Component resolved diagnosis by recombinant allergens in patients with allergies to inhalants

G. Di Spigna[1], P. Ladogana[1], B. Covelli[1], M. Ricciardone[1], S. Salzano[2], D. Spalletti Cernia[1], I. Mormile[1], G. Varriale[1], O. Catapano[3], G. Spadaro[1,4], M. Mormile[5] and L. Postiglione[1,4]

[1]Department of Translational Medical Sciences, University of Naples "Federico II", Naples, Italy; [2]Institute of Experimental Endocrinology and Oncology "G. Salvatore" (National Research Council), Naples, Italy; [3]Italian Association of Pharmacogenomics and Molecular Diagnostics, Ancona, Italy; [4]Center for Basic and Clinical Immunology Research (CISI), University of Naples Federico II, Naples, Italy; [5]Department of Clinical and Surgical Medicine University of Naples "Federico II", Naples, Italy

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Molecular characterization of IgE reactivity of specific individual components of allergenic extracts is now possible due to the technology of recombinant allergens derived from studies of molecular biology of allergic pathology. The identification of the immunoreactivity to single allergenic components in allergic subjects allows to specifically define her/his allergic profile and obtain the so-termed Component Resolved Diagnosis (CRD). Molecular allergens can be classified into those that induce the respiratory allergic reactivity and those that identify the food-related allergic pathology. It is also essential to identify those molecular allergens whose immunoreactivity is able to connect the two clinical conditions: respiratory symptoms and food allergy symptoms. The present study was conducted on 50 patients with a clinical history of hypersensitivity to pollen and/or allergy and positivity to Skin Prick Test. The sera were analyzed in our laboratories and the panel of recombinant allergens was applied in the case of positivity of the specific IgE. Of the 50 patients enrolled, 31 were selected as positive to 4 main pan-allergen Bet v1, Par j2, Art v1 and Phl p1; among these, 14 subjects showed one allergen-specific IgE towards natural extracts of tested foods even in absence of clinical history. CRD allows for an increased accuracy in allergy diagnosis and prognosis and plays an important role in: a) resolving genuine *vs* cross-reactive sensitization in poly-sensitized patients, b) assessing, in selected cases, the risk of severe, systemic *vs* mild, local reactions in food allergy, and c) identifying patients and triggering allergens for specific immunotherapy (ITS). In light of our results, we believe that the transition from a diagnostic based on the use of allergenic extracts to another one based on the use of single allergenic molecules that is able to define the specific allergenic profile of each patient, seems to be able to revolutionize the allergy diagnosis.

Allergic diseases are atopic pathologies the characteristic of which is the presence of a particular class of specific antibodies, IgE. The term 'atopic' indicates the inherited trend of an individual to produce a high level of IgE reacting to a normally harmless antigen, called 'allergen' (1). The allergic inflammation is due to a cellular-mediated response, after binding between allergen and complex specific IgE/high affinity receptor on the surface of basophil and mast cells, through degranulation

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Corresponding Author:
Loredana Postiglione, MD
Department of Translational Medical Sciences,
Center for Basic and Clinical Immunology Research (CISI),
University of Naples "Federico II", Naples, Italy
Tel.: +39 817463016/817463321
e-mail: loredana.postiglione@unina.it

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of soluble mediators, such as hystamine, serotonin, proteolitic enzyme (pre-formed), leukotrienes and prostaglandins (newly synthetized) (2-4); in addition, there is a secretion of cytokines by T lymphocytes, such as IL-2, IL-4, IL-6, which stimulate proliferation of T cells and B-cell activation in plasma cells to produce specific IgE antibodies (5, 6). The pathogenesis of the allergy is multifactorial for the combination among various genetic, environmental, predisposing and competitor factors.

Allergic disease can manifest as respiratory response (asthma or rhinitis), skin reaction (urticaria or dermatitis), gastrointestinal symptoms, or anaphylactic shock. For example, in susceptible hosts, an allergic response can be evoked by repeated inhalation of spores such as Aspergillus (7, 8). These spores become trapped in the thick sputum of asthmatic subjects, causing a cascade of inflammatory reactions in atopic patients, frequently with a consequent pulmonary fibrosis, as in patients with cystic fibrosis (9), asthma (10), chronic diseases of nasal mucosa (11) or some autoimmune diseases (12, 13).

The allergy diagnosis consists of the assessment of clinical forms and of the ability to correlate one case to a pathological condition, through a structured diagnostic process which provides investigations of first level, as *in vivo* investigations, Skin Prick Test, or Patch Test (14); second and third level, as *in vitro* measurement of total and specific IgE on the patient's serum using natural allergenic extracts (15, 16).

The use of recombinant allergens has enriched the *in vitro* diagnostic process, and overcomes many of these difficulties; in fact, they have a defined allergen composition, are quantifiable and standardized (17). The concept of using defined allergenic molecules compared to extract (mixture of allergenic and non-allergenic components) for the diagnosis of type I reactions, is defined Component Resolved Diagnosis (CRD) (18). Thanks to new acquisitions in the field of molecular allergology, allergies could soon be framed as a process of sensitization towards a group of allergen with defined biochemical functions (19). The use of combinations among recombinant allergen, resulting from the sum of relevant antigens of certain allergenic sources, is essential to obtain reliable and reproducible diagnostic tests. A positive reaction to a potential cross-reactive allergen can allow to predict allergic reaction to all sources containing immunologically-related molecules (20-23). However, some inconvenience is possible; in fact, some recombinant allergens possess a minor binding ability for specific IgE compared to the corresponding natural allergens; this is due to the existence of multiple allergenic isoforms with different binding affinities to the antibodies, and also little variations of the amino-acidic sequence can edit the binding site (24). Some of these isoforms, lacking epitopes, have extremely low allergenicity, but they are able to induce response in the T lymphocytes, essential requirement for the induction of tolerance; and the same effect is obtained by the fragmentation in non-anaphylactic portions (25). The clinical use of these hypo-allergenic recombinant molecules allows to reduce the risk of anaphylaxis during immune-specific treatment (ITS) (26). The WAO-ARIA-GA^{2}LEN consensus document on Molecular-based allergy (MA) diagnostics provides a practical guide for the indication, determination and interpretation of MA diagnostics for clinicians trained in allergology (27).

The aim of this study is to highlight the presence, in serum of patients with allergic manifestations towards inhalant/pollen, of specific IgE to the main molecular component of the most common pollens, such as Bet v1, Par j2, as a deeper, correlating diagnostic allergen extract and respective recombinant allergens (28, 29).

MATERIALS AND METHODS

Study subjects

This study was conducted on 50 patients (31 males and 19 females; mean age 35.6 years) and 20 healthy control subjects (12 males and 8 females; mean age 30.6 years) enrolled in the A.O.U. "Federico II", Naples, Italy. All the subjects enrolled gave informed consent to participate in the study. All the patients presented a clinical history of hypersensitivity to pollen and/or allergy (oral allergy syndrome with symptoms: rhinitis, conjunctivitis, asthma, gastrointestinal symptoms, contact urticaria, dermatitis, anaphylaxis, atopic eczema; reactivity to known allergen sources) and positivity to Skin Prick Test and Spirometry Test. All control subjects underwent the same *in vivo* and *in vitro* determinations as the patients – an open oral

challenge test and rubbing test – and the results of both assays were negative. Spirometry is used to diagnose asthma and other condition that affect breathing. The sera were analyzed in our laboratories.

Skin Prick Test

Skin Prick Test is an essential test procedure to confirm sensitization in IgE-mediated allergic disease in all subjects and was performed on the forearms of all individuals. The recommended method of prick testing includes the appropriate use of specific allergen extracts, positive and negative controls, interpretation of the tests after 15-20 min of application, with a positive result defined as a wheal ≥3 mm diameter. It can help to confirm the diagnosis of a suspected type I allergy. The test is minimally invasive, inexpensive, results are immediately available and, when carried out by trained health professionals, reproducible.

Component Resolved Diagnosis

The level of awareness towards the main inhalant allergen was evaluated by specific IgE Assay (ImmunoCAP 250; Phadia, Sweden). The principle of this test is a sandwich immunoassay using a solid phase, "ImmunoCap", which consists of a cellulose derivative content in a capsule; this solid phase has an approximately 150-fold higher capacity than a traditional coated tube. The polymer inside the 'cap' irreversibly binds the allergens keeping their structure intact, then the allergen reacts with specific IgE of the serum. Fluorescence of eluate is used, by which it derives the concentration (through a standard curve) expressed in kUA/L. For the reliability of the results provided at the end of the reaction, both total IgE and specific IgE control samples were inserted.

Statistical analysis

For the assessment of the significance of the results obtained, Variance Analysis was used for unpaired data with significance $P \leq 0.05$. Fisher's Exact Test was used to evaluate recombinant allergens *vs* food natural allergens.

RESULTS

In the sera of patients with suspected allergy to major inhalant allergens, awareness of pollen allergen extract was assessed; subsequently, for testing positive patients, the respective recombinant components were assessed. The panel of recombinant allergens was applied in the case of positivity of the specific IgE (≥0.50 kUA/L) according to Table I.

Table I. *Deepening with recombinant allergens as a function of the relative extractive allergens*

Extractive Allergen	Recombinant Allergen
Birch (t3, t4, t7)	Bet v1, Bet v2, Bet v4
Gramineae (g5, g6, g8)	Phl p1, Phl p5b, Phl p7, Phl p12
Pellitory (w19, w21)	Par j2
Composite (w6)	Art a1

Table II. *Cross-reactivity between inhalant allergen and foods*

Inhalant Recombinant Allergen	Cross-reactivity Foods
Birch (Bet v1)	Apple, peach, pear, apricot, plum, walnut, cherry, banana, fennel, carrot, strawberry
Pellitory (Par j2)	Basil, cherry
Gramineae (Phl p1-Phl p5)	Kiwi, wheat, plum, peach, apricot, cherry
Composite (Art a1)	Apple, fennel, carrot, walnut, banana

In some patients sera, we found high concentration of specific IgE for inhalant recombinant allergens, the so-called 'pan-allergens', present in numerous extracts, despite their taxonomic distance. In these sera, the possible sensitization to some foods was evaluated. The tested foods are shown in Table II.

A panel of 9 natural and purified allergens and 9 recombinant allergens was used to establish the profiles of molecular sensitization. The levels of the specific IgE ≥ 0.50 kUA/L (natural allergens) or ≥ 1.00 kUA/L (recombinant) were considered positive. The molecular allergo-gram obtained with the recombinant allergen in the 50 enrolled patients highlighted the presence of allergen-specific IgE compared to the chosen recombinants in most samples. The positivity in many patients to pan-allergens, such as Bet v1 and Par j2, suggested us the opportunity to evaluate even the possible sensitization to some foods that express the recombinant molecules under study. We used a panel of 14 natural extracts for foods, and the concentration of the specific IgE ≥ 0.50 kUA/L is considered positive.

Measurement in vitro of specific IgE to inhalant extractive (pollen) and recombinant allergen

Gramineae. Considering the cross-reaction of various grass families, we used three extractive allergens for screening tests: g5, g6, g8 resulted positive in 64%, 62%, 66% of patients, respectively. These patients were tested with recombinant allergens and showed positivity to the major allergen of grass - Phl p1 in 65% of the cases (Fig. 1). Some patients were positive also to profilines and/or calcium-binding proteins, providing information for therapeutic decisions.

Pellitory. 74% of patients showed sensitization to this allergen, w19 and w21, so concentrated as to

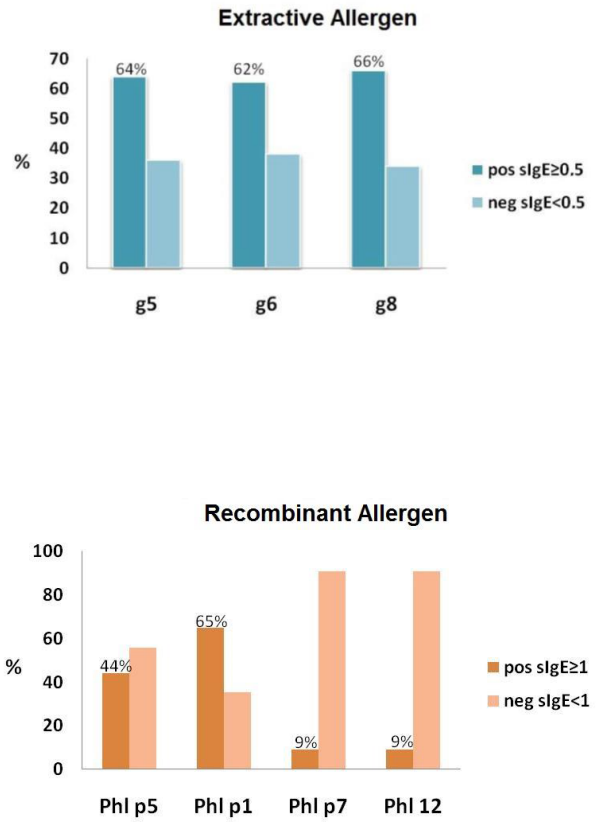

Fig. 1. *Reactivity profiles of specific IgE for gramineae: positivity percentage for extractive and recombinant allergens in patients. The levels of the specific IgE ≥ 0.50 kUA/L (natural allergens) or ≥ 1.00 kUA/L (recombinant) were considered positive – Gramineae*

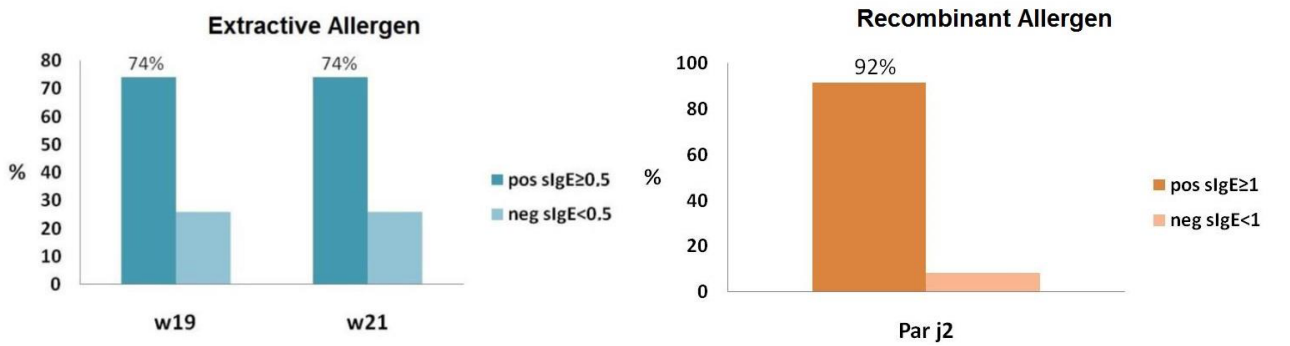

Fig. 2. *Reactivity profiles of specific IgE for pellitory: positivity percentage for extractive and recombinant allergens in patients. The levels of the specific IgE ≥ 0.50 kUA/L (natural allergens) or ≥ 1.00 kUA/L (recombinant) were considered positive – Pellitory*

perform deepening tests according to the molecular recombinant allergen chosen, Par j2, to which 92% of patients resulted positive (Fig. 2).

Composite. The percentage (48%) of patients positive to extractive allergen w6 was lower, and 42% of these resulted positive to the recombinant Art v1 (Fig. 3).

Tree- Birch. Patients positive to extractive allergens t3, t4, t7 were 22%, 22%, 20%, respectively, of whom 19% were positive to recombinant allergen Bet v1 (Fig. 4). Bet v1 was chosen as a marker of specific sensitivity towards birch (5 positive patients) and resulted in potential responsiveness to ITS, and two markers of specific sensitivity: a profiling (Bet v2, with a positive response in 4 patients) and a calcium-binding protein (Bet v4, positive in 3 patients).

Measurement in vitro of specific IgE of natural allergen for foods: cross-reactivity

Of 50 patients enrolled, 31 (62%) were selected as positive to 4 main pan-allergens Bet v1, Par j2, Art v1 and Phl p1. Among these, 14 (45%) subjects showed one allergen-specific IgE towards natural extracts of tested foods even in absence of clinical history. These data were evaluated by Fisher's Exact Test (Table III). Remarkable significance was noticed, as the subjects resulting positive to the main recombinant

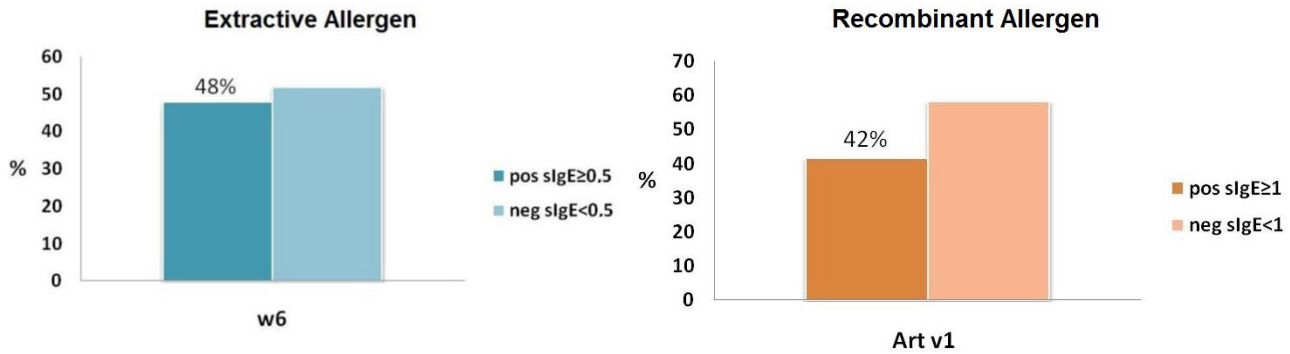

Fig. 3. *Reactivity profiles of specific IgE for composites: positivity percentage for extractive and recombinant allergens in patients. The levels of the specific IgE ≥ 0.50 kUA/L (natural allergens) or ≥ 1.00 kUA/L (recombinant) were considered positive – Composite*

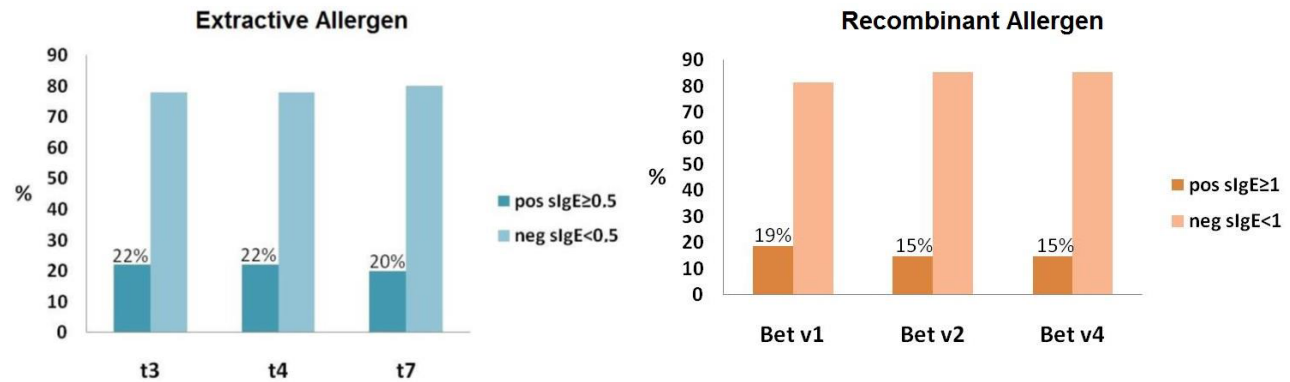

Fig. 4. *Reactivity profiles of specific IgE for tree birch: positivity percentage for extractive and recombinant allergens in patients. The levels of the specific IgE ≥ 0.50 kUA/L (natural allergens) or ≥ 1.00 kUA/L (recombinant) were considered positive – Tree birch*

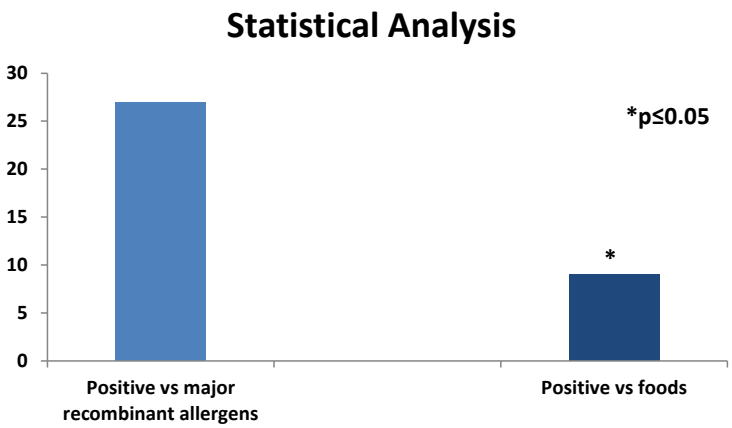

Fig. 5. *Difference between recombinant allergens and specific IgE in patients positive to tested foods*

Table III. *Inhalant recombinant allergen and food natural extractive allergen: positivity in N=14 asymptomatic patients.*

	Recombinant Allergen				Food Natural Allergen													*p*
Pz	Betv1	Phlp1	Parj2	Artv1	f95	f94	f237	f255	f242	f92	f256	f276	f31	f84	f269	f44	f49	f4	
2	4.52	0.01	8.74		26.1	18.7	13.8	19.1	15.2	0.95	11.6	5.51	1.27	1.01		8.92	18.2	1.03	0.0023
9		4.56	10.7		3.02			5.5											0.45
10			58.3		10.4														Nd
18		4.61	25.8		20.6			16											Nd
19			39.3		3.54			3.44											Nd
20			8.15		12.9												9.83		Nd
23		1.6	4.67		1.50	0.89	0.77	1.04	1.15		0.6	0.47	0.9			0.54	1.02		0.67
25		32.00	1.45	1.13	3.50	1.37	0.55	1.08	1.48	0.78	0.95	0.72	9.35			0.98	0.99	0.93	0.31
26	15.40	9.13	30.60	1.94	3.50	1.36	1.08	0.96	2.15	0.56	1.21		1.33	0.49		1.00	1.25	0.94	0.019
29		5.69	8.4		16.00				10.8										0.045
42	6.89	4.84	15.7		0.55	0.93			0.46				0.38				0.56	0.65	0.011
43		7.52	27.9	9.04										3.1					Nd
45	3.58			1.03									0.43						Nd
50		20.50	93.90	3.32	4.76	4.82	3.41	5.12	5.16	3.22	4.75	5.27	5.56	4.63	0.42	4.32	3.48	4.96	>0.001

pan-allergens resulted positive to natural extracts of the tested foods. The statistical analysis shows a significative difference ($p \leq 0.05$) (Fig. 5).

Evaluation of candidate patients for Specific Immune Therapy

The analysis of molecular allergens resulted useful to study subjects to candidate for immunotherapy. Recent studies on animal models of molecular allergens of gramineae suggest, in case of positivity, at least one of the major allergens Phlp1 and Phlp5; there is a good possibility of response to ITS, while the response is poor in case of positivity only to Phlp7 and Phlp12 (Fig. 6). In the results outlined, therefore, at least 15% of the examined subjects would be possible candidates for specific immunotherapy. Instead, for a correct prescription of ITS for pellitory, it is necessary to confirm the

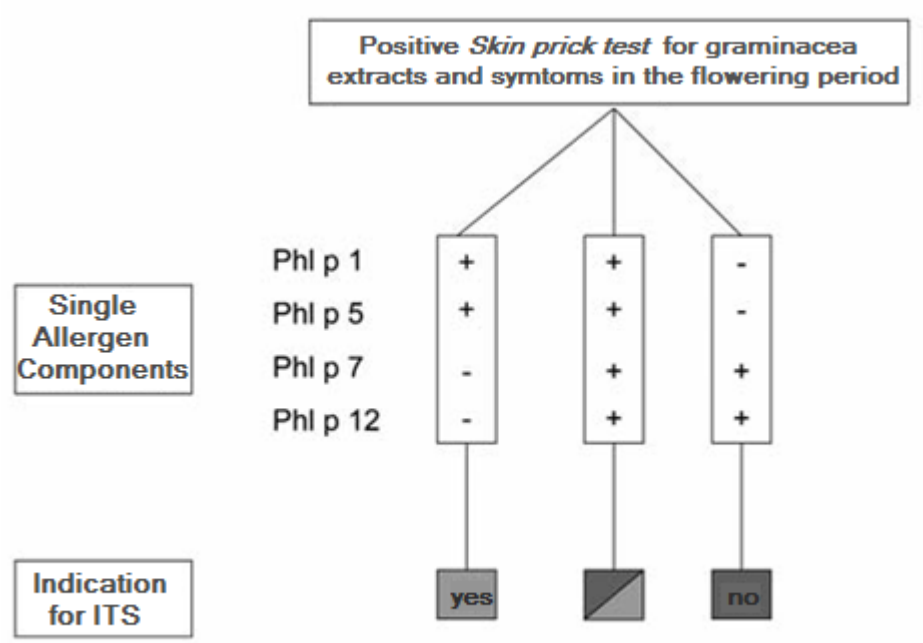

Fig. 6. *ITS and allergen recombinant: algorithm*

positivity to the specific IgE for major allergen Parj2 (positive to 37% of cases).

DISCUSSION

The analysis of the data obtained indicates a good correlation between clinical history, Skin Prick Test and specific IgE levels, evaluated *in vitro* using a panel of natural allergen extracts. CRD with recombinant allergens shows the presence of specific IgE against some molecular components of the pollen. The diagnostic tests, enriched with recombinant tests, allow to design specific immunologic profile, that constitutes a useful tool to better manage any poly-sensitized patient and to contribute to a personal appropriate therapeutic treatment.

The identification of Pan-allergen helped us to understand how patients can develop allergy symptoms, often serious, after contact with allergens from different sources and apparently not correlated. A correct diagnosis is difficult with one source containing several different allergens, however recombinant pan-allergen may be predictive of food allergy. Furthermore, the use of a single allergen component allows progress of the appropriateness of specific immunotherapy ITS. In fact, it is very important to select the right patients for ITS by researching the allergen responsible for the symptomatology.

CRD allows to choose patients for immunotherapy, especially thanks to the detection of those who are allergic to pan-allergen and of the cross-reactions between related species. ITS with extracts often shows low efficacy due to the levels of only 30-40% of antigenic proteins; the recombinant is a purified molecule, so it is 100% efficient for optimal doses, and the use of hypo-allergenic forms of recombinant is possible to reduce adverse reaction. These molecules of new application are missing from certain epitopes recognized by IgE, but they are able to induce response in T-helper lymphocytes, but not to trigger a considerable allergic reaction making ITS safer (30).

Allergies have a common pathogenetic mechanism in which various factors interact by diversifying the clinical manifestation. This mechanism is recallable to release vasoactive amine after interaction with the allergen – immune-globulins of E class and relative receptor activation on basophils/mast cells, with effects on various organs and systems. These

pathological reactions affect children and adults of both sexes in an equivalent way. Therefore, although allergic manifestations have general features regarding how they were triggered, their clinical heterogeneity often poses problems with diagnostic framing.

The transition from a diagnostic based on the use of allergenic extracts to another one based on the use of single allergenic molecules that is able to define the specific allergenic profile of each patient (CRD), seems to be able to revolutionize the allergy diagnosis. The use of recombinant allergen allows to selected candidate patients to specific hypo-sensitization therapy, as well as to make a better diagnostic framing by identifying the allergenic source responsible for the rising awareness by distinguishing between mono-sensitized and poly-sensitized subjects. We can then discriminate whether the poly-awareness is due to co-awareness, that is more primary sensitivity towards major or minor allergens present in the different allergenic sources, or cross-reactivity towards homologue molecules present in different species, sometimes not taxonomically related, which are often clinically meaningless.

Today we no longer have an allergen classification based on distinction among pollen, mold, epithelia, dust mites, etc., but a new classification based on different families of molecules, such as PR-10 proteins (Bet v1 homologues), major allergen of grass and epithelia, lipid-transfer proteins, tropomyosins, storage proteins of dried fruit, calcium-binding proteins, profilins and carbohydrate determinants (CCD).

The present study shows a significative incidence of food allergy in patients without clinical history for these events, but affected by pollen allergy in presence of positivity for a principal pan-allergen such as Bet v1, Par j2, Art v1 and Phl p1.

The laboratory plays the most important role in this revolution and, in close collaboration with the clinical side, should evaluate and validate new diagnostic systems so that they become really important and necessary to the allergic diagnostics, avoiding producing reports of dubious and not immediate interpretation. The task of the laboratory is therefore to provide one allergo-gram, specific for each patient, accompanied by an interpretative report that provides the clinician with useful elements for a correct diagnosis and a better therapeutic route to improve the life expectancy of allergic patients.

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