

The spectrum of olive pollen allergens. From structures to diagnosis and treatment



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ABSTRACT

Olive tree is one of the main allergy sources in Mediterranean countries. The identification of the allergenic repertoire from olive pollen has been essential for the development of rational strategies of standardization, diagnosis, and immunotherapy, all of them focused to increase the life quality of the patients. From its complex allergogram, twelve allergens – Ole e 1 to Ole e 12 – have been identified and characterized to date. Most of them have been cloned and produced as recombinant forms, whose availability have allowed analyzing their three-dimensional structures, mapping their T-cell and B-cell epitopes, and determining the precise allergenic profile of patients for a subsequent patient-tailored immunotherapy. Protein mutant, hypoallergenic derivatives, or recombinant fragments have been also useful experimental tools to analyze the immune recognition of allergens. To test these molecules before using them for clinic purposes, a mouse model of allergic sensitizations has been used. This model has been helpful for assaying different prophylactic approaches based on tolerance induction by intranasal administration of allergens or hypoallergens, used as free or integrated in different delivery systems, and their findings suggest a promising utilization as nasal vaccines. Exosomes – nanovesicles isolated from bronchoalveolar lavage fluid of tolerogenic mice – have shown immunomodulatory properties, being able to protect mice against sensitization to Ole e 1.

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1. Introduction

Olive (*Olea europaea*) pollen is one of the most important causes of pollinosis in the Mediterranean basin, and reaches the first position in several Spanish and Italian provinces where the cultivation of the olive tree is very intensive [1–3]. The origin of this long-life tree seems to be Asia Minor, where the birthplace of the cultivated olive tree took place six millennia ago. From there it spread to Europe and North Africa, and in the sixteenth century to the American Continent, Mexico, California, Peru, Chile and Argentina. More recently the olive tree has continued to expand and today is farmed in South Africa, Australia, Japan and China. The olive tree, which presents around 3000 varieties, is source of wood, fruit and oil, the latter being much appreciated because of its healthy properties [4]. The olive pollen displays the suitable conditions to be allergenic: it is small (around 18 µm), spread by the wind (anemophilous), and their daily pollen counts reach in many areas values higher than 5000 grains/m³, maintaining these levels for weeks [3–7]. Allergy to olive pollen is well documented

in countries such as Spain or Italy where the pollination season – from middle of April to the end of June – lead to a high number of affected individuals and frequently severe symptoms. An interesting feature of the olive pollinosis is the high dependence on the gradient of pollen counts, a fact that strongly influences the sensitization of patients to different allergens: thus, the prevalence of several allergens, minor in certain populations living in low pollen-count regions, turn into major allergens in areas of raised pollen charge [8–13]. To make matters worse, these minor allergens are related to severity and/or persistence of symptoms. The olive tree belongs to the Oleaceae family which also includes ash (*Fraxinus* L.), privet (*Ligustrum* L.), lilac (*Syringa* L.), forsythia (*Forsythia* L.), and jasmine (*Jasminum* L.) genus, some of which were also reported as cause of allergy processes. Interestingly, ash pollen has been recently recognised as a significant allergy inducer in central Europe and clinicians are paying attention to the study of its allergens [14].

The protein extract of olive pollen presents a complex and varied allergogram: at least twenty IgE-reacting protein bands appear in Western blots immunostained with sera from olive allergic individuals [9,10,15–21], and patients can be sensitized to very different combinations of them. In Fig. 1A, a diversity of allergenic profiles obtained after SDS-PAGE and immunoblotting with sera from allergic patients are shown. Immunoblotting results agree

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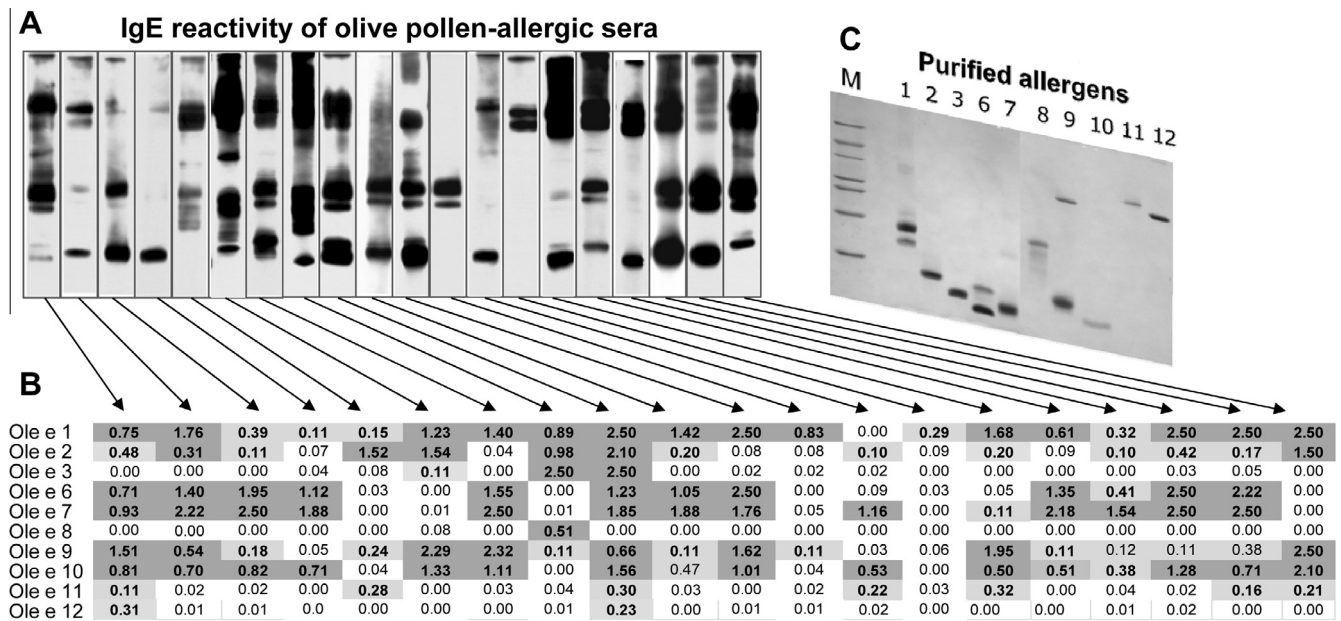


Fig. 1. Allergic profile of olive pollen-allergic patients and purified allergens. Immunoblotting of sera from twenty olive pollen allergic patients shows very different IgE-binding profiles (A), which correlate well with ELISA values obtained with purified allergens (B) SDS-PAGE stained with Coomassie Blue of purified allergens from olive pollen (C). Ole e 1 shows the glycosylated (20 kDa) and non-glycosylated (18.5 kDa) forms. Ole e 6 shows the bands that correspond to different conformations. Ole e 9 corresponds to a mixture of N-terminal and C-terminal domains. ELISA values are given in optical density units; dark, light, and no shadow correspond to >0.5, 0.1–0.5, and <0.1 (negative), respectively.

with the data obtained from ELISA experiments (Fig. 1B) by using purified allergens [13,22,23]. At present, twelve allergenic proteins (Ole e 1 to Ole e 12) with diverse clinical incidence have been identified in olive pollen [11,24] (Fig. 1C), and the use of the powerful proteomic tools promises new future findings.

2. Identification and characterization of allergens

The knowledge of the allergenic profiles of a biological source is a prerequisite for any improvement in the design of protocols for a correct diagnosis and an adequate immunotherapy. The use of purified allergens, rather than the extracts currently available, increases the possibility of identifying the sensitizing antigen. The big efforts carried out in the past two decades to identify the most significant allergens of olive pollen have been essential to know which is the allergen or allergens responsible for the sensitization of each particular patient (Table 1).

2.1. Sensitivity to Ole e 1 indicates genuine sensitization to Oleaceae pollens

From pioneering studies on olive pollen allergy, a major IgE-binding protein was detected [15,16,18,25,26]. It was named Ole e 1 [27] and characterized as a glycosylated protein [28,29]. To date no biological function has been assigned to this allergen, but it seems to be involved, by homology to the cysteine-rich extracellular protein LAT52, to pollen hydration and/or germination processes [30]. Ole e 1 is an abundant protein of the pollen and probably this is the reason because it displays very high values of allergenic incidence: most of the population allergic to olive pollen presents around 80% of prevalence for Ole e 1. It is the best characterized allergen from olive, specifically expressed in pollen and therefore not present in olive fruit. The protein has a length of 145 amino acids (16,331 kDa) with a partially N-glycosylated Asn at position 111. Both protein and the oligosaccharide show a high degree of polymorphism and contribute to the allergenicity of the molecule [31–34]. The ultrastructural location of Ole e 1

has been determined [35,36] and suggests the involvement of the allergen in secretion routes that would be in agreement with a suggested biological role in fertilization processes. The association between the IgE response to Ole e 1 and the DR7-DQ2 haplotype [37], and the analysis of the T-cell and B-cell epitopes have been also reported [38–40].

Allergenic counterparts of Ole e 1 have been reported in other pollens, and they constitute the ubiquitous Ole e 1-like family. The proteins exhibit a broad degree of amino acid sequence identity, being high (>80%) among Oleaceae members (Fra e 1 from ash, Lig v 1 from privet, and Syr v 1 from lilac) [21,35,41–45] and low (25–60%) within not botanically related species such as ryegrass (*Lolium perenne*), Lambs' quarter (*Chenopodium album*), English plantain (*Plantago lanceolata*), rice (*Oriza sativa*), maize (*Zea mays*), tomato (*Lycopersicum esculentum*), birch (*Betula verrucosa*), rapeseed (*Brassica napus*), Canary grass (*Phalaris coerulescens*) or mouse-ear cress (*Arabidopsis thaliana*) [21]. The high similarity among Oleaceae members of the Ole e 1-like family explains their high cross-reactivity reported, although there are patients specifically reactive to a single member of the family [11]. The character of Ole e 1 as a marker of primary sensitization to Oleaceae pollens has been demonstrated [46]. Ole e 1 [29,47], Oleaceae-homologous proteins [39], as well as several mutants [48] have been recombinantly produced and used to study diverse immunological properties of the allergen (Fig. 2).

2.2. Panallergens: profilin and polcalcain

Several allergens are involved in vital functions and therefore are ubiquitously distributed in many biological sources. These pan-allergens are responsible for the polysensitization observed in many patients. Their conserved structures allow the allergen-specific IgE cross-react with homologues belonging to the family. Profilin has been isolated from olive pollen and identified as an allergen. It has named Ole e 2 [49]. Molecular and immunologic properties of Ole e 2 do not significantly differ from those of other profilins, exhibiting a prevalence of around 15% in patients allergic

Table 1

Features of the allergens identified in olive pollen and fruit.

Allergen	Tissue	Family	Ref. ²	pI ³	Molecular mass ⁴ (kDa)	Glycosyl ⁵	Enzymatic Activity ⁶	Cross-react ⁷
Ole e 1	Pollen	Ole e 1-like	[27]	5.5–6.5 ^e	16.3/18.5 ^m	Yes	ND	Oleaceae ^a
Ole e 2	Pollen	Profilin	[49]	5.1 ^t	14–16 ^s	No	No	Panallergen ^b
Ole e 3	Pollen	Polcalcin	[55]	4.3 ^e	9.2 ^m	No	No	Panallergen ^c
Ole e 5	Pollen	Superoxide dismutase	[71]	5.7 ^t	16 ^s	ND	ND	ND
Ole e 6	Pollen	Unknown ¹	[72]	4.2 ^e	5.8 ^m	No	No	Oleaceae ^a
Ole e 7	Pollen	LTP	[58]	>9 ^e	9.9–10.3 ^m	No	No	Oleaceae ^a
Ole e 8	Pollen	Four EF-hand	[74]	4.5 ^t	18.8 ^m	No	No	Moderate ^d
Ole e 9 ⁸	Pollen	1,3-glucanase	[59]	4.8–5.4 ^e	46.4 ^m	Yes	Yes	Yes
Ole e 10	Pollen	CBM-43	[68]	5.8 ^t	10.8 ^m	No	No	Yes
Ole e 11	Pollen	Pectin methylesterase	[77]	6.3–9.3 ^e	39.6 ^m	Yes	Yes	Moderate ^e
Ole e 12	Pollen	Isoflavone reductase-like (PCBER)	[79]	4.8–5.7 ^e	33.9 ^m	No	Yes	ND
Ole e 13	Fruit	Thaumatin-like	[80]	4.3–4.8 ^e	23 ^s	No	No	ND

¹ Ole e 6 is a small cysteine-rich protein which is not assigned to any known protein family.² Only the references corresponding to allergen identification are indicated.³ Isoelectric point (pI) were determined experimentally (^e) or calculated from the amino acid sequence (^t).⁴ Molecular masses were obtained by mass spectrometry (^m) or SDS-PAGE (^s). For Ole e 1, Mw of glycosylated and non glycosylated forms are indicated.^{5,6,7} ND = Not determined.⁷ Ole e 1, Ole e 6, and Ole e 7 exhibit cross-reactivity only with homologous counterparts from taxonomically closely related species^a; the profilin Ole e 2 is a panallergen involved in the pollen-fruit-latex syndrome^b; Ole e 3 is a panallergen whose cross-reactivity is exclusively restricted to pollens^c; Ole e 8 exhibits moderate cross-reactivity intra-species to Ole e 3 and inter-species to other *Oleaceae* and *Juniperus oxycedrus* homologues^d; Ole e 11 exhibits cross-reactivity to the pectin methylesterase from *Salsola kali*^e.⁸ Ole e 4 seems to be a degradation product of Ole e 9.

to olive pollen. Ole e 2 has been sequenced and produced in *Escherichia coli* and displays 70–90% identity with other allergenic profilins [50], that goes along with the cross-reactivity data [49,51–53].

Ole e 3 is a small pollen-specific allergen that binds calcium ions, thus premiering the “polcalcin” family [54,55]. This is a widely distributed panallergen family with high amino acid sequence conservativeness (71–96% similarity, 62–90% identity). Therefore, members of this family exhibit high degree of cross-reactivity. Ole e 3 has been assayed for their IgE cross-reactivity with counterparts from lilac and chenopod [56]. When compared the IgE- and IgG-binding capacity with four-EF hand calcium binding proteins such as calmodulin and Ole e 8, they show no competitive epitopes [57].

2.3. Allergens as markers of severe allergy

Ole e 7 (10 kDa) and Ole e 9 (46 kDa) belong to well known biochemical protein families: non-specific lipid transfer proteins (LTPs) [58] and 1,3-β-glucanases [59], which are included into the pathogenesis-related (PR) protein families PR-14 and PR-2, respectively. Interestingly, the prevalence of these allergens is strongly dependent on the geographic area of sensitization. They exhibit a scarce clinical incidence (frequently under 10%) in zones where the airborne levels of olive tree pollen are low, such as the cities from the continental platforms of Mediterranean countries (e.g. Madrid and Navarra). However, in places where the pollen counts reach up to 5000 per m³ (certain regions of Andalucía such as Jaén and Córdoba, or Apulia in Italy), Ole e 7 and Ole e 9 become highly prevalent allergens with sensitization rates of 50–60% among the allergenic population (Fig. 3) [14,58,60]. In addition, these allergens have been shown as markers of more severe allergy symptoms and therefore the diagnosis of sensitization to them is especially relevant in places of high charge of airborne olive pollen. As it has been shown throughout an epidemiological study performed by using a broad panel of purified allergens and a high number of clinic groups from Hospital Allergy Services (VEGETALIA network) [61], the component-resolved diagnosis can be decisive in these cases as these allergens are present in very low amounts in the pollen grain and the whole protein extract is not efficient enough in skin prick test (SPT) or *in vitro* IgE-binding quantification techniques to detect positive responses. One additional trouble is the highly variable concentration of Ole e 9 in the pollen, which reaches up to 161-fold from batch to batch [60,62].

Ole e 9 is composed of two well defined domains: an N-terminal domain with catalytic activity as 1,3-β-glycosylase and a C-terminal domain with carbohydrate-binding capacity [24]. Their specific cDNAs have been separately expressed in *Pichia pastoris* rendering two recombinant polypeptides that conserve the IgE and IgG immunodominant epitopes [63–65]. The recombinant form of the C-terminus of Ole e 9 has been used for the analysis of the three-dimensional structure of the molecule as well as to demonstrate its glycan-binding capability [24,66]. Since 1,3-β-glucanases are widely spread proteins in plants, a polyclonal antibody specific for the N-terminal domain of Ole e 9, as well as sera from olive pollen allergic patients, were used to detect the possible cross-reactivity of the allergen with ash and birch pollen, tomato, potato (*Solanum tuberosum*), bell-pepper (*Capsicum annuum*), banana and latex (*Hevea brasiliensis*), obtaining positive results [64].

In spite of belonging to a large family of lipid-transfer proteins, Ole e 7 together with Par j 1 from pellitory (*Parietaria judaica*) does not show significant sequence similarity with representative allergenic LTPs from foods such as Pru p 3 from peach (*Prunus persica*), and therefore both allergens seem to be not included in the LTP syndrome [67]. However, its putative cross-reactivity with other vegetable sources deserves a deep immunologic study.

Ole e 10 (10 kDa) is an independent protein with a significant amino acid sequence similarity to the C-terminal domain of Ole e 9, which explains its capability to bind β-1,3-glucans and the existence of inter- and intra-species cross-reactivity [68,69]. The involvement in the carbohydrate binding and its co-localization with callose (β-1,3-glucan) during pollen tube growth has been analyzed [69]. These results have allowed describing a new family of carbohydrate-binding modules (CBM-43), suggesting its participation in the carbohydrate metabolism during the pollen germination and pollen tube growth and assigning a potential regulatory role to this C-terminal domain. An association of Ole e 10 with bronchial asthma has been reported [11]. In addition, these two molecules show cross-reactivity with allergenic extracts from other plants, such as latex, fruits, and other vegetable foods [64,68].

2.4. Other allergens from olive pollen

Other olive pollen allergens have been identified along the last 15 years but their relevance has not been completely elucidated, either by the low prevalence in the analyzed population or because

Natural allergen	Engineering produced allergens	Expression host	References	Major goals
Ole e 1	Fusion protein GST-Ole e 1	Bacteria <i>E. coli</i>	29	• First recombinant olive pollen allergen
	Allergen isoforms ^a Ole e 1/3c Ole e 1/5c	Yeast <i>P. pastoris</i>	39,47 93 46 94,103 112	• Equivalence to the natural Ole e 1, epitope mapping studies and disulphide bridges assignment • Marker of <i>Oleaceae</i> sensitization • Standardization and <i>In vivo</i> diagnosis • Mouse model of allergic sensitization
	Homologues ^b rSyr v 1/rLig v 1/rFra e 1	Yeast <i>P. pastoris</i>	42,43,96 44,45	• Establishment of Ole e 1-like family in <i>Oleaceae</i> • Clinic study with rFra e 1 in a population from Strasbourg
	Polypeptide mutants 135Δ10/140Δ5/Y141A/OB ⁵⁵⁻⁵⁸ Glycan mutants mOle e 1	Yeast <i>P. pastoris</i>	98 40 48,95	• Role of major glycan moiety • Confirmation of an immunodominant epitope at the C-terminal end • Animal model studies
	Allergen derived-fragments GST-F1/GST-F2/GST-F3/GST-F4/GST-F5	Bacteria <i>E. coli</i>	40	• Epitope mapping
Ole e 2	rOle e 2	Bacteria <i>E. coli</i>	50	• Structural studies
	Homologues rChe a 2 ^c , rSin a 4 ^d	Bacteria <i>E. coli</i>	52,53 14	• Cross-reactivity studies • EXPO study
Ole e 3	rOle e 3	Bacteria <i>E. coli</i>	55	• Equivalence to natural Ole e 3 • Ca ²⁺ -binding studies
	Homologues rOle e 3/rSyr v 3	Bacteria <i>E. coli</i>	56 57	• Polcalcin family establishment • Cross-reactivity studies
	rOle e 3	Plant <i>A. thaliana</i>	88	• Stable transgenic lines.
Ole e 5	Fusion protein GST-Ole e 5	Bacteria <i>E. coli</i>	71	• Allergenic studies
Ole e 6	rOle e 6	Yeast <i>P. pastoris</i>	73 92	• 3D-structure determination (NMR) • Immunological equivalence to natural Ole e 6
Ole e 8	rOle e 8	Bacteria <i>E. coli</i>	57,74 75,76	• Intra- and inter-cross-reactivity studies • Ca ²⁺ -binding analysis
	rOle e 8	Plant <i>A. thaliana</i>	88	• Stable transgenic line
Ole e 9	Recombinant fragment N-terminal Domain	Yeast <i>P. pastoris</i>	59,65 64 100 112	• Enzymatic activity • Pollen/food/latex cross-reactivity • Occupational allergy • Prophylactic treatment in mice
	Recombinant fragment C-terminal Domain	Yeast <i>P. pastoris</i>	59,24 63 65,66 112	• Intra-cross-reactivity studies: Ole e 9-Ole e 10 • Novel family of carbohydrate-binding protein (CBM-43) • 3D-structure determination (NMR) • Prophylactic treatment in mice
Ole e 10	rOle e 10	Yeast <i>P. pastoris</i>	91	• N-terminal truncated
	rOle e 10	Baculovirus <i>S. frugiperda</i>	87	• Ole e 10 integrity
Ole e 11	rOle e 11	Yeast <i>P. pastoris</i>	77,78	• Epidemiologic studies
Ole e 12	rOle e 12	Bacteria <i>E. coli</i>	79	• Association to peach allergy

Fig. 2. Engineering produced allergens from olive pollen, expression hosts, and major goals. Recombinant expression systems used for the olive pollen allergens, as well as the type of molecule obtained, either the intact allergen or different derivatives, are indicated. ^aDifferent isoforms obtained after PCR cloning and DNA sequencing. ^bOle e 1-like homologues from *Oleaceae* family; they could be considered as natural isoforms of Ole e 1 due to the high sequence identity (>88%). ^crChe a 2 from *Chenopodium album* has been used as a profilin model for comparing to that from olive. ^dProfilins from mustard and pollens have been included in this studies.

of an incomplete characterization. However, some of them could have a potential relevance considering aspects as association to other allergenic sources, cross-reactivities, and symptoms triggered in the patients. Ole e 4 and Ole e 5 (32 kDa and 16 kDa,

respectively) have been isolated and partially characterized by Boluda et al. [70]. The latter is a Cu/Zn superoxide dismutase with a prevalence of 39% in the olive pollen-allergic population [71]. On the other hand, sequences of several peptides from Ole e 4 show

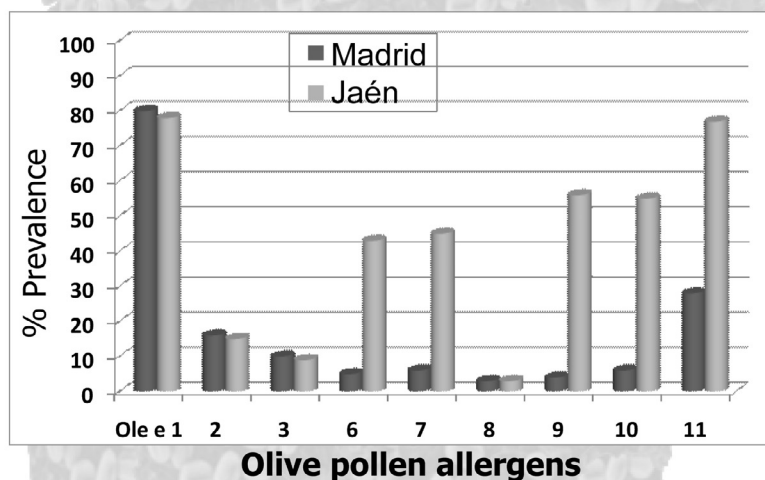


Fig. 3. Prevalence of the olive pollen allergen repertoire. IgE specific to the purified allergens from olive pollen is measured in patients from two Spanish regions, Madrid (dark grey) with low airborne pollen levels and Jaén (light grey) with high levels of pollen grains during pollination season.

high similarity to the N-terminal domain of Ole e 9. The spontaneous separation of both domains during the storage of the pollen extract or due to the isolation conditions (unpublished results), could point to Ole e 4 as a degradation product of Ole e 9. A high incidence of 80% has been reported for this allergen with a very small population of olive-allergic patients [70].

Ole e 6 (50 amino acids length, 5.8 kDa) is a very small cysteine-enriched protein [72], whose three-dimensional structure has been determined [73]. The prevalence of Ole e 6 is also dependent on the geographical area in a similar way than Ole e 7, Ole e 9, and Ole e 10 (Fig. 3). This allergen has been produced in *P. pastoris* and the molecule conserves the immunological properties of the natural protein. Interestingly, no homologues have been reported in other allergenic sources.

Ole e 8 (175 amino acids length, 18 kDa) is a calcium-binding allergen with four EF-hand sites and a very low prevalence (5%) [74,75]. It has not amino acid sequence relation with Ole e 3 apart of the calcium binding sites and no cross-reactivity has been found between them [56]. This allergen could have counterparts in other pollens, such as *Juniperus oxycedrus* [76], although the allergen described Jun o 2, with four “EF-hand” sites and 18 kDa, has not sequence similarity with Ole e 8 out of the calcium-binding sites.

Two years ago we have identified a new allergen: Ole e 11, a pectin methylesterase [77], which has been cloned, sequenced and produced in *P. pastoris*. Pectin methylesterases are widely distributed in the plant kingdom and to date only one allergenic

sequence of this protein family has been reported: Sal k 1 in Russian thistle (*Salsola kali*) pollen [78]. The allergen displays >70% sequence identity with Ole e 11. The analysis of the potential cross-reactivity among pectin methylesterases is of great interest especially when statistically significant differences were found between the prevalence to Sal k 1 in allergic patients suffering pollinosis and those sensitized to food and pollen (11.5 vs 24.3, $p < 0.001$, respectively) [61]. These studies open the door to another potential family of proteins implicated in food and pollen cross-reactivity.

Finally Ole e 12 [79], an isoflavone reductase-like protein, is an olive pollen protein that has been identified as a minor allergen with a prevalence lower than 10% even in populations with high pollen counts, but reaching levels higher than 25% when the pollinosis is associated to peach allergy.

2.5. The first allergen in olive fruit

Despite widespread consumption, scarce data on olive fruit allergy has been reported. We have found an olive-oil mill worker who suffered allergy symptoms after inhalation of particles derived of the processing of olive fruit [80]. The responsible agent was identified as an IgE-reactive 23 kDa protein of the olive fruit pulp, which showed amino acid sequence homology to thaumatin-like proteins present in plants. The isolated protein

was able to induce nasal obstruction, sneezing and runny nose when a nasal challenge test was performed in the patient [80].

3. Milestones of recombinant allergens in olive pollen allergy

From a clinical point of view, one of the aspects more relevant to consider in the allergy field is the achievement of good biological materials for the diagnosis and immunotherapy of allergic individuals. Current forms of diagnosis and immunotherapy are performed using crude extracts consisting of a mixture of different allergenic and non-allergenic components. Thus, a successful diagnosis is not attained in the 100% of the patients, and anaphylactic effects during the treatment or risks of new sensitizations are not discarded. Considering olive pollen as a raw material, many factors could be responsible for an erroneous diagnosis and a failed treatment. First of all, the high number of olive varieties growing in different cultivars [81] renders a high variability in the amount of several pollen allergens as well as their isoforms such as Ole e 9, from batch to batch [62]. These differences could undoubtedly have clinical relevance [62,82]. Secondly, some important allergens as Ole e 7 could be misrepresented in the allergenic extract and their contents could not be enough to detect an immunologic response in diagnosis protocols [9,12–14,61]. The replacement of the natural extracts with purified allergens, isolated from natural sources or recombinantly produced, can contribute to solve these problems [14]. In addition, the production of allergenic mutants, with altered immunologic properties could be feasible (Fig. 2).

The application of the genetic engineering to recombinant production of allergens with a structural and immunologic resemblance to their natural counterparts offers numerous advantages over the natural forms. Their use pretends to select a particular isoform from a polymorphic allergen [83], split an allergen in their respective domains [63,64], increase the yield of production of a minor allergen [84], change the allergenic or biochemical properties of a protein [85] or solve a tricky purification, especially when the allergens are going to be used for research or clinic purposes. The substitution by the recombinant forms has to be supported by the good quality of the protein and a high yield in its production, and both are requirements mainly dependent on the choice of the expression system [86]. In the last 15 years many molecules derived from olive pollen allergens have been produced using different expression systems such as the bacteria *E. coli* [29,50,55,70,73], the methylotrophic yeast *P. pastoris* [47,62,63,76], insect cells using Baculovirus as expression vector [87], or stable transgenic lines of *A. thaliana* [88].

3.1. Selection of the optimal system

The selection of an efficient host to produce a recombinant allergen is not a trivial task. This is the reason why the same allergen has been produced in several systems trying to get the best molecule. Ole e 1 is a good example of how the selection of an accurate expression system can lead to the success or the fail of its recombinant production. It was firstly produced in bacteria as a fusion protein linked to the glutathione-S-transferase (GST) [29]. An active protein, from an immunologic point of view, was obtained, but the yield after solubilizing from inclusion bodies and disrupting the linkage of both proteins was too low. However, when a eukaryotic host, the yeast *P. pastoris*, was employed, a high yield of a soluble secreted and proper folded protein was recovered [47]. *P. pastoris* has been used for the production of more than 600 recombinant proteins [89], many allergens among them- and fulfill main advantages as its simplicity, the availability of strong promoters to drive the gene expression, and the ability to add eukaryotic post-translational modifications [90]. This is the reason why allergens with a complex folding maintained by disulphide

bridges such as Ole e 1, Ole e 6, the N- and C-terminal domain of Ole e 9, and Ole e 10 [21,47,62,63], or glycosylated as Ole e 1, N-terminal of Ole e 9, and Ole e 11 [47,63,75] have been preferably expressed in yeast cells and obtained with a high yield. On the contrary, the bacteria *E. coli* has been successfully used to produced allergens as Ole e 2 (profilin), Ole e 3 (polcalcin) and Ole e 8 [50,55,72], as free proteins, and Ole e 5 as a fusion protein [69], because they are small proteins without disulphide bonds or post-translational modifications.

Even considering *P. pastoris* as a suitable system for many proteins, an alternative host sometimes has been necessary when the low yield in the purification or degradation were affecting to the recombinant allergen. Thus, Ole e 10 has been produced with good yield and intact structure in *Spodoptera frugiperda* insect cells using baculovirus as expression vector [87] whereas a truncated form lacking the N-terminal end was produced in *P. pastoris* [91]. Finally, *A. thaliana* has been employed with Ole e 3 and Ole e 8 rendering good and promising results in terms of native conformation and yield [88]. These stable transgenic lines of plants could offer an optimal approach for producing a protein with the appropriate post-translational modifications as the natural one or for designing edible allergenic vaccines.

The high yield obtained in several olive pollen allergen productions [92] has allowed to determine the three-dimensional structures of Ole e 6 [72] and the C-terminal domain of Ole e 9 [65], the assignment of disulphide bridges of Ole e 1 [93] and Ole e 9 [62], and the inclusion of Ole e 1 in studies of allergen standardization [94,95].

3.2. Polymorphism studies

Other important advantage of the recombinant approach has been the production of different Ole e 1 isoforms derived from the cDNA clones, rOle e 1/3c and rOle e 1/5c [39], as well as homologues from different members of the Oleaceae family, rSyr v 1 from lilac and rLig v 1 from privet [96], and rFra e 1 from ash [44], considered as natural isoforms by their high sequence identity with Ole e 1. Their availability has allowed establishing differences in the IgE-binding capabilities of different isoforms [39,40]. The recombinant production of Fra e 1, major allergen from ash pollen, allowed testing the relevance of this allergen in central Europe [44].

3.3. Genetically engineering allergens

Several strategies involve the targeted modifications of allergen-specific DNA in order to altering properties of the intact molecule in a predictable way (Fig. 2). Polypeptide fragments derived from Ole e 1 have been recombinantly obtained as fusion peptides (GST-peptides) to complete the analysis of the epitope mapping of Ole e 1. This strategy allowed the localization of an IgE immunodominant epitope in the C-terminal end of the allergen [40]. Deletion and substitution mutants generated from Ole e 1, maintaining the T-cell epitopes intact, have been produced as hypoallergenic mutants [48]. A synthetic T-cell peptide spanning the amino acid segment 109–130 of Ole e 1 (OE_{109–130}) has been used in *in vivo* and *in vitro* tests in order to choose those most suitable molecules for immunotherapy [48,97]. A non glycosylated mutant, with the Asn of the consensus site for N-glycosylation substituted by Gln, has been prepared in order to study the structural and immunologic relevance of the glycan moiety [98]. Recently, a mutant consisting of Ole e 1 with an IgE-binding region replaced by a fragment of a non allergenic homologue (BB18) from birch pollen was designed and produced in *P. pastoris* as soluble protein [99]. The recombinant molecule displayed reduced allergenicity and maintained T-cell reactivity compared to wild-type protein.

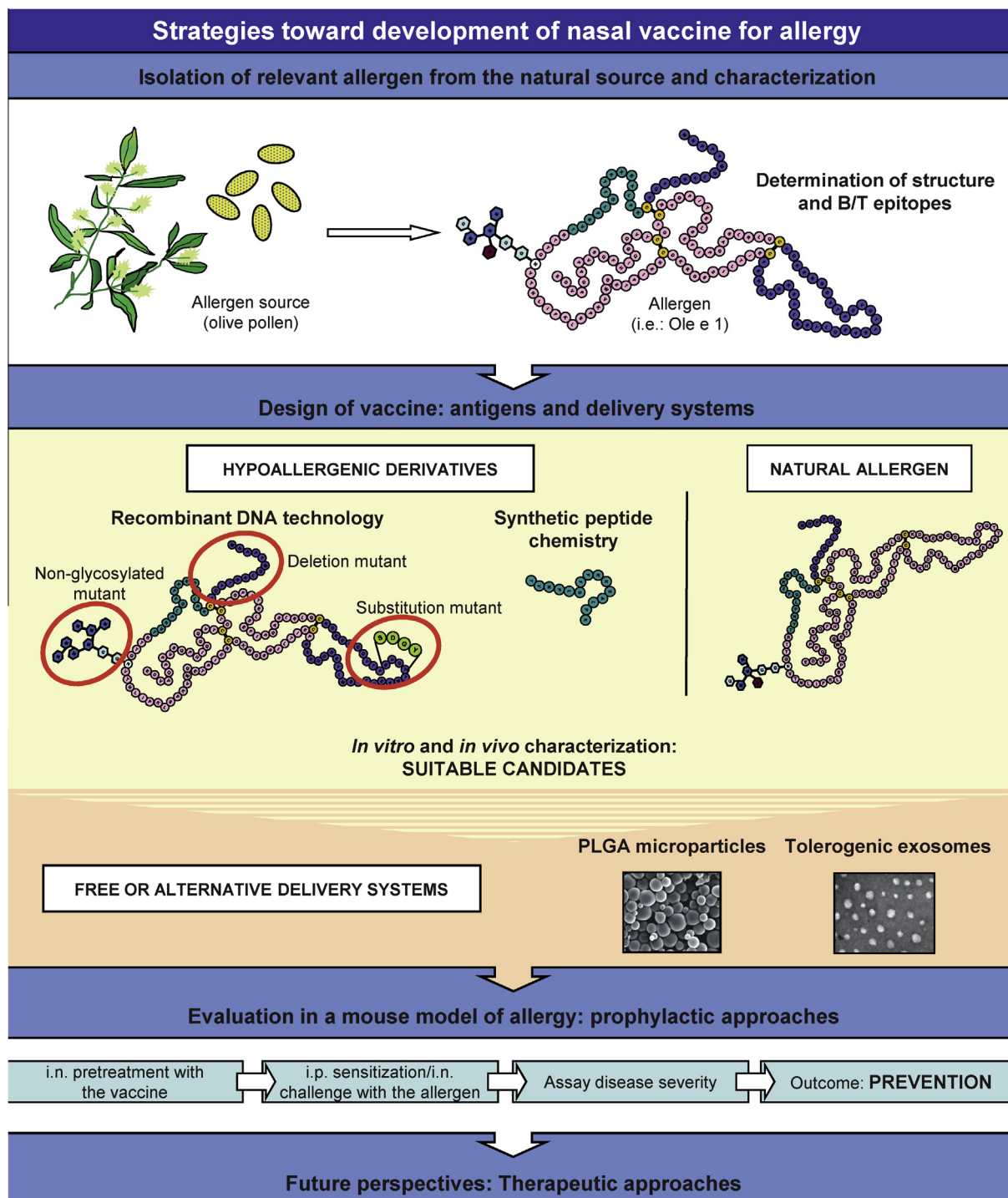


Fig. 4. From olive pollen allergen to vaccine: the design of a nasal vaccine against allergy. The prerequisite for these studies is the isolation of the relevant allergen from olive pollen. Ole e 1 was herein chosen as allergen model. Based on the molecular and immunological data, hypoallergenic allergen derivatives were produced by recombinant DNA technology or synthetic peptide chemistry. Reduced allergenic activity was evaluated *in vitro* and *in vivo* by different methods to select the best candidate vaccines. In addition, the suitability of different delivery systems for antigen administration was analyzed. Their efficacy and safety were further assayed in a suitable mouse model of Ole e 1 allergy previously established. Different prophylactic nasal vaccines, based on these Ole e 1 derivatives were tested as free or in delivery systems.

Both N- and C-terminal domains of Ole e 9 have been independently produced in order to analyze their biochemical features and their allergenic contributions of both domains as independent diagnostic values in olive pollen allergy [64]. With this strategy the presence of the N-terminal catalytic domain as a cross-reactive molecule in other pollens, vegetables and latex [63], and its ability to generate an occupational sensitization during its manipulation [100], have been demonstrated.

4. Component-resolved-diagnosis: towards a more accurate determination of the sensitization profile of olive pollen allergic patients

Polysensitization is a general feature in the allergic population from the Mediterranean area suffering pollinosis. The coexistence of many trees, weeds and grasses species, and the presence of pan-allergens and minor allergens in the extracts result in a complex

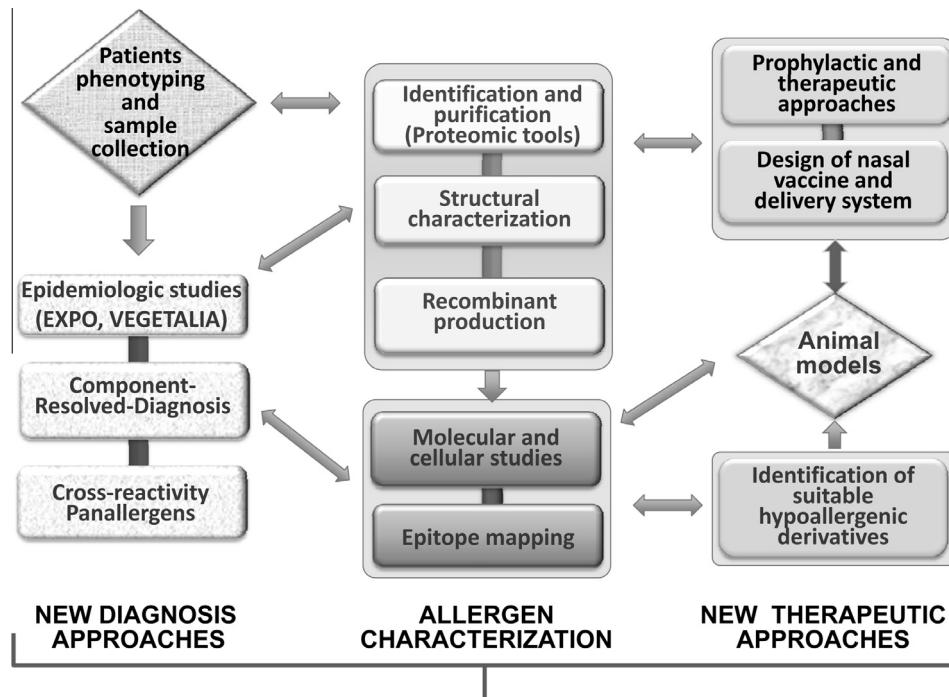


Fig. 5. Final remarks. A summary with the major goals of our research is shown. Allergen characterization, diagnosis strategies and immunotherapy approaches are the three main blocks that sustain this research.

diagnosis and often in an unsuccessful specific immunotherapy. The use of purified allergens, either natural or recombinant, can contribute to optimize the management of these patients and clarify the strategy of treatment [101,102].

This problem is especially severe in Spain where grasses, olive, Russian thistle, chenopod, mugwort (*Artemisia vulgaris*), plantain, and pellitory are common species [24]. Olive is the second cause of allergy after grasses in our country [14]. However, olive pollen counts are not homogeneously distributed along different geographical locations but a strong pollen grains gradient is detected. Whereas in the South of Spain the olive pollen can reach daily peaks of 10,000 grains/m³ during the pollination season, the populations from the center of the country barely attain 1000 counts/m³. These differences contrast with those of grasses, which show more homogeneous profiles in most of the Spanish regions. This particularity made the diagnosis of patients allergic to olive pollen much more complex since in areas of low pollen counts the patients are mainly sensitized against the major and specific marker Ole e 1 whereas minor allergens can become major allergens when the pollen counts are high enough.

An epidemiological study, “EXPO”, has risen in Spain as a cooperative multicenter research network, in which a component-resolved diagnosis has been a useful tool to analyze the associations between allergen-gradient exposure and patient sensitization [13,14]. A big population sample of pollen-allergic patients (900 patients) selected from an area where olive pollinosis plays a dominant role, as well as a full panel of purified allergens from olive pollen and a high-capacity screening methodology (ADVIA-centaur) have allowed extracting important conclusions. Whereas Ole e 1 sensitization reaches a saturation profile in terms of prevalence of around 80% [14,103] (Fig. 3), those values of minor allergens changed along with the pollen gradient, from 6% for Ole e 7 and 3% for Ole e 9 in the area of Madrid, to 43% and 55%, respectively, in Jaén. In the extremely exposed areas, patients monosensitized to Ole e 7 have been reported, which constitutes a different and unusual way to become allergic to olive pollen [104]. Moreover, the risk of severe symptoms as asthma was twice as high in patients from this population mainly

due to the sensitization to minor allergens. This correlation between symptoms and Ole e 9- and Ole e 7-specific-IgE levels makes them clinically relevant marker predictors of severity and force to introduce changes in the standardization strategy for the allergy vaccination in these patients [60]. In the other hand, the prevalence of the two pollen panallergens, profilin and polcalcin, seems to be not linked to the pollen gradient. Ole e 2 is recognized by 15% and 13% in Madrid and Jaén, respectively, and Ole e 3, by 9% and 8%.

Other interesting observation is derived from the fact that several patients submitted to immunotherapy present a clinic condition more sensitive and less tolerant at the recommended allergen dose. This association seems to be linked mainly to Ole e 7 and Ole e 9 sensitization, but not to pollen panallergens polcalcin or profilin. In this sense, Ole e 7 has been associated with an increased risk of plant-derived food anaphylaxis as well [105]. In summary, the component-resolved diagnosis represents a more accurate definition of the sensitization profile of these patients and thus will lead to better intervention.

5. New concepts for allergy vaccine development: a model using olive pollen allergens

Despite current allergen-specific immunotherapy by subcutaneous route (SCIT) can protect against respiratory allergic diseases, it has not free of several disadvantages including long treatment duration, anaphylactic side-effects and limited efficacy [106]. Therefore, new and improved vaccines are required for the treatment, prevention and cure of allergic diseases. Of particular interest in the setting of mass immunization are needle-free mucosal vaccines to induce tolerance with easier and faster delivery, improved safety and compliance, decreased cost and reduced pain compared to SCIT [107–109]. In this concern, allergen-specific sublingual immunotherapy (SLIT) has been approved as an alternative to SCIT in many countries, and standardized vaccines based on aqueous biological extracts from natural allergen sources are available as either drops or fast-dissolving tablets [109–110]. Nevertheless,

the main disadvantages of SLIT vaccines is the requirement of high-doses of allergens (at least 50- to 100-fold more than for SCIT), hold under the tongue for 1–2 min prior to swallowing, to reach similar levels of efficacy.

Promising alternative approaches are nasal vaccines since they offer significant advantages: as the oral route, it is a practical site for easy self-administration of vaccines, but requires much lower doses and induces more potent both local and systemic responses than any other mucosal route of immunization [110–111]. Nevertheless, two mucosal vaccines have reached the marketplace and products are still treated with caution, particularly when live organisms are utilized. Our studies have been focused to the design of nasal allergy vaccines based on the induction of allergen-specific tolerance with either purified allergens or allergen derivatives (recombinant mutants or polypeptide fragments), alone or in a delivery system (Fig. 4). For preclinical testing of the potential nasal vaccines, two mouse models of allergic sensitization were established, involving both systemic sensitization to and subsequent nasal challenge with the allergen to achieve both airway inflammation and high levels of serum-specific IgE [97,112]. In all our attempts, Ole e 1 and Ole e 9 allergens were selected as antigen models.

In a first prophylactic approach, low doses of a nasal vaccine based on a mixture of the two recombinant domains of Ole e 9 was able to prevent airway inflammation induced by the allergen, but not specific IgE response in serum [112]. Several prophylactic approaches have been designed for Ole e 1 and its derivatives. Intranasal (i.n.) pretreatment with an immunodominant OE_{109–130} of Ole e 1 lacking IgE-binding capacity, protected mice against subsequent sensitization to the allergen as efficiently as the whole protein [97]. In this pre-clinical model, three i.n. administrations of the peptide were enough for inhibiting the outcome of specific IgE response in serum, Th2-cytokines production and airway inflammation, and induced long lasting protective effect. Another strategy was to encapsulate the OE_{109–130} into poly (lactide-co-glycolide) microparticles in order to improve uptake by antigen-presenting cells [113]. In mice, i.n. pretreatment with few micrograms of the vaccine prevented subsequent sensitization to the allergen [113].

Finally, other prophylactic approach have been designed taking the advantage of the exosomes to transfer functional proteins, mRNA and miRNAs between cells, in particular immune cells, resulting in the induction, amplification and/or modulation of the immune response [114]. Exosomes are nanovesicles secreted into extracellular environment by a variety of cell types [114] which composition can be manipulated for tailoring with new properties [115]. Exosomes-based vaccines have been proposed as a very attractive and promising strategy in various human diseases, especially in cancer [114]. For allergy, we have shown that exosomes derived from bronchoalveolar lavage of Ole e 1-tolerized mice were efficient to protect animals against allergic sensitization to Ole e 1 [116] and the non-related allergen Bet v 1 [117], in contrast to exosomes derived from naïve mice. In addition, long lasting protective effect was also observed [116]. Altogether, these prophylactic studies emphasize the high potential of nasal vaccines for allergy since they suppressed the most important clinical features of this disease. However, exosomes could be a more suitable vaccine for allergy because of their properties. The possibility of using prophylactic vaccines for early prevention in atopic individuals or children at risk has been proposed, but further therapeutic studies will be required.

6. Concluding remarks

To complete the identification of the antigen panel triggering allergic reactions to olive pollen as well as the improvements of its diagnosis are the primary goals of our research. This could allow

simplifying and increasing the efficiency and safety in the treatment and prevention of these diseases. Pollen from olive tree, with great allergenic relevance in the Mediterranean area, has been deeply studied in terms of the allergen content. The research has been focused on the analysis of species-sensitization markers, allergens involved in multiple sensitizations, and the establishment of correlations between allergen sensitization and symptoms development. The difficulty inherent to handle biological materials and to isolate the natural allergens has required the use of recombinant production systems for many of them. This production has allowed the knowledge about the structural and immunologic mechanisms underlying the allergic processes. The availability of recombinant allergens and hypoallergenic derivatives and the development of new formats and routes of administration have made necessary the use of animal models for the immunological analysis of all the designed strategies. Finally, the intranasal induction of tolerance by means of nasal vaccines based on exosomes open a new and promising field of study. All of major approaches accomplishing in this research are summarized in Fig. 5. The future investigations on this field are open to achieve further safer diagnosis and new therapeutic discoveries. The use of allergy vaccines using allergen-modified molecules, new delivery systems and alternative routes of administration will improve the everyday life of the allergic patient as well as unravel the immunological mechanisms underlying immunotherapy to pursue the development of new therapeutic strategies.

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