proteins, PR-17). Some examples are: β-1,3-glucanases, chitinases, thaumatin-like proteins, proteinase inhibitors, peroxidases, Bet v 1 homologs (also known as PR-10), lipid transfer proteins (LTPs, PR-14), defensins, thionins or oxalate oxidases (58).

1.2.4.4 Cross-Reactive Carbohydrate Determinants (CCDs)

As said before, type I Hypersensitivity reactions mediated by IgE are mainly caused by proteins. However, it is known that IgE antibodies can also react to carbohydrates binding to carrier proteins (120).

In eukaryotic glycoproteins, the main IgE binding oligosaccharides are complex N-glycans, known as "cross-reactive carbohydrate determinants" (or CCDs) (120). They are composed by two molecules of N-acetylglucosamine and one distal mannose with the attachment of carbohydrate residues. Structural modifications on them, not found on human tissues but in plants, insects, helminths and molluscs, represent an immunogenic target for sIgE. sIgE against CCDs in patients allergic to pollen can induce cross-reactivity with food or inhaled allergens, insect venoms and latex (121,122). Inversely, insect venoms induce CCD sIgE and so, in non-atopic patients with insect venom allergy can be found positive *in vitro* results for pollen, food or latex (123). They would act as "mimickers of allergy". Importantly, N-glycans do not suppose important allergy triggering but can interfere with *in vitro* diagnostic generating false positive results (124,125) conducing to inappropriate dietary restrictions, prohibitions or unjustified immunotherapy (126). To overcome this problem and improve the specificity of *in vitro* tests, strategies such as the preferential use of recombinant allergens (that can lack CCDs in comparison with natural purified), serum CCD inhibition and serum CCD antibodies are applied in molecule-based allergy diagnosis (127). To detect anti-CCD sIgE, MUXF is used as an allergen containing CCD as well as natural glycoproteins such as bromelain, peroxidase from horseradish, or ascorbate oxidase (126). Nevertheless, glycosylated allergen extracts are still used because of the limited availability of certain components, of which not always are recombinant. The possible artifact of CCDs on purified natural proteins always has to be considered (128).

1.2.5 Treatment

The main step on food allergy management is an exclusion diet avoiding offending foods. However, strict avoidance can be a problem due to the abstention of the whole nutrients comprised on the food which could have nutritional implications. Moreover, this can significantly alter eating behaviours, taste preferences and limited dietary choices, decreasing patients quality of life (129). For this reason, an accurate allergy diagnosis is essential to define the most appropriate treatment for each subject (130).

However, drugs provide the treatment of clinical reactions: **Adrenalin** (epinephrine, to treat systemic manifestations and it will be key to prevent death on anaphylaxis (131);

Antihistamines (H1 receptor blockers on the treatment of localized FA symptoms and H2 receptor blockers to treat gastrointestinal symptoms (40)); **Corticosteroids** (to downregulate symptoms induced by immunologic reaction). Other medicines can be used to treat allergic disease such as: sodium cromoglycate, anti-leukotrienes (montelukast), topic anti-cholinergic drugs or inhaled β2 adrenergic receptor agonists (8).

During the last decades, biological treatments have been developed for the treatment of food allergy (132): **IgE targeted therapies** (Omalizumab (27,133–135) used as monotherapy might increase the tolerated dose in various foods in patients with FA (136)); **IL-4- and IL-5-targeted therapies** (For Dupilumab (137) active clinical trials are focused on studying its effect on the management of FA (138,139); **Alarmin-Targeted Therapies** (Alarmins as IL-33, IL-25, and TSLP are implicated on the development and upkeeping of FA. i.e. Etokimab (140)).

IMMUNOTHERAPY

Based on the understanding of how desensitization is produced, food allergen-specific immunotherapy (FAIT) is being under investigation (40). The main objective of FAIT is achieving desensitization or no clinical allergic response to the specific foods (i.e., tolerance), although in some cases, patients only achieve an increase on the onset dose necessary to initiate the allergic response (141). Nevertheless, this already represents an increase on safety against accidental exposures. In short, CD4+ T cells change the allergic phenotype to a regulatory or anergic one, increasing the amount of Treg, which produce TGF-β1. This reduces the inflammatory response after allergen exposure, consequently sIgE levels decrease and sIgG4 increase (40). Different FAIT modalities have been included in the EAACI guidelines (142): **oral (OIT)**, **sublingual (SLIT)** and **epicutaneous (EPIT)** (143). In OIT and SLIT, the allergen dose is gradually raised. Currently, OIT is recommended on lasting allergy to peanut, cow's milk and hen's egg in 4-5 years children (144). In most cases, FAIT adverse effects are mild symptoms as oropharyngeal pruritus or abdominal pain, which may depend on the dose (145). A SLIT peach extract with quantified Pru p 3 (50μg Pru p 3/mL) from ALK-ABELLÓ (Madrid, Spain) is currently commercially available, with demonstrated efficacy and safety (146–148). Moreover, in patients with systemic symptoms, SLIT demonstrated efficacy and not only for Pru p 3, but also for Ara h 9 in patients with concomitant allergy to peanut (148,149). Positive results have also been demonstrated by using fresh peelings and quantifying Pru p 3 to obtain peach extracts to use for SLIT (150,151). Recently, a Pru p 3-epitope based SLIT treatment using Pru p 3 T-cell peptides bound to oligodeoxyribonucleotide with CpG motifs has been reported (152) with promising

results, inducing specific T_H1/Treg response. Interestingly, a group of patients in which a SLIT with commercial peach-juice was performed, safeness and effectiveness has also been reported (153).

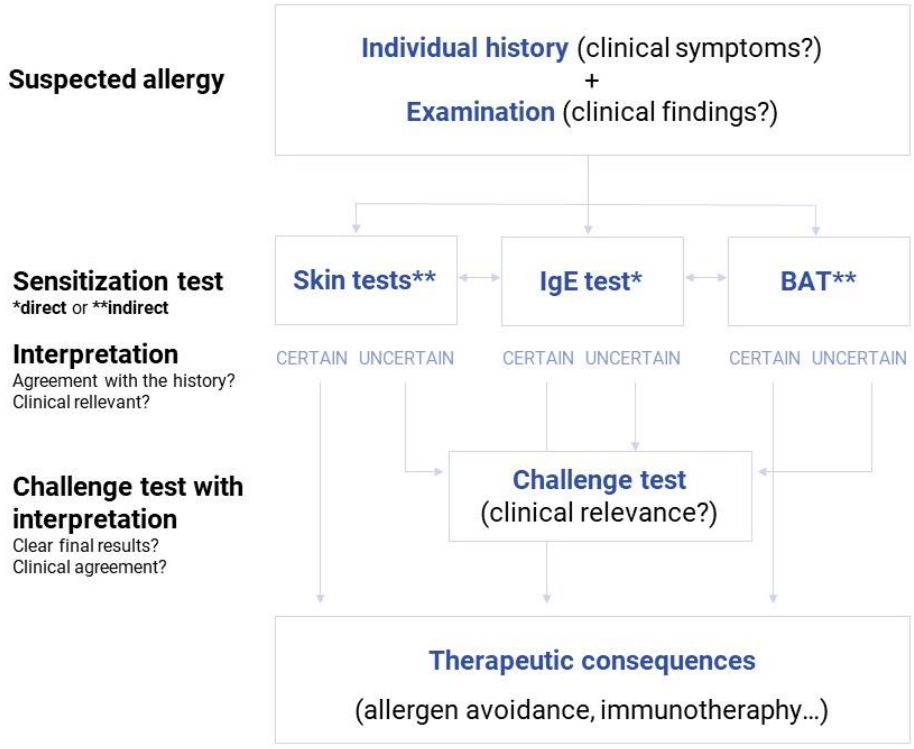
1.2.6 Diagnostic

A precise diagnostic is essential to focus on an adequate treatment for food-allergic patients, ensuring that only strictly necessary foods are avoided. Matricardi *et al*. (8) designed a diagnostic algorithm (**Figure 2**) to guide professionals on this process, starting with an exhaustive anamnesis, clinical history, and physical examination, followed by anamnesis-directed, IgE sensitization tests *in vivo* (SPT) or *in vitro* (serum IgE detection or Basophil activation tests (BAT)). However, sIgE presence indicates sensitivity but not allergy and so, these results should be compared and validated by clinical history. When results are not clear, challenge tests are required, which are still considered the gold standard in FA diagnosis. A clear outcome will inform about clinical relevance to provide an adequate therapeutic intervention (8,154).

1.2.6.1 Clinical History

A rigorous patient clinical history/anamnesis is essential in the diagnosis of food allergy. This thesis is not focused on this part of the diagnosis and so, it will be roughly described hereby. It includes patient's account of the symptoms, severity and frequency of them, familiar antecedent of allergic disease and seasonal variances. Data on impact on patients' quality of life is also important, specially in those reporting gastrointestinal symptoms. Particularly in FA, is important to take in consideration the food allergens class (1 or 2). Class 1 allergens are related to rapid or not onset of the symptoms after ingestion and frequently, multiple organs are involved. Moreover, can be produced by both cook or uncooked foods. It will be rare to find an isolated asthma in this kind of FA although it can produce asthma associated to other symptoms (rash, angioedema or vomiting). Class 2 allergens are pollen-derived allergens that cross-react. Usually associated to mild symptoms on the oral cavity which immediately appear after ingesting raw fruit or vegetables. The amount of ingested food; how long did symptoms appear after ingestion, or the presence of cofactors is information which needs to be registered. It can certainly be difficult to differentiate symptoms produced by foods from other causes, so complementary tests will be necessary to obtain a definitive diagnostic (2).

Figure 2. Diagnostic work-out for IgE mediated/type I hypersensitivity reactions.



Adapted from Matricardi *et al*. (8).

1.2.6.2 Allergen Specific IgE Detection: *In Vivo* And *In Vitro* Studies

The presence of allergen specific IgE (sIgE) can be evaluated *in vivo* through skin testing (sIgE bound on FcεRI on mast cells) or *in vitro,* either on serum/plasma samples or tight on the surface of FcεRI receptors in the surface of basophils (2).

SKIN TESTING

Skin tests provide information on effector cell-bound sIgE and can be done by two ways: **skin prick tests** (SPT) or **intradermal**. SPT is usually the most frequently used for offering enough specificity and sensitivity although, in some cases, such as venom hypersensitivity or penicillin allergy, the intradermal is required for offering an increased sensitivity. These tests require specific training on adequate performance and interpreting the results (5). Systemic reactions are common on intradermal tests while SPT is considered as a safe procedure in which associated anaphylaxis is rare and only a few cases have been described (130,155–157).

- INTRADERMAL SKIN TESTING

  Consists of injecting a low dose of allergen (100 to 1000-fold less concentrated that in SPT), using a needle producing a 2-3 mm bleb in the dermis. Usually performed in the forearm (5).

- SKIN PRICK TESTS

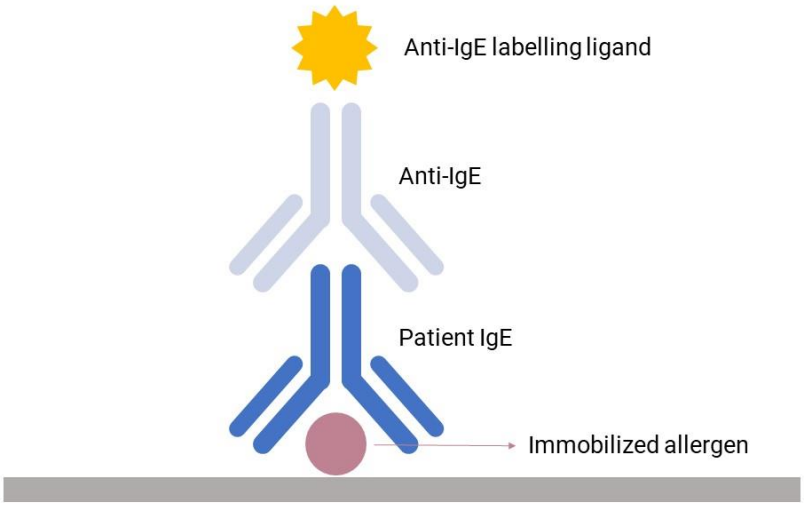
  SPT is considered the primary tool for allergists to detect allergen sensitization since it is cheap, fast and does not require specific equipment (158). SPT are usually performed on the forearm or in the back (considered to be more reactive) by applying a drop of the allergen extract on previously cleaned skin. Then a needle or a lancet penetrates the drop at a 90º angle, through the skin, creating a small break on the epidermis which permits allergen penetration. The size of the wheal is measured 15-20 minutes after, a 3 mm diameter is the minimum to consider a result as positive (154). To avoid potential false negative results, a positive control with histamine (10 mg/mL) is used. Antihistamine drugs or drugs with antihistaminic properties as tricyclic antidepressant can induce false negative results thus treatment has to be stopped prior testing. Oral corticosteroids do not inhibit SPT results. Saline serum or allergen diluent is used to assess false positive results. False positive results can occur in dermographism and SPTs should not be performed in the presence of severe eczema. Despite its wide use, SPT has some limitations (154): it is operator-dependent, not all allergen extracts are available and not all are of sufficient quality (159), antihistamine treatment must be stopped a few days before and the manifestation of severe symptoms occur in rare cases (160). It is necessary the use of standardized, validated and well-conserved allergen extracts to elude false negative results. It has been proved that the use of prick-prick tests with fresh food and vegetables (*prick-by-prick*) is more sensitive to confirm food allergy diagnosis than SPT with commercial extracts (161). It is known that the size of a positive SPT corresponds with the likelihood of allergy to that food but not to the severity of the clinical reaction (162). Moreover, a positive SPT, reveals the presence of allergen-specific IgE but does not mean that the patient is allergic (163). Also, it is not well known but exists, the interference from certain molecules with the subject's test result. For example, when histidine is present on the extract, in certain conditions as bacterial presence (with histidine decarboxylase), the conversion to histamine can be increased providing false positive results (130).

SERUM sIgE

Allergen-specific IgE in serum and plasma can be measured *in vitro* in the laboratory by immunoassays, overcoming risks and logistical limitations of *in vivo* testing. Various quantitative and semi-quantitative immunoassays have been developed since the first description of IgE in 1967 (164), early radioimmunoassays, and later mostly enzyme-immunoassay, chemiluminescence, fluorescence/enzyme-immunoassay and immunoblotting assay (158). Allergens are attached on allergo-sorbent solid phases (i.e., sephadex or paper; polymer of cellulose enclosed in a capsule, streptavidin-covered polystyrene ball conjugated with streptavidin) or liquid-phase labelled allergen contained in reaction vessels (36,165). The patient's serum will be added and incubated. If this serum contains allergen-specific IgE, this will bind to the allergen-linked solid phase (see **Figure 3**). After washing to clear out unbound antibodies from the serum, a labelled (i.e., with a chromophore enzyme, fluorophore or radioiodine) human anti-IgE-Fc is added to bind to the allergen-specific IgE from the patient that is bound to the allergen. For the process, buffer solution for constant pH values are used. The detection of this human anti-IgE will provide the result for this assay by using a Calibration system and a Data processing system.

The response/signal measured is proportional with the quantity of allergen sIgE from the sample that has bound to the allergen. Variations against the different manufacturer's assays are found on the solid phase used, on the allergen concentration, or on the detection system (2). Thus, test results are not always equivalent and direct comparisons between test outcomes must be handled with care (158,166). No internationally accepted calibration standards exist for sIgE (36). A single calibration based on total serum IgE is used to interpolate sIgE from all allergens (Heterologous calibration based on total IgE) when both total and specific IgE are diluted in parallel, although allergen-specific calibrations can be done. It is based on a reference calibrated curve for total IgE to the official WHO standard (WHO 11/234) generated for each assay. In general, sIgE levels range from a limit of quantification of 0.01 kU_A/L to 100 kU_A/L. Thus, serum with higher sIgE has to be measured on a diluted form to be quantified (36,158,166). IgE distribution is not linear but logarithmic. Some assays show that sIgE levels heterogeneously calibrated proportionally correspond to total IgE, for this reason, can be compared to improve proportion. The ratio sIgE to total IgE can be particularly interesting for *in* vitro tests interpretation when total IgE levels are extremely low or high (167). This ratio is also found on effector cells and when total IgE values are normalized (given in relative, i.e., ratio sIgE to total IgE in percent). Kleine-Tebbe and Jakob (36) explained that, by doing these calculi, a better concordance between relative (%) sIgE proportion and the quantitative analysis of sensitization tests as BAT and SPT can be expected.

Figure 3. General scheme of immunoassays used for the measurement of allergen specific IgE in serum.



In some cases, a reverse IgE immunoassay can be performed to overcome interferences of other serum antibodies. For example, in patients receiving immunotherapy non-IgE isotype antibodies such as IgG are found in serum samples could produce a competitive inhibition giving false results. All, or at least almost all, IgE antibodies from serum are captured by a solid-phase in which anti-IgE is found in molar excess. Following this step, allergen sIgE is detected with a quantified amount of labelled allergen. The advantage of this assay is the measurement of mainly high-affinity sIgE which presume to be more relevant compared to classic immunoassays. It requires important amounts of anti-IgE to bind all the IgE from the serum and show an important bias due to the fact that depends on the fraction of specific IgE (8).

CELL-BASED ASSAYS

- BASOPHIL ACTIVATION TEST (BAT)

Basophils can be found in peripheral blood while mast cells are localised in tissues, making blood basophils an accessible sample (168). BAT shows the degranulation/cell activation after stimulation with increasing concentrations of the allergen by flow cytometry, through the expression of basophil activation markers like CD63 and CD203c in cell surface. Basophils can be identified by flow cytometry with different antibody combinations as: CD123+/HLADR-, CRTH2+, CD203c+, or CD193+. CD63 is the most validated marker although CD203c has demonstrated to be a solid test (169). CD63 is found inside mast cells and basophils as well as in the same lysosome secreting histamine (168,170). Although its role is not well understood it is directly correlated with

the release of histamine into the cell supernatant (171,172). CD203c can be used for basophil identification and also activation marker, and its expression increases after cell manipulation or non-degranulating stimulation (173). It has been observed a variation on CD203c reactivity during the day which has to be taken into consideration when blood sampling (174).

The expression of CD63 has been shown to directly correlate with histamine release and it is considered to be the more clinically validated activation marker (170,171,173).

Some variables can significantly affect the dose-dependent basophil activation and should be taken into consideration for analytical techniques (36): total amount of IgE bound to cells; the ratio of sIgE to total IgE (1% is enough for half-maximal activation of cells); the ratio of low- to high-affinity IgE antibodies; clonality (Number of epitope-specific antibodies); affinity (binding strength between allergen and sIgE); avidity (total amount of multivalent sIgE binding sites binding strongly to the allergenic molecule) (175). FcεRI receptors from the effector cells surface are stabilized by IgE (176) and so, the number of them and the amount of cell-bound IgE is regulated (177).

As BAT assess IgE cross-linking using live cells stimulated with the allergen, they can be more informative than sIgE measurement in serum in terms of IgE functionality and clinical relevance. Compared to provocation tests, BAT is presented as a good alternative, being more comfortable and safer for the patient, less invasive and cheaper. For this reason, its performance may be a good option when clinical data does not match with SPT or serum sIgE results (168).

BAT has demonstrated to be useful in indicating the phenotype of patients sensitized to food, insect venoms, drugs and for chronic urticaria. BAT shows higher specificity compared to IgE sensitization tests in food allergy (178,179) and is useful to monitor the response of immunomodulatory treatments or the natural resolution of food allergies. Nevertheless, it requires deep analytical and clinical validation, standard procedures and quality guarantee to insure reliability and reproducibility of the results (168,172). BAT confers two types of information towards the allergen, known as basophil reactivity (BR) and basophil sensitivity (BS). BR is defined as the proportion of basophils expressing CD63 in comparison with the negative control (% of CD63+ basophils) and supports the presence of biologically relevant IgE sensitization to the tested allergen (168). It is determined by the state of the basophil and the cellular response after the IgE signal. BS is determined by the BR and the affinity of free and bounded sIgE for the allergen (172). The response after graded allergen concentrations are fitted to a curve of reactivity vs allergen concentration from which will be determined the concentration of allergen producing 50% of the maximum response. BS can be expressed as EC_{50} or as CD-sens (the inverse of EC_{50} multiplied by 100) and it has been reported to correlate with

the severity of certain food allergic reactions (168,180). Both activation parameters (BR and BS) are distinct but interdependent and regulated by Syk kinases (168,181). The measurement of the area under curve (AUC) combines both parameters and it is useful when responses do not fit to the common dose-response curve, as in anergy states from the basophils in allergen immunotheraphy (AIT) (182). Finally, ROC curves are used on the analysis and validation of BAT with new allergens (172).

BAT not only assesses IgE cross-linking of IgE with FcεRI on the basophil surface, but also to non-IgE stimulus like the bacterial tripeptide N-formilmetionil-leucil-fenilalanina (fMLP) (which can be used as a positive control on the test) (168,183). The sIgE-FcεRI complex depends on the affinity of the sIgE for the allergen. Frequently produces a bell-shaped dose-response curve although can vary due to the complexity of antigens and allergen epitopes affinity for the sIgE, the density of epitope sIgE on the basophil cover and intrinsic characteristics of the basophils. These factors will vary the optimal allergen concentration to activate basophils among subjects (168). Functionality of basophils have to be ensured in all cases. Both IgE and non-IgE signaling controls (anti-IgE or anti-FcεRI, and fMLP, respectively) are used as positive controls of basophil reactivity.

When basophils only get activated after fMLP but not anti- IgE or FcεRI controls, are named as "non-responders", which is found in approximately 10% of the population, and the BAT outcome must be interpreted within this context. Moreover, non-sensitized donors should be tested with the allergen preparations used to prove specificity (168).

BAT results can be altered by multiple factors. The medication on the patient, as oral steroids (taken during at least 3 weeks before the test) and ciclosporin A should be avoided, but not oral antihistamines and topic steroids (184,185). The time between blood draw and the test performance should not be more than 4 hours, or ideally less than 24 h to avoid a decrease on reactivity (172). Exceptionally, it is known that is possible to obtain a positive result after 2 days (186). Moreover, blood samples should be collected for no more than 1 year from the last exposure to the allergen (172,187,188).

Basophils are preferentially tested in heparin-stabilized blood. Blood stabilized with EDTA or acid-citrate dextrose show limited basophil degranulation unless after adding calcium (189). The material used for the stimulation are preferable standardized extracts, recombinant or native purified allergens or fresh parenteral drugs solutions. It will be prepared following standard procedures at non-toxic concentrations for basophils (less than 1%w/v) and more than four sequential log dilution responses should be done (172). Finally, the staining antibodies used and the flow cytometric analyses should be taken in consideration (168).

- MAST CELL ACTIVATION TESTS (MAT)

It is known that basophils are effector cells involved in allergy disease, however, mast cells (MC) are considered to be the main effector cells in the allergic response (190). For this reason, a novel mast cell-based assay (MAT) has been developed with the aim to improve the diagnosis of IgE-mediated allergy assessing MC reactivity towards the allergen *in vitro*. This test consists on PBMCs isolation from human peripheral blood. Posteriorly, CD117+ progenitor cells are magnetically separated and cultured till mature MC are obtained. Mature MC are incubated overnight with patient's serum and later, activated with the allergen. Degranulation is quantified by measuring CD63 upregulation by flow cytometry. Bahri and colleagues (191), described in 2018 a reproducible and robust MAT protocol. They could demonstrate allergen-specific and dose dependent degranulation (for (CD63 and CD107a) for peanut, grass pollen, and Hymenoptera (wasp venom) allergy. Other studies have demonstrated MAT specificity on the diagnostic of peanut allergy (192). Alternatively, MAT can be performed using the cell line LAD2 (193).

1.2.6.3 Serum Tryptase

The serum tryptase quantification is used to diagnose allergic reactions related with acute mast cell activation, such as anaphylaxis. The concentration of tryptase in mast cells is 300-700 times higher than basophils (2). In anaphylaxis, serologic levels increase from 30 minutes to 4 hours after symptoms. Besides, in life-threatening reactions can be found 24 hours after the reaction (2,194). The EAACI guidelines (70) recommend its measurement 30 to 120 minutes after the reaction has started, and at least 24 hours after symptoms resolution for baseline tryptase evaluation. Although it is a specific test which will not be useful to make an emergency diagnostic it will let the confirmation of the anaphylaxis diagnostic. If the levels of serum tryptase (from 30 to 120 minutes after the beginning of the reaction) increase (1.2 x baseline) +2 μ g/L would confirm the diagnosis of anaphylaxis (70,195,196). However, some anaphylactic events can be produced without detectable tryptase levels, particularly in children and food-induced episodes (195).

1.2.6.4 Oral Challenge

As said earlier in the text, *in vitro* and *in vivo* testing identifies sensitization, when clinical history is not conclusive, oral challenge despite its drawbacks is required. The Double-

blind placebo-controlled food challenge (DBPCFC) is considered the gold standard on FA diagnosis.

It consists of the oral administration of the food which is suspected to be the cause of the FA, under standardized and controlled conditions. Its objective is to prove if a certain food is the origin of the allergy (197). Also, can be used to assess tolerability after food allergy resolution as well as identifying the limit dose of responsiveness (197,198).

This process requires a wide logistic planification which includes healthcare facilities (doctor, nurse, adapted hospital environment). The challenge has its own risks of inducing an allergic reaction that can be severe. Before starting the oral food challenge (OFC), the patient should avoid the challenged food at least, two weeks before and being stable in the basis of allergic disease (not having had an allergic reaction) (198). It is preferably to perform the challenge under fastening conditions (198) and physician should take in consideration that the reintroduction of long-time excluded food to which IgE is detectable may produce severe reactions (199).

1.2.7 Molecular Allergy

1.2.7.1 Component Resolved Diagnosis (CRD)

In 1999, Valenta et al. (200) described the concept of component-resolved diagnosis (CRD), based on the use of individual recombinant-allergen (either recombinant or naturally purified) for diagnostic purpose. Until then, allergy diagnostic had been based on the use of allergen whole extracts.

Allergen extracts are generally aqueous preparations of the unprocessed allergen source. Frequently, performed at neutral or close pH, deffated and dialysed (8). When diagnostic is based on allergenic extracts, an important variability can be found between manufacturers (201–203). Depending on the standards used for the extraction, the composition from the extract can variate (130). Due to their physic-chemical characteristics, some important molecules (as prolamins) are lost during the process of extraction resulting on very low concentrations of allergen if the manufacturer does not add an extra amount into the extract (204). In addition, lipid soluble allergens are optimally extracted at neutral pH while food allergens whose exposure route is the stomach (with low pH) are not properly represented (8,205,206). In the EU, commercialized allergen extracts are standardized by measuring the complete IgE binding potency (but not from specific allergen). For this reason, it is not always possible to compare or interchange data from extracts from different manufacturers (130).

During the last decades, allergology has accomplished a technological revolution and the identification and characterisation of new allergens has increased. A large number of allergens named as components (synonyms: allergen molecules, single allergens), have been generated by molecular biology techniques, found as *recombinant* (expressed as "r") or *purified* from the allergenic source obtaining native molecular proteins (expressed as "n") (207).

To improve allergy diagnosis, components have been introduced in most immunoassays. This new approach offers a more precise diagnostic and may aid the clinical management of the patients, towards "Precision allergology". CRD may allow a deeper phenotyping and thus personal treatment of allergic patients (8). CRD-based assays permit an extensive analysis of the sensitization and cross-reactivity profiles of allergens, improving the efficiency of IgE mediated allergy diagnosis.

What can be statistically associated to less than 1% of the population can be critical for an individual patient, highlighting the importance of detecting the focus on these patients. For example, Pru p 3 should not be used as the "marker" to diagnose allergy to all LTPs: it has been revealed individual sensitization patterns to LTPs from different sources with molecular tests (208). The recent advances suppose a challenge for the traditional diagnosing strategies, optimizes the selection of food oral challenges, evades life-threatening reactions and improves food avoidance recommendations. CRD aids at an initial diagnostic either monitoring IgE levels on allergy-resolution processes (207). In addition, it is related to a better assay performance (sensitivity and specificity). Low abundance molecules from extracts can be represented on molecular profiles (8) although extracts contain some allergenic molecules not available on an isolated form, as an example, TLP Pru p 2 in peach extract (209). Molecular diagnosis has frequently the risk of overinterpreting the results, nevertheless, like allergological studies *in vitro* based on whole extracts, CRD (210) provides a sensitization profile which needs to be contrasted with clinical history.

The use of components (either native or recombinant) can improve the clinical performance of immunoassays in different ways. In some cases, recombinant proteins differ on their structure when compared with the natural one because of the presence of incorrect disulphide bridges or the absence of post-translational modifications (130). Each has its own advantages and disadvantages. While recombinant allergens can be synthetized with CCDs or not depending on the production system used, purified allergens will contain a mix of CCDs and isoforms. As Matricardi *et al*. (8) explain, allergenic components can be used as a mixture of all the available components from a common allergenic source in substitution of the natural allergen extract mixture, however its benefits are questionable, since this may not include all relevant allergens

and it would be expensive. The common use of allergen molecules is individually for allergen-specific IgE detection in singleplex (as individual reagents) or in multiplex immunoassays. Another option is the use of equal mixtures of selected molecular allergens representative of allergen specific sensitizations for sIgE detection. Individual allergenic molecules have also been added to an allergenic extract to increase its sensitivity and enhance its limit of quantification (spiking) (8). Noteworthy, molecular and extract-based diagnosis can coexist and so, discrepancies between extract and component sIgE results (qualitative and quantitative) sometimes occur, due to multiple factors. In some cases, the allergen extract is positive but its molecular allergens are negative, if the offending allergen is not available in a single format but also, when low sIgE levels, the Limit of quantification (LoQ) of the extract is lower than the molecule. In some others, the extract is negative while the allergen molecule is positive, if some molecules are underrepresented on the extract or the sensitivity is lower in the extract (higher LoQ) compared to the molecule, if sIgE levels are low. Also, allergen extracts can be positive, but their components are negative, when recognition profiles are limited to minor and highly cross-reactive components but not major ones on panallergen or CCD sensitized. In some patients the extracts show lower sIgE than the components: this can occur on highly sensitized patients as extract-based immunoassays contain limited amounts of molecules. Consequently, the total amount of individual sIgE components is higher than the corresponding extract sIgE (lower levels on extracts). This may also be produced when exist partial cross-reactivity between allergens from different protein families. In conclusion, coexistence of both diagnostic tools is still required today, but needs to be wisely chosen and interpreted (8). Finally, two principal trends on the use of CRD on allergological work up are seen: some professionals support the integration of the new techniques to the traditional ones (top down) while a second group defend CRD as a unique strategy (bottom-up). On the first case, diagnosis is based on singleplex (one assay per sample) performed by doctor's choice after clinical examination and anamnesis. The second one uses multiplex (various assays per sample) microarrays, which provide a wide sensitization profile of the patient, before asking for corresponding symptoms. The EAACI suggested to combine them both in a "U-shape" methodology. Clinical history and physical examination are done and SPT or sIgE detection with extracts. According to all this information, sIgE to selected molecules are examined and cross-reactions will be detected in this last step (211).

1.2.7.2 Singleplex and Multiplex Immunoassays

Evaluation of sensitization, i.e., allergen sIgE, in serum can be approached by using singleplex and multiplex immunoassays, with whole extracts and/or components.

Singleplex immunoassays consist in the measurement of one analyte per analysis while multiplex allow two or more analytes to be tested in one single analysis.

The singleplex analysers are the most frequently used for the high throughput allergological routine work up. They use total IgE calibration curves, have good precision, reproducibility and their limit of quantification is 0.1 kU_A/L.

The IMMUNOCAP® SYSTEM (Thermofisher Scientific, Uppsala, Sweden) is one of the most frequently used worldwide *in vitro* automatized singlepex immunoassay for the measurement of allergen-specific IgE in serum, largely validated with clinical practice. It is a fluorescence enzyme-immunoassay which has a high efficiency allowing different loads of samples depending on the equipment (158). It has been frequently used as a reference assay when testing new diagnostic platforms (120,212). Its solid phase is based on a three-dimensional encapsulated cellulose polymer on which the allergen is linked by a covalent bound, designed as a sandwich assay (213). ImmunoCAP® quantifies sIgE with a 0.01 kU_A/L detection limit, although 0.35 kU_A/L has been traditionally considered the positivity cut-off for most allergens.

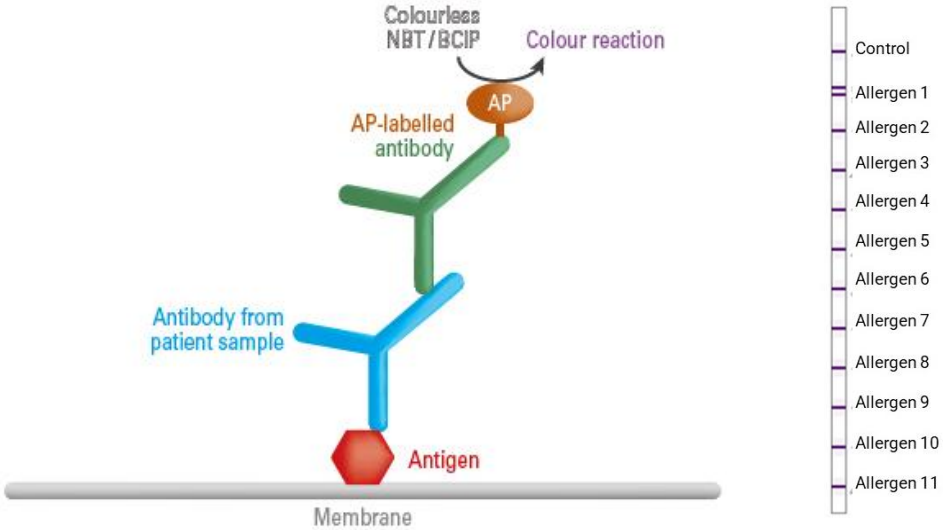
Recently, single allergen tests (*singleplex*) have been followed by chips/microarrays containing multiple allergens using a little amount of patient's serum (*multiplex*). Multiplex assays permit in a single analysis the individual detection of various specific antibody isotypes and its semi-quantification (214). Noteworthy, these new systems with multiple components and/or extracts from different sources and different protein types available facilitate the detection of cross-reactivity profiles in patients. The quantity of antibody detected depends of several factors: IgE and non-IgE antibody concentration, epitope specificity, affinity and IgE specific activity among others (8,214,215). **Table 3** (extracted from Matricardi *et al*. (8)) describes the most relevant advantages and limitations of both singleplex and multiplex assays, respectively.

The IMMUNOCAP ISAC® - ImmunoSorbent Allergen Chip (Thermofisher Scientific, Uppsala, Sweden) was the first multiplex assay launched to the market around 15 years ago. The current version consists of an allergen biochip containing 112 molecular allergens from 51 allergenic sources (216) bound on a glass slide array. The allergen molecules are dotted in triplets and immobilized on a polymer coated slide by covalent bindings. When the sample contains sIgE antibodies, those will bind the immobilized allergens. This complex is detected by a fluorescence labelled anti-IgE antibody, quantified by a laser scanner. It is a semi-quantitative test and results are given in ISAC Standardized Units (ISU), providing sIgE levels ranging from 0.3 to 100 ISU (217). On respect to LTPs, the allergens of interest for this thesis, Nine LTPs are available on it: Ara h 9 (peanut), Cor a 8 (hazelnut), Jug r 3 (walnut), Pru p 3 (peach), Tri a 14 (wheat), Art 3 (mugwort), Ole e 7 (olive tree), Par j 2 (parietaria), Pla a 3 (plane tree). The ImmunoCAP

ISAC® has lower sensitivity compared to ImmunoCAP®, given the lower amount of allergen bound to the solid phase (218).

The EUROLINE® immunoassay (EUROIMMUN AG A PerkinElmer company. Lübek, Germany), which plays a special role in this thesis, is a multiplex system to detect sIgE by immunoblot. It has been developed for a wide range of allergic profiles. It requires only a small amount of serum to assess sensitization to a wide number of allergens, automatization is possible although not mandatory (158). This multiparameter test provides semiquantitative information by measuring bands intensity corresponding to allergen specific IgE antibody given in system classes 0 to 6. The classes from the Enzyme-Allergo-Sorbent Test (EAST) are transformed into concentrations expressed in kU_A/L: **Class 0:** <0.35; **Class 1:** ≥0.35 to <0.7; **Class 2:** ≥0.7 to <3.5; **Class 3:** ≥3.5 to <17.5; **Class 4:** ≥17.5 to < 50; **Class 5:** ≥50 to <100; **Class 6:** ≥100. A membrane on which allergens are coated is used as a solid phase (**Figure 4**). When the sample contains specific antibodies, these will bind the allergens. An alkaline phosphatase (AP) labelled antibody (conjugate) binding the allergen-membrane complex, catalyses a colour reaction with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). If the sample contains antibodies, a dark line will appear on the particular allergen place and its intensity will be proportional to the IgE antibody concentration from the tested sample. Each membrane strip contains a control band which indicates that the test has been performed successfully. It can be performed either semi or fully automated or manual (219).

Figure 4. Euroline® multiparameter assay principles and strip representation.



From EUROIMMUN AG website (219).

Table 3. Advantages and limitations of singleplex and multiplex assay for sIgE testing.

	Singleplex IgE Antibody Assay	**Multiplex IgE Antibody Assay**
Performance Related Advantages (pro)	▪ Increased assay analytical sensitivity (lower Limit of Quantitation, LoQ) ▪ Potentially more precise quantitation and precision, facilitating comparisons between different allergen reagents (extracts vs. molecules) ▪ More established internal and external quality control measures (proficiency testing)	▪ Increased speed of analysis and reduced result turnaround time ▪ Conservation of sample volume facilitating paediatric testing
Assay Design and Cost Related Advantages (pro)	▪ Traceable of allergen-specific IgE values to a total human IgE International Reference Preparation ▪ Similar units for total IgE and allergen-specific IgE due to heterologous calibration (permits calculation of allergen-specific IgE/total IgE ratio) ▪ Global availability in many countries ▪ In case of limited number of samples more cost efficient ▪ Minimizes unneeded testing	▪ Greater simplicity ▪ Reduced cost due to fewer required reagents ▪ Reduced technician intervention ▪ Optimal design applications for point-of-care tests
Performance Limitations (con)	▪ More costly due to increased need for reagents ▪ More technical intervention ▪ Limited answers in case of few samples per subject ▪ Expensive in case of large scale screening (i.e., multisensitized subjects)	▪ Potentially lower analytical sensitivity for each analyte specificity measured (higher limit of detection, LoD) ▪ Reduced ability to accurately quantitate each IgE antibody ▪ Encouragement of abusive testing which involves the measurement of unwanted or unneeded IgE antibody specificities
Assay Design and Cost Related Limitations (con)	▪ More serum required, particularly in case of many samples ▪ Potentially slower analysis ▪ Likely more sophisticated assay format	▪ Less global availability ▪ Cost of the new instrumentation and reagents ▪ Greater challenge in managing different levels of non-specific binding ▪ Enhanced challenges in optimizing, balancing and standardizing assay reagents and assay quality control ▪ Potential greater inter-lot variability

From Matricardi *et al.* (8).

Recently, another multiplex assay has been developed, ALEX² Allergy Explorer (MacroArray Diagnostics GmbH, Vienna, Austria) which currently measures total and specific IgE against 295 allergen molecules and extracts. Allergens bound to nano-particles are shaped on a solid-phase, conforming a macroscopic array. If the sample contains antibodies, these will bind to the allergens. An IgE-antihuman labelled enzyme will chain the allergen-sIgE complex before adding a substrate. A coloured precipitated reaction will be proportional to the sIgE concentration on the sample (colorimetric test) (220,221).

Diagnostic tools in allergy try to reproduce the *in vivo* conditions, when sIgE binds to the antigenic epitopes from the allergens. Nevertheless, it is not fully possible to reproduce in experimental conditions what occurs in live organisms. sIgE will bind allergens when chemical and physical conditions are allowed: protein conformation, chemical groups charge and the access to the targeted epitopes influenced by environmental factors. For all this, combining different conditions and procedures would increase the number of epitopes available for sIgE detection (130).

1.2.7.3 Imunoassay Performance Evaluation

To test the efficacy of a method, sensitivity and specificity parameters have been described as useful variables to analyse the performance and predictive capacity to detect the presence and severity of clinical diseases. Sensitivity and specificity performance of diagnostic tests are usually depicted as receiver operating characteristics (ROC) curves.

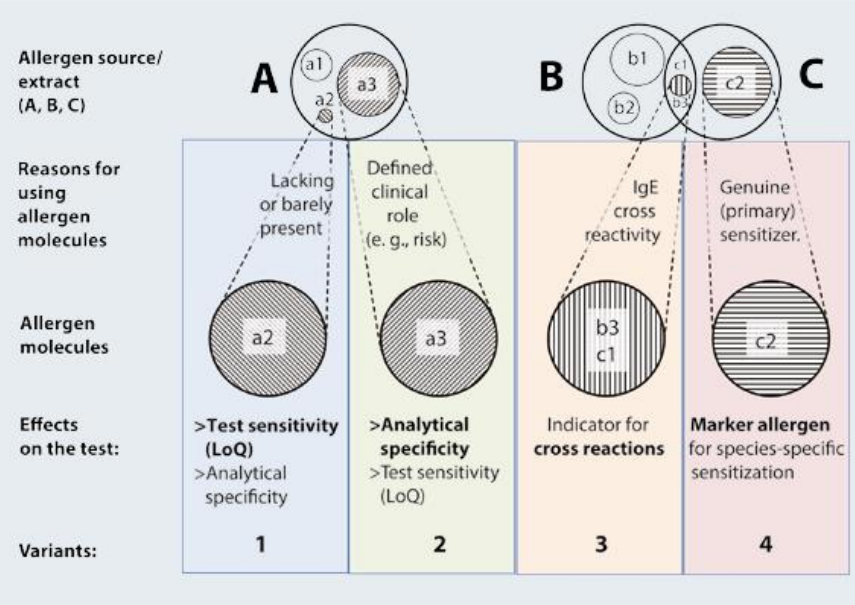
The analytical *sensitivity* would be the lowest cut-off and represents the slope of the calibration curve of the assay (8,36). It is provided by harmonized variables:

- **Limit of blank (LoB):** signal of the sample without the analyte of interest (i.e., sIgE).
- **Limit of detection (LoD):** signal of the lowest detectable sIgE in the sample.
- **Limit of quantification (LoQ):** signal with the lowest sIgE in the sample with a predefined precision.

The analytical *specificity* considers a targeted, selective sIgE detection (discarding the detection of antibodies of other classes). It is increased with the use of single molecules on which are only detected sIgE (on the use of allergen extracts an entire IgE repertoire are used). This would not only increase specificity but also sensitivity (and decrease LoQ) as well as indicate serological cross-reactivity and mark genuine (“primary”)

sensitization (summarized in **Figure 5**). These analytical characteristics described and their capacity of predicting the clinical relevance or severity introduce two new terms: diagnostic sensitivity or specificity (154).

Figure 5. Universal arguments for the methodological use of components.



From Jakob *et al.* (218).

Besides the diagnostic efficiency of the diagnostic tool (i.e., analytical sensitivity, specificity and other parameters) an individualized interpretation based on the clinical symptoms must be done to get what is known as a clinical diagnostic criterion. Isolated molecular-based sensitization results are not enough to make proper predictions on the clinical outcome, some items must be taken in consideration: sensitivity of the test (proportion of positives among allergic individuals), specificity (proportion of – in healthy individuals), clinical cross-reactivity indicators (symptoms not caused by the primary sensitization), clinical reaction predictions (positive predictive value, negative predictive value, thresholds, likelihood ratio…) (8).

Using allergen molecules on laboratory tests may increase the proportion of positive tests on allergic population and the proportion of negatives in healthy individuals. Moreover, it might resolve cross-reactive and, in some cases, reflect clinical symptoms to sources that did not cause the primary sensitization and improve prediction markers as positive predictive value (PPV), negative predictive value (NPV), likelihood ratios

(LR), etc. Furthermore, they may improve the identification of specific reactivity patterns that could be related to different symptoms severity (8,36).

The key question on allergy diagnosis *in vitro* tests is: "which is the clinical relevance of the sIgE values detected by the assay?". A positive IgE result is not a proof of allergic disease and will only be relevant when related with symptoms from anamnesis or at challenge (8).

Importantly, allergen specific IgE levels provided by the immunoassays are not predictive of clinical reactivity or reaction severity, despite conflicting results have been published (51,222,223). Nevertheless, high sIgE concentrations correlate with increased risk of reactions (224). The importance of stablishing sIgE cut-offs to provide reasonable clinical indications in the assessment of food allergy has been extensively reported (225–227). The cut-off for most common immunoassays used to quantify serum sIgE (e.g., ImmunoCAP® ThermoFisher Scientific, Uppsala, Sweden), has traditionally been set at 0.35 kU_A/L; and it is still used in many clinical lab settings, despite the reports showing that the cut-off may differ depending on factors such as the allergenic source and patient age (226) and the technical detection limit is 0.010 KU_A/L. Little evidence has been reported on the clinical relevance of sIgE levels between 0.1 and 0.35 KU_A/L and it is matter of discussion in the field.

1.3 LTP Syndrome

1.3.1 Location and function

Non-specific Lipid Transfer Proteins (nsLTP) play a role in lipid transport across cell membranes and plant defence, for which have been classified as type 14 pathogenesis-related proteins (PR-14). nsLTPs present antimicrobial activity against pathogenic fungi and bacteria from plants and antiviral activity (60). Thus, nsLTPs are gene outcomes produced by the plant which are secreted and assembled on the external part of cell walls in aerial organs (LTP Type 1) or roots (LTP Type 2) (228). In fact, they can be expressed in epidermal tissues all over the plant: seeds, stems, fruits, flowers, roots, leaves or siliques (60). nsLTPs can be found on a wide range of allergenic sources such as fruit but also grains, nuts, vegetables, pollens or natural rubber latex (60). It is particular the fact that in kiwifruit (Act d 10 and Act c 10) and tomato (Sola l 6 and Sola l 7) LTPs are found on the seeds next to the pulp (208,229) while for most plant-foods is more usual to find nsLTPs in fruit's peel (where more abundant) and pulp. Heretofore, many nsLTPs have been described as food allergens but inhalant LTP-allergens have also been found in tree and grass pollen with clinical relevance (230). Until September 2023, in the Allergome database (http://www.allergome.org/), 183 LTPs and its isoforms have been registered.

Plant foods show variable concentrations of nsLTPs, depending on cultivar, storage, and maturity conditions (8). In peach cultivars, Pru p 3 (peach LTP, *Prunus persica*) amount varies depending on harvest and ripening moments, but also due to climatic conditions behaviours and phytochemical treatments, when compared different cultivars from different years (231).

1.3.2 Structural characteristics

nsLTPs are polypeptides of 91-95 aminoacids with a conserved motif of eight cysteines. Two types are identified according to their molecular weight: 7 kDa (nsLTP type 1, LTP1) and 9 kDa (nsLTP type 2, LTP2). nsLTPs (see **Figure 6**) also contain compact domains composed of 4 α-helices with rigid tertiary structure, held by 4 conserved disulphide bounds and a compact folding which forms a hydrophobic cavity through the whole molecule able to accommodate distinct ligands (8). Most of the allergens belong to LTP1 family (228,230). nsLTPs are distributed in different phylogenetic families and some LTPs share about 30% sequence identity, although tertiary structure (which would characterise the conformational epitope) is strongly conserved (60,230,232). Due to these

characteristics and the frequently reported cross-reactivity, LTPs are considered panallergens.

Figure 6. Crystal structure of Pru p 3, the LTP from peach.

Image obtained from https://www.uniprot.org/ database. Published by Pasquato and collaborators in 2006 (233), obtained by a X-ray diffraction (2.35Å) method.

The disulphide bridges from nsLTPs are responsible of their resistance in low pH, heat treatment and digestive proteolytic attack, which has been related with the induction of severe symptoms in many patients (8,102). The resistance of Pru p 3 over pepsin and chymotrypsin compared to trypsin has been reported (234). That last one, was able to generate different peptides: high molecular weight ones (full protein, still containing the disulphide bridges) which could be recognised by patient's sera, and small molecular weight ones which were not reactive. It is known how food-processing can affect allergenicity and antigenicity on foods and that physical processing may affect three-dimensional structures destroying epitopes or generating new ones. Nevertheless, it was demonstrated that microwaving at 140ºC and ultrasounds over peach peel did not reduce Pru p 3 sIgE binding (235).

1.3.3 LTPs Allergenicity and LTP Syndrome

LTPs are the chief allergens from the *Rosaceae* family, specially from the *Prunoideae* subfamily which would include peach, plum, cherry and apricot (58). **Pru p 3**, the main allergen of peach was the first nsLTP identified and characterized (236,237). In the

Mediterranean area, Pru p 3 is considered the prototype marker for LTP syndrome (later described) and the verified sensitizer for LTP allergy (230). Actually, LTP sensitization in Southern Europe is ruled out by testing Pru p 3 (238). It seems to be in some cases a precursor for other LTP sensitizations (239–241). It is the most frequently recognised allergen in the nsLTP family being Pru p 3 sIgE levels frequently greater than for other LTPs (230). However, although not frequently, some patients recognise other LTPs without being sensitized to Pru p 3 (8), i.e. Tri a 14 (242–244).

Pru p 3 allergen is a basic protein of 91 aminoacids with a molecular weight of 9.178 kDa (8). Three major conformational IgE epitopes on Pru p 3 were recognised by García-Casado in 2003 (245): Pru p $3_{11–25}$, Pru p $3_{31–45}$ and Pru p $3_{71–80}$ which would be responsible of cross-reactivity. These are shared with other fruits (apple, apricot, plum, cherry, orange, strawberry, grape) with a sequence identity from 62% to 81% (102,245). Also, a high degree of sIgE cross reactivity has been observed between *Rosaceae* fruits and citrus fruits, grape, tomato, vegetables (asparagus, lettuce), nuts, corn, onion, carrot and rice and spelt (partial) (102). Moreover, two immunodominant T-cell-reactive regions (Pru p $3_{12–27}$ and Pru p $3_{57–72}$) which induce IL-4 production after allergen stimulation have been described (246). These peptides maintain the ability to sensitize or induce allergic reaction, binding IgE and T-cell, even after thermal and proteolytic treatments (234,247). R39, T40, and R44 residues of Pru p 3 epitopes were demonstrated to be necessary for binding with sIgE (245).

The peach peel contains a 220-fold major proportion of Pru p 3 than the pulp, especially the peach fuzz (8,248). Additionally, when peach extracts from pulp and peel on a proteomic assay are compared: aminoacid sequences were identic for peel and pulp, although two methylated sites on the R_{18} and R_{32} from the arginine residues from peel Pru p 3 were found. These modifications may alter their binding ability and modulate their biological functions (249).

On the other side, Pru p 3 contains a ligand composed by an alkaloid bound to a lipid tail (phytosphingosine) collocated into the Pru p 3 cavity. It was suggested that this lipid-ligand carrier from the Pru p 3 could play a critical role as an adjuvant modulating the T_H2 response (250). Pru p 3 without the lipid-ligand was able to induce sensitization but the Pru p 3 lipid-ligand complex induced a stronger IgE production in mouse models. This would intensify the susceptibility to peach allergy and a lower grade of Pru p 3 exposure would be enough to induce sensitization (250).

Due to the broad IgE cross-reactivity among LTPs from different allergenic sources, patients being sensitized to multiple LTPs from non-related botanical sources is common, designating this phenomenon as **LTP Syndrome** (251). Some authors consider

that Pru p 3 must be present, followed by two more foods non-taxonomically related (51).

In contrast, botanically unrelated LTPs, as nuts and cereals, seem to be related to LTP sensitization and severe symptoms without Pru p 3: Tri a 14, the wheat LTP has been related to exercise-induced anaphylaxis (252) as well as Baker's asthma (243). Moreover, Bernardi *et al.* reported (208) the presence of heterogenicity in kiwi fruit epitopes. Over new LTPs from kiwifruit, the absence of conserved sequence regions in all LTP1 tested was reported, discarding the idea of one identical epitope existence, particularly when sequence identity is low. A micro-heterogenicity on the sequence was observed, mainly in distantly related LTPs which could be the reason of heterogenic epitope patterns and IgE recognition.

1.3.4 Geographical distribution and prevalence

Considering patients sensitized exclusively to LTP allergen, **LTP syndrome** would be the most frequent type of food allergy in the Mediterranean Basin, in adults and adolescents (49,253). Nevertheless, LTP sensitization has been reported around the world: China (254,255), Australia (256) and central Europe (257–259). Moreover, in Spain and Italy, geographical prevalence distribution also varies between southern and northern areas (253). The reasons to explain variable sensitization prevalence among different geographical areas are still unknown. One hypothesis to explain this fact is the presence of homologous pollen-allergens producing primary sensitization in pollen endemic areas such as trees (plane tree and olive) and weed (mugwort, pellitory or ragweed) although their relevance is not clear (230,255,260).

More than 90% of vegetal food allergic patients in the Mediterranean area are sensitized to LTPs, for this reason, years ago, studies focused on LTP allergy were limited to Southern Europe. However, nowadays some case reports and studies using component-resolved diagnosis (CRD) outside this area, are increasing (230). LTP sensitizations out from the Mediterranean area are less frequent and those are considered minor allergens: many isolated case reports related with allergic reactions to LTPs have been published (256,259,261,262). Occupational allergies due to LTP have also been registered in non-Mediterranean areas, as Baker's Asthma related to Tri a 14 (wheat LTP) in central Europe (263). Reported data suggest that LTP sensitization outside Mediterranean basin coexists with an extensive reactivity to allergens from the same source which on storage proteins cases can induce severe reactivity: sensitization patterns to LTP differ from Mediterranean serotype (230). In birch endemic areas, patients presented co-sensitization to PR-10 and LTP while only a few were only sensitized to LTP (264). Also,

Cor a 8 (Hazelnut's LTP) in symptomatic children, negative to Pru p 3, has been reported in an area where birch sensitization was frequent (265). In contrast, in Japan, a low prevalence on Pru p 3 sensitization was related to consuming habits since the peel is frequently removed (266). Using CRD on studies allows a better understanding on sensitization profiles in non-Mediterranean countries.

1.3.5 Pollen influence on LTP sensitization

It has been suggested in some reports that, in addition to a primary sensitization through Pru p 3 followed by cross-reactivity with LTPs from foods and pollens, LTPs from pollen could also induce primary sensitization inducing cross-reactivity to food LTPs (267). It was proposed that LTP sensitization could be originated by the exposition to endemic pollens in Southern Europe and that the geographic difference on LTPs from pollen could be related with the different prevalence of FA induced by LTP (230). The pollen LTPs identified according the IUIS allergen nomenclature database are: plane tree (*Platanus acerifolia*, Pla a 3) and mugwort (*Artemisia vulgaris*, Art v 3) with more than 45% sequence identity with Pru p 3; pellitory (*Parietaria judaica*, Par j 1/2), olive tree (*Olea europaea*, Ole e 7), ragweed (*Ambrosia artemisiifolia*, Amb a 6), Thale Cress (*Arabidopsis thaliana*, Ara t 3) and field mustard (*Brassica rapa*, Bra r 3) with less than 35% sequence identity (102).

In the Mediterranean area, mugwort pollen-related allergy to peach has been studied in Art v 3 and Pru p 3 sIgE positive patients to define which of the two LTPs can be the primary sensitization agent. Reported results show that both allergens can induce primary sensitization in these patients (268–271). Sanchez-López J. *et al.* (272) showed that Art v 3 can induce respiratory symptoms. Moreover, other studies reported that Art v 3 and Pru p 3 were both allergens that could induce peanut allergy, compared to Ara h 9 (254). For plane tree, Lauer *et al*. (273) demonstrated that, although Pru p 3 plays an important role on LTP-allergy pathomechanism, Pla a 3 has demonstrated to be biologically active in Pru p 3 negative patients suffering from peach allergy, although being characterised as a minor allergen. Scala *et al*. reported (274) that respiratory symptoms mediated by Pla a 3 and Art v 3 were related with sensitization to LTPs from foods and Art v 3 could block IgE binding LTPs from foods in 50-100% of the tested subjects. More than 60% of their Pru p 3 negative subjects were positive to Pla a 3 or Art v 3, and frequently were mono-sensitized discarding other pollen allergens from plane or mugwort.

LTP-allergic patients in the Mediterranean basin, are frequently -sensitized to LTPs from plant foods (240). Contrarily, in Central and Northern Europe LTP sensitization goes along co-sensitization with birch pollen allergens which will cross-react, as explained (8,275). In Northern Spain, two clinical profiles of peach allergic patients were described: systemic symptoms after peach were present on Pru p 3 sensitized patients while OAS was related with the profilin from birch or Bet v1 family sensitization (276). For this reason, it has been considered the potential protective role of birch sensitization by observing in high birch-sensitization areas that LTP sensitization is low and conversely (51,274).

1.3.6 Clinical manifestations

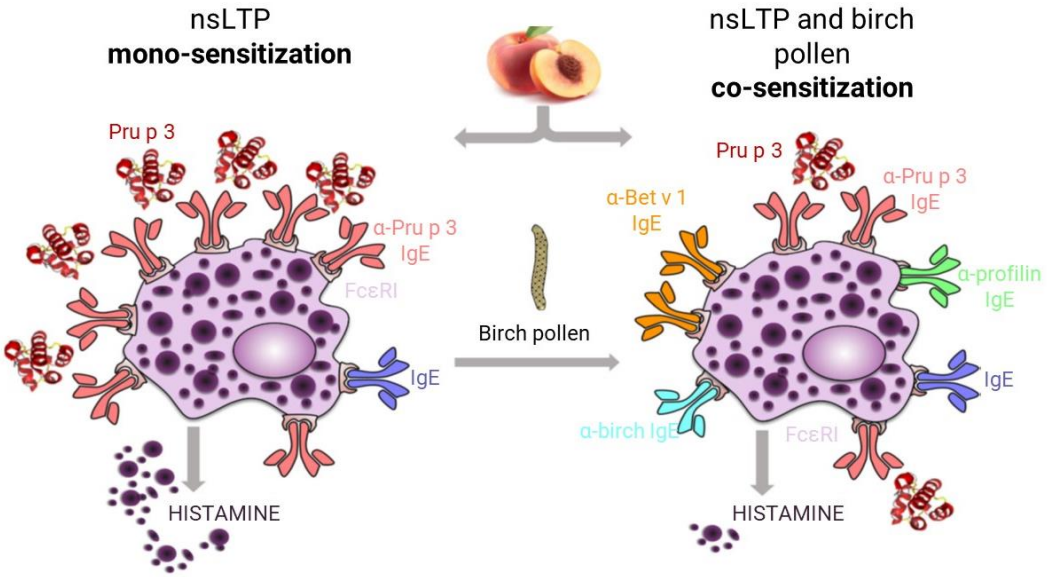
As exposed beforehand, three sensitization routes are possible for LTP: gastrointestinal tract, respiratory tract, and cutaneous. Differentiating them may be useful to predict clinical reactivity (240,250,277). First, Pru p 3, the peach LTP, seems to pass the intestinal epithelium by a fast-transcellular route, without a proteolytic mechanism neither altering the integrity of the tight junctions, inducing the production of alarmins (TSLP, IL-33 and IL-25). This would remark the importance of Pru p 3 as a gastrointestinal primary sensitizer (278). Secondly, LTP sensitization also seems to involve respiratory tract. Although some studies suggested Art v 3 and Pla a 3, as primary sensitizers in some patients as previously explained, inhaled Pru p 3 has been suggested as a primary sensitizer in airways (279). Also, the wheat LTP Tri a 14, is known to be involved *Baker's Asthma* respiratory allergy by respiratory tract sensitization (206,243). Third, LTP is notably plentiful in peach fuzz and peel which would have its logic considering that as the peel is a protective barrier from the fruit, the quantity of defence proteins is higher (248,249). LTP is especially found in fresh picked peaches and is mostly removed during washing and packing procedures. In Italy, it has been described that many employees working on these procedures have to wear gloves while working to avoid contact urticaria produced by peaches (57). Additionally, it was demonstrated in mice models that Pru p 3 is capable of inducing sensitization through skin (250) and the fact that many patients present an allergic reaction the first time they eat peach suggests that skin exposure could be a principal sensitization route to peach.

LTP allergy, i.e., LTP syndrome, is characterised for patients presenting a highly variable clinical expression, that can range from asymptomatic (280) to anaphylactic shock (260).

Contact urticaria (CU) induced by peach is the most frequent symptom as an isolated clinical presentation and a typical clinical manifestation from LTP allergy. CU was reported to be present in 60% of the patients, of a studied group in Spain (49) as well as

in Italy (281). OAS is also a frequent symptom in LTP allergy (49,51,282). Gastrointestinal symptomatology, increasing in prevalence and not always reported by patients if not specifically asked for, is usually followed by systemic symptoms. Often, LTP-induced symptoms are systemic and severe (276,283) especially when peel and pulps are both eaten by the patients (248). In Italy, LTPs were reported to be the main cause of food-induced anaphylaxis although the proportion of anaphylaxis cases compared to all LTP sensitized patients was lower than for other culprit foods (nuts or shrimp) (260,284). Additionally, mono-sensitization to LTP correlates with a more severe clinical reactivity (230). This could be explained by the fact that IgE receptors are mostly occupied by LTP sIgE which would induce a more efficient cross-linking of the FcεRI and effector cell activation (**Figure 7**).

Figure 7. Effects of Pru p 3 sensitization on mast cell activation.



Adapted from Scheurer *et al.* (230). Mono-sensitization (left) mediated by high density sIgE induce mediator release increasing the probability of severe reactions. Co-sensitization (right) tot non-related allergens induce less efficient mediator release.

It has been reported that severe symptoms are related to patients sensitized only to food LTP, without pollen sensitization, which might be a protective factor (247,274). Minor allergens as Pla a 3 or Art v 3, have been associated to complex high olive pollen prevalence, leading to a Pru p 3 sensitization, has been suggested (285). recognition patterns in LTP food-allergic patients (286) related with "pollen food syndrome induced

by LTP", presenting sensitization to Art v 3/Pla a 3 and Pru p 3 (287). Recently, the implication of Ole e 7 as a primary sensitization in regions with

Also, it seems that Pru p 3 sensitized children present symptoms in an earlier age compared to the ones sensitized to non-LTP pollen-related allergens and develop allergic reactions to a higher number of plant-foods (223). Interestingly, it has been reported that LTPs from fruits and vegetables could induce respiratory symptoms (288).

The influence on cofactors in LTP clinical reactivity is also known (51). LTPs have been reported to be the most frequent food allergens causing clinical reactivity induced by cofactors, Cofactor-enhanced food allergy (CEFA) (74). While some patients need a cofactor to present clinical manifestations some others present mild symptoms which are aggravated in the presence of the cofactor (51). Anaphylaxis seems to be the most frequent symptom on patients suffering from CEFA (74,289). Exercise seems to play a role in LTP sensitized patients on amplifying the severity of the symptoms (75) as well as alcoholic beverages and non-steroidal anti-inflammatory medicines (74). An association between chronic urticaria and intolerance to NSAIDs with LTP sensitization has been seen, in which both factors could make mast cells more excitable (290). Pascal, Muñoz, *et al*. (291) studied the response *in vitro* of NSAIDs in presence of Pru p 3, by using BAT. They reported higher reactivity and sensitivity profiles in basophils from patients without FDNIA and that NSAIDs would modify the basophil sensitivity (decreasing it) to Pru p 3 (291).

Clinical manifestations spectrum is variable and currently, the management of patients with LTP Syndrome is complex. It is seen in clinical practice that many food-allergic patients avoid eating specific foods although sensitization has not been proved. However, Asero R. *et al* reported in 2018 that more than 25% of their studied patients had had allergic reactions to previously tolerated foods concluding that would be reasonable to recommend to these allergic patients to avoid *Rosacea* and tree nuts, advising each patient on an individual basis (292). Cofactors presence could justify only a part of the patient's clinical reactivity (51) while pollinosis seems to be a severity protective factor (247,274).

High Pru p 3 sIgE concentrations correlate with increased risk of reactions (293). However, Pru p 3 sensitization has been related with severe systemic reactions in several studies regardless the sIgE values (284). Ciprandi *et al.* (294) described Pru p 3 sIgE levels variation as an age-dependent event, reporting an increase from infancy to young adulthood (highest from 21 to 30 years) and posteriorly decreased. It has been described that values are inversely related with early onset peach allergy (223). Similar results have been observed for other non-Rosaceae foods; patients with severe reactions showed low levels while asymptomatic ones had elevated sIgE (222). Moreover, Pastorello *et al*.

stablished 2.69 kU_A/L as a cut-off level on rPru p 3 sIgE to discriminate patients with risk of peach induced allergic symptoms (227), but other authors have found overlapped values between allergic and tolerant (222). Nevertheless, Pru p 3 allergic patients have also been reported with sIgE levels <0.35 KU_A/L ("low levels") (295) and also this is a matter of work of this thesis (296).

On the other hand, clinical implications between detecting one or more nsLTP sensitization is not clear, neither the relationship between sensitization profile nor clinical reactivity. A study in Barcelona in 2012 from our group (51), evidenced the high cross reactivity and wide distribution of LTPs. The patients reacted to a median of 4 foods from an extensive panel of plant foods all of them positive for Pru p 3. It was confirmed the difficulty of finding the culprit food, frequent in LTP syndrome. In 2015, Scala *et al.* (274) analysed reactivity profiles and clinical behaviour in a group of patients and reported that immunoreactive patients to five or more nsLTP (without PR-10 nor Profilin pan-allergens sensitization) were more likely to have systemic reactions. Interestingly, a previous study (245) identified on the levels of sIgE from LTP sensitized patients a predictable hierarchical order with peach at the top, followed by apple, walnut, hazelnut and peanut without correlation with the clinical reactivity to each food.

As Pru p 3, the peach LTP, has been considered the primary sensitizer in the Mediterranean area (238), the *in vitro* measurement of sIgE to Pru p 3 is frequently used in allergy diagnostics as a marker of LTP sensitization. As explained, although Pru p 3 shares many IgE epitopes with other nsLTPs, inhibition studies show that it does not diminish all IgE reactivity for other food and plant LTPs. By implication, not all LTP sensitizations can be detected with Pru p 3 (297,298). This finding draws attention to the need of a broader diagnostic approach including the testing of multiple nsLTPs. Today, one of the challenges for an accurate diagnosis and patient management of nsLTP-based food allergy is the somehow limited diversity of commercially available allergens to allow picturing a broad sensitization map of the patient and assessing its clinical relevance to guide diet avoidance and/or immunotherapy approaches.

This emphasizes the pressing need for further improvement of molecular diagnostic tools. Nowadays, multiplex immunoassays allow the screening for sensitization towards multiple components and/or extracts from different allergenic sources with low sample volumes at the same time.

Despite headways on the understanding of food allergy; knowledge in diagnosis, treatment or also epidemiology have some interrogates that should be answered. It is still not clear the management of Pru p 3-sensitized patients tolerating LTPs from foods. The variability over sensitization profiles on LTP -sensitized patients, the diverse cross-reactivity among LTPs and the wide spectrum on clinical manifestations and their

severity, enlighten the necessity of finding predictable sensitization clusters which could allow a better diagnosis but also phenotypes related to observable clinical manifestations from the disease improving a personalised treatment. An interesting field is whether sensitization profiles could be biomarkers predictive of disease progression.

Chapter II
Hypothesis

HYPOTHESIS

Patients with LTP Syndrome show a wide range of sensitization to plant foods taxonomically related or not, and a diverse profile of clinical symptoms, from mild to severe or even fatal. Component based *in vitro* diagnostic tools are useful on diagnosis and clinical management of LTP-syndrome patients. Nevertheless, a deeper knowledge and optimisation of current *in vitro* immunoassays could allow the identification of diverse phenotypical profiles among these patients offering a more personalised clinical management as well as improving their quality of life. Indeed, new diagnostic tools are needed to better manage the LTP syndrome patients. A good knowledge of the spectrum of sensitization and potential clinical relevance to LTPs of multiple allergenic sources in LTP syndrome would improve the therapeutic intervention of these patients.

Chapter III
Objectives

PRINCIPAL OBJECTIVE

The main objective of this thesis work is to improve on the utility of the *in vitro* tools used for the diagnosis of LTP syndrome as well as the clinical management of these patients.

SPECIFIC OBJECTIVES

Objective 1. To improve on the utility of the currently available *in vitro* tools for the diagnosis and clinical management of patients with LTP syndrome.

1.1. To assess the usefulness of considering low levels of Pru p 3 sIgE in the diagnosis of LTP syndrome.

1.2. To identify patterns of sensitization (clusters) to currently commercially available LTPs in LTP syndrome patients to aid at diagnosis and clinical management.

Objective 2. To improve the currently available molecular allergy based *in vitro* tools for the diagnosis and clinical management of patients with LTP syndrome.

2.1. To develop and validate a novel multiplex assay containing multiple LTPs from a wide range of taxonomically related and unrelated allergenic sources for the diagnosis and clinical management of patients with LTP syndrome (LTP-strip).

2.2. To describe the molecular sensitization profile of a real-life cohort of patients with LTP syndrome of our area using the LTP-strip.

Chapter IV
Material and methods

4.1 Patient selection

All patients included in this thesis project are adults and derive from the Allergy Department, ICR, Hospital Clínic de Barcelona. Allergological diagnosis in routine clinical practice consists of a detailed anamnesis, followed by skin test and/or *in vitro* tests, the later performed at the Immunology Department of the same hospital. For routine clinical practice, Pru p 3 (at least) is used for screening of LTP sensitization in the allergological work up.

To reach **Objective 1**, clinical and serological data from patients studied for suspicion of LTP allergy from 2012 to 2019 were retrospectively collected and analyzed after the approval of the Ethical Committee of Hospital Clinic (HCB/2020/0373).

4.1.1 Clinical Relevance of Pru p 3 sIgE Low Levels

To assess Objective 1.1, Pru p 3 sensitized patients (f420, ImmunoCAP® (Thermofisher Scientific, Upsala, Sweden), sIgE >0.1 kU_A/L) were selected (n=496) and classified in two groups: (**grLOW=group Low levels**) values from 0.1 to 0.34 kU_A/L and (**grB= group High levels**) values ≥ 0.35 kU_A/L. Data on sIgE to peach extract (f95) and other commercialised nsLTP sIgE by ImmunoCAP® (Thermofisher Scientific, Upsala, Sweden) were also registered when available: rAra h 9 (peanut, f427), nCor a 8 (hazelnut, f425), rJug r 3 (walnut, f442), rMal d 3 (apple, f435), rTri a 14 (wheat, f433). Given the reported possible interference of CCD sensitization to the detection of low levels of Pru p 3 sIgE, sIgE to MUXF3 (o214) was also evaluated. Sensitization to other plant food allergens was assessed by microarray ImmunoCAP® ISAC (Thermo Fisher Scienfitic, Sweden). Patients sensitized to other panallergens (PR-10; TLP; Profilin) besides LTP were excluded from analysis.

4.1.2 Cluster Identification on LTP Sensitized Patients

To satisfy Objective 1.2, a subset of patients that had available data on sIgE of all commercialised plant food nsLTP (tested by ImmunoCAP® system, n=307) were grouped and analyzed. Data on sIgE and clinical relevance on the following LTPs was included: rPru p 3 (f420, peach), rMal d 3 (f435, apple), rTri a 14 (f433, wheat), rJug r 3 (f442, walnut), nCor a 8 (f425, hazelnut) and rAra h 9 (f427, peanut) were selected. Patients sensitized to other panallergens (PR-10; TLP; Profilin) were excluded.

Patients were grouped according to the number of recognised LTPs, considering a 0.1 kU_A/L cut-off for sIgE: positive for 1 LTP (**Group 1**), positive for 2 LTPs (**Group 2**), positive for 3 LTPs (**Group 3**), positive for 4 LTPs (**Group 4**), positive for 5 LTPs (**Group 5**) and positive for all 6 LTPs (**Group 6**). The prevalence of the sensitization patterns detected as well as clinical relevance was evaluated.

4.1.3 LTP Strip - Multiplex Assay Evaluation

To reach the **Objective 2** and test the diagnostic performance of the newly designed LTP immunoblot patients diagnosed of LTP syndrome at the allergy clinic which accepted blood extraction and prick-by-prick testing for multiple allergenic sources were recruited. Inclusion criteria were (1) the presence of food-allergic reactions with at least two different plant-foods, not taxonomically related, (2) sensitization to LTPs from peach (Pru p 3) and/or hazelnut (Cor a 8) and (3) no sensitization to any non-LTP plant-food allergens (profilin, PR-10, thaumatin-like protein Act d 2 and storage proteins) tested with ImmunoCAP® ISAC and/or ImmunoCAP® (ThermoFisher Scientific). Healthy non-allergic individuals as controls were included: without clinical history of allergy disease, without food and respiratory allergen sensitization and with good tolerance to vegetal foods. The Ethical Committee of Hospital Clinic approved the study (HCB/2016/0361).

4.2 Clinical characterization

Demographic and clinical data was retrospectively recorded from clinical history for all the patients by experienced allergists from medical records and by patient interview in systematic questionnaires specially designed for each objective of the study (**Figure 8** for Objective 1.2. and **Figure 9** for Objective 2.1.). Allergy symptoms compatible with IgE food allergy were classified as: local (gastrointestinal symptoms –GI- including functional dyspepsia, crampy abdominal pain, nausea, vomiting and/or diarrhoea, Oral Allergy Syndrome –OAS- and contact urticaria -CU-) and systemic (generalized urticaria and/or angioedema –U/AE-, anaphylaxis-AN-). Tolerance (–TOL-) and avoidance (-AV-; due to medical advice, fear or dislike) were also recorded as well as the involvement of cofactors, including exercise, alcohol, non-steroidal anti-inflammatory drugs (NSAIDs) and/or menstruation. The time period accepted for considering a potential relationship with food-allergic reaction was 2 hours after food ingestion following clinical practice guidelines.

Figure 8. Anamnestic data questionnaire for the cluster identification study.

Plantilla de recollida de dades per l'estudi d'identificació de clústers en pacients sensibilitzats a LTP

Nom i cognoms: **NHC:** **Any de naixement:**

Has tingut alguna vegada símptoms ingerint el següents vegetals?																					
Fruites										Hortalisses							Verdures				
Plàtan		Kiwi		Meló		Raïm		Altres		Enciam		Tomàquet		Altres		Mongeta verda		Altres			
Si	No	Si	No	Si	No	Si	No			Si	No	Si	No			Si	No				

ALIMENT	Has ingerit alguna vegada aquest aliment?			T'ha provocat mai algun tipus de reacció?											
				Quin tipus de reacció vas tenir?							Quan va tenir la reacció, recorda si havia pres...?				
	si	no	no ho recorda	Picor a la boca	Erupcions pel cos, inflamació de llavis i parpelles	Mals de panxa, vòmit, diarrea	Ofec	Mareig o pèrdua de coneixement	No recorda	Ibuprofeno, Nolotil, Enantyum, Voltaren	Exercici, alcohol	Res	No ho recorda
Préssec pell													
Préssec polpa													
Poma pell													
Poma polpa													
Nou													
Cacahuet													
Avellana													
Ametlla													
Anacard													
Pistatxo													
Pipes de gírsol													

Clinical symptoms were registered, and prick tests were performed.

Figure 9. Anamnestic data questionnaire used for the LTP-strip validation study.

FIS: PI16/00344.
Síndrome LTP: identificación y caracterización clínica de los epítopos inmunodominantes B y T y su presentación antigénica HLA.
PI: Dra. Mariona Pascal Capdevila; Dr. Joan Bartra Tomàs

IDENTIFICATION OF THE PATIENT	
Name + Family name	
Date of birth	
City of birth	
City of living	
Date of consultation	
Medication	○ Statin ○ Proton Pomp Inhibitor ○ ACE inhibitor ○ Beta blocker ○ NSAID ○ Antihistaminica ○ Omalizumab ○ Immunotherapy; please define: …… …… ○ Other relevant medication; please define: ……
Relevant comments	

Page 1 | 6

Clinical symptoms were registered, and prick tests were performed. Page 1 from 6.

Figure 9. Anamnestic data questionnaire.

FIS: PI16/00344.
Síndrome LTP: identificación y caracterización clínica de los epítopos inmunodominantes B y T y su presentación antigénica HLA.
PI: Dra. Mariona Pascal Capdevila; Dr. Joan Bartra Tomàs

Allergen	Tolerance (Please define if fruit was tolerated with or without peel)			CU	OAS	GID	U/ AE	ANAPH (grade)	SHOCK	No exposure	Avoidance			Cofactor dependent	
	YES/ NO	W/ peel	W/o peel								Medical Advice	Afraid	Doesn't like it	Yes (which?)	No
Banana															
Tomato															
Walnut															
Lentil															
Melon															
Wheat															
Mustard															
Corn															
Chickpea															
Peanut															
Hazelnut															
Peach															
Apple															
Green bean															
Artemisia															
Prune															
Almond															

Page 2 | 6

Continues. Page 2 from 6.

Figure 9. Anamnestic data questionnaire.

FIS: PI16/00344.
Síndrome LTP: identificación y caracterización clínica de los epítopos inmunodominantes B y T y su presentación antigénica HLA.
PI: Dra. Mariona Pascal Capdevila; Dr. Joan Bartra Tomàs

Allergen	Tolerance (Please define if fruit was tolerated with or without peel)			CU	OAS	GID	U/ AE	ANAPH (grade)	SHOCK	No exposure	Avoidance			Cofactor dependent	
	YES/ NO	W/ peel	W/o peel								Medical Advice	Afraid	Doesn't like it	Yes (which?)	No
Cherry															
Kiwi															
Lettuce															
Orange															
Cypress															
Parietaria															
Peas															
Sesame															
Pine nut															
Olivetree															
Plane tree															
Allergic Reactions to other Plant Food(s)															
1															
2															
3															
4															

Page 3 | 6

Continues. Page 3 from 6.

Figure 9. Anamnestic data questionnaire.

FIS: PI16/00344.
Síndrome LTP: identificación y caracterización clínica de los epítopos inmunodominantes B y T y su presentación antigénica HLA.
PI: Dra. Mariona Pascal Capdevila; Dr. Joan Bartra Tomàs

	Yes	No
Seasonal rhinoconjunctivitis?		
(Allergic) Asthma? (Recurrent attacks of breathlessness and wheezing)		
Remarks		

Continues. Page 4 from 6.

Figure 9. Anamnestic data questionnaire.

FIS: PI16/00344.
Síndrome LTP: identificación y caracterización clínica de los epítopos inmunodominantes B y T y su presentación antigénica HLA.
PI: Dra. Mariona Pascal Capdevila; Dr. Joan Bartra Tomàs

Allergen	Prick test (max Ø)	Prick by Prick (max Ø)
Banana		
Tomato Peel		
Tomato Pulp		
Walnut		
Lentil		
Melon		
Wheat		
Mustard		
Corn		
Chickpea		
Peanut		
Hazelnut		
Peach Peel		
Peach Pulp		
Apple Peel		
Apple Pulp		
Green bean		
Artemisia		
Almond		
Prune Peel		
Prune Pulp		
Cherry Peel		
Cherry Pulp		
Kiwi Peel		
Kiwi Pulp		
Fresh Lettuce		
Frozen Lettuce		
Orange		
Cypress		
Parietaria		
Peas		

Page 5 | 6

Continues. Page 5 from 6.

Figure 9. Anamnestic data questionnaire-

FIS: PI16/00344.
Síndrome LTP: identificación y caracterización clínica de los epítopos inmunodominantes B y T y su presentación antigénica HLA.
PI: Dra. Mariona Pascal Capdevila; Dr. Joan Bartra Tomàs

Allergen	Prick test (max Ø)	Prick by Prick (max Ø)
Sesame		
Pine nut		
Olivetree		
Plane tree		
Histamine		

Continues. Page 6 from 6.

4.3 Skin prick tests

Prick tests were performed with a 1-mm single peak lancet (Alk-Abelló, Madrid, Spain). Histamine 10 mg/mL and phosphate buffered saline serum were used as positive and negative controls respectively. A 3 mm mean weal diameter was considered positive, and results were recorded after 15 minutes following EAACI guidelines (154,299–301).

1. Data on SPT outcome with commercial peach extract enriched with Pru p 3 (ALK-Abelló, Madrid, Spain) and commercial whole extract to peach, walnut, hazelnut, peanut, apple and wheat (LETI Pharma S.L., Madrid, Spain) were registered either to corroborate the sensitization on patients participating in the analysis of the clinical relevance of low levels of Pru p 3 sIgE (Objective 1.1) or to corroborate sensitization on the patients participating on the cluster's identification study (Objective 1.2).
2. For Objective 2.1, fresh fruits to perform prick by prick tests were used as previously described to validate the new multiplex assay. Foods (**Table 4**, PbP) had been previously prepared as 1 cm^3, under extremely hygienic conditions to avoid contamination. Prepared slices (**Figure 10**) were preserved in cryotubes and frozen (-25ºC). Foods were thawed at room temperature the same day of testing (**Figure 11)**. All patients and controls had been tested with the same stock of plant foods. The number of PbP tested plant food varied among patients due to several circumstances as fresh food availability (i.e. cherry) and latter introduction. The majority was tested in at least 36 patients (**Table 16**). Only for pea and sesame the number of patients was reduced (pea: 14, sesame: 15).

Figure 10. Fresh food cut and distributed in cryotubes before frosting.

Figure 11. Prick by Prick performance

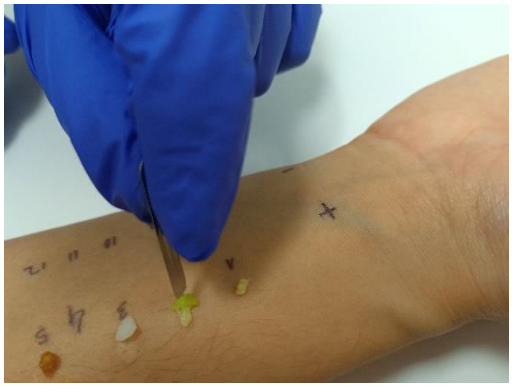


Table 4. Recombinant nsLTP proteins included on the LTP-strip immunoassay.

Allergen	Uniprot ID/NCBI	Allergenic source	SPT	PbP	BAT	ImmunoCAP component
Act d 10	P86137	Kiwi	Kiwi extract	Kiwi pulp	x	
Lac s 1-1*	A1E2H4	Lettuce	Lettuce extract	Lettuce	x	
Lac s 1-2*	A1E2H5	Lettuce	Lettuce extract	Lettuce	x	
Cuc m LTP	A0A1S3B5F4	Melon	Melon extract	Melon pulp next to peel	x	
Cor a 8	Q9ATH2	Hazelnut	Hazelnut extract	Hazelnut		Cor a 8 (f425)
Ara h 9	B6CG41	Peanut	Peanut extract	Peanut		Ara h 9 (f427)
Len c 3	A0AT29	Lentil	Lentil extract	Lentil (boiled)		
Pha v 3.0101*	D3W146	Green bean	Green bean extract	Green bean	x	
Pha v 3.0201*	D3W147	Green bean	Green bean extract	Green bean	x	
Pis s 3	C0HJR7	Pea	Pea extract	Mashed peas		
Jug r 3	C5H617	Walnut	Walnut extract	Walnut		Jug r 3 (f442)
Mus a 3	A0A804IQP6	Banana	Banana extract	Banana pulp		
Tri a 14*	Q8GZB0	Wheat	Wheat extract	Wheat flour		
Tri a 14.0201*	D2T2K2	Wheat	Wheat extract	Wheat flour		
Zea m 14	P19656-2	Corn	Corn extract	Corn flour		
Mal d 3	Q5J026	Apple	Apple extract	Apple peel		Mal d 3 (f435)
Pru av 3	Q9M5X8	Cherry	Cherry extract	Cherry peel		
Pru du 3*	B6CQU2	Almond	Almond extract	Almond	x	
Pru du 3.0101*	C0L0I5	Almond	Almond extract	Almond	x	
Pru p 3	P81402	Peach	Peach extract	Peach peel / pulp	x	Pru p 3 (f420)
Sola l 3	P93224	Tomato	Tomato extract	Tomato peel		
Sola l 6	A0A3Q7F7X3	Tomato	Tomato extract	Tomato peel		
Sola l 7	A0A3Q7EJP1	Tomato	Tomato extract	Tomato peel		
Ses i LTP 1	A5JUZ7	Sesame	Sesame extract	Mashed sesame		
Ses i LTP 2	A5JUZ8	Sesame	Sesame extract	Mashed sesame		
Ses i LTP 3	A5JUZ9	Sesame	Sesame extract	Mashed sesame		

Allergen	Uniprot ID/NCBI	Allergenic source	SPT	PbP	BAT	ImmunoCAP component
Ses i LTP 4	A5JV00	Sesame	Sesame extract	Mashed sesame		
Ses i LTP 5	A5JV01	Sesame	Sesame extract	Mashed sesame		
CCD			-			MUXF3 (o214)

Description of the recombinant nsLTPs used in this study with Uniprot ID or NCBI gene bank Isoforms are marked as asterisks and proteins which are not mentioned on IUIS are highlighted with a grey background. The sources corresponding with each LTP are described. The commercially available extracts (Leti, Spain) and fresh foods used for skin tests (SPT, PbP) are included. The molecules tested on Basophil activation test are marked. The corresponding molecule on ImmunoCAP® and its reference is also included.

3. SPT with commercial whole extracts provided by LETI Pharma (Madrid, Spain) for the allergenic sources corresponding to the allergens included on the evaluated multiplex assay were also tested (**Table 4**, SPT) and registered on a questionnaire (**Figure 9)** (Objective 2.1).

4.4 Serum allergen sIgE immunoassays

4.4.1 IMMUNOCAP® and IMMUNOCAP® ISAC

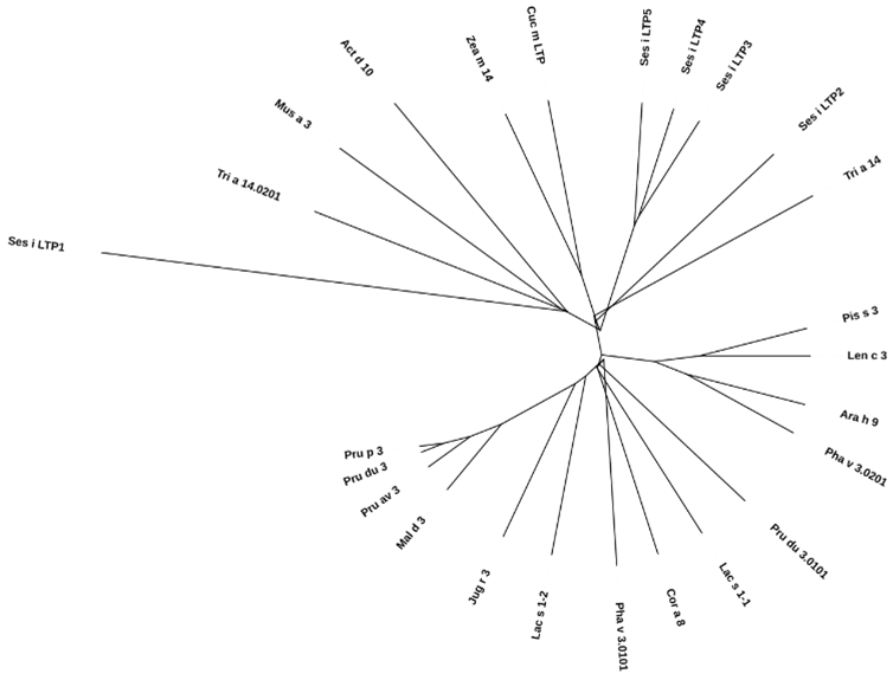
Serum total IgE (KU/L) and peach extract sIgE (f95); **rPru p 3** (f420), **rMal d 3** (f435), **rTri a 14** (f433), **rJug r 3** (f442), **nCor a 8** (f425) and **rAra h 9** (f427) sIgE values from singleplex ImmunoCAP® and multiplex ImmunoCAP® ISAC (both ThermoFisher Scientific, Uppsala, Sweden) were used. Manufacturer's protocols were used to perform the assay. Values > 0.1 kU_A/L for ImmunoCAP® and ≥ 0.3 ISU (ISAC standardized units) for ImmunoCAP® ISAC were considered positive.

4.4.2 THE LTP-STRIP IMMUNOBLOT ASSAY

For Objective 2, serum samples from individuals included in the study cohort were tested with the LTP-strip, a multiplex immunoblot assay, from EUROIMMUN Medizinische Labordiagnostika AG (Lübeck, Germany) to detect sIgE towards a wide panel of the major offending food nsLTPs in our area. This multiplex immunoblot assay was especially designed for this study and contains 7 membrane segments with 28 immobilized recombinant nsLTP allergens from 18 allergenic sources: 17 plant food allergens (including selected isoforms) as well as a CCD sensitization marker; see allergen details on **Table 4**, **Table 5** and **Figure 12**. Proteins were expressed and purified

as previously described (302) with some modifications. The proteins contained an N-terminal 6x Histidine-tag and were isolated by immobilized metal-affinity chromatography and size-exclusion chromatography. The protein quality was assessed by SDS-PAGE and mass spectrometry. The purity of the proteins is >95%.

Figure 12. Guide tree based on amino acid sequence data.



The guide tree data was generated by Clustal W (303,304). The graphical presentation was edited in iTOL (305).

Table 5. Sequence identity percentages (%) from the proteins included on the strips.

ALLERGEN	Ses i LTP1	Tri a 14.0201	Mus a 3	Act d 10	Cuc m LTP	Zea m 14	Ses i LTP5	Ses i LTP3	Ses i LTP4	Ses i LTP2	Tri a 14	Len c 3	Pis s 3	Pha v 3.0201	Ara h 9	Pru du 3.0101	Lac s 1-1	Cor a 8	Pha v 3.0101	Lac s 1-2	Jug r 3	Mal d 3	Pru av 3	Pru p 3	Pru du 3
Ses i LTP1	100	24	21	24	19	28	24	24	27	24	26	29	24	25	25	24	22	19	22	26	21	24	26	25	25
Tri a 14.0201	24	100	46	43	42	46	42	43	43	54	44	49	46	43	44	47	47	41	44	48	37	48	47	47	46
Mus a 3	21	46	100	42	51	47	42	47	41	43	38	43	41	46	43	40	46	41	42	52	46	43	41	40	40
Act d 10	24	43	42	100	51	52	46	38	43	46	44	52	46	51	56	51	51	42	43	50	40	46	43	47	46
Cuc m LTP	19	42	51	51	100	60	51	46	48	52	42	49	55	54	52	51	54	49	49	56	56	56	59	58	54
Zea m 14	28	46	47	52	60	100	52	49	46	53	57	56	53	59	62	53	54	47	53	56	56	55	54	58	57
Ses i LTP5	24	42	42	46	51	52	100	70	75	52	45	53	49	52	56	51	49	46	44	59	52	50	51	49	50
Ses i LTP3	24	43	47	38	46	49	70	100	75	53	43	52	45	47	47	51	44	44	38	57	49	50	50	50	51
Ses i LTP4	27	43	41	43	48	46	75	75	100	56	48	59	53	52	52	55	54	48	44	60	48	53	49	50	51
Ses i LTP2	24	54	43	46	52	53	52	53	56	100	46	54	54	51	53	55	49	42	46	57	49	53	51	52	53
Tri a 14	26	44	38	44	42	57	45	43	48	46	100	56	46	40	44	43	47	43	44	48	44	46	44	46	47
Len c 3	29	49	43	52	49	56	53	52	59	54	56	100	78	67	68	54	53	55	54	61	60	62	57	60	59
Pis s 3	24	46	41	46	55	53	49	45	53	54	46	78	100	65	67	52	54	58	54	56	58	57	57	59	57
Pha v 3.0201	25	43	46	51	54	59	52	47	52	51	40	67	65	100	73	60	53	54	62	66	63	66	62	65	63
Ara h 9	25	44	43	56	52	62	56	47	52	53	44	68	67	73	100	57	56	55	62	56	58	63	62	62	58
Pru du 3.0101	24	47	40	51	51	53	51	51	54	55	43	54	52	60	57	100	57	61	51	62	59	59	57	58	59
Lac s 1-1	22	47	46	51	54	54	49	44	54	49	47	53	54	53	56	57	100	56	56	65	57	58	57	57	57
Cor a 8	19	41	41	42	49	47	46	44	48	42	43	55	55	54	55	61	56	100	57	57	58	59	59	59	63
Pha v 3.0101	22	44	42	43	49	53	44	38	44	46	44	54	54	62	62	51	56	57	100	56	51	63	62	62	63
Lac s 1-2	26	48	52	50	56	60	59	57	60	57	48	61	56	66	56	62	65	57	56	100	63	67	66	66	67
Jug r 3	21	37	46	40	56	56	52	49	48	49	44	60	58	63	58	55	53	58	51	63	100	68	63	64	63
Mal d 3	24	48	43	41	59	56	50	50	53	53	46	62	57	66	63	59	57	62	63	67	68	100	82	80	79
Pru av 3	26	47	41	43	54	55	51	50	49	51	47	57	57	62	62	63	58	59	63	66	63	82	100	88	89
Pru p 3	25	47	40	47	54	58	49	50	51	52	46	60	59	65	62	58	57	59	62	66	64	80	88	100	95
Pru du 3	25	46	40	46	54	57	50	51	51	53	47	59	57	63	58	59	57	63	63	67	63	79	89	95	100

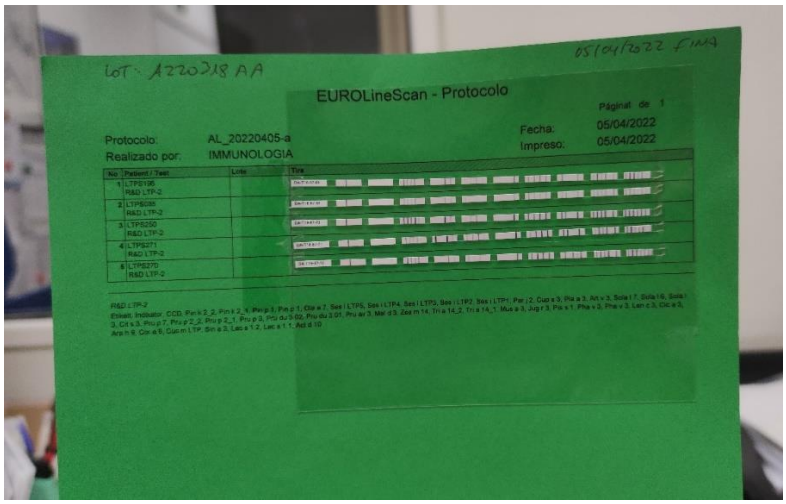
The strip includes LTPs described as allergens in the literature but not commercially available for routine testing as individual components, as well as different LTP isoforms like the five from sesame (Ses i LTP 1-5) which were chosen based on database and literature research (306), and two isoforms for Tri a 14 (wheat LTP), Pru du 3 (almond LTP) , Pha v 3 (green bean LTP) and Lac s 1 (lettuce LTP) (243,307,308).

The assay set up was done as described before (309). Briefly, the immunoblot test strips were incubated overnight with 100 μL of serum diluted 1:11 in universal buffer at room temperature (RT) according to manufacturer's instructions. All reagents were included on a kit provided by EUROIMMUN AG. The strips were washed with diluted universal buffer three times and incubated 1 hour at RT (room temperature) with enzyme conjugate (alkaline phosphatase-labelled anti-human IgE antibody) (**Figure 13**). Afterwards, strips were washed three times again and incubated 10 min at RT with chromogen substrate solution. The reactions were stopped with distilled water and, after placing on evaluation protocol, strips were dried on air and finally evaluated with "EuroLineScan" software by EUROIMMUN AG (**Figure 14**).

Figure 13. EUROIMMUN-LTP strips performance.

Conjugate incorporation previous to 1 hour incubation.

Figure 14. Evaluation protocol sheet.



Evaluation protocol sheet, with incubated strips, ready to scan with the "EuroLineScan" software by EUROIMMUN AG.

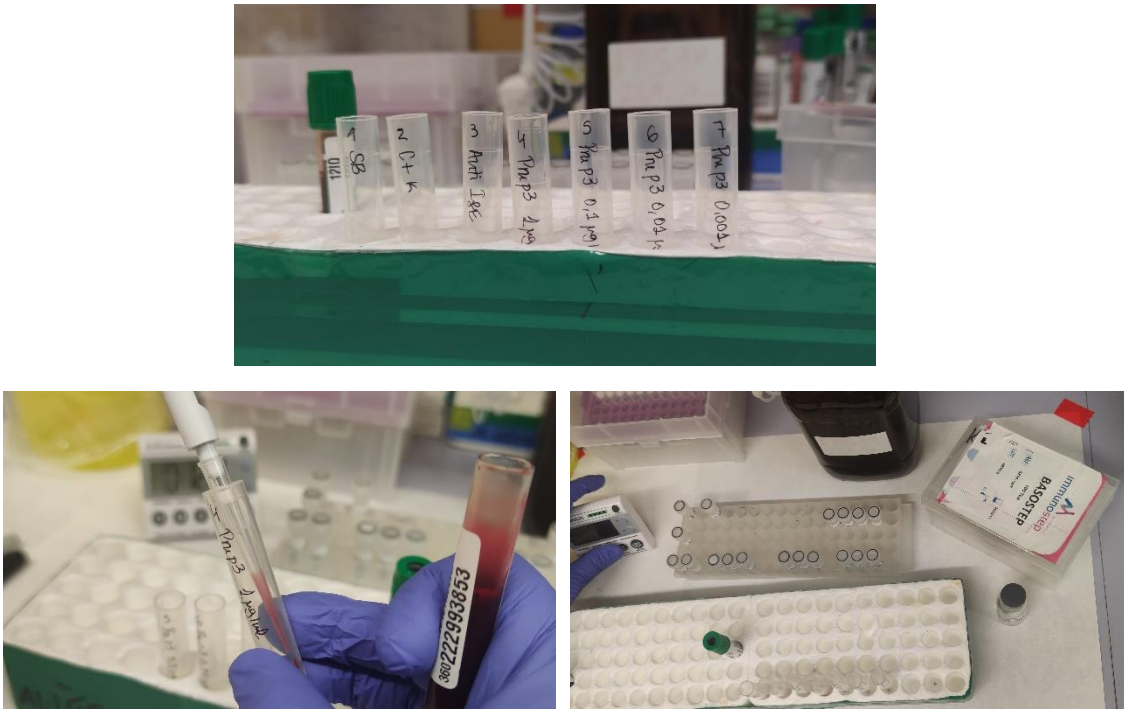
4.5 Basophil activation test (BAT)

After patient informed consent, 10 mL of heparinized peripheral blood was obtained and immediately taken to the laboratory for Basophil Activation Testing (BAT) using the Flow2CAST™ kit (Bühlmann Laboratories AG, Switzerland) and following manufacturer's procedures (**Figure 15**). Stimulation buffer provided by the manufacturer was used as negative control to evaluate basal/spontaneous degranulation. Stimulation control, a monoclonal anti-FcεRI antibody (Bühlmann Laboratories AG, Switzerland) and fMLP (N-Formylmethionyl-leucyl-phenylalanine, Bühlmann Laboratories AG, Switzerland) were used as positive controls. A mixture of anti-CD63-FITC and anti-CCR3-PE monoclonal antibodies were used to stain the samples. Basophils were identified by flow cytometry (FACS-Canto II, BD Biosciences, Germany) and those CD63+ were considered activated. Samples were discarded when less than 200 basophils could be acquired. A percentage of activated basophils ≥15% (previously subtracting basal CD63+ basophils) was considered a positive test according to the manufacturer's recommendations for food allergens. Basophil Reactivity (BR) corresponding to the number of basophils responding to a stimulus was calculated as the post stimulus %CD63 expression minus basal %CD63 expression and was represented as %CD63. Basophil sensitivity (BS) was calculated as CD-sens: inversion x 100 of EC50, the concentration inducing 50% of maximum response. The purpose of performing BAT in this project was to assess the functionality of the sIgE detected in the serum of the patients studied.

1- To test the functionality of the low levels of sIgE from the study cohort of **Objective 1.1**, BAT was perfomed with purified **Pru p 3** (1 mg/mL, Bial Aristegui, Bilbao, Spain) at 25 ng/mL, 12.5 ng/mL, 5 ng/mL and 2.5 ng/mL final concentrations to a subset of patients from grLOW (0.1-0.35 KU_A/L Pru p 3 sIgE) and grB (≥0.35 KU_A/L Pru p 3 sIgE).

2- To complete the analytical validation of the new LTP-strip assay (**Objective 2.1),** BAT was performed with several purified LTPs (**Pru p 3** - Peach; **Pru du 3** and **Pru du 3.0101** - Almond; **Pha v 3.0101** and **Pha v 3.0201** – Green bean; **Lac s 1-1** and **Lac s 1-2** -Lettuce; **Cuc m** – Melon; **Act d 10** – Kiwi) provided by Euroimmun AG at 1 μg/mL, 0.1 μg/mL, 0.01 μg/mL and 0.001 μg/mL final concentrations in both nsLTP sensitized individuals and healthy donors of the study cohort. Performing BAT to all LTPs included in the strip at several concentrations was not feasible in our hands, therefore several were selected based on clinical criteria and novelty. For basophil activation test, the subset patients prick-tested were recontacted and a new blood sample obtained. The timeframe between tests was not longer than 2 months. In our hands it was only feasible to test several proteins at a time per each patient in BAT guarantying the correct performance of the test. Thus we selected the proteins to use in BAT based on several premises: 1) we wanted to test nsLTPs which were clinically relevant in patients of our area with LTP allergy with little or no data reported in the literature to gain knowledge and experience on them in terms of basophil activation (i.e., first we started with Lac s 1, Pru du 3 and Pha v 3 and then when proteins were available for BAT we added Act d 10, Cuc m LTP, this is why we have less individuals tested for these 2 proteins), 2) we also wanted to compare the functional impact of different isoforms of a particular nsLTP that had been included in the LTP-strip (Lac s 1-1 vs Lac s 1-2, Pru du 3 vs Pru du 3.0101 and Pha v 3.0101 and Pha v 3.0201), 3) we tested also all the individuals with Pru p 3 as a reference of basophil activation since we are familiar with the basophil activation performance with this protein in LTP allergic patients. Thus, we could have data on the activation of new LTPs compared to the activation caused by Pru p 3.

Figure 15. BAT performance in our laboratory.



4.6 Statistical analysis

In order to obtain an adequate sample size in the study of the utility of the currently available *in vitro* tools , we used the data available at our centre that met the requirements between 2012 (the first data available to us in our computerized database) and 2019, the time to start the analysis. For the performance of the skin tests (PbP) on the LTP-strips analysys, testing 37 patients was logistically possible and reasonable for us. Finally, in the analysis of the LTP-strips, all the 202 strips provided by the manufacturer of the immunoblot were used.

All data was analysed using Microsoft Excel 14.0 (Washington, USA) and GraphPad Prism 8.0.2 software (Inc, CA, USA) for the statistical analysis. sIgE centralization and dispersion measurements were calculated considering a quantitative and asymmetric distribution: median and interquartile range (IQR) were calculated. Free distribution was considered on our analysis so non-parametric tests were used to verify heterogenicity between our variables. Quantitative data as sIgE levels, %CD63 expression or CD-sens when two independent groups, were compared using Mann Withney U-test. For 3 or more groups was used Kruskall-Wallis test. Qualitative data

distributed in contingency tables was compared using Chi-squared test or Yate's continuity corrected Chi-square test, except for smaller than 5 values in more than 80% boxes in which Fisher's exact test for small simple size was used. P values lower than 0.05 were considered statistically significant. On Clusters analysis (Objective 1.2), the most frequent bivariate protein association was calculated and analysed with Spearman's correlation coefficient (r_s).

The outcome of the multiparameter immunoblot assay (Objective 2) was qualitatively compared (positive/negative test result) to the PbP outcome. Thus, strip results were classified into four groups as follows: true positive (TP, Euroline+; PbP+), false positive (FP, Euroline+; PbP-), true negative (TN, Euroline-, PbP-); false negative (FN, Euroline-; PbP+). The Microsoft Excel 14.0 (Microsoft, USA), Add-In Analyse-it 5.90 (Analyse-it Software, Ltd.) and GraphPad Prism 8.0.2 software (Inc., CA, USA) were used for the statistical analysis. BAT results were presented as %CD63 for each individual allergen concentration. The maximal %CD63 at an allergen concentration of 1 μg/mL were shown as Box plots calculated with Analyse-it Software, Ltd. (5.11 / 2.30 (Win) / 2018 Operating system, Windows). Basophil sensitivity for each individual allergen and patient were expressed as CD-sens (100 / EC_{50}) calculated as previously described by Santos (168) and Di Veroli (310). Only dose-dependent curves with an inflection point could be analyzed.

Chapter V
Results

5.1 Clinical relevance of low Pru p 3 sIgE in patients with peach allergy

This part of the study was designed to achieve the **Objective 1** (in short, to improve on the utility of the currently available *in vitro* tools for diagnosis and clinical management of LTP syndrome), specifically **Objective 1.1** (assessing the usefulness of considering low levels of Pru p 3 sIgE).

The results from this part of the thesis work have been published in the following cited paper (**Annex**, original version):

Low Levels Matter: Clinical Relevance of Low Pru p 3 sIgE in Patients With Peach Allergy.

Balsells-Vives S, San Bartolomé C, Casas-Saucedo R, Ruano-Zaragoza M, Rius J, Torradeflot M, Bartra J, Munoz-Cano R and Pascal M. Front. Allergy (2022) 3:868267 DOI: 10.3389/falgy.2022.868267

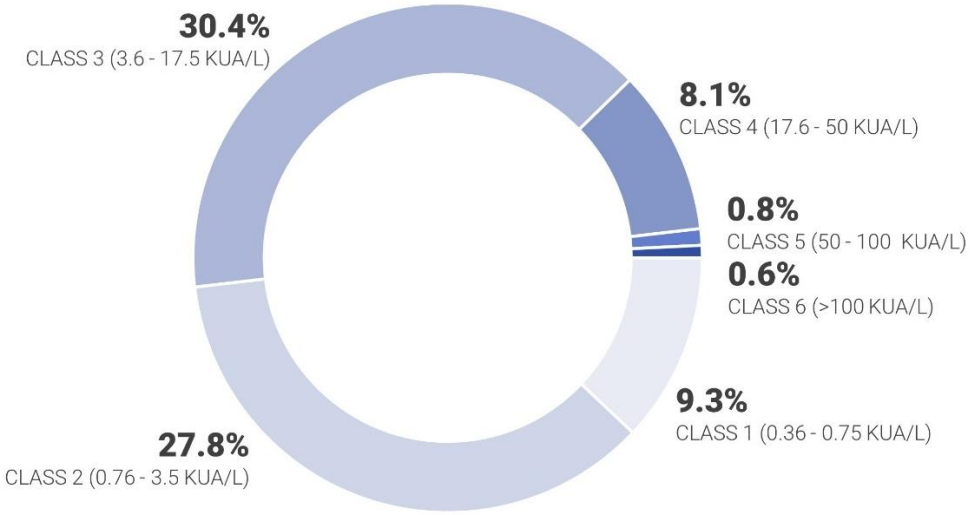
Journal information: **Frontiers in Allergy.**

Recently created journal, not impact factor yet.

5.1.1 Study population

A total of 496 subjects with Pru p 3 sIgE≥0.1 kU_A/L were recorded between 2012-2019. 284 (57.3%) were women with a median [IQR] age of 42 [17-92] years. Of them, 114 (23.0%) had Pru p 3 sIgE values between 0.1-0.34 kU_A/L (**grLOW = group Low Levels**) and 382 (77.0%) had values ≥0.35 kU_A/L (**grB = group High Levels**). A major proportion of the patients in GrB, had Pru p 3 sIgE values between 0.76 and 17.5 kU_A/L, corresponding to classes 2 and 3 from the EAST classification (**Figure 16**).

Figure 16. GrB distribution among EAST classification.



Levels in grB are distributed following the ImmunoCAP® Enzyme-Allergo-Sorbent Test (EAST) Classes Classification (1 to 6). The number of patients on each group and their prevalence are represented.

A relatively high percentage of patients were allergic to peach in both groups (44.7%-grLOW and 59.9%-grB; p>0.05) with similar peach-related symptoms although U/AE was more frequent in grLOW (p=0.020). Peach avoidance was statistically superior in grLOW (p <0.0001) (**Table 6**).

Table 6. Clinical relevance frequencies among studied patients.

	GrLOW n=114	**GrB** n=328	**P value**
Peach allergic	44.7%	59.9%	ns
Peach tolerant	20.2%	25.9%	ns
Peach avoidance	35.1%	14.1%	****
Peach-related symptoms			
Local	50.4%	55.1%	ns
CU	21.9%	25.1%	ns
OAS	23.7%	24.6%	ns
GI	4.4%	5.5%	ns
Systemic	22.8%	25.4%	ns
U/AE	21.2%	17.5%	*
AN	1.9%	8.1%	ns

GrLOW: Pru p 3 sIgE from 0.1-0.34 kU_A/L; GrB: Pru p 3 sIgE >0.35 kU_A/L. CU: contact urticaria; OAS: oral allergy syndrome; GI: gastrointestinal symptoms; U/AE: generalized urticaria or angioedema; AN: anaphylaxis. Chi-squared test and Fisher's exact test were used to obtain p. Patients from the group AV (avoid) were not included on the statistical analysis from the whole group because tolerance or allergy could not be guaranteed. Asterisks express significance of p value * 0.01 to 0.05, ** 0.001 to 0.01, *** 0.0001 to 0.001, **** <0.0001 and ns non-significant.

5.1.2 sIgE levels evaluation

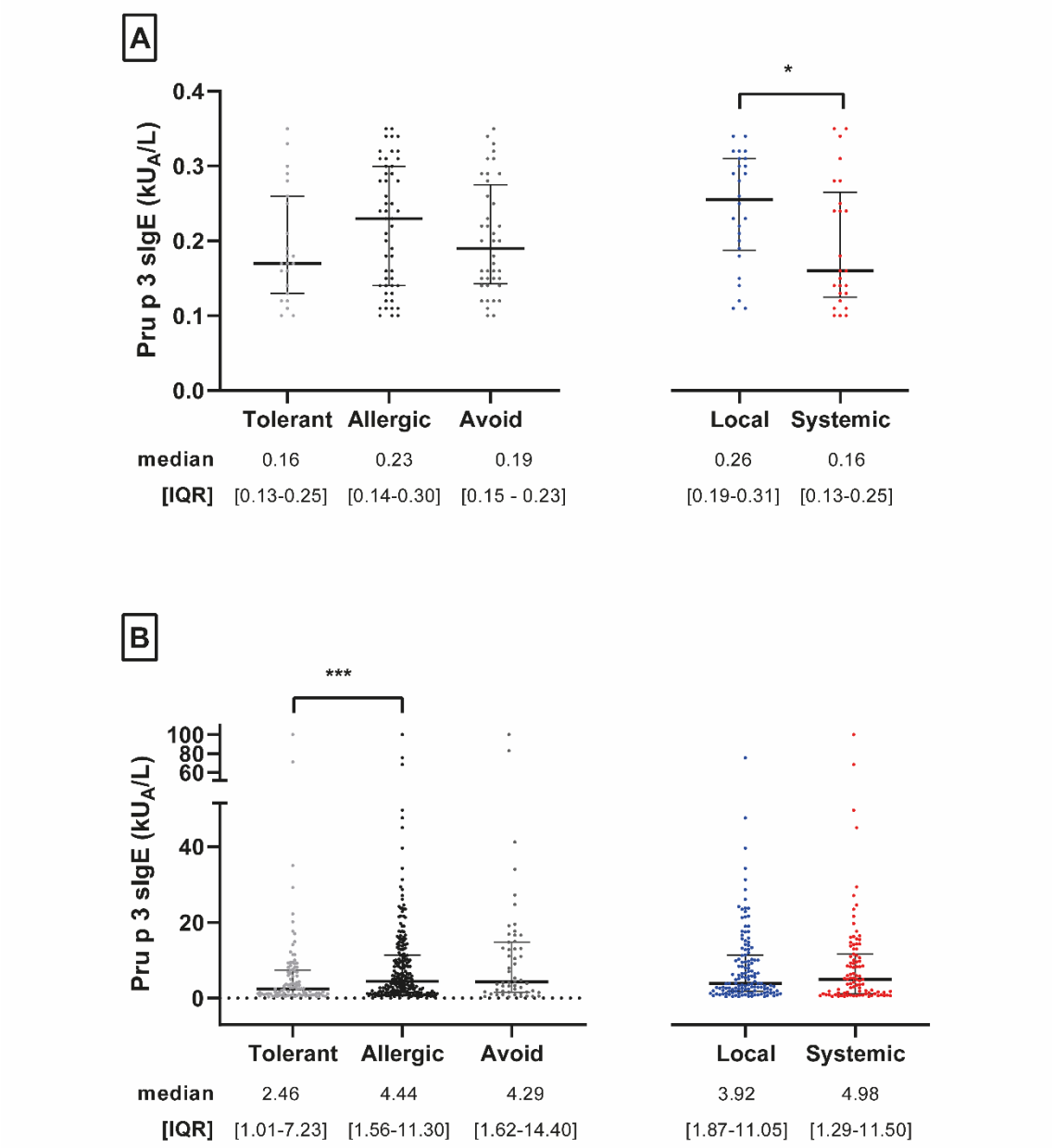
Peach sIgE values were higher in grB, as well as Pru p 3/total IgE ratio ($p<0.05$), whereas no differences were observed in Pru p 3/Peach sIgE (ratio) (**Table 7**). In grLOW (**Figure 17A**), Pru p 3 sIgE was higher in patients with local compared to systemic symptoms ($p=0.0385$). In grB (**Figure 17B**), Pru p 3 sIgE was higher in allergic compared to tolerant ($p=0.0009$). The medians from the ratios Pru p 3/peach sIgE were superior to 1 for either grLOW or grB. Moreover, when classifying patients according to their clinical symptoms, no statistically significant differences were found. Pru p 3/Total IgE ratios were lower than 1% in grLOW unlike grB. In both groups, these ratios were statistically higher ($p<0.0001$) in allergic compared to tolerant (**Table 8**).

Table 7. Pru p 3 sIgE values distribution among groups.

Pru p 3 sIgE levels, n (%) kU_A/L	**Peach sIgE** median [IQR] kU_A/L	**Pru p 3 sIgE** median [IQR] kU_A/L	**Pru p 3/ Peach sIgE** median [IQR]	**Pru p 3/ Total sIgE** median [IQR]	**Pru p 3 sIgE on CCD+ (n from total analysed; %)** median [IQR] kU_A/L
GrLOW [0.1 - 0.34] 114 (23.0%)	0.20 [0.14-0.28]	0.19 [0.07-0.26]	1.16 [0.92-1.46]	0.00 [0.00-0.01]	0.29 [0.22-0.31] ns (7/80; 8.8%)
GrB [≥0.35] 382 (77.0%)	3.73 [1.35-10.28]	3.37 [1.16-9.67]	1.19 [1.04-1.38]	0.03 [0.01-0.07]	16.30 [4.58 – 20.85] * (19/226; 8.4%)
	***	***	ns	***	

GrLOW (Pru p 3 sIgE from 0.1-0.34 kU_A/L) and grB (Pru p 3 sIgE >0.35 kU_A/L). Pru p 3, peach and Pru p 3/Peach ratio sIgE median and IQR (interquartile range) results are included as well as + CCD sIgE frequencies and their Pru p 3 sIgE median [IQR]. Differences between grLOW and grB were statistically evaluated with Mann-Withney U-test. Also differences on Pru p 3 sIgE levels between CCD+ and CCD-. Asterisks express significance of p value * 0.01 to 0.05, ** 0.001 to 0.01, *** 0.0001 to 0.001, **** <0.0001 and ns as non-significant.

Figure 17. Pru p 3 sIgE values distribution among symptoms.



Median and IQR (interquartile range) values from grLOW (A) and grB (B) according to tolerance/allergenicity or avoidance and local vs systemic. Mann Whitney U-test was used to test p. Asterisks express significance of p value * 0.01 to 0.05, ** 0.001 to 0.01, *** 0.0001 to 0.001, **** <0.0001 and ns non-significant.

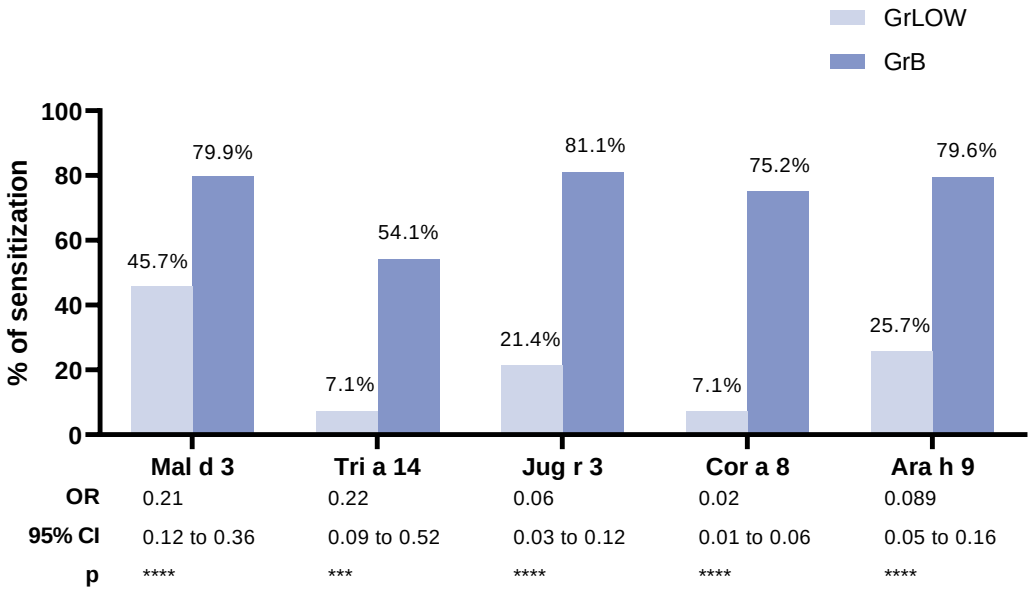
Table 8. Pru p 3/Peach sIgE and Pru p 3/Total IgE ratio median and IQR values according to tolerance/allergenicity or avoidance and symptoms classification.

	Pru p 3/Peach sIgE median [IQR] kU_A/L		**Pru p 3/Total sIgE** median [IQR] kU_A/L	
symptoms classification	**GrLOW**	**GrB**	**GrLOW**	**GrB**
Allergic	1.13 [0.88-1.37]	1.19 [1.04-1.36]	0.00 [0.00-0.01]	0.03 [0.01-0.07]
Tolerant	1.27 [0.77-2.18]	1.29 [1.02-1.60]	0.00 [0.00-0.00]	0.01 [0.00-0.04]
Avoid	1.38 [1.18-1.79]	1.16 [1.05-1.16]	0.00 [0.00-0.00]	0.04 [0.01-0.08]
	Ns	*ns*	**	***
Local	1.13 [0.88-1.21]	1.21 [1.09-1.42]	0.01 [0.00-0.01]	0.03 [0.01-0.06]
Systemic	1.13 [0.92-1.42]	1.16 [1.01-1.29]	0.00 [0.00-0.01]	0.04 [0.01-0.09]
	Ns	*ns*	*Ns*	*ns*
CU	1.16 [1.00-1.40]	1.15 [1.00-1.31]	0.01 [0.00-0.01]	0.03 [0.01-0.07]
OAS	1.15 [1.00-1.49]	1.23 [1.08-1.43]	0.00 [0.00-0.01]	0.03 [0.01-0.07]
GI	0.72 [0.37-1.21]	1.16 [1.03-1.46]	0.00 [0.00-0.01]	0.03 [0.01-0.05]
U/AE	1.13 [0.89-1.40]	1.15 [1.02-1.25]	0.00 [0.00-0.01]	0.04 [0.01-0.08]
AN	1.29 [1.20-138]	1.21 [0.99-1.43]	0.01 [0.01-0.02]	0.04 [0.02-0.12]
	Ns	*ns*	*Ns*	*ns*

GrLOW: Pru p 3 sIgE from 0.1-0.34 kU_A/L; GrB: Pru p 3 sIgE >0.35 kU_A/L. Local (CU: contact urticaria; OAS: oral allergy syndrome; GI: gastrointestinal symptoms), systemic symptoms (U/AE: generalized urticaria or angioedema; AN: anaphylaxis). Mann Whitney U-test and Kruskal-Wallis test were used to verify significance. Patients from the group AV (avoid) were not included on the statistical analysis because tolerance or allergy could not be guaranteed. Asterisks express significance of p value * 0.01 to 0.05, ** 0.001 to 0.01, *** 0.0001 to 0.001, **** <0.0001 and ns non-significant.

Sensitization to other LTPs was analysed in 70 patients from grLOW and 318 from grB (**Table 9** and **Figure 18**). In grLOW, 64.3 % of patients were positive for one or more LTPs, in grB, 95.9%. In grLOW, sensitizations to other LTPs were statistically less frequent. Mal d 3, Ara h 9 and Jug r 3 were the most frequent sensitizations and Tri a 14 the less one in both groups.

Figure 18. Co-sensitization to other LTPs.



Represented as % of sensitization to Mal d 3, Tri a 14, Jug r 3, Cor a 8 and Ara h 9. OR: Odds ratio (<1 inverse or >1 direct association); 95% CI: 95% Confidence interval. Fisher's exact test and OR (95% CI) as an association measurement were used to test statistical significance. Asterisks express significance of p value * 0.01 to 0.05, ** 0.001 to 0.01, *** 0.0001 to 0.001, **** <0.0001 and ns non-significant.

Table 9. Sensitization to LTPs from other allergenic sources.

	Total Sensitized	Mal d 3	Tri a 14	Jug r 3	Cor a 8	Ara h 9
GrLOW (n=70)	45 (64.3%)	32 (45.7%)	5 (7.1%)	15 (21.4%)	5 (7.1%)	18 (25.7%)
GrB (n=318)	307 (96.2%)	254 (79.9%)	172 (54.1%)	258 (81.1%)	239 (75.2%)	253 (79.6%)

The total patients analysed are included for GrLOW and GrB. From them, the total sensitized to one or more non-Pru p 3 LTPs and the percentage they represent are included. Additionally, the number and percentage of sensitized per LTP (Mal d 3, Tri a 14, Jug r 3, Cor a 8, Ara h 9) are described.

5.1.3 CCD co-sensitization

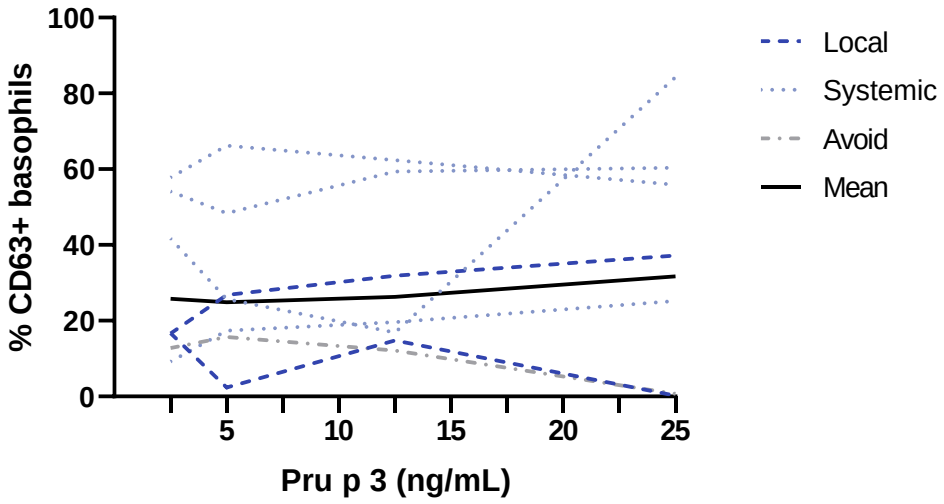
CCD reactive sIgE may cause false positive results in Pru p 3 measurements by binding the test cellulose matrix (120). In our series, CCD sensitization data was available for 80 (70.2%) patients of grLOW and 226 (59.2%) of grB. In grLOW, of the 7 CCD+ (8.7%), 5 avoided eating peach, 1 tolerated and 1 referred local symptoms. In grB, of the 19 CCD+

(8.4%), 4 avoided the ingestion of peach, 3 tolerated, 6 had local and 6 systemic symptoms (2 anaphylaxis). Tolerant and allergic frequencies were not statistically different between CCD+ or negative. In grB, were found significant differences on sIgE to Pru p 3 from CCD+ compared to CCD –, being higher on CCD+ (**Table 7**).

5.1.4 Basophil activation tests

nPru p 3 BAT was performed to 12 patients per group as previously reported (295). All in grB were BAT+, being 3 (25%) tolerant and 9 (75%) allergic (5 local/4 systemic reactions). In grLOW, 7 (58.3%) were BAT+ (summarized in **Figure 19**): 6 (85.7%) allergic (2 local/4 systemic reactions) and 1 (14.3%) avoided peach. In BAT-: 2 (40%) were tolerant and 3 (60%) allergic (2 local/1 systemic reactions) (**Table 10**). The median [IQR] for Pru p 3 sIgE for grLOW was 0.26 [0.10-0.28] KU_A/L. The ratio Pru p 3/peach sIgE median was 0.99 [0.79-1.09]. Additionally, from these BAT- patients were 0.21 [0.18-0.23] (Pru p 3 sIgE) and 0.98 [0.97-0.99] (Pru p 3/peach sIgE ratio).

Figure 19. Basophil activation test under Pru p 3 *stimuli* in positive patients with low levels (grLOW).



Basophil reactivities (%CD63+) under Pru p 3 stimulation (25 ng/mL, 12.5 ng/mL, 5 ng/mL and 2.5 ng/mL) are depicted and differentiated according to clinical relevance: local (dark blue dashed line), systemic symptoms (blue dotted line) and avoidance (grey row-dotted lines). The mean value is also represented (black line).

Table 10. Characteristics and BAT results of the grLOW patients (n=12) tested under a Pru p 3 stimulation.

	BAT	EC50	% CD63+ basophils ng/mL Pru p 3				Peach Symptoms	Pru p 3 sIgE KU_A/L	Ratio Pru p 3/peach sIgE
			2.5	5	12.5	25			
P1	-	3.29	0.60	0.40	0.20	1.40	TOL	0.26	NA
P2	-	3.71	0.60	0.50	0.00	0.20	TOL	0.20	0.00
P3	-	32.48	0.20	0.00	0.00	0.00	CU, OAS	0.25	0.96
P4	-	∞	0.00	0.00	0.00	0.00	OAS	0.30	0.73
P5	+	0.04	16.60	26.80	31.90	37.20	OAS	0.34	0.97
P6	+	0.13	16.70	2.40	14.80	0.20	GID	0.22	NA
P7	-	∞	0.00	0.00	0.00	0.00	AN	0.26	1.00
P8	+	0.02	41.70	25.70	16.90	84.30	U/AE	0.12	0.52
P9	+	0.00	57.80	66.20	62.40	55.90	U/AE (exercise)	0.12	1.09
P10	+	0.09	9.20	17.40	19.60	25.20	U/AE	0.26	1.18
P11	+	0.00	54.10	48.40	59.40	60.40	SHOCK	0.29	1.07
P12	+	0.22	12.80	15.70	12.10	0.70	AVOID	0.28	0.43

%CD63+: % of activated basophils. EC50: the concentration inducing 50% of maximum response. Tolerance (TOL), local (CU: contact urticaria; OAS: oral allergy syndrome; GI: gastrointestinal symptoms) and systemic symptoms (U/AE: generalized urticaria or angioedema; AN: anaphylaxis). In parentheses the presence of cofactors is detailed. Pru p 3 and Pru p 3/peach sIgE are included.

BAT reactivity (BR, %CD63+ basophils) was not statistically different between groups (BR median: 17.8% grLOW/ 27.3% grB), neither when only allergic patients of each group were compared. In grLOW, BR was significantly higher on allergic individuals versus tolerant ones (p=0.0286), and on those having systemic symptoms when comparing local ones (p=0.0286). No statistically significant differences on basophil sensitivity (CD-sens expressed as the inversion and multiplication by 100 of EC50, the concentration inducing 50% of maximum response) were found between groups, although being higher in grLOW (CD-sens median: 819.0 grLOW/ 75.4 grB).

5.2 Molecular sensitization profiles detection on LTP sensitized patients

This part of the study was designed to obtain the **Objective 1**, specifically **Objective 1.2**.

Briefly, we sought to improve on the utility of the currently available *in vitro* tools specifically identifying common patterns of sensitization to currently commercially available LTPs in a group of patients with LTP syndrome.

For this purpose, patients with available data on sIgE s from all commercialised plant food nsLTP on the ImmunoCAP® system were analyzed as detailed before.

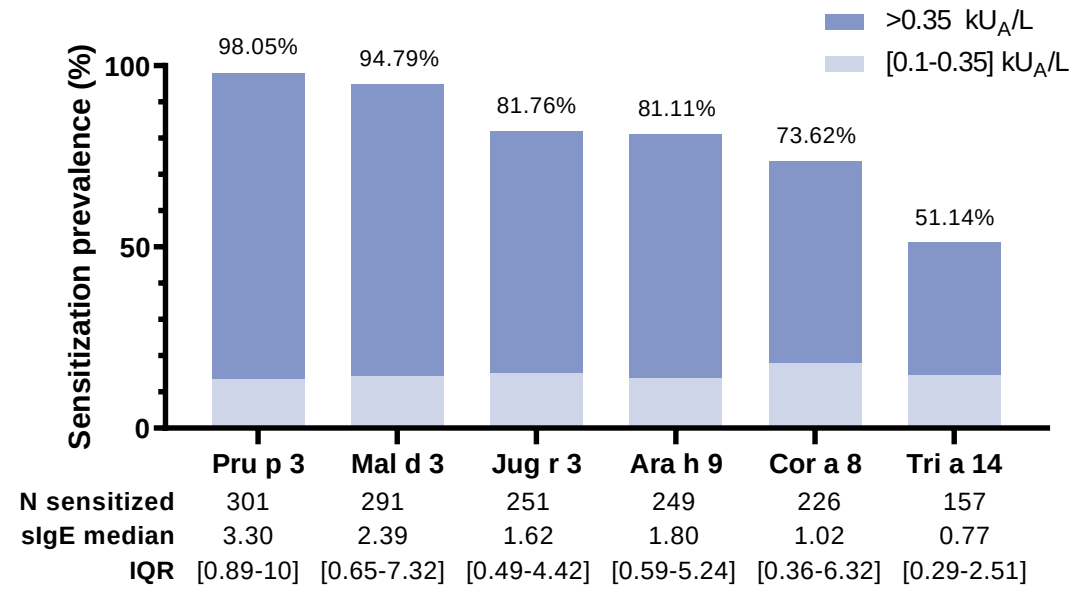
5.2.1 Study population

A total of 307 patients [median (range)]: 41 (17-85) years, 179 women (58.3 %) were selected.

Pru p 3 was the most prevalent LTP, followed by Mal d 3 (**Figure 20**). For both LTPs, most of the sIgE levels were major than 0.35 kU_A/L (Pru p 3: 84.7%, Mal d 3: 80.5%). An average of 45.5 patients (14.8%) had sIgE levels from [0.1-0.35] kU_A/L. Pru p 3 had the lowest proportion of low sIgE levels (13.4%) while Cor a 8 the highest (17.9%).

Only 51.1% of the subjects (157/307) were sensitized to Tri a 14. Sensitization prevalence order from highest to lowest was almost the same than for the sIgE levels (considering median and IQR): Pru p 3 followed by Mal d 3 had the major sensitizations and sIgE values while Tri a 14 followed by Cor a 8 presented the lowest, being sIgE levels significantly different ($p<0.001$). Jug r 3 and Ara h 9 have practically the same prevalence (81.8% and 81.1%) and close sIgE values (median [IQR]: 1.6[0.5-4.4] and 1.8[0.6-5.2] kU_A/L) ($p=0.4768$). Six patients had Pru p 3 sIgE <0.1 kU_A/L, among these, 4 were monosensitized to Tri a 14, 1 was sensitized to Cor a 8 and Ara h 9, and the other one was sensitized to Mal d 3 and Cor a 8.

Figure 20. Prevalence of sensitized patients for each of the analysed LTPs.

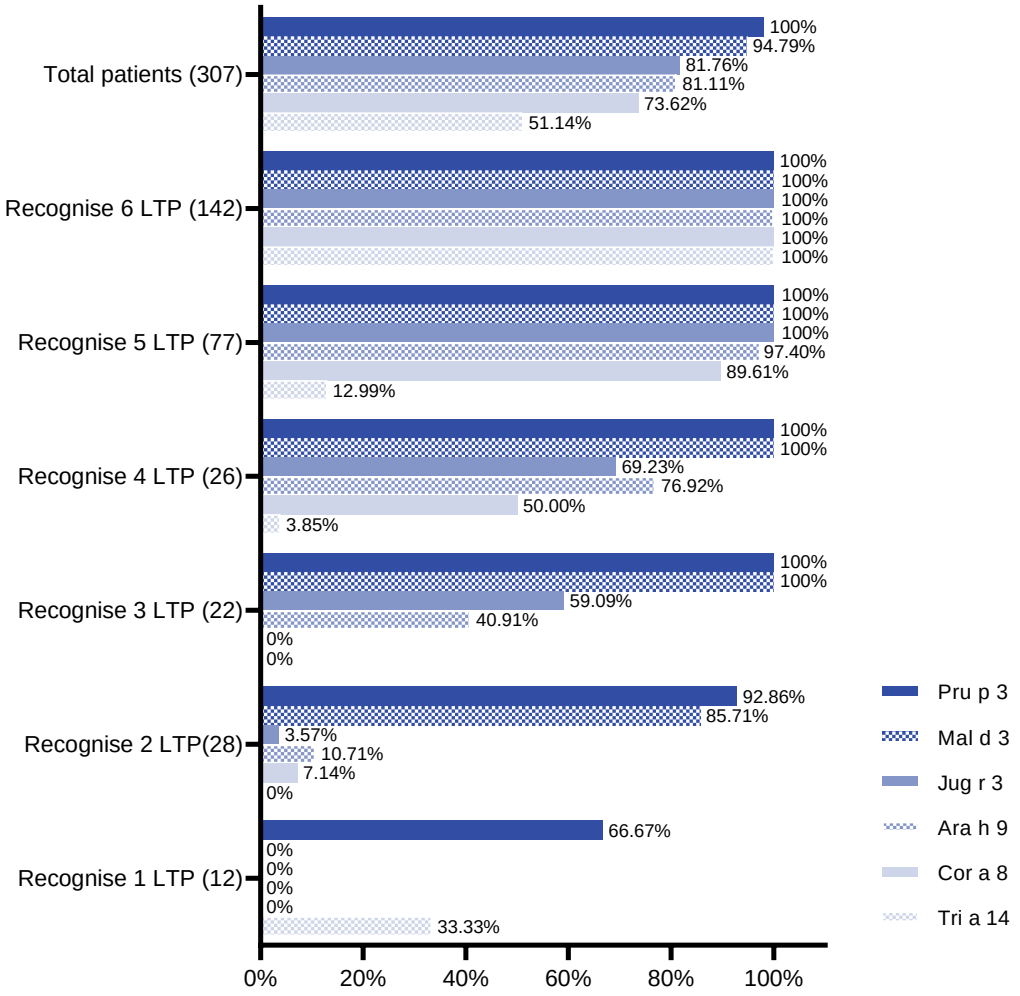


Above each bar the percentage of sensitized (≥0.1 kU_A/L) from the total (n=307) per protein is given, while under the graph is seen the absolute value they represent. sIgE median and IQR (interquartile range) from all sensitized are given per LTP. Each bar is divided in two according to sIgE levels major (blue) and minor (light blue) 0.35 kU_A/L respectively: Pru p 3, 84.7% (260/307) and 13.3% (41/307); Mal d 3, 80.4% (247/307) and 14.3% (44/307); Jug r 3,

66.8% (205/307) and 15.0% (46/307); Ara h 9, 67.4% (207/307) and 13.7% (42/307); Cor a 8, 55.7% (171/307) and 17.9% (55/307); Tri a 14, 36.5% (112/307) and 14.6% (45/307).

Analysed subjects recognised an average of 4.8 LTPs per person. From all 307 (**Figure 21**), a minority of the participants (12 patients) were monosensitized (Group 1, 3.9%), being positive to Pru p 3 (8/12) and Tri a 14 (4/12). Pru p 3 and Mal d 3 were considerably the most prevalent LTPs in low reactivity groups as Group 2 (92.9% and 85.7% respectively) or Group 3 (100% both). Moreover, 100% of the multireactive population were Pru p 3, Mal d 3 and Jug r 3 +. Interestingly, 142 (46.2%) of them recognised the entire assortment of LTPs tested and patients positive to 5 or 6 LTPs (71% of the population) recognised Ara h 9 in more than 97% of the cases.

Figure 21. LTP sensitization distribution.



LTP recognition distributed against the 6 groups and the total of patients on them. Each bar represents the sensitization frequency (%) from the studied LTPs (Pru p 3, Mal d 3, Jug r 3, Ara h 9, Cor a 8, Tri a 14).

5.2.2 Clinical characterization

Clinical information could be obtained in 305 patients. The associations between clinical profiles and LTP sensitizations are summarized in **Table 11**. Pru p 3 and Jug r 3 are the LTPs producing a major proportion of allergy on sensitized. Interestingly, Mal d 3 is the second protein with more sensitized patients (seen in **Figure 20**) and a relatively high percentage (45.7%) of them tolerate apple. From these, 82/132 (62.1%) were allergic to peach. Tri a 14 is the LTP with less sensitized patients (51.1%): 79.5% tolerate wheat and 11.5% are allergic. In allergic-to-wheat patients, more than 80% had local symptoms, specially GID.

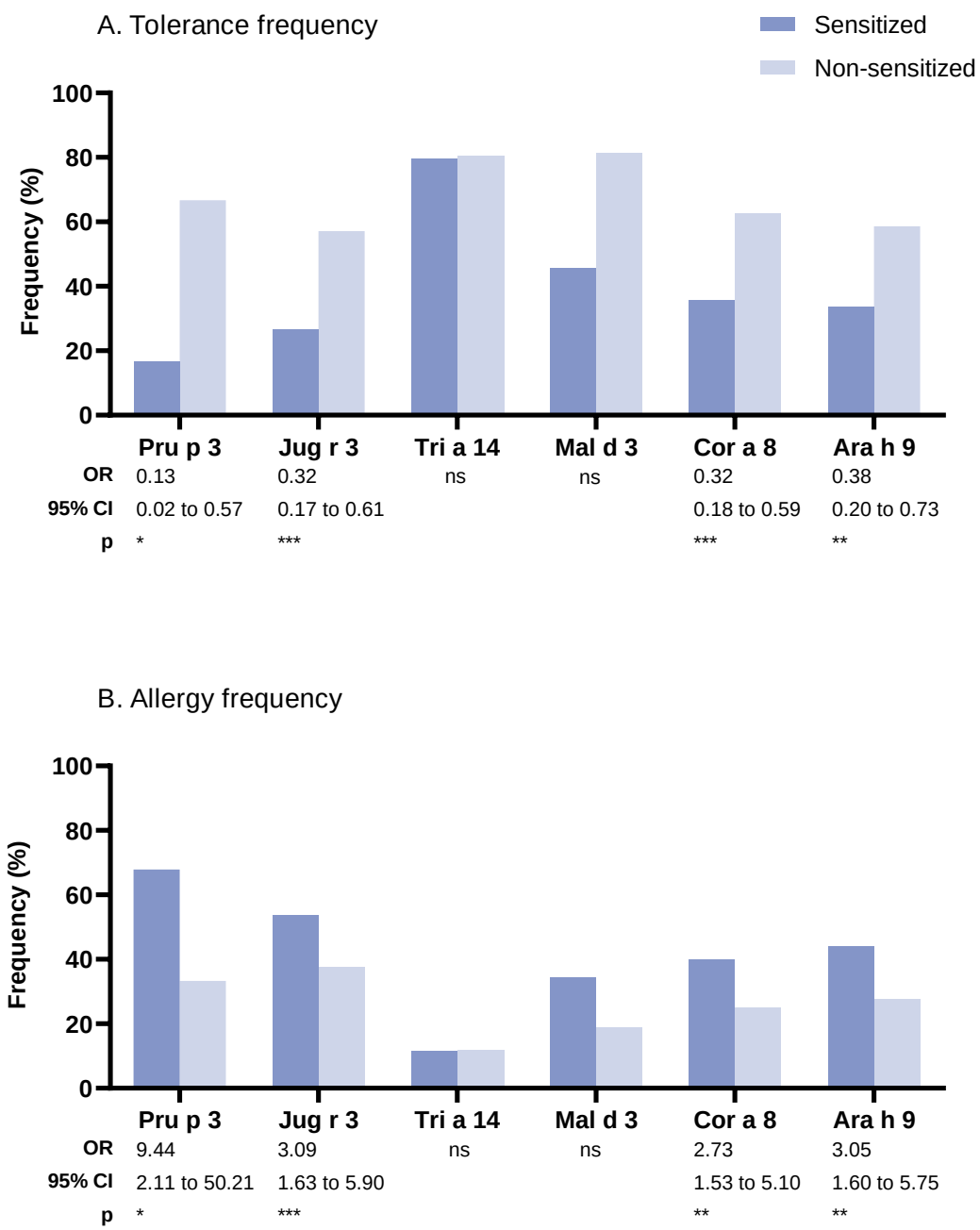
Table 11. Clinical characterization against LTPs.

	Avoid	Tolerant	Allergic	CU	OAS	GI	U/AE	RESP	AN
	% from all sensitized			% from all allergic					
Pru p 3	17.1	16.4	67.9	33.0	34.0	9.4	40.4	0.5	19.7
Mal d 3	19.7	45.7	18.7	5.6	72.2	70.4	53.7	1.9	27.8
Jug r 3	19.7	26.5	53.8	1.5	45.5	13.4	43.3	1.5	19.4
Ara h 9	25.1	33.6	43.7	0.9	44.4	22.2	40.7	0.9	13.9
Cor a 8	24.9	35.6	49.6	1.1	39.3	19.1	39.3	0.0	21.3
Tri a 14	8.3	79.5	11.5	0.0	16.7	66.7	11.1	0.0	11.1

Are represented the percentage of sensitized patients to each protein with allergy clinic, tolerance or avoidance. Moreover, allergic patients are distributed according to their clinical reactivity: Local (CU: contact urticaria; OAS: oral allergy syndrome; GI: gastrointestinal symptoms), systemic symptoms (U/AE: generalized urticaria or angioedema; AN: anaphylaxis). When various symptoms affected one of the subjects, all were included in the table.

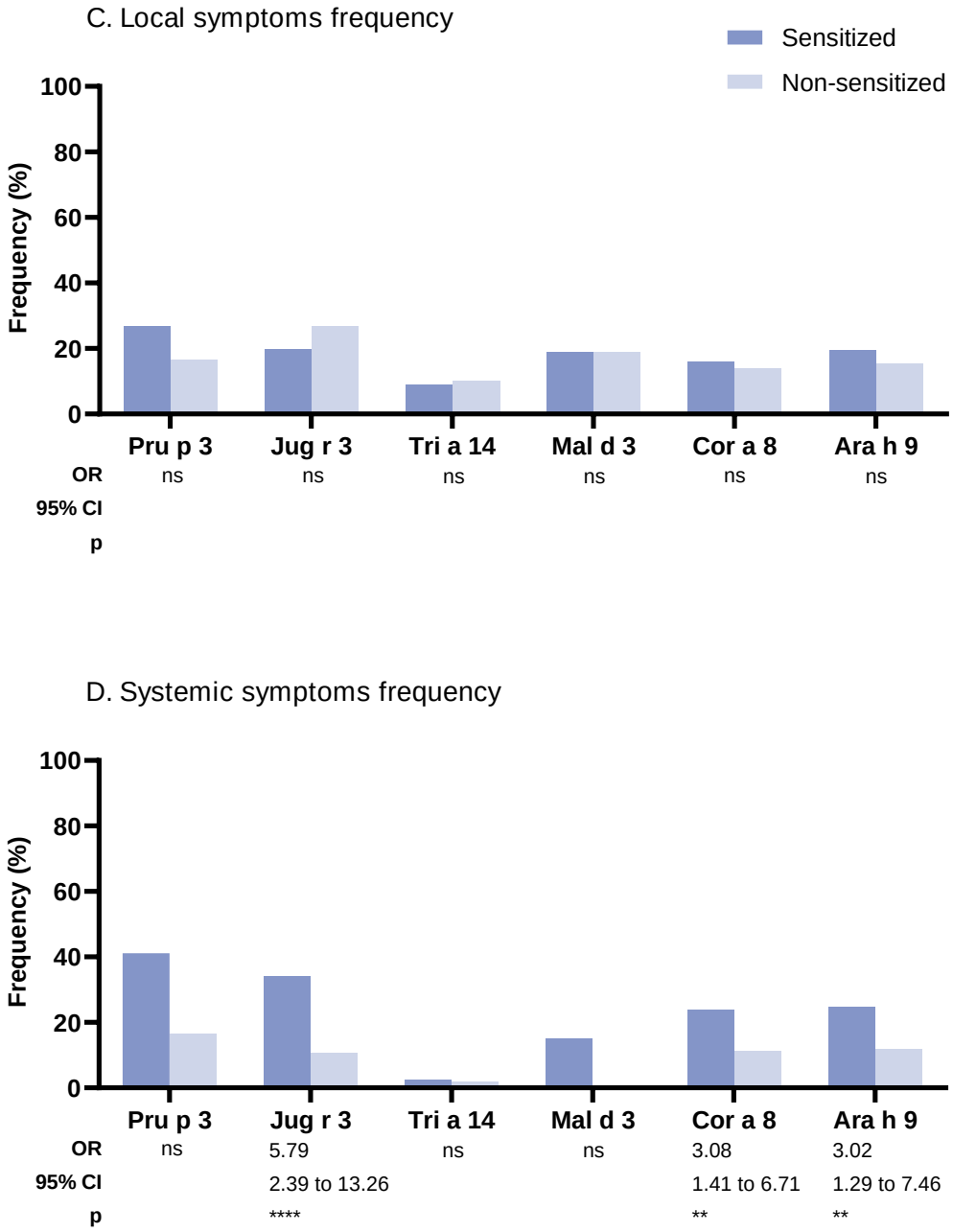
Tolerance and allergenicity frequencies (**Figure 22 and 23**) were compared in subjects sensitized or not to each studied LTP. A clinical history of systemic symptoms was significantly associated with nuts sensitization: Jug r 3, Cor a 8, Ara h 9. Jug r 3 and Cor a 8 sensitized patients had statistically more GI and AN symptoms. Moreover, U/AE was more frequent on Jug r 3 sensitized. Pru p 3 did not present differences on systemic symptoms when comparing sensitized (123/299) or not (1/6) from which 14% of the patients being symptomatic had sIgE levels under 0.35 kU_A/L (**Tables 12** and **13**).

Figure 22. Frequency of symptoms on sensitized patients.



Comparison between sensitized and non-sensitized for each of the proteins when classified by tolerance (A) and allergenicity (B). OR: Odds ratio (<1 inverse or >1 direct association); 95% CI: 95% confidence interval. Yate's continuity corrected chi-square test and Fisher's exact test were used to obtain p values. Asterisks express significance of p value * 0.01 to 0.05, ** 0.001 to 0.01, *** 0.0001 to 0.001, **** <0.0001 and ns as non-significant.

Figure 23. Frequency of symptoms on sensitized patients.



Comparison between sensitized and non-sensitized for each of the proteins when classified by local (C) or systemic (D) symptoms. OR: Odds ratio (<1 inverse or >1 direct association); 95% CI: 95% confidence interval. Yate's continuity corrected chi-square test and Fisher's exact test were used to obtain p values. Asterisks express significance of p value * 0.01 to 0.05, ** 0.001 to 0.01, *** 0.0001 to 0.001, **** <0.0001 and ns as non-significant.

Table 12. Comparison between sensitized and non-sensitized for each of the proteins when classified by local symptoms.

Allergen	CU		OAS		GID	
	Sensitized	N/Sensitized	Sensitized	N/Sensitized	Sensitized	N/Sensitized
Pru p 3	22.4%	16.7%	23.1%	0.0%	6.4%	0.0%
OR (95% CI)	*ns*		*ns*		*ns*	
p						
Jug r 3	0.8%	0.0%	24.5%	28.6%	7.2%	0.0%
OR (95% CI)	*ns*		*ns*		*∞ (1.40 to ∞)*	
p					*	
Tri a 14	0.0%	0.7%	1.9%	2.0%	7.7%	8.1%
OR (95% CI)	*ns*		*ns*		*ns*	
p						
Mal d 3	1.0%	0.0%	13.5%	0.0%	13.1%	18.8%
OR (95% CI)	*ns*		*ns*		*ns*	
p						
Cor a 8	0.4%	0.0%	15.6%	13.8%	7.6%	1.3%
OR (95% CI)	*ns*		*ns*		*7.49 (1.25 to 79.82)*	
p					*	
Ara h 9	0.4%	0.0%	19.4%	15.5%	9.7%	3.4%
OR (95% CI)	*ns*		*ns*		*ns*	
p						

Percentage of Sensitized versus N/Sensitized (non-sensitized) for each protein and local symptoms. CU, contact urticaria; OAS, oral allergy syndrome; GID, gastrointestinal disease. Statistically compared with OR: Odds ratio (<1 inverse or >1 direct association); 95% CI: 95% confidence interval. Yate's continuity corrected chi-square test and Fisher's exact test were used to obtain p values. Asterisks express significance of p value * 0.01 to 0.05, ** 0.001 to 0.01, *** 0.0001 to 0.001, **** <0.0001 and ns as non-significant.

Table 13. Comparison between sensitized and non-sensitized for each of the proteins when classified by systemic symptoms.

	U/AE		AN		RESP	
	Sensitized	**N/Sensitized**	**Sensitized**	**N/Sensitized**	**Sensitized**	**N/Sensitized**
Pru p 3	27.4%	16.7%	13.4%	0.0%	0.3%	0.0%
OR (95% CI)	*ns*		*ns*		*ns*	
p						
Jug r 3	23.3%	7.1%	10.4%	1.8%	0.8%	1.8%
OR (95% CI)	*5.00 (1.78 to 13.41)*		*7.77 (1.38 to 81.63)*		*ns*	
p	*		*			
Tri a 14	1.3%	0.7%	1.3%	1.3%	0.0%	0.0%
OR (95% CI)	*ns*		*ns*		*ns*	
p						
Mal d 3	10.0%	0.0%	5.2%	0.0%	0.3%	0.0%
OR (95% CI)	*ns*		*ns*		*ns*	
p						
Cor a 8	15.6%	8.8%	8.4%	1.3%	0.0%	1.3%
OR (95% CI)	*ns*		*8.49 (1.45 to 89.91)*		*ns*	
p			*			
Ara h 9	17.8%	10.3%	6.1%	1.7%	0.4%	0.0%
OR (95% CI)	*ns*		*ns*		*ns*	
p						

Percentage of Sensitized versus N/Sensitized (non-sensitized) for each protein and systemic symptom. U/AE; generalized urticaria or angioedema; RESP, respiratory symptoms; AN, anaphylaxis. Statistically compared with OR: Odds ratio (<1 inverse or >1 direct association); 95% CI: 95% confidence interval. Yate's continuity corrected chi-square test and Fisher's exact test were used to obtain p values. Asterisks express significance of p value * 0.01 to 0.05, ** 0.001 to 0.01, *** 0.0001 to 0.001, **** <0.0001 and ns as non-significant.

5.2.3 nsLTP sensitization profiles: Cluster identification

Most frequently paired allergen combinations were analysed (**Table 14**). A statistically significant and strong relationship was seen between all proteins except with Tri a 14. Tri a 14 shows the weakest relationship with the rest of nsLTP, sharing prevalence with other allergens in around 150 (49%) patients. Highest association could be seen between Pru p 3-Mal d 3 (290 patients, r_s=0.9334). Also, Jug r 3- Pru p 3, Mal d 3-Jug r 3 and Pru p 3-Ara h 9.

Table 14. Bivariate protein association.

Pru p 3	Jug r 3	Tri a 14	Mal d 3	Cor a 8	Ara h 9	
301 (r_s) *p*	251 (0.8543) ****	153 (0.5981) ****	290 (0.9334) ****	224 (0.7883) ****	248 (0.8670) ****	Pru p 3
	251 (r_s) *p*	152 (0.6683) ****	250 (0.9014) ****	217 (0.849) ****	229 (0.8411) ****	Jug r 3
		157 (r_s) *p*	153 (0.6534) ****	144 (0.6094) ****	151 (0.661) ****	Tri a 14
			291 (r_s) *p*	225 (0.8132) ****	246 (0.8641) ****	Mal d 3
				226 (r_s) *p*	217 (0.8337) ****	Cor a 8
					249 (r_s) *p*	Ara h 9

Combination frequency between LTPs in absolute numbers are represented in the table. Spearman's correlation coefficient (r_s) from sIgE values are represented inside the brackets, considering 95% confidence interval. r_s values above 0.7 reflect a strong positive correlation. Asterisks express significance of p value * 0.01 to 0.05, ** 0.001 to 0.01, *** 0.0001 to 0.001, **** <0.0001 and ns as non-significant.

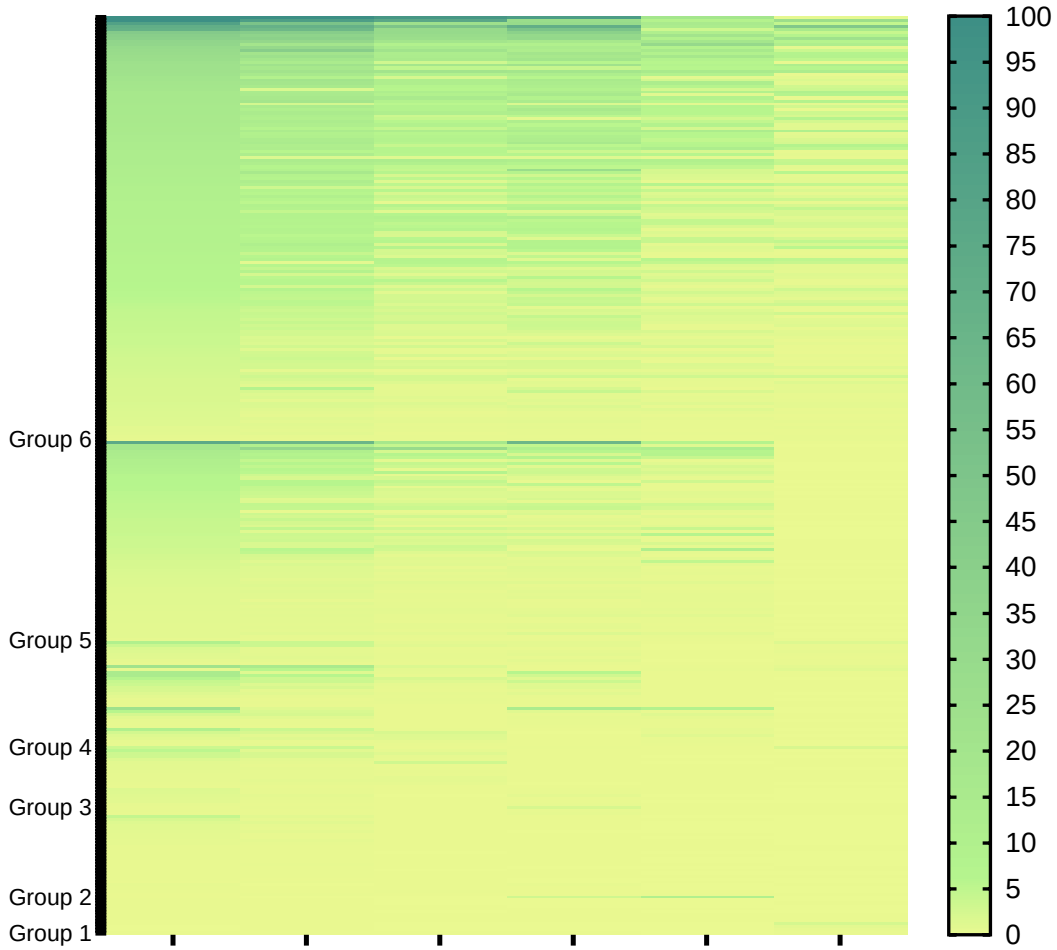
After analysing the protein combinations available on our group of patients, 17 different clusters could be found (**Table 15**): 142/307 (46.2%) recognised all 6 nsLTP (Cluster Q) (also seen in **Figure 21**). The second major cluster was found in Group 5 (Cluster N) in which 67/307 (21.8%) recognised Pru p 3, Jug r 3, Mal d 3, Cor a 8, Ara h 9. Straightaway in Group 2, 23/307 (7.5%) recognised Pru p 3 and Mal d 3 (Cluster C). Contrarily other combinations from Group 2 as Pru p 3 and Jug r 3 (Cluster E); Mal d 3 and Cor a 8 (Cluster F); Ara h 9 and Cor a 8 (Cluster G) or Pru p 3, Tri a 14, Mal d 3, Ara h 9 (Cluster M) in Group 4 were only observed in one subject (0.3%). Pru p 3 appears in 14 from 17 clusters obtained. Following it, Mal d 3 appears in 12/17, Ara h 9 in 9/17, Jug r 3 in 8/17, Cor a 8 in 7/17. Finally, Tri a 14 appeared in only 5/17 clusters. Single sIgE levels slightly increase from group 1 to 6 being statistically significant in all cases ($p<0.001$) (**Figure 24**).

Table 15. Protein Combination Clusters obtained from the different reactivity groups.

	Combination profiles (N, % from total subjects)				
Group 1 (12)	**A** Pru p 3 (8; 2.60%)	**B** Tri a 14 (4; 1.30%)			
Group 2 (28)	**C** Pru p 3, Mal d 3 (23, 7.49%)	**D** Pru p 3, Ara h 9 (2, 0.65%)	**E** Pru p 3, Jug r 3 (1, 0.33%)	**F** Mal d 3, Cor a 8 (1, 0.33%)	**G** Cor a 8, Ara h 9 (1, 0.33%)
Group 3 (22)	**H** Pru p 3, Jug r 3, Mal d 3 (13, 4.23%)	**I** Pru p 3, Mal d 3, Ara h 9 (9, 2.93%)			
Group 4 (26)	**J** Pru p 3, Jug r 3, Mal d 3, Ara h 9 (12, 3.91%)	**K** Pru p 3, Mal d 3, Cor a 8, Ara h 9 (7, 2.28%)	**L** Pru p 3, Jug r 3, Mal d 3, Cor a 8 (6, 1.95%)	**M** Pru p 3, Tri a 14, Mal d 3, Ara h 9 (1, 0.33%)	
Group 5 (77)	**N** Pru p 3, Jug r 3, Mal d 3, Cor a 8, Ara h 9 (67, 21.82%)	**O** Pru p 3, Jug r 3, Tri a 14, Mal d 3, Ara h 9 (8, 2.61%)	**P** Pru p 3, Jug r 3, Mal d 3, Tri a 14, Cor a 8 (2, 0.65%)		
Group 6 (142)	**Q** Pru p 3, Mal d 3, Jug r 3, Tri a 14, Cor a 8, Ara h 9 (142, 46.25%)				

The different LTP combination profiles (A to Q) obtained on each group are represented: Group 1 (positive for 1 LTP), Group 2 (positive for 2 LTPs), Group 3 (positive for 3 LTPs), Group 4 (positive for 4 LTPs), Group 5 (positive for 5 LTPs) and Group 6 (positive for all 6 LTPs). Also, the number of patients and the percentage they represent (from all 307 in the study).

Figure 24. sIgE distribution against clusters.



	Pru p 3	Mal d 3	Jug r 3	Ara h 9	Cor a 8	Tri a 14
Group 1	0.11 [0.04-0.14]	0.06 [0.05-0.07]	0.01 [0.00-0.03]	0.01 [0.00-0.04]	0.00 [0.00-0.01]	0.02 [0.00-0.20]
Group 2	0.23 [0.16-0.53]	0.17 [0.11-0.26]	0.03 [0.01-0.05]	0.04 [0.01-0.07]	0.01 [0.00-0.03]	0.00 [0.00-0.00]
Group 3	0.68 [0.32-1.07]	0.33 [0.24-0.52]	0.13 [0.06-0.34]	0.07 [0.02-0.17]	0.03 [0.01-0.05]	0.02 [0.00-0.04]
Group 4	1.55 [0.32-3.42]	0.98 [0.29-1.84]	0.20 [0.09-0.37]	0.25 [0.11-0.94]	0.11 [0.05-0.22]	0.02 [0.00-0.04]
Group 5	2.91 [0.97-4.67]	1.65 [0.81-3.81]	0.70 [0.41-1.80]	0.71 [0.42-1.62]	0.39 [0.22-0.90]	0.02 [0.00-0.05]
Group 6	8.5 [3.36-16.05]	6.64 [2.41-12.55]	2.96 [1.40-7.11]	3.81 [1.54-9.84]	1.73 [0.67-5.18]	0.91 [0.31-2.80]
p	****	****	****	****	****	****

The heat map represents sIgE levels (x axis) for the 6 studied LTPs (Pru p 3, Mal d 3, Jug r 3, Ara h 9, Cor a 8 and Tri a 14) when patients (y axis) positive at least for one LTP, are grouped into the 17 reactivity clusters obtained. Patients were first classified by groups (1 to 6) and clusters (A to Q) in each, ordering then sIgE levels from low to high. Moreover, sIgE levels (kU_A/L, median [IQR]) for each LTP were compared between groups (represented below the graph) with Kruskal-Wallis test. Asterisks express significance of p value * 0.01 to 0.05, ** 0.001 to 0.01, *** 0.0001 to 0.001, **** <0.0001 and ns as non-significant.

5.2.4 Clinical symptoms and clusters

The frequency of symptoms to peach was compared on high reactivity patients being sensitized to 4 or more LTPs (243 subjects, 79.7%), and low reactivity ones, being sensitized to less than 4 LTPs (62 subjects, 20.3%) (**Table 16**). Tolerance to peach was statistically higher on low reactivity patients. The same analysis was done choosing the most severe symptom to any of the studied LTPs for each patient: no differences were found when comparing tolerant and allergic individuals. Indeed, local symptoms were statistically superior on <4 LTPs group while systemic on >4 LTPs groups (p= 0.0253).

Table 16. Symptoms frequency on multiple reactivity groups.

	Peach symptoms			All foods symptoms		
	<4 LTPs (%)	**≥4 LTPs** (%)	**p**	**<4 LTPs** (%)	**≥4 LTPs** (%)	**p**
Tolerant	25.8	14.0	*	16.1	9.5	ns
Allergic	64.5	67.9	ns	83.9	90.1	ns
Avoidance	9.7	18.1	ns	0.0	0.4	ns
Local	29.0	25.9	ns	33.9	19.3	*
Systemic	35.5	42.0	ns	50.0	70.8	*
OAS	32.1	25.6	ns	-	-	-
GI	3.6	8.5	ns	-	-	-
CU	23.2	27.6	ns	-	-	-
U/AE	28.6	33.7	ns	-	-	-
RESP	0.0	0.5	ns	-	-	-
AN	10.7	17.1	ns	-	-	-

The patients were separated in high reactivity group (4 or more positive LTPs) and low reactivity group (less than 4 positive LTP). Symptoms to peach were compared. Allergy, tolerance or avoidance. Moreover, allergic ones were compared according to their clinical reactivity: Local (CU: contact urticaria; OAS: oral allergy syndrome; GI: gastrointestinal symptoms), systemic symptoms (U/AE: generalized urticaria or angioedema; AN: anaphylaxis). The same comparison was done over the frequency of the most severe symptom from all 6 foods implicated. P values were obtained after chi-squared and Fisher's exact test. Asterisks express significance of p value * 0.01 to 0.05, ** 0.001 to 0.01, *** 0.0001 to 0.001, **** <0.0001 and ns as non-significant.

From our 12 mono-sensitized patients: 3 were tolerant to all six tested foods (2 were Tri a 14 +; 1 was Pru p 3 +), 5 had local symptoms for some of them (1 was Tri a 14 +; 4 were

Pru p 3+) and 4 had had systemic reaction for at least one LTP (1 was Tri a 14+; 3 were Pru p 3+). In addition, 6 patients were negative for Pru p 3 being: 2 of them tolerant to all studied foods (both were Tri a 14 +); 2 local (1 was Tri a 14 + and 1 was Mal d 3 and Cor a 8+) and 2 systemic symptoms (one as Tri a 14+ and the other was Cor a 8 and Ara h 9 +) to at least one of the foods containing the studied LTPs.

5.3 Improving *in vitro* detection of sensitization to Lipid Transfer Proteins: LTP Strip, a new molecular multiplex IgE assay

This part of the study was designed to achieve the **Objective 2**. In short, to develop and validate a novel multiplex assay containing multiple LTPs from a wide range of taxonomically related and unrelated allergenic sources for the diagnosis and clinical management of patients with LTP syndrome.

The results from this part of the study have been published in the following cited paper (**Annex**, original version):

Improving *in vitro* detection of sensitization to Lipid Transfer Proteins: A new molecular multiparameter IgE assay

Balsells-Vives S, Flügge U, Brix B, Weimann Y, Peralta T, San Bartolomé C, Araujo-Sánchez G, Casas-Saucedo R, Torradeflot M, Lara R, Munoz-Cano R, Bartra J, Suer W, Pascal M. Mol Nutr Food Res. 2023 May 17:e2200906. DOI: 10.1002/mnfr.202200906

Journal information: **Molecular Nutrition & Food Research**

Impact factor (2022):5.2.

Journal Citation Reports (Clarivate, 2023): 34/142 (Food Science & Technology (Science)).

5.3.1 Patient's characterization

38 adults (age median [IQR]: 43 [34-50] years; female: 27 (71%)) with LTP syndrome diagnosis were included in this analysis (**Tables 17,18** and **19**).

The reactivities to the plant-food allergenic sources found by PbP are reported in **Table 20.** All patients showed a positive PbP to peach peel, whereas 13 (34%) of them also reacted to the pulp. Other most frequent sensitizations found were apple peel (92%), peanut (89%), hazelnut (87%), green bean (87%) followed by cherry peel (86%), corn (84%), melon (81%), walnut (79%), tomato peel (71%), almond (68%), lettuce (66%) and kiwi pulp (66%). Although, pea was only tested in 14 patients, the sensitization was quite high (71%). Wheat, peach pulp, banana, lentil and sesame showed low sensitization (< 40 %).

5.3.2 EUROLINE-LTP Immunoassay validation

In the EUROLINE-LTP immunoblot assay (from now on LTP-Strip), each of the recombinant nsLTPs could evoke a positive sIgE response confirming a suitable epitope presentation (**Figure 25**). The measured sIgE intensities are presented in EAST classes (**Fig. 25**), spanning from low sIgE reactivity for Ses i LTP 1 and Tri a 14, to highly reactive components such as Pru p 3, Pru av 3, and Mal d 3.

Two subjects (patients 2+3) showed IgE sensitization to every recombinant protein included in the LTP-strip while all other subjects recognized at least four nsLTPs from different allergenic sources, although not following any particular pattern. Most patients were sensitized to allergens of the *Rosacae* family, like Pru p 3, Pru av 3, Pru du 3 and Mal d 3. Isoforms (as i.e. Pru du 3) showed different IgE reactivity patterns justifying the need of working with various homologous forms of the allergens. Ses i LTP 5 is the most frequently recognized isoform (26/38) from the sesame LTPs. Interestingly, 29 patients had high sIgE levels for the LTP from melon (Cuc m LTP).

The analytical performance of the LTP-strip was assessed considering the PbP data as the reference test result, due to logistic and practical limitation to challenge the patients for all allergenic sources *via* OFC. As whole food may contain more LTP than processed extracts the PbP got preference over SPT (see **Table 21** and **22**).

Table 17. Anamnestic data from the patients tested.

					Banana		Tomato		Walnut		Lentil		Melon		Wheat	
Patient	PR-10	Profilin	Storage proteins	Act d 2 TLP	PbP	S	PbP	S	PbP	S	PbP	S	PbP	S	PbP	S
1	<0.3	<0.3	<0.3	<0.3	3	TOL	5	TOL	20	AN	5	TOL	6	TOL	6	TOL
2	<0.3	<0.3	<0.3	<0.3	4	OAS, U/AE	7	OAS, AN	1	OAS, U/AE	0	TOL	3	OAS, U/AE	4	TOL
3	<0.3	<0.3	<0.3	<0.3	0	GID	6	OAS	4	OAS	0	TOL	0	X	0	TOL
4	<0.3	<0.3	<0.3	<0.3	2	OAS	3	TOL	25	X	4	TOL	4	GID	8	TOL
5	<0.3	<0.3	<0.3	<0.3	4	GID, U/AE	3	TOL	2	OAS, GID	2	TOL	3	TOL	3	TOL
6	<0.3	<0.3	<0.3	<0.3	0	TOL	11	GID	4	AN	0	TOL	3	TOL	6	TOL
7	<0.3	<0.3	<0.3	<0.3	2	TOL	5	AN	11	OAS	5	TOL	9	TOL	4	TOL
8	<0.3	<0.3	<0.3	<0.3	10	AN	7	TOL	8	AN	0	TOL	7	TOL	3	TOL
9	<0.3	<0.3	<0.3	<0.3	0	TOL	2	TOL	7	X	0	TOL	4	TOL	0	TOL
10	<0.3	<0.3	<0.3	<0.3	4	TOL	4	TOL	13	OAS, U/AE	3	TOL	5	U/AE	2	TOL
11	<0.3	<0.3	<0.3	<0.3	6	TOL	14	TOL	10	X	0	TOL	7	TOL	0	TOL
12	<0.3	<0.3	<0.3	<0.3	3	TOL	7	TOL	4	X	0	GID	6	TOL	0	GID
13	<0.3	<0.3	<0.3	<0.3	4	TOL	5	TOL	4	X	0	TOL	3	TOL	2	TOL
14	<0.3	<0.3	<0.3	<0.3	0	TOL	4	TOL	5	OAS	3	TOL	4	TOL	0	TOL
15	<0.3	<0.3	<0.3	<0.3	4	TOL	5	TOL	10	U/AE, AN	3	TOL	3	TOL	5	TOL
16	<0.3	<0.3	<0.3	<0.3	2	TOL	9	TOL	5	TOL	0	TOL	5	U/AE, CU	0	TOL
17	<0.3	<0.3	<0.3	<0.3	0	TOL	3	CU	2	OAS	0	TOL	4	OAS	2	GID
18	<0.3	<0.3	<0.3	<0.3	3	U/AE	4	TOL	11	OAS	2	GID	3	TOL	4	GID
19	<0.3	<0.3	<0.3	<0.3	2	TOL	2	TOL	3	TOL	2	TOL	6	TOL	0	TOL

PR-10, Profilin, Storage proteins, TLP reactivities. Symptoms (S) and PbP (Prick by prick). CU: contact urticaria; OAS: oral allergy syndrome; GI: gastrointestinal symptoms; U/AE: generalized urticaria or angioedema; AN: anaphylaxis. (Patient 1 to 19, Part 1)

Table 17. Anamnestic data from the patients tested. Continuation.

					Banana		Tomato		Walnut		Lentil		Melon		Wheat	
Patient	PR-10	Profilin	Storage proteins	Act d 2 TLP	PbP	S	PbP	S	PbP	S	PbP	S	PbP	S	PbP	S
20	<0.3	<0.3	<0.3	<0.3	4	GID	5	TOL	6	U/AE	2	TOL	6	TOL	3	TOL
21	<0.3	<0.3	<0.3	<0.3	1	TOL	3	TOL	4	OAS	0	TOL	3	U/AE	3	TOL
22	<0.3	<0.3	<0.3	<0.3	3	TOL	3	TOL	7	X	0	TOL	7	TOL	3	TOL
23	<0.3	<0.3	<0.3	<0.3	0	TOL	0	TOL	5	AN	0	TOL	3	TOL	0	TOL
24	<0.3	<0.3	<0.3	<0.3	0	X	0	TOL	5	OAS	0	TOL	3	X	0	TOL
25	<0.3	<0.3	<0.3	<0.3	0	X	10	TOL	6	AN	0	TOL	9	OAS	0	TOL
26	<0.3	<0.3	<0.3	<0.3	3	TOL	2	TOL	6	TOL	3	TOL	5	TOL	2	TOL
27	<0.3	<0.3	<0.3	<0.3	0	TOL	4	TOL	5	AN	3	TOL	6	OAS, CU	2	TOL
28	<0.3	<0.3	<0.3	<0.3	0	TOL	0	TOL	0	TOL	0	TOL	0	TOL	0	TOL
29	<0.3	<0.3	<0.3	<0.3	0	X	5	AN	5	X	0	TOL	0	U/AE	0	TOL
30	<0.3	<0.3	<0.3	<0.3	0	AN	1	X	10	OAS	6	X	9	OAS	5	GID
31	<0.3	<0.3	<0.3	<0.3	2	TOL	4	TOL	8	X	0	TOL	9	TOL	3	TOL
32	<0.3	<0.3	<0.3	<0.3	0	TOL	3	TOL	6	X	0	TOL	8	TOL	2	TOL
33	<0.3	<0.3	<0.3	<0.3	2	TOL	4	OAS, GID	6	OAS	0	TOL	2	GID	6	TOL
34	<0.3	<0.3	<0.3	<0.3	0	OAS	0	OAS	5	X	6	TOL	6	CU, AN	0	TOL
35	<0.3	<0.3	<0.3	<0.3	0	TOL	0	GID	0	X	0	TOL	0	TOL	0	TOL
36	<0.3	<0.3	<0.3	<0.3	0	TOL	2.5	U/AE	2	OAS, GID, U/AE	0	TOL	3	TOL	0	TOL
37	<0.3	<0.3	<0.3	<0.3	0	TOL	2	TOL	1	TOL	1	TOL	0	TOL	0	TOL
38	<0.3	<0.3	<0.3	<0.3	0	TOL	0	TOL	0	TOL	0	GID	0	TOL	0	GID

PR-10, Profilin, Storage proteins, TLP reactivities. Symptoms (S) and PbP (Prick by prick). CU: contact urticaria; OAS: oral allergy syndrome; GI: gastrointestinal symptoms; U/AE: generalized urticaria or angioedema; AN: anaphylaxis. (Patient 20 to 38, Part 1)

Table 18. Anamnestic data from the patients tested (part 2).

Patient	Corn		Peanut		Hazelnut		Peach		Apple		Green bean		Cherry		Lettuce	
	PbP	S	PbP	S	PbP	S	PbP	S	PbP	S	PbP	S	PbP	S	PbP	S
1	9	TOL	10	OAS, U/AE	10	OAS, U/AE	5	TOL	8	TOL	10	TOL	5	TOL	4	TOL
2	10	OAS	7	OAS	10	OAS	10	CU, OAS, U/AE	8	OAS	5	OAS	10	OAS	6	OAS
3	7	TOL	5	OAS	5	OAS	11	GID	10	GID	15	TOL	7	X	3	GID
4	8	TOL	7	X	10	X	12	AN	12	U/AE, AN	9	TOL	5	X	5	GID, U/AE
5	4	TOL	10	X	5	OAS, GID	5	CU, OAS, GID	5	OAS, GID	5	OAS	5	OAS, GID	3	OAS
6	5	TOL	14	AN	8	AN	6	OAS, CU	6	AN	9	TOL	7	X	4	TOL
7	9	OAS, GID	8	OAS	11	OAS	6	X	10	OAS, GID	10	TOL	10	X	6	OAS, GID
8	10	TOL	10	X	16	X	12	X	10	TOL	8	TOL	10	TOL	2	TOL
9	2	TOL	6	X	4	U/AE	13	X	7	X	10	OAS	0	X	10	TOL
10	9	TOL	10	X	7	OAS, U/AE	11	TOL	10	TOL	9	TOL	6	X	10	TOL
11	10	TOL	14	X	11	AN	13	AN	13	TOL	16	AN	7	X	14	TOL
12	19	GID	4	X	9	X	7	CU	6	X	7	AN	Not tested	TOL	9	X
13	4	TOL	4	X	12	TOL	4	TOL	6	TOL	5	TOL	5	TOL	4	TOL

Symptoms (S) and PbP (Prick by prick). CU: contact urticaria; OAS: oral allergy syndrome; GI: gastrointestinal symptoms; U/AE: generalized urticaria or angioedema; AN: anaphylaxis. (Patient 1 to 13, Part 2)

Figure 18. Anamnestic data from the patients tested (part 2). Continuation.

Patient	Corn		Peanut		Hazelnut		Peach		Apple		Green bean		Cherry		Lettuce	
	PbP	S	PbP	S	PbP	S	PbP	S	PbP	S	PbP	S	PbP	S	PbP	S
14	5	TOL	9	U/AE	20	OAS	9	CU	10	TOL	11	TOL	20	TOL	0	TOL
15	9	TOL	12	AN	6	TOL	4	CU	4	TOL	9	TOL	4	TOL	2	TOL
16	7	TOL	5	OAS	9	AN	5	CU, OAS	7	TOL	4	TOL	4	OAS	4	X
17	7	TOL	3	OAS, GID	7	TOL	3	OAS, GID, CU	7	TOL	5	TOL	11	GID	3	TOL
18	5	TOL	10	GID	16	U/AE	10	TOL	4	TOL	3	GID	3	GID	0	TOL
19	3	TOL	0	TOL	3	TOL	10	AN	11	TOL	6	TOL	11	TOL	6	AN
20	8	U/AE	4	U/AE	5	U/AE	4	GID	7	GID	6	TOL	4	TOL	3	TOL
21	5	X	8	GID	4	TOL	8	AN	10	U/AE	5	TOL	5	GID	3	TOL
22	8	TOL	9	X	7	X	6	OAS	8	TOL	10	TOL	8	X	3	TOL
23	5	TOL	6	X	12	X	6	OAS	5	GID	6	TOL	4	TOL	2	TOL
24	6	TOL	4	OAS	9	TOL	6	AN	4	OAS	8	TOL	0	X	0	TOL
25	10	AN	7	AN	3	AN	10	AN	11	AN	9	AN	Not tested	U/AE	6	AN
26	2	TOL	14	OAS	6	TOL	9	OAS	9	TOL	8	TOL	5	TOL	0	TOL

Symptoms (S) and PbP (Prick by prick). CU: contact urticaria; OAS: oral allergy syndrome; GI: gastrointestinal symptoms; U/AE: generalized urticaria or angioedema; AN: anaphylaxis. (Patient 14 to 26, Part 2)

Figure 18. Anamnestic data from the patients tested (part 2). Continuation.

Patient	Corn		Peanut		Hazelnut		Peach		Apple		Green bean		Cherry		Lettuce	
	PbP	S	PbP	S	PbP	S	PbP	S	PbP	S	PbP	S	PbP	S	PbP	S
27	6	TOL	3	U/AE	3	X	4	CU, OAS, U/AE	10	TOL	3	TOL	5	TOL	5	GID
28	0	TOL	3	TOL	0	TOL	14	U/AE	8	TOL	0	TOL	9	OAS	0	TOL
29	5	TOL	0	AN	0	X	5	AN	0	U/AE	0	TOL	0	X	3	AN
30	8	TOL	7	X	10	OAS	20	AN	18	X	10	X	10	X	4	X
31	7	OAS	6	AN	7	X	12	X	6	TOL	14	TOL	3	X	2	GID
32	10	X	7	GID	6	GID	9	TOL	8	TOL	9	TOL	4	TOL	6	X
33	7	GID	9	OAS	5	X	9	CU	6	TOL	9	TOL	5	GID	5	GID
34	3	OAS	5	X	3	AN	10	AN, CU	11	AN	11	U/AE	9	OAS	2	TOL
35	0	TOL	5	X	0	GID	7	AN, CU	2	GID	0	TOL	5	X	0	GID
36	6	TOL	2	TOL	2	GID, U/AE	3	TOL	3	X	3	TOL	3.5	TOL	3	GID
37	0	TOL	5	OAS	5	TOL	6	U/AE	2	TOL	0	TOL	2	TOL	0	TOL
38	0	TOL	0	TOL	0	TOL	3	AN	3	OAS	0	GID	0	OAS	0	GID

Symptoms (S) and PbP (Prick by prick). CU: contact urticaria; OAS: oral allergy syndrome; GI: gastrointestinal symptoms; U/AE: generalized urticaria or angioedema; AN: anaphylaxis. (Patient 27 to 38, Part 2)

Table 19. Anamnestic data from the patients tested (part 3).

Patient	Kiwi		Almond		Peas		Sesame	
	PbP	S	PbP	S	PbP	S	PbP	S
1	4	TOL	9	U/AE	6	TOL	8	TOL
2	6	OAS	6	CU	3	OAS	1	OAS
3	0	OAS	0	TOL	Not tested		Not tested	
4	7	TOL	3	X	0	TOL	4	TOL
5	5	OAS	6	OAS, GID	4	TOL	2	TOL
6	5	TOL	0	TOL	Not tested		Not tested	
7	3	X	5	OAS	3	OAS, GID	0	TOL
8	11	X	10	X	Not tested		Not tested	
9	6	TOL	4	X	Not tested		Not tested	
10	3	TOL	7	OAS, U/AE	0	TOL	0	TOL
11	7	TOL	6	X	Not tested		Not tested	
12	3	X	6	AN	Not tested		Not tested	
13	4	TOL	3	OAS, U/AE	3	TOL	0	OAS, U/AE
14	3	TOL	10	OAS	Not tested		Not tested	
15	4	TOL	3	TOL	4	TOL	3	TOL
16	4	OAS	1	TOL	Not tested		Not tested	
17	4	TOL	3	TOL	3	TOL	2	TOL
18	2	TOL	3	U/AE	Not tested		Not tested	
19	6	TOL	5	TOL	Not tested		Not tested	
20	4	TOL	5	U/AE	Not tested		Not tested	

Symptoms (S) and PbP (Prick by prick). CU: contact urticaria; OAS: oral allergy syndrome; GI: gastrointestinal symptoms; U/AE: generalized urticaria or angioedema; AN: anaphylaxis. (Patient 1 to 20, Part 3)

Table 20. Anamnestic data from the patients tested (part 3). Continuation.

	Kiwi		Almond		Peas		Sesame	
Patient	PbP	S	PbP	S	PbP	S	PbP	S
21	0	TOL	3	TOL	3	TOL	0	TOL
22	7	TOL	13	U/AE	Not tested		Not tested	
23	3	OAS	0	X	2	TOL	0	TOL
24	3	X	0	TOL	Not tested		Not tested	
25	0	U/AE	Not tested		Not tested		Not tested	
26	3	TOL	5	TOL	Not tested		Not tested	
27	3	OAS	3	TOL	2	GID	0	TOL
28	0	TOL	2	TOL	Not tested		Not tested	
29	0	OAS	0	X	Not tested		Not tested	
30	4	OAS	15	OAS	Not tested		Not tested	
31	2	TOL	0	X	Not tested		Not tested	
32	3	TOL	9	GID	Not tested		Not tested	
33	2	TOL	0	U/AE	3	GID	0	TOL
34	0	OAS	3	TOL	Not tested		Not tested	
35	0	TOL	0	X	Not tested		Not tested	
36	1.5	TOL	3	GID, U/AE	0	TOL	0	TOL
37	0	TOL	0	TOL	Not tested		Not tested	
38	0	OAS	0	TOL	Not tested		0	TOL

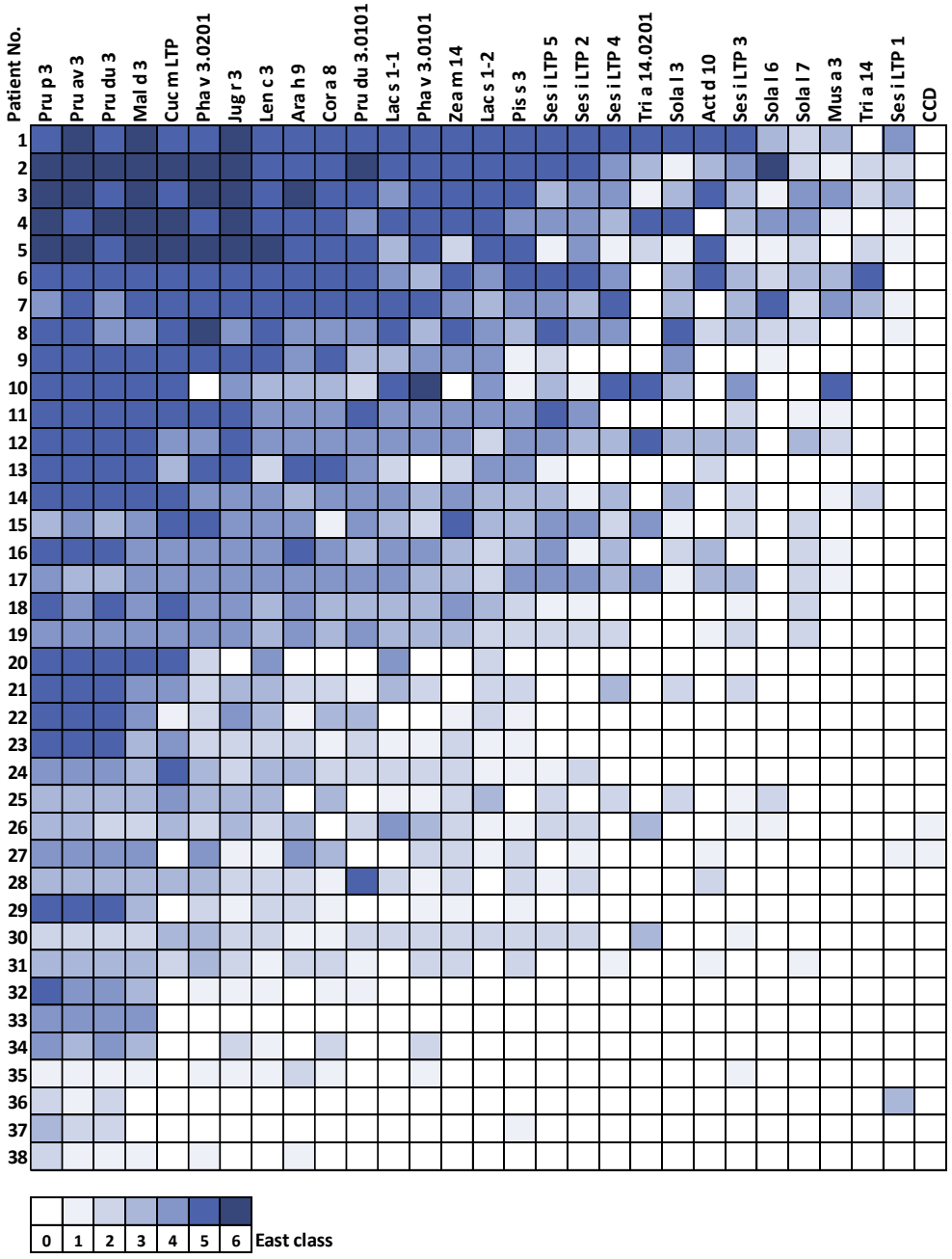
Symptoms (S) and PbP (Prick by prick). CU: contact urticaria; OAS: oral allergy syndrome; GI: gastrointestinal symptoms; U/AE: generalized urticaria or angioedema; AN: anaphylaxis. (Patient 21 to 38, Part 3)

Table 21. Plant-food sensitization detected by prick-by-prick test.

Allergen source	n (total)	PbP Positive	
		n	%
Peach peel	38	38	100%
Apple peel	38	35	92%
Peanut	38	34	89%
Hazelnut	38	33	87%
Green Bean	38	33	87%
Cherry Peel	36	31	86%
Corn	38	32	84%
Melon	37	30	81%
Walnut	38	30	79%
Peas	14	10	71%
Tomato peel	38	27	71%
Almond	37	25	68%
Kiwi pulp	38	25	66%
Lettuce	38	25	66%
Wheat	38	15	39%
Peach pulp	38	13	34%
Banana	38	13	34%
Lentil	38	10	26%
Sesame	15	3	20%

Total of patients tested with prick by prick for each food (n total). Number and percentage of positives on Prick by prick (PbP).

Figure 25. Heatmap of sIgE reactivities against nsLTPs of the LTP strip.



Measured by the nsLTP immunoblot assay in 38 patient sera. nsLTPs recognized from the same individual are shown in rows. The level of sIgE reactivity is indicated in different shades of blue. Every shade is exemplary for a class (Class: 0 negative; Class:1-6 positive). The nsLTPs are arranged by the total sum of the sIgE reactivity from all patients (left: highest sIgE levels, right: lowest sIgE levels).

Table 22. Prick by Prick outcome and anamnestic data.

Allergenic source (number of patients tested)	Prick by prick POSITIVE				Prick by prick NEGATIVE			
	Total positive	Allergic	Tolerant	Avoid	Total negative	Allergic	Tolerant	Avoid
Peach peel (38)	38	27 (71%)	7 (18%)	4	0	0 (0%)	0 (0%)	0
Walnut (mashed) (38)	30	17 (57%)	3 (10%)	10	8	4 (50%)	3 (38%)	1
Almond (mashed) (37)	25	14 (56%)	7 (28%)	4	12	1 (8%)	7 (58%)	4
Peach Pulp (38)	11	6 (55%)	3 (27%)	2	27	22 (81%)	3 (11%)	2
Peanut (mashed) (38)	34	18 (53%)	3 (9%)	13	4	1 (25%)	3 (75%)	0
Hazelnut (mashed) (38)	33	17 (52%)	8 (24%)	8	5	2 (40%)	2 (40%)	1
Lettuce (38)	25	11 (44%)	10 (40%)	4	13	3 (23%)	10 (77%)	0
Banana (38)	13	5 (42%)	8 (62%)	0	25	4 (16%)	18 (72%)	3
Apple peel (38)	36	14 (39%)	18 (50%)	4	2	1 (50%)	1 (50%)	0
Pea (mashed) (14)	9	3 (33%)	6 (67%)	0	5	1 (20%)	4 (80%)	0
Melon pulp (37)	31	10 (32%)	20 (65%)	1	6	2 (33%)	4 (67%)	0
Cherry peel (36)	31	9 (29%)	12 (39%)	10	5	1 (20%)	1 (20%)	3
Tomato peel (38)	26	7 (27%)	19 (73%)	0	12	3 (25%)	8 (67%)	1
Corn flour (38)	32	8 (25%)	22 (69%)	2	6	0 (0%)	6 (100%)	0
Green bean (38)	33	8 (24%)	24 (73%)	1	5	1 (20%)	4 (80%)	0
Kiwi pulp (38)	25	6 (24%)	15 (60%)	4	13	5 (38%)	8 (62%)	0
Wheat flour (38)	15	2 (13%)	13 (87%)	0	23	3 (13%)	20 (87%)	0
Lentil (boiled) (38)	10	0 (0%)	9 (90%)	1	28	3 (11%)	25 (89%)	0
Sesame (mashed) (15)	3	0 (0%)	3 (100%)	0	12	2 (17%)	10 (83%)	0

Patients are grouped according to PbP results (positive/negative). On each, are classified by being allergic or tolerant. Some patients could not be classified due to avoidance (for medical recommendation, fear or dislike).

Table 23. LTP-strip outcome and anamnestic data.

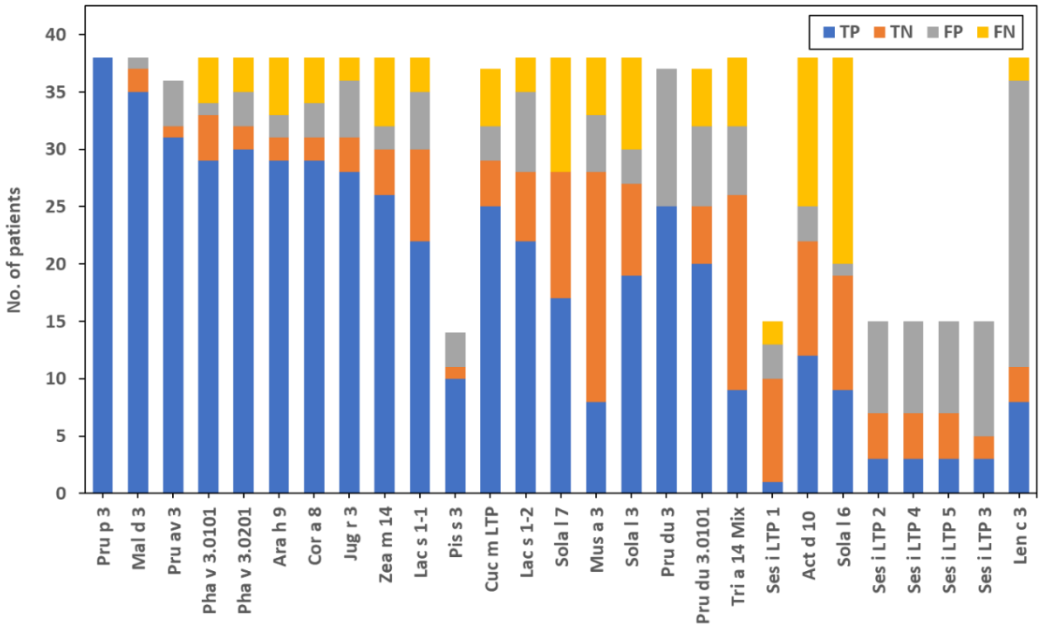
LTP Allergen	LTP-strip POSITIVE				LTP-strip NEGATIVE			
	N	Allergic	Tolerant	Avoid	N	Allergic	Tolerant	Avoid
Pru p 3	38	27 (71%)	7 (18%)	4	0	0 (0%)	0 (0%)	0
Jug r 3	33	20 (61%)	3 (9%)	10	5	1 (20%)	3 (60%)	1
Ara h 9	31	18 (58%)	3 (10%)	10	7	1 (14%)	3 (43%)	3
Mus a 3	12	6 (50%)	6 (50%)	0	26	3 (12%)	20 (77%)	3
Cor a 8	32	16 (50%)	7 (22%)	9	6	3 (50%)	3 (50%)	0
Pru du 3.0101	28	14 (50%)	8 (29%)	6	10	1 (10%)	7 (70%)	2
Sola l 6	11	5 (45%)	6 (55%)	0	27	5 (19%)	21 (78%)	1
Mal d 3	36	14 (39%)	18 (50%)	4	2	1 (50%)	1 (50%)	0
Pru du 3	38	15 (39%)	14 (37%)	8	0	0 (0%)	0 (0%)	0
Lac s 1-1	27	10 (37%)	14 (52%)	3	11	4 (36%)	6 (55%)	1
Cuc m LTP	29	10 (36%)	18 (64%)	1	9	2 (22%)	6 (67%)	1
Sola l 7	16	5 (31%)	11 (69%)	0	22	5 (23%)	16 (73%)	1
Lac s 1-2	28	10 (36%)	15 (54%)	3	10	4 (40%)	5 (50%)	1
Pis s 3	30	5 (17%)	24 (80%)	1	8	0 (0%)	8 (100%)	0
Sola l 3	17	5 (29%)	12 (71%)	0	21	5 (24%)	15 (71%)	1
Pru av 3	38	11 (29%)	14 (37%)	13	0	0 (0%)	0 (0%)	0
Act d 10	14	4 (29%)	9 (64%)	1	24	7 (29%)	14 (58%)	3
Pha v 3.0101	30	8 (27%)	22 (73%)	0	8	1 (13%)	6 (75%)	1
Ses i LTP 1	9	1 (11%)	7 (78%)	1	29	1 (3%)	27 (93%)	1
Pha v 3.0201	33	8 (24%)	25 (76%)	0	5	1 (20%)	3 (60%)	1
Zea m 14	28	7 (25%)	20 (71%)	1	10	1 (10%)	8 (80%)	1
Ses i LTP 2	22	2 (9%)	19 (86%)	1	16	0 (9%)	15 (94%)	1
Ses i LTP 4	18	2 (11%)	15 (83%)	1	20	0 (0%)	19 (95%)	1
Ses i LTP 5	24	2 (8%)	21 (88%)	1	14	0 (0%)	13 (93%)	1

LTP Allergen	LTP-strip POSITIVE				LTP-strip NEGATIVE			
	N	Allergic	Tolerant	Avoid	N	Allergic	Tolerant	Avoid
Tri a 14.0201	11	2 (18%)	9 (82%)	0	27	3 (11%)	24 (89%)	0
Ses i LTP 3	21	2 (10%)	18 (86%)	1	17	0 (0%)	16 (94%)	1
Len c 3	34	2 (6%)	31 (94%)	0	4	1 (20%)	3 (60%)	0
Tri a 14	6	0 (0%)	5 (100%)	0	32	4 (16%)	27 (84%)	0

Patients are grouped according to LTP-strips results (positive/negative). N= number of patients. On each, are classified by being allergic or tolerant. Some patients could not be classified due to avoidance (for medical recommendation, fear or dislike).

Strip reactivity (positive/negative) was classified qualitatively in comparison to PbP outcome (**Figure 26**).

Figure 26. nsLTP immunoblot assay performance in comparison to PbP results.



For qualitative analysis of the sIgE levels they were subdivided into 4 groups: true positive (TP, blue), true negative (TN, orange), false positive (FP, grey) and false negative (FN, yellow). The allergens are sorted in the descending order according to the relative amount of true positive and true negative reactions.

Table 24. Qualitative analysis of the sIgE reactivity of the nsLTP immunoblot assay in comparison to the PbP outcome.

nsLTP	n (total)	TP	TN	FP	FN	TP+TN
Pru p 3	38	38 (100%)	0 (0%)	0 (0%)	0 (0%)	38 (100%)
Mal d 3	38	35 (92%)	2 (5%)	1 (3%)	0 (0%)	37 (97%)
Pru av 3	36	31 (86%)	1 (3%)	4 (11%)	0 (0%)	32 (89%)
Pha v 3.0101	38	29 (76%)	4 (11%)	1 (3%)	4 (11%)	33 (87%)
Pha v 3.0201	38	30 (79%)	2 (5%)	3 (8%)	3 (8%)	32 (84%)
Ara h 9	38	29 (76%)	2 (5%)	2 (5%)	5 (13%)	31 (82%)
Cor a 8	38	29 (76%)	2 (5%)	3 (8%)	4 (11%)	31 (82%)
Jug r 3	38	28 (74%)	3 (8%)	5 (13%)	2 (5%)	31 (82%)
Zea m 14	38	26 (68%)	4 (11%)	2 (5%)	6 (16%)	30 (79%)
Lac s 1-1	38	22 (58%)	8 (21%)	5 (13%)	3 (8%)	30 (79%)
Pis s 3	14	10 (71%)	1 (7%)	3 (21%)	0 (0%)	11 (79%)
Cuc m LTP	38	25 (66%)	4 (11%)	3 (8%)	5 (13%)	29 (76%)
Lac s 1-2	38	22 (58%)	6 (16%)	7 (18%)	3 (8%)	28 (74%)
Sola l 7	38	17 (45%)	11 (29%)	0 (0%)	10 (26%)	28 (74%)
Mus a 3	38	8 (21%)	20 (53%)	5 (13%)	5 (13%)	28 (74%)
Sola l 3	38	19 (50%)	8 (21%)	3 (8%)	8 (21%)	27 (71%)
Pru du 3	37	25 (68%)	0 (0%)	12 (32%)	0 (0%)	25 (68%)
Pru du 3.0101	37	20 (54%)	5 (14%)	7 (19%)	5 (14%)	25 (68%)
Tri a 14 Mix	38	9 (24%)	17 (45%)	6 (16%)	6 (16%)	26 (68%)
Ses i LTP 1	15	1 (7%)	9 (60%)	3 (20%)	2 (13%)	10 (67%)
Act d 10	38	12 (32%)	10 (26%)	3 (8%)	13 (34%)	22 (58%)
Sola l 6	38	9 (24%)	10 (26%)	1 (3%)	18 (47%)	19 (50%)
Ses i LTP 2	15	3 (20%)	4 (27%)	8 (53%)	0 (0%)	7 (47%)
Ses i LTP 4	15	3 (20%)	4 (27%)	8 (53%)	0 (0%)	7 (47%)
Ses i LTP 5	15	3 (20%)	4 (27%)	8 (53%)	0 (0%)	7 (47%)
Ses i LTP 3	15	3 (20%)	2 (13%)	10 (67%)	0 (0%)	5 (33%)

nsLTP	n (total)	TP	TN	FP	FN	TP+TN
Len c 3	38	8 (21%)	3 (8%)	25 (66%)	2 (5%)	11 (29%)

Total number of analysed for each allergen are included. From these, sIgE levels were subdivided into 4 groups: true positive (TP), true negative (TN), false positive (FP) and false negative (FN) results, in absolute number and percentage. The allergens are sorted according to the sum of true positive and true negative (TP+TN) reactions.

The proportion of correctly classified (TP and TN) subjects exceeds 70% for the majority (17/28) of the recombinant nsLTPs (**Table 23**). Particularly good performance is shown by Pru p 3 (100%) and Mal d 3 (97%), Pru av 3 (89%), Pha v 3.0101/0201 (87%, 84%), Ara h 9 (82%), Cor a 8 (82%) and Jug r 3 (82%). Interestingly, the newly introduced nsLTPs, Cuc m LTP and Ses i LTP1 correlate well with the PbP outcome (76% and 67% agreement, respectively).

Even though, many patients were sensitized against Pru du 3 and Len c 3 in the immunoblot assay without a positive PbP outcome.

To validate the reliability of the measured IgE reactivities in the cohort of patients tested, sera of 28 healthy blood donors were analysed with the LTP-strips. These subjects had no clinical history of food or respiratory allergy, were Pru p 3 sIgE negative (<0.1 KU_A/L ImmunoCAP, ThermoFisher Scientific) and /or had negative SPT to peach peel extract (Leti Pharma, Madrid, Spain).

28 healthy donors were tested with the LTP-strips and no IgE reactivity above the cut-off with the tested LTPs was found. Only sera from 4 individuals reacted close to the cut-off (0.35 kU_A/L) for certain proteins (Jug r 3 (2/28), Pru av 3 (1/28), Sola l 7 (1/28), Pru du 3 (1/28) and Pha v 3.0201(1/28), **Table 24**).

Table 25. sIgE reactivities in classes against nsLTPs measured by LTP-strips in sera of 28 healthy donors.

#Blood donor	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
CCD	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ses i LTP 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tri a 14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mus a 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sola l 7	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sola l 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ses i LTP 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Act d 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sola l 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tri a 14.0201	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ses i LTP 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ses i LTP 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ses i LTP 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pis s 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lac s 1-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zea m 14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pha v 3.0101	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lac s 1-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pru du 3.0101	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cor a 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ara h 9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Len c 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Jug r 3	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pha v 3.0201	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cuc m LTP	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mal d 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pru du 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pru av 3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pru p 3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Number 1 indicates class 1 or superior, number 0 indicates class 0.

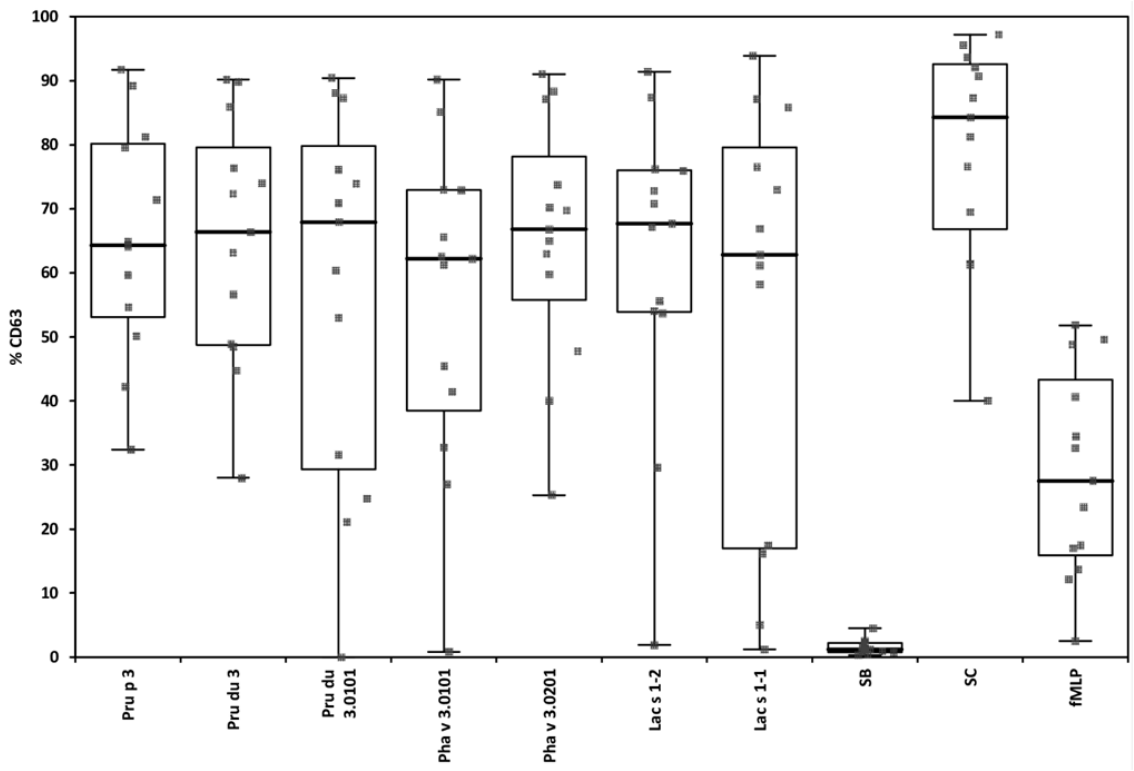
5.3.3 Recombinant nsLTP induced basophil activation in BAT

Nine recombinant nsLTPs (Pru p 3, Lac s 1-1, Lac s 1-2, Pha v 3.0101, Pha v 3.0201, Pru du 3, Pru du 3.0101, Act d 10, Cuc m LTP) of the LTP-strips frequently recognized by LTP allergic patients were used for basophil activation testing (BAT) in a subset of individuals (n=16; 13 patients + 3 healthy donors) to assess their functionality, i.e., ability to crosslink receptor-bound sIgE and cause cell activation (assessed as CD63 expression by flow cytometry).

All proteins were able to induce the expression of CD63 on the cell surface, and thus, are all allergenically active molecules. At the highest allergen dose, the median of %CD63 for all tested recombinant proteins is above 60% (**Figure 27**). All patients showed a high basophil reactivity to Pru p 3, Pru du 3 and Pha v 3.0201.

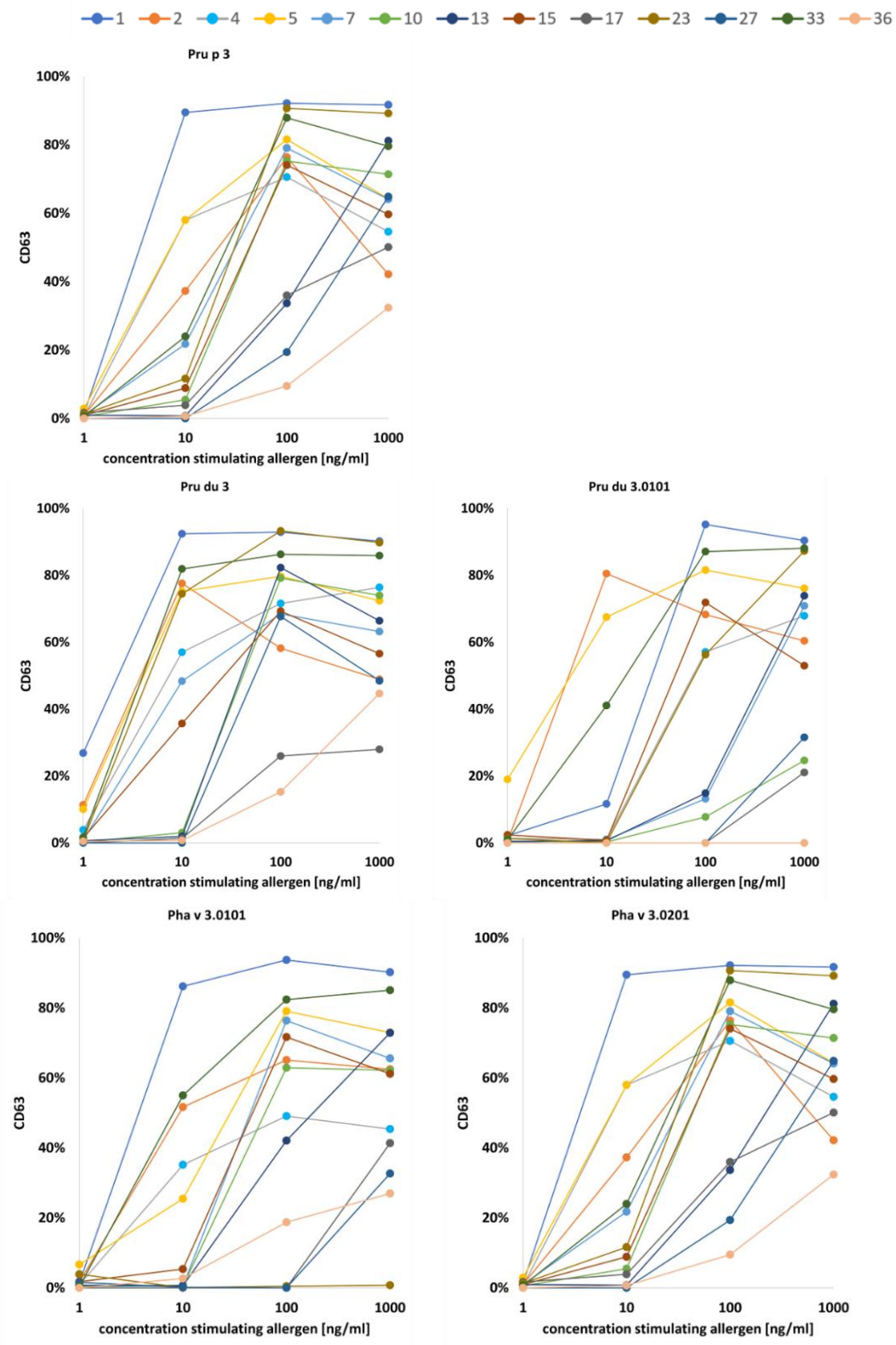
Pru du 3 induced CD63 expression at very low allergen concentrations followed by Lac s 1-2, Lac s 1-1, Pru p 3 and Pha v 3.0101 (**Figure 28**). These results show that trace amounts of allergens induce basophil activation which could trigger an allergic reaction.

Figure 27. Box plot of the BAT results for selected nsLTPs with a stimulation concentration of 1 µg/mL.



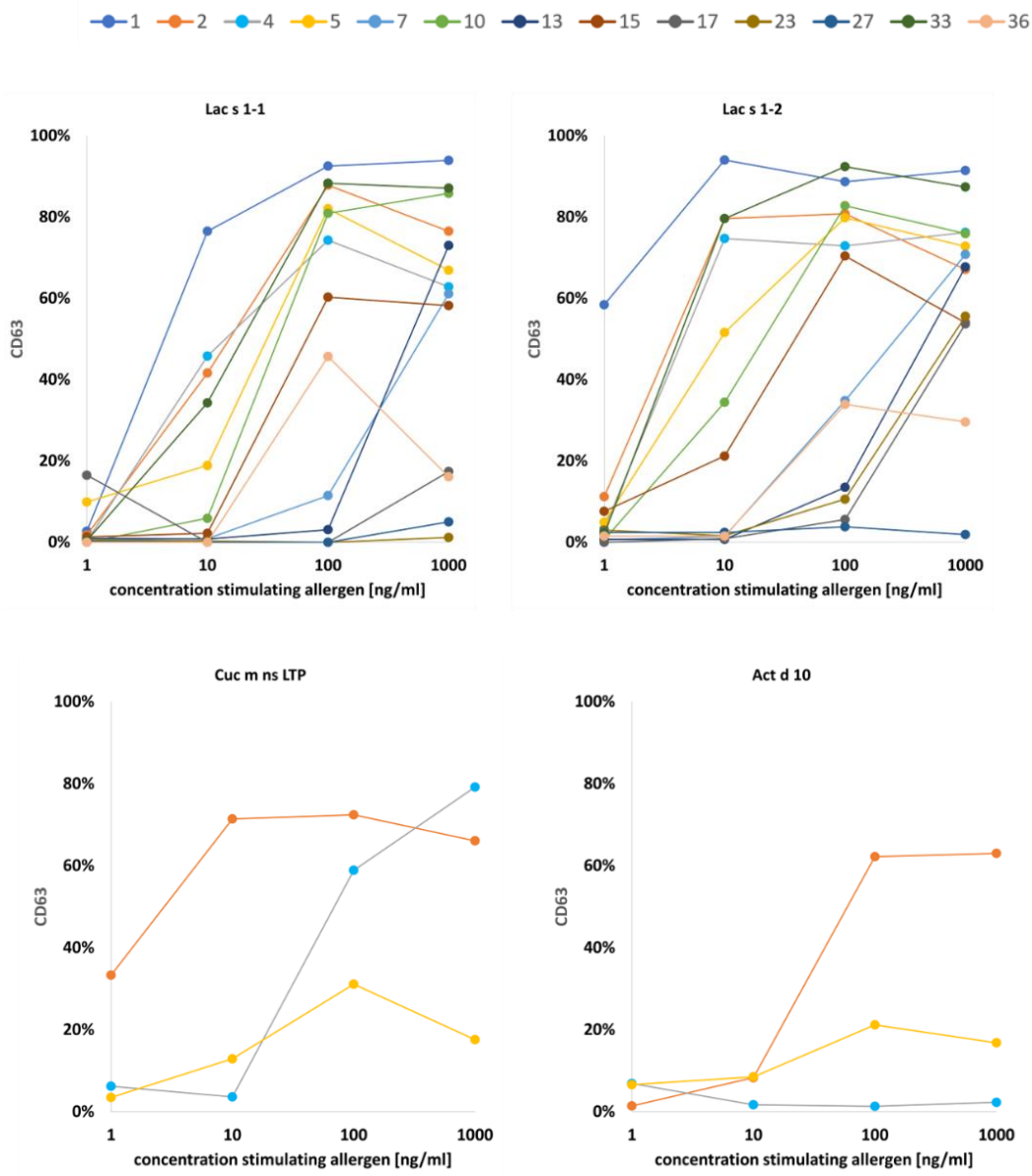
The activation is presented as percentage of CD63 positive out of the basophilic cells. Stimulation with anti-FcεRI antibody (SC, specific IgE control), fMLP (unspecific cell activator) and stimulation buffer (SB, Negative control) is shown.

Figure 28. BAT results for a selected set of allergens and patients (Part 1).



The graphs indicate CD63 expression for the tested allergens Pru p 3 - Peach; Pru du 3.0101 and Pru du 3.2 - Almond; Pha v 3.1 and Pha v 3.2 – Green bean at 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL and 0.001 µg/mL final concentrations, on a total of 13 patients.

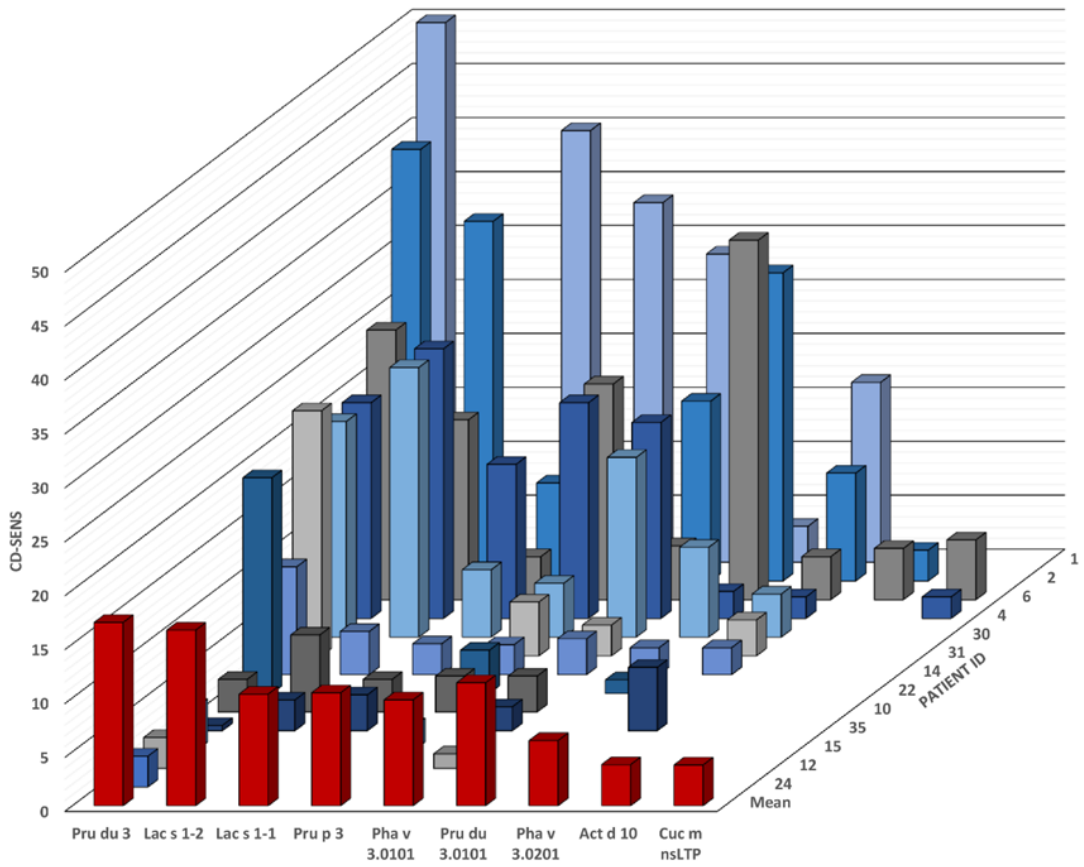
Figure 29. BAT results for a selected set of allergens and patients (Part 2).



The graphs indicate CD63 expression for the tested allergens Lac s 1.1 and Lac s 1.2 -Lettuce; Cuc m – Melon; Act d 10 – Kiwi at 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL and 0.001 µg/mL final concentrations, on a total of 13 patients for lettuce and 3 for melon and kiwi.

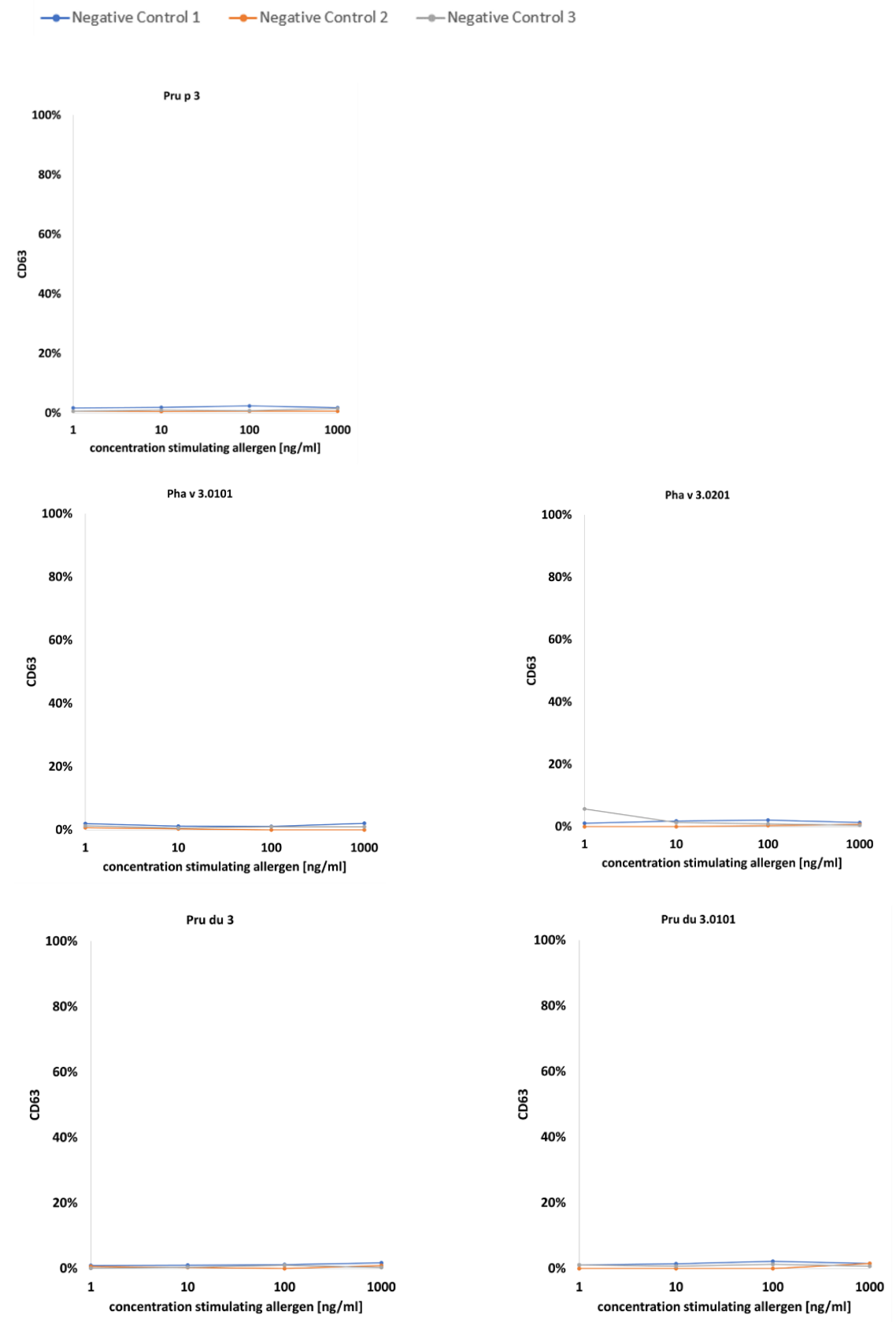
Based on the slope of the dose-response curves (**Figure 28 and 29**) the CD-sens values, as a marker for basophil sensitivity, were estimated (168) and averaged for the selected allergens (**Figure 30**). In **Figure 31** is presented the BAT data for healthy controls, showing no activation.

Figure 30. CD-sens values distribution.



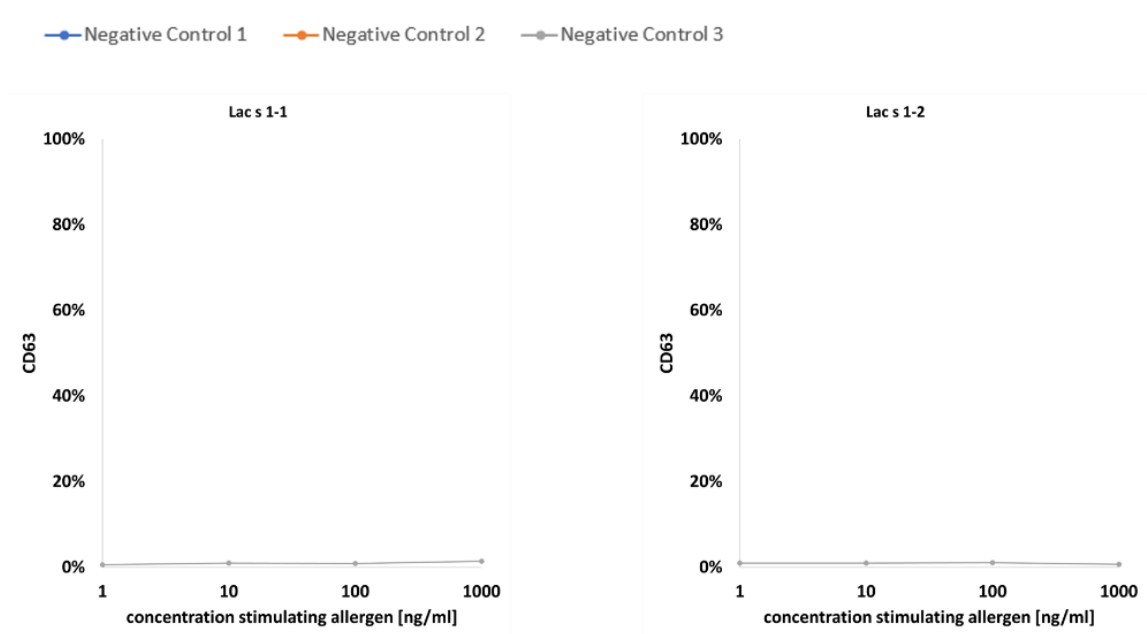
CD-sens values of the BAT for a selected set of allergens and patients. In front the mean CD-sens value is shown in red.

Figure 31. BAT results on negative controls (Part 1).



CD63 expression for the tested allergens (Pru p 3, Pru du 3, Pru du 30101, Pha v 3.0101, Pha v 3.0201) over negative controls are represented (n=3).

Figure 32. BAT results on negative controls (Part 2).



CD63 expression for the tested allergens (Lac s 1-1 and Lac s 1-2) over negative controls are represented (n=3).

In this study, the CD-sens was used as a criterion for the allergy-inducing property of the target protein. For Act d 10 and Cuc mLTP only 3 sera were tested. Thus, the results for these two allergens should be treated like a first indication which needs further confirmation by a larger cohort.

Pru du 3 induced the highest basophil sensitivity in the selected patients followed by Lac s 1-2, Lac s 1-1, Pru p 3 and Pha v 3.0101, although, the basophil reactivity differed significantly between all patients. Patient 1, i.e., shows a very strong basophil response to Pru du 3, Pru p 3, Pha v 3.0101, Pru du 3.0101 and Act d 10, whereas for patient 17 the CD-sens values were very low and only Pru du 3 and Pru p 3 elicited a slight basophil stimulation.

The patient ID from **Figure 29** is consistent with the heat map in **Figure 25**. The patients (1-38) are ordered by their sIgE level response in the LTP-strips. A low ID means high sIgE reactivity. Patient 1, 2, 4 and 5 showed the highest basophil reactivity which correlates with LTP-strips results.

5.3.4 Comparison of LTP-strips with the ImmunoCAP *in vitro* assay

To verify the data from the LTP-strip, the results were compared to an established largely clinically validated *in vitro* assay based on sIgE determination, the ImmunoCAP® system (ThermoFisher Scientific). The degree of concordance between the EAST classes from EUROLINE® (semi-quantitative) vs ImmunoCAP® (quantitative) was analyzed for the 5 plant food LTPs (Pru p 3, Mal d 3, Cor a 8, Jug r 3 and Ara h 9) commercially available in this platform, by Spearman's rank correlation (**Table 25**). There is a positive relationship between the two diagnostic tests for the examined components. The Spearman's rank coefficient varied from 0.6-0.89. Nevertheless, the informative value of this correlation is limited because of the small case number.

Table 26. Correlation between ImmunoCAP and Euroline data.

LTP	r_s	p-Value	n (total)
Pru p 3	0.875	<0.0001	38
Ara h 9	0.890	<0.0001	24
Cor a 8	0.602	0.0019	24
Jug r 3	0.698	0.0001	24
Mal d 3	0.857	<0.0001	33

Were considered EAST-Classes via Spearman's rank correlation. r_s: Spearman's rank correlation coefficient; p: significance level (α=0,05).

5.4 Characterization of a LTP syndrome cohort with the LTP-strip

This part of the study was designed to obtain the **Objective 2**, specifically **Objective 2.2**.

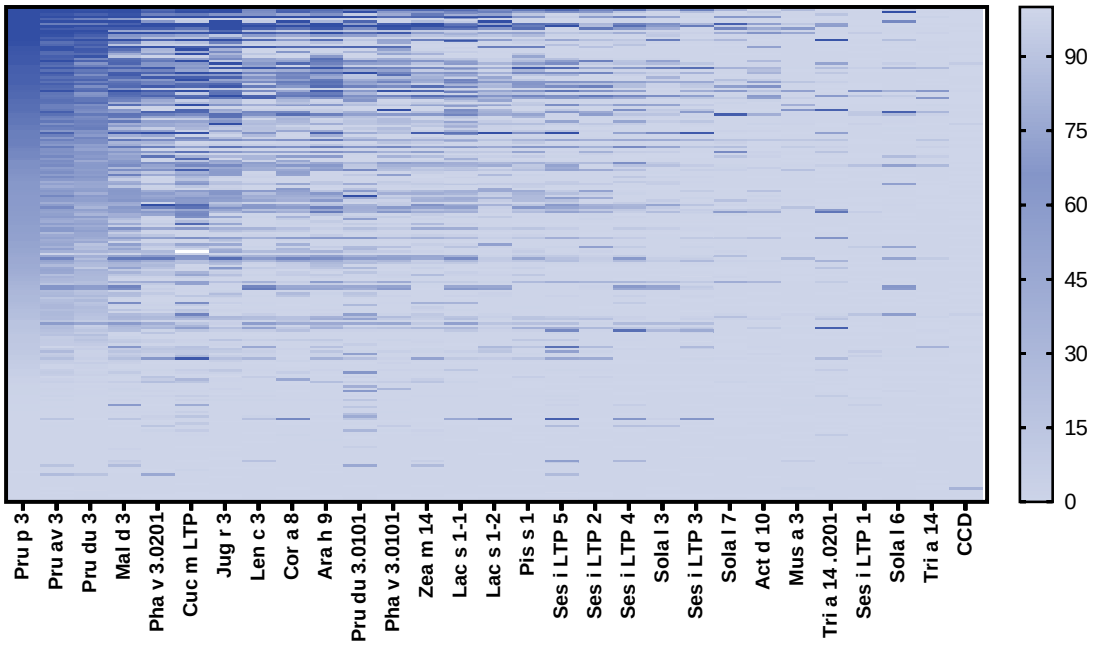
In brief, we aimed to describe the molecular sensitization profile of a real-life cohort of patients with LTP syndrome of our area using the LTP-strip containing a wider spectrum of allergenic sources of relevance in our area.

5.4.1 Patient's characterization

A total of 202 adult subjects [125 women (61.8%); mean [range] age of 46.4 [22-89] years-old diagnosed of LTP allergy in the Allergy Department were retrospectively recruited from a clinical database.

Sensitization to all plant-food LTP allergenic sources included in the LTP strips is depicted in **Figure 32**. The most prevalent sensitizations were Pru p 3 (94.1%), Pru av 3 (93.1%), Pru du 3 (93.1%) and Mal d 3 (92.1%). In addition, the highest sIgE levels (and so, EAST Classes, useful to illustrate sIgE levels gradient as in **Table 26**) were also found on these LTPs, following the same order: Pru p 3, Pru av 3, Pru du 3.1 and Mal d 3 ($p<0.0001$), so sensitization prevalence was positively correlated with sIgE levels. Interestingly, high sIgE levels range from ≥50 to < 100 kU_A/L (Class 5).

Figure 33. LTP strips heat map.



sIgE levels from the 202 patients analysed (y-axis) with EUROIMMUN-LTP strips are represented.

Table 27. Frequency of LTP plant-food sensitization by the LTP strips.

Allergen	Total sensitized		Number of strips analysed n=202 Patients EUROLINE Classes of specific IgE							sIgE Median [IQR]
	N	%	0	1	2	3	4	5	6	
Pru p 3	190	(94.1%)	12	10	18	24	34	89	15	50.9 [10.4-79.0]
Pru av 3	188	(93.1%)	14	14	15	18	42	90	9	46.4 [8.5-76.3]
Pru du 3	188	(93.1%)	14	16	18	19	47	83	5	38.5 [3.5-70.0]
Mal d 3	186	(92.1%)	16	18	18	25	47	69	9	30.6 [3.1-65.4]
Pha v 3.0102	168	(83.2%)	34	18	27	31	36	49	7	11.4 [0.6-54.5]
Cuc m LTP	166	(82.2%)	36	18	19	29	32	54	14	17.4 [0.5-64.5]
Jug r 3	161	(79.7%)	41	21	23	26	34	45	12	10.4 [0.4-51.8]
Len c 3	159	(78.7%)	43	28	33	22	35	39	2	3.1 [0.3-34.6]
Cor a 8	153	(75.7%)	49	22	30	21	38	39	3	3.4 [0.3-46.3]
Ara h 9	151	(74.8%)	51	24	29	22	28	44	4	3.1 [0.3-45.1]
Pru du 3.0101	147	(72.8%)	55	19	22	31	40	30	5	6.5 [0.3-37.2]
Pha v 3.0101	146	(72.3%)	56	28	31	23	30	32	2	2.1 [0.3-28.1]
Zea m 14	144	(71.3%)	58	24	31	32	31	24	2	2.1 [0.3-21.4]
Lac s 1-2	139	(68.8%)	63	31	36	28	25	16	3	0.7 [0.3-11.4]
Lac s 1-1	134	(66.3%)	68	29	18	24	32	30	1	0.7 [0.3-29.3]
Pis s 1	134	(66.3%)	68	32	21	34	24	23	0	0.7 [0.2-16.4]
Ses i LTP 5	116	(57.4%)	86	28	23	16	20	27	2	0.4 [0.2-15.4]
Ses i LTP 2	110	(54.5%)	92	31	27	14	24	14	0	0.3 [0.2-3.5]
Ses i LTP 4	91	(45.0%)	111	19	11	22	20	19	0	0.3 [0.2-8.5]
Sola l 3	87	(43.1%)	115	21	18	22	14	12	0	0.3 [0.2-2.4]
Ses i LTP 3	74	(36.6%)	128	18	16	22	11	7	0	0.2 [0.2-1.7]
Sola l 7	71	(35.1%)	131	21	21	9	12	8	0	0.2 [0.2-0.6]
Act d 10	67	(33.2%)	135	19	20	9	11	8	0	0.2 [0.2-0.5]
Mus a 3	53	(26.2%)	149	23	15	6	6	3	0	0.2 [0.2-0.3]
Tri a 14 .0201	41	(20.3%)	161	10	3	10	7	11	0	0.2 [0.2-0.3]
Ses i LTP 1	34	(16.8%)	168	16	2	12	3	1	0	0.2 [0.0-0.2]
Sola l 6	32	(15.8%)	170	6	7	4	6	8	1	0.2 [0.0-0.2]
Tri a 14	30	(14.9%)	172	12	8	4	4	2	0	0.2 [0.0-0.2]
CCD	14	(6.9%)	188	9	2	3	0	0	0	0.2 [0.0-0.2]

The number of sensitized patients and the percentage they represent are included on the table. Also, sIgE median and IQR (interquartile range) for each allergen. This LTP strips provide semiquantitative information by measuring bands intensity corresponding to specific IgE antibody given in system classes 0 to 6. The classes from the Enzyme-Allergo-Sorbent Test (EAST) were transformed into concentrations expressed in kU_A/L: Class 0 < 0.35; Class 1: ≥0.35 to < 0.7; Class 2: ≥0.7 to < 3.5; Class 3: ≥3.5 to < 17.5; Class 4: ≥17.5 to < 50; Class 5: ≥50 to < 100; Class 6 ≥ 100. Class 1 or superior was the positivity criteria.

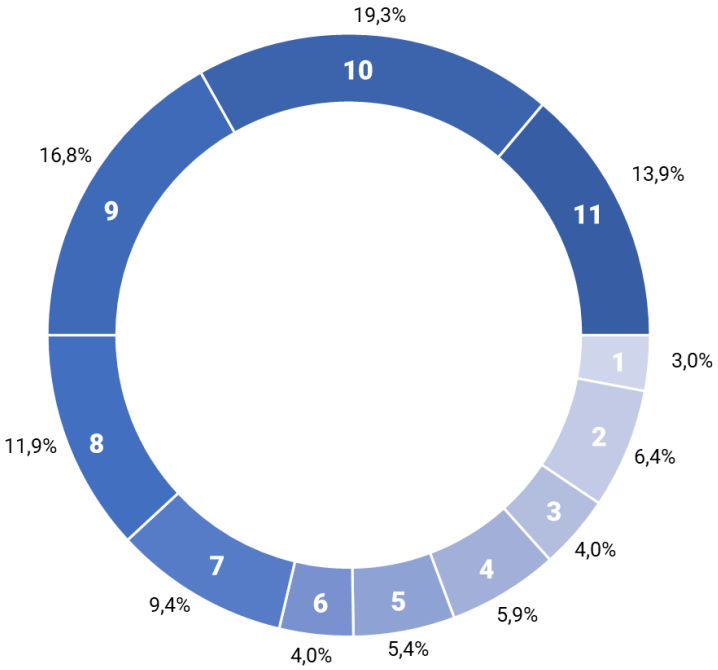
The LTPs producing a minor sensitization were Tri a 14 (30/202), Sola l 6 (32/202) and Ses i LTP 1 (34/202). As previously described, wheat and sesame have a high proportion of tolerant sensitized. CCD sensitization was observed in 6.9% of the tested samples.

Twelve patients (6%) were negative for Pru p 3. For these, the most recognised LTPs were Mal d 3 (7 patients) and Pru av 3, Zea m 14, Tri a 14.0201, Len c 3, Lac s 1-2 (3 patients each). CCD sensitization was discarded for all them.

5.4.2 Taxonomic families recognition

Allergens included on LTP strip were classified according to their taxonomic family, on a total of 11 families: *Rosaceae* (Pru p 3, Mal d 3, Pru av 3, Pru du 3 and Pru du 3.0101), *Fabaceae* (Ara h 9, Len c 3, Pha v 3.0101 and Pha v 3.0201, Pis s 3), *Pedaliaceae* (Ses i LTP 1, Ses i LTP 2, Ses i LTP 3, Ses i LTP 4 and Ses i LTP 5), *Asteraceae* (Lac s 1-1 and Lac s 1-2), *Poaceae* (Zea m 14, Tri a 14 and Tri a 14.0201), *Solanaceae* (Sola l 3, Sola l 6 and Sola l 7), *Juglandaceae* (Jug r 3), *Cucurbitaceae* (Cuc m LTP), *Musaceae* (Mus a 3), *Fagaceae* (Cor a 8), *Actinidiaceae* (Act d 10).

Figure 34. Diversity on family recognition.



The graph classifies patients according to the number of families they are sensitized to, from 1 to 11 (percentage of patients they represent on each case).

Patients recognized a median [range] of 8.5 [1-11] families, and mainly 10 (19.3%), 9 (16.8%) and 11 (13.9%) families (**Figure 33**). Some taxonomic families included more than

one allergen, being the prevalence of sensitization to *Rosaceae* the major: most of subjects (88.1%) recognized them 4 while 2.5% (5/202) were not sensitized to any of them. Additionally, at least 97.5% of the patients recognized an allergen from the *Rosaceae* family. For the *Fabaceae* family, 63.4% of the subjects were sensitized to the 4 LTPs included and 89.6% recognised at least, one LTP from this taxonomic family (detailed in **Table 27**).

Table 28. Distribution of sensitization on each taxonomic family according to the number of recognised LTPs.

Number of recognised allergens	POACEAE (n/%)		SOLANACEAE (n/%)		ROSACEA (n/%)		FABACEAE (n/%)		PEDALIACEAE (n/%)	
0	52	25.7%	93	46.0%	5	2.5%	21	10.4%	55	27.2%
1	98	48.5%	47	23.3%	3	1.5%	15	7.4%	33	16.3%
2	52	25.7%	43	21.3%	7	3.5%	21	10.4%	30	14.9%
3			19	9.4%	9	4.5%	17	8.4%	24	11.9%
4					178	88.1%	128	63.4%	40	19.8%
5									20	9.9%
Total individuals sensitized	**150**	**74.3%**	**109**	**54.0%**	**197**	**97.5%**	**181**	**89.6%**	**147**	**72.8%**

Sensitization prevalence is expressed as absolute number and the percentage they represent (total n=202).

Finally, polysensitization was observed in 98.5% (199/202) of the cases, from which 185 (92.0%) recognised 5 or more LTPs. Three subjects recognised all the 28 LTPs included in the EUROIMMUN-LTP strip. Patients recognised an average of 16.6 LTPs. Only 3 patients were monosensitized (P1: Cuc m +; P2: Tri a 14.0201+; P3: Ses i LTP 5+).

5.4.3 Clinical characterization

A heterogeneous pattern of symptoms was observed depending on the food involved (**Table 28**).

Table 29. Distribution of symptoms on foods over sensitized.

Allergenic source (total sensitized)	TOLERANT (%)	ALLERGIC (%)	AVOIDANCE (%)	CU (%)	OAS (%)	GI (%)	U/AE (%)	AN (%)
Walnut (161)	0.0	73.3	26.7	0.0	23.0	4.3	19.3	8.7
Peach (190)	15.3	67.4	17.4	27.9	19.5	5.8	21.6	14.2
Peanut (151)	27.2	41.1	31.8	0.0	17.2	6.0	15.9	8.6
Hazelnut (153)	28.8	38.6	32.7	0.0	15.7	4.6	13.7	9.2
Lettuce (134)	51.5	35.8	12.7	1.5	8.2	16.4	4.5	6.7
Banana (53)	60.4	32.1	7.5	0.0	11.3	11.3	7.5	7.5
Apple (186)	48.4	29.0	22.6	0.5	11.3	8.1	8.6	6.5
Melon (166)	63.3	25.3	11.4	2.4	14.5	5.4	4.2	0.6
Tomato (87)	64.4	21.8	13.8	2.3	9.2	3.4	3.4	4.6
Almond (188)	44.1	19.7	36.2	0.5	7.4	3.2	8.5	3.2
Corn (144)	74.3	13.9	11.8	0.7	5.6	3.5	2.8	2.1
Cherry (188)	55.9	12.2	32.4	0.0	5.3	3.7	2.1	1.6
Kiwi (67)	68.7	11.9	19.4	0.0	6.0	4.5	0.0	1.5
Pine nut (19)	68.4	10.5	21.1	0.0	5.3	0.0	5.3	0.0
Green bean (168)	79.2	10.1	10.7	1.2	3.0	1.2	1.8	3.0
Wheat (30)	80.0	10.0	10.0	0.0	3.3	0.0	3.3	3.3
Lentil (159)	84.9	7.5	7.5	0.0	2.5	4.4	0.0	0.6
Peas (134)	88.1	6.0	6.0	0.0	1.5	2.2	3.0	0.0
Sesame (34)	91.2	2.9	5.9	0.0	2.9	0.0	0.0	0.0

Percentage of sensitized presenting symptoms according to the offending food. *AN*: anaphylaxis. *CU*: contact urticaria. *GI*: gastrointestinal symptoms. *OAS*: Oral Allergy Syndrome. *U/AE*: generalized urticaria and/or angioedema.

Most individuals reported multiple symptoms with the different foods tested. The main culprit foods detected by LTP-strips were (% allergic with confirmed sensitization) walnut (73.3%) and peach (67.4%). For walnut, OAS (23%) and U/AE (19.3%) were the most frequently reported symptoms. Interestingly, any of the Jug r 3 sensitized reported tolerance. For peach, CU (27.9%), U/AE (21.6%) and OAS (19.5%) were the most frequent symptoms; 15.3% were tolerant. A high frequency of tolerance on sensitized subjects was found for some food allergens: Sesame (31/34; 91.2%), Pea (118/134; 88.1%), Lentil (135/159; 84.9%) and Wheat (24/30; 80.0%). Moreover, in 25 patients (12.2%) could be

registered the involvement of cofactors: exercise (13 patients), NSAIDs (9 patients), alcohol (1) or period (1). In 3 patients, the cofactor involved could not be properly described by means of the clinical history. Lettuce was the food with a more frequent implication of cofactors (10 patients) (**Table 29**).

Table 30. Culprit foods and cofactor involvement.

Culprit food (total patients w/ cofactor)	**Exercise**	**NSAID**	**Period**	**Alcohol**	**Not specified**
Lettuce (10)	5	4	-	-	1
Tomato (6)	3	-	-	1	2
Walnut (5)	2	2	-	-	1
Peanut (4)	3	-	-	-	1
Almond (4)	1	2	-	-	1
Apple (3)	2	-	1	-	-
Green bean (2)	1	-	-	-	1
Pine nut (2)	1	1	-	-	-
Wheat (2)	2	-	-	-	-
Banana (2)	1	1	-	-	-
Peas (1)	-	1	-	-	-
Corn (1)	-	-	-	-	1
Lentil (1)	-	1	-	-	-
Peach (1)	1	-	-	-	-
Hazelnut (0)	1	-	-	-	1
Melon (0)	-	-	-	-	-
Mustard (0)	-	-	-	-	-
Chickpea (0)	-	-	-	-	-
Cherry (0)	-	-	-	-	-
Kiwi (0)	-	-	-	-	-
Orange (0)	-	-	-	-	-
Sesame (0)	-	-	-	-	-

Total individuals reporting cofactor influence per food.

Chapter VI
Discussion

LTP is the most frequent cause of plant food allergy in our area, alike Southern Europe. Patients with LTP Syndrome show a wide range of sensitization to plant foods taxonomically related or not, and a diverse profile of clinical symptoms, from mild to severe or even fatal. Component based *in vitro* diagnostic tools are useful on diagnosis and clinical management of LTP-syndrome patients. A deeper knowledge and optimisation of *in vitro* immunoassays could allow the identification of diverse phenotypical profiles among these patients offering a more personalised clinical management as well as improving their quality of life. Thus, with this thesis work we aimed to improve on the utility of the *in vitro* tools used for the diagnosis of LTP syndrome as well as the clinical management of these patients.

Currently, multiple *in vitro* tools are available for LTP syndrome diagnosis, being Pru p 3 the most frequent LTP used for screening. Nevertheless, despite the advances in the field with the introduction of molecular allergology and multiplex tools, similarly to other food allergies, identifying clinical relevance and patients risk stratification are still a challenge.

Hereby, we demonstrate the clinical relevance of low sIgE levels of Pru p 3 (from 0.1 to 0.35 kU_A/L) on a group of patients (grLOW) compared to another group (grB) with sIgE Pru p 3 ≥ 0.35 kU_A/L. Over the cohort analysed the ratio Pru p 3/Peach was similar in both groups and superior to 1, which would confirm a sensitization due to Pru p 3 (167). About 45% of our patients of grLOW are allergic, 1.9% anaphylactic, highlighting the importance of considering Pru p 3 sIgE > 0.1 kU_A/L as potentially clinically relevant, despite 0.35 kU_A/L has traditionally been used as the cut-off. Indeed, BAT reactivity (similar in both groups) demonstrated the functionality of these sIgE. Besides the theory reported by Kleine-Tebbe and Jakob (36) exposing that a 0.01 or greater ratio of specific IgE to total IgE, translated as a fraction of 1% of bound total IgE, is enough for basophil half-maximal activation, we observe basophil activation with a lower percentage. Thus, reliable quantitative detection of sIgE and the ratios analysis of specific and total IgE on these patients is relevant for an accurate diagnosis (167,311). Despite the limitations of our study, i.e., retrospective, with not all patients challenged due to logistic limitations, and the fact that avoidance may have caused sIgE concentrations to decrease in patients with a history of a severe reaction, we believe that modifying the cut-off from 0.35 to 0.1 kU_A/L on such a broadly used *in vitro* test as Pru p 3 sIgE (ImmunoCAP) on the screening for LTP syndrome/allergy significantly improves the allergological work-up (**Objective 1.1**).

Our results revealed that Pru p 3 sIgE levels were higher on patients with local symptoms compared with those with systemic in our population, and the reason is not definite. Little is known about the real correlation between LTP sIgE levels and symptoms severity, and conflicting results have been published (51,222,223). It has been reported that high Pru p 3 sIgE concentrations correlate with an increased risk of reactions (293). Ciprandi *et al*. (294) described Pru p 3 sIgE levels variation as an age-dependent event. They reported an increase from infancy to young adulthood (highest from 21 to 30 years) that posteriorly decreased. Also, values have been inversely related with an early onset peach allergy (223). Moreover, it has been described that, mono-sensitization to LTP correlates with a more severe clinical reactivity (230) which could be explained by the fact that IgE receptors are mostly occupied by LTP sIgE, which would induce a more efficient cross-linking of the Fc ε RI and effector cell activation, but not actually related to sIgE levels as the authors explained in the cited report. Actually, a lower frequency of co-sensitization to other LTPs was found on grLOW although sensitization profiles (peanut, walnut, and apple) were similar in both the groups.

In previous studies from our group and collaborators (291,312,313), also a trend to lower levels of sIgE have been observed in those groups with severe symptoms compared with those with mild symptoms. From our point of view, this might be explained by the differential affinity of sIgE to the antigen and differential efficiency on the cross-linking in effector cells in which the ratio of sIgE to total IgE of 0.01 is enough for half-maximal activation of the effector cells.

The interference of CCD sensitization on the detection of low levels of sIgE to Pru p 3 by ImmunoCAP was described some years ago (120). Nevertheless, it doesn't seem to alter our observations, given that CCD sensitization was similarly distributed in both the groups, ruling out that Pru p 3 sIgE low levels detected were merely artifacts of CCD interaction not deserving clinical consideration.

The analysis of the molecular sensitization profiles (clusters) to commercial nsLTP (singleplex) of the LTP syndrome patients recruited also translated in useful observations for the routine clinical practice.

From all the nsLTPs tested, Pru p 3 was the most prevalent, with only a minority of non-reactive patients (6/307, 1.9%), supporting that peach represents the primary source of sensitization to LTP on our population (314). Accordingly, Pru p 3 appears in 14/17 clusters obtained, supporting the previously reported that peach could contain most of the allergenic epitopes of LTPs from other plant derived foods but not in all cases (274)(314). Nevertheless, other similar studies report 18% of the subjects negative for Pru p 3 (however, tested on a microarray and so, semi-quantitative) and thus question Pru

p 3 being the archetypal nsLTP with the presence of all potential binding epitopes (274). Of the 18% of Pru p 3 negative patients, 40 (38%) were Jug r 3 positive and 66 (63%) sensitized to pollen LTPs: Art v 3 and/or Pla a 3 positive. In our study, when Pru p 3 was negative, patients were mostly sensitized to Tri a 14. Indeed, we did not assess sensitization to pollen LTPs in our patients.

An order in sensitization prevalence among the six tested LTPs was found, as well as in sIgE median values (from highest to lowest): Pru p 3, Mal d 3, Jug r 3 and Ara h 9, Cor a 8 and Tri a 14. Interestingly, a previous study identified on the levels of sIgE from LTP sensitized patients a predictable hierarchical order with peach at the top, followed by apple, walnut, hazelnut and peanut without correlation with the clinical reactivity to each food (245). Moreover, LTPs sIgE levels statistically increased together with co-sensitization (from group 1 to 6). Previous studies confirmed an increase on reactivity and risk of allergic reactions (222,274). Asero *et al*. described in 2004 a relationship between high Pru p 3 sIgE levels and cross-reactivity to plant-derived foods other than *Rosaceae* (314).

Pru p 3 and Mal d 3 presented a high association (r_s) on the different clusters (94.5%) most likely due to their high sequence identity (80.22%) producing probable cross-reactions (247,297). In fact, 45.7% of sensitized to Mal d 3 were tolerant to apple, from which 62.1% were allergic to peach.

Results, also suggest an unusual behaviour of Tri a 14, which has been previously related to exercise-induced anaphylaxis (252) as well as Baker's asthma (243). Previous studies report variable cross-reactivities between Tri a 14 and Pru p 3 (315). In our study, Tri a 14 shows the weakest association with other LTPs appearing in only 5 of the 17 clusters obtained. Most of those patients were tolerant to wheat (79.5%), but the allergic ones presented GID (66.7% from allergic). As some authors previously described, exists the possibility of more severe symptoms when wheat intake is associated to cofactors although not being reflected in our population (51,289,316). Under wheat-LTP allergy suspicion, Tri a 14 should always be tested besides Pru p 3 or any other LTP, since sensitization to it has been found independently from other LTPs.

While in our study, the shared epitope recognition theory is boost by 46.2% of the patients recognising all 6 plant-food LTP, in 2015 the population of Scala *et al.* (274) recognising all nsLTP was only of 15%. However, the mentioned report uses a multiplex assay instead of singleplex and includes not only plant-food but also pollen LTPs (Pla a 3 and Art v 3), thus studies are not fully comparable. The implication of pollen LTPs on LTP-syndrome has been extensively studied (273,274). A novel study exposes the difficulty of Pla a 3 and Art v 3 (over 3 tested sera on sIgE inhibition experiments) to

fully inhibit Pru p 3 (317). Also, Pla a 3 and Art v 3 share a smaller amount of allergenic epitopes compared to Pru p 3, and so, the authors question the evidence of these pollen-LTPs to act as a primary sensitizer. The observed data and the fact that our LTP allergic patients are not tested for pollen-sensitization neither respiratory symptoms are analysed, explain why our studies are not completely comparable.

Sensitized to LTPs (from peach and nuts) were statistically more frequently allergic as well as non-sensitized were more frequently tolerant. This association between clinical reactivity and test results would indicate that, being ruled out sensitization to other common plant food panallergens, LTPs are the cause of the allergic reaction in the studied patients and co-sensitization to other unknown/undescribed allergens is unlikely. Nuts-LTP sensitization was related to more severe reactions. A previous study on LTP allergic patients (222) reported that the most frequent culprit food causing symptoms on a group of patients testing a wide range of food allergens (Walnut, hazelnut, peanut, tomato, rice and corn) belong to the *Rosaceae* family. Moreover, *Rosaceae* LTPs (Pru p 3 and Mal d 3) were the most frequently associated to other allergenic sources.

The analysis of sensitization patterns including 6 LTPs led to a substantial number of possible protein combinations clusters, 57 different ones. Of them, only 17 (29.8%) were found in at least one patient and 11 of them (%) in more than two patients. Thus, 40 possible clusters were not found in our studied cohort, meaning that on multiple LTP reactivity, molecular sensitization profiles exist (**Objective 1.2**). The most frequent cluster (46.25%) was the one including all plant food LTPs considered (Mal d 3 + Pru p 3 + Tri a 14 + Cor a 8 + Ara h 9 + Jug r 3). The second most frequent cluster (21.82%) included all 5 LTPs, except Tri a 14 . In these groups of patients showing higher diversity of allergen recognition (i.e., ≥4 LTPs) a significantly major proportion of severe symptoms was observed. At once, milder symptoms were more frequent on low reactivity groups (<4LTPs). Tolerance to peach was also associated to low reactivity groups (<4 LTP) in the present study. This trend of number of proteins recognized and clinical reactivity had been previously described elsewhere (274).

Our observations are limited by the fact that clinical data is retrospectively obtained and the fact that patients were selected based on the criteria of availability of data on the 6 commercially available plant food LTPs. We could find a multireactive tendence profile on our LTP-syndrome studied cohort. Multireactivity groups correlated with sIgE levels and were associated to more severe symptoms. Prospective studies with systematic allergological workup are required to validate these observations. Identifying one or various clusters of recognition of LTPs would aid at diagnosis and management of LTP

syndrome with the possibility to associate concrete sensitization profiles to predictable clinical reactivity and so, assessing the patient on a more accurate diet exclusion and precautionary measures.

Developing a validated novel multiplex assay containing multiple LTPs from varied allergenic sources (LTP-strip) aimed to improve the currently available *in vitro* tools for diagnosis and clinical management of LTP syndrome. From our experience in the field, multiple sensitizations can only be assessed today by using whole extracts, with their known limitations, and clinical history. Thus, the LTP-strip was specially designed including the most relevant and frequent allergenic sources in LTP-syndrome in our area with the aim to cover the clinical need of assessing sIgE sensitization to a broader panel of nsLTPs from taxonomically related and unrelated allergenic sources. Indeed, not only identifying sensitization to as many allergenic sources is remarkable at diagnosis but also reliably identifying those that the patient is not sensitized to is of utmost interest for the clinical management (diet intervention) of the patient.

The diagnostic performance of the LTP-strip was validated in a cohort of patients with diagnosed LTP-syndrome from our area (**Objective 2.1**). We defined PbP as the reference for diagnosis since oral challenge to all allergenic sources for all patients was not feasible in our hands and PbP sensitivity has been described superior to commercial extracts for SPT (318,319). A good concordance with the PbP outcome was found. Despite being a rather small cohort of patients, since this was intended as a validation study of a novel *in vitro* tool, the high diversity of sIgE recognition profiles among them emphasizes the convenience of a broad panel of nsLTP in a multiparametric test for the diagnosis and clinical characterization of LTP syndrome. Indeed, most patients were sensitized to *Rosaceae* LTPs (Pru p 3, Mal d 3, Pru av 3, Pru du 3), however sensitization to other plant food which are not closely related cannot be assumed by taxonomy also frequently occurs. The inclusion of several isoforms of one protein has also provided *in vitro* individual IgE-binding properties and cellular activation, leading to different sensitization pattern. Importantly, we demonstrate hereby that sensitization to nsLTP not included in current commercially available multiplex tests are frequently recognized by LTP allergic patients and thus should be considered (i.e., lettuce, green bean, almond, etc.).

All individuals of the cohort showed sensitization to peach peel when PbP-tested, the approach considered as the gold standard for the analysis, and all of them showed positive sIgE reactivity in the immunoblot assay to at least 4 different nsLTPs. A subset of nine nsLTP allergens were further analyzed by BAT. In all patients that IgE sensitization was detected in the LTP strip, basophil activation was observed upon

stimulation with the corresponding nsLTP, meaning that the serum specific IgE detected with the strip corresponded with IgE functionality at basophil level. Also, to our knowledge it is the first time to report BAT data on the performance of nsLTPs from lettuce, green bean, almond, melon and kiwi.

When compared to PbP, the proportion of correctly classified subjects exceeds 70% for the majority of the recombinant nsLTPs in the immunoblot assay. Pru p 3 and Mal d 3 correlate very well with the PbP outcome (100% and 97% agreement, respectively). Considering the different implementations and allergen presentation between PbP testing and *in vitro* detection of sIgE, this correlation between the two assays systems is actually very good.

Some proteins (i.e., Pru du 3 and Len c 3) show a high amount of false positive reactions when comparing the immunoblot sIgE levels and the PbP outcome. Interestingly, the BAT results for patient 23 and patient 33 which where both PbP negative for almond seeds, clearly showed an activation for Pru du 3 supporting the results from the immunoblot assay. Nevertheless, we looked further into possible explanation for potential false positive sIgE reactivity. On the one hand, nsLTP share a high amino acid sequence similarity which results in a high degree of cross-reactivity (e.g., Pru du 3 and Pru p 3 have a sequence similarity of 94,5%) (205). Pru du 3 shares three major IgE-binding epitopes which are reported for Pru p 3 in previous studies (273) . Interestingly, the isoform Pru du 3.0101 only has one shared epitope. These findings were supported by the heatmap and by the BAT data, where Pru du 3 shows similar reactivity to Pru p 3 whereas Pru du 3.0101 deviates from this pattern. To what extend the cross-reactivity may be clinically relevant has to be further investigated. Even though the cross-reactivity has to be considered, it does not explain the negative PbP outcome. The latter may be caused by the different antigen presentation of the two test systems. Whereas the immunoblot contains exclusively recombinant proteins, fresh fruits, nuts and vegetables were used for the PbP. Plant-based food shows variable concentrations of nsLTPs, depending on cultivar, storage, maturity conditions and processing (8). This fact is very significant, since the fresh food source that we were testing may not contain the nsLTP or have it in a very low quantity with the consequent false negative of PbP. Also, the PbP test with lentil, e.g., was done with canned lentils which have been cooked in advance. Previous studies showed reduced IgE-binding capacity after heating of rLen c 3 (320). Maybe the amount of IgE-reactive Len c 3 was too low to induce an allergic reaction in the PbP.

In another previous study, the nsLTP of almond tree (Pru du 3) could be detected in the almond leaf and the epicarp/mesocarp which coats the almond seeds. Like our own

findings, the prick test with 3 types of almond seeds had a negative outcome. Presumably, the LTP quantity in the seeds is reduced compared to other tissues (321).

In this study novel LTPs which are not described as allergens yet where included. Cuc m LTP and the Ses i LTP 1-5 do not have an especially high amino acid sequence similarity to Pru p 3 (~ 50%). Nevertheless, a lot of patients showed a Cuc m LTP sensitization. The sIgE reactivity for Cuc m LTP in the immunoblot assay was even higher than for Pru p 3 in a few patients. The clinical relevance for this data must be further analyzed. Previous studies also hinted for LTP as culprit in melon allergy (322). The LTPs of sesame were recognized by a fair number of subjects. The interpretation of these data is difficult as there was just a small number of patients which was pricked (PbP) with sesame and just 6 of them had a positive outcome.

Indeed, there are some limitations in our study. We highlight that we are considering PbP results as the reference test whereas oral challenge outcome is the real gold standard for food allergy diagnosis, but this approach was not logistically feasible for this project in our hands. Thus, we selected a possibilistic approach to be able to test sensitization to a broad panel of allergenic sources and also, we believed based on our experience and reported data on the field of allergology that diversity of proteins to assess sensitization would be more preserved in PbP than in commercial SPT extracts. The analysis of the outcome of prick by prick and the LTP strip with the anamnestic data shows that the performance of the *in vitro* approach with LTP Euroline for sensitization screening was superior to PbP for particular allergen sources like lentil, sesame, Jug r 3, Cuc m LTP and Tri a 14.02. Thus, it is noteworthy to have as many high quality recombinant nsLTP from a broad range of allergenic sources as possible to improve LTP syndrome diagnosis and management.

The selection of our nsLTP allergic patients was according to Pru p 3 sensitizations, considering it to be a universal LTP for the diagnosis of LTP sensitized patients in general, independently of the allergenic source. Only serum samples from the Mediterranean area were included, which is representative for Southern Europe but means the generalization of the data to non-Mediterranean areas is limited. Furthermore, the PbP was only conducted in patients with LTP syndrome, and negative controls were only skin tested by peach-peel and ImmunoCAP tested to confirm absence of LTP sensitization. As this was a pilot study, only a small cohort was used for preliminary results. Indeed, all these observations have to be confirmed with larger cohorts of LTP allergic individuals tested and with as much oral food challenges as possible.

Finally, the LTP strip was used to analyse the molecular sensitization profile and clinical characterization in a larger cohort of 202 patients with LTP syndrome (manuscript in preparation, **Objective 2.2**).

Having a broad panel of allergens on the multiplex diagnostic tool, allowed the identification of senisitization profiles on LTP-allergic on a wider panel of plant-food allergens. Regarding symptoms, a heterogeneous pattern was seen depending on the culprit food. Walnut and Peach were the main offending cause of allergy in this cohort, also described in the second part of the thesis (using singleplex, Objective 1.2) but also reported in previous studies (274,292,323). Only 15.3% tolerated peach consumption. Inversely, wheat, was tolerated by 80% of the patients (common with the results in part 2) and <20% of the tested samples demonstrated sensitization to Tri a 14 (14.9%) and Tri a 14.0201 (20.0%). Stands out that fact that, Zea m 14 (sharing *Poaceae* taxonomic family with Tri a 14; 56.7% percent identity -extracted from personal database-) is prevalent in 71.3% of the studied patients and that 74.3% of them are tolerant to corn. Not many information has been described from this LTP.

OAS and U/AE were the most frequently reported symptoms as previously described for LTP syndrome (49,51,276,282,283). Interestingly, GI disorders were specially found on lettuce sensitized, which is the food with a major cofactor implication. Availability of lettuce LTP would aid at diagnosis of these patients, something that might not be obvious from clinical history of patients are not specifically inquired for GI symptoms.

Is interesting the fact that, a median of 63.3% of the sensitized, were tolerant to the allergenic source. As explained in the introduction from this thesis, Asero *et al.* reported in 2018 (292) that a non-negligible proportion of the patients studied (25%) reacted to tolerated foods. In 2021, this fact was observed again by a spanish group (324): 13% of LTP-sensitized patients developed allergy 10 years later, mainly to *Rosaceae* fruits and nuts, specially on those who had been previously allergic to other LTPs.

Rosaceae was the most dominant family on our cohort, as described in other studies for Mediterranean basin countries (51) related to high sIgE levels. As previously noted (314) Pru p 3 frequently leads sensitization to other vegetal foods. However, non-Pru p 3 sensitized patients existed (5.9%: Mal d 3 and Pru av 3, but also Zea m 14, Tri a 14.0201, Len c 3, Lac s 1-2) which emphasizes that fact that heterogenous patterns are also present and that *Rosacea* sensitization does not represent all the LTP-allergic population. Non-Rosacea LTPs may have variable IgE-binding epitopes (208).

Most predominant sensitizations (expressed as high prevalence) were positively correlated with high sIgE levels from the semiquantitative tool, mainly 50 to 100 kU_A/L: Rosaceae (Pru p 3, Pru av 3, Pru du 3 and Mal d 3) followed by Pha v 3.0102 (green bean,

83.2%), Cuc m (melon, 82.2%), Jug r 3 (walnut, 79.7%) and Len c 3 (lentil, 78.7%). These high sensitization prevalences were also observed in the validation analysis from the strips (part 3 from the thesis, also on (325)). It is interesting the case of Cuc m LTP, with high sensitization prevalence and its sequence similarity with Pru p 3 is 53.85% (personal database): in some cases its sIgE reactivity was higher than for Pru p 3. Len c 3, was also surprisingly prevalent in agreement with the results from the previous section, in which was found a 89.5% of sensitization. Also, one Pru p 3 negative patient presented positive Len c 3, which remarks the usefulness of its presence on the LTP-strips.

High IgE levels have been related to a major risk of clinical reactivity (224), also for Pru p 3 (293). In our studied cohort, major allergens as Pru p 3 and Jug r 3, demonstrated this fact, but not other predominant *Rosaceae* allergens as Pru du 3, Pru av 3 or Mal d 3 (the last one, also showed an important proportion of tolerant in Part 2). For these LTPs, co-sensitization was frequent. For *Rosaceae* allergens (apple, apricot and plum) were described conserved epitopes associated to IgE-binding cross-reactivity which did not necessarily coexisted with cross-allergenicity (326). Pru av 3 (cherry) also demonstrated a high sequence identity with Pru p 3 (327) and was described as a possible major allergen in south European patients in which its allergenicity has been demonstrated (328).

A median of 8.5 taxonomic families were recognised by the studied patients, being led by *Rosaceae* but also *Fabaceae*. Multiple reactivity was evident, recognising 92.0% of the patients 5 or more LTPs. In addition, our studied cohort, demonstrated how wide can co-sensitization in LTP syndrome be, affecting an extensive range of non-taxonomically related families emphasizing the usefulness of having a diagnostic tool that allows obtaining a broader profile of sensitization to plant-food LTPs, letting clinicians a better management of patients with LTP Syndrome.

Finally, the clinical management of the patients with LTP Syndrome is complicated due to their diverse sensitization profiles and broad range of clinical reactivities. Indeed, allergy symptoms are encountered despite Pru p 3 sIgE low levels. Two main sensitization clusters (all 6 LTP+ and 5 LTP+/Tri a 14-) are dominant in our group of patients and a reactivity to 4 or more LTPs is associated to be more likely to develop severe symptoms. Further, the utility of a newly developed multiplex assay with a wider range of LTPs from taxonomically related and un-related sources has been demonstrated on a cohort of patients with LTP syndrome of our area. Test validation with prick by prick and anamnestic data revealed the high-quality recombinant LTPs used for the performed strips and also BAT assays showed its functionality. When testing it over a larger cohort, could be reported A trend to polysensitization to multiple taxonomic

families is observed, despite mainly *Rosaceae*. These observations, allow the identification of phenotypical profiles among patients with LTP syndrome optimizing the use and interpretation of the available *in vitro* immunoassays, offering a deeper knowledge of their sensitization profile and improving the clinical management.

Chapter VII
Conclusions

The conclusions derived from this thesis are the following ones:

1. Currently available *in vitro* tools for LTP-syndrome diagnosis can be improved.

2. Regardless of patients with low sIgE to Pru p 3 (peach LTP) may represent a minority in our daily practice, this sensitization can be clinically relevant, with up to 20% of systemic reactions. Therefore, Pru p 3 sensitizations should be carefully evaluated even when sIgE levels are low.

3. Almost half of the patients with allergy to Lipid Transfer Proteins (LTPs) studied are sensitized to all the commercially available plant food LTPs (Peach-Pru p 3, Apple-Mal d 3, Wheat-Tri a 14, Walnut-Jug r 3, Hazelnut-Cor a 8, Peanut-Ara h 9). The second more frequent cluster of LTPs lacks only the LTP from wheat (Tri a 14). Severity on symptoms increase in concordance with immunoreactivity.

4. Absence of Pru p 3 (peach LTP) sensitization is rare among our patients with allergy to Lipid Transfer Proteins. In that case, in our cohort, patients are mostly sensitized to Tri a 14 (wheat LTP). Thus, Tri a 14 has to be tested independently besides Pru p 3 or other LTPs.

5. The new multiplex assay containing multiple LTPs from varied allergenic sources designed (the LTP-strip) can be successfully used for detection of specific IgE against nsLTPs in patients with LTP-syndrome of our area. The qualitative performance of the LTP-strip when compared to Prick-by-prick and anamnestic data was valuable.

6. Len c 3 (lentil LTP), Pha v 3 (green bean LTP) and Cuc m (melon LTP) allergens demonstrated a high sensitization prevalence and diagnostic performance over our LTP sensitized patients, standing out the necessity of introducing new LTP molecules such these ones on routinary allergy diagnosis tools.

7. A wider scope of LTP plant food testing in the allergological work up, not only has the value of the true positives that improve patient diagnosis but also offers the value of the true negatives, which represent allergenic sources that could be potentially tolerated and consequently improve their clinical management and quality of life.

8. The LTP-strip tested in a cohort of 200 individuals with a clinical history of allergy to Lipid Transfer Proteins shows the need and usefulness to test multiple taxonomically unrelated plants-foods besides the current commercially available ones.

Bibliography

1. Abbas AK, Lichtman AH, Pillai S, Baker DL, Baker A. Inmunología celular y molecular. 10th ed. Elsevier Inc; 2022.

2. Holgate S, Church M, Broide D, Martinez F. Allergy. 4th ed. Elsevier; 2012.

3. Ozdemir C, Akdis M, Akdis CA. T regulatory cells and their counterparts: Masters of immune regulation. Clin Exp Allergy. 2009;39(5):626–39. Available from https://doi.org/ 10.1111/j.1365-2222.2009.03242.x.

4. Horton C, Shanmugarajah K, Fairchild PJ. Harnessing the properties of dendritic cells in the pursuit of immunological tolerance. Biomed J. 2017;40(2):80–93. Available from: http://dx.doi.org/10.1016/j.bj.2017.01.002

5. European Academy of Allergy and Clinicial Immunology. Global Atlas oF Global Atlas of Allergy. 2014.

6. Thomas WR. Innate affairs of allergens. Clin Exp Allergy. 2013;43(2):152–63. Available from https://doi.org/ DOI: 10.1111/j.1365-2222.2012.04059.x.

7. Grabenhenrich L, Lange L, Härtl M, Kalb B, Ziegert M, Finger A, et al. The component-specific to total IgE ratios do not improve peanut and hazelnut allergy diagnoses. J Allergy Clin Immunol. 2016 Jun; 137(6):1751-1760.e8. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674916003766

8. Matricardi PM, Kleine-Tebbe J, Hoffmann HJ, Valenta R, Hilger C, Hofmaier S, et al. EAACI Molecular Allergology User's Guide. Pediatr Allergy Immunol. 2016 May ;27:1–250. Available from: http://doi.wiley.com/10.1111/pai.12563

9. Hilger C, Fischer J, Wölbing F, Biedermann T. Role and Mechanism of Galactose-Alpha-1,3-Galactose in the Elicitation of Delayed Anaphylactic Reactions to Red Meat. Curr Allergy Asthma Rep [Internet]. 2019 Jan 23;19(1):3. Available from: http://link.springer.com/10.1007/s11882-019-0835-9

10. Johansson SG. The discovery of immunoglobulin e and its role in allergy. Chem Immunol Allergy. 2014;100:150-4. Available from https://doi.org/10.1159/000358621.

11. Beyer K, Morrowa E, Li X-M, Bardina L, Bannon GA, Burks AW, et al. Effects of cooking methods on peanut allergenicity. J Allergy Clin Immunol. 2001 Jun;107(6):1077–81. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674901535575

12. Mills Enc, Jenkins Ja, Alcocer Mjc, Shewry Pr. Structural, Biological, and Evolutionary Relationships of Plant Food Allergens Sensitizing via the Gastrointestinal Tract. Crit Rev Food Sci Nutr. 2004 Sep 10; 44 (5) : 379–407. Available from https://doi.org/10.1080/10408690490489224

13. Reeck GR, de Haën C, Teller DC, Doolittle RF, Fitch WM, Dickerson RE, et al. "Homology" in proteins and nucleic acids: a terminology muddle and a way out of it. Cell. 1987 Aug 28;50(5):667. Available from: http://www.ncbi.nlm.nih.gov/pubmed/362134

14. Radauer C. Navigating through the Jungle of Allergens: Features and Applications of Allergen Databases. Int Arch Allergy Immunol. 2017;173(1):1–11. Available from: https://www.karger.com/Article/FullText/471806

15. Pomés A, Davies JM, Gadermaier G, Hilgerd C, Holzhausere T, Lidholmf J, et al. WHO/IUIS Allergen Nomenclature: Providing a common language. Mol Immunol. 2019;100(1968):3–13. Available from https://doi.org/ DOI: 10.1016/j.molimm.2018.03.003.

16. Radauer C, Goodman RE, Pomés A. ALLERGEN NOMENCLATURE [Internet]. Allergen Nomenclature Subcomitee. 1984 [cited 2023 Sep 27]. Available from: www.allergen.org

17. Pabst O, Mowat AM. Oral tolerance to food protein. Mucosal Immunol. 2012 May 8;5(3):232–9. Available from: http://dx.doi.org/10.1038/mi.2012.4

18. Yokanovich LT, Newberry RD, Knoop KA. Regulation of oral antigen delivery early in life: Implications for oral tolerance and food allergy. Clin Exp Allergy. 2021 Jan 5;cea.13823. Available from: https://onlinelibrary.wiley.com/doi/10.1111/cea.13823

19. Coombes JL, Siddiqui KRR, Arancibia-Cárcamo C V., Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-β -and retinoic acid-dependent mechanism. J Exp Med. 2007;204(8):1757–64. Available from https://doi.org/ DOI: 10.1084/jem.20070590.

20. Palomares O, Akdis M, Martín-Fontecha M, Akdis CA. Mechanisms of immune regulation in allergic diseases: the role of regulatory T and B cells. Immunol Rev . 2017 Jul;278(1):219–36. Available from: http://doi.wiley.com/10.1111/imr.12555

21. Kucuksezer UC, Ozdemir C, Cevhertas L, Ogulur I, Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy and allergen tolerance. Allergol Int. 2020;69(4):549–60. Available from https://doi.org/ DOI: 10.1016/j.alit.2020.08.002.

22. Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszcz M, Blaser K, et al. IL-10 and TGF-β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. Eur J Immunol [Internet]. 2003 May;33(5):1205–14. Available from: http://doi.wiley.com/10.1002/eji.200322919

23. Meiler F, Klunker S, Zimmermann M, Akdis CA, Akdis M. Distinct regulation of IgE, IgG4 and IgA by T regulatory cells and toll-like receptors. Allergy Eur J Allergy Clin Immunol. 2008;63(11):1455–63. Available from https://doi.org/10.1111/j.1398-9995.2008.01774.x

24. van de Veen W, Stanic B, Yaman G, Wawrzyniak M, Söllner S, Akdis DG, et al. IgG4 production is confined to human IL-10–producing regulatory B cells that suppress antigen-specific immune responses. J Allergy Clin Immunol [Internet]. 2013 Apr;131(4):1204–12. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674913001504

25. De Benedetto A, Rafaels NM, McGirt LY, Ivanov AI, Georas SN, Cheadle C, et al. Tight junction defects in patients with atopic dermatitis. J Allergy Clin Immunol [Internet]. 2011;127(3):773-786.e7. Available from: http://dx.doi.org/10.1016/j.jaci.2010.10.018

26. Soyka MB, Wawrzyniak P, Eiwegger T, Holzmann D, Treis A, Wanke K, et al. Defective epithelial barrier in chronic rhinosinusitis: The regulation of tight junctions by IFN-γ and IL-4. J Allergy Clin Immunol. 2012;130(5):1087-1096.e10. Available from https://doi.org/ 10.1016/j.jaci.2012.05.052

27. Krempski JW, Dant C, Nadeau KC. The origins of allergy from a systems approach. Ann Allergy, Asthma Immunol [Internet]. 2020 Nov;125(5):507–16. Available from:

https://doi.org/10.1016/j.anai.2020.07.013

28. Akdis M, Burgler S, Crameri R, Eiwegger T, Fujita H, Gomez E, et al. Interleukins, from 1 to 37, and interferon-γ: Receptors, functions, and roles in diseases. J Allergy Clin Immunol. 2011;127(3):701-721.e70. Available from: https://doi.org/10.1016/j.jaci.2010.11.050

29. Scanlon ST, McKenzie ANJ. Type 2 innate lymphoid cells: New players in asthma and allergy. Curr Opin Immunol [Internet]. 2012;24(6):707–12. Available from: http://dx.doi.org/10.1016/j.coi.2012.08.009

30. Licona-Limón P, Kim LK, Palm NW, Flavell RA. TH2, allergy and group 2 innate lymphoid cells. Nat Immunol. 2013;14(6):536–42. Avalable from: https://doi.org/ 10.1038/ni.2617

31. Abbas AK, Lichtman AH, Pillai S, Baker DL, Baker A. Inmunología celular y molecular. 9th ed. Barcelona: Elsevier España, S.L.U; 2018. 608 p.

32. Gurram RK, Zhu J. Orchestration between ILC2s and Th2 cells in shaping type 2 immune responses. Cell Mol Immunol. 2019;16(3):225–35. Available from: http://dx.doi.org/10.1038/s41423-019-0210-8

33. Matthias J, Zielinski CE. Shaping the diversity of Th2 cell responses in epithelial tissues and its potential for allergy treatment. Eur J Immunol. 2019 Sep 11;49(9):1321–33. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/eji.201848011

34. Pelly VS, Kannan Y, Coomes SM, Entwistle LJ, Rückerl D, Seddon B, et al. IL-4-producing ILC2s are required for the differentiation of TH2 cells following Heligmosomoides polygyrus infection. Mucosal Immunol. 2016 Nov 17;9(6):1407–17. Available from: http://www.nature.com/articles/mi20164

35. He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, et al. Circulating Precursor CCR7loPD-1hi CXCR5+ CD4+ T Cells Indicate Tfh Cell Activity and Promote Antibody Responses upon Antigen Reexposure. Immunity. 2013 Oct;39(4):770–81. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1074761313004299

36. Kleine-Tebbe J, Jakob T. Molecular allergy diagnostics using IgE singleplex determinations: methodological and practical considerations for use in clinical routine. Allergo J Int. 2015 Oct 28;24(6):185–97. Available from: http://link.springer.com/10.1007/s40629-015-0067-z

37. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils. J Allergy Clin Immunol [Internet]. 2010 Feb;125(2):S73–80. Available from: http://dx.doi.org/10.1016/j.jaci.2009.11.017

38. Johansson SG., Bieber T, Dahl R, Friedmann PS, Lanier BQ, Lockey RF, et al. Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. J Allergy Clin Immunol. 2004 May;113(5):832–6. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674904009303

39. Lin CH. Food allergy. Curr Opin Gastroenterol. 2019 Mar;35(2):114–8. Available from: https://journals.lww.com/00001574-201903000-00011

40. De Martinis M, Sirufo MM, Viscido A, Ginaldi L. Food Allergy Insights: A Changing Landscape. Arch Immunol Ther Exp (Warsz)]. 2020 Apr 1;68(2):8. Available from:

http://link.springer.com/10.1007/s00005-020-00574-6

41. Sampson HA. Update on food allergy☆. J Allergy Clin Immunol . 2004 May;113(5):805–19. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674904011455

42. Warren CM, Jiang J, Gupta RS. Epidemiology and Burden of Food Allergy. Curr Allergy Asthma Rep [Internet]. 2020 Feb 14;20(2):6. Available from: http://link.springer.com/10.1007/s11882-020-0898-7

43. Nwaru BI, Hickstein L, Panesar SS, Muraro A, Werfel T, Cardona V, et al. The epidemiology of food allergy in Europe: a systematic review and meta-analysis. Allergy. 2014 Jan;69(1):62–75. Available from: http://doi.wiley.com/10.1111/all.12305

44. Turner PJ, Jerschow E, Umasunthar T, Lin R, Campbell DE, Boyle RJ. Fatal Anaphylaxis: Mortality Rate and Risk Factors. J Allergy Clin Immunol Pract. 2017 Sep;5(5):1169–78. Available from: https://doi.org/10.1016/j.jaip.2017.06.031

45. Iweala OI, Choudhary SK, Commins SP. Food Allergy. Curr Gastroenterol Rep. 2018 May 5;20(5):17. Available from: http://link.springer.com/10.1007/s11894-018-0624-y

46. Sicherer SH, Sampson HA. Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. J Allergy Clin Immunol. 2014 Feb;133(2):291-307.e5. Available from: http://dx.doi.org/10.1016/j.jaci.2013.11.020

47. Lyons SA, Clausen M, Knulst AC, Ballmer-Weber BK, Fernandez-Rivas M, Barreales L, et al. Prevalence of Food Sensitization and Food Allergy in Children Across Europe. J Allergy Clin Immunol Pract. 2020 Sep;8(8):2736-2746.e9. Available from: https://linkinghub.elsevier.com/retrieve/pii/S2213219820303718

48. Lyons SA, Burney PGJ, Ballmer-Weber BK, Fernandez-Rivas M, Barreales L, Clausen M, et al. Food Allergy in Adults: Substantial Variation in Prevalence and Causative Foods Across Europe. J Allergy Clin Immunol Pract. 2019 Jul;7(6):1920-1928.e11. Available from: https://linkinghub.elsevier.com/retrieve/pii/S2213219819302697

49. Ojeda PM, Sastre J, Olaguibel JM, Chivato T. Alergólogica 2015: A national survey on allergic diseases in the adult Spanish population. J Investig Allergol Clin Immunol. 2018;28(3):151–64.

50. Sánchez-López J, Gázquez V, Rubira N, Valdesoiro L, Guilarte M, Garcia-Moral A, et al. Food allergy in Catalonia: Clinical manifestations and its association with airborne allergens. Allergol Immunopathol (Madr). 2017 Jan;45(1):48–54. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0301054616300970

51. Pascal M, Muñoz-Cano R, Reina Z, Palacín A, Vilella R, Picado C, et al. Lipid transfer protein syndrome: clinical pattern, cofactor effect and profile of molecular sensitization to plant-foods and pollens. Clin Exp Allergy. 2012 Oct;42(10):1529–39. Available from: http://doi.wiley.com/10.1111/j.1365-2222.2012.04071.x

52. Bartra J, García-Moral A, Enrique E. Geographical differences in food allergy. Bundesgesundheitsblatt - Gesundheitsforsch - Gesundheitsschutz. 2016 Jun 13;59(6):755–63. Available from: http://link.springer.com/10.1007/s00103-016-2357-0

53. Du Toit G, Roberts G, Sayre PH, Bahnson HT, Radulovic S, Santos AF, et al. Randomized Trial of Peanut Consumption in Infants at Risk for Peanut Allergy. N Engl J Med. 2015 Feb 26;372(9):803–13. Available from: http://www.nejm.org/doi/10.1056/NEJMoa1414850

54. Hong X, Hao K, Ladd-Acosta C, Hansen KD, Tsai H-J, Liu X, et al. Genome-wide association study identifies peanut allergy-specific loci and evidence of epigenetic mediation in US children. Nat Commun. 2015 May 24;6(1):6304. Available from: http://www.nature.com/authors/editorial_policies/license.html#terms

55. Ober C, Yao T-C. The genetics of asthma and allergic disease: a 21st century perspective. Immunol Rev. 2011 Jul;242(1):10–30. Available from: http://doi.wiley.com/10.1111/j.1600-065X.2011.01029.x

56. Werfel T, Asero R, Ballmer-Weber BK, Beyer K, Enrique E, Knulst AC, et al. Position paper of the EAACI: food allergy due to immunological cross-reactions with common inhalant allergens. Allergy. 2015 Sep;70(9):1079–90. Available from: http://doi.wiley.com/10.1111/all.12666

57. Asero R, Antonicelli L. Does Sensitization to Foods in Adults Occur Always in the Gut? Int Arch Allergy Immunol. 2011;154(1):6–14. Available from: https://www.karger.com/Article/FullText/319203

58. Breiteneder H, Ebner C. Molecular and biochemical classification of plant-derived food allergens. J Allergy Clin Immunol. 2000 Jul;106(1):27–36. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674900724213

59. Valenta R, Hochwallner H, Linhart B, Pahr S. Food Allergies : The Basics. Gastroenterology. 2015;148(6):1120-1131.e4. Available from: http://dx.doi.org/10.1053/j.gastro.2015.02.006

60. Finkina EI, Melnikova DN, Bogdanov I V, Ovchinnikova T V. Plant Pathogenesis-Related Proteins PR-10 and PR-14 as Components of Innate Immunity System and Ubiquitous Allergens. Curr Med Chem. 2017;24(17):1772–87. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27784212

61. Izadi N, Luu M, Ong P, Tam J. The Role of Skin Barrier in the Pathogenesis of Food Allergy. Children. 2015 Sep 2;2(3):382–402. Available from: http://www.mdpi.com/2227-9067/2/3/382

62. Lack G. Epidemiologic risks for food allergy. J Allergy Clin Immunol. 2008 Jun;121(6):1331–6. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674908007781

63. Berin MC, Sampson HA. Food allergy: an enigmatic epidemic. Trends Immunol. 2013 Aug;34(8):390–7. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624763/pdf/nihms412728.pdf

64. De Benedetto A, Qualia CM, Baroody FM, Beck LA. Filaggrin Expression in Oral, Nasal, and Esophageal Mucosa. J Invest Dermatol. 2008 Jun;128(6):1594–7. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0022202X15339051

65. Chan SMH, Turcanu V, Stephens AC, Fox AT, Grieve AP, Lack G. Cutaneous lymphocyte antigen and α4β7 T-lymphocyte responses are associated with peanut allergy and tolerance in children. Allergy 2012 Mar;67(3):336–42. Available from: http://doi.wiley.com/10.1111/j.1398-9995.2011.02765.x

66. Brunner M, Waltzer M. Absortion of Undigested proteins in human beings. The absortion of

unaltered fish proteins in adults. Arch Intern Med. 1928 Aug 1;42(2):172. Available from: http://archinte.jamanetwork.com/article.aspx?doi=10.1001/archinte.1928.00130200024002

67. Vethachalam S, Persaud Y. Contact Urticaria. StatPearls. 2020. Available from: http://www.ncbi.nlm.nih.gov/pubmed/31751080

68. Zuberbier T, Aberer W, Asero R, Abdul Latiff AH, Baker D, Ballmer-Weber B, et al. The EAACI/GA^{2}LEN/EDF/WAO guideline for the definition, classification, diagnosis and management of urticaria. Allergy. 2018 Jul;73(7):1393–414. Available from: http://doi.wiley.com/10.1111/all.13397

69. Zuberbier T, Abdul Latiff AH, Abuzakouk M, Aquilina S, Asero R, Baker D, et al. The international EAACI/GA^{2}LEN/EuroGuiDerm/APAAACI guideline for the definition, classification, diagnosis, and management of urticaria. Allergy. 2022 Mar 20;77(3):734–66. Available from: https://onlinelibrary.wiley.com/doi/10.1111/all.15090

70. Muraro A, Worm M, Alviani C, Cardona V, DunnGalvin A, Garvey LH, et al. EAACI guidelines: Anaphylaxis (2021 update). Allergy. 2022 Feb;77(2):357–77. Available from: https://onlinelibrary.wiley.com/doi/10.1111/all.15032

71. Cardona V, Ansotegui IJ, Ebisawa M, El-Gamal Y. World allergy organization anaphylaxis guidance 2020. World Allergy Organ J. 2020 Oct 30;13(10):100472Available from: https://doi.org/10.1016/j.waojou.2020.100472

72. Brockow K, Kneissl D, Valentini L, Zelger O, Grosber M, Kugler C, et al. Using a gluten oral food challenge protocol to improve diagnosis of wheat-dependent exercise-induced anaphylaxis. J Allergy Clin Immunol. 2015 Apr;135(4):977-984.e4. Available from: https://linkinghub.elsevier.com/retrieve/pii/S009167491401197X

73. Christensen MJ, Eller E, Mortz CG, Brockow K, Bindslev-Jensen C. Wheat-Dependent Cofactor-Augmented Anaphylaxis: A Prospective Study of Exercise, Aspirin, and Alcohol Efficacy as Cofactors. J Allergy Clin Immunol Pract. 2019 Jan;7(1):114–21. Available from: https://linkinghub.elsevier.com/retrieve/pii/S2213219818304197

74. Cardona V, Luengo O, Garriga T, Izquierdo A. Co-factor-enhanced food allergy. Allergy. 2012;39:1316–8. Available at: https://doi.org/10.1111/j.1398-9995.2012.02877.x.

75. Ansley L, Bonini M, Delgado L, Giacco S Del, Toit G Du, Khaitov M, et al. Pathophysiological mechanisms of exercise-induced anaphylaxis : an EAACI position statement. Allergy. 2015;70:1212–21. Available at: https://doi.org/10.1111/all.12677

76. Saeki K, Endo K, Yamasaky H. Histamine release by inorganic cations from mast cell granules isolated by different procedures. Jpn J Pharmacol. 1972;22(1):27–32. Available from: http://joi.jlc.jst.go.jp/JST.Journalarchive/jphs1951/22.27?from=CrossRef

77. Matsuo H, Morimoto K, Akaki T, Kaneko S, Kusatake K, Kuroda T, et al. Exercise and aspirin increase levels of circulating gliadin peptides in patients with wheat-dependent exercise-induced anaphylaxis. Clin Exp Allergy. 2005 Apr;35(4):461–6. Available from: http://doi.wiley.com/10.1111/j.1365-2222.2005.02213.x

78. Suzuki Y, Ra C. Analysis of the mechanism for the development of allergic skin inflammation and the application for its treatment: Aspirin modulation of IgE-dependent mast cell activation: Role of

aspirin-induced exacerbation of immediate allergy. J Pharmacol Sci. 2009;110(3):237–44. Available at: https://doi.org/10.1254/jphs.08R32FM

79. Ribó P, Guo Y, Aranda J, Ainsua-Enrich E, Navinés-Ferrer A, Guerrero M, et al. Mutation in KARS: A novel mechanism for severe anaphylaxis. J Allergy Clin Immunol . May;147(5):1855-1864.e9; Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674920324325

80. Breiteneder H, Mills ENC. Molecular properties of food allergens. J Allergy Clin Immunol [Internet]. 2005 Jan;115(1):14–23. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674904026788

81. Radauer C, Bublin M, Wagner S, Mari A, Breiteneder H. Allergens are distributed into few protein families and possess a restricted number of biochemical functions. J Allergy Clin Immunol. 2008 Apr;121(4):847-852.e7. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674908001632

82. Liu J-J, Sturrock R, Ekramoddoullah AKM. The superfamily of thaumatin-like proteins: its origin, evolution, and expression towards biological function. Plant Cell Rep. 2010 May 5;29(5):419–36. Available from: http://link.springer.com/10.1007/s00299-010-0826-8

83. Hsieh L-S, Moos M, Lin Y. Characterization of apple 18 and 31 kd allergens by microsequencing and evaluation of their content during storage and ripening. J Allergy Clin Immunol. 1995 Dec;96(6):960–70. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674995702342

84. Hauser M, Roulias A, Ferreira F, Egger M. Panallergens and their impact on the allergic patient. Allergy, Asthma Clin Immunol. 2010 Dec 18;6(1):1. Available from: http://aacijournal.biomedcentral.com/articles/10.1186/1710-1492-6-1

85. Santos A, Van Ree R. Profilins: Mimickers of Allergy or Relevant Allergens. Int Arch Allergy Immunol. 2011;155(3):191–204. Available from: https://www.karger.com/Article/FullText/321178

86. Radauer C, Willerroider M, Fuchs H, Hoffmann-Sommergruber K, Thalhamer J, Ferreira F, et al. Cross-reactive and species-specific immunoglobulin E epitopes of plant profilins: an experimental and structure-based analysis. Clin Exp Allergy. 2006 Jul;36(7):920–9. Available from: http://doi.wiley.com/10.1111/j.1365-2222.2006.02521.x

87. Dunwell JM, Culham A, Carter CE, Sosa-Aguirre CR, Goodenough PW. Evolution of functional diversity in the cupin superfamily. Trends Biochem Sci]. 2001 Dec;26(12):740–6. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0968000401019818

88. López-Torrejón G, Salcedo G, Martín-Esteban M, Díaz-Perales A, Pascual CY, Sánchez-Monge R. Len c 1, a major allergen and vicilin from lentil seeds. J Allergy Clin Immunol . 2003 Dec;112(6):1208–15. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674903021961

89. Sanchez-Monge R, Lopez-Torrejon G, Pascual CY, Varela J, Martin-Esteban M, Salcedo G. Vicilin and convicilin are potential major allergens from pea. Clin Exp Allergy. 2004 Nov;34(11):1747–53. Available from: http://doi.wiley.com/10.1111/j.1365-2222.2004.02085.x

90. Pascual CY, Fernandez-Crespo J, Sanchez-Pastor S, Padial MA, Diaz-Pena JM, Martin-Muñoz F, et al. Allergy to lentils in Mediterranean pediatric patients. J Allergy Clin Immunol. 1999 Jan;103(1):154–8. Available from:

https://linkinghub.elsevier.com/retrieve/pii/S0091674999705397

91. D'Avino R, Bernardi ML, Wallner M, Palazzo P, Camardella L, Tuppo L, et al. Kiwifruit Act d 11 is the first member of the ripening-related protein family identified as an allergen. Allergy. 2011 Jul; 66 (7): 870–7. Available from: http://doi.wiley.com/10.1111/j.1398-9995.2011.02555.x

92. Biedermann T, Winther L, Till SJ, Panzner P, Knulst A, Valovirta E. Birch pollen allergy in Europe. Allergy. 2019 Apr 14;74(7): all.13758. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/all.13758

93. Gepp B, Lengger N, Bublin M, Hemmer W, Breiteneder H, Radauer C. Chimeras of Bet v 1 and g 1 reveal heterogeneous IgE responses in patients with birch pollen allergy. J Allergy Clin Immunol. 2014 Jul;134(1):188–94. Available from: http://dx.doi.org/10.1016/j.jaci.2013.12.1073

94. Vieths S, Scheurer Ss, Ballmer-Weber B. Current Understanding of Cross-Reactivity of Food Allergens and Pollen. Ann N Y Acad Sci. 2006 Jan 24;964(1):47–68. Available from: http://doi.wiley.com/10.1111/j.1749-6632.2002.tb04132.x

95. Guhsl EE, Hofstetter G, Hemmer W, Ebner C, Vieths S, Vogel L, et al. Vig r 6, the cytokinin-specific binding protein from mung bean (V igna radiata) sprouts, cross-reacts with Bet v 1-related allergens and binds IgE from birch pollen allergic patients' sera. Mol Nutr Food Res. 2014 Mar;58(3):625–34. Available from: https://onlinelibrary.wiley.com/doi/10.1002/mnfr.201300153

96. Moreno FJ, Clemente A. 2S Albumin Storage Proteins: What Makes them Food Allergens? Open Biochem J. 2008 Feb 6;2:16–28. Available from: http://benthamopen.com/ABSTRACT/TOBIOCJ-2-16

97. Shewry PR, Halford NG, Belton PS, Tatham AS. The structure and properties of gluten: an elastic protein from wheat grain. Bailey AJ, Macmillan J, Shrewry PR, Tatham AS, editors. Philos Trans R Soc London Ser B Biol Sci. 2002 Feb 28;357(1418):133–42. Available from: https://royalsocietypublishing.org/doi/10.1098/rstb.2001.1024

98. Tatham AS, Shewry PR. Allergy to wheat and related cereals. Clin Exp Allergy. 2008 Sep;38(11):1712–26. Available from: http://doi.wiley.com/10.1111/j.1365-2222.2008.03101.x

99. Oda Y, Matsunaga T, Fukuyama K, Miyazaki T, Morimoto T. Tertiary and Quaternary Structures of 0.19 α-Amylase Inhibitor from Wheat Kernel Determined by X-ray Analysis at 2.06 Å Resolution † , ‡. Biochemistry. 1997 Nov; 36 (44) : 13503–11. Available from: https://pubs.acs.org/doi/10.1021/bi971307m

100. Salcedo G, Quirce S, Diaz-Perales A. Wheat allergens associated with Baker's asthma. J Investig Allergol Clin Immunol. 2011;21(2):81–92; quiz 94. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21462797

101. Radauer C, Breiteneder H. Evolutionary biology of plant food allergens. J Allergy Clin Immunol. 2007 Sep;120(3):518–25. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674907014224

102. Salcedo G, Sánchez-Monge R, Barber D, Díaz-Perales A. Plant non-specific lipid transfer proteins: An interface between plant defence and human allergy. Biochim Biophys Acta - Mol Cell Biol Lipids. 2007 Jun;1771(6):781–91. Available from:

https://linkinghub.elsevier.com/retrieve/pii/S1388198107000029

103. Kamath SD, Bublin M, Kitamura K, Matsui T, Ito K, Lopata AL. Cross-reactive epitopes and their role in food allergy. J Allergy Clin Immunol. 2023 May;151(5):1178–90. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674923001483

104. Pomés A, Schulten V. Cross-reactivity in allergy: A double-edged sword. Allergy. 2020 Jan 8;75(1):9–11. Available from: https://onlinelibrary.wiley.com/doi/10.1111/all.13993

105. Matsuo H, Yokooji T, Taogoshi T. Common food allergens and their IgE-binding epitopes. Allergol Int. 2015 Oct;64(4):332–43. Available from: https://linkinghub.elsevier.com/retrieve/pii/S132389301500146X

106. Sampson HA, O'Mahony L, Burks AW, Plaut M, Lack G, Akdis CA. Mechanisms of food allergy. J Allergy Clin Immunol. 2018 Jan;141(1):11–9. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674917318092

107. Aalberse RC, Crameri R. IgE-binding epitopes: a reappraisal. Allergy. 2011 Oct;66(10):1261–74. Available from: https://onlinelibrary.wiley.com/doi/10.1111/j.1398-9995.2011.02656.x

108. Aalberse RC. Assessment of allergen cross-reactivity. Clin Mol Allergy. 2007 Dec 9;5(1):2. Available from: https://clinicalmolecularallergy.biomedcentral.com/articles/10.1186/1476-7961-5-2

109. Aalberse R. Assessment of sequence homology and cross-reactivity. Toxicol Appl Pharmacol. 2005 Sep 1;207(2):149–51. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0041008X05002255

110. Lee CH, Salio M, Napolitani G, Ogg G, Simmons A, Koohy H. Predicting Cross-Reactivity and Antigen Specificity of T Cell Receptors. Front Immunol. 2020 Oct 22;11:565096. Available from: https://www.frontiersin.org/articles/10.3389/fimmu.2020.565096/full

111. Westernberg L, Schulten V, Greenbaum JA, Natali S, Tripple V, McKinney DM, et al. T-cell epitope conservation across allergen species is a major determinant of immunogenicity. J Allergy Clin Immunol. 2016 Aug;138(2):571-578.e7. Available from: https://linkinghub.elsevier.com/retrieve/pii/S009167491600083X

112. McKenna OE, Asam C, Araujo GR, Roulias A, Goulart LR, Ferreira F. How relevant is panallergen sensitization in the development of allergies? Pediatr Allergy Immunol. 2016 Sep 22;27(6):560–8. Available from: https://onlinelibrary.wiley.com/doi/10.1111/pai.12589

113. Asam C, Hofer H, Wolf M, Aglas L, Wallner M. Tree pollen allergens—an update from a molecular perspective. Allergy. 2015 Oct 6;70(10):1201–11. Available from: https://onlinelibrary.wiley.com/doi/10.1111/all.12696

114. Price A, Ramachandran S, Smith GP, Stevenson ML, Pomeranz MK, Cohen DE. Oral Allergy Syndrome (Pollen-Food Allergy Syndrome). Dermatitis. 2015 Mar;26(2):78–88. Available from: https://journals.lww.com/01206501-201503000-00004

115. Reese G, Ayuso R, Lehrer SB. Tropomyosin: An Invertebrate Pan–Allergen. Int Arch Allergy Immunol. 1999;119(4):247–58. Available from: https://www.karger.com/Article/FullText/24201

116. Hatzler L, Panetta V, Lau S, Wagner P, Bergmann RL, Illi S, et al. Molecular spreading and predictive

value of preclinical IgE response to Phleum pratense in children with hay fever. J Allergy Clin Immunol. 2012 Oct;130(4):894-901.e5. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674912009591

117. Iqbal J. Skeletal morphofunctional considerations and the pituitary-thyroid axis. Front Biosci. 2009;1(1):92. Available from: https://imrpress.com/journal/FBE/1/1/10.2741/E9

118. Masilamani M, Commins S, Shreffler W. Determinants of Food Allergy. Immunol Allergy Clin North Am. 2012 Feb;32(1):11–33. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0889856111001159

119. van Loon LC, Pierpoint WS, Boller T, Conejero V. Recommendations for naming plant pathogenesis-related proteins. Plant Mol Biol Report. 1994 Sep;12(3):245–64. Available from: http://link.springer.com/10.1007/BF02668748

120. Hemmer W, Altmann F, Holzweber F, Gruber C, Wantke F, Wöhrl S. ImmunoCAP cellulose displays cross-reactive carbohydrate determinant (CCD) epitopes and can cause false-positive test results in patients with high anti-CCD IgE antibody levels. J Allergy Clin Immunol. 2018;141(1):372-381.e3. Avalable from: https://doi.org/10.1016/j.jaci.2017.04.028.

121. Ebo DG, Hagendorens MM, Bridts CH, De clerck LS, Stevens WJ. Sensitization to cross-reactive carbohydrate determinants and the ubiquitous protein profilin: mimickers of allergy. Clin Exp Allergy Exp Allergy. 2004 Jan;34(1):137–44. Available from: http://doi.wiley.com/10.1111/j.1365-2222.2004.01837.x

122. Mertens M, Amler S, Moerschbacher BM, Brehler R. Cross-reactive carbohydrate determinants strongly affect the results of the basophil activation test in hymenoptera-venom allergy. Clin Exp Allergy. 2010 Jun 7;40(9):1333–45. Available from: http://doi.wiley.com/10.1111/j.1365-2222.2010.03535.x

123. Kochuyt A-M, Van Hoeyveld EM, Stevens EAM. Prevalence and clinical relevance of specific immunoglobulin E to pollen caused by sting- induced specific immunoglobulin E to cross-reacting carbohydrate determinants in Hymenoptera venoms. Clin Exp Allergy. 2005 Apr;35(4):441–7. Available from: http://doi.wiley.com/10.1111/j.1365-2222.2005.02217.x

124. Mari A, Ooievaar-de Heer P, Scala E, Giani M, Pirrotta L, Zuidmeer L, et al. Evaluation by double-blind placebo-controlled oral challenge of the clinical relevance of IgE antibodies against plant glycans. Allergy. 2008 Jun 28;63(7):891–6. Available from: http://doi.wiley.com/10.1111/j.1398-9995.2008.01703.x

125. Altmann F. Coping with cross-reactive carbohydrate determinants in allergy diagnosis. Allergo J Int. 2016;25(4):98–105. Available from: http://doi.wiley.com/10.1007/s40629-016-0115-3.

126. Aberer W, Holzweber F, Hemmer W, Koch L, Bokanovic D, Fellner W, et al. Inhibition of cross-reactive carbohydrate determinants (CCDs) enhances the selectivity of in vitro allergy diagnosis. Allergol Sel. 2017 Jul 1;1(2):141–9. Available from: https://www.dustri.com/article_response_page.html?artId=16042&doi=10.5414/ALX01638E&L=0

127. Holzweber F, Svehla E, Fellner W, Dalik T, Stubler S, Hemmer W, et al. Inhibition of IgE binding to cross-reactive carbohydrate determinants enhances diagnostic selectivity. Allergy. 2013 Oct

21;68(10):1269–77. Available from: https://onlinelibrary.wiley.com/doi/10.1111/all.12229

128. Villalta D, Conte M, Asero R, Da Re M, Stella S, Martelli P. Isolated IgE reactivity to native walnut vicilin-like protein (nJug r 2) on ISAC™ microarray is due to cross-reactive carbohydrate epitopes. Clin Chem Lab Med. 2013 Jan 1;51(10):1991–5. Available from: https://www.degruyter.com/doi/10.1515/cclm-2013-0027

129. Skypala IJ, McKenzie R. Nutritional Issues in Food Allergy. Clin Rev Allergy Immunol. 2019 Oct 15;57(2):166–78. Available from: http://link.springer.com/10.1007/s12016-018-8688-x

130. Alessandri C, Ferrara R, Bernardi ML, Zennaro D, Tuppo L, Giangrieco I, et al. Diagnosing allergic sensitizations in the third millennium: why clinicians should know allergen molecule structures. Clin Transl Allery. 2017 Dec 17;7(1):21. Available from: http://ctajournal.biomedcentral.com/articles/10.1186/s13601-017-0158-7

131. Keet C. Recognition and Management of Food-Induced Anaphylaxis. Pediatr Clin North Am . 2011 Apr;58(2):377–88. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0031395511000083

132. Fiocchi A, Vickery BP, Wood RA. The use of biologics in food allergy. Clin Exp Allergy. 2021 Aug 2;51(8):1006–18. Available from: https://onlinelibrary.wiley.com/doi/10.1111/cea.13897

133. Sirufo MM, Ginaldi L, De Martinis M. Successful Treatment With Omalizumab in a Child With Asthma and Urticaria: A Clinical Case Report. Front Pediatr. 2019 Jun 5;7:213. Available from: https://www.frontiersin.org/article/10.3389/fped.2019.00213/full

134. Vazquez-Ortiz M, Turner PJ. Improving the safety of oral immunotherapy for food allergy. Pediatr Allergy Immunol. 2016 Mar 22;27(2):117–25. Available from: https://onlinelibrary.wiley.com/doi/10.1111/pai.12510

135. Fiocchi A, Pecora V, Valluzzi RL, Fierro V, Mennini M. Use of biologics in severe food allergies. Curr Opin Allergy Clin Immunol. 2017 Jun;17(3):232–8. Available from: https://journals.lww.com/00130832-201706000-00012

136. Zuberbier T, Wood RA, Bindslev-Jensen C, Fiocchi A, Chinthrajah RS, Worm M, et al. Omalizumab in IgE-Mediated Food Allergy: A Systematic Review and Meta-Analysis. J Allergy Clin Immunol Pract. 2023 Apr;11(4):1134–46. Available from: https://linkinghub.elsevier.com/retrieve/pii/S221321982201296X

137. Harb H, Chatila TA. Mechanisms of Dupilumab. Clin Exp Allergy [Internet]. 2020 Jan 30;50(1):5–14. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/cea.13491

138. Rial MJ, Barroso B, Sastre J. Dupilumab for treatment of food allergy. J Allergy Clin Immunol Pract. 2019 Feb;7(2):673–4. Available from: https://linkinghub.elsevier.com/retrieve/pii/S221321981830463X

139. Clinical trial from Regeneron Pharmaceuticals. Study in Pediatric Subjects With Peanut Allergy to Evaluate Efficacy and Safety of Dupilumab as Adjunct to AR101 (Peanut Oral Immunotherapy). September 25, 2018.

140. Chinthrajah S, Cao S, Liu C, Lyu S-C, Sindher SB, Long A, et al. Phase 2a randomized, placebo-

controlled study of anti–IL-33 in peanut allergy. JCI Insight. 2019 Nov 14;4(22). Available from: https://insight.jci.org/articles/view/131347

141. Berin MC, Mayer L. Can we produce true tolerance in patients with food allergy? J Allergy Clin Immunol. 2013 Jan;131(1):14–22. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674912018374

142. Muraro A, Roberts G, Halken S, Agache I, Angier E, Fernandez-Rivas M, et al. EAACI guidelines on allergen immunotherapy: Executive statement. Allergy Eur J Allergy Clin Immunol. 2018;73(4):739–43. Available from: https://doi.org/10.1111/all.13420.

143. Chiang D, Berin MC. An Examination of Clinical and Immunologic Outcomes in Food Allergen Immunotherapy by Route of Administration. Curr Allergy Asthma Rep. 2015 Jun 4;15(6):35. Available from: http://link.springer.com/10.1007/s11882-015-0536-y

144. Chu DK, Wood RA, French S, Fiocchi A, Jordana M, Waserman S, et al. Oral immunotherapy for peanut allergy (PACE): a systematic review and meta-analysis of efficacy and safety. Lancet. 2019 Jun;393(10187):2222–32. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0140673619304209

145. Duca B, Patel N, Turner PJ. GRADE-ing the Benefit/Risk Equation in Food Immunotherapy. Curr Allergy Asthma Rep. 2019 Jun 25;19(6):30. Available from: http://link.springer.com/10.1007/s11882-019-0862-6

146. Fernández-Rivas M, Garrido Fernández S, Nadal JA, Alonso Díaz de Durana MD, García BE, González-Mancebo E, et al. Randomized double-blind, placebo-controlled trial of sublingual immunotherapy with a Pru p 3 quantified peach extract. Allergy. 2009 Jun;64(6):876–83. Available from: http://doi.wiley.com/10.1111/j.1398-9995.2008.01921.x

147. Palomares F, Gomez F, Bogas G, Campo P, Perkins JR, Diaz-Perales A, et al. Immunological Changes Induced in Peach Allergy Patients with Systemic Reactions by Pru p 3 Sublingual Immunotherapy. Mol Nutr Food Res. 2018 Feb;62(3):1700669. Available from: http://doi.wiley.com/10.1002/mnfr.201700669

148. Gomez F, Bogas G, Gonzalez M, Campo P, Salas M, Diaz-Perales A, et al. The clinical and immunological effects of Pru p 3 sublingual immunotherapy on peach and peanut allergy in patients with systemic reactions. Clin Exp Allergy]. 2017 Mar;47(3):339–50. Available from: http://doi.wiley.com/10.1111/cea.12901

149. Beitia JM, Vega Castro A, Cárdenas R, Peña-Arellano MI. Pru p 3 Sublingual Immunotherapy in Patients with Lipid Transfer Protein Syndrome: Is It Worth? Int Arch Allergy Immunol. 2021 Feb 15;1–8. Available from: https://www.karger.com/Article/FullText/512613

150. Garrido-Fernández S, García BE, Sanz ML, Echechipía S, Lizaso MT, Tabar AI. Are basophil activation and sulphidoleukotriene determination useful tests for monitoring patients with peach allergy receiving sublingual immunotherapy with a Pru p 3-enriched peach extract? J Investig Allergol Clin Immunol. 2014;24(2):106–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24834773

151. García BE, González-Mancebo E, Barber D, Martín S, Tabar AI, Díaz de Durana AMD, et al. Sublingual immunotherapy in peach allergy: monitoring molecular sensitizations and reactivity to apple fruit

and Platanus pollen. J Investig Allergol Clin Immunol. 2010;20(6):514–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21243936

152. Rodriguez MJ, Mascaraque A, Ramos-Soriano J, Torres MJ, Perkins JR, Gomez F, et al. Pru p 3-Epitope-based sublingual immunotherapy in a murine model for the treatment of peach allergy. Mol Nutr Food Res. 2017 Oct;61(10):1700110. Available from: http://doi.wiley.com/10.1002/mnfr.201700110

153. Navarro B, Alarcón E, Claver Á, Pascal M, Díaz-Perales A, Cisteró-Bahima A. Oral immunotherapy with peach juice in patients allergic to LTPs. Allergy, Asthma Clin Immunol. 2019 Dec 24;15(1):60. Available from: https://aacijournal.biomedcentral.com/articles/10.1186/s13223-019-0374-x

154. Dramburg S, Hilger C, Santos AF, de las Vecillas L, Aalberse RC, Acevedo N, et al. EAACI Molecular Allergology User's Guide 2.0. Pediatr Allergy Immunol. 2023 Mar 26;34(S28). Available from: https://onlinelibrary.wiley.com/doi/10.1111/pai.13854

155. Haktanir Abul M, Orhan F. Anaphylaxis after prick-to-prick test with fish. Pediatr Int. 2016 Jun;58(6):503–5. Available from: https://onlinelibrary.wiley.com/doi/10.1111/ped.12856

156. Pitsios C, Dimitriou A, Stefanaki EC, Kontou-Fili K. Anaphylaxis during skin testing with food allergens in children. Eur J Pediatr. 2010 May 23;169(5):613–5. Available from: http://link.springer.com/10.1007/s00431-009-1070-5

157. Yılmaz Ö, Atıcı S, Ertoy Karagöl Hİ, Topal E, Bakırtaş A, Demirsoy MS, et al. A Case of Anaphylaxis to Legumes During Prick to Prick Test. Asthma Allergy Immunol. 2016 Aug 9;14(1):36–40. Available from: http://aai.org.tr/index.php/aai/article/view/28

158. Wongpiyabovorn J, Suratannon N, Boonmee S, Chatchatee P. Comparison of specific IgE detection by immunoblotting and fluorescence enzyme assay with in vivo skin prick test. Asian Pacific J Allergy Immunol. 2018;36(3):159–65. Available from: http://apjai-journal.org/wp-content/uploads/2017/09/AP-270217-0035.pdf

159. Heinzerling L, Mari A, Bergmann K, Bresciani M, Burbach G, Darsow U, et al. The skin prick test – European standards. Clin Transl Allergy. 2013 Jan;3(1):3. Available from: https://onlinelibrary.wiley.com/doi/10.1186/2045-7022-3-3

160. Foong R-X, Dantzer JA, Wood RA, Santos AF. Improving Diagnostic Accuracy in Food Allergy. J Allergy Clin Immunol Pract. 2021 Jan;9(1):71–80. Available from: https://linkinghub.elsevier.com/retrieve/pii/S2213219820310862

161. Ortolani C, Ispano M, Pastorello EA, Ansaloni R, Magri GC. Comparison of results of skin prick tests (with fresh foods and commercial food extracts) and RAST in 100 patients with oral allergy syndrome. J Allergy Clin Immunol. 1989 Mar;83(3):683–90. Available from: https://linkinghub.elsevier.com/retrieve/pii/0091674989900833

162. Wainstein BK, Yee A, Jelley D, Ziegler M, Ziegler JB. Combining skin prick, immediate skin application and specific-IgE testing in the diagnosis of peanut allergy in children. Pediatr Allergy Immunol. 2007 May;18(3):231–9. Available from: https://onlinelibrary.wiley.com/doi/10.1111/j.1399-3038.2007.00517.x

163. Peters RL, Gurrin LC, Allen KJ. The predictive value of skin prick testing for challenge-proven food

allergy: A systematic review. Pediatr Allergy Immunol. 2012 Jun;23(4):347–52. Available from: https://onlinelibrary.wiley.com/doi/10.1111/j.1399-3038.2011.01237.x

164. Ishizaka K, Ishizaka T. Identification of gamma-E-antibodies as a carrier of reaginic activity. J Immunol. 1967 Dec;99(6):1187–98. Available from: http://www.ncbi.nlm.nih.gov/pubmed/4168663

165. Ansotegui IJ, Melioli G, Canonica GW, Caraballo L, Villa E, Ebisawa M, et al. IgE allergy diagnostics and other relevant tests in allergy, a World Allergy Organization position paper. World Allergy Organ J. 2020 Feb;13(2):100080. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1939455119312360

166. Sicherer SH, Wood RA. Allergy Testing in Childhood: Using Allergen-Specific IgE Tests. Pediatrics. 2012 Jan 1;129(1):193–7. Available from: http://pediatrics.aappublications.org/cgi/doi/10.1542/peds.2011-2382

167. Pascal M, Moreno C, Dávila I, Tabar AI, Bartra J, Labrador M, et al. Integration of in vitro allergy test results and ratio analysis for the diagnosis and treatment of allergic patients (INTEGRA). Clin Transl Allergy. 2021 Sep 16; 11(7):e12052. Available from: https://doi.org/10.1002/clt2.12052.

168. Santos AF, Alpan O, Hoffmann H. Basophil activation test: Mechanisms and considerations for use in clinical trials and clinical practice. Allergy. 2021 Aug 27;76(8):2420–32. Available from: https://onlinelibrary.wiley.com/doi/10.1111/all.14747

169. Ocmant A, Peignois Y, Mulier S, Hanssens L, Michils A, Schandené L. Flow cytometry for basophil activation markers: The measurement of CD203c up-regulation is as reliable as CD63 expression in the diagnosis of cat allergy. J Immunol Methods. 2007 Mar;320(1–2):40–8. Available from: https://linkinghub.elsevier.com/retrieve/pii/S002217590600367X

170. Knol EF, Mul FPJ, Jansen H, Calafat J, Roos D. Monitoring human basophil activation via CD63 monoclonal antibody 435. J Allergy Clin Immunol . 1991 Sep;88(3):328–38. Available from: https://linkinghub.elsevier.com/retrieve/pii/0091674991900945

171. Ebo DG, Bridts CH, Mertens CH, Hagendorens MM, Stevens WJ, De Clerck LS. Analyzing histamine release by flow cytometry (HistaFlow): A novel instrument to study the degranulation patterns of basophils. J Immunol Methods. 2012 Jan;375(1–2):30–8. Available from: http://dx.doi.org/10.1016/j.jim.2011.09.003

172. Hoffmann HJ, Santos AF, Mayorga C, Nopp A, Eberlein B, Ferrer M, et al. The clinical utility of basophil activation testing in diagnosis and monitoring of allergic disease. Allergy. 2015 Nov;70(11):1393–405. Available from: http://doi.wiley.com/10.1111/all.12698

173. MacGlashan D. Expression of CD203c and CD63 in human basophils: relationship to differential regulation of piecemeal and anaphylactic degranulation processes. Clin Exp Allergy. 2010 Jul 13;40(9):1365–77. Available from: http://doi.wiley.com/10.1111/j.1365-2222.2010.03572.x

174. Ando N, Nakamura Y, Ishimaru K, Ogawa H, Okumura K, Shimada S, et al. Allergen-specific basophil reactivity exhibits daily variations in seasonal allergic rhinitis. Allergy. 2015 Mar 22;70(3):319–22. Available from: https://onlinelibrary.wiley.com/doi/10.1111/all.12552

175. Lund G, Willumsen N, Holm J, Christensen LH, Würtzen PA, Lund K. Antibody repertoire complexity

and effector cell biology determined by assays for IgE-mediated basophil and T-cell activation. J Immunol Methods. 2012 Sep;383(1–2):4–20. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0022175912001597

176. MacGlashan D, Xia HZ, Schwartz LB, Gong J. IgE-regulated loss, not IgE-regulated synthesis, controls expression of FcepsilonRI in human basophils. J Leukoc Biol. 2001 Aug;70(2):207–18. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11493612

177. MacGlashan D. IgE and FceRI Regulation. Clin Rev Allergy Immunol. 2005;29(1):049–60. Available from: http://link.springer.com/10.1385/CRIAI:29:1:049

178. Santos AF, Douiri A, Bécares N, Wu S-Y, Stephens A, Radulovic S, et al. Basophil activation test discriminates between allergy and tolerance in peanut-sensitized children. J Allergy Clin Immunol. 2014 Sep;134(3):645–52. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674914007283

179. Ocmant A, Mulier S, Hanssens L, Goldman M, Casimir G, Mascart F, et al. Basophil activation tests for the diagnosis of food allergy in children. Clin Exp Allergy. 2009 Aug;39(8):1234–45. Available from: https://onlinelibrary.wiley.com/doi/10.1111/j.1365-2222.2009.03292.x

180. Santos AF, Du Toit G, O'Rourke C, Becares N, Couto-Francisco N, Radulovic S, et al. Biomarkers of severity and threshold of allergic reactions during oral peanut challenges. J Allergy Clin Immunol. 2020 Aug;146(2):344–55. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674920304905

181. MacGlashan DW. Relationship between spleen tyrosine kinase and phosphatidylinositol 5' phosphatase expression and secretion from human basophils in the general population. J Allergy Clin Immunol. 2007 Mar;119(3):626–33. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674906020161

182. Patil SU, Shreffler WG. Immunology in the Clinic Review Series; focus on allergies: basophils as biomarkers for assessing immune modulation. Clin Exp Immunol. 2012 Jan;167(1):59–66. Available from: http://doi.wiley.com/10.1111/j.1365-2249.2011.04503.x

183. Hoffmann HJ, Knol EF, Ferrer M, Mayorga L, Sabato V, Santos AF, et al. Pros and Cons of Clinical Basophil Testing (BAT). Curr Allergy Asthma Rep. 2016 Aug 13;16(8):56. Available from: http://dx.doi.org/10.1007/s11882-016-0633-6

184. Sturm GJ, Kranzelbinder B, Sturm EM, Heinemann A, Groselj-Strele A, Aberer W. The basophil activation test in the diagnosis of allergy: technical issues and critical factors. Allergy. 2009 Sep;64(9):1319–26. Available from: http://doi.wiley.com/10.1111/j.1398-9995.2009.02004.x

185. Iqbal K, Bhargava K, Skov PS, Falkencrone S, Grattan CE. A positive serum basophil histamine release assay is a marker for ciclosporin-responsiveness in patients with chronic spontaneous urticaria. Clin Transl Allergy. 2012 Jan;2(1):19. Available from: https://onlinelibrary.wiley.com/doi/10.1186/2045-7022-2-19

186. Mukai K, Gaudenzio N, Gupta S, Vivanco N, Bendall SC, Maecker HT, et al. Assessing basophil activation by using flow cytometry and mass cytometry in blood stored 24 hours before analysis. J Allergy Clin Immunol. 2017 Mar;139(3):889-899.e11. Available from:

https://linkinghub.elsevier.com/retrieve/pii/S0091674916306133

187. Kvedariene V, Kamey S, Ryckwaert Y, Rongier M, Bousquet J, Demoly P, et al. Diagnosis of neuromuscular blocking agent hypersensitivity reactions using cytofluorimetric analysis of basophils. Allergy. 2006 Mar;61(3):311–5. Available from: https://onlinelibrary.wiley.com/doi/10.1111/j.1398-9995.2006.00978.x

188. Fernández TD, Torres MJ, Blanca-López N, Rodríguez-Bada JL, Gomez E, Canto G, et al. Negativization rates of IgE radioimmunoassay and basophil activation test in immediate reactions to penicillins. Allergy. 2009 Feb;64(2):242–8. Available from: https://onlinelibrary.wiley.com/doi/10.1111/j.1398-9995.2008.01713.x

189. Sousa N, Martínez-Aranguren R, Fernández-Benitez M, Ribeiro F, Sanz ML. Comparison of basophil activation test results in blood preserved in acid citrate dextrose and EDTA. J Investig Allergol Clin Immunol. 2010;20(6):535–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21243940

190. Reber LL, Hernandez JD, Galli SJ. The pathophysiology of anaphylaxis. J Allergy Clin Immunol. 2017 Aug;140(2):335–48. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674917310205

191. Bahri R, Custovic A, Korosec P, Tsoumani M, Barron M, Wu J, et al. Mast cell activation test in the diagnosis of allergic disease and anaphylaxis. J Allergy Clin Immunol. 2018 Aug;142(2):485-496.e16. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674918303087

192. Santos AF, Couto-Francisco N, Bécares N, Kwok M, Bahnson HT, Lack G. A novel human mast cell activation test for peanut allergy. J Allergy Clin Immunol. 2018 Aug;142(2):689-691.e9. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674918305189

193. Farazuddin M, Ludka N, Baker J. The use of LAD2 cells in the mast cell activation test (MAT): a potential tool for food allergy diagnosis. J Allergy Clin Immunol. 2021 Feb;147(2):AB88. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674920320996

194. Brown SG, Blackman KE, Heddle RJ. Can serum mast cell tryptase help diagnose anaphylaxis? Emerg Med Australas. 2004 Apr;16(2):120–4. Available from: https://onlinelibrary.wiley.com/doi/10.1111/j.1742-6723.2004.00562.x

195. Vitte J, Amadei L, Gouitaa M, Mezouar S, Zieleskiewicz L, Albanese J, et al. Paired acute-baseline serum tryptase levels in perioperative anaphylaxis: An observational study. Allergy. 2019 Mar 21;all.13752. Available from: https://onlinelibrary.wiley.com/doi/10.1111/all.13752

196. Valent P, Akin C, Arock M, Brockow K, Butterfield JH, Carter MC, et al. Definitions, Criteria and Global Classification of Mast Cell Disorders with Special Reference to Mast Cell Activation Syndromes: A Consensus Proposal. Int Arch Allergy Immunol. 2012;157(3):215–25. Available from: https://www.karger.com/Article/FullText/328760

197. Calvani M, Bianchi A, Reginelli C, Peresso M, Testa A. Oral Food Challenge. Medicina (B Aires). 2019 Sep 27;55(10):651. Available from: https://www.mdpi.com/1648-9144/55/10/651

198. Sampson HA, Gerth van Wijk R, Bindslev-Jensen C, Sicherer S, Teuber SS, Burks AW, et al. Standardizing double-blind, placebo-controlled oral food challenges: American Academy of Allergy, Asthma & Immunology–European Academy of Allergy and Clinical Immunology PRACTALL

consensus report. J Allergy Clin Immunol. 2012 Dec;130(6):1260–74. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674912016636

199. David TJ. Anaphylactic shock during elimination diets for severe atopic eczema. Arch Dis Child. 1984 Oct 1;59(10):983–6. Available from: https://adc.bmj.com/lookup/doi/10.1136/adc.59.10.983

200. Valenta, Lidholm, Niederberger, Hayek, Kraft, Grönlund. The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). Clin Exp Allergy. 1999 Jul;29(7):896–904. Available from: http://doi.wiley.com/10.1046/j.1365-2222.1999.00653.x

201. van Kampen V, de Blay F, Folletti I, Kobierski P, Moscato G, Olivieri M, et al. Evaluation of commercial skin prick test solutions for selected occupational allergens. Allergy. 2013 May;68(5):651–8. Available from: http://doi.wiley.com/10.1111/all.12116

202. Kespohl S, Maryska S, Zahradnik E, Sander I, Brüning T, Raulf-Heimsoth M. Biochemical and immunological analysis of mould skin prick test solution: current status of standardization. Clin Exp Allergy. 2013 Nov;43(11):1286–96. Available from: http://doi.wiley.com/10.1111/cea.12186

203. Rossi R, Monasterolo G, Passalacqua G. The biological potency of different extracts for sublingual immunotherapy assessed by skin prick tests. Eur Ann Allergy Clin Immunol. 2010 Jun;42(3):112–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20648773

204. Morita E, Chinuki Y, Takahashi H. Recent advances of in vitro tests for the diagnosis of food-dependent exercise-induced anaphylaxis. J Dermatol Sci. 2013 Sep;71(3):155–9. Available from: http://dx.doi.org/10.1016/j.jdermsci.2013.04.010

205. Akkerdaas J, Finkina EII, Balandin SV V., Santos Magadán S, Knulst A, Fernandez-Rivas M, et al. Lentil (Lens culinaris) Lipid Transfer Protein Len c 3: A Novel Legume Allergen. Int Arch Allergy Immunol. 2012;157(1):51–7. Available from: https://www.karger.com/Article/FullText/324946

206. San Bartolomé C, Muñoz-Cano R, Rius J, Casas-Saucedo R, Balsells S, Egri N, et al. Detection of Wheat Lipid Transfer Protein (Tri a 14) Sensitization: Comparison between Currently Available Diagnostic Tools. Int Arch Allergy Immunol. 2022;183(1):75–9. Available from: https://www.karger.com/Article/FullText/517963

207. Borres MP, Maruyama N, Sato S, Ebisawa M. Recent advances in component resolved diagnosis in food allergy. Allergol Int. 2016 Oct;65(4):378–87. Available from: http://dx.doi.org/10.1016/j.alit.2016.07.002

208. Bernardi ML, Giangrieco I, Camardella L, Ferrara R, Palazzo P, Panico MR, et al. Allergenic Lipid Transfer Proteins from Plant-Derived Foods Do Not Immunologically and Clinically Behave Homogeneously: The Kiwifruit LTP as a Model. PLoS One. 2011 Nov 17;6(11):e27856. Available from: https://dx.plos.org/10.1371/journal.pone.0027856

209. Tuppo L, Alessandri C, Pomponi D, Picone D, Tamburrini M, Ferrara R, et al. Peamaclein - A new peach allergenic protein: similarities, differences and misleading features compared to Pru p 3. Clin Exp Allergy. 2013 Jan;43(1):128–40. Available from: http://doi.wiley.com/10.1111/cea.12028

210. Incorvaia C, Mauro M, Ridolo E, Makrì E, Montagni M, Ciprandi G. A Pitfall to Avoid When Using an Allergen Microarray: The Incidental Detection of IgE to Unexpected Allergens. J Allergy Clin Immunol Pract. 2015 Nov;3(6):879–82. Available from:

https://linkinghub.elsevier.com/retrieve/pii/S2213219814004449

211. Matricardi PM, Kleine-Tebbe J. Molecular Allergology between Precision Medicine and the Choosing Wisely initiative. Clin Exp Allergy. 2016 May;46(5):664–7. Available from: https://onlinelibrary.wiley.com/doi/10.1111/cea.12679

212. Ollert M, Weissenbacher S, Rakoski J, Ring J. Allergen-specific IgE measured by a continuous random-access immunoanalyzer: interassay comparison and agreement with skin testing. Clin Chem. 2005 Jul;51(7):1241-9. Available from http://doi.wiley.com/10.1373/clinchem.2004.046565..

213. Ewan PW, Coote D. Evaluation of a capsulated hydrophilic carrier polymer (the ImmunoCAP) for measurement of specific IgE antibodies. Allerg. 1990 Jan;45(1):22–9. Available from: http://doi.wiley.com/10.1111/j.1398-9995.1990.tb01080.x

214. Hamilton RG, Matsson PN, Hovanec-Burns DL, Van Cleve M, Chan S, Kober A, et al. Analytical Performance Characteristics, Quality Assurance and Clinical Utility of Immunological Assays for Human IgE Antibodies of Defined Allergen Specificities. (CLSI-ILA20-A3). J Allergy Clin Immunol 2015 Feb;135(2):AB8. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674914027420

215. Hamilton RG, MacGlashan DW, Saini SS. IgE antibody-specific activity in human allergic disease. Immunol Res. 2010 Jul 12;47(1–3):273–84. Available from: http://link.springer.com/10.1007/s12026-009-8160-3

216. Gadisseur R, Chapelle J-P, Cavalier E. A new tool in the field of in-vitro diagnosis of allergy: preliminary results in the comparison of ImmunoCAP© 250 with the ImmunoCAP© ISAC. Clin Chem Lab Med. 2011 Jan 1;49(2):277–80. Available from: https://www.degruyter.com/document/doi/10.1515/CCLM.2011.052/html

217. Thermo Fisher Scientific Inc. Principles of the test procedure [Internet]. Cited: 2021 June 22. Available from: http://www.recaptulandodigital.com.br/da/Products/Allergy-testing-products/ImmunoCAP-ISAC/Test-Principle-ImmunoCAP-ISAC/.

218. Jakob T, Forstenlechner P, Matricardi P, Kleine-Tebbe J. Molecular allergy diagnostics using multiplex assays: methodological and practical considerations for use in research and clinical routine. Allergo J Int. 2015 Dec 17;24(8):320–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27069843

219. AG EML. Immunoblot [Internet]. Cited 2021 June 30. Available from: https://www.euroimmun.com/products/techniques/immunoblot/

220. GmbH MD. ALEX Allergy Explorer [Internet]. Cited 2021 June 30. Available from: https://www.macroarraydx.com/products/alex

221. Francesca B, Mirella DR, Enrico S, Paola M, Mariaelisabetta C, Ignazio B, et al. Evaluation of a new multiplex assay for allergy diagnosis. Clin Chim Acta. 2019;493(February):73–8. Available from: https://doi.org/10.1016/j.cca.2019.02.025

222. Asero R, Arena A, Cecchi L, Conte M., Crivellaro M, Emiliani F, et al. Are IgE Levels to Foods other than Rosaceae Predictive of Allergy in Lipid Transfer Protein-Hypersensitive Patients? Int Arch

Allergy Immunol. 2011;155(2):149–54. Available from: https://www.karger.com/Article/FullText/318864

223. Pastorello EA, Farioli L, Stafylaraki C, Mascheri A, Scibilia J, Pravettoni V, et al. Anti-rPru p 3 IgE Levels Are Inversely Related to the Age at Onset of Peach-Induced Severe Symptoms Reported by Peach-Allergic Adults. Int Arch Allergy Immunol. 2013;162(1):45–9. Available from: https://www.karger.com/Article/FullText/350467

224. Frischmeyer-Guerrerio PA, Rasooly M, Gu W, Levin S, Jhamnani RD, Milner JD, et al. IgE testing can predict food allergy status in patients with moderate to severe atopic dermatitis. Ann Allergy, Asthma Immunol. 2019 Apr;122(4):393-400.e2. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1081120619300018

225. Nilsson SF, Lilja G, Järnbert-Pettersson H, Alm J. Relevance of low specific IgE levels to egg, milk and peanut in infancy. Clin Exp Allergy. 2019 Mar;49(3):308–16. Available from: http://doi.wiley.com/10.1111/cea.13273

226. Cuomo B, Indirli GC, Bianchi A, Arasi S, Caimmi D, Dondi A, et al. Specific IgE and skin prick tests to diagnose allergy to fresh and baked cow's milk according to age: a systematic review. Ital J Pediatr. 2017 Dec 12;43(1):93. Available from: http://ijponline.biomedcentral.com/articles/10.1186/s13052-017-0410-8

227. Pastorello EA, Farioli L, Pravettoni V, Scibilia J, Mascheri A, Borgonovo L, et al. Pru p 3-Sensitised Italian Peach-Allergic Patients Are Less Likely to Develop Severe Symptoms When Also Presenting IgE Antibodies to Pru p 1 and Pru p 4. Int Arch Allergy Immunol. 2011;156(4):362–72. Available from: https://www.karger.com/Article/FullText/324440

228. Egger M, Hauser M, Mari A, Ferreira F, Gadermaier G. The Role of Lipid Transfer Proteins in Allergic Diseases. Curr Allergy Asthma Rep. 2010 Sep 26;10(5):326–35. Available from: http://link.springer.com/10.1007/s11882-010-0128-9

229. Martín-Pedraza L, González M, Gómez F, Blanca-López N, Garrido-Arandia M, Rodríguez R, et al. Two nonspecific lipid transfer proteins (nsLTPs) from tomato seeds are associated to severe symptoms of tomato-allergic patients. Mol Nutr Food Res. 2016;60(5):1172–82. Available from https://doi.org/10.1002/mnfr.201500782

230. Scheurer S, van Ree R, Vieths S. The Role of Lipid Transfer Proteins as Food and Pollen Allergens Outside the Mediterranean Area. Curr Allergy Asthma Rep. 2021 Feb 3;21(2):7. Available from: http://link.springer.com/10.1007/s11882-020-00982-w

231. Brenna O V., Pastorello EA, Farioli L, Pravettoni V, Pompei C. Presence of Allergenic Proteins in Different Peach (Prunus persica) Cultivars and Dependence of Their Content on Fruit Ripening. J Agric Food Chem. 2004 Dec;52(26):7997–8000. Available from: https://pubs.acs.org/doi/10.1021/jf0491052

232. Yeats TH, Rose JKC. The biochemistry and biology of extracellular plant lipid-transfer proteins (LTPs). Protein Sci. 2008 Feb;17(2):191–8. Available from: http://doi.wiley.com/10.1110/ps.073300108

233. Pasquato N, Berni R, Folli C, Folloni S, Cianci M, Pantano S, et al. Crystal Structure of Peach Pru p 3,

the Prototypic Member of the Family of Plant Non-specific Lipid Transfer Protein Pan-allergens. J Mol Biol. 2006 Feb;356(3):684–94. Available from: https://linkinghub.elsevier.com/retrieve/pii/S002228360501466X

234. Cavatorta V, Sforza S, Aquino G, Galaverna G, Dossena A, Pastorello EA, et al. In vitro gastrointestinal digestion of the major peach allergen Pru p 3, a lipid transfer protein: Molecular characterization of the products and assessment of their IgE binding abilities. Mol Nutr Food Res. 2010 Oct;54(10):1452–7. Available from: http://doi.wiley.com/10.1002/mnfr.200900552

235. Garino C, Zitelli F, Travaglia F, Daniel J, Cravotto G. Evaluation of the Impact of Sequential Microwave/Ultrasound Processing on the IgE Binding Properties of Pru p 3 in Treated Peach Juice. J Agric Food Chem. 2012;60:8755–8762. Available from: http://doi.wiley.com/10.1021/jf302027e

236. Pastorello EA, Farioli L, Pravettoni V, Ortolani C, Ispano M, Monza M, et al. The major allergen of peach (Prunus persica) is a lipid transfer protein. J Allergy Clin Immunol. 1999 Mar;103(3):520–6. Available from: https://linkinghub.elsevier.com/retrieve/pii/S009167499970480X

237. Sánchez-Monge R, Lombardero M, García-Sellés FJ, Barber D, Salcedo G. Lipid-transfer proteins are relevant allergens in fruit allergy. J Allergy Clin Immunol. 1999 Mar;103(3):514–9. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674999704793

238. Asero R, Piantanida M, Pinter E, Pravettoni V. The clinical relevance of lipid transfer protein. Clin Exp Allergy. 2018 Jan;48(1):6–12. Available from: https://onlinelibrary.wiley.com/doi/10.1111/cea.13053

239. Rossi CM, Lenti MV, Merli S, Licari A, Marseglia GL, Di Sabatino A. Immunotherapy with Pru p 3 for food allergy to peach and non-specific lipid transfer protein: a systematic review. Clin Mol Allergy. 2023 May 31;21(1):3. Available from: https://clinicalmolecularallergy.biomedcentral.com/articles/10.1186/s12948-023-00184-5

240. Skypala IJ, Asero R, Barber D, Cecchi L, Diaz Perales A, Hoffmann-Sommergruber K, et al. Non-specific lipid-transfer proteins: Allergen structure and function, cross-reactivity, sensitization, and epidemiology. Clin Transl Allergy. 2021 May 18;11(3). Available from: https://onlinelibrary.wiley.com/doi/10.1002/clt2.12010

241. Asero R, Pravettoni V, Scala E, Villalta D. Lipid transfer protein allergy: A review of current controversies. Clin Exp Allergy. 2022 Feb 28;52(2):222–30. Available from: https://onlinelibrary.wiley.com/doi/10.1111/cea.14049

242. Pastorello EA, Farioli L, Conti A, Pravettoni V, Bonomi S, Iametti S, et al. Wheat IgE-Mediated Food Allergy in European Patients: α-Amylase Inhibitors, Lipid Transfer Proteins and Low-Molecular-Weight Glutenins. Int Arch Allergy Immunol. 2007;144(1):10–22. Available from: https://www.karger.com/Article/FullText/102609

243. Palacin A, Quirce S, Armentia A, Fernández-Nieto M, Pacios LF, Asensio T, et al. Wheat lipid transfer protein is a major allergen associated with baker's asthma. J Allergy Clin Immunol. 2007 Nov;120(5):1132–8. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674907013589

244. Battais F, Mothes T, Moneret-Vautrin DA, Pineau F, Kanny G, Popineau Y, et al. Identification of IgE-

binding epitopes on gliadins for patients with food allergy to wheat. Allergy. 2005 Jun;60(6):815–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15876313

245. García-casado G, Pacios LF, Díaz-perales A, Sánchez-monge R, Lombardero M, García-selles FJ, et al. Identification of IgE-binding epitopes of the major peach allergen Pru p 3. Clin Immunol. 2003;112(3):599–605.

246. Pastorello EA, Monza M, Pravettoni V, Longhi R, Bonara P, Scibilia J, et al. Characterization of the T-Cell Epitopes of the Major Peach Allergen Pru p 3. Int Arch Allergy Immunol. 2010;153(1):1–12. Available from: https://www.karger.com/Article/FullText/301573

247. Salcedo G, Sanchez-Monge R, Diaz-Perales A, Garcia-Casado G, Barber D. Plant non-specific lipid transfer proteins as food and pollen allergens. Clin Exp Allergy Exp Allergy. 2004 Sep;34(9):1336–41. Available from: http://doi.wiley.com/10.1111/j.1365-2222.2004.02018.x

248. Fernández-Rivas, Cuevas. Peels of Rosaceae fruits have a higher allergenicity than pulps. Clin Exp Allergy. 1999 Sep;29(9):1239–47. Available from: http://doi.wiley.com/10.1046/j.1365-2222.1999.00628.x

249. Larocca M, Martelli G, Grossi G, Padula MC, Riccio P, Rossano R. Peel LTP (Pru p 3) – the major allergen of peach – is methylated. A proteomic study. Food Chem. 2013 Dec;141(3):2765–71. Available from: http://dx.doi.org/10.1016/j.foodchem.2013.04.082

250. Tordesillas L, Cubells-Baeza N, Gómez-Casado C, Berin C, Esteban V, Barcik W, et al. Mechanisms underlying induction of allergic sensitization by Pru p 3. Clin Exp Allergy. 2017 Nov;47(11):1398–408. Available from: http://doi.wiley.com/10.1111/cea.12962

251. Pastorello EA, Robino AM. Clinical role of lipid transfer proteins in food allergy. Mol Nutr Food Res . 2004 Oct;48(5):356–62. Available from: http://doi.wiley.com/10.1002/mnfr.200400047

252. Pastorello EA, Farioli L, Stafylaraki C, Scibilia J, Mirone C, Pravettoni V, et al. Wheat-dependent exercise-induced anaphylaxis caused by a lipid transfer protein and not by ω-5 gliadin. Ann Allergy, Asthma Immunol. 2014 Apr;112(4):386-387.e1. Available from: https://linkinghub.elsevier.com/retrieve/pii/S108112061400026X

253. Asero R, Antonicelli L, Arena A, Bommarito L, Caruso B, Crivellaro M, et al. EpidemAAITO: Features of food allergy in Italian adults attending allergy clinics: a multi-centre study. Clin Exp Allergy. 2009 Apr;39(4):547–55. Available from: http://doi.wiley.com/10.1111/j.1365-2222.2008.03167.x

254. Ma S, Nie L, Li H, Wang R, Yin J. Component-Resolved Diagnosis of Peanut Allergy and Its Possible Origins of Sensitization in China. Int Arch Allergy Immunol. 2016;169(4):241–8. Available from: https://www.karger.com/Article/FullText/446156

255. Gao Z-S, Yang Z-W, Wu S-D, Wang H-Y, Liu M-L, Mao W-L, et al. Peach allergy in China: A dominant role for mugwort pollen lipid transfer protein as a primary sensitizer. J Allergy Clin Immunol. 2013 Jan;131(1):224-226.e3. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674912011906

256. Murad A, Katelaris CH, Baumgart K. A case study of apple seed and grape allergy with sensitisation to nonspecific lipid transfer protein. Asia Pac Allergy. 2016;6(2):129. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3269605&tool=pmcentrez&rendertype

=abstract

257. Azofra J, Berroa F, Gastaminza G, Saiz N, Gamboa PM, Vela C, et al. Lipid Transfer Protein Syndrome in a Non-Mediterranean Area. Int Arch Allergy Immunol. 2016;169(3):181–8. Available from: https://www.karger.com/Article/FullText/445893

258. Skypala IJ, Cecchi L, Shamji MH, Scala E, Till S. Lipid Transfer Protein allergy in the United Kingdom: Characterization and comparison with a matched Italian cohort. Allergy. 2019 2019 Jul;74(7):1340-1351. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/all.13747

259. Gülsen A, Jappe U. Lipid transfer protein sensitization in an apple-allergic patient: a case report from northern Europe. Eur Ann Allergy Clin Immunol. 2018 Mar;51(2):80-83. Available from: http://www.eurannallergyimm.com/cont/journals-articles/676/volume-lipid-transfer-protein-sensitization-appleallergic.asp

260. Asero R, Pravettoni V. Anaphylaxis to plant-foods and pollen allergens in patients with lipid transfer protein syndrome. Curr Opin Allergy Clin Immunol. 2013 Aug;13(4):379–85. Available from: https://insights.ovid.com/crossref?an=00130832-201308000-00011

261. Schäd SG, Trcka J, Vieths S, Scheurer S, Conti A, Bröcker E-B, et al. Wine Anaphylaxis in a German Patient: IgE-Mediated Allergy against a Lipid Transfer Protein of Grapes. Int Arch Allergy Immunol. 2005;136(2):159–64. Available from: https://www.karger.com/Article/FullText/83324

262. Ukleja-Sokołowska N, Zacniewski R, Gawrońska-Ukleja E, Żbikowska-Gotz M, Lis K, Sokołowski Ł, et al. Food-dependent, exercise-induced anaphylaxis in a patient allergic to peach. Int J Immunopathol Pharmacol. 2018 Jan 1;32:205873841880315. Available from: http://journals.sagepub.com/doi/10.1177/2058738418803154

263. Sander I, Rozynek P, Rihs H-P, van Kampen V, Chew FT, Lee WS, et al. Multiple wheat flour allergens and cross-reactive carbohydrate determinants bind IgE in baker's asthma. Allergy. 2011 Sep;66(9):1208–15. Available from: http://doi.wiley.com/10.1111/j.1398-9995.2011.02636.x

264. Blankestijn MA, Knulst AC, Knol EF, Le T-M, Rockmann H, Otten HG, et al. Sensitization to PR-10 proteins is indicative of distinctive sensitization patterns in adults with a suspected food allergy. Clin Transl Allergy. 2017 Dec 23;7(1):42. Available from: https://ctajournal.biomedcentral.com/articles/10.1186/s13601-017-0177-4

265. Flinterman AE, Akkerdaas JH, den Hartog Jager CF, Rigby NM, Fernandez-Rivas M, Hoekstra MO, et al. Lipid transfer protein–linked hazelnut allergy in children from a non-Mediterranean birch-endemic area. J Allergy Clin Immunol. 2008 Feb;121(2):423-428.e2. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674907019513

266. Asaumi T, Sato S, Yanagida N, Takahashi K, Mori Y, Okazaki F, et al. IgE-specific Pru p 4 negatively predicts systemic allergy reaction to peach among Japanese children. Allergol Int. 2019 Oct;68(4):546–8. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1323893019300668

267. Zuidmeer L, van Ree R. Lipid transfer protein allergy: primary food allergy or pollen/food syndrome in some cases. Curr Opin Allergy Clin Immunol. 2007 Jun;7(3):269–73. Available from: https://insights.ovid.com/crossref?an=00130832-200706000-00010

268. Pastorello EA, Pravettoni V, Farioli L, Rivolta F, Conti A, Ispano M, et al. Hypersensitivity to mugwort

(Artemisia vulgaris) in patients with peach allergy is due to a common lipid transfer protein allergen and is often without clinical expression. J Allergy Clin Immunol. 2002 Aug;110(2):310–7. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674902000969

269. Lombardero M, Garcia-Selles FJ, Polo F, Jimeno L, Chamorro MJ, Garcia-Casado G, et al. Prevalence of sensitization to Artemisia allergens Art v 1, Art v 3 and Art v 60 kDa. Cross-reactivity among Art v 3 and other relevant lipid-transfer protein allergens. Clin Exp Allergy]. 2004 Sep;34(9):1415–21. Available from: http://doi.wiley.com/10.1111/j.1365-2222.2004.02053.x

270. Asero R. In patients with LTP syndrome food-specific IgE show a predictable hierarchical order. Eur Ann Allergy Clin Immunol. 2014 Jul;46(4):142–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25053631

271. Ma S, Yin J, Jiang N. Component-resolved diagnosis of peach allergy and its relationship with prevalent allergenic pollens in China. J Allergy Clin Immunol. 2013 Sep;132(3):764–7. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674913006647

272. Sánchez-López J, Tordesillas L, Pascal M, Muñoz-Cano R, Garrido M, Rueda M, et al. Role of Art v 3 in pollinosis of patients allergic to Pru p 3. J Allergy Clin Immunol. 2014 Apr;133(4):1018-1025.e3. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674913012219

273. Lauer I, Miguel-Moncin MS, Abel T, Foetisch K, Hartz C, Fortunato D, et al. Identification of a plane pollen lipid transfer protein (Pla a 3) and its immunological relation to the peach lipid-transfer protein, Pru p 3. Clin Exp Allergy. 2007 Feb;37(2):261–9. Available from: http://doi.wiley.com/10.1111/j.1365-2222.2007.02653.x

274. Scala E, Till SJ, Asero R, Abeni D, Guerra EC, Pirrotta L, et al. Lipid transfer protein sensitization: reactivity profiles and clinical risk assessment in an Italian cohort. Allergy. 2015 Aug;70(8):933–43. Available from: http://doi.wiley.com/10.1111/all.12635

275. Gomez F, Aranda A, Campo P, Diaz-Perales A, Blanca-Lopez N, Perkins J, et al. High Prevalence of Lipid Transfer Protein Sensitization in Apple Allergic Patients with Systemic Symptoms. Hogan SP, editor. PLoS One. 2014 Sep 11;9(9):e107304. Available from: https://dx.plos.org/10.1371/journal.pone.0107304

276. Gamboa PM, Cáceres O, Antepara I, Sánchez-Monge R, Ahrazem O, Salcedo G, et al. Original article: Two different profiles of peach allergy in the north of Spain. Allergy. 2007 Mar 12;62(4):408–14. Available from: http://doi.wiley.com/10.1111/j.1398-9995.2006.01284.x

277. Cubells-Baeza N, Gómez-Casado C, Tordesillas L, Ramírez-Castillejo C, Garrido-Arandia M, González-Melendi P, et al. Identification of the ligand of Pru p 3, a peach LTP. Plant Mol Biol. 2017 May 15;94(1–2):33–44. Available from: http://link.springer.com/10.1007/s11103-017-0590-z

278. Tordesillas L, Gómez-Casado C, Garrido-Arandia M, Murua-García A, Palacín A, Varela J, et al. Transport of Pru p 3 across gastrointestinal epithelium - an essential step towards the induction of food allergy? Clin Exp Allergy. 2013 Dec;43(12):1374–83. Available from: http://doi.wiley.com/10.1111/cea.12202

279. Garcia BE, Lombardero M, Echechipia S, Olaguibel JM, Diaz-Perales A, Sanchez-Monge R, et al. Respiratory allergy to peach leaves and lipid-transfer proteins. Clin Exp Allergy. 2004

Feb;34(2):291–5. Available from: http://doi.wiley.com/10.1111/j.1365-2222.2004.01871.x

280. Pascal M, Vazquez-Ortiz M, Folque MM, Jimenez-Feijoo R, Lozano J, Dominguez O, et al. Asymptomatic LTP sensitisation is common in plant-food allergic children from the Northeast of Spain. Allergol Immunopathol (Madr). 2016 Jul;44(4):351–8. Available from: http://dx.doi.org/10.1016/j.aller.2015.10.003

281. Asero R. Peach-Induced Contact Urticaria Is Associated with Lipid Transfer Protein Sensitization. Int Arch Allergy Immunol. 2011;154(4):345–8. Available from: https://www.karger.com/Article/FullText/321827

282. Pastorello EA, Ortolani C, Farioli L, Pravettoni V, Ispano M, Borga Å, et al. Allergenic cross-reactivity among peach, apricot, plum, and cherry in patients with oral allergy syndrome: An in vivo and in vitro study. J Allergy Clin Immunol. 1994 Oct;94(4):699–707. Available from: https://linkinghub.elsevier.com/retrieve/pii/0091674994901775

283. Fernández-Rivas M. Clinically relevant peach allergy is related to peach lipid transfer protein, Pru p 3, in the spanish population*1. J Allergy Clin Immunol. 2003 Oct;112(4):789–95. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674903020165

284. Asero R, Antonicelli L, Arena A, Bommarito L, Caruso B, Colombo G, et al. Causes of Food-Induced Anaphylaxis in Italian Adults: A Multi-Centre Study. Int Arch Allergy Immunol. 2009;150(3):271–7. Available from: https://www.karger.com/Article/FullText/222679

285. Oeo-Santos C, Navas A, Benedé S, Ruíz-León B, Díaz-Perales A, Vogel L, et al. New insights into the sensitization to nonspecific lipid transfer proteins from pollen and food: New role of allergen Ole e 7. Allergy. 2020 Apr 6;75(4):798–807. Available from: https://onlinelibrary.wiley.com/doi/10.1111/all.14086

286. Palacín A, Gómez-Casado C, Rivas LA, Aguirre J, Tordesillas L, Bartra J, et al. Graph Based Study of Allergen Cross-Reactivity of Plant Lipid Transfer Proteins (LTPs) Using Microarray in a Multicenter Study. Stumbles PA, editor. PLoS One. 2012 Dec 14;7(12):e50799. Available from: https://dx.plos.org/10.1371/journal.pone.00507.99

287. Flores E, Cervera L, Sanz ML, Diaz-Perales A, Fernández J. Plant Food Allergy in Patients with Pollinosis from the Mediterranean Area. Int Arch Allergy Immunol.2012;159(4):346–54. Available from: https://www.karger.com/Article/FullText/338282

288. Barber D, de la Torre F, Lombardero M, Antépara I, Colas C, Dávila I, et al. Component-resolved diagnosis of pollen allergy based on skin testing with profilin, polcalcin and lipid transfer protein pan-allergens. Clin Exp Allergy. 2009 Nov;39(11):1764–73. Available from: http://doi.wiley.com/10.1111/j.1365-2222.2009.03351.x

289. Romano A, Scala E, Rumi G, Gaeta F, Caruso C, Alonzi C, et al. Lipid transfer proteins: the most frequent sensitizer in Italian subjects with food-dependent exercise-induced anaphylaxis. Clin Exp Allergy. 2012 Nov;42(11):1643–53. Available from: https://onlinelibrary.wiley.com/doi/10.1111/cea.12011

290. Asero R. Hypersensitivity to lipid transfer protein is frequently associated with chronic urticaria. Eur Ann Allergy Clin Immunol. 2011 Feb;43(1):19–21. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/21409857

291. Pascal M, Muñoz-Cano R, Milà J, Sanz ML, Diaz-Perales A, Sánchez-López J, et al. Nonsteroidal anti-inflammatory drugs enhance IgE-mediated activation of human basophils in patients with food anaphylaxis dependent on and independent of nonsteroidal anti-inflammatory drugs. Clin Exp Allergy. 2016 Aug;46(8):1111–9. Available from: https://onlinelibrary.wiley.com/doi/10.1111/cea.12735

292. Asero R, Piantanida M, Pravettoni V. Allergy to LTP: to eat or not to eat sensitizing foods? A follow-up study. Eur Ann Allergy Clin Immunol. 2018 Mar;50(04):156. Available from: http://www.eurannallergyimm.com/cont/journals-articles/592/volume-allergy-ltp-sensitizing-foods.asp

293. Kattan JD, Sicherer SH. Optimizing the Diagnosis of Food Allergy. Immunol Allergy Clin North Am. 2015 Feb;35(1):61–76. Available from: https://linkinghub.elsevier.com/retrieve/pii/S088985611400109X

294. Ciprandi G, De Amici M, Di Martino ML, Barocci F, Comite P. The impact of age on Pru p 3 IgE production in Italy. Asia Pac Allergy. 2017;7(1):42. Available from: https://synapse.koreamed.org/DOIx.php?id=10.5415/apallergy.2017.7.1.42

295. Decuyper II, Pascal M, Van Gasse AL, Mertens C, Díaz-Perales A, Araujo G, et al. Performance of basophil activation test and specific IgG4 as diagnostic tools in nonspecific lipid transfer protein allergy: Antwerp-Barcelona comparison. Allergy. 2019 Mar;75(3):616-624. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/all.14040

296. Balsells-Vives S, San Bartolomé C, Casas-Saucedo R, Ruano-Zaragoza M, Rius J, Torradeflot M, et al. Low Levels Matter: Clinical Relevance of Low Pru p 3 sIgE in Patients With Peach Allergy. Front Allergy. 2022 Apr 5;3:868267. Available from: https://www.frontiersin.org/articles/10.3389/falgy.2022.868267/full

297. Morales M, López-Matas MÁ, Moya R, Carnés J. Cross-reactivity among non-specific lipid-transfer proteins from food and pollen allergenic sources. Food Chem. 2014 Dec;165:397–402. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0308814614008176

298. Tordesillas L, Sirvent S, Díaz-Perales A, Villalba M, Cuesta-Herranz J, Rodríguez R, et al. Plant Lipid Transfer Protein Allergens: No Cross-Reactivity between Those from Foods and Olive and *Parietaria* Pollen. Int Arch Allergy Immunol. 2011;156(3):291–6. Available from: https://www.karger.com/Article/FullText/323503

299. Dreborg ES, Frew A, Frew A. Position paper: Allergen standardization and skin tests. The European Academy of Allergology and Clinical Immunology. Allergy [Internet].. 1993;48(14 Suppl):48–82. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8342740

300. Hoffmann-Sommergruber K, de las Vecillas L, Dramburg S, Hilger C, Matricardi P, Santos A. EAACI Molecular Allergology User´s Guide. John Wiley & Sons Ltd, editor. Zurich: European Academy of Allergy and Clinical Immunology (EAACI); 2022.

301. Garriga T, Guilarte M, Luengo O, Guillén M, Labrador-Horrillo M, Fadeeva T, et al. Frozen fruit skin prick test for the diagnosis of fruit allergy. Asian Pacific J allergy Immunol. 2010 Dec;28(4):275–8.

Available from: http://www.ncbi.nlm.nih.gov/pubmed/21337912

302. Pokoj S, Lauer I, Fötisch K, Himly M, Mari A, Enrique E, et al. Pichia pastoris is superior to E. coli for the production of recombinant allergenic non-specific lipid-transfer proteins. Protein Expr Purif 2010 Jan;69(1):68–75. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1046592809002149

303. McWilliam H, Li W, Uludag M, Squizzato S, Park YM, Buso N, et al. Analysis Tool Web Services from the EMBL-EBI. Nucleic Acids Res. 2013 Jul 1;41(W1):W597–600. Available from: http://academic.oup.com/nar/article/41/W1/W597/1095852/Analysis-Tool-Web-Services-from-the-EMBLEBI

304. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol. 2011 Jan 11;7(1):539. Available from: https://onlinelibrary.wiley.com/doi/10.1038/msb.2011.75

305. Ciccarelli FD, Doerks T, von Mering C, Creevey CJ, Snel B, Bork P. Toward Automatic Reconstruction of a Highly Resolved Tree of Life. Science. 2006 Mar 3;311(5765):1283–7. Available from: https://www.science.org/doi/10.1126/science.1123061

306. Choi AM, Lee SB, Cho SH, Hwang I, Hur C-G, Suh MC. Isolation and characterization of multiple abundant lipid transfer protein isoforms in developing sesame (Sesamum indicum L.) seeds. Plant Physiol Biochem. 2008 Feb;46(2):127–39. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0981942807002033

307. Hartz C, del Mar San Miguel-Moncín M, Cisteró-Bahíma A, Fötisch K, Metzner KJ, Fortunato D, et al. Molecular characterisation of Lac s 1, the major allergen from lettuce (Lactuca sativa). Mol Immunol. 2007 Apr;44(11):2820–30. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0161589007000430

308. Röder M, Vieths S, Holzhauser T. Sensitive and specific detection of potentially allergenic almond (Prunus dulcis) in complex food matrices by Taqman® real-time polymerase chain reaction in comparison to commercially available protein-based enzyme-linked immunosorbent assay. Anal Chim Acta. 2011 Jan;685(1):74–83. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0003267010014340

309. Di Fraia M, Arasi S, Castelli S, Dramburg S, Potapova E, Villalta D, et al. A new molecular multiplex IgE assay for the diagnosis of pollen allergy in Mediterranean countries: A validation study. Clin Exp Allergy . 2019 Mar;49(3):341–9. Available from: http://doi.wiley.com/10.1111/cea.13264

310. Di Veroli GY, Fornari C, Goldlust I, Mills G, Koh SB, Bramhall JL, et al. An automated fitting procedure and software for dose-response curves with multiphasic features. Sci Rep. 2015 Dec 1;5(1):14701. Available from: http://www.nature.com/articles/srep14701

311. Casas ML, Esteban Á, González-Muñoz M, Labrador-Horrillo M, Pascal M, Teniente-Serra A. Proyecto VALIDA: Validation of ALlergy In vitro Diagnostics Assays (Herramientas y recomendaciones para la valoración de las pruebas in vitro en el diagnóstico de la alergia). Adv Lab Med. 2020 Jul 27;1(4):20200022. Available from: https://doi.org/10.1515/almed-2020-0022

312. Muñoz-Cano R, Pascal M, Bartra J, Picado C, Valero A, Kim D-K, et al. Distinct transcriptome profiles differentiate nonsteroidal anti-inflammatory drug–dependent from nonsteroidal anti-inflammatory

drug–independent food-induced anaphylaxis. J Allergy Clin Immunol. 2016 Jan;137(1):137–46. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674915007988

313. Bogas G, Muñoz-Cano R, Mayorga C, Casas R, Bartra J, Pérez N, et al. Phenotyping peach-allergic patients sensitized to lipid transfer protein and analysing severity biomarkers. Allergy. 2020 Dec 6;75(12):3228–36. Available from: https://onlinelibrary.wiley.com/doi/10.1111/all.14447

314. Asero R, Mistrello G, Roncarolo D, Amato S. Relationship between peach lipid transfer protein specific IgE levels and hypersensitivity to non-Rosaceae vegetable foods in patients allergic to lipid transfer protein. Ann Allergy, Asthma Imunol.. 2004 Feb;92(2):268–72. Available from: http://dx.doi.org/10.1016/S1081-1206(10)61559-1

315. Tordesillas L, Pacios LF, Palacin A, Quirce S, Armentia A, Barber D, et al. Molecular basis of allergen cross-reactivity: Non-specific lipid transfer proteins from wheat flour and peach fruit as models. Mol Immunol. 2009 Dec;47(2–3):534–40. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0161589009006270

316. Skypala IJ. Food-Induced Anaphylaxis: Role of Hidden Allergens and Cofactors. Front Immunol. 2019 Apr 3;10. Available from: https://www.frontiersin.org/article/10.3389/fimmu.2019.00673/full

317. Asero R, Brusca I, Cecchi L, Pignatti P, Pravettoni V, Scala E, et al. Why lipid transfer protein allergy is not a pollen-food syndrome: novel data and literature review. Eur Ann Allergy Clin Immunol. 2022 Aug;54(05):198. Available from: http://www.eurannallergyimm.com/cont/journals-articles/1134/volume-lipid-transfer-protein-allergy-pollenfood.asp

318. Rancé F, Juchet A, Brémont F, Dutau G. Correlations between skin prick tests using commercial extracts and fresh foods, specific IgE, and food challenges. Allergy . 1997 Oct;52(10):1031–5. Available from: https://onlinelibrary.wiley.com/doi/10.1111/j.1398-9995.1997.tb02427.x

319. Valenta R, Karaulov A, Niederberger V, Zhernov Y, Elisyutina O, Campana R, et al. Allergen Extracts for In Vivo Diagnosis and Treatment of Allergy: Is There a Future? J Allergy Clin Immunol Pract. 2018 Nov;6(6):1845-1855.e2. Available from: https://linkinghub.elsevier.com/retrieve/pii/S2213219818305695

320. Finkina EI, Melnikova DN, Bogdanov I V., Matveevskaya NS, Ignatova AA, Toropygin IY, et al. Impact of Different Lipid Ligands on the Stability and IgE-Binding Capacity of the Lentil Allergen Len c 3. Biomolecules. 2020 Dec 13;10(12):1668. Available from: https://www.mdpi.com/2218-273X/10/12/1668

321. Garrido-Fernández S, Garcia BE, Sanz ML, Ariz S, Tabar A. The role of lipid transfer proteins in the almond tree and almond fruit as contact and airborne allergens. J Investig Allergol Clin Immunol. 2009;19(1):61–3. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19274932

322. Sokolova A, Bartolomé B, Sampaio G, Mascarenhas MI, Carreiro H. Severe multiple plant food allergy with LTP identification as melon allergen. Clin Transl Allergy. 2011 Dec 12;1(S1):P1. Available from: https://ctajournal.biomedcentral.com/articles/10.1186/2045-7022-1-S1-P1

323. Randomized Trial of Peanut Consumption in Infants at Risk for Peanut Allergy. N Engl J Med. 2016 Jul 28;375(4):398–398. Available from: http://www.nejm.org/doi/10.1056/NEJMx150044

324. Betancor D, Gomez-Lopez A, Villalobos-Vilda C, Nuñez-Borque E, Fernández-Bravo S, De las Heras

Gozalo M, et al. LTP Allergy Follow-Up Study: Development of Allergy to New Plant Foods 10 Years Later. Nutrients. 2021 Jun 24;13(7):2165. Available from: https://www.mdpi.com/2072-6643/13/7/2165

325. Balsells-Vives S, Flügge U, Brix B, Weimann Y, Peralta T, San Bartolomém C, et al. Improving In Vitro Detection of Sensitization to Lipid Transfer Proteins: A New Molecular Multiplex IgE Assay. Mol Nutr Food Res. 2023 Jul 2;67(14). Available from: https://onlinelibrary.wiley.com/doi/10.1002/mnfr.202200906

326. Borges J-P, Barre A, Culerrier R, Granier C, Didier A, Rougé P. Lipid transfer proteins from Rosaceae fruits share consensus epitopes responsible for their IgE-binding cross-reactivity. Biochem Biophys Res Commun. 2008 Jan;365(4):685–90. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0006291X07024175

327. Scheurer S, Pastorello EA, Wangorsch A, Kästner M, Haustein D, Vieths S. Recombinant allergens Pru av 1 and Pru av 4 and a newly identified lipid transfer protein in the in vitro diagnosis of cherry allergy. J Allergy Clin Immunol. 2001 Apr;107(4):724–31. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674901491251

328. Scheurer S, Lauer I, Foetisch K, Moncin MSM, Retzek M, Hartz C, et al. Strong allergenicity of Pru av 3, the lipid transfer protein from cherry, is related to high stability against thermal processing and digestion. J Allergy Clin Immunol. 2004 Oct;114(4):900–7. Available from: https://linkinghub.elsevier.com/retrieve/pii/S009167490401677X

Annexes

frontiers | Frontiers in Allergy

BRIEF RESEARCH REPORT
published: 05 April 2022
doi: 10.3389/falgy.2022.868267

Low Levels Matter: Clinical Relevance of Low Pru p 3 sIgE in Patients With Peach Allergy

Sara Balsells-Vives[1], *Clara San Bartolomé*[1,2], *Rocío Casas-Saucedo*[1,3,4], *María Ruano-Zaragoza*[1,3,4], *Josefina Rius*[2], *Maria Torradeflot*[2], *Joan Bartra*[1,3,4]*, *Rosa Munoz-Cano*[1,3,4†] *and Mariona Pascal*[1,2,4†]

[1] *Clinical and Experimental Respiratory Immunoallergy (IRCE), Institut D'Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS), Barcelona, Spain,* [2] *Department of Immunology, Centre de Diagnòstic Biomedic (CDB), Hospital Clinic de Barcelona, Barcelona, Spain,* [3] *Allergy Section, Department of Pneumology, Institut Clinic Respiratori (ICR), Hospital Clinic de Barcelona, Barcelona, Spain,* [4] *Spanish Research Network on Allergy (ARADyAL, Red Nacional de Alergia–Asma, Reacciones Adversas y Alérgicas-), Instituto de Salud Carlos III, Madrid, Spain*

OPEN ACCESS

Edited by:
Joana Vitte, INSERM UMRUA11 Institut Desbrest d'Épidémiologie et de Santé Publique (IDESP), France

Reviewed by:
Mayte Villalba, Complutense University of Madrid, Spain
Domingo Barber, Universidad CEU San Pablo, Spain

***Correspondence:**
Joan Bartra
jbartra@clinic.cat

[†] *These authors have contributed equally to this work*

Specialty section:
This article was submitted to Allergens, a section of the journal Frontiers in Allergy

Received: *02 February 2022*
Accepted: *28 February 2022*
Published: *05 April 2022*

Citation:
Balsells-Vives S, San Bartolomé C, Casas-Saucedo R, Ruano-Zaragoza M, Rius J, Torradeflot M, Bartra J, Munoz-Cano R and Pascal M (2022) Low Levels Matter: Clinical Relevance of Low Pru p 3 sIgE in Patients With Peach Allergy. Front. Allergy 3:868267. doi: 10.3389/falgy.2022.868267

Many clinical lab settings still use 0.35 KU_A/L as the cut-off for serum specific-IgE (sIgE) immunoassays, while the detection limit is 0.1 KU_A/L. The clinical relevance of -low-level sIgE (0.1–0.35 KU_A/L) remains controversial. Pru p 3 sIgE is considered to be the main routine tool for assessing lipid transfer protein (LTP) sensitization. We aimed to evaluate the clinical relevance of Pru p 3 sIgE low levels in a population diagnosed with LTP allergy. Adults diagnosed with LTP allergy and Pru p 3 sIgE $\geq$ 0.1 KUA/L between 2012 and 2019 were included. Clinical data were reviewed. nPru p 3 basophil activation test (BAT) was performed and basophil reactivity (BR) and sensitivity (BS) correlated with the peach allergy symptoms. Pru p 3 sIgE from 496 subjects was recorded, 114 (23.0%) between 0.1 and 0.34 KU_A/L (grLOW), the rest $\geq$ 0.35 KU_A/L (grB). A total of 44.7% in grLOW and 59.9% in grB were allergic. Urticaria was more frequent in grLOW. In grLOW, Pru p 3 sIgE was higher in patients with local compared with systemic symptoms. In grB, Pru p 3 sIgE was higher in allergic patients. Pru p 3/Total IgE ratios were higher in allergic vs. tolerant in both groups. In BAT, BR was similar in both groups. In grLOW, it was higher on allergic compared with tolerant ($p = 0.0286$), and on those having systemic vs. local symptoms ($p = 0.0286$). BS showed no significant difference between groups. Patients with low levels represent a non-negligible fraction and around 45% are peach allergic. BAT showed functional sIgE in them. Pru p 3 sensitizations should be carefully evaluated even when sIgE levels are low.

Keywords: serum specific-IgE, low levels, lipid transfer protein, clinical relevance, BAT

INTRODUCTION

Allergen-specific IgE (sIgE) levels cannot be used as individual predictors of clinical reactivity or severity, although high-sIgE concentrations correlate with increased risk of reactions (1). The importance of establishing sIgE cut-offs to provide clinical relevance in the assessment of food allergy has been extensively reported (2–4). The cut-off for the most common immunoassays used to quantify serum sIgE (e.g., ImmunoCAP® ThermoFisher Scientific, Uppsala), has traditionally been 0.35 KU_A/L; and it is still used in many clinical lab settings, despite the reports showing

that the cut-off may differ depending upon the factors, such as the allergenic source and patient age (3). Indeed, the technical detection limit for the *in vitro* singleplex fluorescence enzyme-immunoassay ImmunoCAP® (ThermoFisher Scientific) is 0.10 KU_A/L. Little evidence has been reported on the clinical relevance of sIgE levels between 0.1 and 0.35 KU_A/L and it is a matter of discussion in the field.

Lipid transfer proteins (LTPs) are widely cross-reacting panallergens related to complex clinical profiles regarding severity and food triggers (5, 6). LTPs are the most important cause of plant food allergy in adults and children in the Mediterranean, but indeed emerging in other areas (6). Pru p 3, the peach LTP, is considered to be the prototype protein, and routinely used as the main marker to assess LTPs sensitization (7). High Pru p 3 sIgE has been related with systemic reactions and a higher prevalence of hazelnut, peanut, and walnut allergy (4, 8). Pastorello et al. established Pru p 3 2.69 KU_A/L to discriminate patients at risk of reactions (4), but other authors have found overlapped values between allergic and tolerant (9). Nevertheless, Pru p 3 allergic patients have also been reported with sIgE levels < 0.35 KU_A/L (10). We aimed to evaluate the clinical relevance of low levels of Pru p 3 sIgE by ImmunoCAP®.

MATERIALS AND METHODS

Study Population

Adult patients evaluated in the Allergy Section of Hospital Clinic (Barcelona, Spain) between 2012 and 2019 with an LTPs food allergy and Pru p 3 sIgE $\geq$ 0.1 KU_A/L were selected. Serum samples obtained following routine practice were analyzed in the Immunology Department of the same hospital. Pru p 3 sIgE (by ImmunoCAP®, Thermo Fisher Scientific) is measured per protocol in all LTPs allergic patients regardless of the presence of symptoms with peach. Sensitization to other plant food allergens was analyzed by microarray ImmunoCAP® ISAC (Thermo Fisher Scientific.) Patients sensitized to other panallergens (PR-10; TLP; Profilin) were excluded. The study was approved by the local ethic committee (HCB/2020/0373).

Clinical Characterization

Demographical and epidemiological data were retrospectively recorded from clinical history. Peach allergy symptoms were classified as: local (gastrointestinal symptoms–GI-, Oral Allergy Syndrome–OAS-, and contact urticaria–CU-) and systemic (generalized urticaria and/or angioedema–U/AE-, anaphylaxis-AN-). Peach tolerance (–TOL-) and peach avoidance (-AV-; due to medical advice, fear, or dislike) were also recorded and also the involvement of cofactors, including exercise, alcohol, non-steroidal anti-inflammatory drugs (NSAIDs), and/or menstruation.

Basophil Activation Test

Pru p 3 basophil activation test (BAT) was performed in some patients to assess sIgE functionality. Briefly, after the patient informed consent, 10 ml of heparinized peripheral blood was obtained and immediately taken to the laboratory for BAT using the Flow2CAST™ kit (Bühlmann Laboratories AG, Switzerland) and following the manufacturer's procedures. Purified Pru p 3 (1 mg/ml, Bial Aristegui, Bilbao, Spain) was tested at 25, 12.5, 5, and 2.5 ng/ml final concentrations. Basophils were identified by flow cytometry (FACS-Canto II, BD Biosciences, Germany). A minimum of 500 basophils was gated and those CD63+ were defined as activated ($\geq$15% was considered a positive test). Basophil reactivity (BR, i.e., number of basophils responding to a stimulus) was calculated as the CD63+ expression post-stimulus minus basal CD63+ expression, represented as % CD63+. Basophil sensitivity (BS) is calculated as CD-sens, i.e., inversion of EC50 (concentration inducing 50% of maximum response) $\times$ 100 (11).

Statistical Analysis

Pru p 3 sIgE centralization and dispersion measurements were calculated considering a quantitative and asymmetric distribution. Free distribution was considered in our analysis so non-parametric tests were used to verify heterogenicity between our variables. Quantitative data were compared using the Mann Whitney *U*-test or the Kruskal–Wallis test. Qualitative data were compared using the chi-squared test and Fisher's exact test for a small sample size. *P* values lower than 0.05 were considered statistically significant. The GraphPad Prism 8.0.2 software (Inc., CA, USA) was used for the statistical analysis.

RESULTS

Groups Characterization

A total of 496 subjects with Pru p 3 sIgE $\geq$ 0.1 KU_A/L were recorded between 2012 and 2019. A total of 284 (57.3%) subjects were women, median [Interquartile range, IQR] age of 42 (17–92) years. Of 496 subjects, 114 (23.0%) had Pru p 3 sIgE between 0.1

TABLE 1 | Clinical picture.

	grLOW $n = 114$	grB $n = 328$	*P* value
Peach allergic	44.7%	59.9%	*ns*
Peach tolerant	20.2%	25.9%	*ns*
Peach avoidance	35.1%	14.1%	****
Peach-related symptoms			
Local	50.4%	55.1%	*ns*
CU	21.9%	25.1%	*ns*
OAS	23.7%	24.6%	*ns*
GI	4.4%	5.5%	*ns*
Systemic	22.8%	25.4%	*ns*
U/AE	21.2%	17.5%	*
AN	1.9%	8.1%	*ns*

*Clinical relevance frequencies among studied patients. grLOW, Pru p 3 sIgE from 0.1 to 0.34 KU_A/L; grB, Pru p 3 sIgE >0.35 KU_A/L; CU, contact urticaria; OAS, oral allergy syndrome; GI, gastrointestinal symptoms; U/AE, generalized urticaria or angioedema; AN, anaphylaxis. Chi-squared test and Fisher's exact test for small simple size were used to test p (*0.01 to 0.05, ****< 0.0001 and ns, non-significant). Patients avoiding peach were not included on the symptom statistical analysis because tolerance or allergy could not be guaranteed.*

TABLE 2 | Pru p 3 sIgE values distribution.

classification	Peach sIgE median [IQR] KU_A/L	Pru p 3 sIgE median [IQR] KU_A/L	Pru p 3/Peach sIgE median [IQR]	Pru p 3/Total sIgE median [IQR]	Pru p 3 sIgE on CCD+ median [IQR] KU_A/L
grLOW [0.1–0.35]	0.20 [0.14–0.28]	0.19 [0.07–0.26]	1.16 [0.92–1.46]	0.00 [0.00–0.01]	0.29 [0.22–0.31] *ns*
grB [≥0.35]	3.73 [1.35–10.28]	3.37 [1.16–9.67]	1.19 [1.04–1.38]	0.03 [0.01–0.07]	16.30 [4.58–20.85] *p**
	*p****	*p****	*ns*	*p****	

*IgE values distribution among groups: grLOW (Pru p 3 sIgE from 0.1 to 0.34 KU_A/L) and grB (Pru p 3 sIgE >0.35 KU_A/L). Pru p 3, peach and Pru p 3/Peach ratio sIgE median and IQR (interquartile range) results are included as well as CCD+ Pru p 3 sIgE median [IQR]. Differences between grLOW/grB and between CCD+/CCD- Pru p 3 sIgE in grLOW/grB were statistically evaluated with the Mann–Whitney–test (*0.01 to 0.05, ***0.0001 to 0.001, ns, non-significant).*

and 0.34 KU_A/L (grLOW = group low levels) and 382 (77.0%) ≥ 0.35 KU_A/L (grB = group high levels).

44.7% of patients of grLOW and 59.9% in grB were allergic ($p > 0.05$), with similar peach-related symptoms and a higher presence of local symptoms. However, U/AE was more frequent in grLOW ($p = 0.020$). Peach avoidance was statistically superior in grLOW ($p < 0.0001$) (**Table 1**).

Pru p 3 sIgE Levels and Symptoms

Peach sIgE values were higher in grB, as well as Pru p 3/total IgE ratio ($p < 0.05$), whereas no differences were observed in Pru p 3/Peach sIgE (ratio) between groups (**Table 2**). In grLOW (**Figure 1A**), Pru p 3 sIgE was higher in patients with local compared to systemic symptoms ($p = 0.0385$). In grB (**Figure 1B**), Pru p 3 sIgE was higher in allergic compared to tolerant ($p = 0.0009$). The medians from the ratios Pru p 3/peach sIgE were superior to 1 for either grLOW or grB. Moreover, when classifying patients according to their clinical symptoms, no statistically significant differences were found. Pru p 3/Total IgE ratios were lower than 1% in grLOW, unlike grB. In both groups, these ratios were statistically higher ($p < 0.0001$) in allergic compared to tolerant (**Supplementary Table 1**).

Co-sensitization

Co-sensitization to other LTPs was analyzed in 70 patients of grLOW and 318 of grB (**Supplementary Figure 1**; **Supplementary Table 2**). In grLOW, co-sensitization was statistically less frequent (64.3 vs. 95.9%). Mal d 3, Ara h 9, and Jug r 3 were the most frequent ones, and Tri a 14 the rarest in both groups.

Cross-reactive carbohydrate determinants (CCD) reactive sIgE may cause false-positive results in Pru p 3 measurements by binding the test cellulose matrix (12). CCD sensitization data were available for 80 (70.2%) patients of grLOW and 226 (59.2%) of grB. In grLOW, of the 7 CCD+ (8.7%), 5 avoided eating peach, 1 tolerated and 1 referred local symptoms. In grB, of the 19 CCD+ (8.4%), four avoided the ingestion of peach, three tolerated, six had local, and six systemic symptoms (two anaphylaxis). Tolerant and allergic frequencies were not statistically different between CCD+ and negative (**Table 2**). In grB, were found significant differences on sIgE to Pru p 3 from CCD+ compared with CCD–, being higher on CCD+.

Basophil Activation Test Results

nPru p 3 BAT was performed on 12 patients per group as previously reported (10). All in grB were BAT+, being 3 (25%) tolerant and 9 (75%) allergic (5 local/4 systemic reactions). In grLOW (**Table 3**), 7 (58.3%) were BAT+: 6 (85.7%) allergic (2 local/4 systemic reactions) and 1 (14.3%) avoided peach. In BAT-: 2 (40%) were tolerant and 3 (60%) allergic (2 local/1 systemic reactions). The median [IQR] for Pru p 3 sIgE for grLOW was 0.26 [0.10–0.28] KU_A/L. The ratio Pru p 3/peach sIgE median was 0.99 [0.79–1.09]. In addition, from these BAT- patients were 0.21 [0.18–0.23] (Pru p 3 sIgE) and 0.98 [0.97–0.99] (Pru p 3/peach sIgE ratio). BAT reactivity (BR, %CD63+ basophils) was not statistically different between groups (BR median: 17.8% grLOW/ 27.3% grB), neither when only allergic patients of each group were compared. In grLOW, BR was significantly higher on allergic individuals vs. tolerant ones ($p = 0.0286$), and on those having systemic symptoms vs. local ($p = 0.0286$). No statistically significant differences in basophil sensitivity were found between groups, although being higher in grLOW (CD-sens median: 819.0 grLOW/ 75.4 grB).

CONCLUSION

In summary, the ratio Pru p 3/Peach was similar in both groups and superior to 1, which would confirm a sensitization due to Pru p 3 on our population (13). About 45% of our patients of grLOW are allergic, highlighting the importance of considering Pru p 3 sIgE > 0.1 as potentially clinically relevant, despite 0.35 has traditionally been used as the cut-off, BAT reactivity (similar in both groups) demonstrated the presence of functional sIgE in patients with low levels.

Besides the theory reported by Kleine-Tebbe and Jakob (14) exposing that a 0.01 or greater ratio of specific IgE to total IgE, translated as a fraction of 1% of bound total IgE, is enough for basophil half-maximal activation, we observe basophil activation with a lower percentage. Thus, reliable quantitative detection of sIgE and the ratios analysis of specific and total IgE on these patients is relevant for an accurate diagnosis (13, 15).

A definite answer for the reason why Pru p 3 sIgE levels are higher on patients with local symptoms compared with those with systemic is not clear. Little is known about the real correlation between LTP sIgE levels and symptoms severity,

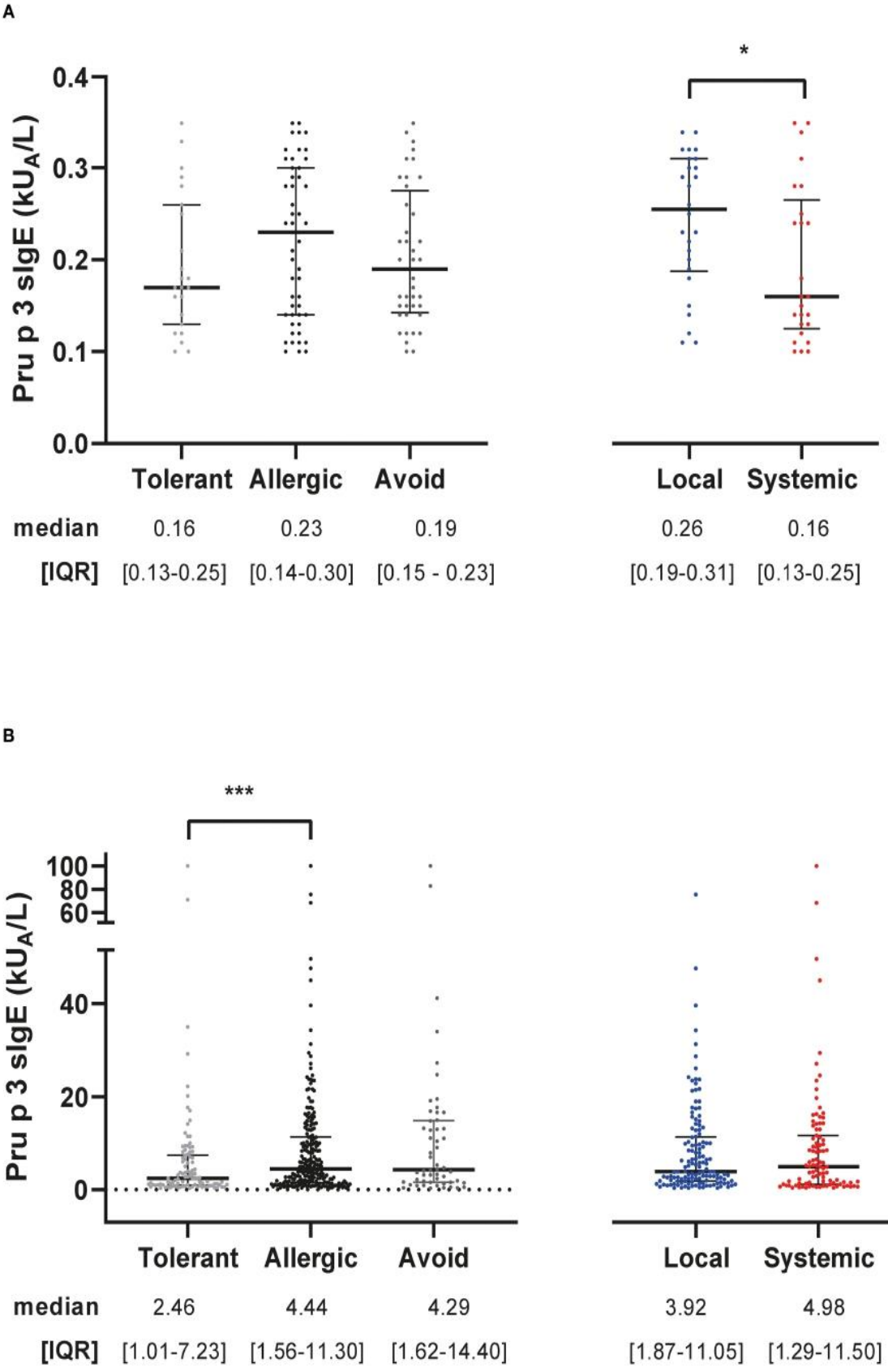


FIGURE 1 | Levels of Pru p 3 sIgE per group. Pru p 3 sIgE distribution, median, and IQR (interquartile range) values from grLOW **(A)** and grB **(B)** according to symptom classification (tolerance vs. allergy, local vs. systemic). The Mann–Whitney test was used to test *p* (*0.01 to 0.05, ***0.0001 to 0.001).

TABLE 3 | Characteristics and BAT results of the allergic patients from grLOW.

	BAT	EC50	% CD63+				Symptoms	Pru p 3 sIgE (KU_A/L)	Ratio Pru p 3/peach sIgE
			2.5	5	12.5	25			
			ng/mL Pru p 3						
P1	-	3.29	0.60	0.40	0.20	1.40	TOL	0.26	NA
P2	-	3.71	0.60	0.50	0.00	0.20	TOL	0.20	20
P3	-	32.48	0.20	0.00	0.00	0.00	CU, OAS	0.25	0.96
P4	-	-	0.00	0.00	0.00	0.00	OAS	0.30	0.73
P5	+	0.04	16.60	26.80	31.90	37.20	OAS	0.34	0.97
P6	+	0.13	16.70	2.40	14.80	0.20	GID	0.22	NA
P7	-	-	0.00	0.00	0.00	0.00	AN	0.16	1.00
P8	+	0.02	41.70	25.70	16.90	84.30	U/AE	0.12	0.52
P9	+	0.00	57.80	66.20	62.40	55.90	U/AE (exercise)	0.12	1.09
P10	+	0.09	9.20	17.40	19.60	25.20	U/AE	0.26	1.18
P11	+	0.00	54.10	48.40	59.40	60.40	SHOCK	0.29	1.07
P12	+	0.22	12.80	15.70	12.10	0.70	AVOID	0.28	0.43

Characteristics and BAT results of the grLOW patients (n = 12) tested under a Pru p 3 stimulation. %CD63+, % of activated basophils; EC50, the concentration inducing 50% of maximum response. Tolerance (TOL), local (CU, contact urticaria; OAS, oral allergy syndrome; GI, gastrointestinal symptoms) and systemic symptoms (U/AE, generalized urticaria or angioedema; AN, anaphylaxis). In parentheses the presence of cofactors is detailed. Pru p 3 and Pru p 3/peach sIgE are included. A ratio ≥1 indicates a greater proportion of sIgE Pru p 3 compared to peach sIgE. NA, not available.

and conflicting results have been published (9, 16, 17). It has been reported that high Pru p 3 sIgE concentrations correlate with an increased risk of reactions (18). Ciprandi et al. (19) described Pru p 3 sIgE levels variation as an age-dependent event. They reported an increase from infancy to young adulthood (highest from 21 to 30 years) that posteriorly decreased. Also, values have been inversely related with an early onset peach allergy (16).

Moreover, it has been described that mono-sensitization to LTP correlates with a more severe clinical reactivity (20) which could be explained by the fact that IgE receptors are mostly occupied by LTP sIgE, which would induce a more efficient cross-linking of the FcεRI and effector cell activation, but not actually related to sIgE levels.

In the previous studies from our group and collaborators (21–23), a trend to lower levels of sIgE has been observed in those groups with severe symptoms compared with those with mild symptoms. From our point of view, we think that this might be explained by the differential affinity of sIgE to the antigen and differential efficiency on the cross-linking in effector cells in which the ratio of sIgE to total IgE of 0.01 is enough for half-maximal activation of the effector cells.

CCD sensitization was similarly distributed in both the groups, ruling out that low levels detected were merely artifacts of CCD interaction not deserving clinical consideration.

Finally, a lower co-sensitization to other LTPs was found on grLOW although sensitization profiles (peanut, walnut, and apple) were similar in both the groups. This study has some limitations, besides being retrospective. Mainly, oral food challenges could not be done to confirm food diagnosis due to logistic limitations; and the fact that avoidance may have caused sIgE concentrations to decrease in patients with a history of a severe reaction.

In conclusion, our data show that, regardless of patients with low Pru p 3 sIgE may represent a minority in our daily practice, this sensitization can be clinically relevant, with up to 20% of systemic reactions. Therefore, Pru p 3 sensitizations should be carefully evaluated even when sIgE levels are low.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospital Clínic de Barcelona Ethical Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SB-V has contributed to the acquisition, analysis, and interpretation of data, as well as drafting the manuscript for publication. RC-S and MR-Z have contributed to the acquisition of clinical data. CS, JR, and MT contributed to the performance of laboratory tests. JB, RM, and MP have contributed to the design and interpretation of the data and critically revised. All authors have participated

sufficiently in the work, approved the final version, agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/falgy.2022.868267/full#supplementary-material

REFERENCES

1. Frischmeyer-Guerrerio PA, Rasooly M, Gu W, Levin S, Jhamnani RD, Milner JD, et al. IgE testing can predict food allergy status in patients with moderate to severe atopic dermatitis. *Ann Allergy Asthma Immunol.* (2019) 122:393–400.e2. doi: 10.1016/j.anai.2019.01.001
2. Nilsson SF, Lilja G, Järnbert-Pettersson H, Alm J. Relevance of low specific IgE levels to egg, milk and peanut in infancy. *Clin Exp Allergy.* (2019) 49:308–16. doi: 10.1111/cea.13273
3. Cuomo B, Indirli GC, Bianchi A, Arasi S, Caimmi D, Dondi A, et al. Specific IgE and skin prick tests to diagnose allergy to fresh and baked cow's milk according to age: a systematic review. *Ital J Pediatr.* (2017) 43:93. doi: 10.1186/s13052-017-0410-8
4. Pastorello EA, Farioli L, Pravettoni V, Scibilia J, Mascheri A, Borgonovo L, et al. Pru p 3-sensitised Italian Peach-allergic patients are less likely to develop severe symptoms when also presenting IgE antibodies to Pru p 1 and Pru p 4. *Int Arch Allergy Immunol.* (2011) 156:362–72. doi: 10.1159/000324440
5. Skypala IJ, Bartra J, Ebo DG, Antje Faber M, Fernández-Rivas M, Gomez F, et al. The diagnosis and management of allergic reactions in patients sensitized to non-specific lipid transfer proteins. *Allergy.* (2021) 76:2433–46. doi: 10.1111/all.14797
6. Skypala IJ, Asero R, Barber D, Cecchi L, Diaz Perales A, Hoffmann-Sommergruber K, et al. Non-specific lipid-transfer proteins: allergen structure and function, cross-reactivity, sensitization, and epidemiology. *Clin Transl Allergy.* (2021) 11:e12010. doi: 10.1002/clt2.12010
7. Asero R, Piantanida M, Pinter E, Pravettoni V. The clinical relevance of lipid transfer protein. *Clin Exp Allergy.* (2018) 48:6–12. doi: 10.1111/cea.13053
8. Rossi RE, Monasterolo G, Canonica GW, Passalacqua G. Systemic reactions to peach are associated with high levels of specific IgE to Pru p 3. *Allergy.* (2009) 64:1795–6. doi: 10.1111/j.1398-9995.2009.02133.x
9. Asero R, Arena A, Cecchi L, Conte M, Crivellaro M, Emiliani F, et al. Are IgE levels to foods other than rosaceae predictive of allergy in lipid transfer protein-hypersensitive patients? *Int Arch Allergy Immunol.* (2011) 155:149–54. doi: 10.1159/000318864
10. Decuyper II, Pascal M, Van Gasse AL, Mertens C, Díaz-Perales A, Araujo G, et al. Performance of basophil activation test and specific IgG4 as diagnostic tools in nonspecific lipid transfer protein allergy: Antwerp-Barcelona comparison. *Allergy.* (2019) 75:616–24. doi: 10.1111/all.14040
11. Hoffmann HJ, Santos AF, Mayorga C, Nopp A, Eberlein B, Ferrer M, et al. The clinical utility of basophil activation testing in diagnosis and monitoring of allergic disease. *Allergy.* (2015) 70:1393–405. doi: 10.1111/all.12698
12. Hemmer W, Altmann F, Holzweber F, Gruber C, Wantke F, Wöhrl S. ImmunoCAP cellulose displays cross-reactive carbohydrate determinant (CCD) epitopes and can cause false-positive test results in patients with high anti-CCD IgE antibody levels. *J Allergy Clin Immunol.* (2018) 141:372–81.e3. doi: 10.1016/j.jaci.2017.04.028
13. Pascal M, Moreno C, Dávila I, Tabar AI, Bartra J, Labrador M, et al. Integration of *in vitro* allergy test results and ratio analysis for the diagnosis and treatment of allergic patients (INTEGRA). *Clin Transl Allergy.* (2021) 11:e12052. doi: 10.1002/clt2.12052
14. Kleine-Tebbe J, Jakob T. Molecular allergy diagnostics using IgE singleplex determinations: methodological and practical considerations for use in clinical routine. *Allergo J Int.* (2015) 24:185–97. doi: 10.1007/s40629-015-0067-z
15. Casas ML, Esteban Á, González-Muñoz M, Labrador-Horrillo M, Pascal M, Teniente-Serra A. Proyecto VALIDA: Validation of ALlergy *In vitro* Diagnostics Assays (Herramientas y recomendaciones para la valoración de las pruebas *in vitro* en el diagnóstico de la alergia). *Adv Lab Med.* (2020) 1:20200022. doi: 10.1515/almed-2020-0022
16. Pastorello EA, Farioli L, Stafylaraki C, Mascheri A, Scibilia J, Pravettoni V, et al. Anti-rPru p 3 IgE levels are inversely related to the age at onset of peach-induced severe symptoms reported by peach-allergic adults. *Int Arch Allergy Immunol.* (2013) 162:45–9. doi: 10.1159/000350467
17. Pascal M, Muñoz-Cano R, Reina Z, Palacín A, Vilella R, Picado C, et al. Lipid transfer protein syndrome: clinical pattern, cofactor effect and profile of molecular sensitization to plant-foods and pollens. *Clin Exp Allergy.* (2012) 42:1529–39. doi: 10.1111/j.1365-2222.2012.04071.x
18. Kattan JD, Sicherer SH. Optimizing the diagnosis of food allergy. *Immunol Allergy Clin North Am.* (2015) 35:61–76. doi: 10.1016/j.iac.2014.09.009
19. Ciprandi G, De Amici M, Di Martino ML, Barocci F, Comite P. The impact of age on Pru p 3 IgE production in Italy. *Asia Pac Allergy.* (2017) 7:42. doi: 10.5415/apallergy.2017.7.1.42
20. Scheurer S, van Ree R, Vieths S. The role of lipid transfer proteins as food and pollen allergens outside the Mediterranean area. *Curr Allergy Asthma Rep.* (2021) 21:7. doi: 10.1007/s11882-020-00982-w
21. Pascal M, Muñoz-Cano R, Milà J, Sanz ML, Diaz-Perales A, Sánchez-López J, et al. Nonsteroidal anti-inflammatory drugs enhance IgE-mediated activation of human basophils in patients with food anaphylaxis dependent on and independent of nonsteroidal anti-inflammatory drugs. *Clin Exp Allergy.* (2016) 46:1111–9. doi: 10.1111/cea.12735
22. Muñoz-Cano R, Pascal M, Bartra J, Picado C, Valero A, Kim D-K, et al. Distinct transcriptome profiles differentiate nonsteroidal anti-inflammatory drug–dependent from nonsteroidal anti-inflammatory drug–independent food-induced anaphylaxis. *J Allergy Clin Immunol.* (2016) 137:137–46. doi: 10.1016/j.jaci.2015.05.042
23. Bogas G, Muñoz-Cano R, Mayorga C, Casas R, Bartra J, Pérez N, et al. Phenotyping peach-allergic patients sensitized to lipid transfer protein and analysing severity biomarkers. *Allergy.* (2020) 75:3228–36. doi: 10.1111/all.14447

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

RESEARCH ARTICLE

Molecular Nutrition & Food Research
www.mnf-journal.com

Improving In Vitro Detection of Sensitization to Lipid Transfer Proteins: A New Molecular Multiplex IgE Assay

*Balsells-Vives Sara, Flügge Ulrike, Brix Bettina, Weimann Yvonne, Peralta Teresa, San Bartolomé Clara, Araujo-Sánchez Giovanna, Casas-Saucedo Rocío, Torradeflot María, Lara Rocío, Munoz-Cano Rosa, Bartra Joan, Suer Waltraud, and Pascal Mariona**

Scope: LTP-syndrome is characterized by sensitization (IgE) to multiple non-specific lipid transfer proteins (nsLTPs) with a variable clinical outcome. The treatment is primarily based on offending food avoidance. However, the determination of Pru p 3-specific IgE is currently the main diagnostic tool to assess sensitization to nsLTPs. Herein, the study evaluates improvement of LTP-syndrome diagnosis and clinical management using a new IgE multiplex-immunoblot assay with a high diversity of food nsLTPs.

Methods and results: An EUROLINE-LTP strip with 28 recombinant nsLTPs from 18 allergenic sources is designed. In total the study investigates 38 patients with LTP-syndrome and compares results from the nsLTPs (LTP-strip) with the respective food extracts of Prick-by-prick (PbP) testing. The agreement exceeds 70% for most nsLTPs, e.g., Pru p 3 (100%), Mal d 3 (97%), Pru av 3 (89%), Pha v 3 isoforms (87%/84%), Ara h 9 (82%), Cor a 8 (82%), and Jug r 3 (82%). The functionality and allergenic relevance of nine recombinant nsLTPs are proven by Basophil activation testing (BAT).

Conclusions: The new IgE multiplex-immunoblot nsLTP assay shows a good diagnostic performance allowing culprit food assessment. Negative results from LTP-strip may indicate potentially tolerable foods, improving diet intervention and patients' quality of life.

1. Introduction

Non-specific lipid transfer proteins (nsLTPs) are the most frequent cause of plant food allergy in adults living in the Mediterranean area.[1,2] Current studies indicate a growing clinical relevance of nsLTP sensitization in Northern Europe[3,4] and also in certain areas of Australia[5] and China.[6–9] These proteins are highly conserved and widely distributed throughout the plant kingdom, representing an important family of panallergens.[4,10,11]

Their high degree of sequence and structural homology leads to cross reactivity amongst several nsLTPs[12] from taxonomically related or not plant foods and pollen, thus sensitization to multiple nsLTP is frequent not always having the same clinical relevance all of them if any[13] (the so called LTP syndrome[14]).

nsLTPs fold into a very compact structure enabling heat stability and resistance

B.-V. Sara, S. B. Clara, A.-S. Giovanna, C.-S. Rocío, M.-C. Rosa, B. Joan, P. Mariona
Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS)
Universitat de Barcelona
Barcelona 08036, Spain
E-mail: mpascal@clinic.cat

F. Ulrike, B. Bettina, W. Yvonne, S. Waltraud
EUROIMMUN AG A PerkinElmer Company
23560 Lübeck, Germany

P. Teresa
Department of Clinical Immunology and Rheumatology
Pontificia Universidad Católica de Chile
8331150 Santiago, Chile

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/mnfr.202200906

DOI: 10.1002/mnfr.202200906

S. B. Clara, T. María, L. Rocío, P. Mariona
Immunology Department
Centre de Diagnòstic Biomèdic
Hospital Clínic de Barcelona
Barcelona 08036, Spain

A.-S. Giovanna, C.-S. Rocío, M.-C. Rosa, B. Joan
Allergy Department
Institut Clinic Respiratori (ICR)
Hospital Clinic
Universitat de Barcelona
Barcelona 08036, Spain

C.-S. Rocío, M.-C. Rosa, B. Joan, P. Mariona
RETIC Asma
Reacciones Adversas a Fármacos y Alergia (ARADyAL) and RICORS Red de Enfermedades Inflamatorias (REI)
Instituto de Salud Carlos III
Madrid 28029, Spain

B. Joan, P. Mariona
Departament de Medicina
Facultat de Medicina i Ciències de la Salut
Campus Clínic
Universitat de Barcelona (UB)
c. Casanova, 143, Barcelona 08036, Spain

to proteolytic digestion.[15] These features are often associated with the induction of severe allergic symptoms. However, the clinical expression varies from asymptomatic to severe anaphylaxis or anaphylactic shock.[13] Indeed, in some cases, the allergic reactions are modulated or even only occur with the influence of cofactors (cofactor-enhanced food anaphylaxis).[13,16,17] The clinical impact of single or multiple sensitizations against nsLTPs is not fully described. Some reports indicate that patients sensitized to five or more nsLTPs are more likely to have systemic reactions.[2] Two main sensitization routes have been described on LTP sensitization: gastrointestinal tract[18] and respiratory tract.[19,20] However, it was also demonstrated in mice models that Pru p 3 is capable of inducing sensitization through skin.[21] And the fact that many patients present an allergic reaction the first time they eat peach suggests that skin exposure could be a principal sensitization route to peach.

Routine diagnosis of nsLTP food allergy is currently based on anamnesis followed by sensitization studies either in vivo (skin tests) and/or in vitro (determination of serum specific IgE-levels [sIgE] towards the offending foods, whole extracts and/or components) and in some cases, when there is not a convincing history, on the result of an oral food challenge tests. As Pru p 3, the peach LTP, has been considered the primary sensitizer in the Mediterranean area,[22] the in vitro measurement of sIgE to Pru p 3 is frequently used in allergy diagnostics as a marker of LTP sensitization. nsLTPs are highly cross-reactive with particular hierarchies of sensitization depending on the primary sensitization but without a universal pattern of cross-reactivity.[3]

Although Pru p 3 shares many IgE epitopes with other nsLTPs, inhibition studies show that it does not diminish all IgE reactivity for other food and plant LTPs. By implication, not all LTP sensitizations can be detected with Pru p 3.[23,24] This finding draws attention to the need of a broader diagnostic approach including the testing of multiple nsLTPs.

Similar to other food allergies, avoidance is currently the main management for this condition, although it may compromise the patient's nutritional status and quality of life.[25,26] Commercialized sublingual immunotherapy with Pru p 3 has shown promising results and demonstrated efficacy and safety on LTP allergy treatment.[27–29]

Today, one of the challenges for an accurate diagnosis and patient management of nsLTP-based food allergy is the somehow limited diversity of commercially available allergens to allow picturing a broad sensitization map of the patient and assessing its clinical relevance to guide diet avoidance and/or immunotherapy approaches.

This emphasizes the pressing need for further improvement of molecular diagnostic tools. Nowadays, multiplex immunoassays allow the screening for sensitization towards multiple components and/or extracts from different allergenic sources with low sample volumes at the same time. A new multiplex immunoblot assay has been specifically designed by EUROIMMUN AG (Lübeck, Germany) with various recombinant nsLTPs from the main plant-food allergenic source frequently encountered as offending foods in the Mediterranean area (EUROLINE-LTP). The aim of the present study is to develop a robust efficient in vitro tool to assess LTP sensitization in patients diagnosed with LTP syndrome in the allergy clinic. Hereby, we provide the analytical validation of this new assay.

2. Experimental Section

2.1. Recruitment and Characterization

This study included 38 patients diagnosed of LTP food allergy at the Allergy Department of Hospital Clinic (Barcelona, Spain) between June 2017 and February 2020 and 28 healthy non-allergic individuals as controls. Inclusion criteria were 1) the presence of food-allergic reactions with at least two different plant-foods, not taxonomically related, 2) sensitization to LTPs from peach (Pru p 3) and/or hazelnut (Cor a 8), and 3) no sensitization to any other plant-food allergen (profilin, PR-10, thaumatin-like protein Act d 2, and storage proteins) tested with ImmunoCAP ISAC and/or ImmunoCAP (ThermoFisher Scientific). Food-allergy diagnosis in these patients was established based on a clinical history of symptoms compatible with IgE food allergy (urticaria, oral allergy syndrome (OAS), gastrointestinal disorders (GID), including functional dyspepsia, crampy abdominal pain, nausea, vomiting and/or diarrhea, and/or anaphylaxis). The time period accepted for considering a potential relationship with food-allergic reaction was 2 h after food ingestion. Demographic and clinical data were recorded by experienced allergists from medical records and by patient interview in systematic questionnaire. The patients were cited for blood extraction and prick-by-prick testing. For basophil activation test, the subset patients tested were recontacted and a new blood sample obtained. The timeframe between test was not longer than 2 months. The Ethical Committee of Hospital Clinic approved the study (HCB/2016/0361) and all patients signed the informed consent.

2.2. Serum Specific IgE Detection to nsLTPs by EUROLINE-LTP

All serum samples from individuals included in the study cohort were tested with the EUROLINE-LTP strips (EUROIMMUN Medizinische Labordiagnostika AG [Lübeck, Germany]). The multiplex immunoblot assay was especially designed for this study and contained seven membrane chips with 28 immobilized recombinant nsLTP allergens from 18 allergenic sources: 17 plant food allergens (including selected isoforms) as well as a CCD sensitization marker; see allergen details on **Tables 1** and S1, Supporting Information and Figure S1, Supporting Information. Proteins were expressed and purified as previously described[30] with some modifications: the proteins contained an N-terminal 6x Histidine-tag and were isolated by immobilized metal-affinity chromatography and size-exclusion chromatography. The protein quality was assessed by SDS-PAGE and mass spectrometry. The purity of the proteins was >95%.

The strip included LTPs described as allergens in the literature but not available for routine testing, as well as different LTP isoforms like the five from sesame (Ses i LTP 1–5) which were chosen based on database and literature research,[31] and two isoforms for Tri a 14 (wheat LTP), Pru du 3 (almond LTP), Pha v 3 (green bean LTP), and Lac s 1 (lettuce LTP).[32–34]

This multiplex immunoblot assay provided semiquantitative information by measuring bands intensity correlating to sIgE concentration range typified by the EAST class system, from 0 to 6. Class 1 or superior was the positivity criteria ($\geq$0.35 $kU_A\ L^{-1}$) following manufacturer's recommendation. The assay set up was

Table 1. Recombinant nsLTP proteins included on the membrane strips of the multiplex immunoblot assay.

Allergen	Uniprot ID/NCBI	Allergenic source	PbP	BAT	ImmunoCAPcomponent
Act d 10	P86137	Kiwi	Kiwi pulp	x	
Lac s 1-1*	A1E2H4	Lettuce	Lettuce	x	
Lac s 1–2*	A1E2H5	Lettuce	Lettuce	x	
Cuc m LTP	A0A1S3B5F4	Melon	Melon pulp next to peel	x	
Cor a 8	Q9ATH2	Hazelnut	Hazelnut		Cor a 8 (f425)
Ara h 9	B6CG41	Peanut	Peanut		Ara h 9 (f427)
Len c 3	A0AT29	Lentil			
Pha v 3.0101*	D3W146	Green bean	Green bean	x	
Pha v 3.0201*	D3W147	Green bean	Green bean	x	
Pis s 3	C0HJR7	Pea	Mashed peas		
Jug r 3	C5H617	Walnut	Walnut		Jug r 3 (f442)
Mus a 3	A0A804IQP6	Banana	Banana pulp		
Tri a 14*	Q8GZB0	Wheat	Wheat flour		
Tri a 14.0201*	D2T2K2	Wheat	Wheat flour		
Zea m 14	P19656-2	Corn	Corn flour		
Mal d 3	Q5J026	Apple	Apple peel		Mal d 3 (f435)
Pru av 3	Q9M5x8	Cherry	Cherry peel		
Pru du 3*	B6CQU2	Almond	Almond	x	
Pru du 3.0101*	C0L0I5	Almond	Almond	x	
Pru p 3	P81402	Peach	Peach peel/pulp	x	Pru p 3 (f420)
Sola l 3	P93224	Tomato	Tomato peel		
Sola l 6	A0A3Q7F7x3	Tomato	Tomato peel		
Sola l 7	A0A3Q7EJP1	Tomato	Tomato peel		
Ses i LTP 1	A5JUZ7	Sesame	Mashed sesame		
Ses i LTP 2	A5JUZ8	Sesame	Mashed sesame		
Ses i LTP 3	A5JUZ9	Sesame	Mashed sesame		
Ses i LTP 4	A5JV00	Sesame	Mashed sesame		
Ses i LTP 5	A5JV01	Sesame	Mashed sesame		
CCD					CCD

Description of the recombinant nsLTPs used in this study with Uniprot ID or NCBI gene bank identifier, native source used for PbP. Isoforms are marked as asterisks and proteins which are not mentioned on IUIS were highlighted with a grey background. BAT, Basophil activation test; PbP, prick by prick.

done as described before.[35] Briefly, the EUROLINE-LTP strips were incubated overnight with 100 μL of serum diluted 1:11 in universal buffer at room temperature (RT) according to manufacturer's instructions. All reagents were included on a kit provided by EUROIMMUN AG. The strips were washed with diluted universal buffer three times and incubated 1 h at RT with enzyme conjugate (alkaline phosphatase-labeled anti-human IgE antibody). Afterwards, strips were washed three times again and incubated 10 min at RT with chromogen substrate solution. The reactions were stopped with distilled water and, after placing on evaluation protocol, strips were dried on air and finally evaluated with "EuroLineScan" software by EUROIMMUN AG.

2.3. Prick by Prick Testing

Prick by prick test (PbP) with fresh plant foods as well as canned lentils, obtained from a local market in Barcelona (Table 1) were performed following EAACI's guidelines.[36,37] Results were obtained after 15 min of testing. A 3 mm mean weal diameter was considered positive. Histamine 10 mg mL^{-1} and phosphate buffered saline were used as positive and negative controls, respectively. All plant foods had been previously prepared in 1 cm^3 cubes and stored frozen (−25 °C) and thawed at room temperature the same day of testing. All patients and controls had been tested with the same stock of plant foods. The number of PbP tested plant food varies among patients. The majority was tested in at least 36 patients (**Table 2**). Only for pea and sesame the number of patients was reduced (pea: 14, sesame: 15). Both plant foods were less common allergic sources and therefore only included in a smaller set of patients.

2.4. Serum Specific IgE Detection by Immunocap

Serum specific IgE to hazelnut (Cor a 8), peanut (Ara h 9), walnut (Jug r 3), peach (Pru p 3), apple (Mal d 3), and wheat (Tri a 14) nsLTP have been tested with ImmunoCAP (ImmunoCAP, ThermoFisher Scientific, Uppsala, Sweden; Table 1). sIgE ≥0.1 kU_A L^{-1} was considered positive.

Table 2. Plant-food sensitization detected by prick-by-prick test.

Allergen source	*n* (total)	PbP Positive	
		n	%
Peach peel	38	38	100%
Apple peel	38	35	92%
Peanut	38	34	89%
Hazelnut	38	33	87%
Green bean	38	33	87%
Cherry peel	36	31	86%
Corn flour	38	32	84%
Melon pulp	37	30	81%
Walnut	38	30	79%
Peas (mashed)	14	10	71%
Tomato peel	38	27	71%
Almond	37	25	68%
Kiwi pulp	38	25	66%
Lettuce	38	25	66%
Wheat flour	38	15	39%
Peach pulp	38	13	34%
Banana	38	13	34%
Lentil (boiled)	38	10	26%
Sesame (mashed)	15	3	20%

2.5. Basophil Activation Test

Heparinized peripheral blood obtained and immediately taken to the laboratory from the patients was used for Basophil Activation Test (BAT) procedure (using the kit Flow2 CAST from Bühlmann Laboratories AG, Switzerland).[38] The positive control used was the "Stimulation control" reagent of the Flow2CAST kit for BAT (Bühlmann Laboratories AG, Switzerland), which corresponded to a monoclonal anti-FcϵRI antibody.

Certain purified nsLTPs (Pru p 3; Pru du 3 and Pru du 3.0101; Pha v 3.0101 and Pha v 3.0201; Lac s 1-1 and Lac s 1–2; Cuc m LTP; Act d 10) provided by EUROIMMUN Medizinische Labordiagnostika AG (Lübeck, Germany) were used on 1, 0.1, 0.01, and 0.001 µg mL^{-1} final concentrations in 13 nsLTP sensitized individuals and three healthy donors of the study cohort. At least 500 basophils were acquired and the expression of CD63 identified by flow cytometry, using a FACS-Canto II flow cytometer (Biosciences, Heidelberg, Germany) and the FACSDiva software.

2.6. Statistical Analysis

The diagnostic performance of the EUROLINE-LTP strips was determined relative to PbP results. PbP was considered the gold standard in in vivo sensitization test, due to the impossibility to perform oral food challenges (OFC) with all plant foods included in this study.

The outcome of the multiplex immunoblot assay was qualitatively compared (positive/negative test result) to the PbP outcome. Thus, strip results were classified into four group as follows: true positive (TP, Euroline: positive; PbP: positive), false positive (FP, Euroline: positive; PbP: negative), true negative (TN, Euroline: negative, PbP: negative); false negative (FN, Euroline: negative; PbP: positive). The Microsoft Excel 14.0 (Microsoft, USA), Add-In Analyse-it 5.90 (Analyse-it Software, Ltd.), and GraphPad Prism 8.0.2 software (Inc., CA, USA) were used for the statistical analysis.

BAT results were presented as %CD63 for each individual allergen concentration. The maximal %CD63 at an allergen concentration of 1 µg mL^{-1} were shown as Box plots calculated with Analyse-it Software, Ltd. (5.11 / 2.30 (Win) / 2018 Operating system, Windows). Basophil sensitivity for each individual allergen and patient were expressed as CD-sens (100/EC_{50}) calculated as previously described by Santos et al.[39] and Di Veroli et al.[40] Only dose-dependent curves with an inflection point could be analyzed.

3. Results

3.1. Patient Characterization

Thirty eight adults (age median [IQR]: 43[34–50] years; female: 27 (71%)) with LTP syndrome diagnosis were included in this analysis (Table S2, Supporting Information).

The reactivities to the plant-food allergenic sources found by PbP are reported in Table 2. All patients showed a positive PbP to peach peel, whereas 13 (34%) of them also reacted to the pulp. Other most frequent sensitizations found were apple peel (92%), peanut (89%), hazelnut (87%), green bean (87%) followed by cherry peel (86%), corn (84%), melon (81%), walnut (79%), tomato peel (71%), almond (68%), lettuce (66%), and kiwi pulp (66%). Although pea was only tested in 14 patients, the sensitization was quite high (71%). Wheat, peach pulp, banana, lentil, and sesame showed low sensitization (<40%).

3.2. EUROLINE-LTP Immunoassay Validation

In the EUROLINE-LTP immunoblot assay, each of the recombinant nsLTPs could evoke a positive sIgE response confirming a suitable epitope presentation (**Figure 1**). The measured sIgE intensities are presented in EAST classes (Figure 1), spanning from low sIgE reactivity for Ses i LTP1 and Tri a 14, to highly reactive components such as Pru p 3, Pru av 3, and Mal d 3.

Two subjects (patients 2 + 3) showed IgE sensitization to every recombinant protein included in the EUROLINE-LTP strip while all other subjects recognized at least four nsLTPs from different allergenic sources, although not following any particular pattern. Most patients were sensitized to allergens of the *Rosacae* family, like Pru p 3, Pru av 3, Pru du 3, and Mal d 3. Isoforms (as, i.e., Pru du 3) showed different IgE reactivity patterns justifying the need of working with various homologous forms of the allergens. Ses LTP 5 is the most frequently recognized isoform (26/38) from the sesame LTPs. Interestingly, 29 patients had high sIgE levels for the LTP from melon (Cuc m LTP).

The analytical performance of the EUROLINE-LTP strip was assessed considering the PbP data as the reference test result, due to logistic and practical limitation to challenge the patients for all allergenic sources via OFC. As whole food may contain more LTP than processed extracts the PbP got preference over SPT (see Tables S3, S4, Supporting Information).

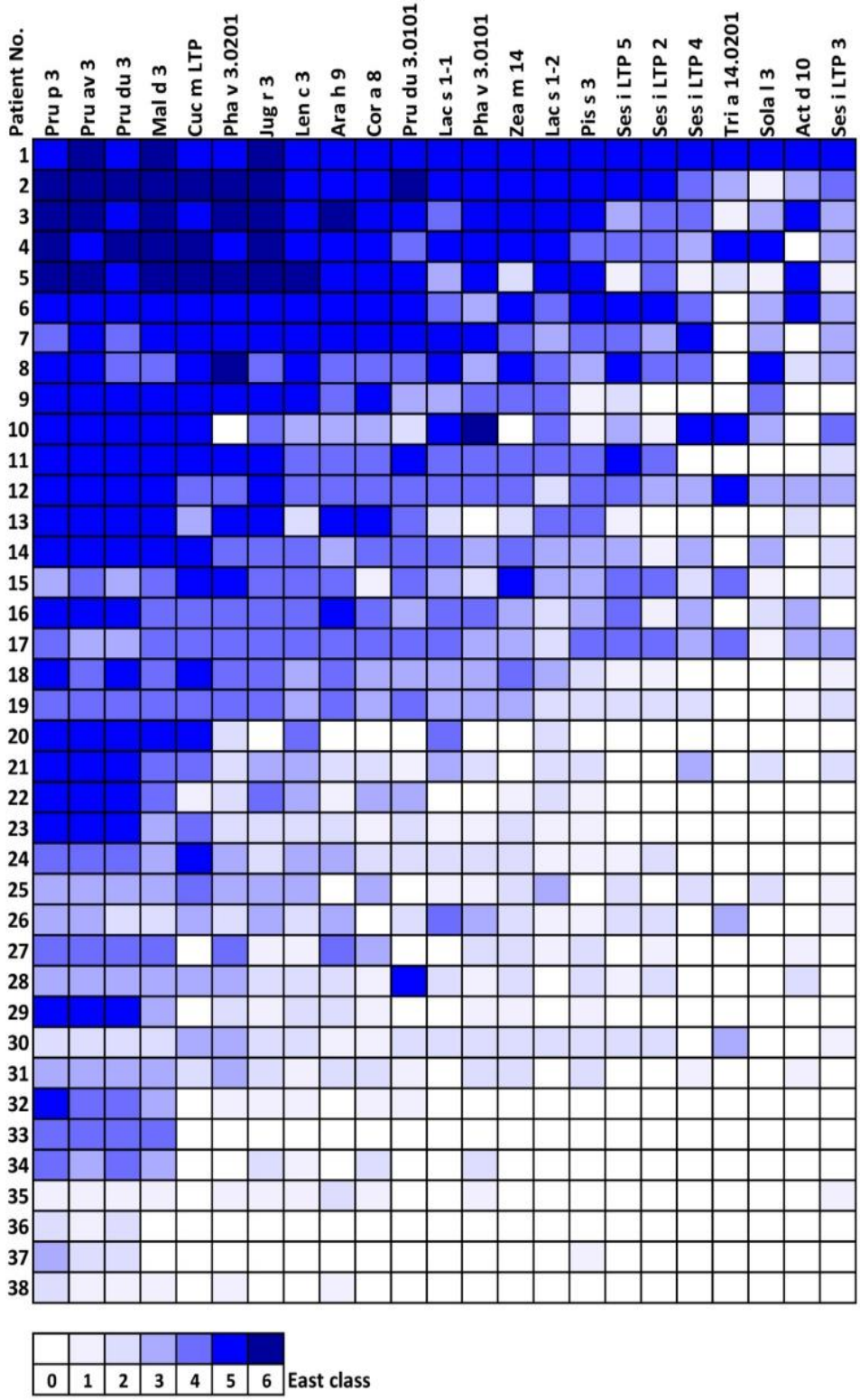


Figure 1. Heatmap of sIgE reactivities against nsLTPs measured by the nsLTP immunoblot assay in 38 patient sera. nsLTPs recognized from the same individual are shown in rows. The level of sIgE reactivity is indicated in different shades of blue. Every shade is exemplary for a class (Class: 0 negative; Class: 1–6 positive). The nsLTPs are arranged by the total sum of the sIgE reactivity from all patients (left: highest sIgE levels, right: lowest sIgE levels).

Strip reactivity (positive/negative) was classified qualitatively in comparison to PbP outcome (**Figure 2**).

The proportion of correctly classified (TP and TN) subjects exceeds 70% for the majority (17/28) of the recombinant nsLTPs (Table S5, Supporting Information). Particularly good performance is shown by Pru p 3 (100%) and Mal d 3 (97%), Pru av 3 (89%), Pha v 3.0101/0201 (87%, 84%), Ara h 9 (82%), Cor a 8 (82%), and Jug r 3 (82%). Interestingly, the newly introduced nsLTPs, Cuc m LTP, and Ses i LTP1 correlate well with the PbP outcome (76% and 67% agreement, respectively).

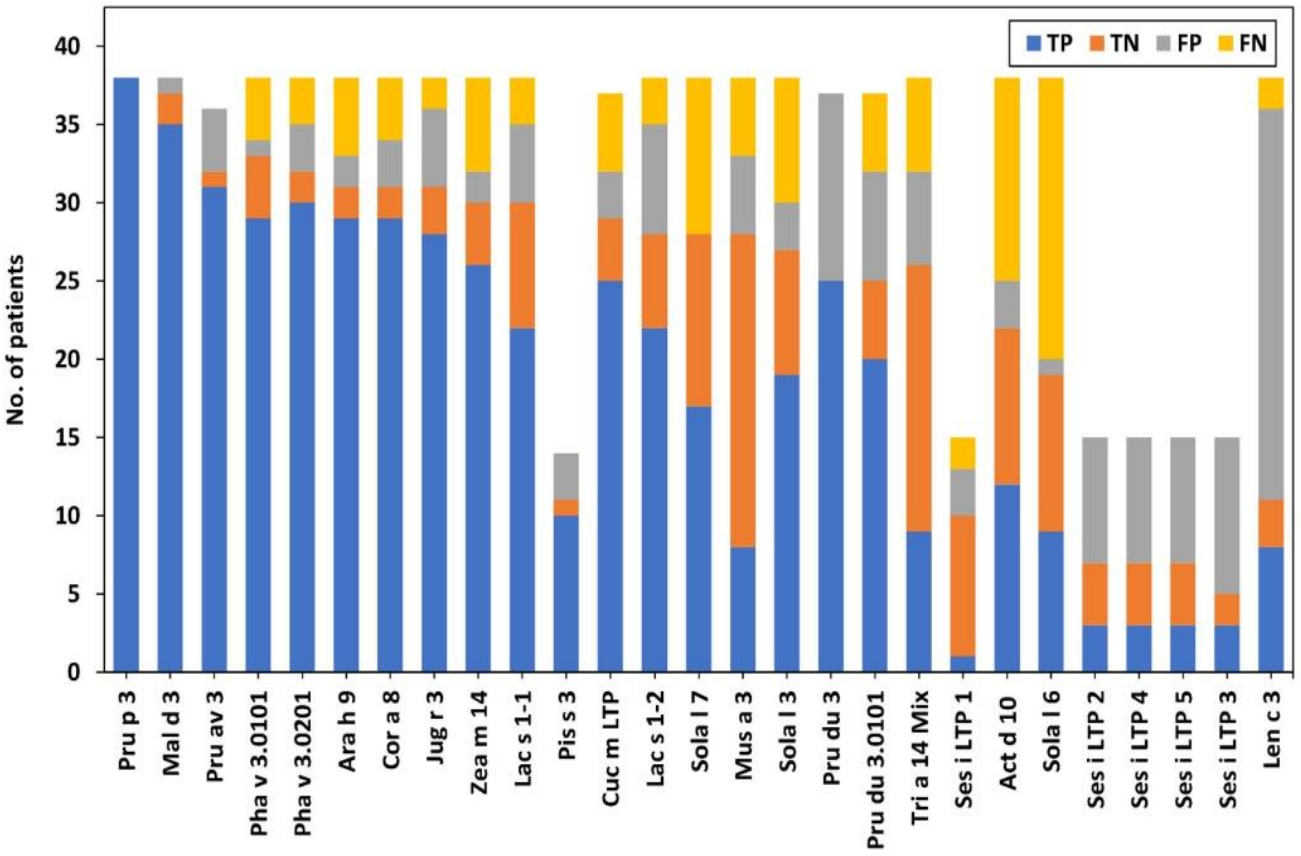


Figure 2. nsLTP immunoblot assay performance in comparison to PbP results. For qualitative analysis of the sIgE levels they were subdivided into four groups: true positive (TP, blue), true negative (TN, orange), false positive (FP, grey) and false negative (FN, yellow). The allergens are sorted in the descending order according to the relative amount of true positive and true negative reactions.

Even though, many patients were sensitized against Pru du 3 and Len c 3 in the immunoblot assay without a positive PbP outcome.

To validate the reliability of the measured IgE reactivities in the cohort of patients tested, sera of 28 healthy blood donors were analyzed with the EUROLINE-LTP strips. These subjects had no clinical history of food or respiratory allergy, were Pru p 3 sIgE negative (<0.1 $KU_A\ L^{-1}$ ImmunoCAP, ThermoFisher Scientific) and/or had negative SPT to peach peel extract (Leti Pharma, Madrid, Spain).

28 healthy donors were tested with the EUROLINE-LTP strips and no IgE reactivity above the cut-off with the tested LTPs was found. Only sera from four individuals reacted close to the cut-off (0.35 kUA L^{-1}) for certain proteins (Jug r 3 (2/28), Pru av 3 (1/28), Sola l 7 (1/28), Pru du 3 (1/28), and Pha v 3.0201 (1/28), Table S6, Supporting Information).

3.3. Recombinant nsLTPs Induced Allergic Reaction in BAT

Nine recombinant nsLTPs (Pru p 3, Lac s 1-1, Lac s 1–2, Pha v 3.0101, Pha v 3.0201, Pru du 3, Pru du 3.0101, Act d 10, Cuc m LTP) of the EUROLINE-LTP strips frequently recognized by LTP allergic patients were used for basophil activation testing (BAT) in a group of individuals ($n = 16$; 13 patients + 3 healthy donors) to assess their functionality, i.e., ability to crosslink receptor-bound sIgE and cause cell activation (assessed as CD63 expression by flow cytometry). In our hands it was only feasible to test several proteins at a time per each patient in BAT guarantying the correct performance of the test. Thus we selected the proteins to use in BAT based on several premises: 1) we wanted to test nsLTPs which were clinically relevant in patients of our area with LTP allergy with little or no data reported in the literature to gain knowledge and experience on them in terms of basophil activation (i.e., first we started with Lac s 1, Pru du 3, and Pha v 3 and then when proteins were available for BAT we added Act d 10, Cuc m LTP, this is why we have less individuals tested for these two proteins), 2) we also wanted to compare the functional impact of different isoforms of a particular nsLTP that had been included in the EUROLINE LTP strip (Lac s 1-1 vs Lac s 1–2, Pru du 3 vs Pru du 3.0101, and Pha v 3.0101 and Pha v 3.0201), 3) we tested also all the individuals with Pru p 3 as a reference of basophil activation since we are familiar with the basophil activation performance with this protein in LTP allergic patients. Thus, we could have data on the activation of new LTPs compared to the activation caused by Pru p 3.

Basophils were stimulated with four concentrations (1, 0.1, 0.01, and 0.001 μg mL^{-1}) for each protein.

All proteins were able to induce the expression of CD63 on the cell surface, and thus, are all allergenically active molecules. At the highest allergen dose, the median of %CD63 for all tested recombinant proteins is above 60% (Figure S2, Supporting Information). All patients showed a high basophil reactivity to Pru p 3, Pru du 3, and Pha v 3.0201.

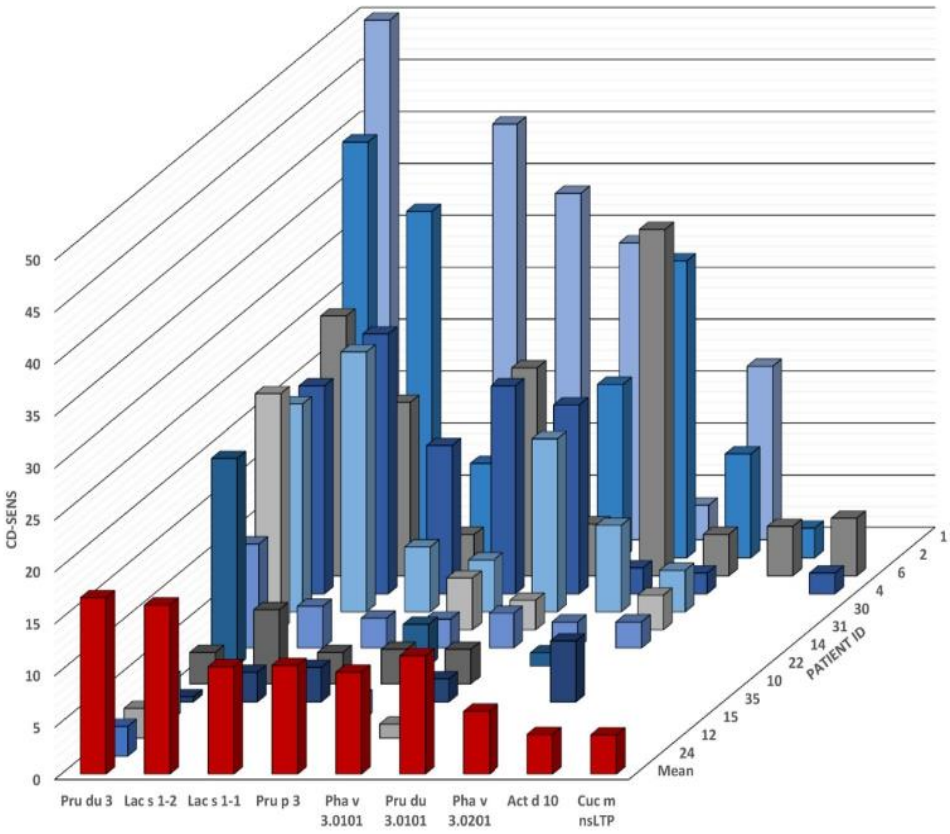


Figure 3. CD-sens values distribution. CD-sens values of the BAT for a selected set of allergens and patients. In front the mean CD-sens value is shown in red.

Pru du 3 induced CD63 expression at very low allergen concentrations followed by Lac s 1–2, Lac s 1-1, Pru p 3, and Pha v 3.0101 (Figure S3, Supporting Information). These results show that trace amounts of allergens induce basophil activation which could trigger an allergic reaction.

Based on the slope of the dose–response curves (Figure S3, Supporting Information) the CD-sens values, as a marker for basophil sensitivity, were estimated[39] and averaged for the selected allergens (**Figure 3**). In Figure S4, Supporting Information is presented the BAT data for healthy controls, showing no activation.

In this study, the CD-sens was used as a criterion for the allergy-inducing property of the target protein. For Act d 10 and Cuc mLTP only three sera were tested. Thus, the results for these two allergens should be treated like a first indication which needs further confirmation by a larger cohort.

Pru du 3 induced the highest basophil sensitivity in the selected patients followed by Lac s 1–2, Lac s 1-1, Pru p 3, and Pha v 3.0101, although, the basophil reactivity differed significantly between all patients. Patient 1, i.e., shows a very strong basophil response to Pru du 3, Pru p 3, Pha v 3.0101, Pru du 3.0101, and Act d 10, whereas for patient 17 the CD-sens values were very low and only Pru du 3 and Pru p 3 elicited a slight basophil stimulation.

The patient ID from Figure 3 is consistent with the heat map in Figure 1. The patients (1–38) are ordered by their sIgE level response in the EUROLINE-LTP strips. A low ID means high sIgE reactivity. Patient 1, 2, 4, and 5 showed the highest basophil reactivity which correlates with EUROLINE-LTP strips results.

Table 3. Correlation between immunoCAP and Euroline data considering EAST-classes via Spearman's rank correlation.

LTP	r_s	p-value	n (total)
Pru p 3	0.875	<0.0001	38
Ara h 9	0.890	<0.0001	24
Cor a 8	0.602	0.0019	24
Jug r 3	0.698	0.0001	24
Mal d 3	0.857	<0.0001	33

r_s, Spearman's rank correlation coefficient; p, significance level ($\alpha = 0.05$).

3.4. Comparison of EUROLINE-LTP Strips with an Established In Vitro Assay

To verify the data from the EUROLINE-LTP strip, the results were compared to an established in vitro assay based on sIgE determination, the ImmunoCAP system (ThermoFisher Scientific). The degree of concordance between the EAST classes from EUROLINE (semi-quantitative) versus ImmunoCAP (quantitative) was analyzed for five well-characterized LTPs (Pru p 3, Mal d 3, Cor a 8, Jug r 3, and Ara h 9) by Spearman's rank correlation (**Table 3**). There is a positive relation between the two diagnostic tests for the examined components. The Spearman's rank coefficient varies from 0.6 to 0.89. The informative value of this correlation is limited because of the small case number.

4. Discussion

In this study the diagnostic performance of a new multiplex nsLTP immunoblot assay in a cohort of patients with diagnosed LTP-syndrome from the Mediterranean area was evaluated and a good concordance with the PbP outcome was found. The assay was especially designed including the most relevant and frequent allergenic sources in LTP-syndrome in our area with the aim to cover the clinical need of assessing sIgE sensitization to a broader panel of nsLTPs from taxonomically related and unrelated allergenic sources compared to the already commercially available tests to improve the allergological management of these patients.

An accurate allergy diagnosis is crucial to define the most appropriate treatment for each subject.[41] The use of a multiplex test may facilitate the implementation of a more precise molecular diagnosis coupled with an improved clinical management and diet intervention of the patients. Although sIgE reactivity does not always correlate with the clinical reactivity and it refers to sensitization, a reliable in vitro tool providing data on true presence and absence of nsLTP sensitization to a broad panel of allergenic sources could be a valuable aid for the clinical management of patients with LTP-syndrome, addressing specifically the diet intervention.[2]

Despite being a rather small cohort of patients, since this is intended as a validation study of a novel in vitro tool, the high diversity of sIgE recognition profiles among them emphasizes the convenience of a broad panel of nsLTP in a multiparametric test for the diagnosis and clinical characterization of LTP syndrome. Indeed, most patients were sensitized to *Rosaceae* LTPs (Pru p 3, Mal d 3, Pru av 3, Pru du 3), however sensitization to other plant food which are not closely related cannot be assumed by taxonomy also frequently occurs. The inclusion of several isoforms of one protein has also provided relevant information. Isoforms of one protein (e.g., Pru du 3, Lac s 1, Pha v 3) possessed individual IgE-binding properties and cellular activation, leading to different sensitization pattern. Importantly, we demonstrate hereby that sensitization to nsLTP not included in current commercially available multiplex tests are frequently recognized by LTP allergic patients and thus should be considered (i.e., lettuce, green bean, almond, etc.).

All individuals of the cohort showed sensitization to peach peel when PbP-tested, the approach considered as the gold standard for the analysis, and all of them showed positive sIgE reactivity in the immunoblot assay to at least four different nsLTPs. A subset of nine nsLTP allergens was further analyzed by BAT. In all patients that IgE sensitization was detected in the EUROLINE-LTP strip, basophil activation was observed upon stimulation with the corresponding nsLTP, meaning that the serum specific IgE detected with the strip corresponded with IgE functionality at basophil level. Also, to our knowledge it is the first time to report BAT data on the performance of nsLTPs from lettuce, green bean, almond, melon, and kiwi.

When compared to PbP, the proportion of correctly classified subjects exceeds 70% for the majority of the recombinant nsLTPs in the immunoblot assay. Pru p 3 and Mal d 3 correlate very well with the PbP outcome (100% and 97% agreement, respectively). Considering the different implementations and allergen presentation between PbP testing and in vitro detection of sIgE, this correlation between the two assays systems is actually very good.

Some proteins (i.e., Pru du 3 and Len c 3) show a high amount of false positive reactions when comparing the immunoblot sIgE levels and the PbP outcome. Interestingly, the BAT results for patient 23 and patient 33 which where both PbP negative for almond seeds, clearly showed an activation for Pru du 3 supporting the results from the immunoblot assay. Nevertheless, we looked further into possible explanation for potential false positive sIgE reactivity. On the one hand, nsLTP share a high amino acid sequence similarity which results in a high degree of cross-reactivity (e.g., Pru du 3 and Pru p 3 have a sequence similarity of 94.5%).[42] Pru du 3 shares three major IgE-binding epitopes which are reported for Pru p 3 in previous studies.[43] Interestingly, the isoform Pru du 3.0101 only has one shared epitope. These findings were supported by the heatmap and by the BAT data, where Pru du 3 shows similar reactivity to Pru p 3 whereas Pru du 3.0101 deviates from this pattern. To what extend the cross-reactivity may be clinically relevant has to be further investigated. Even though the cross-reactivity has to be considered, it does not explain the negative PbP outcome. The latter may be caused by the different antigen presentation of the two test systems. Whereas the immunoblot contains exclusively recombinant proteins, fresh fruits, nuts, and vegetables were used for the PbP. Plant-based food shows variable concentrations of nsLTPs, depending on cultivar, storage, maturity conditions, and processing.[44] This fact is very significant, since the fresh food source that we were testing may not contain the nsLTP or have it in a very low quantity with the consequent false negative of PbP. Also, the PbP test with lentil, e.g., was done with canned lentils which have been cooked in advance. Previous studies showed reduced IgE-binding capacity after heating of rLen c 3.[45] Maybe the amount of IgE-reactive Len c 3 was too low to induce an allergic reaction in the PbP.

In another previous study, the nsLTP of almond tree (Pru du 3) could be detected in the almond leaf and the epicarp/mesocarp which coats the almond seeds. Similar to our own findings, the prick test with three types of almond seeds had a negative outcome. Presumably, the LTP quantity in the seeds is reduced compared to other tissues.[46]

In this study novel LTPs which are not described as allergens yet where included. Cuc m LTP and the Ses i LTP 1–5 do not have an especially high amino acid sequence similarity to Pru p 3 (≈50%). Nevertheless, a lot of patients showed a Cuc m LTP sensitization. The sIgE reactivity for Cuc m LTP in the immunoblot assay was even higher than for Pru p 3 in a few patients. The clinical relevance for this data has to be further analyzed. Previous studies also hinted for LTP as culprit in melon allergy.[47] The LTPs of sesame were recognized by a fair number of subjects. The interpretation of these data is difficult as there was just a small number of patients which was pricked (PbP) with sesame and just six of them had a positive outcome.

There are some limitations in our study. We highlight that we are considering PbP results as the reference test whereas oral challenge outcome is the real gold standard for food allergy diagnosis, but this approach was not logistically feasible for this project in our hands. Thus we selected a possibilistic approach to be able to test sensitization to a broad panel of allergenic sources and also we believed based on our experience and reported data

on the field of allergology that diversity of proteins to assess sensitization would be more preserved in PbP than in commercial SPT extracts. The analysis of the outcome of prick by prick and the LTP strip with the anamnestic data shows that the performance of the in vitro approach with LTP Euroline for sensitization screening was superior to PbP for particular allergen sources like lentil, sesame, Jug r 3, Cuc m LTP and Tri a 14.02. Thus, it is noteworthy to have as many high quality recombinant nsLTP from a broad range of allergenic sources as possible to improve LTP syndrome diagnosis and management.

The selection of our nsLTP allergic patients was according to Pru p 3 sensitization, considering it to be an universal LTP for the diagnosis of LTP sensitized patients in general, independently of the allergenic source. Only serum samples from the Mediterranean area were included, which is representative for Southern Europe but means the generalization of the data to non-Mediterranean areas is limited. Furthermore, the PbP was only conducted in patients with LTP syndrome, and negative controls were only skin tested by peach-peel and ImmunoCAP tested to confirm absence of LTP sensitization. As this was a pilot study, only a small cohort was used for preliminary results. Indeed, all these observations have to be confirmed with larger cohorts of LTP allergic individuals tested and with as much oral food challenges as possible.

In conclusion, our results revealed that this new multiplex test could be successfully used for detection of specific IgE against nsLTPs. In this rather small validation cohort, the qualitative performance of the LTP-strip when compared to PbP was valuable. A wider scope of assessing nsLTP plant food sensitizations, not only has the value of the true positives that improve patient diagnosis but also offers the value of the true negatives, which represent allergenic sources that could be potentially tolerated by patients and consequently improve their clinical management and quality of life.[48–50]

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The present study has been supported by the Instituto de Salud Carlos III of the Ministry of Economy and Competitiveness (ISCIII.Madrid, Spain). FIS grant: PI16/00344.

Conflicts of Interest

B.B., U.F., W.S., and Y.W. are employees of EUROIMMUN AG A PerkinElmer company (Lübeck, Germany). Other authors have no conflicts of interest to declare.

Author Contributions

S.B.V. and U.F. have contributed to acquisition, analysis, and interpretation of data, as well as drafting the manuscript for publication. R.C.S., T.P., and G.A.S. have contributed to the acquisition of clinical data. M.T., C.S.B., R.L. contributed to the performance of laboratory tests. B.B., Y.W., S.W., J.B., R.M.C., and M.P. have contributed on the design and interpretation of the data and critically revised and approved the final version of the manuscript. All authors have participated sufficiently in the work, approved the final version, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

Keywords

allergy, Basophil activation test, in vitro testing, lipid transfer proteins, multiplex immunoblot assay, plant food, serum specific IgE

Received: December 30, 2022
Revised: April 11, 2023
Published online:

[1] P. M. Ojeda, J. Sastre, J. M. Olaguibel, T. Chivato, *J. Investig. Allergol. Clin. Immunol.* **2018**, *28*, 151.
[2] E. Scala, S. J. Till, R. Asero, D. Abeni, E. C. Guerra, L. Pirrotta, R. Paganelli, D. Pomponi, M. Giani, O. De Pità, L. Cecchi, *Allergy* **2015**, *70*, 933.
[3] I. J. Skypala, R. Asero, D. Barber, L. Cecchi, A. Diaz Perales, K. Hoffmann-Sommergruber, E. A. Pastorello, I. Swoboda, J. Bartra, D. G. Ebo, M. A. Faber, M. Fernández-Rivas, F. Gomez, A. P. Konstantinopoulos, O. Luengo, R. van Ree, E. Scala, S. J. Till, *Clin. Transl. Allergy* **2021**, *11*, 1.
[4] S. Scheurer, R. van Ree, S. Vieths, *Curr. Allergy Asthma Rep.* **2021**, *21*, 7.
[5] A. Murad, C. H. Katelaris, K. Baumgart, *AsiaPac. Allergy* **2016**, *6*, 129.
[6] S. Ma, L. Nie, H. Li, R. Wang, J. Yin, *Int. Arch. Allergy Immunol.* **2016**, *169*, 241.
[7] S. Ma, J. Yin, N. Jiang, *J. Allergy Clin. Immunol.* **2013**, *132*, 764.
[8] Z.-S. Gao, Z.-W. Yang, S.-D. Wu, H.-Y. Wang, M.-L. Liu, W.-L. Mao, J. Wang, G. Gadermaier, F. Ferreira, M. Zheng, R. van Ree, *J. Allergy Clin. Immunol.* **2013**, *131*, 224.
[9] Z. Gao, W.-Y. Fu, Y. Sun, B. Gao, H.-Y. Wang, M. Liu, F.-M. Luo, X. Zhou, J. Jin, L. Zhao, S. Wu, Y. Liu, L. Wu, X. Wang, N.-B. Tang, B.-H. Guo, Y. Feng, J. Y. Zhou, G. Gadermaier, F. Ferreira, S. A. Versteeg, R. van Ree, *Allergy* **2019**, *74*, 284.
[10] I. J. Skypala, J. Bartra, D. G. Ebo, M. Antje Faber, M. Fernández-Rivas, F. Gomez, O. Luengo, S. J. Till, R. Asero, D. Barber, L. Cecchi, A. Diaz Perales, K. Hoffmann-Sommergruber, E. Anna Pastorello, I. Swoboda, A. P. Konstantinopoulos, R. Ree, E. Scala, *Allergy* **2021**, *76*, 2433.
[11] E. I. Finkina, D. N. Melnikova, I. V. Bogdanov, T. V. Ovchinnikova, *Acta Naturae* **2016**, *8*, 47.
[12] R. Asero, G. Mistrello, D. Roncarolo, S. Amato, G. Caldironi, F. Barocci, R. van Ree, *Allergy* **2002**, *57*, 900.
[13] M. Pascal, R. Muñoz-Cano, Z. Reina, A. Palacín, R. Vilella, C. Picado, M. Juan, J. S. López, M. Rueda, G. Salcedo, A. Valero, J. Yagüe, J. Bartra, J. Sánchez-López, M. Rueda, G. Salcedo, A. Valero, J. Yagüe, J. Bartra, J. S. López, M. Rueda, G. Salcedo, A. Valero, J. Yagüe, J. Bartra, *Clin. Exp. Allergy* **2012**, *42*, 1529.
[14] E. A. Pastorello, A. M. Robino, *Mol. Nutr. Food Res.* **2004**, *48*, 356.
[15] S. Scheurer, I. Lauer, K. Foetisch, M. S. M. Moncin, M. Retzek, C. Hartz, E. Enrique, J. Lidholm, A. Cistero-Bahima, S. Vieths, *J. Allergy Clin. Immunol.* **2004**, *114*, 900.

[16] M. L. Bernardi, I. Giangrieco, L. Camardella, R. Ferrara, P. Palazzo, M. R. Panico, R. Crescenzo, V. Carratore, D. Zennaro, M. Liso, M. Santoro, S. Zuzzi, M. Tamburrini, M. A. Ciardiello, A. Mari, *PLoS One* **2011**, *6*, e27856.
[17] V. Cardona, O. Luengo, T. Garriga, A. Izquierdo, *Allergy* **2012**, *39*, 1316.
[18] L. Tordesillas, C. Gómez-Casado, M. Garrido-Arandia, A. Murua-García, A. Palacín, J. Varela, P. Konieczna, J. Cuesta-Herranz, C. A. Akdis, L. O'Mahony, A. Díaz-Perales, *Clin. Exp. Allergy* **2013**, *43*, 1374.
[19] B. E. Garcia, M. Lombardero, S. Echechipia, J. M. Olaguibel, A. Diaz-Perales, R. Sanchez-Monge, D. Barber, G. Salcedo, A. I. Tabar, *Clin. Exp. Allergy* **2004**, *34*, 291.
[20] C. San Bartolomé, R. Muñoz-Cano, J. Rius, R. Casas-Saucedo, S. Balsells, N. Egri, M. Ruano-Zaragoza, C. de la Cruz, J. Bartra, M. Pascal, *Int. Arch. Allergy Immunol.* **2022**, *183*, 75.
[21] L. Tordesillas, N. Cubells-Baeza, C. Gómez-Casado, C. Berin, V. Esteban, W. Barcik, L. O'Mahony, C. Ramirez, L. F. Pacios, M. Garrido-Arandia, A. Díaz-Perales, *Clin. Exp. Allergy* **2017**, *47*, 1398.
[22] R. Asero, M. Piantanida, E. Pinter, V. Pravettoni, *Clin. Exp. Allergy* **2018**, *48*, 6.
[23] M. Morales, M. Á. López-Matas, R. Moya, J. Carnés, *Food Chem.* **2014**, *165*, 397.
[24] L. Tordesillas, S. Sirvent, A. Díaz-Perales, M. Villalba, J. Cuesta-Herranz, R. Rodríguez, G. Salcedo, *Int. Arch. Allergy Immunol.* **2011**, *156*, 291.
[25] I. J. Skypala, R. McKenzie, *Clin. Rev. Allergy Immunol.* **2019**, *57*, 166.
[26] R. A. Wood, *Chem. Immunol Allergy* **2015**, *101*, 87.
[27] M. Fernández-Rivas, S. Garrido Fernández, J. A. Nadal, M. D. Alonso Díaz de Durana, B. E. García, E. González-Mancebo, S. Martín, D. Barber, P. Rico, A. I. Tabar, *Allergy* **2009**, *64*, 876.
[28] F. Gomez, G. Bogas, M. Gonzalez, P. Campo, M. Salas, A. Diaz-Perales, M. J. Rodriguez, A. Prieto, D. Barber, M. Blanca, M. J. Torres, C. Mayorga, *Clin. Exp. Allergy* **2017**, *47*, 339.
[29] F. Palomares, F. Gomez, G. Bogas, P. Campo, J. R. Perkins, A. Diaz-Perales, M. J. Rodriguez, A. Prieto, D. Barber, M. J. Torres, C. Mayorga, *Mol. Nutr. Food Res.* **2018**, *62*, 1700669.
[30] S. Pokoj, I. Lauer, K. Fötisch, M. Himly, A. Mari, E. Enrique, M. del M. S. Miguel-Moncin, J. Lidholm, S. Vieths, S. Scheurer, *Protein Expr. Purif.* **2010**, *69*, 68.
[31] A. M. Choi, S. B. Lee, S. H. Cho, I. Hwang, C.-G. Hur, M. C. Suh, *Plant Physiol. Biochem.* **2008**, *46*, 127.
[32] A. Palacin, S. Quirce, A. Armentia, M. Fernández-Nieto, L. F. Pacios, T. Asensio, J. Sastre, A. Diaz-Perales, G. Salcedo, *J. Allergy Clin. Immunol.* **2007**, *120*, 1132.
[33] C. Hartz, M. del Mar San Miguel-Moncín, A. Cisteró-Bahíma, K. Fötisch, K. J. Metzner, D. Fortunato, J. Lidholm, S. Vieths, S. Scheurer, *Mol. Immunol.* **2007**, *44*, 2820.
[34] M. Röder, S. Vieths, T. Holzhauser, *Anal. Chim. Acta* **2011**, *685*, 74.
[35] M. Di Fraia, S. Arasi, S. Castelli, S. Dramburg, E. Potapova, D. Villalta, S. Tripodi, I. Sfika, A. M. Zicari, V. Villella, S. Perna, A. Travaglini, P. L. Verardo, P. M. Matricardi, *Clin. Exp. Allergy* **2019**, *49*, 341.
[36] K. Hoffmann-Sommergruber, L. de las Vecillas, S. Dramburg, C. Hilger, P. Matricardi, A. Santos, *EAACI Molecular Allergology User´s Guide, European Academy Of Allergy And Clinical Immunology (EAACI)*, Zurich **2022**.
[37] E. S. Dreborg, A. Frew, A. Frew, *Allergy* **1993**, *48*, 48.
[38] M. Pascal, R. Muñoz-Cano, J. Milà, M. L. Sanz, A. Diaz-Perales, J. Sánchez-López, A. García-Moral, M. Juan, A. Valero, J. Yagüe, C. Picado, J. Bartra, *Clin. Exp. Allergy* **2016**, *46*, 1111.
[39] A. F. Santos, O. Alpan, H. Hoffmann, *Allergy* **2021**, *76*, 2420.
[40] G. Y. Di Veroli, C. Fornari, I. Goldlust, G. Mills, S. B. Koh, J. L. Bramhall, F. M. Richards, D. I. Jodrell, *Sci. Rep.* **2015**, *5*, 14701.
[41] C. Alessandri, R. Ferrara, M. L. Bernardi, D. Zennaro, L. Tuppo, I. Giangrieco, M. Tamburrini, A. Mari, M. A. Ciardiello, *Clin. Transl. Allergy* **2017**, *7*, 21.
[42] J. Akkerdaas, E. I. I. Finkina, S. V. V. Balandin, S. Santos Magadán, A. Knulst, M. Fernandez-Rivas, R. Asero, R. van Ree, T. V. V. Ovchinnikova, *Int. Arch. Allergy Immunol.* **2012**, *157*, 51.
[43] I. Lauer, M. S. Miguel-Moncin, T. Abel, K. Foetisch, C. Hartz, D. Fortunato, A. Cistero-Bahima, S. Vieths, S. Scheurer, *Clin. Exp. Allergy* **2007**, *37*, 261.
[44] P. M. Matricardi, J. Kleine-Tebbe, H. J. Hoffmann, R. Valenta, C. Hilger, S. Hofmaier, R. C. Aalberse, I. Agache, R. Asero, B. Ballmer-Weber, D. Barber, K. Beyer, T. Biedermann, M. B. Bilò, S. Blank, B. Bohle, P. P. Bosshard, H. Breiteneder, H. A. Brough, L. Caraballo, J. C. Caubet, R. Crameri, J. M. Davies, N. Douladiris, M. Ebisawa, P. A. Elgenmann, M. Fernandez-Rivas, F. Ferreira, G. Gadermaier, M. Glatz, et al., *Pediatr. Allergy Immunol.* **2016**, *27*, 1.
[45] E. I. Finkina, D. N. Melnikova, I. V. Bogdanov, N. S. Matveevskaya, A. A. Ignatova, I. Y. Toropygin, T. V. Ovchinnikova, *Biomolecules* **2020**, *10*, 1668.
[46] S. Garrido-Fernández, B. E. Garcia, M. L. Sanz, S. Ariz, A. Tabar, *J. Investig. Allergol. Clin. Immunol.* **2009**, *19*, 61.
[47] A. Sokolova, B. Bartolomé, G. Sampaio, M. I. Mascarenhas, H. Carreiro, *Clin. Transl. Allergy* **2011**, *1*, P1.
[48] F. Sievers, A. Wilm, D. Dineen, T. J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Söding, J. D. Thompson, D. G. Higgins, *Mol. Syst. Biol.* **2011**, *7*, 539.
[49] H. McWilliam, W. Li, M. Uludag, S. Squizzato, Y. M. Park, N. Buso, A. P. Cowley, R. Lopez, *Nucleic Acids Res.* **2013**, *41*, W597.
[50] F. D. Ciccarelli, T. Doerks, C. von Mering, C. J. Creevey, B. Snel, P. Bork, *Science* **2006**, *311*, 1283.