Chapter

Fundamentals of Molecular Allergy: From Bench to Bedside

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Abstract

This chapter describes the fundamentals of molecular allergy diagnosis and raises the concept of allergens, allergenic components, and recombinant allergens. In addition, the authors review quality aspects related to the laboratory methodology. In the last part of the chapter, the different singleplex and multiplex platforms currently used for molecular diagnosis are compared. Finally, the diagnostic systems' challenges, strengths, and pitfalls are discussed to understand their clinical impact. Undoubtedly, this chapter will be handy for the background knowledge for health personnel, allergists/immunologists, and clinical laboratory personnel to guide the selection of diagnostic tests for allergy as well as their interpretation and therapeutic approach.

Keywords: molecular allergy, laboratory tests, allergens, allergy diagnostic

1. Introduction

Allergies are one of the most prevalent diseases affecting almost one billion people worldwide [1]. The traditional approaches for identifying allergen-specific IgE have undergone a revolution as a result of the growing need for the best diagnostic techniques, technological advancements, and understanding of allergen structure and obtention. These technical and scientific advances are the fundamentals of molecular diagnosis and precision medicine in allergy [2, 3].

2. Principles of laboratory testing for molecular allergy

Immunoglobulin E (IgE) is one of the five immunoglobulin isotypes described in humans and is considered to mediate hypersensitivity type I reactions and be the main soluble molecule involved in allergy pathology [4]. This immunoglobulin has historically been recognized as a biomarker for allergic processes. Due to its feasibility to detect and measure in serum samples, several laboratory methods focus on the identification of total IgE (tIgE), and specific IgE (sIgE) [5].

Measurements of tIgE and sIgE are based on antigen-antibody reactions. For tIgE detection, an anti-IgE antibody (detection antibody) will bind to the fragment crystallizable region in the immunoglobulin E. For sIgE, the serum sample is incubated

with the allergen-coated surface before incubation with the detection antibody, thus allowing allergen-specific IgE to be detected. Finally, the reaction is detected according to the platform methodology: radiation, colorimetry, fluorometry, or chemiluminescence [6–8].

2.1 Units and equivalences

Serum IgE is usually found in very low concentrations ranging <1μg/mL. Most immunoassay systems now use a total calibration curve that is associated with the World Health Organization (WHO) IgE standard and reported in arbitrary units; for better comprehension, tIgE is reported in IU/ml or kIU/L, which is equivalent to 2.4 ng/ml; [9] while sIgE is reported in kUA/L (kUA/L kilo mass units of allergenspecific antibody per unit volume) [10]

2.2 Methodologies for tIgE and sIgE determination

The evolution of methods for IgE diagnostic comprises methods like Radio-Immuno-Sorbent-Test (RAST), Paper-Radio-Immuno-Sorbent-Test (PRIST), and Enzyme-Linked Immuno-Sorbent-Assay (ELISA), gave rise to more reliable, safe, and automatized methods. A deeper revision of these methodologies could be found in [6].

2.3 Current methodologies used for sIgE determination

2.3.1 Enzyme-linked-immuno-sorbent assay (ELISA)

ELISA protocols are based on colorimetric reactions. Allergen is bound to the plate, then the sample of the patient containing IgE is incubated, allowing it to react with the allergen, forming the first antigen-antibody complex. Then a secondary antibody linked to an oxidizing enzyme binds to the previously formed complex, and by addition of the substrate, the color begins to develop. Finally, the plate is read in a spectrophotometer to detect the absorbance, which is proportional to the sIgE concentration (**Figure 1**) [11].

2.3.2 Immunoblot

For immunoblot-based methods, antigens are bound to a polymeric membrane acting as the solid phase, allowing IgE to interact with the different allergens. Then a phosphatase alkaline-linked secondary antibody is added to the reaction. Finally, the substrate precipitates leaving colored marks in the spots where patient´s IgE reacted with allergen (**Figure 1**) [12].

2.3.3 Chemiluminescence

The method for chemiluminescence platforms is very similar to ELISA. Alkaline phosphatase, which is linked to the secondary antibody, produces chemiluminescence signals when it reacts with its substrate, the phosphate ester of adamantyl dioxetane. In this method, the intensity of chemiluminescence is proportional to sIgE concentration (**Figure 1**) [13].

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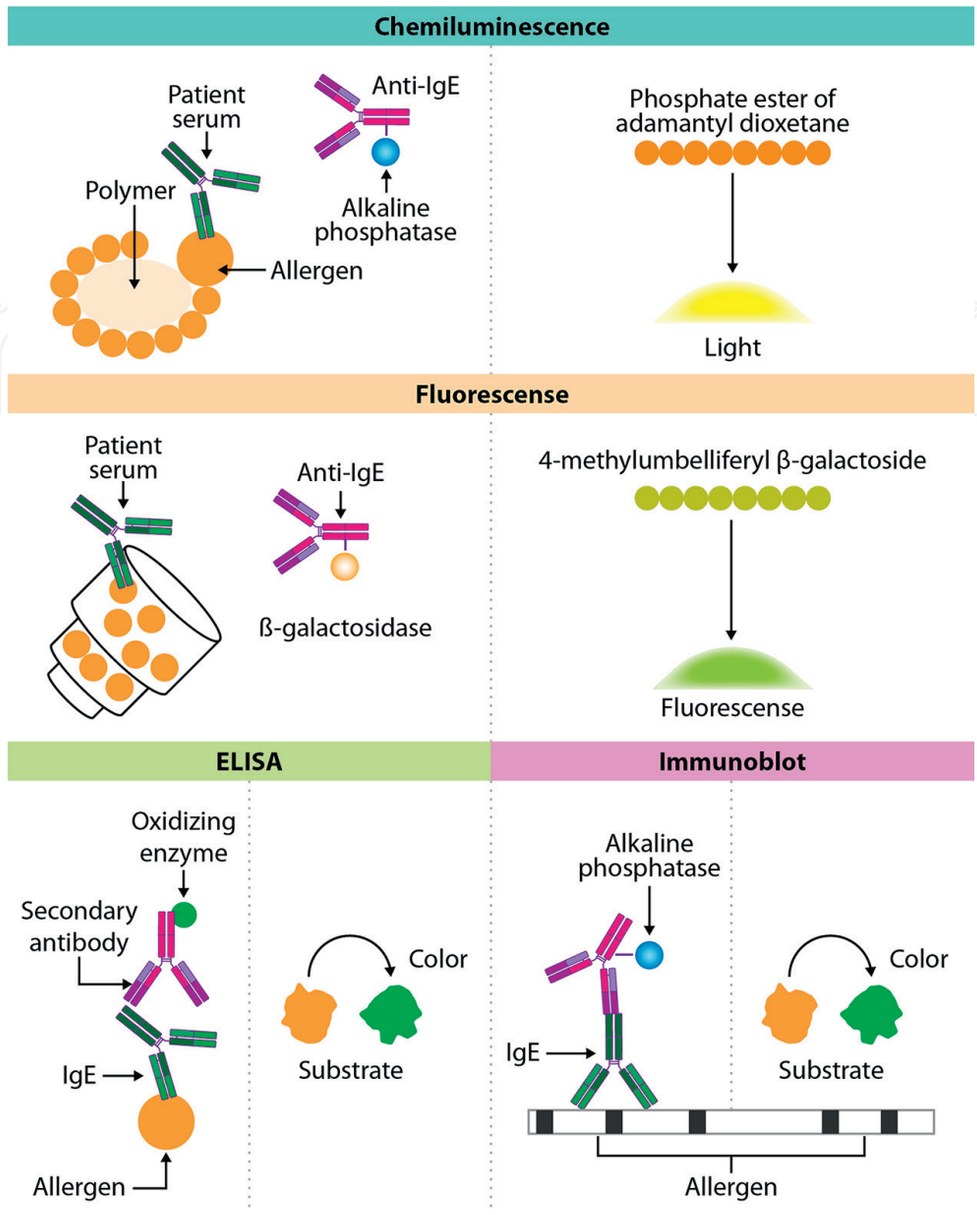

Figure 1.
Fundamentals of current methods for IgE detection. Different techniques are used to determine tIgE and sIgE in patients' samples; all of them are based on Ag-Ab reaction.

2.3.4 Fluoro-enzyme-immunoassay (FEIA)

In FEIA techniques, the secondary antibody is coupled to galactosidase, which reacts with 4-methylumbelliferyl βgalactoside to generate fluorescence proportional to the amount of specific IgE in the sample (**Figure 1**) [14].

3. Singleplex platforms for IgE determinations

Singleplex platforms permit the determination and quantification of tIgE or sIgE. In the case of sIgE determinations, these instruments identify one allergen per reaction.

Singleplex devices usually include the following components: [2, 14]

1. An allergen platform in solid or liquid phase.

2. A reaction container in which human serum or controls are exposed to anti-IgE detection reagent.

3. Calibration, data acquisition, processing, and analysis systems.

3.1 ImmunoCAP Phadia by ThermoFisher

It was the first automated platform using FEIA as the operating principle, showing high concordance with RAST in its results. Phadia 250 has a processing capacity of 60 tests per hour and allows the use of native and recombinant allergenic components which are grouped within the ImmunoCAP line, such as grass pollens, weed pollens, tree pollens, microorganisms, animal proteins, and mites, among others [15, 16].

3.2 Immulite by Siemens

Immulite is an IgE detection platform based on chemiluminescence. This equipment determines a variety of allergens from animals, drugs, food, grasses, insects, mites, mold, parasites, trees, and weeds, among others. It also includes a panel of 26 recombinant allergenic components. Immulite 2000 is capable of processing up to 200 results per hour and with a sensitivity of up to 0.1 kU/L [17, 18].

3.3 Hytec 288 by Hycor Biomedical

Hytec 288 is an immunoassay instrument based on ELISA. This platform offers the determination of single allergens and allergen mixture from drugs, food, grasses/weeds, animal proteins, among others. This equipment could perform up to 288 tests per run [19].

These three platforms are the leaders in the global market and exhibit excellent analytic sensitivity, precision, reproducibility, and linearity in total and allergen-specific IgE assays, but some variability in allergen-specific IgE quantitative estimates [16, 19].

4. Multiplex platforms for IgE determinations

Multiplex immunoassays allow for the identification of IgE sensitization repertoires against a diverse set of allergens. In contrast to singleplex platforms, the results are semiquantitative and not interchangeable. Characteristics of both platforms can be seen in **Table 1.**

4.1 Immuno solid-phase allergen chip (ISAC), by Thermo Fisher

The immuno solid-phase allergen chip (ImmunoCAP-ISAC) was the first multiplex platform designed and approved for IgE identification. This platform is based on the FEIA on-chip methodology, which can identify up to 112 allergenic components from 48 different allergen sources in approximately 4 hours. The ISAC system employs ISAC standardized units (ISU-E) ranging from 0.3 to 100 ISU-E, equivalent

	Singleplex	Multiplex
Results	**Quantitative**	**Semi quantitative**
Allergens tested per run	Depends on platform	Up to 178
Test result time	60 per hour- 200 per hour for individual tests	4h for whole panels
Cost	Cheap (if testing for few allergens)	Expensive
Personnel	Laboratory Technician	High-trained Laboratory Technician

Table 1.
Comparison between singleplex and multiplex platforms.

to 0.3–100 kUa/L, to categorize sIgE concentration into four groups: undetectable or very low (0.3 kUA/L), low (0.3 to 13 kUA/L), moderate to high (13 to 153 kUA/L), and very high (153 kUA/L) (**Figure 2**) [11, 20].

4.2 EUROLINE by Euroimmune

Euroline is a semiquantitative based immunoblot instrument. It provides pre-coated membranes for detecting sIgE from various allergen sources. These precoated membrane panels are tailored to the clinically relevant allergens in the regions where these are commercialized. Interestingly, this platform offers reagents for diminishing cross-reactive carbohydrate determinants (CCD), improving sensitivity. The number of allergens detectable in one membrane varies depending on the panel in use (8–45 allergens). The results can be obtained in a lapse of approximately two hours [21].

4.3 Allergy Explorer (ALEX) by Macro Array Diagnostics

The Allergy Explorer platform was the first to use an ELISA-based methodology to determine tIgE and sIgE levels for 117 extracts and 178 recombinant allergens at

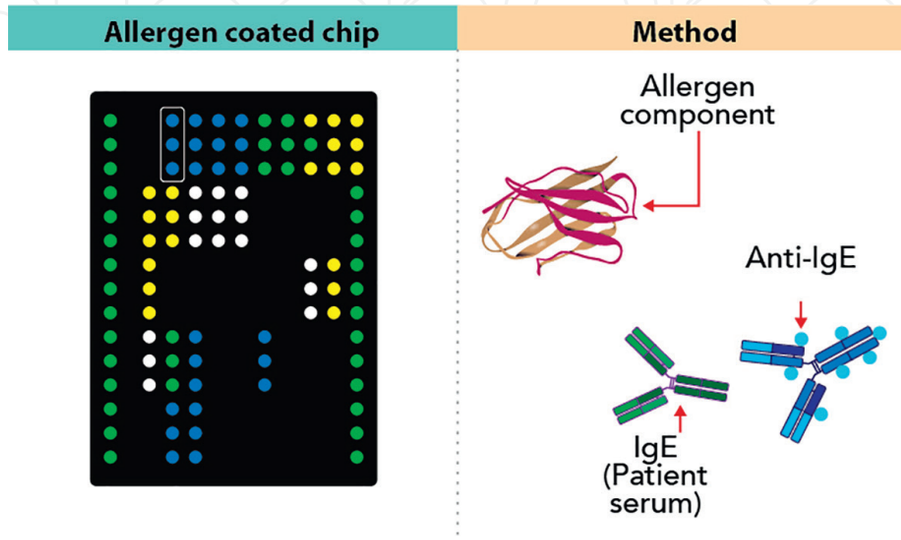

Figure 2.
Immuno solid-phase allergen chip (ISAC). Multiplex immunoassay based on FEIA methodology.

the same time. This device can block the determination of clinically irrelevant sIgE directed against CCD. The platform has manual and automated processing formats, with the capacity to analyze up to 50 patients in an approximate time of 4 hours. ALEX contains pre-designed panels by a group of symptoms or group of allergens, such as grass pollens, dander allergens, epithelium of animals, mites and cockroaches, molds, and yeasts.

The results of the tests are presented graphically, including the allergen's name, the specific allergen component or extract, the biological function, and the reported sIgE concentration in kUA/L. The final report includes a demonstration of possible cross-sensitization as well as interpretation and medical follow-up recommendations for the treating physician. ALEX employs a classification based on the concentration of sIgE obtained: Negative or uncertain (0.3 kUA/L), low (0.3 to 1 kUA/L), moderate (1–5 kUA/L), high (5–15 kUA/L), and very high (> 15 kUA/L) (See **Figure 3**) [22].

These three instruments evaluate the eight most common allergen families: Bet v 1-related protein (PR-10); Venom group 5 allergen family; Cupin Superfamily; EF-hand domain (Ca++ binding proteins); Expansin C-terminal domain; Lipocalin; Profilin; and Prolamin superfamily [20–22].

Although evaluated in different allergic diseases with patients sensitized to different allergens its performance, sensitivity and specificity have been reviewed and tested by different authors (**Table 2**) [8, 23, 24].

5. Allergens, allergenic extracts, and allergen components.

As mentioned above, laboratory diagnosis relies on antigen-antibody reactions, with the allergen defining the IgE specificity. Therefore, it is essential to emphasize the concepts of allergen, source, and obtention methods.

5.1 Allergens

Allergens are any molecule that binds to IgE antibodies [25]. Allergens are immunogenic antigens that induce a robust Th2 response, characterized by high IL-4 and IL-13 production with secretion of IgE [26].

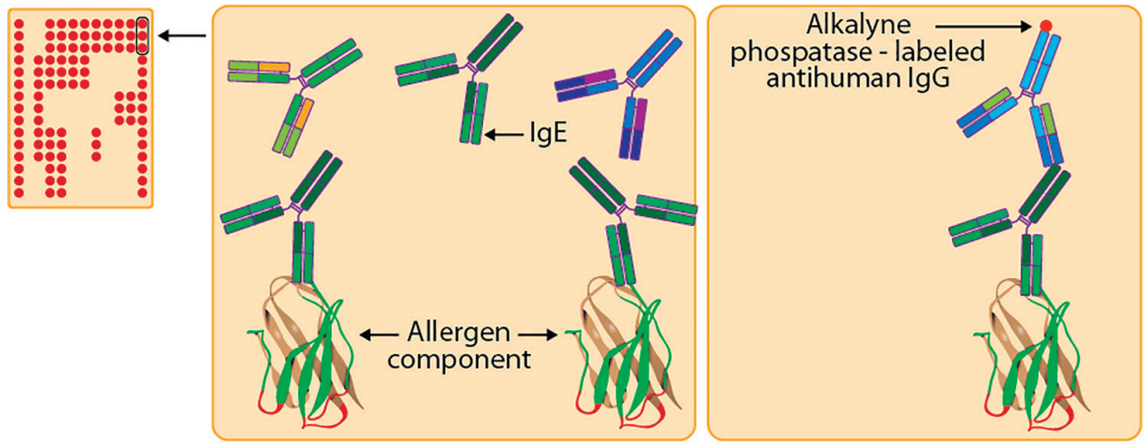

Figure 3.
Allergy Explorer (ALEX). Multiplex immunoassay based on ELISA technique.

	ALEX	ISAC	EUROLINE
Allergens or components tested per run	117–178	112	Up to 45
Test result time	4 h	4 h	3h (time optimized), 3.5 h (time/volume optimized), 14–26 h (volume/time optimized)
Sensitivity	93%*	86%**	31–88.9%***
Specificity	100%*	100%**	70–96.7%***
Methodology	ELISA	Fluorescence	Blot

**Evaluated in tree nut allergy.*
***Systematic review.*
****Compared to skin prick test.*

Table 2.
Comparison of multiplex platforms.

5.2 Allergen extracts

Allergen extracts (AEs) are complex mixtures of allergenic and nonallergenic molecules, including proteins, lipids, saccharides, nucleic acids, lipids, low molecular weight metabolites, pigments, and salts. AEs are obtained from natural sources such as pollens, animals, and insects, using physical methods (grinding) or chemical methods (solvents). Based on their intended application, allergen extracts should be characterized and subjected to quality control. As a result, validated assays must be developed to ensure the presence of relevant allergens for diagnostic or therapeutic applications (**Figure 4**) [27, 28].

5.3 Allergen components

Allergen components are isolated proteins derived from a purified extract of a specific allergenic source. These allergens, whether native or recombinant, are generally homogeneous and subject to stringent quality control [14].

Recombinant allergens are the most effective approach for obtaining allergen components. These highly pure allergens are produced by biotechnology; the process begins with cDNA obtention from mRNA through reverse transcription. Then, the cDNA may be modified (point mutations, chimeras/hybrids, fragmentation, oligomerization) to obtain the most accurate allergen molecule. Subsequently, the cDNA is inserted into expression vectors, usually *E. coli*. or *P. pastoris*, to express the protein and obtain the recombinant allergen. The allergen is then isolated, purified, evaluated, and validated for its usage in diagnostic platforms or to be used as a hypoallergenic allergens for immunotherapy (**Figure 5**) [29].

5.4 Structural importance of allergens

5.4.1 Proteins

Proteins constitute the vast majority of allergens, but only a few allergens bind IgE antibodies in the serum of most allergic patients. These molecules are known as "major allergens." A major allergen is defined as an antigen that binds to IgE in 50%

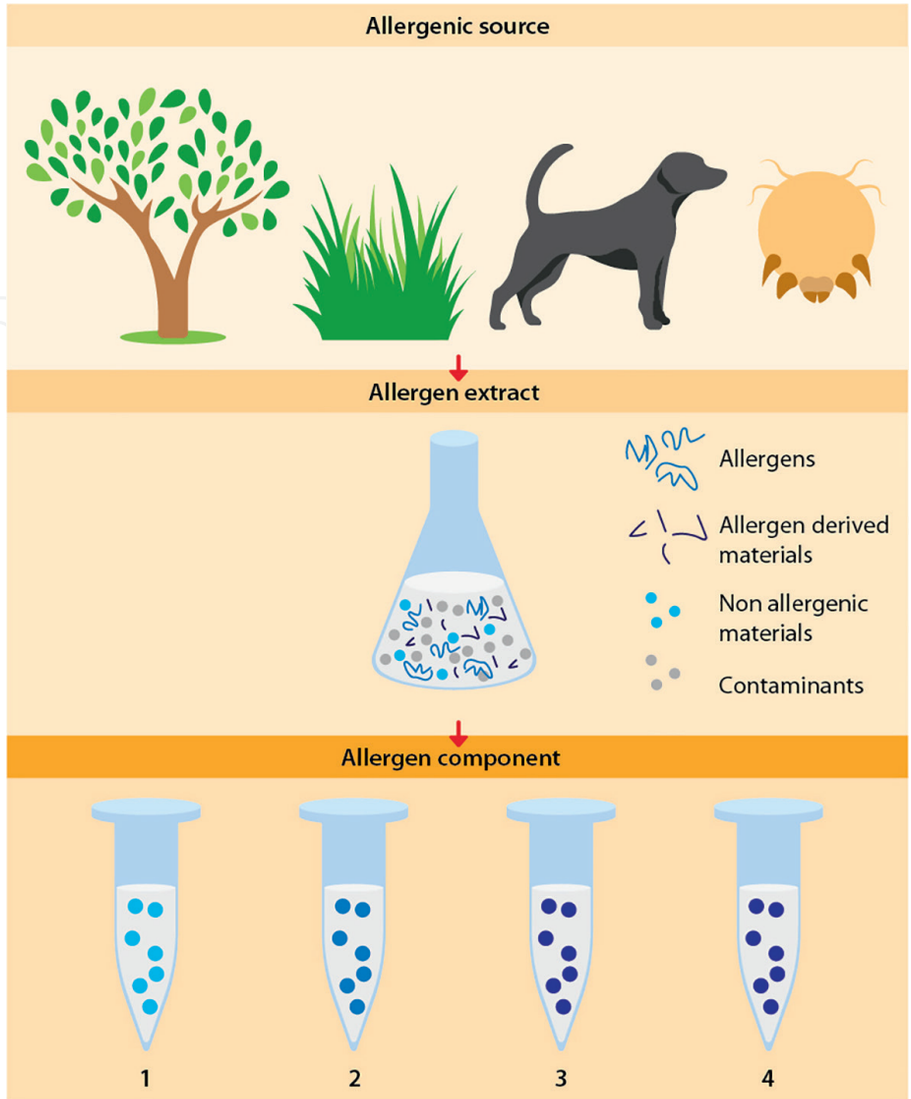

Figure 4.
Obtention of allergen extracts and allergen components from allergenic sources. Different techniques are used to obtain allergen extracts from diverse allergen sources. Most allergen extracts contain sensitizing allergen, allergen-derived materials, non-allergenic components, and contaminants. Following the obtention of allergen extracts, allergen components are isolated and purified, and protein characterization is performed.

or more of clinically allergic patients' serum. Other antigens that account for less than half of IgE binding are known as "minor allergens." Identifying major allergens has aided in understanding the immune response during allergic reactions, sensitization in atopy, and diagnostic applications.

5.4.2 Carbohydrates

Specific IgE antibodies for oligosaccharides are present in some patients, these antibodies cause numerous cross-reactions in vitro, given the designation cross-reactive carbohydrate determinants (CCDs). However, in recent years, oligosaccharide epitopes have been implicated in allergic sensitization, acute allergic reactions, and not just cross-reactions; consequently, characterization and discovery of glycan allergens have been a challenge. Currently, there are about approximately 20 oligosaccharides found in pollens, venoms, nematodes, worms, and ticks that are distributed in five glycans groups and have been shown to be significant for allergic disease [30].

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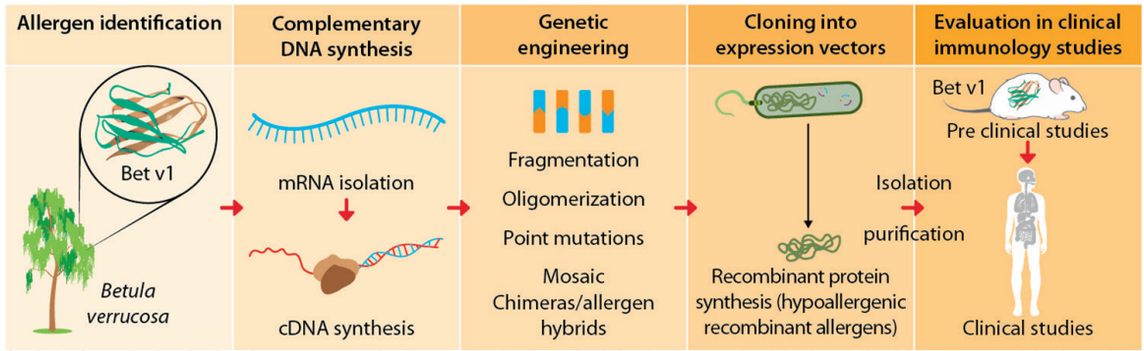

Figure 5.
Obtention of recombinant allergens. Recombinant allergens are obtained by isolating the mRNA from the allergenic source. Then transcribed into cDNA and inserted in bacteria or yeasts to allow its expression. Finally, clinical validation is needed to be used for diagnosis in vitro or in vivo.

5.4.3 Major groups

Group A. Cross-reactive carbohydrate determinants. Most CCDs are N-glycans, characterized by a basic structure of two GlcNAc with two or three terminal mannose residues. Allergens with these glycans are *Ole e* 1, *Api g* 5, *Bla g* 2 [30, 31].

Group B. Mammalian non-human oligosaccharides. The glycan structures described in this group are the disaccharide galactose-alpha-1,3-galactose, and the monosaccharide N-glycolyl neuraminic acid. These glycans are related to anaphylaxis and could be fond in red meat, tick bites, and some monoclonal antibodies [32, 33].

5.4.4 Minor groups

Group C. Oligosaccharides with O-linkage. O-glycans are oligosaccharides attached to serine or threonine residues on a protein and sometimes to tyrosine, hydroxylysine, or hydroxyproline. Examples of allergens expressing O-glycans are *Art v* 1, *Amb a* 4 [32].

Group D: Oligosaccharide Epitopes expressed on Schistosomes and other Helminths. These oligosaccharides have a single terminal galactose or N-acetylgalactosamine residue (GalNAc), keeping a molecular similarity to CCDs. Their clinical significance is still under study since alpha-1, 3-fucose epitope could be implicated with a paradoxical protective effect in asthmatic patients [34].

Group E. Short-chain galactooligosaccharides (GOS). GOS are usually produced by bacterial beta-galactosidase and occur naturally in milk processed with prebiotics. They are typically a chain of 2 to 6 galactose molecules attached to glucose and have been recognized in allergic reactions [30, 32].

5.4.5 Lipids

Lipid antigens are much less understood than carbohydrate antigens, they have been shown a direct effect on allergenic potential and cause allergic responses. For example, lipids delay the enzymatic digestion of Ara h 8 allowing this molecule to reach the intestinal immune system and favoring sensitization. Conversely, lipid-associated allergens such as Der p 2, Der p 5, and Der p 7 have been related to increased asthma symptoms and severe allergic reactions [35]. Thus, the application of sIgE determination against lipids is limited.

6. Interpretation, clinical applications, and limitations for molecular allergy

Even though the first cases of pollen-induced hay fever were documented in the early 1800s, it was not until 100 years later that a relationship to a serum factor called reagin was discovered (IgE) [4]. In the mid-1960s, allergy diagnosis was primarily relied on skin testing, and allergen extracts were far from standardized. However, developing recombinant allergens and starting allergen cloning between 1988 – 1995 created new opportunities for studying and diagnosing allergy disorders [29, 36, 37]. Molecular allergy is the practical application of these advances, allowing us to manage patients with high accuracy, and leading into the era of precision medicine.

6.1 Singleplex *vs*. multiplex immunoassays

As previously discussed, singleplex assays allow detection of IgE antibodies specific to the allergens identified in the patient's clinical history. A multiplex platform, in contrast, enables defining a person's IgE reaction to the whole range of allergens arrayed on a chip.

The main benefit of the singleplex immunoassays is that it measures the allergen-specific IgE antibody level in kilounits per liter (kUA/L) based on a total IgE calibration system that can be traced back to a human reference preparation from the WHO. The assay has high precision and reproducibility, reporting values as low as 0.1 kUA/L (range, 0.1–100 kUA/L), without interference of allergen-specific IgG antibodies.

Compared to multiplex immunoassays, singleplex assays have fewer allergen molecules available, give an incomplete IgE reactivity profile with just one or a few tests, are more expensive if more than one measurement needs to be taken, and need a larger amount of serum [38]. In contrast, multiplex assays are semiquantitative and provide a comprehensive IgE pattern using only a small volume of serum, which could be useful in the evaluation of polysensitized patients; but are only available in laboratories with high-end machinery with highly trained personnel, delaying results by days or weeks **(Table 3)** [39, 40].

Molecular immunoassays have some advantages over *in vivo* assays, such as the ability to be performed regardless of extensive skin disease or medications used, minor pain or anxiety- provoking in children, little patient cooperation required, and no risk to the patient. The fact that the whole allergen of a fresh allergen is more sensitive than purified allergen components is one of the limitations of molecular diagnosis compared to *in vivo* evaluation, this is particularly important if the goal is to perform allergen-specific immunotherapy [39, 40]. In contrast, advances in molecular allergy have enabled the development of vaccines based on recombinant DNA technology and synthetic peptide chemistry that could be monitored with sIgE or sIgG determinations throughout treatment [41].

6.2 Clinical allergy *vs*. sensitization

The majority of allergens, but not all, are sensitizing, which is defined as the capacity to induce allergen-specific IgE antibodies. Non-sensitizing allergens can only cause allergic symptoms if the individual has been sensitized to a cross-reactive allergen [3]. Cross-reactivity defines an antigen attribute intrinsically related to the allergen molecular characteristics that determine immune recognition by IgE.

	Singleplex immunoassays	**Multiplex Immunoassays**
Number of allergens	Limited, selected according to clinical history.	Complete profile of allergens, useful in polysensitized patients.
Preferred for cross- reactivity suspicion	No	Yes
Preferred for immunotherapy selection	No	Yes
Preferred for patients with well-known sensitization history	Yes	No
Cross-reactive carbohydrate determinants (CCDs) evaluation	No	Yes

Table 3.
Variables to consider when the molecular diagnosis is selected for the clinician: Singleplex vs. Multiplex instruments.

Identification of cross-reactivity is critical to detect patients with a high risk of anaphylaxis for example in peanut and tree nuts or seeds allergy. Other cases of cross-reactivity are latex and food, that is, banana, avocado, kiwi, and chestnut; and cross-reactivity between shellfish and insects due to chitins, specifically tropomyosin in dust mites

The precise point at which a sensitizing allergen causes clinical symptoms is determined by several factors such as quantity, exposure route, antigen structural characteristics, genetics, microbiota, innate or adaptative immune interactions, and microenvironment, among others. Thus, identifying an IgE-mediated mechanism is a critical step that directs avoidance measures and suitable pharmacological treatment. However, positive skin tests or specific IgE assay results do not always indicate that an allergen is causing symptoms; the clinical significance of allergen exposure and its relationship to symptoms must be established by examining the patient's medical history.

6.3 Allergen extracts *vs*. recombinant allergens

Although diagnostic assays based on purified recombinant allergens are becoming more popular, extracts from natural allergen sources continue to be widely used. The composition of an allergenic extract has a significant influence on the results of any IgE-based immunoassay.

Allergen extracts used in some platforms are made up of a variety of allergens, some of which have little or no clinical significance, such as carbohydrate epitopes in peanut or timothy grass pollen, which might result in false positive findings [38]. The use of allergenic extracts allows to precisely detect the specificity of the IgE in a patient's sample, but also permits the evaluation of only clinically relevant components from allergenic sources.

In the other hand, protein characterization of allergens has been fundamental to understand IgE cross-reactivity data in the absence of allergen-antibody complexes. Some of the benefits of recombinant allergens include increased diagnostic accuracy, the ability to distinguish genuine sensitization from cross-reactivity, the ability to evaluate the type and risk of an allergic reaction, and the ability to select patients and suitable allergens for immunotherapy [29, 42].

7. Conclusions

Personalized therapy based on genetic, immunologic, and functional endotyping, defined as the examination of a biological or pathological process, including therapeutic response through biomarkers determination, is part of the new treatment advances for allergy patients known as precision medicine. As previously discussed, a correct diagnosis is critical in these therapeutical approaches. In the case of molecular allergy, the choice of testing is influenced by several variables, including test accessibility, clinical history, technical constraints, type of allergen, immunoassay accuracy, single or multiplex platforms, and most importantly, the clinical question that the analysis pretends to resolve.

Finally, despite molecular diagnosis is an excellent tool for selecting the appropriate allergens for immunotherapy, minimizing potential test-related complications, evaluating polysensitization with difficult interpretation, and possibly predicting clinical outcomes. Unfortunately, the high cost, access limited in low-income countries, restricted availability due to regulatory affairs in others, and a lack of sufficient clinical studies with recombinant allergens keep molecular allergy out of reach for routine use, but with a promising future once these limitations are overcome.

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Conflict of interest

The authors declare no conflict of interest.

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