Molecular allergology: a clinical laboratory tool for precision diagnosis, stratification and follow-up of allergic patients

Abstract: Identification of the molecular culprits of allergic reactions leveraged molecular allergology applications in clinical laboratory medicine. Molecular allergology shifted the focus from complex, heterogeneous allergenic extracts, e.g. pollen, food, or insect venom, towards genetically and immunologically defined proteins available for in vitro diagnosis. Molecular allergology is a precision medicine approach for the diagnosis, stratification, therapeutic management, follow-up and prognostic evaluation of patients within a large range of allergic diseases. Exclusively available for in vitro diagnosis, molecular allergology is nonredundant with any of the current clinical tools for allergy investigation. As an example of a major application, discrimination of genuine sensitization from allergen cross-reactivity at the molecular level allows the proper targeting of the culprit allergen and thus dramatically improves patient management. This review aims at introducing clinical laboratory specialists to molecular allergology, from the biochemical and genetic bases, through immunological concepts, to daily use in the diagnosis and management of allergic diseases.

Keywords: allergy diagnosis; hypersensitivity; molecular allergen; immunoglobulin E; in vitro diagnosis

Introduction

Allergic diseases are noncommunicable diseases affecting up to 30 % of the general population, with age-related and regional variations [1, 2]. The dramatic increase in the prevalence of allergic diseases since the mid-20th century has been paralleled by mechanistic, diagnostic and therapeutic progress, supported by novel biomarkers.

Allergy is defined as a hypersensitivity reaction (clinical manifestation) induced by an exogenous antigen (allergen) able to elicit an adaptive immune response (sensitization) in a predisposed host (genetic background, barrier tissue alterations) [3, 4].

At the molecular level, allergenicity is defined as the ability of an antigen (therefore called allergen) to induce a type 2 adaptive immune response with immunoglobulin (Ig) E production, and to cross-link IgE-FcεRI complexes at the surface of mast cells. The allergen which initiates the sensitization phase and induces the aggregation of IgE-FcεRI complexes at the surface of mast cells must contain at least two IgE binding sites (epitopes). Like other antigens, most allergens are proteins [5]. Proteins with demonstrated allergenicity have been identified only in a limited number of protein families [6]. Thus, the widely held view that any protein can behave as an allergen is obsolete.

Humans encounter potential allergens as components of the native products causing the allergic reaction, which are complex mixtures called allergenic sources: pollen grains, house dust mites’ feces, processed or raw foods etc.
Allergic extracts, obtained by physico-chemical processing of allergenic sources, have been employed in allergy diagnosis and treatment for more than a century [7, 8]. Access to the molecular level of allergenic proteins was gained progressively since the end of the 20th century. Denoted as molecular allergens (MA), these protein tools have revolutionized the in vitro diagnosis (IVD) of IgE-mediated allergic diseases (Figure 1).

Improved diagnostic specificity and sensitivity, risk and therapeutic stratification, and prediction of the response to allergen immunotherapy (AIT) illustrate some of the day-to-day applications of MA. From the IVD perspective, MA support standardization, accuracy, and innovative approaches using machine learning and artificial intelligence. This review provides the clinical laboratory specialist with an introduction to the concepts, applications, and current developments of molecular allergology.

Pathophysiology of IgE-mediated allergic diseases

IgE-mediated mechanisms underlie major allergic diseases, such as food allergy, allergic asthma, atopic dermatitis, eosinophilic esophagitis, drug allergy, and venom allergy. The natural history of IgE-mediated allergy is divided into the sensitization phase, which is asymptomatic, and the effector phase, when immediate hypersensitivity reactions occur (Figure 2).

The sensitization phase comprises antigen transport across the epithelial border, followed by phagocytosis, digestion and antigen presentation by dendritic cells to naive T cells. The subsequent orientation of T cells towards the T helper (Th) 2 profile depends on interleukin (IL)-4 predominance in the microenvironment. Cooperation between activated Th2 cells and naive B cells results in B cell activation, differentiation into plasma cells and production of antibodies directed to the initial antigen. IL-4 and other type 2 cytokines support isotype switch and enhanced IgE production. Following their secretion form plasma cells, IgE bind through their crystallizable (constant) fragment Fc to the high affinity receptor for the Fc of IgE (FcεRI) expressed on mast cells and basophils. FcεRI-bound IgE are long-lived at the surface of mast cells. Mast cells are sentinel cells strategically located beneath the epithelia and in close contact with vasculature, where they can sense the reintroduction of the cognate allergen. Mast cell cytoplasm is filled with secretory granules, a specialized compartment containing preformed, active mediators. The effector phase is triggered by the subsequent reintroduction of the cognate allergen: its recognition and binding by the IgE-FcεRI complex at the plasma membrane of the mast cell induces the aggregation of IgE-FcεRI complexes, followed by a signal transduction cascade culminating with protease kinase C activation and rapid exocytosis of the contents of the secretory granules. Histamine, other vasoactive amines, proteases, and other granule mediators act immediately, inducing the typical symptoms of allergy: either local (e.g. sneezing, wheezing, facial edema) or systemic (e.g. vasodilation and hypotension,

Figure 1: Concepts of allergenic source, allergen extract, molecular allergen, and IgE epitope. The allergenic source is the native product causing the allergic reaction (e.g. peanut, mold, cat dander). An allergen extract is a preparation obtained from an allergen source and with the aim of performing allergy diagnosis and/or treatment. Molecular components of an increasing number of allergenic sources and extracts are available for in vitro diagnosis as purified, recombinant, or synthetic peptides. Analysis at the epitope level, i.e. at the level of the 7–12 aminoacid sequence or conformational motif recognized by specific IgE, is not yet available outside clinical research studies.
urticaria). This early phase of IgE-mediated allergy is followed by a late-phase reaction, which consists of an inflammatory reaction with leukocyte recruitment and activation following the release of arachidonic acid-derived lipid mediators, de novo cytokine synthesis, and tissue remodeling [9]. Mast cells do not die following degranulation; instead, they replenish the contents of their secretory granules in 48–72 h and are thus able to perform multiple cycles of degranulation [10].

Allergen-basophil interactions are similar and lead to basophil activation and degranulation of preformed mediators, most of which are similar to those stored in mast cell secretory granules, but the low numbers of basophils and their location within the vascular compartment make their real contribution to allergic reactions still elusive.

**MA nomenclature and databases**

More than one thousand MA have been cleared by the Joint Allergen Subcommittee Nomenclature acting on behalf of the International Union of Immunological Societies (IUIS) and the World Health Organization (WHO) [11]. Other MA have been reported in the literature, without a formal submission to the IUIS/WHO Subcommittee. MA available for IgE assays worldwide are presented in the Diagnostic Allergen Database (DADB), a systematic database recently developed by the Clinical and Laboratory Standards Institute (CLSI) [12]. Along with molecular allergens, DADB also contains extracts which are employed worldwide for allergy testing.

For a MA to be included in the IUIS/WHO Nomenclature, structural and functional requirements include resolution of at least a partial sequence, production either as a recombinant or as a purified protein, and display allergenicity defined as IgE binding with functional relevance, evaluated by symptom elicitation, skin test, or *in vitro* functional assays of basophil activation. Comparison of structural, physico-chemical and biological activity of the recombinant protein and its natural counterpart purified from the allergenic source is usually provided, aiming at the highest similarity between the two [8].
MA, thus defined by their nucleotide sequence and their IgE-binding and functional effects, are named with the first three letters of the genus name and the first letter of the species name of the corresponding allergenic source, followed by a number that usually indicates the order of characterization of the molecule [13]. For example, Fel d 1 was the first allergen characterized in cat (Felis domesticus, now Felis catus) epithelium, while Api m 2 was the second allergen characterized in honeybee (Apis mellifera) venom [11]. Additional letters may be necessary to avoid confusion between MA. For example, tomato (Solanum lycopersicum) allergens are named Sola 1 to Sola 17 [11]. In some cases, homologous allergens from different sources have received the same number. The gibberellin-regulated protein (GRP) family is an example. Following the identification of the peach (Prunus persica) allergenic GRP peamaclene and its entry in the nomenclature as Pru p 7, homologs characterized later were named Cit s 7 (Citrus sinensis, sweet orange), Pun g 7 (Punica granatum, pomegranate), Cup s 7 (Cupressus sempervirens, funeral cypress), Cap a 7 (Capsicum annuum, bell pepper) etc [11, 14].

For IVD use, MA production relies on either purification from the natural allergenic source (natural purified MA), or sequence-based synthesis using expression vectors (recombinant MA) or peptide synthesis (synthetic MA) (Figure 1). Prefixes “n”, “r” and “s” indicate the purified, recombinant or synthetic nature of a MA, e.g. the honeybee allergen phospholipase A2 is available as either nApi m 1 purified from honeybee venom, or rApi m 1 synthesized within an expression vector, while honeybee melittin is available as sApi m 4. Commercially available recombinant MA are usually obtained using the bacterial Escherichia coli expression vector, and thus do not undergo the post-translational modifications, most notably glycosylation, that can be present in their natural counterpart. Since IgE binding to carbohydrates is virtually always devoid of clinical relevance but may induce a detectable IgE test result, it is important to distinguish between natural and recombinant or synthetic MA [15].

Within the same allergenic source, MA may display variants and/or isoallergens, with variants defined as harboring a limited number of aminoacid substitutions but sharing a 90 % or higher sequence identity, while isoallergens share at least 67 % aminoacid sequence identity, similar molecular weight, and the same biological function [13].

### Biological functions of MA and pathophysiological implications

MA belong to some 180 biochemical families [8]. Proteases, pathogenesis-related (PR) proteins, transport proteins, storage proteins, structural proteins are typical examples of MA.

MA were initially considered immunologically inert proteins, targeted by an excessive or inappropriate adaptive immune response in a predisposed host. Harmless pollen grains were invoked as a typical example. Over the past decade, the biological function of many MA was discovered and shown to interact with the host’s immune response, either through direct activation of immune sentinel mechanisms, or indirectly, via epithelial or other tissue damage [5, 16–21]. Consequently, the concept of the “innocuous”, “harmless”, or “inert” allergen has been barred from the latest version of the allergen nomenclature [5, 22].

As an example, the cysteine protease Der p 1, a representative member of the mite group 1 allergens, targets the intercellular tight junctions of the airway epithelium disrupting intercellular adhesion proteins like occludin, activates protease activated receptors-2 (PAR-2) expressed by epithelial cells, degrades host structural proteins like fibrinogen and thrombin, and stimulates nociceptive neurons, resulting in increased permeability of epithelial barriers, epithelial production of danger signals such as the alarmins interleukin (IL)-33 and thymic stromal lymphopoietin (TSLP), thus initiating type 2 innate immunity and inflammation and facilitating Der p 1 and other allergens delivery across the epithelium [20, 21, 23, 24].

Ligand-binding MA display additional immune cross-talk mechanisms as a function of the nature or conformation of the ligand, or of ligand-induced variations in allergen conformation [25–29]. The lipocalin family, prominent mammalian allergens comprising airborne Can f 1 and Fel d 7 from dog and cat and ingested Bos d 5 beta-lactoglobulin from cow’s milk, are innocuous and display bacteriostatic effects when properly charged with a physiological ligand, but disrupt plasma membranes of airway and intestinal epithelia and exert pro-type 2 inflammatory effects when insufficiently loaded [30–33].

### Diagnostic relevance of MA

Proper identification of the genuine sensitizer, defined as the allergenic source that has induced the IgE response in the considered individual [34], is needed for adequate patient management, such as AIT choice, exclusion regimens, or lifestyle changes. In parallel, potential cross-reactivity among distinct allergenic sources must be investigated in each patient to predict the expected spectrum and severity of clinical reactions to apparently unrelated allergenic sources, AIT efficacy and tolerance, and outgrowth of the allergic
phenotype in children [35]. MA are the first tools enabling such precision medicine approaches (Figure 3).

Marker MA display expression, protein homology and IgE binding restricted to a species, genus, or other taxonomically limited group of allergenic sources. As an example, Fel d 1 is a marker MA for cat epithelium sensitization, since it is only bound by the corresponding IgE [36]; Cup a 1 is a marker MA for Cupressaceae pollen sensitization, since it is bound by IgE synthesized in response to a limited group of homologous pectate lyases mainly expressed in pollen from cypress, juniper, and Japanese cedar [14]; phospholipase A1 Ves v 1 is a marker MA for common wasp venom sensitization [37]; beta-lactoglobulin Bos d 5 is a marker MA for cow milk sensitization [34]. Table 1 presents a selection of marker MA frequently used in the clinical laboratory [11, 22, 34, 36, 38, 39].

Cross-reactive MA are distinct allergens containing epitopes which are bound by IgE molecules sharing the same specificity [34]. Cross-reactivity usually occurs when IgE antibodies induced by MA from the genuine sensitizer allergenic source recognize and bind shared epitopes present on MA from other allergenic sources. Clinically relevant examples include the pollen-food syndromes, which manifest as food-induced symptoms in people with pollen allergy, due to shared epitopes between pollen and plant food molecules, the cross-reactivity between peanut and tree nuts, the cross-reactivity between mammalian allergens [40–42]. Cross-reactivity is rather a continuum than a discrete categorization, e.g. the cat marker allergen Fel d 1 cross-reacts with orthologues from other Felidae, but in the vast majority of patients it is a reliable marker for genuine cat sensitization.

Cross-reactive allergens usually belong to the same biochemical family. Within a biochemical family, a sequence identity percentage of 40 % or higher is usually associated with observable cross-reactivity [40]. The degree of cross-reactivity at the molecular level, hence the prevalence of clinically relevant cross-reactivity at the allergenic source level, vary from one biochemical family to another. Allergen families which cross-react almost always and are widely distributed within allergenic sources are called “pan-allergens”. For example, polcalcins are pollen panallergens, with IgE induced by one polcalcin virtually binding any other polcalcin and resulting in multiple skin and blood positive tests with pollen allergenic extracts.

Plant, fungal and animal MA usually belong to distinct families that do not exhibit cross-reactivity, but clinically relevant exceptions exist, especially among fungal, mite and insect allergens. For example, tropomyosins from invertebrate animals form an animal panallergen family with extended cross-reactivity. Clinically relevant tropomyosin cross-reactivity applies to insects (e.g. Bla g 7 for German cockroach Blattella germanica), house dust mites (e.g. Der p 10 from the European house dust mite Dermatophagoides pteronyssinus), crustaceans (e.g. Pen a 1 from the shrimp Penaeus azteca), nematodes (e.g. Ani s 3 from the fish

Figure 3: Illustration of marker, cross-reactive, major, minor and immunodominant molecular allergens. Marker allergens are only found in one or a few closely related allergic sources. Prominent examples include Fel d 1 (cat) and Can f 5 (dog). Demonstration of IgE to a marker allergen indicates genuine sensitization to the corresponding allergenic source. Cross-reactive allergens usually belong to shared biochemical families and are recognized by IgE raised against other members of the same family. The prevalence of sensitization defines major allergens (sensitization present in more than 50 % of patients allergic to the corresponding source) and minor allergens (sensitization is present in less than 50 % of patients allergic to the corresponding source). Immunodominant allergens induce the most abundant production of specific IgE. A closely related concept is that of immunodominant epitope(s) of a molecular allergen.
Table 1: Selected examples of molecules employed as marker allergens in the clinical laboratory.

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Biochemical family</th>
<th>Distribution</th>
<th>Exposure</th>
<th>Cross-reactivity</th>
<th>Examples of clinically relevant members</th>
<th>Examples of application in the clinical laboratory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plants</td>
<td>Beta-expansins</td>
<td>Grass pollen</td>
<td>R</td>
<td>+++ (grasses)</td>
<td>Phl p 1 (timothy grass pollen), Cyn d 1 (Bermuda grass pollen)</td>
<td>Genuine sensitization to grass pollen.</td>
</tr>
<tr>
<td></td>
<td>Common olive group 1</td>
<td>Oleaceae tree pollen: olive, ash</td>
<td>R</td>
<td>Ash and olive tree pollen</td>
<td>Ole e 1 (olive tree pollen), Fra e 1 (ash tree pollen)</td>
<td>Genuine sensitization to olive or ash tree pollen.</td>
</tr>
<tr>
<td></td>
<td>Rubber elongation factor</td>
<td>Para rubber tree latex</td>
<td>C</td>
<td>No</td>
<td>Hev b 1 (latex)</td>
<td>Genuine sensitization to latex.</td>
</tr>
<tr>
<td></td>
<td>Pectate lyases (Cupressaceae)</td>
<td>Pollen</td>
<td>R</td>
<td>++ (Cupressaceae pollen)</td>
<td>Cup a 1 (Arizona cypress pollen), Cry j 1 (Japanese cedar pollen), Jun a 1 (mountain cedar pollen)</td>
<td>Genuine sensitization to Cupressaceae pollen.</td>
</tr>
<tr>
<td>Animals</td>
<td>Phospholipases A2</td>
<td>Apid venom</td>
<td>I</td>
<td>+++ (Apids)</td>
<td>Api m 1 (honeybee), Bom t 1 (bumble bee)</td>
<td>Sensitization to Apid (honeybee, bumble bee) venom.</td>
</tr>
<tr>
<td></td>
<td>Phospholipases A1</td>
<td>Vespid venom</td>
<td>I</td>
<td>+ (Vespids)</td>
<td>Ves v 1 (yellowjacket), Pol d 1 (paperwasp), Vesp v 1 (Asian hornet)</td>
<td>Sensitization to Vespid (wasps, hornets) venom.</td>
</tr>
<tr>
<td></td>
<td>House dust mite group 1</td>
<td>House dust mites</td>
<td>R</td>
<td>++</td>
<td>Der f 1 (American house dust mite), Der p 2 (European house dust mite)</td>
<td>Genuine sensitization to house dust mites. Early-life sensitization is predictive of later asthma development. Members of the extended cysteine protease family comprising other clinically relevant allergens without cross-reactivity with mite group 1.</td>
</tr>
<tr>
<td></td>
<td>Secretoglobins</td>
<td>Felids</td>
<td>R</td>
<td>++ (Felids)</td>
<td>Fel d 1 (domestic cat)</td>
<td>Genuine sensitization to cat dander.</td>
</tr>
<tr>
<td></td>
<td>Kallikrein</td>
<td>Male dogs</td>
<td>R</td>
<td>±</td>
<td>Can f 5 (prostatic dog kallikrein)</td>
<td>Genuine sensitization to (male) dog dander. Cross-reactive with human ortholog, hence employed for IVD of allergy to human seminal liquid.</td>
</tr>
<tr>
<td></td>
<td>Caseins</td>
<td>Milk</td>
<td>F</td>
<td>++ (milk)</td>
<td>Bos d 8 (mix of bovine caseins), Bos d 9, Bos d 10</td>
<td>Genuine sensitization to milk.</td>
</tr>
<tr>
<td></td>
<td>Ovomucoid</td>
<td>Bird egg white</td>
<td>F</td>
<td>+++ (bird egg white)</td>
<td>Gal d 1 (hen’s egg)</td>
<td>Genuine sensitization to bird egg.</td>
</tr>
<tr>
<td>Fungi</td>
<td>Mitogillin</td>
<td>Aspergillus fumigatus</td>
<td>R</td>
<td>± (closely related species)</td>
<td>Asp f 1 (Aspergillus fumigatus)</td>
<td>Genuine sensitization to A. fumigatus.</td>
</tr>
<tr>
<td></td>
<td>Unnamed</td>
<td>Alternaria alternata</td>
<td>R</td>
<td>± (closely related species)</td>
<td>Alt a 1 (Alternaria alternata), Ulo c 1 (Ulocladium chartarum, formerly Alternaria chartarum)</td>
<td>Genuine sensitization to A. alternaria.</td>
</tr>
</tbody>
</table>

C, contact; F, food exposure (ingestion); I, injection (insect sting, iatrogenic etc.); R, respiratory exposure (inhalation); S, skin exposure (transcutaneous). References [11, 22, 34, 36, 38, 39].

parasitic nematode Anisakis simplex, mollusks (e.g. Hel as 1 from the garden snail Helix aspersa) and fish tropomyosins such as Sal s 4 from the Atlantic salmon Salmo salar [11, 34, 43, 44].

Tropomyosins also illustrate the lack of allergenicity in proteins showing a sequence identity of 70 % or greater with human orthologs. Indeed, tropomyosin is a ubiquitous actin-binding protein found in invertebrate and vertebrate cells [45]. However, tropomyosins from evolutionarily close species share high sequence identity, implying that sensitization to mammalian tropomyosins would require a loss of tolerance to the human (self) protein. Thus, allergy to mammalian meat does not involve mammalian tropomyosin as an allergen.

Table 2 presents a selection of well-characterized families of allergens relevant for clinical cross-reactivity and clinical laboratory assessment [11, 22, 34, 40, 46–50].

Labeling a clinical reaction as cross-reactivity rather than genuine sensitization affects the way it will be handled, since IgE to multiple cross-reactive MA is associated with decreased efficacy and increased prevalence of adverse reactions during AIT. On the other hand, demonstrated IgE to a cross-reactive MA may explain clinical reactions to apparently unrelated
<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Biochemical family</th>
<th>Distribution</th>
<th>Exposure</th>
<th>Cross-reactivity</th>
<th>Examples of clinically relevant members</th>
<th>Examples of clinical relevance and applications in the clinical laboratory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plants</td>
<td>PR-10</td>
<td>Pollen, edible plant organs</td>
<td>F, R</td>
<td>++</td>
<td>Bet v 1 (birch pollen), Pru p 1 (peach), Cor a 1 (hazel pollen and hazelnut), Api g 1 (celery root)</td>
<td>Pollen-food syndromes mainly presenting as oral allergy syndrome with raw plant food in temperate regions.</td>
</tr>
<tr>
<td></td>
<td>nsLTP</td>
<td>Pollen edible plant organs</td>
<td>F, R</td>
<td>+</td>
<td>Art v 3 (mugwort), Pla a 3 (London plane tree pollen), Ole e 7 (olive pollen), Cor a 8 (hazelnut), Pru p 3 (peach)</td>
<td>Selective food cross-reactivity (raw and processed plant food) with potentially severe presentation, more frequent in Mediterranean regions.</td>
</tr>
<tr>
<td></td>
<td>Profilins</td>
<td>Pollen, edible plant organs</td>
<td>F, R</td>
<td>+++</td>
<td>Bet v 2 (birch pollen), Phl p 12 (timothy grass pollen), Cuc m 2 (muskmelon), Hev b 8 (latex), Mus a 1 (banana), Pru p 4 (peach)</td>
<td>Pollen-food syndromes mainly presenting as oral allergy syndrome with raw plant food; primary food allergy possible. Increased risk of asthma in pollen-sensitized individuals.</td>
</tr>
<tr>
<td></td>
<td>Polcalcins</td>
<td>Pollen</td>
<td>R</td>
<td>+++</td>
<td>Bet v 4 (birch pollen), Phl p 7 (timothy grass pollen)</td>
<td>Extensive pollen and plant food reactivity. Increased risk of asthma in pollen-sensitized individuals.</td>
</tr>
<tr>
<td></td>
<td>GRP</td>
<td>Pollen, edible plant organs</td>
<td>F, R</td>
<td>++</td>
<td>Cup s 7 (funeral cypress pollen), Cry j 7 (Japanese cedar pollen), Pru p 7 (peach), Cit s 7 (sweet orange), Cap a 7 (bellpepper)</td>
<td>Pollen-food syndrome with raw and processed plant food. Severe reactions following plant food ingestion, with or without a cofactor.</td>
</tr>
<tr>
<td></td>
<td>Seed storage proteins</td>
<td>Peanut, pulses, tree nuts, grains</td>
<td>F</td>
<td>±</td>
<td>Ara h 2, Ara h 6 (peanut 2S-albumins), Ana o 3 (cashew nut 2S-albumin), Ses i 1 (sesame 2S-albumin), Cor a 9 (11S-globulin), Gly m 4 (soy 7S-vicilin)</td>
<td>Food sensitization and food allergy. Complex patterns of cross-reactivity, with some storage proteins employed as marker allergens for genuine sensitization to the corresponding allergenic source (e.g., Ara h 2 and Ara h 6, Ana o 3, Ses i 1) Severe reactions to plant food. The allergenicity of peanut and hazelnut oleosins increases upon roasting.</td>
</tr>
<tr>
<td></td>
<td>Oleosins</td>
<td>Peanut, tree nuts, grains</td>
<td>F</td>
<td>±</td>
<td>Ara h 10, Ara h 11, Ara h 14 (peanut), Cor a 12, Cor a 15 (hazelnut), Ses i 4, Ses i 5 (sesame)</td>
<td>Severe reactions to plant food. The allergenicity of peanut and hazelnut oleosins increases upon roasting.</td>
</tr>
<tr>
<td>Animals</td>
<td>Lipocalins</td>
<td>Mammals, insects</td>
<td>R, F</td>
<td>++</td>
<td>Bos d 5 (cow's milk beta-lactoglobulin), Can f 1, Can f 2, Can f 6 (dog), Fel d 4, Fel d 7 (cat), Bla g 4 (German cockroach)</td>
<td>Elucidation of genuine and cross-reactive sensitization to furry animals. Respiratory sensitization and allergy. Milk allergy. Minor allergy: airborne in mammalian dander allergy, ingested in (undercooked) meat allergy. May present as meat allergy in cow's milk allergic patients, as cat-pork syndrome (reactions upon underdone pork ingestion in cat-allergic individuals) or bird-egg syndrome (inhaled or ingested primary sensitization).</td>
</tr>
<tr>
<td></td>
<td>Serum albumins</td>
<td>Mammals, birds</td>
<td>R, F</td>
<td>+++ (low between mammals and birds)</td>
<td>Bos d 6 (cow), Can f 3 (dog), Fel d 2 (cat), Sus s 1 (pig), Gal d 5 (chicken meat and hen's egg)</td>
<td>Minor allergy: airborne in mammalian dander allergy, ingested in (undercooked) meat allergy. May present as meat allergy in cow's milk allergic patients, as cat-pork syndrome (reactions upon underdone pork ingestion in cat-allergic individuals) or bird-egg syndrome (inhaled or ingested primary sensitization).</td>
</tr>
<tr>
<td></td>
<td>Beta-parvalbumins</td>
<td>Fish, reptiles</td>
<td>F</td>
<td>+++</td>
<td>Cyp c 1 (common carp), Gad c 1 (Baltic cod), Cro p 1 (crocodile)</td>
<td>Genuine sensitization to fish. Food sensitization and food allergy.</td>
</tr>
</tbody>
</table>
allergenic sources. In terms of IgE binding affinity, a gradient is usually observed from the sensitizing MA (e.g. Der p 10 tropomyosin in HDM-allergic patients) to cross-reactive MA (e.g. shrimp Pen a 1 or cockroach Bla g 7 tropomyosins), that may affect their functional relevance for IgE-FcεRI binding and cross-linking, mast cell degranulation and clinical expression [34, 51, 52].

The prevalence of sensitization to MA is another criterion for MA interpretation. Major allergens are defined as inducing detectable sensitization in 50 % or greater of patients allergic to the corresponding allergenic source. Minor allergens are those with detectable IgE in less than 50 % in the considered population. In line with this categorization, an immunodominant allergen is defined as being recognized by a majority of IgE directed against the corresponding allergenic source. For example, peanut storage protein Ara h 2 is a major peanut allergen, while peanut structural protein profilin Ara h 5 is a minor one. Ara h 2 is also an immunodominant allergen [53].

Major allergens are not always marker allergens, and vice-versa; moreover, being a minor or a major allergen may depend on the considered population. As an example, the dog kallikrein Can f 5 is a marker allergen for genuine sensitization to dog, but a minor allergen in dog-allergic patients. Members of the PR-10 family are major allergens of Rosaceae fruit such as apple, peach, and pear, in temperate and Northern regions, but they are minor allergens for the same fruit in Mediterranean regions. The explanation resides in geographical variations of allergen exposure: in most cases, sensitization to fruit PR-10 results from cross-reactivity with the primary sensitizers which are Fagales pollen PR-10, such as Bet v 1 from birch pollen. Fagales trees (e.g. birch, beech, hazel, alder, oak etc) are highly prevalent in temperate and Northern regions but much less so in Mediterranean regions [34, 54].

**MA and clinical presentation: phenotype, severity, and persistence**

Several airborne MA, mainly from grass pollen, house dust mites, and animal dander, have been shown to associate with airway disease phenotypes [55]. As an example, sensitization to major cat and dog allergens Fel d 1, Can f 1 and Can f 2 are associated with an increased risk of asthma in adults [56]. Lipocalins, especially cat lipocalin Fel d 4, predict a phenotype of asthma, rather than allergic rhinitis, and persistent type 2 inflammation biomarkers [33, 49].

MA sensitization patterns and levels may be associated to more severe allergic reactions. IgE to heat-stable MA such as storage proteins Ara h 2 and Ara h 6 in peanut and Cor a 9 and Cor a 14 in hazelnut are associated with a higher risk of severe reactions [38, 39].

In patients with a diagnosis of allergy, MA investigation may help evaluate the risk of persistence of the allergic disease. Cow’s milk allergic children with high levels of
casein Bos d 8 and egg-allergic children with high levels of ovomucoid Gal d 1 are more likely to have persistent allergy [57–59]. Prediction of future development of allergic diseases has been dramatically improved by MA utilization. It is now demonstrated that sensitization to MA from house dust mites, such as Der p 1, or from grass pollen, such as Phl p 5, at preschool age is a predictor of asthma at 11–15 years [60–63].

**Analytical aspects of MA-specific IgE testing**

MA are only available for IVD, presumably because of the price and regulatory constraints required for *in vivo* testing. Two test formats are in use for MA: singleplex tests, which measure IgE to individual MA, and multiplex tests, which detect IgE binding to dozens or hundreds of MA [64]. The former are quantitative tests, while the latter are usually considered as semi-quantitative [65, 66] (Figure 4).

Singleplex methods for IgE to MA are similar to conventional allergen-specific IgE assays initially derived from the “RAST” (Radio-AllergoSorbent Assay), currently relying on fluorescence or chemiluminescence [67, 68]. Nowadays, singleplex IgE assays can be calibrated by interpolation against the WHO total IgE international standard, allowing for result expression as kilounits of allergen-specific IgE per liter and improving intermethod variability (kUA/L) [65, 68, 69]. The lower limit of quantitation (LOQ) for MA singleplex assays is the same as for other specific IgE, usually 0.10 kUA/L across manufacturers [70]. The historical “positivity” threshold of 0.35 kUA/L is sometimes still employed by certain clinicians in search of greater specificity, or in the context of comparing historical cohorts of patients with allergic diseases lacking a clear dose-response correlation, such as allergy to wasp and bee stings.

The first allergen multiplex method was designed in 2002 by an academic team in Vienna, Austria, as a protein microarray comprising 74 MA that could be assessed in parallel using a single serum sample of 20 μL [71]. This test was subsequently developed and commercialized as 103–112 MA microarrays, with research versions of up to 180 allergens. Allergen multiplex investigation was hailed as a breakthrough in clinical research and for patient diagnosis and long-term prediction, despite the test not being cleared for clinical use, or not being commercialized, in all countries [60, 62–64, 72–78]. Since 2016–2018, technical improvements of the multiplex technology included covalent allergen binding to polystyrene nanobeads followed by nanobead coupling to a nitrocellulose reaction matrix as an alternative to direct coating of MA to a functionalized glass reaction support, and the introduction of an enzymatic amplification step [79–81]. This approach allowed optimization of each allergen individually, combined immobilization of MA and allergenic extracts on the same support, an increase of the total number of allergens (MA and extracts) to 300, and better sensitivity and linearity of the results [81–83]. The multiplex investigation is increasing [66], and various other...

Figure 4: Principles of singleplex and multiplex allergen *in vitro* diagnostic assays. Singleplex assessment of serum specific IgE with fluorimetric or chemiluminescent methods, derived from the pioneering “RAST” method, is employed in most cases of *in vitro* diagnosis and follow-up of Ig-mediated allergy. Multiplex methods based on miniaturized assays of 100–300 molecules and extracts have been developed since the turn of the 21st century.
multiplex methods are being developed by manufacturers and academic teams [86]. Compared to singleplex methods, multiplexes usually have a slightly higher LOQ, at 0.35 or 0.30 units. The results of IgE binding are expressed in units that differ from one platform to another [81]. For a given MA, the overall correlation between singleplex and multiplex IgE results is good, but quantitative comparison is not recommended [81–83, 87, 88].

Interlaboratory variability is low, even for multiplex methods [75, 89]. The analytical performance of MA-specific IgE testing is similar to that of conventional IgE testing using allergen extracts [53, 69, 89, 90]. Notably, intermethod agreement and the effect of IgG interference may not be improved by the use of MA, since they depend on the principle of the method employed by a given manufacturer, rather than on the MA itself [68, 91, 92].

The single analyte strategy is applicable for quality assessment of MA-specific IgE measured with the same method [69]. Worldwide IgE antibody proficiency programs (external quality assessment schemes) are available to clinical laboratories for both singleplex and multiplex methods, such as College of American Pathologists (CAP) Diagnostic Allergy SE Survey in the USA, United Kingdom National External Quality Assessment Service for Immunology (UKNEQAS) Immunology Proficiency Survey in the UK, Reference Institute for Bioanalytics (RfB) and Society for Promoting Quality Assurance in Medical Laboratories e. V. (INSTAND e. V) in Germany, Société Suisse d’Allergologie et Immunologie (SSAI) IgE totales et IgE spécifiques d’allergènes Survey in Switzerland, among others [12, 93].

Besides IgE tests, MA can be employed for the measurement of specific IgG4 or IgG binding [92, 94]. MA-specific IgG4 levels increase during successful AIT [95–97]. Functional tests with MA, such as basophil activation tests, may improve the sensitivity of detection and the predictive value for the diagnosis and severity of allergic diseases [38, 98].

Diagnosis of IgE-mediated allergy: clinical history, allergic extracts and MA-assisted diagnosis

The diagnosis of IgE-mediated allergy requires the association of a convincing clinical history of immediate hypersensitivity reaction to a culprit allergenic source and a demonstrated sensitization to the same allergenic source (Figure 5). Personalized mapping of sensitization at the molecular level is obtained with MA.

Clinical history

Anamnesis must confirm the typical presentation of immediate hypersensitivity: symptoms occur generally minutes after exposure to the allergenic source, and not later than 4 h; symptoms are similar for subsequent exposure to the same allergenic source; symptoms do not occur outside exposure to the allergenic source. While taking the clinical history, a suspected allergenic source needs to be identified, in order to proceed with targeted investigations.

Demonstration of sensitization: allergic extracts

Sensitization to this allergenic source is first assayed with allergic extracts, either through skin tests evaluating the degranulation of dermal mast cells, or as blood tests for specific IgE [99]. A positive skin test and/or the detection of circulating specific IgE to extracts of the culprit allergenic source confirms sensitization.

Nowadays, most blood tests for assessing specific IgE are quantitative. For some common food allergenic extracts, in defined populations, quantitative dose-response values can be established, linking the concentration of specific IgE measured with a given method to the risk of experiencing an allergic reaction upon a food challenge test [65, 68, 100, 101]. For both skin tests and blood IgE tests, investigations of the suspected allergenic source usually start with the corresponding allergenic extract.

The allergenic extract should ideally contain all allergenic proteins found in the allergenic source, in comparable proportion as in the source. However, most allergen extracts are obtained through aqueous extraction, meaning that amphiphilic proteins are at risk of being lost from the extract, e.g. oleosins from peanut and tree nuts. Small-size proteins can also be lacking, e.g. the GRP peamaclein Pru p 7, a 7 kDa allergen from peach often involved in severe allergic reactions, is underrepresented or even missing from peach extracts for IVD [41]. In other cases, proteins are easily denatured within the allergenic extract, e.g. PR-10 proteins from hazelnut, leading to a decrease in the diagnostic sensitivity of the extract.

Allergenic extracts, being a mixture of proteins, may also contain MA that have not yet been identified and characterized. In this case, diagnostic sensitivity of IgE detection with allergen extracts is greater than with MA.

Last but not least, extracts from taxonomically related or even unrelated allergenic sources may share homologous, cross-reactive proteins that may decrease the diagnostic
specificity of the measure of IgE to allergenic extract. IgE cross-reactivity can be observed in IVD and skin tests with extracts, and in vivo upon exposure to allergenic sources, with potential clinical implications. For example, birch-allergic patients possess IgE directed against the birch pollen allergen Bet v 1. Bet v 1 belongs to the PR-10 protein family which contains homologous proteins found in the pollen of Fagales trees (such as birch, alder, hazel, oak) and in numerous plant foods (Rosaceae fruit, hazelnut, peanut, tomato, celery etc). In some cases, IgE to Bet v 1 often bind also to Bet v 1-like proteins found in hazelnut, apple, peach etc., and can induce symptoms including perioral and oral itching and swelling upon ingestion of the fresh culprit foods (oral allergy syndrome due to pollen-food IgE cross-reactivity) [34].

The natural variability of allergenic source materials is another well-known cause of allergenic extract heterogeneity, even within the production of a single manufacturer, is. Examples include variations in the amount and biological activity (potency of allergen-induced mast cell or basophil degranulation) of allergenic proteins in pollen depending on temperature, hygrometry, and air pollution; in plant foods, depending on the same factors plus the cultivar; in house dust mites and molds depending on the culture conditions [102]. When extracts from different manufacturers are compared, heterogeneity in terms of protein concentration and potency may be striking [103]. The same issues pertain to therapeutic allergenic extracts, which are employed for AIT and expected to cover a patient’s spectrum of IgE to allergenic proteins from the allergenic source [102]. Figure 3 illustrates the concepts of allergenic source, allergen extract, and MA.

Despite their inherent variability, the lack of standardization from one manufacturer to another, and an increasing regulatory pressure, commercialized allergenic extracts are still considered as the most sensitive first-line test for detecting IgE sensitization. As of 2024, allergenic extracts are widely employed and recommended as the first-line investigation of IgE sensitization [99, 104, 105].

MA-assisted diagnosis of IgE-mediated allergy

MA are usually employed for second-line investigations, either as a complement for in-depth characterization of sensitization detected to a given allergenic extract, or as a means of improving the sensitivity of IgE detection in patients who exhibit negative allergen extract tests despite a convincing clinical history [34, 104].

Depending on the considered MA and allergen source, molecular profiling contributes to the stratification of the
allergic disease, as previously explained: risk of severity, risk of persistence, risk of developing allergic diseases later in life.

It has been shown that the accuracy of allergy diagnosis is improved by a comprehensive molecular investigation, however, this scientific incentive is not always met in real life due to regulatory and economic issues [106, 107].

Less than 150 MA are currently available for IVD. Thorough investigation of most common plant foods, cow’s milk, tree and grass pollen, mites, and animal dander can be performed. Confirmation and differential diagnosis of allergy to honeybee (Apis mellifera) venom and/or to common wasp (Vespula vulgaris) also rely on MA testing (reviewed in [34]). On the other hand, notoriously under-served categories are fungi, insects, fish, meat, and many allergenic sources outside the temperate climate regions.

**Strategy for the use and interpretation of MA-specific IgE**

Guidance for MA use and interpretation is available as a comprehensive resource in a recent position paper [34]. Three examples of MA application in daily practice are presented below.

**Case 1 (original):**

**Clinical history:** A 27-year-old woman with a history of immediate reactions with generalized urticaria and dizziness induced by the consumption of Rosaceae fruit (apple, peach, plum, and cherry). She also reports spring rhinoconjunctivitis during the past 4 years.

**Additional anamnesis:** The patient was born and raised in a Mediterranean region, before moving to a Northern European region. Food-induced reactions are observed with raw as well as heat-processed fruit.

**Skin testing:** The patients reacted on skin testing with birch pollen, ash tree pollen, grass pollen, peach and apple extracts.

**Reasoning:**

1. Fruit-induced reactions: the association of Rosaceae fruit-induced immediate reactions with spring pollinosis in a Northern European region is suggestive of pollen-food cross-reactivity (“pollen-food syndrome”) due to birch pollen major allergen Bet v 1 cross-reacting with homologous fruit allergens (e.g., Pru p 1 from peach and Mal d 1 from apple). However, Bet v 1-related pollen-food syndrome usually presents as a mild reaction with perioral involvement, rather than as a systemic reaction, and is induced by raw, but not heat-processed fruit, since Bet v 1 and its homologs are heat-labile. Given that the patient has lived in a Mediterranean region for the major part of her life and experiences systemic reactions upon consumption of heat-processed fruit, a primary sensitization to peach via the heat-stable non-specific lipid transfer protein (nsLTP) Pru p 3 is suspected.

2. Spring pollinosis: all three sources of pollen reacting on skin testing can be involved. Marker pollen allergens are required to identify the genuine sensitization(s). Potential cross-reactivity via the profilin panallergen family is suspected and can be tested using the birch profilin Bet v 2.

**Blood IgE testing:** First aim: distinguish between Bet v 1 and Pru p 3 sensitization as a molecular substrate for food-induced reactions. Second aim: identify the pollen culprit for spring rhinoconjunctivitis as Bet v 1 and/or Ole e 1 and/or Phl p 1 + Phl p 5 (respectively, birch, ash tree and grass pollen marker MA) and check pollen cross-reactivity with Bet v 2 (or Phl p 12), cross-reactivity markers for the plant panallergen family of profilins.

IgE reactivity as determined by a singleplex platform: IgE Bet v 1 and IgE Phl p 1 + Phl p 5<0.10 kUA/L (indetectable); IgE Pru p 3=5.35 kUA/L; IgE Ole e 1=3.20 kUA/L; IgE Bet v 2=2.14 kUA/L.

**Conclusion:** Fruit allergy with a molecular substrate of heat-stable nsLTP sensitization (Pru p 3), associated with genuine sensitization to ash tree pollen (Ole e 1) and with profilin cross-reactivity to birch and grass pollen (Bet v 2).

**Management:** Exclusion of culprit foods, prescription and explanation of appropriate emergency medication (epinephrine autoinjector) and seasonal antihistamines, yearly follow-up. Screening for a potential mast cell condition through baseline tryptase determination may be considered [108]. AIT to ash pollen may be considered, after optimization of the antihistamine treatment and depending on the local availability of the therapeutic extract [109, 110].

**Case 2 (original):**

**Clinical history:** A 55-year-old man, transient dizziness following an unidentified Hymenoptera insect sting while sitting in the grass during a summer hike.

**Additional anamnesis:** The hike took place 3 months prior to the allergist appointment. The patient was stung by a wasp one month after the hike and experienced only local swelling of the affected arm. The culprit insect for the second sting was formally identified as a yellowjacket (common wasp, Vespula vulgaris) thanks to a picture taken by the patient’s daughter.

**Reasoning:** Hymenoptera-induced systemic reactions are eligible for venom allergen immunotherapy (VIT) since they may be life-threatening. The choice of the appropriate VIT (yellowjacket Vespula vulgaris, paperwasp Polistes spp,
or honeybee *Apis mellifera*) is based on the identification of the genuine venom sensitizer. Phospholipase A2 Api m 1 and icarapin Api m 10 are marker allergens for honeybee venom sensitization, as they are devoid of cross-reactivity with Vespid (wasps and hornets) venom allergens. Vespid venom allergen group 5 (Ves v 5, Pol d 5) and group 1 (Ves v 1) are marker allergens or genuine sensitization to Vespid venom. Cross-reactivity between Vespid venom group 5 allergens prevents the use of Ves v 5 and Pol d 5 as differential markers of sensitization to *Vespula* vs. sensitization to *Polistes* venom. A clear quantitative predominance (ratio>50%) of Ves v 5 or Pol d 5 IgE concentration may help identify the primary venom sensitizer [111]. Cross-reactive carbohydrate determinants such as MUXF3 may contribute to the positivity of IgE measured with honeybee and *Vespula*, but not *Polistes* venom extracts [112].

**Skin testing:** Skin prick tests with the three venom extracts were negative. Titrated intradermal testing was positive at 0.01 µg and at 0.001 µg for honeybee venom extract, at 0.001 µg for *Vespula* venom extract, and negative for *Polistes* spp venom extract.

**Blood IgE testing:** Honeybee venom extract IgE=15.3 kUA/L, IgE to Api m 1=14.1 kUA/L, IgE to Api m 10<0.10 kUA/L; *Vespula* venom extract=1.32 kUA/L, IgE to Ves v 5 and IgE to Ves v 1<0.10 kUA/L (indetectable), *Polistes* spp venom extract=0.45 kUA/L. Baseline tryptase determination=5 µg/L (within the normal reference range).

**Conclusion:** Genuine sensitization to honeybee venom confirmed by the presence of IgE to Api m 1. Absence of proof for genuine sensitization to *Vespula* and *Polistes* venom. Additional investigation of the molecular substrate of cross-reactivity between honeybee and *Vespid* venoms can be performed using MA Api m 4 and Api m 5, and cross-reactive carbohydrate MUXF3. An underlying mast cell disorder is unlikely given the normal baseline tryptase.

**Management:** Instauration of VIT using honeybee venom extract with appropriate monitoring.

**Case 3 (original):**

**Clinical history:** A 12-year-old boy living in a temperate region, pharyngeal itching after eating two roasted peanuts.

**Additional anamnesis:** The patient had experienced generalized urticaria after eating hazelnut chocolate. The packaging mentioned “may contain peanut”. No other health complaint, no asthma, no allergic rhinitis, no history of atopic dermatitis.

**Skin testing:** Positive SPT to peanut and hazelnut. All other common food and airborne allergens were SPT-negative.

**Reasoning:** Peanut allergy is the most probable diagnosis, and genuine sensitization to peanut needs confirmation. Peanut storage proteins Ara h 2 and Ara h 6 (2S albumins) are mandatory, Ara h 1 (7S-icillin) and Ara h 3 (11S-globulin) are optional. Possible cross-reactivity between peanut and hazelnut needs investigation, potential culprits being PR-10 proteins (Ara h 8 and Cor a 1), LTP (Ara h 9 and Cor a 8), and profilins (Ara h 5 and Cor a 2, non-available for IVD, Bet v 2 or Pru p 4 can be used). However, if the patient were sensitized to PR-10 or to profilins, SPT to pollens would be positive. Therefore, testing only for LTP is suggested.

**Blood IgE testing:** IgE Ara h 2=1.82 kUA/L, IgE Ara h 6=1.20 kUA/L, IgE Ara h 1, Ara h 3 and Ara h 9<0.10 kUA/L, IgE Cor a 8<0.10 kUA/L.

**Interpretation:** This sensitization pattern confirms genuine peanut sensitization but does not bring solid proof of LTP-induced cross-reactivity between peanut and hazelnut, as the concentration of IgE to Cor a 8 is very low. Genuine sensitization to hazelnut needs investigation with storage proteins Cor a 14 (2S albumin) and Cor a 9 (11S globulin).

**Blood IgE testing:** IgE Cor a 9=18.3 kUA/L, IgE Cor a 14<0.1 kUA/L.

**Conclusion:** Genuine cosensitization to peanut (2S albumins Ara h 2 and Ara h 6) and hazelnut (11S-globulin Cor a 9).

**Management:** Oral food challenge to determine the reaction threshold for peanut and hazelnut, followed by oral AIT.

**Comments:** This case illustrates the stepwise approach for MA-assisted diagnosis, with only the most probable hypothesis tested after the SPT. Had the initial reasoning been confirmed (genuine sensitization to peanut and LTP cross-reactivity to hazelnut), the second step of MA investigation could have been spared. However, this approach posed the risk of misidentifying genuine hazelnut sensitization. Ideally, all marker allergens for peanut and hazelnut should have been assayed in the first step: Ara h 2 and Ara h 6, Cor a 9 and Cor a 14, Ara h 1 and Cor a 3. Of note, in this patient IgE to allergenic extracts (peanut and hazelnut) were not measured, resulting in additional difficulty for proper quantitative interpretation of MA IgE values.

**Conclusions**

MA availability for clinical laboratories reshaped the diagnostic, prognostic and follow-up management of IgE-mediated allergic reactions, resulting in the current precision medicine approach in allergology. While the screening step is still mainly performed using allergenic extracts or MA mixes [34, 113], investigation of the molecular substrate is usually performed when sensitization to a given allergenic source
is identified. Marker MA single out genuine sensitization, cross-reactive MA describe cross-reactivity patterns, and selected MA from common allergenic sources inform on the risk of severity, persistence, later development of allergic diseases, andAIT eligibility, choice thereof, and therapeutic response. Each of these steps requires constant collaboration between clinical and laboratory specialists [34]. The ideal setting comprises allergists, clinical laboratory specialists, and basic science specialists addressing the MA field from different perspectives, ultimately providing the best care for the allergic patients [114]. Cross-sectional allergology units based on multidisciplinary education and practice within healthcare facilities and coordination with patient associations are increasingly set up in Europe [115, 116], foreseeing an increase in companion laboratory methods such as MA assessment.

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