

Improving In Vitro Detection of Sensitization to Lipid Transfer Proteins: A New Molecular Multiplex IgE Assay

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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

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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 ($\geq 0.35 \text{ kU}_A \text{ L}^{-1}$) 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	ImmunoCAP component
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 μ L 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 cm³ 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 Labor-diagnostika 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/EC₅₀) 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 *Rosaceae* 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).

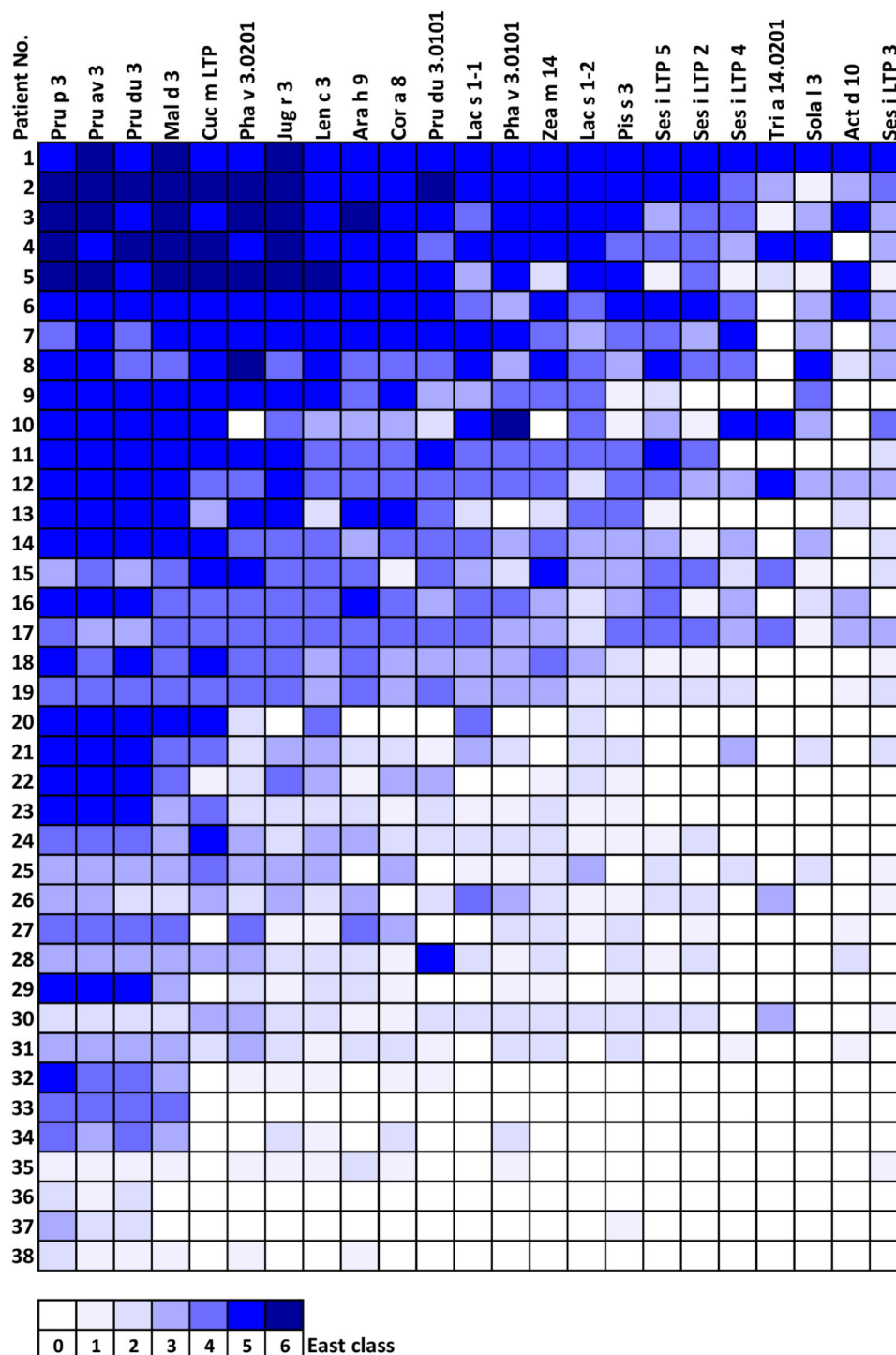


Figure 1. Heatmap of sIgE reactivities against nsLTPs measured by the nsLTP immunoblot assay in 38 patient sera. nsLTPs recognized from the same individual are shown in rows. The level of sIgE reactivity is indicated in different shades of blue. Every shade is exemplary for a class (Class: 0 negative; 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 perfor-

mance 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).

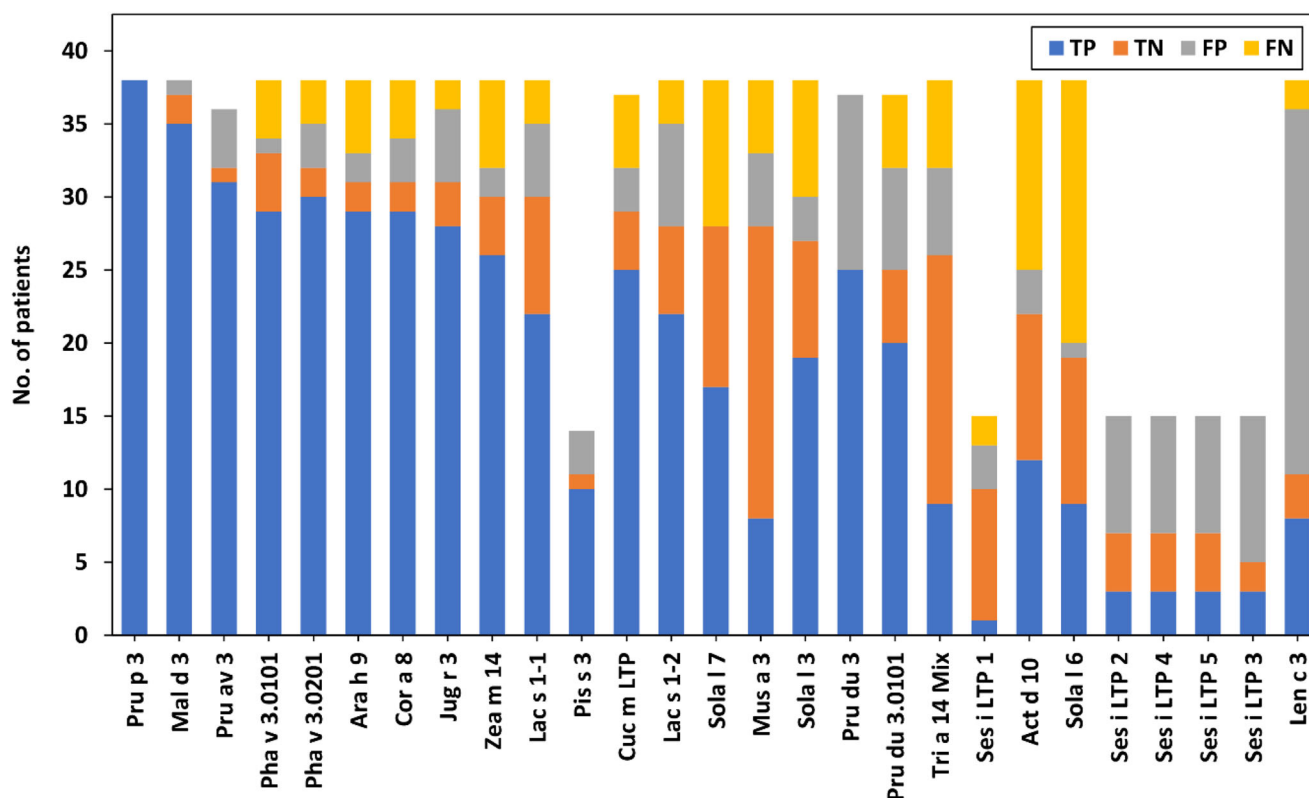


Figure 2. nsLTP immunoblot assay performance in comparison to PbP results. For qualitative analysis of the sIgE levels they were subdivided into four groups: true positive (TP, blue), true negative (TN, orange), false positive (FP, grey) and false negative (FN, yellow). The allergens are sorted in the 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 ($<0.1 \text{ KU}_A \text{ L}^{-1}$ 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 guaranteeing 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 $0.001 \mu\text{g mL}^{-1}$) 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.

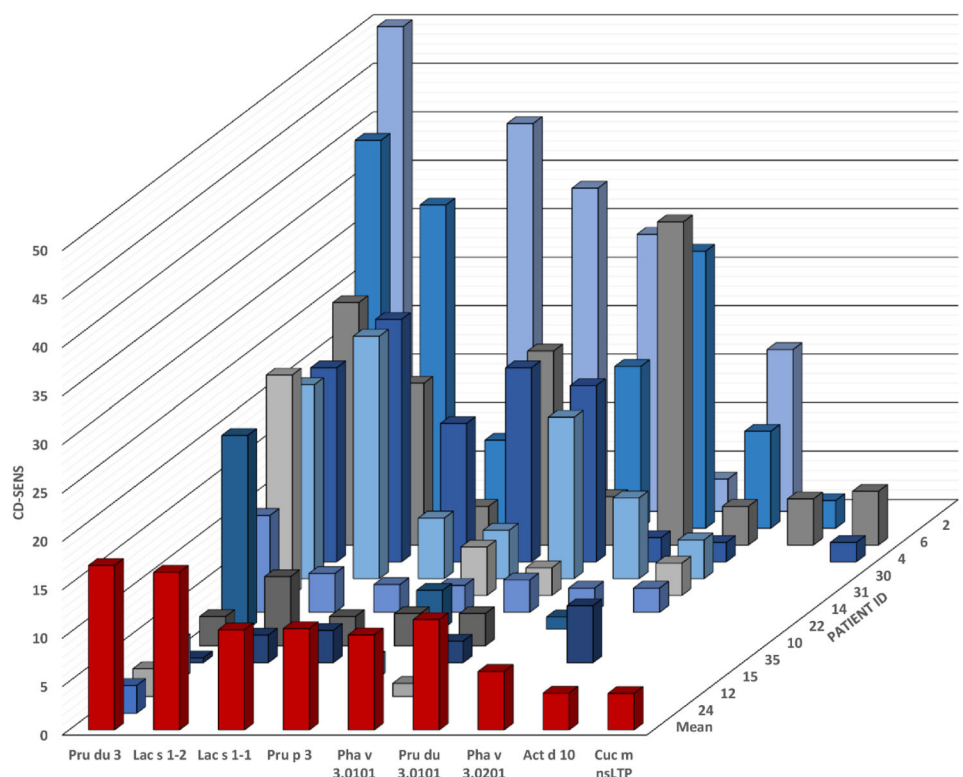


Figure 3. CD-sens values distribution. CD-sens values of the BAT for a selected set of allergens and patients. In front the mean CD-sens value is shown in red.

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 were 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 extent 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 were 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.

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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

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