



Microarray Immunodiagnostics for Aeroallergens

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Abstract

Purpose of Review The impact of new technologies, especially multiplexed molecular allergy diagnostics based on allergen arrays, on the management of complex patients with respiratory allergies has been important.

Recent Findings Currently, the detailed characteristics of the IgE profile of the patient, such as sensitization to genuine or cross-reacting components or the sensitization to potentially harmful allergens, allow an allergist to tailor treatment in the context of precision medicine rules.

Summary A number of relevant articles have been published in recent years on this topic, and, in this review, the new added values of allergen array-based diagnostics are reported.

Keywords Allergen source · Allergen · Laboratory assays · Single-plexed · Multiplexed · Aeroallergens

Introduction

Since the discovery of the first allergens produced by genetic engineering [1], it was evident that new tools for allergy diagnosis were available. Within few years, molecular allergens were used in clinical trials and then in clinics. In early 2000, the development of allergen array [2] further modified the strategy of using molecules in the description of the IgE profile of the patients. This new strategy was deeply integrated in the new precision medicine approach, due to the very accurate characterization of the patient's phenotype. In the same years, personalized medicine, focused on the patient's endotype, was proposed [3]. Nowadays, the integration of precision medicine (based on the fine IgE profile of the patient) with personalized medicine (based on the patient's clinical endotype)

seems to be the winning strategy. Molecular allergy diagnosis (MAD) has been extensively used in patients with food, latex, and hymenoptera sensitization [4]. But probably in patient with a sensitization to aeroallergens—when an allergy immunotherapy (AIT) approach is possible, MAD will probably represent a gold standard in a near future.

In this work, we describe the different MAD approach used in patients sensitized to inhalant allergens and discuss the possible role of MAD in the identification of the best AIT. Along this line, we will consider “allergen sources,” the original material (pollen, dust, etc.); “extracts from allergens sources,” the raw material used for skin prick test and specific IgE assay; “allergens (but also components),” the single molecules that has been shown to display an allergenic activity in vitro and/or in vivo.

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The Allergic Diseases of the Respiratory Tract

Rhinitis, Rhinoconjunctivitis, and Rhinosinusitis Rhinitis is an inflammation of the nasal mucosae caused by allergic, non-allergic, infectious, hormonal, and occupational factors. Although allergic rhinitis is considered the most frequent type of chronic rhinitis, other factors account for a percentage of the case variable between 30 and 70% (see the ARIA workshop report). Rhinitis can occur alone or be associated with conjunctivitis and/or sinusitis. In some cases, hypertropia of the mucosa up to the nasal polyps can be detected in patients with chronic allergic rhinitis. Rhinitis can be associated with sensitization to seasonal allergens (such as pollens) or perennial allergens (such as mites, pets, and molds).

Symptoms associated with bronchospasm include shortness of breath, wheezing, and coughing as well as chest tightness. Additionally, allergic asthma can be caused by seasonal and perennial allergens. However, other non-aeroallergens (such as certain food or drugs) can be involved in exacerbation of asthma.

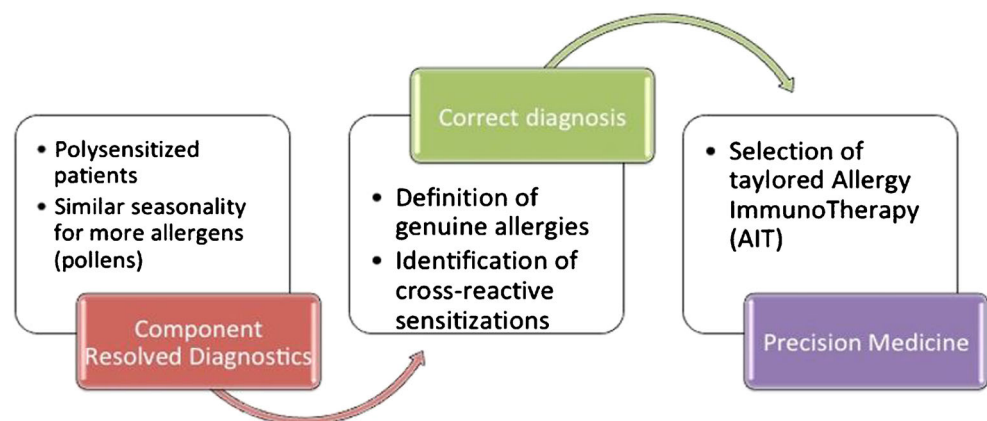
Finally, both rhinitis and asthma can be associated with locoregional (mouth) or systemic symptoms in the case of a pollen-food syndrome. Therefore, an aerogenic sensitization to birch (in particular, to its main component Bet v 1) can cause oral allergy syndrome (OAS) after eating a fresh apple, containing Mal d 1, a component highly homologous to Bet v 1. Of note, due to its susceptibility to heat, Mal d 1 does not cause OAS if apples are eaten after cooking.

The In vitro Diagnosis of Respiratory Allergy

The diagnosis of allergic respiratory diseases includes a detailed clinical history with particular attention to the seasonality of consistent symptoms and the relationship between exposure to airborne allergens and symptom onset as well as a

diagnostic test to demonstrate the IgE sensitization to relevant allergens (mainly skin prick tests and/or serum specific IgE assays). Patients may be sensitized to a single source or multiple allergenic sources, and there is increasing evidence that polysensitized individuals account for up to 75% of allergic patients (according to the different studied populations) [5] and that their proportion is constantly increasing over time [6]. Skin prick tests (SPT) are suggested as first-line diagnostic tools for allergic diseases [7], as they give faster results, are less invasive, they are cheaper than serological tests, and have good diagnostic accuracy [8, 9]. For polysensitized patients, SPT may not be conclusive in confirming or ruling out a real association between positive results for given allergens and exposure to them [10]. Therefore, second-level tests [4] are often useful to better diagnose allergic respiratory diseases in these polysensitized patients: the dosage of serum IgE concentrations for extracts of allergenic sources (i.e., pollens, molds, mites); third-level tests [4] for their single allergenic components (i.e., genuine and cross-reactive allergenic molecules of given allergenic sources); and multiplex molecular allergens assays, which include Thermo Fisher ISAC [11], Mdx ALEX [12], FABER [9], Euroimmun Multiplex immunoblot assay [13] that are all commercially available, while Microtest [14] and MedAll [15] do not seem to be distributed. The possibility to detect single allergenic components gives the clinician the opportunity to make a more precise diagnosis of allergic diseases using the approach defined as the “component resolved diagnosis” (CRD) [16, 17]; indeed, distinguishing between genuine and cross-reactive allergenic sources may help the clinician in defining the most likely causal allergen exposure and allergic symptoms relation and rule out nonrelevant skin prick test positivities, particularly in polysensitized patients. This approach is particularly important when AIT strategies are available, and choosing the right allergenic target for any single patient is a good, practical and easy-to-apply example of “precision medicine” [18]. In Fig. 1, the clinical relevance of CRD is summarized.

Fig. 1 The pathway from the patient to precision medicine in an allergy diagnosis



The Molecular Diagnosis

Although allergies related to inhalant allergens were described in the late 1800s, and the first *in vivo* assays, such as the skin prick test, were used in the first decades of 1900s, it was not until the 1960s that two different groups from Japan [19] and Sweden [20] identified IgE, which is the class of antibodies that was directly involved in a large fraction of allergic manifestations. From that moment, relevant laboratory tests (such as the famous radioallergen sorbent test or RAST [21]) were developed and improved, and, in late 1990s, highly automated random-access instruments were available for large-scale screening of IgE sensitization. In those years, hundreds of extracts from allergen sources were commercially available, including inhalants, food, molds, and drugs. However, it was evident for some decades that these extracts (such as those from the pollen of Birch) were constituted by a complex mixture of molecules, some of which were allergens, while others were non-allergenic components. In the same years, different groups started isolating single allergens by biochemical methods or cloned the allergen gene in *E. coli*, obtaining a protein with structural and functional characteristics corresponding to the original component [22] with the obvious exception of posttranslational modification including glycosylation that were absent. This aspect was immediately considered a significant advantage for the use of allergens in molecular allergy diagnosis; indeed, IgE to cross-reacting carbohydrate determinants (CCD) [23], which are frequently observed in patients with inhalant allergy, were unable to bind to the component derived from *E. coli*, but were able to bind to the same molecule obtained by extractive methods or expressed in some insect cells or plant cells. This sort of “non-specific” reactivity was carefully considered in recent protocols of AIT in certain climates [24]. Along this line, the risk of incorrect IgE test results was also described for certain reagents [25•].

In the late 1990s, the first attempts to improve allergy diagnosis by using components were successful, and, a few years ago, another approach based on the use of arrays of allergens on a single chip was developed [26]. Similar to other innovative approaches, the use of allergen arrays was immediately accepted from a fraction of the allergist community but was refused or not considered by others.

Therefore, allergists that were against a systematic use of allergen arrays sustained the virtual uselessness of a large panel of allergens in the large majority of allergic patients, argued the costs and were annoyed by the possibility that unexpected sensitization could be found. Allergists who were in favor of the use of arrays, in contrast, sustained the importance of obtaining a panel of allergens larger than that derived from SPT and from specific IgE testing, particularly in those patients where a clear polysensitization was evident from first-line diagnostics, a pollen-food syndrome was suspected, and a

significant effect of cross-reacting components and of pan-allergens was expected.

These two different approaches were recently identified as the top-down and bottom-up approaches for molecular allergen diagnostics and allergen arrays used at the end of the diagnostic process or at the beginning of the diagnostic process, respectively [27••]. Currently, the two processes can be mixed in different manners; for example, a bottom-up approach allows the definition of the patient’s phenotype (based on the specific IgE profile), and the subsequent top-down approach allows the definition of the patient’s endotype based on the presence of asthma, rhinitis, etc. [3•]. A comprehensive allergy diagnosis must consider those two characteristics to design a modern and efficient therapeutic proposal.

From a practical point of view, a molecular allergen (also known as a component) can be differently classified, as shown in Table 1. Allergens and components can be sorted in different manners; notably, all these characteristics can be mixed together, and, for this reason, the interpretation of a complex allergen array is sometimes a challenge for skilled allergists. A comprehensive view of the problem is presented from both a structural biology [29••] and a more practical point of view [30•].

Laboratory Technologies Available

At present, two different laboratory approaches based on slightly different technologies are available to allergists: the so-called single-plexed and multiplexed approaches.

In the former approach, one or more molecular components are identified by the allergist as relevant in the diagnostic process; the costs will be under control, but only what is searched for is found. In the latter approach, the use of allergen

Table 1 A functional classification of components

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- | |
|---|
| a) Nature (inhalant, food, contact, hymenoptera) |
| b) Reactivity (genuine if only associated with a given allergen source, cross-reactive if associated with different allergen sources and pan-allergen, if extensively distributed in nature) |
| c) Risk (allergen characterized by a low risk reaction, profilins, polcalcins, and another by a higher risk of reaction, lipid transfer proteins, 2S albumins, 7S and 11S globulins, tropomyosins, etc.) |
| d) Major or minor antigens (if the frequency of a given component is > 50 or < 50%, respectively, of subjects sensitized to the allergen source extracts) [28] |
| e) Pathogenic or nonpathogenic allergens (if the sensitization to a component is associated with a clear manifestation of the disease or the disease may be inconsistently associated to relevant symptoms) |
| f) Resistant to heat, low pH, and peptidase (if the component is able to maintain its allergenicity after cooking and/or digestion in the gastroenteric tract). |
| g) Geographic distribution of the allergen and its sensitization patterns |
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arrays (preconstituted and unchangeable) increases the costs but allows the description of an exhaustive IgE profile.

For single-plexed technology, reagents from Thermo Fisher ImmunoCAP, Siemens, and Hycor represent the very large majority of assays performed worldwide. For multiplexed technology, Thermo Fisher ImmunoCAP ISAC, which is constituted only by molecular components, was a standard reagent since 2007 [11, 31]. In 2017, MADx developed ALEX, which is a macroarray of molecular components that were spotted together with extractive allergens [12•]. Therefore, ISAC is a pure third-level reagent and utilizes the information from SPT and/or specific IgE for the interpretation of the results of molecular components [4], while ALEX is a combination of the second and third levels for the presence of both extractive allergens and molecular components on the same chip. The strategy to be used in the decision of which is the best approach is still a matter of debate, and the author's opinion is shown in Table 2.

The Interpretation of a Molecular Allergy Tool in Practice

It is evident that few allergists are aware of the characteristics of all components, and, for this reason, a strategy for interpretation could be useful. In interpreting laboratory results related to IgE, it should always be considered that laboratory tools are able to describe the profile of sensitization of the patient (the so-called phenotype), while the endotype (namely, the synthesis of sensitization and clinical signs and symptoms) is defined

by the allergist. Then, in the case of a single-plexed approach, allergists should first consider whether the results of the SPT already performed were confirmed by the IgE assays. This is true in the large majority cases but not in all. Second, considering the therapeutic plan for that patient, the allergist should request for the relevant panel of components for a given positive specific IgE test. For example, in the presence of a positive IgE to grasses (such as the Phleum pratense, Phl p 1 and Phl p 5, genuine and major components, as well as Phl p 7 and Phl p 12, cross-reacting components, a polcalcin and a profilin, respectively) should be evaluated at the molecular level. Indeed, it has been suggested that AIT is more effective in the presence of genuine sensitization, while cross-reactive sensitization has a poorer possibility of success [32, 33]. Similar algorithms can be defined for all the other situations [24]. A skilled allergist will recognize certain positive allergens on the SPT that were negative by genuine molecular components and were associated with a cross-reaction (and, then, they will not have a real clinical relevance). The interpretation of allergen arrays is more complex, but, as stated before, these tests are indicated in polysensitized patients, in pollen-food syndrome, etc. Therefore, the interpretation of ISAC will be performed considering the results of the SPT and/or of the specific IgE tests. Additionally, in this case, the concordance between the two diagnostic tools should be verified. In the case of inhalant allergies, genuine components will be identified and will be considered the proofs of a specific sensitization to a given allergen (Table 3). If this evidence is supported by clinical signs and symptoms, the patient will be considered allergic, and a therapeutic plan will be

Table 2

Situation	Option	Single-plexed		Multiplexed			Use	Comment
		Specific IgE	Molecular components	ISAC	ALEX	EL		
Mono-sensitized seasonal rhinitis	1	Yes	NN	NN	NN	NN	For medical treatment only	
	2	Yes	Yes	No	No	No	For AIT	1
Polysensitized seasonal rhinitis	1	Yes	No	Yes	No	Yes*	Similar results in all cases	1, 3
	2	No	No	No	Yes	Yes*		2, 3
Chronic rhinitis, nasal polyps	1	Yes	No	Yes	No	Yes*	Similar results in all cases	1, 3
	2	No	No	No	Yes	Yes*		2, 3
Mono-sensitized asthma	1	Yes	NN	NN	NN	NN	For medical treatment only	
	2	Yes	Yes	No	No	No	For AIT	1
	3	No	No	No	Yes	Yes*		2, 3
Polysensitized asthma	1	Yes	No	Yes	No	Yes*	For AIT	1, 3
	2	No	No	No	Yes	Yes*		2, 3
Pollen-food syndromes	1	Yes	No	Yes	No	Yes*	Similar results in all cases	1, 3
	2	No	No	No	Yes	Yes*		2, 3

(1) Frequently requires double access to the lab (the first for the specific IgE test and the second for the molecular components); (2) in general requires single access to the lab. (3) yes*: Euroline (EL) strips contain both extracts and components and can be used in selected situations when the IgE profile can be described with few reagents

Table 3 Components belonging to the first 10 allergen families in ISAC, ALEX, and Euroline

		ISAC	ALEX	Euroline
Bet v 1-related protein (PR-10)	<i>Actinidia deliciosa</i> (green kiwi)	Act d 8	—	—
	<i>Alnus glutinosa</i> (alder)	Aln g 1	Aln g 1	—
	<i>Apium graveolens</i> (celery)	Api g 1	Api g 1	—
	<i>Arachis hypogaea</i> (peanut)	Ara h 8	Ara h 8	—
	<i>Betula pendula</i> (white birch)	Bet v 1	Bet v 1	Bet v 1
	<i>Corylus avellana</i> (hazel)	Cor a 1	Cor a 1	—
	Glycine max (soybean)	Gly m 4	Gly m 4	—
	<i>Daucus carota</i> (carrot)	—	Dau c 1	—
	<i>Malus domestica</i> (apple)	Mal d 1	Mal d 1	—
	<i>Prunus persica</i> (peach)	Pru p 1	—	—
Venom group 5 allergen family	<i>Polistes dominulus</i> (European paper wasp)	Pol d 5	Pol d 5	Pol d 5
	<i>Vespa vulgaris</i> (yellow jacket)	Ves v 5	Ves v 5	Ves v 5
Cupin superfamily	<i>Anacardium occidentale</i> (cashew)	Ana o 2	—	—
	<i>Arachis hypogaea</i> (peanut)	Ara h 1	Ara h 1	Ara h 1
	<i>Arachis hypogaea</i> (peanut)	Ara h 3	Ara h 3	Ara h 3
	<i>Corylus avellana</i> (hazel)	Cor a 9	Cor a 9	—
	<i>Corylus avellana</i> (hazel)	—	Cor a 11	—
	Glycine max (soybean)	Gly m 5	Gly m 5	—
	Glycine max (soybean)	Gly m 6	Gly m 6	—
	<i>Juglans regia</i> (English walnut)	Jug r 2	Jug r 2	—
EF hand domain (Ca ⁺⁺ -binding proteins)	<i>Betula pendula</i> (white birch)	Bet v 4	—	—
	<i>Gadus callarias/morhua</i> (Baltic cod)	Gad c 1	Gad m 1	—
	<i>Penaeus monodon</i> (black tiger shrimp)	Pen m 4	—	—
	<i>Phleum pratense</i> (Timothy grass)	Phl p 7	Phl p 7	Phl p 7
Expansin, C-terminal domain	<i>Cynodon dactylon</i> (Bermuda grass)	Cyn d 1	—	—
	<i>Phleum pratense</i> (Timothy grass)	Phl p 1	Phl p 1	Phl p 1
	<i>Phleum pratense</i> (Timothy grass)	Phl p 2	Phl p 2	—
Lipocalin	<i>Blatella germanica</i> (cockroach)	—	Bla g 4	—
	<i>Bos domesticus</i> (domestic cattle)	—	Bos d 2	—
	<i>Bos domesticus</i> (domestic cattle)	Bos d 5	Bos d 5	Bos d 5
	<i>Canis familiaris</i> (domestic dog)	Can f 1	Can f 1	—
	<i>Canis familiaris</i> (domestic dog)	Can f 2	Can f 2	—
	<i>Equus caballus</i> (horse)	Equ c 1	Equ c 1	—
	<i>Felis domesticus</i> (domestic cat)	Fel d 4	Fel d 4	—
	<i>Mus musculus</i> (mouse)	Mus m 1	Mus m 1	—
Profilin	<i>Betula pendula</i> (white birch)	Bet v 2	Bet v 2	—
	<i>Hevea brasiliensis</i> (Para rubber tree)	Hev b 8	Hev b 8	—
	<i>Mercurialis annua</i> (annual mercury)	Mer a 1	—	—
	<i>Phleum pratense</i> (Timothy grass)	Phl p 12	Phl p 12	Phl p 12
	<i>Phoenix dactylifera</i>	—	Pho d 2	—
	<i>Olea europaea</i>	—	Ole e 2	—
Prolamin superfamily	<i>Anacardium occidentale</i>	—	Ana o 3	—
	<i>Arachis hypogaea</i> (peanut)	Ara h 2	Ara h 2	Ara h 2
	<i>Arachis hypogaea</i> (peanut)	Ara h 6	Ara h 6	Ara h 6
	<i>Arachis hypogaea</i> (peanut)	Ara h 9	Ara h 9	Ara h 9
	<i>Artemisia vulgaris</i> (mugwort)	Art v 3	Art v 3	—
	<i>Bertholletia excelsa</i> (Brazil nut)	Ber e 1	Ber e 1	—
	<i>Brassica/Sinapis</i> spp.	—	Sin a 1	—
	<i>Corylus avellana</i> (hazel)	Cor a 8	Cor a 8	—

Table 3 (continued)

	ISAC	ALEX	Euroline
<i>Corylus avellana</i> (hazel)	–	Cor a 14	–
<i>Fagopyrum esculentum</i>	–	Fag e 2	–
Glycine max (soybean)	–	Gly m 8	–
<i>Juglans regia</i>	Jug r 1	Jug r 1	–
<i>Macadamia integrifolia</i>	–	Mac i 2S	–
<i>Brassica/Sinapis</i> spp.	–	Sin a 1	–
<i>Papaver somniferum</i>	–	Psp s 2S	–
<i>Sesamum indicum</i>	–	Ses i 1	–

proposed. When ALEX results are achieved, a direct comparison between allergen extracts and allergen components (in other words, direct evidence of the presence of a specific or a cross-reacting sensitization) can be made. Then, the rules will remain the same: a pharmacologic treatment and/or AIT will be proposed to the patient according to the published algorithm. Of note, ALEX has a CCD inhibitor that virtually blocks all nonspecific recognition of extractive and purified molecules that still express carbohydrate chains on their surface [25•]. For this reason, any interference due to anti-CCD antibodies is avoided.

In the presence of polysensitized patients, it sometimes occurs that more than 25% of allergens and components are positive. In this case, not all these results can be easily explained; indeed, grasses share components with weeds and trees; mites share components with crustaceans, snails, and shellfishes; and cod share components with many other fishes, such as salmon and trout. These cross-reactions can be observed and explained at the molecular level. However, the allergists must define whether these sensitizations can also be associated with specific symptoms to identify an allergy and design a treatment.

In consideration of the real difficulties related to a comprehensive interpretation of allergen arrays (112 components in ISAC and 282 allergens and components in ALEX), in recent years, a number of informatic tools have been developed to support the interpretation of molecular allergy results, especially the results of allergen arrays. For example, with the use of machine learning, the results of how an allergen microarray can be associated with different diseases were evaluated [34]. For the interpretation of ISAC results, Allergenius [35], which is an expert system, a new and improved version of this tool was recently developed and implemented for the interpretation of ALEX results. Informatic support for allergy diagnosis management is expected to be relevant both in the analytical phase in the laboratory (for quality controls, evaluation of the coherence of the results, and comparison with similar patterns in a given regional environment) and to aid allergists' understanding of the complex relationships that govern component characteristics. For this reason, artificial intelligence tools will

probably become a standard in future procedures for allergy diagnosis and treatment. However, the role of allergists will remain determinant because the association between an IgE profile and relevant symptoms is the central issue in any clinical allergy process.

The Added Value of Molecular Allergy Diagnosis for AIT in Respiratory Allergy

In recent years, many authors have noted that, following an analysis of the IgE repertoire by MBD, the prescription of AIT could be modified. In 2007, Valenta et al. suggested that sensitization to genuine components could be the best target for AIT [33]. However, in the absence of published controlled studies finalized to this aim—that, at least in the Author knowledge, do not seem to be not even waited in a near future, the only evidence we have is the work of Schmid-Grendelmeier [32], who showed in an observational study that people with genuine sensitizations had better results than the group with IgE directed to cross-reacting components. Other authors have associated MAD with the prescription of AIT. Sastre et al. [36] showed that, in more than 50% of patients, the allergen microarray results significantly changed the indication or the selection of allergens for immunotherapy. This finding is in agreement with the observations by Passalacqua et al. [31] AIT was first prescribed to 32 out of 318 polysensitized patients with respiratory symptoms. Following MBD, the prescription was changed in 3 of those patients by adding a new extract, and it was newly prescribed in 85 additional patients. In another study group, Moreno et al. [37] demonstrated that within a group of 1263 polysensitized patients, 922 (73%) would have been candidates for AIT to treat a mixture of grass and olive pollens based on available clinical data and skin prick test results. In 56.8% of patients, there was no concordance in the composition of AIT that would be selected before and after investigators received the in vitro data. In a large group of pediatric patients, Stringari et al. [38] showed that after MBD, the SPT-based decision on AIT prescription or composition was changed in 277 (42%) of 651 and in 315 (48%) of

651 children according to the European or American approach, respectively. Recently, it was shown that a small panel of extracts/components adapted to include those allergens prevalent in the geographical area in question appears to be sufficient for more effective immunotherapy, also leading to an improved cost-benefit ratio [39]. To further describe the complexity of the problem, the European and American approaches should be carefully considered, as discussed [40]. In this context, the European approach always suggests not exceeding the number of three different allergen sources for AIT in the same patient, while the American approach treats patients with a mixture of all the positive allergens. Of course, advantages and disadvantages are apparent in these two approaches.

Conclusion

The large number of extracts and components available and the two laboratory tools (single-plexed and multiplexed) have modified the tools that allergists have in their hands for an accurate description of the IgE profile of the patient and for the administration of therapies (from drugs to AIT). The more complex the diagnostics are the more helpful is a deep knowledge of the molecular allergy and its rules. However, modern medicine is moving to a precision medicine approach (Fig. 1), and due to this innovation, allergy diagnosis and treatment will further improve the quality of their activities.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflicts of interest relevant to this manuscript.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- Of major importance

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