

# Molecular diagnosis for allergen immunotherapy



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The extensive use of allergen molecules in birth cohort studies revealed that atopic sensitization is a sequential IgE response to distinct non-cross-reacting molecules from the same allergenic source (ie, molecular spreading), starting with an initiator molecule. This phenomenon reaches different degrees of progression (monomolecular, oligomolecular, and polymolecular) according to the individual atopic propensity and allergen exposure, thus producing an extreme heterogeneity of IgE sensitization profiles in patient populations. In patients with allergic rhinitis, the broader the IgE molecular sensitization profile, the greater is the risk of asthma and other allergic comorbidities, such as oral allergy syndrome. Hence it has been proposed to anticipate immunologic intervention at disease onset (early allergen immunotherapy) or even earlier during the preclinical sensitization stage (allergen immunoprophylaxis). Diagnostic algorithms based on singleplex or multiplex molecular IgE tests allow the discrimination of genuine from cross-reacting sensitization and the selection of the right extracts for allergen immunotherapy composition. Patients with extreme molecular poly-sensitization and greater risk of asthma or other IgE-mediated comorbidities, can be easily identified by means of allergen microarray or macroarray

procedures and might benefit from anti-IgE treatment. IgE molecular tests have opened the era of precision allergology, and their routine use should aim at cost-effectiveness, according to the principles of the Choosing Wisely initiative. (*J Allergy Clin Immunol* 2019;143:831-43.)

**Key words:** Allergen, allergen immunotherapy, allergic rhinitis, asthma, biomarker, component-resolved diagnostics, diagnostic algorithm, IgE, IgE tests, molecular allergology, molecular spreading

Allergen immunotherapy (AIT) is the only curative approach for allergic diseases, targeting their antigen-specific immunologic mechanisms. Since the first treatment, performed more than 100 years ago by Noon,<sup>1</sup> AIT has evolved in all its aspects: allergen content, vehicle, adjuvant, route and schedule of administration, production, distribution, and documentation.<sup>2</sup> Although the conventional routes of administration are subcutaneous immunotherapy (SCIT), sublingual immunotherapy (SLIT), or oral immunotherapy, others, such as the epicutaneous and intralymphatic routes, are under investigation.<sup>3,4</sup>

For more than 90 years, allergens used for the diagnosis and therapy of allergic diseases have been produced through extraction from primary sources. Manufacturing stages are distinguished in native, intermediate, and bulk allergenic preparations. Intermediate extracts often undergo various modifications, including different chemical (eg, conjugation with formaldehyde/glutaraldehyde) and physical (eg, adsorption onto different media and integration into *liposomes/microspheres*) processes, as well as addition of biologically active agents (eg, LPS derivatives).<sup>5</sup> With these methods, the reagent is first manufactured, and the composition of the final product is defined only afterward by using downstream tests. Thus, the exact concentration of allergenic and nonallergenic molecules remains unknown in allergen extracts derived from raw biological sources.<sup>6</sup> Therefore no information on the role of individual allergenic molecules can be obtained by means of detection of IgE reactivity to an allergenic extract.<sup>7</sup>

With regard to the standardization of allergen extracts, several *in vivo* and *in vitro* methods have been proposed.<sup>8</sup> Production of certified reference materials for allergenic products has been initiated by the World Health Organization and the International Union of Immunological Societies.<sup>9</sup> This European Union-funded project led to the introduction of 2 major allergens (Bet v 1 and Phl p 5a) as reference materials by the European Pharmacopoeia Commission.<sup>10</sup> Despite this important success, only a few allergens out of a multitude are covered, and standardization remains a challenging topic for both manufacturers and prescribers. The former also face several regulatory difficulties concerning the registration of allergen

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Supported by the Charité Medical University (P.M.M., S.D., and E.P.) and the Universities of Giessen and Marburg Lung Center (UGMLC; to H.R. and C.S.), the German Center for Lung Research (DZL; 82DZL00502/A2; to H.R. and C.S.), and the Deutsche Forschungsgemeinschaft (DFG)–funded SFB 1021 (C04, to H.R. and C.S.).

Disclosure of potential conflict of interest: P. M. Matricardi is a consultant for Hycor, Euroimmun, and Novartis and has received research funding from the Deutsche Forschungsgemeinschaft (DFG; grant no. MA-4740/1-1), Hycor Biomedical, and Euroimmun and reagents for research from Thermo Fisher and receives speaker's fees from Euroimmun, Thermo Fisher Scientific, Stallergenes-Greer, HAL Allergy. C. Skevaki is a consultant and receives research funding from Hycor Biomedical and receives research funding from Mead Johnson Nutrition (MJN). C. Skevaki and H. Renz have received grants to their institution from Deutsche Zentrum für Lungenforschung (DZL; German Lung Center; no. 82DZL00502/A2), and Deutsche Forschungsgemeinschaft (DFG)–funded SFB 1021. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication October 15, 2018; revised December 5, 2018; accepted for publication December 14, 2018.

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0091-6749/\$36.00

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<https://doi.org/10.1016/j.jaci.2018.12.1021>

Terms in boldface and italics are detailed in the glossary on page 832.

**Abbreviations used**

|        |                                                     |
|--------|-----------------------------------------------------|
| AIP:   | Allergen immunoprophylaxis                          |
| AIT:   | Allergen immunotherapy                              |
| BAMSE: | Children, Allergy, Milieu, Stockholm, Epidemiology  |
| CRD:   | Component-resolved diagnostics                      |
| EAACI: | European Academy of Allergy and Clinical Immunology |
| MAS:   | Multicenter Allergy Study                           |
| OAS:   | Oral allergy syndrome                               |
| SLIT:  | Sublingual immunotherapy                            |
| SPT:   | Skin prick test                                     |

products because they are classified as medicinal products by the European Medicines Agency, irrespective of whether they are used in a diagnostic or therapeutic setting. As such, the registration of allergen extracts is often difficult, especially because of the lack of a standardized approach between regulatory agencies from different regions of the world.<sup>11</sup>

## MOLECULAR ALLERGOLOGY: INNOVATIVE APPROACHES AND OPEN ISSUES

IgE antibodies are not only the major contributor to but also a marker for immediate hypersensitivity reactions and atopic diseases.<sup>12</sup> Since the late 1960s, serologic tests for allergen-specific IgE antibodies have been used to identify triggers of allergic symptoms.<sup>13</sup> Remarkably, the results of

traditional IgE assays based on allergen extracts are strongly affected by the composition of the extract.<sup>14</sup> Therefore IgE assays produced by different companies often produce different quantitative and sometimes even nonconcordant qualitative results (positive vs negative).

In 1988 and 1989, cDNA encoding allergens was cloned for the first time from hornet venom, birch, and house dust mites. This achievement marked the beginning of molecular allergology.<sup>15</sup> Consequently, the precision of diagnostic procedures in allergology has improved significantly through the production of recombinant and purified allergenic molecules for serologic and skin test diagnostics.<sup>16</sup> This goes along with the worldwide trend toward precision medicine, which pursues the goal of providing personalized treatment strategies based on individual genotypes and phenotypes of disease.<sup>17,18</sup> Although the allergen composition and concentration in commercial extracts often varies,<sup>19,20</sup> the use of recombinant/purified single proteins allows us to choose specific molecules of interest according to the clinical history of the patient.

There are several arguments in favor of the targeted use of allergen molecules in diagnostic tests<sup>21-26</sup>: (1) analytic sensitivity is increased, especially in the case of an underrepresentation or total absence of important molecules in the extract and (2) analytic and diagnostic specificity is enhanced, particularly when the presence of IgE antibodies against an allergenic molecule provides additional information on potential risks, possible cross-reactivity versus primary (species-specific and genuine) sensitization, and the stability of recognized allergens to changes

## GLOSSARY

**ALLERGEN IMMUNOPROPHYLAXIS (AIP):** Administration of an allergen preparation to prevent onset of allergic symptoms in healthy children with IgE antibodies against the corresponding allergenic source.<sup>39</sup>

**COSENSITIZATION:** Genuine sensitization to multiple allergen sources, requiring multiple extracts in an immunotherapy prescription. This contrasts with genuine sensitization to an allergenic source, as well as sensitization to cross-reactive components from other allergen sources.

**CROSS-REACTIVITY:** The ability of an antigen to bind with an antibody that was raised to a different antigen. This can occur through multivalent antigens with shared epitopes or epitopes with conformational similarity. More than 70% identity in the primary sequence is considered necessary for clinical cross-reactivity in food allergy. Antibody affinity, which can be affected by protein conformation, affects expression of clinical cross-reactivity.

**EARLY ALLERGEN IMMUNOTHERAPY (e-AIT):** "Allergen immunotherapy started within 6 months after allergic symptom onset".<sup>39</sup>

**IgE INHIBITION TEST:** Varying quantities of an extract are added into an assay to determine the amount necessary to inhibit IgE binding of pooled sera from allergic donors to a solid-phase antigen. This test allows for a direct comparison among multiple different extracts, as well as comparison with national standards.

**INITIATOR MOLECULE:** "The allergenic molecule within an allergenic source responsible for induction of the first IgE antibody response to that allergenic source".<sup>39</sup>

**LIPOSOME:** A spherical membranous structure composed of a phospholipid bilayer and an aqueous core. Liposomes can be phagocytosed by immune cells and are used in vaccine design and drug delivery.

**MICROSPHERES:** A shell comprised of a biodegradable polymer used for controlled drug release.

**MOLECULAR SPREADING:** "Sequential development of antibody (IgE) response to distinct non-cross-reacting molecules from the same antigenic (allergenic) source, starting with an "initiator" (allergenic) molecule".<sup>39</sup>

**NASAL PROVOCATION:** Also known as nasal allergen challenge. An objective measurement of nasal airway allergen responsiveness through intranasal administration of progressively increasing amounts of allergen extract. Nasal symptoms are assessed after each dose of extract, and the procedure is stopped after a predetermined threshold of symptoms is achieved.

**OVERPOWERED IMMUNIZATION (MISMATCH TYPE 2):** "Allergen immunotherapy performed with a preparation containing more molecules of the targeted allergenic source than those to which the patient is IgE sensitized".<sup>27</sup>

**POTENCY:** Allergenicity of an extract. This is often determined by calculating the log dose producing 50 mm of erythema after intradermal skin testing (ID<sub>50</sub>) of subjects maximally reactive to reference concentrates. ID<sub>50</sub> values are used to assign bioequivalent allergy unit (BAU) values. Surrogate *in vitro* potency assays (eg, IgE inhibition tests) are available to assign potency units to manufactured extract lots.

**PR.10 MOLECULES:** Pathogenesis-related protein family 10. Like profilins, PR.10 proteins are known as panallergens because of their presence in a variety of different plant families. They can be found in pollen, as well as other plant tissues, including ingested sources. PR.10 family proteins are plant steroid carriers and include Bet v 1 from birch pollen.

**UNDERPOWERED IMMUNIZATION (MISMATCH TYPE 1):** "Allergen immunotherapy performed with a preparation not containing all the molecules of the targeted allergenic source to which the patient is IgE sensitized".<sup>27</sup>

in heat or pH, which can be considered for clinical recommendations. Therefore IgE tests using individual allergenic molecules are considered a more precise and informative option than those based on allergenic extracts, particularly for polysensitized patients.<sup>27</sup>

A growing spectrum of molecules, representing single allergens of clinical relevance, have been identified, characterized, and produced for commercial *in vitro* assays. The use of component-resolved diagnostics (CRD) allows a detailed molecular profiling of the polyclonal IgE repertoire of the allergic patient.<sup>16,27</sup> Still, the correct interpretation of new and bigger data sets produced within this “allergomics” approach represents a challenge for the clinical daily life of allergists.<sup>20</sup> Therefore several databases of allergenic molecules and advanced Web platforms have been created to make proper information available.<sup>28</sup>

A molecular approach to allergy diagnosis still has some open issues. The list of important allergenic molecules cloned or purified and introduced for diagnostic purposes, although rapidly increasing,<sup>28</sup> is still incomplete.<sup>29</sup> New relevant allergenic molecules, such as Der p 23 for *Dermatophagoides pteronyssinus*<sup>30</sup> and Pru p 7 for peach,<sup>31</sup> have been discovered only recently and made available as diagnostic tests. Furthermore, the costs of IgE assays using individual allergens exceed those of extract-based test methods, and this is even more evident for microarrayed or macroarrayed multiplex tests. It is thus clear that the use of molecular assays in allergology needs to be balanced with a reasonable distribution of the scarce resources of public health systems.<sup>17</sup> This might be particularly relevant for IgE microarrays testing more than 100 individual allergenic molecules in a single analysis.<sup>32</sup>

The American Academy of Allergy, Asthma & Immunology recently published a Choosing Wisely initiative and alerted its members against performing an indiscriminate battery of IgE tests in the evaluation of allergy.<sup>33</sup> It is further underlined that the cost/benefit use of molecular assays in allergy diagnosis must be validated.<sup>17,34</sup> In other words, precision allergology will progressively grow, but it needs to be well integrated in a Choosing Wisely scenario. Given these premises, both the *in vitro* IgE assays based on allergenic extracts and those based on molecules will probably coexist in clinical practice for many years to come.

## NOVEL CONCEPTS FROM MOLECULAR ALLERGOLOGY

### Molecular spreading of the IgE response and the initiator molecule

Cross-sectional studies in patient populations have shown that the number of allergenic molecules recognized by IgE antibodies is greater in older patients.<sup>6,34</sup> Birth cohort studies have prospectively examined the evolution of IgG and IgE responses to allergen molecules within the individual patient since the early years of life. One of the biggest European birth cohorts on atopic diseases, the German Multicenter Allergy Study (MAS), provides insight not only on the onset and evolution of IgE responses in atopic versus healthy children but also on the humoral state before the appearance of IgE or allergic symptoms. Serologic analyses have shown that healthy and atopic children express a broad repertoire of IgG antibodies to a wide array of allergen molecules already at 2 years of age.<sup>35</sup> The intensity and frequency of these

“normal” IgG responses are maximal to animal food allergens, intermediate toward vegetable food allergens, and only minimal when directed against airborne allergens. Interestingly, atopic children show stronger and more frequent IgG responses than their nonatopic peers.<sup>35</sup>

A study zooming in on the IgE response to Bet v 1 and other **PR.10 molecules** revealed a second type or “(pre)-atopic” IgE response. This response, although directed against airborne allergens, is persistent and precedes or accompanies IgE production against the same molecule. This observation led to the assumption that the IgE response to Bet v 1 in children with birch allergy represents the final stage of a broader “abnormal” humoral response involving “(pre)-atopic” IgG antibodies directed to the same antigens.<sup>36</sup> A broader study confirmed and expanded this concept, showing that the “default” IgE response to major allergenic molecules becomes stronger and persistent in atopic subjects with IgE responses to the same molecules from mites, pollen, or molds.<sup>37</sup>

Once an IgE response has emerged, several patterns of evolution have been described in various cohorts. Again, among subsets of children from the MAS cohort, studies showed a sequential broadening of the IgE response to *Phleum pratense* molecules. The broadening evolution from an initial monomolecular through an oligomolecular sensitization pattern toward a polymolecular sensitization pattern could be observed in many, although not all of the children allergic to Timothy grass and it has been determined as **molecular spreading** (Fig 1).<sup>38,39</sup> Interestingly, in almost all children, the initial IgE response is directed against Phl p 1, a sort of **initiator molecule** of this IgE response. Only a few children develop a full-blown polymolecular response to all 8 allergenic molecules of *P pratense*. The molecular spreading process starts often in the preclinical stages of sensitization. IgE to Phl p 1 has been observed up to 5 years before the child starts to perceive first allergic symptoms.<sup>38</sup>

A very similar process has been described in children with IgE sensitization to *D pteronyssinus*.<sup>40</sup> Sensitization started against Der p 1 and/or Der p 2 and/or Der p 23 (defined as group A molecules); then involved Der p 4, Der p 5, Der p 7, and Der p 21 (group B molecules); and ended up with IgE to Der p 11, Der p 14, Der p 15, Der p 18, and clone 16 (group C molecules). This progression could be defined as the ABC march of mite allergy (ie, molecular spreading of the IgE repertoire against *D pteronyssinus* allergens).<sup>41</sup> Moreover, an early IgE sensitization onset, parental hay fever, and greater exposure to mites were associated with a broader polymolecular IgE sensitization pattern. Interestingly, participants reaching the broadest ABC IgE sensitization stage had a significantly greater risk of mite-related allergic rhinitis and asthma than the monomolecularly sensitized participants. Finally, IgE sensitization in healthy children to Der p 1 or Der p 23 at age 5 years or less predicted asthma at school age.<sup>40</sup>

The Manchester Asthma and Allergy Study revealed a similar trend among 235 children whose sera have been tested for IgE toward Timothy grass pollen and mite allergens.<sup>42</sup> The study group identified 3 sensitization slopes related to mite allergy among children aged 5 to 11 years: group 1 sensitization (to Der f 1 and Der p 1), group 2 sensitization (to Der f 2 and Der p 2), and complete mite sensitization. Although children exhibiting group 1 and complete mite sensitization trajectories had a significantly increased risk for asthma, eczema, and rhinitis,

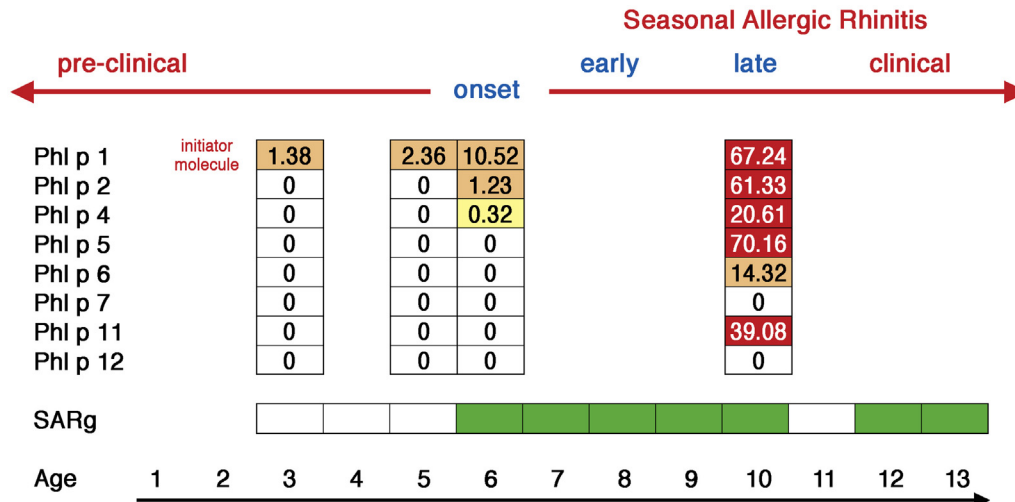


**FIG 1.** Molecular spreading of the IgE response to *P pratense* and implications for allergen-specific immunologic intervention in a child with hay fever (from the MAS birth cohort). This child started having symptoms of seasonal allergic rhinitis at the age of 6 years. The IgE response against *P pratense* started 3 years before with a weak monomolecular sensitization to Phl p 1. The IgE response was stronger and directed also to Phl p 2 and Phl p 4 at disease onset. After disease onset, the IgE response was much stronger and directed also to Phl p 5, Phl p 6, and Phl p 11. In clinical practice, AIT would be normally prescribed at this advanced stage after some years of symptoms (age 10 years). An interesting hypothesis is that AIT would be more efficient if started much earlier, ideally at disease onset (age 6 years; early AIT). Moreover, it could be investigated whether an immune intervention at the earliest preclinical stages (age 3 years in this case) of disease could even better change the natural history of the sensitization and prevent or delay disease onset (AIP). Modified with permission from Matricardi.<sup>39</sup>

the highest risk for asthma (odds ratio, 7.15; 95% CI, 3.80–13.44) was observed among subjects of the complete mite sensitization group. With regard to grass sensitization, a molecular broadening of the IgE response similar to molecular spreading among MAS participants was observed. Interestingly, an early onset of the IgE response to grass molecules was related to asthma and diminished lung function, whereas a late onset appeared to be associated with allergic rhinitis.<sup>42</sup>

In the Swedish Children, Allergy, Milieu, Stockholm, Epidemiology (BAMSE) study, sensitization to Fel d 1 and Can f 1 in childhood and polysensitization to either cat or dog allergen molecules predicted cat and dog allergy cross-sectionally and longitudinally significantly better than IgE to cat or dog extract.<sup>43</sup> Also, in patients with food allergies, the onset and molecular profile of the IgE response in childhood and adolescence seem to be crucial, as shown by the BAMSE population-based birth cohort. Researchers identified 2 distinct phenotypes in the development of peanut allergy before adulthood. Although an early onset of sensitization to Ara h 2 is associated with an increased risk of systemic reactions after peanut exposure, the second phenotype, starting later in childhood and being related to Ara h 8, goes along with nonsystemic reactions after peanut ingestion.<sup>44</sup> When broadening the data analysis to all IgE results against 132 allergen molecules, the authors could further identify 4 risk molecules to which an IgE response was associated with an increased risk of asthma, allergic rhinitis, or both, namely Ara h 1 (peanut), Bet v 1 (birch), Fel d 1 (cat), and Phl p 1 (grass). The results for airborne molecules could be replicated within the Manchester Asthma and Allergy Study cohort, adding Der f 2 (dust mite) and Phl p 5 (grass) as disease predictors.<sup>45</sup>

This novel understanding of the progressive molecular spreading of the immune response, especially to airborne

allergens, led to speculation that an immunologic intervention (AIT) should ideally be anticipated because this might prevent the development of more complex and strong IgE responses.<sup>39</sup> The **initiator molecules**, such as Phl p 1 for grass pollen; Bet v 1 for birch pollen; Der p 1, Der p 2, and Der p 23 for mites; and Fel d 1 for cat might be optimal targets of an **allergen immunoprophylaxis (AIP)** aimed at preventing the onset of allergic rhinitis and asthma in children during a preclinical sensitization stage.<sup>38,39</sup>

### IgE molecular sensitization profiles and their heterogeneity

An Italian cross-sectional study among 176 children with grass pollen allergy showed the vast amount of additional information provided by molecular versus extract-based diagnostic testing. Examining the sera in a classic ELISA based on the allergenic extract of Timothy grass, all patients with grass pollen allergy looked the same from a qualitative point of view. Although exhibiting different degrees of amplitude, they were all positive. When instead using a molecular assay, the alleged homogeneity is replaced by a remarkable diversity of responses. Overall, a series of 39 different IgE sensitization profiles to grass pollen molecules could be observed.<sup>27</sup> This figure increased to 87 in another study examining 1120 children cross-sectionally (Fig 2).<sup>40,46</sup> Both studies included patients sensitized to only 1 molecule (in most cases Phl p 1), as well as those sensitized to all 8 allergenic molecules examined. Differences in the molecular sensitization profile were also linked to clinical phenotypes. To provide an example, Phl p 7 was a reliable biomarker of asthma, whereas Phl p 12 served as a biomarker of oral allergy syndrome (OAS). Sensitization to Phl p 7 was

| Number of molecules | Der p 1 | Der p 2 | Der p 4 | Der p 5 | Der p 7 | Der p 11 | Der p 14 | Der p 15 | Clone 16 | Der p 18 | Der p 21 | Der p 23 | n  | %    |
|---------------------|---------|---------|---------|---------|---------|----------|----------|----------|----------|----------|----------|----------|----|------|
| 1                   |         |         |         |         |         |          |          |          |          |          |          |          | 15 | 12.6 |
| 2                   | *       | *       |         |         |         |          |          |          |          |          |          |          | 10 | 8.4  |
| 0                   |         |         |         |         |         |          |          |          |          |          |          |          | 10 | 8.4  |
| 3                   | *       | *       |         |         |         |          |          |          |          |          |          | *        | 7  | 5.9  |
| 1                   |         |         |         |         |         |          |          |          |          |          |          | *        | 7  | 5.9  |
| 3                   | *       | *       |         | *       |         |          |          |          |          |          |          |          | 5  | 4.2  |
| 3                   | *       | *       | *       |         |         |          |          |          |          |          |          |          | 4  | 3.4  |
| 2                   |         | *       |         |         |         |          |          |          |          |          |          | *        | 4  | 3.4  |
| 1                   | *       |         |         |         |         |          |          |          |          |          |          |          | 4  | 3.4  |
| 7                   | *       | *       | *       | *       | *       |          |          |          |          |          | *        | *        | 3  | 2.5  |
| 4                   | *       | *       | *       |         |         |          |          |          |          |          |          | *        | 3  | 2.5  |
| 4                   | *       | *       |         | *       |         |          |          |          |          |          | *        |          | 3  | 2.5  |
| 2                   | *       |         |         |         |         |          |          |          |          |          |          | *        | 3  | 2.5  |
| 10                  | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 2  | 1.7  |
| 6                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 2  | 1.7  |
| 6                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 2  | 1.7  |
| 5                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 2  | 1.7  |
| 5                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 2  | 1.7  |
| 4                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 2  | 1.7  |
| 4                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 2  | 1.7  |
| 11                  | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 10                  | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 10                  | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 10                  | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 9                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 9                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 9                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 9                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 8                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 8                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 8                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 8                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 7                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 6                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 6                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 6                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 6                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 6                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 5                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 5                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 5                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 5                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 4                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 4                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 4                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 4                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 4                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 4                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 4                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 3                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 3                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 3                   | *       | *       | *       | *       | *       | *        | *        | *        | *        | *        | *        | *        | 1  | 0.8  |
| 1                   |         |         |         | *       |         |          |          |          |          |          |          |          | 1  | 0.8  |

| Number of molecules | rPhl p 1 | rPhl p 2 | rPhl p 4 | rPhl p 5 | rPhl p 6 | rPhl p 7 | rPhl p 11 | rPhl p 12 | n   | %    |
|---------------------|----------|----------|----------|----------|----------|----------|-----------|-----------|-----|------|
| 1                   | *        | *        | *        | *        | *        | *        | *         | *         | 195 | 17.4 |
| 6                   | *        | *        | *        | *        | *        | *        | *         | *         | 70  | 6.3  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 68  | 6.1  |
| 6                   | *        | *        | *        | *        | *        | *        | *         | *         | 57  | 5.2  |
| 6                   | *        | *        | *        | *        | *        | *        | *         | *         | 57  | 5.1  |
| 7                   | *        | *        | *        | *        | *        | *        | *         | *         | 55  | 4.9  |
| 2                   | *        | *        | *        | *        | *        | *        | *         | *         | 54  | 4.8  |
| 0                   | *        | *        | *        | *        | *        | *        | *         | *         | 47  | 4.2  |
| 3                   | *        | *        | *        | *        | *        | *        | *         | *         | 40  | 3.6  |
| 5                   | *        | *        | *        | *        | *        | *        | *         | *         | 38  | 3.4  |
| 2                   | *        | *        | *        | *        | *        | *        | *         | *         | 31  | 2.8  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 29  | 2.6  |
| 5                   | *        | *        | *        | *        | *        | *        | *         | *         | 28  | 2.5  |
| 5                   | *        | *        | *        | *        | *        | *        | *         | *         | 27  | 2.4  |
| 5                   | *        | *        | *        | *        | *        | *        | *         | *         | 24  | 1.9  |
| 2                   | *        | *        | *        | *        | *        | *        | *         | *         | 20  | 1.8  |
| 1                   | *        | *        | *        | *        | *        | *        | *         | *         | 16  | 1.3  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 16  | 1.3  |
| 3                   | *        | *        | *        | *        | *        | *        | *         | *         | 15  | 1.3  |
| 6                   | *        | *        | *        | *        | *        | *        | *         | *         | 13  | 1.2  |
| 3                   | *        | *        | *        | *        | *        | *        | *         | *         | 13  | 1.2  |
| 3                   | *        | *        | *        | *        | *        | *        | *         | *         | 13  | 1.2  |
| 5                   | *        | *        | *        | *        | *        | *        | *         | *         | 12  | 1.1  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 11  | 1.0  |
| 2                   | *        | *        | *        | *        | *        | *        | *         | *         | 10  | 0.9  |
| 2                   | *        | *        | *        | *        | *        | *        | *         | *         | 9   | 0.8  |
| 1                   | *        | *        | *        | *        | *        | *        | *         | *         | 7   | 0.6  |
| 1                   | *        | *        | *        | *        | *        | *        | *         | *         | 7   | 0.6  |
| 3                   | *        | *        | *        | *        | *        | *        | *         | *         | 6   | 0.5  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 6   | 0.5  |
| 8                   | *        | *        | *        | *        | *        | *        | *         | *         | 6   | 0.5  |
| 3                   | *        | *        | *        | *        | *        | *        | *         | *         | 5   | 0.4  |
| 3                   | *        | *        | *        | *        | *        | *        | *         | *         | 5   | 0.4  |
| 2                   | *        | *        | *        | *        | *        | *        | *         | *         | 5   | 0.4  |
| 2                   | *        | *        | *        | *        | *        | *        | *         | *         | 4   | 0.4  |
| 3                   | *        | *        | *        | *        | *        | *        | *         | *         | 4   | 0.4  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 4   | 0.4  |
| 5                   | *        | *        | *        | *        | *        | *        | *         | *         | 4   | 0.4  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 4   | 0.4  |
| 1                   | *        | *        | *        | *        | *        | *        | *         | *         | 3   | 0.3  |
| 3                   | *        | *        | *        | *        | *        | *        | *         | *         | 3   | 0.3  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 3   | 0.3  |
| 3                   | *        | *        | *        | *        | *        | *        | *         | *         | 3   | 0.3  |
| 3                   | *        | *        | *        | *        | *        | *        | *         | *         | 3   | 0.3  |
| 7                   | *        | *        | *        | *        | *        | *        | *         | *         | 3   | 0.3  |
| 2                   | *        | *        | *        | *        | *        | *        | *         | *         | 2   | 0.2  |
| 2                   | *        | *        | *        | *        | *        | *        | *         | *         | 2   | 0.2  |
| 3                   | *        | *        | *        | *        | *        | *        | *         | *         | 2   | 0.2  |
| 1                   | *        | *        | *        | *        | *        | *        | *         | *         | 2   | 0.2  |
| 3                   | *        | *        | *        | *        | *        | *        | *         | *         | 2   | 0.2  |
| 3                   | *        | *        | *        | *        | *        | *        | *         | *         | 2   | 0.2  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 2   | 0.2  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 2   | 0.2  |
| 6                   | *        | *        | *        | *        | *        | *        | *         | *         | 2   | 0.2  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 2   | 0.2  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 2   | 0.2  |
| 5                   | *        | *        | *        | *        | *        | *        | *         | *         | 2   | 0.2  |
| 6                   | *        | *        | *        | *        | *        | *        | *         | *         | 2   | 0.2  |
| 7                   | *        | *        | *        | *        | *        | *        | *         | *         | 2   | 0.2  |
| 1                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 2                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 2                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 5                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 2                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 5                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 3                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 5                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 7                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 3                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 3                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 6                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 5                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 5                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 4                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 5                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |
| 7                   | *        | *        | *        | *        | *        | *        | *         | *         | 1   | 0.1  |

**FIG 2.** Molecular profiles of IgE sensitization to *D pteronyssinus* (A) and *P pratense* (B). Fig 2, A, Profiles of IgE to 12 *D pteronyssinus* molecules in 119 subjects (every line represents 1 profile, and the frequency of each profile is indicated on the right for each line) with an IgE reaction to *D pteronyssinus* at 20 years of age and a complete data set. Absolute frequencies are shown. Profiles are ordered by decreasing frequency. Fig 2, B, Molecular profiles of IgE sensitization ( $\geq 0.35$  KU<sub>A</sub>/L) to *P pratense* molecules: rPhl p 1, rPhl p 2, rPhl p 4, rPhl p 5, rPhl p 6, rPhl p 7, rPhl p 11, and rPhl p 12 in 1120 children with a skin reaction to *P pratense* (wheal  $\geq 3$  mm) and tested for sIgE to all considered molecules. The number of children belonging to that specific code (numbers) and the relative frequency (percentages) are shown. Profiles are ordered by decreasing frequency. Modified with permission from Posa et al<sup>40</sup> (Fig 2, A) and Cipriani et al<sup>46</sup> (Fig 2, B).

also associated with a greater severity of seasonal allergic rhinitis.<sup>46</sup>

A comparison of molecular IgE results to *P pratense* molecules with nasal and conjunctival provocation tests has been performed by a German study group among 101 adult patients with pollinosis. Apart from observing a substantial heterogeneity in sensitization profiles, the group described a positive correlation between the number of recognized molecules and the likelihood of a positive provocation test result. Interestingly, none of the IgE profiles matched exactly the composition of a previously published component-resolved specific immunotherapy containing Phl p 1, Phl p 2, Phl p 5a/b, and Phl p 6.<sup>47</sup>

Similarly, the individual sensitization profiles of 119 patients with house dust mite allergy from the German MAS cohort were extremely heterogenous at age 20 years, with 27 subjects responding to only 1 molecule (monomolecular profile), 50 responding to 2 to 4 molecules (oligomolecular profile), and 32 producing IgE to 5 or more of the 12 molecules (polymolecular profile, Fig 2, A).<sup>40</sup>

A study investigating IgE profiles in 243 sera of patients with *D pteronyssinus* allergy from 4 European countries (Austria, France, Italy, and Sweden) was aimed at determining allergen panels needed for diagnostic purposes. Although more than 50% reacted to more than 1 molecule, the group found that 97% of the participating patients could be given a diagnosis of a combination of Der p 1 and Der p 2.<sup>48</sup> By contrast, a more recent study among 97 patients showed that 16 mite allergic patients had negative test results to Der p 1, Der p 2, and Der p 10. Indeed, 5 patients were monosensitized to Der p 23, and 11 patients with positive skin prick test (SPT) responses, intranasal provocation and IgE results to *D pteronyssinus* extract of UniCAP class 1 or greater showed no IgE to any of the 13 tested components on the Mechanisms of the Development of Allergy chip.<sup>49</sup>

IgE sensitization profiles to dog allergens have been investigated by a Swedish group testing IgE against 6 allergenic molecules of dog dander in 60 children aged 10 to 18 years. Comparing the results with *nasal provocation* test outcomes, the authors concluded that a broader panel of

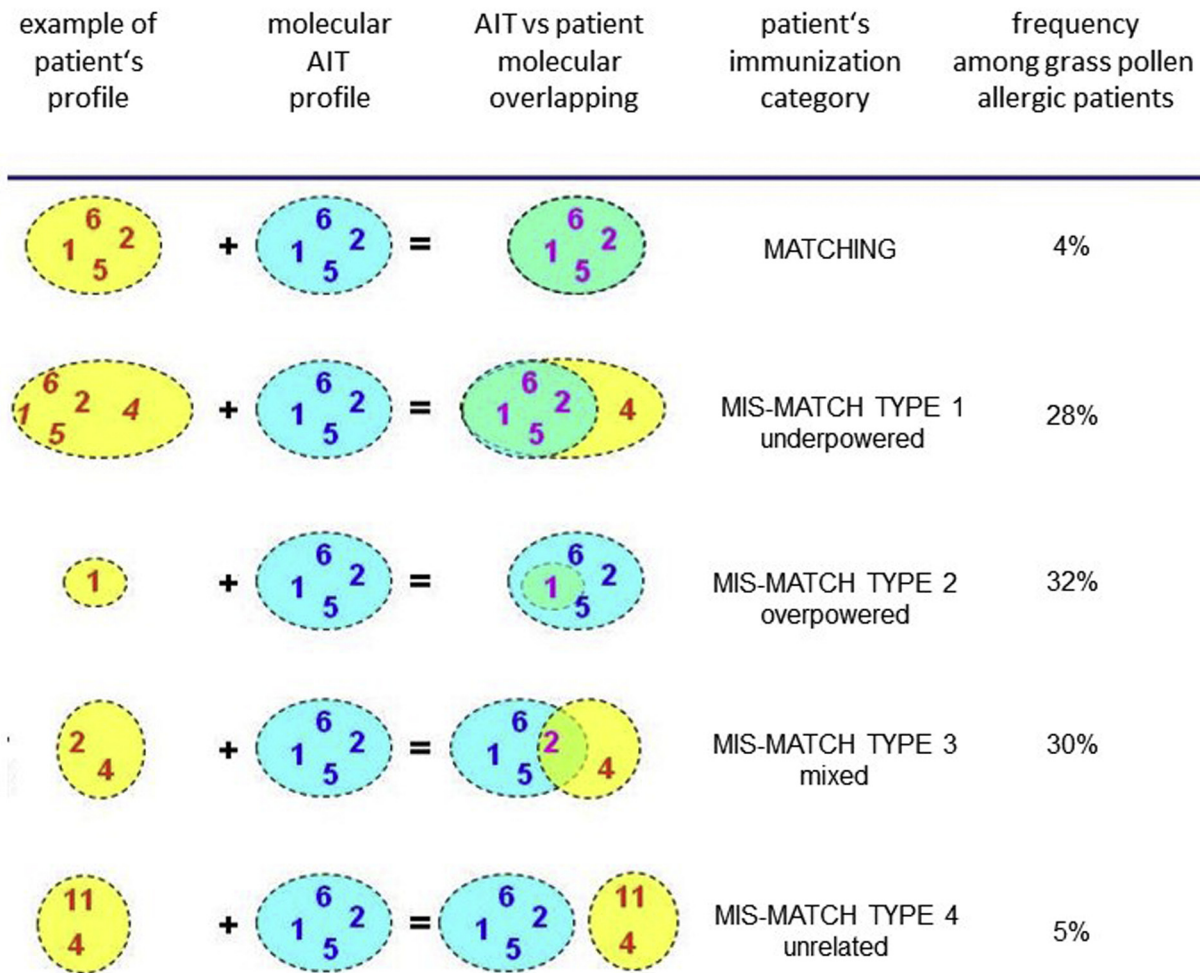


**FIG 3.** Matching and mismatching categories of IgE sensitization to *P. pratense* against specific immunotherapy. Matching molecular profiles of IgE sensitization to *P. pratense* against the composition of a molecularly designed specific immunotherapy preparation in 176 children with grass pollen respiratory allergies. Five categories have been created with all 5 different possible typologies of matching/mismatching between sensitization profiles and composition of the specific immunotherapy preparation. Modified with permission from Tripodi et al.<sup>27</sup>

recognized allergens increases the likelihood of a positive challenge test result.<sup>50</sup>

### AIT composition versus the patient's IgE sensitization profile: Five classes of molecular matching and mismatching

The major requirements for a standardization of allergens are a consistent composition combined with a stable **potency** related to clinical efficacy. The Monograph on Allergen Products, a European regulation, permits a wide range of variation (from 50% to 200% tested by **IgE inhibition tests**) for the representation of individual allergenic molecules in an extract.<sup>51</sup> This means that for a relatively simple allergen extract containing, for example, only 3 major allergenic proteins, there can be some batches with a very low and others with a very high allergenic content, including all intermediate possibilities.

Reflecting these regulations, allergen extracts from different manufacturers differ greatly in their molecular composition and potency, although representing the same allergen sources. This

underlines that allergen extract standardization remains difficult, if not impossible.<sup>52-54</sup> Translating the problem into daily clinical practice, it is not surprising to see divergent SPT wheal reactions for the same pollen or mite species in the same patient elicited by allergen extracts from different manufacturers.<sup>53,54</sup> Similarly, part of the variability of *in vitro* IgE test results to extracts of the same allergen source can probably be attributed to the extract composition.<sup>55</sup> Standardization of allergen extract for diagnosis and therapy is still an open issue in allergology.

Recently, new European regulations put into effect that allergen extracts for *in vivo* diagnostics (eg, SPT reagents) are subject to the same registration procedures as used for equivalent therapeutic products.<sup>56</sup> As this means very high registration costs for each allergen extract used in the clinical routine, the supply of SPT reagents available on the market will soon be reduced to those few extracts corresponding to a registered immunotherapy preparation.<sup>57</sup>

An interesting yet unanswered question is whether sensitization profiles affect the individual outcomes of AIT. Although there is significant interest for patient-tailored

immunotherapy,<sup>58,59</sup> no product has reached the commercial market yet. It is not clear whether patients with different sensitization profiles respond differently to the same allergen-specific immunotherapy. A theoretical approach to this question was used in a study<sup>29</sup> comparing all possible combinations between the molecular sensitization profiles of 176 study participants and the molecular profile of an allergen AIT preparation previously clinically tested for Timothy grass pollen allergy.<sup>60</sup> The 5 categories of molecular matching/mismatching between the IgE sensitization profile of the patient and the molecular composition of an AIT preparation have been thoroughly analyzed and described (Fig 3)<sup>27</sup>:

- **Class 1: Perfect matching**—The molecular composition of the AIT corresponds exactly to the patient's IgE molecular sensitization profile.
- **Class 2: Underpowered immunization**—Some patients were sensitized to more allergenic molecules than contained in the immunotherapy. In this case the efficacy of the immunization might be insufficient.
- **Class 3: Overpowered immunization**—The immunotherapy preparation contains more molecules than the individual patient's sensitization profile. Three consequences could result: a beneficial induction of IgG antibodies and/or prevention of new IgE sensitization,<sup>60</sup> an unwanted IgE sensitization to these molecules,<sup>61</sup> and no effect.<sup>62,63</sup>
- **Class 4: Underpowered/overpowered immunization**—This category includes patients with a mixed (type 1 and type 2) match/mismatch. Also, the expected effects of such a mismatch might be any of the previously mentioned options.
- **Class 5: Unrelated immunization**—In this case the immunization does not fit the sensitization profile of the patient. Logically, this category bears the lowest (if any) expected benefit, as well as the highest risk of unwanted effects.

Yet there has not been an analysis with enough statistical power to test the abovementioned combinations of matching versus mismatching IgE results and immunotherapy outcomes. Given the synergistic role of individual molecules in triggering IgE-mediated degranulation of mast cells, the overall immune response to specific immunotherapy can be linked more to the overall concentration of IgE antibodies directed against an allergenic source than to the nature of the individual molecules that they recognize. Thus far, the effect of multiple sensitization on the efficacy of AIT has been examined only at the extract level.<sup>64-66</sup> A study in polysensitized patients with allergic rhinitis has shown that SLIT for *D pteronyssinus* and/or *D farinae* improved nasal symptoms and rescue medication scores equally well as in monosensitized patients.<sup>67</sup>

## DIAGNOSTIC USE OF MOLECULES FOR AIT PRESCRIPTION

### Comparing outcomes of extract- versus molecular-based IgE tests

Two laboratory strategies targeted to the same objective often produce different results. Ideally, a positive IgE extract assay result should always be associated with a positive outcome in 1 or more of the IgE assays using the extract's individual molecules. Similarly, a negative IgE extract assay result should always correspond to negative outcomes in molecular-based IgE assays

covering all the extract's components. Nevertheless, IgE antibodies of allergic subjects can sometimes react to allergenic extracts of a given source (eg, cat) but not to its molecules (eg, Fel d 1, Fel d 4, or both).<sup>68</sup> Nonconcordant outcomes among extract- and molecular-based IgE assays can have several characteristics and explanations that have been thoroughly described elsewhere<sup>17</sup> and can be summarized as follows (Table I)<sup>29</sup>:

- **Positive extract versus negative molecules**: IgE antibodies can recognize certain molecules that are not included in the used molecular assay. Another possibility is a lower limit of quantitation in the molecular assay when compared with the extract so that low specific IgE levels could be detected only by using the extract-based test.<sup>68</sup>
- **Negative extract versus positive molecules**: IgE antibodies can recognize components in a molecular assay that are missing or underrepresented in the corresponding extract. In the case of low specific IgE levels, a lower analytic sensitivity of the extract assay can also explain this constellation.<sup>69</sup>
- **Positive extract versus negative genuine molecules**: In this case IgE antibodies can recognize only minor and highly cross-reactive components of an allergenic extract but not its genuine, species-specific (major) components. Positive IgE results to many different allergenic extracts can be found often in patients sensitized to panallergens who therefore falsely appear to be highly polysensitized.<sup>70</sup> Similarly, sera of patients sensitized to cross-reactive carbohydrate determinants will only produce positive results to extracts and not to the corresponding molecules because these lack posttranslational modifications, such as cross-reactive carbohydrate determinants.<sup>71</sup>
- **Low positive extract versus very highly positive molecules**: Levels of serum IgE to an allergenic extract can be much lower than the sum of IgE levels to its components. This is most likely due to an underrepresentation of particular molecules recognized by the individual patient.<sup>72</sup> An additional explanation is the potential cross-reactivity between structurally nonrelated allergens (ie, Ara h 1, 2, and 3) from different protein families.

### Advantages of molecular diagnosis and their effect on the doctor's AIT prescription

Molecular diagnosis offers a higher level of definition in identification of the offending allergen source to be used for AIT (Table II).<sup>17,26,27,73</sup> They are mainly based on an improved assay performance and/or on new possibilities of interpretation, such as in terms of risk assessment or differentiation between genuine (primary) sensitization and **cross-reactivity**.<sup>22</sup> Although these arguments clearly support the use of allergen molecules in clinical routine, we need to be careful not to overinterpret results of molecular allergy diagnostics and to respect clear limitations when it comes to predicting clinical outcomes. Specific IgE test results, regardless of whether using extracts or single molecules, always need to be interpreted in the context of the clinical history. Still, guidelines for the use of molecular assays in allergology are essential. Accordingly, the World Allergy Organization,<sup>74</sup> as well as the European Academy of Allergy and Clinical Immunology (EAACI),<sup>29</sup> published consensus documents and a user's guide on molecular allergology, including rationale, methods, and

**TABLE I.** Discrepancies between allergen extract and allergen molecular IgE assay results\*

|                                                     | Extracts     | Molecular                    | Explanations                                                                                                                                                                                                                                                                          |
|-----------------------------------------------------|--------------|------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Absolute disagreement<br>(qualitative differences)  |              |                              |                                                                                                                                                                                                                                                                                       |
| A                                                   | Positive     | Negative                     | (1) Serum IgE binds only to the extract's molecules that are not (yet) available in molecular assays.                                                                                                                                                                                 |
| B                                                   | Negative     | Positive                     | (2) The molecular assay is less analytically sensitive than the extract-based assay.<br>(1) Serum IgE binds to molecules tested as components that were missing or of low abundance in the extract.<br>(2) The extract assay is less analytically sensitive than the molecular assay. |
| Relative disagreement<br>(quantitative differences) |              |                              |                                                                                                                                                                                                                                                                                       |
| C                                                   | Positive     | Negative to major components | Serum IgE binds only to highly cross-reactive, minor allergenic molecules, or CCD determinants.                                                                                                                                                                                       |
| D                                                   | Lower levels | Higher levels                | Serum IgE binds to molecules tested as components being of low abundance in the extract.                                                                                                                                                                                              |

CCD, Cross-reactive carbohydrate determinant.

\*Modified with permission from Matricardi et al.<sup>29</sup>**TABLE II.** Usefulness of molecular diagnosis in prescription of AIT

|   |                                                                                                                                                                                                                                                                                                                |
|---|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1 | Identification of clinically relevant inhalant allergen sources (ie, tree, grass, or weed pollen) is required before allergen-specific immunotherapy.                                                                                                                                                          |
| 2 | In the case of IgE sensitization to plant panallergens (minor allergens profilin and/or polcalcin), diagnostic pollen extracts lack analytic specificity and show positive and thus misleading results.                                                                                                        |
| 3 | Molecular marker allergens for allergen-specific IgE detection help to separate specific sensitizations to tree and/or grass and/or weed pollen from IgE sensitization to pollen panallergens.                                                                                                                 |
| 4 | Candidates for molecular allergy diagnostics to identify specific allergen sensitivities (ie, major allergen markers) are Bet v 1 (birch, hazel, alder, beech, and oak), Ole e 1 (olive and ash), Jun a 1 (cedar), Phl p 1/5 (Pooidea grasses), Amb a 1 (ragweed), Art v 1 (mugwort), and Par j 2 (pellitory). |
| 5 | Allergen extract compositions based on confirmed IgE sensitizations to major allergen markers might facilitate more targeted allergen-specific immunotherapy.                                                                                                                                                  |

\*Reprinted with permission from Klein-Tebbe et al.<sup>73</sup>

diagnostic algorithms to use molecular allergy diagnosis for clinical decisions.

A typical example of the use of molecular diagnosis for the etiological diagnosis and AIT prescription for grass pollen allergy is shown in Fig 4.<sup>29</sup> Patients with allergic rhinitis symptoms during the grass pollen season and a positive SPT/IgE assay response to grass pollen extracts are further investigated to detect serum IgE antibodies to Phl p 1, Phl p 2, Phl p 5, Phl p 7, Phl p 11, and Phl p 12. Identification of 1 or more of IgE antibodies to Phl p 1, Phl p 2, Phl p 5, and/or Phl p 11 is followed by the prescription of grass pollen AIT. Identification of IgE to Phl p 12 (profilin) is followed by further investigation of OAS and it is relevant to better interpret results of SPT/IgE assays with other pollen extracts or vegetables. Identification of IgE to Phl p 7 alerts the doctor of a worse prognosis, a greater severity of the disease, and asthma (Fig 4). Interestingly, it has also been shown that **co-sensitization** to Phl p 5 and Phl p 12 predicts the appearance of

side effects during AIT.<sup>75</sup> Moreover, a molecular classification of pollen-food syndrome associated with seasonal allergic rhinitis has been recently proposed.<sup>76</sup>

Especially in polysensitized patients with pollinosis, clinicians often face a difficult setting for the decision on AIT prescription. This challenge has been recognized and resulted in concrete recommendations for a reasonable diagnostic workup of these complex patients.<sup>73</sup> Particularly in the Mediterranean area, where many botanical sources have overlapping pollination periods, diagnostic algorithms for an optimized immunotherapy candidate selection are fundamental.<sup>77</sup>

A major advantage of CRD in allergology is the possibility of distinguishing a cosensitization that might underlie conditions recently being considered as primary atopic disorders<sup>78</sup> from a cross-sensitization.<sup>79</sup> A study among 651 Italian children with moderate-to-severe pollen-related allergic rhinitis showed that in a significant number of patients presenting with a clinical history and SPT results concordant with pollen allergy, no specific IgE antibodies to the major allergens of the expected source could be found. IgE to profilins, polcalcins, or both could explain 173 (37%) of 464 SPT reactions. When reconsidering the AIT prescription after receiving the results of CRD, the SPT-based decision on specific immunotherapy prescription or composition was changed in 277 (42%) of 651 or 315 (48%) of 651 children according to the European or American approach, respectively.<sup>70</sup> This study shows that the effect of CRD on prescription and composition of AIT is high, especially in geographic areas in which polysensitization airborne allergens is frequent.

Similar conclusions were reached in a study investigating sensitization patterns to major grass and olive pollen allergens in 1263 Spanish patients with seasonal allergic rhinitis and positive SPT responses to grass and olive pollens. Based on a traditional diagnostic approach, 922 (73%) patients would have received an AIT prescription with both grass and olive pollens; however, the investigators changed the composition of AIT based on additional CRD IgE antibody data in 56.8% of the patients.<sup>80</sup>

A recent comparative clinical study has shown that the multiplex analysis of IgE to allergenic molecules is a reliable diagnostic tool, which also has proved to be time efficient by decreasing the number of performed SPTs.<sup>81</sup> Concerning the

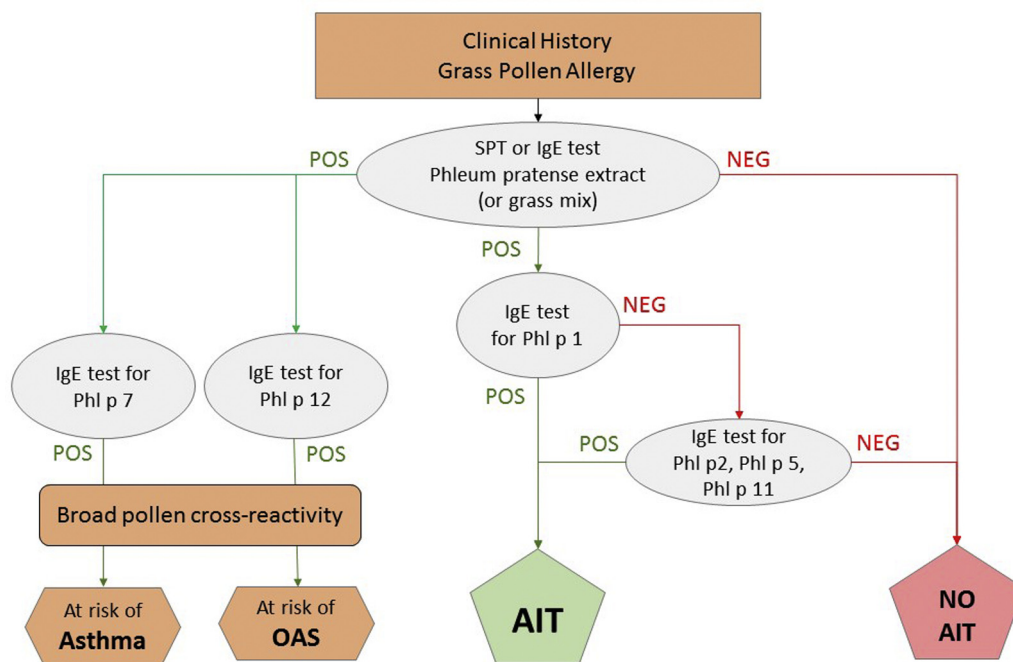


**FIG 4.** Diagnostic algorithm for AIT prescription in patients with grass pollen allergy. Patients with allergic rhinitis symptoms during the grass pollen season and a positive SPT/IgE assay result to grass pollen extracts are further investigated to detect serum IgE antibodies to Phl p 1, Phl p 2, Phl p 5, Phl p 7, Phl p 11, and Phl p 12. Identification of 1 or more IgE antibodies to Phl p 1, Phl p 2, Phl p 5, and/or Phl p 11 is followed by prescription of grass pollen AIT (after considering other factors, such as availability and compliance). Identification of IgE to Phl p 12 (profilin) is followed by further investigation of OAS, and it is relevant to better interpret the results of SPT/IgE assays with other pollen extracts or vegetables. Identification of IgE to Phl p 7 alerts the doctor of a worse prognosis and greater severity of the disease. Courtesy of Dr Jörg Kleine-Tebbe and modified with permission from Matricardi et al.<sup>29</sup>

costs for molecular diagnostic tests, a study among 95 patients from Kazakhstan showed that although the costs for SPT-based diagnosis per patient were lower, component-based serologic diagnosis was more precise in detecting the disease-eliciting allergen. This resulted in a lower need of immunotherapy treatments (119 after CRD vs 275 after extract-based diagnosis), which considerably reduced the total costs for diagnosis and for a 3-year treatment.<sup>82</sup>

### Which tests? Singleplex, microarrays, macroarrays, multiplex customized

Serologic analyses based on biotechnologically engineered allergens can be performed in singleplex assays testing one allergen at a time or in a multiplex approach allowing the simultaneous determination of IgE toward a multitude of molecules. Especially for pediatric patients, a multiplex assay seems attractive because very small amounts of serum (20 µL) are already sufficient to provide a broad set of information.<sup>83</sup> Depending on the diagnostic approach (“from symptoms to molecules” or “from molecules to symptoms”),<sup>29</sup> different test systems are suitable.

- **Microarrays and macroarrays:** Although a multiplex approach produces a broad set of information, it is also relatively costly, and the clinical relevance of its results needs to be evaluated. According to the chosen method (microarray or macroarray), the doctor is confronted with IgE results toward plenty of allergenic molecules: 112

(ImmunoCAP ISAC; Thermo Fisher Scientific, Uppsala, Sweden)<sup>32</sup> or 176 (MeDALL allergen chip)<sup>82-85</sup> in the case of microarrays or up to 282 (ALEX; MADx, Vienna, Austria)<sup>86</sup> in a macroarray setting. Based on this broad information, clinical relevance needs to be assessed by taking a precise clinical history.

- **Singleplex:** If an extensive clinical history is collected before obtaining serologic information and the test assay is being chosen based on the clinical picture, then singleplex assays (ImmunoCAP) might be favorable because they are much more cost-effective and do not provide potentially irrelevant information.<sup>26</sup> On the other hand, the doctor needs to know very clearly which allergenic proteins to assess.
- **Disease-oriented, customized multiplex tests:** An intermediate option between singleplex assays and microarrays or macroarrays are the customized allergen profiles (Euroline; Euroimmun, Lübeck, Germany), which combine allergen extracts and a set of the most relevant related allergenic molecules to distinguish cross-reactivity, genuine sensitization, and possibly assess the risk profile.<sup>87</sup> The assembled allergens are chosen according to the clinical profile (eg, pollen in Northern Europe, pollen in Southern Europe, food allergy in different geographic regions and others) of the patient.

There is still no general rule or guidelines suggesting the use of one or the other test. When choosing, it is extremely important to

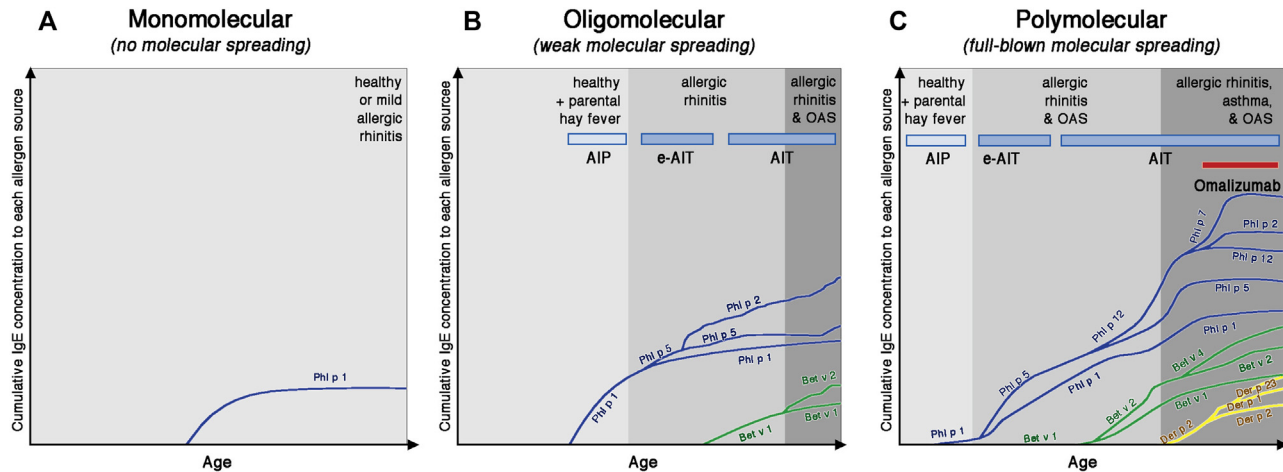


**FIG 5.** Timing and targets of immunologic intervention according to the stage of the IgE response. **A**, Patient with persistent monomolecular sensitization (not undergoing molecular spreading). **B**, Patient with modest molecular spreading, reaching an oligomolecular sensitization to 1 allergen source only. **C**, Patient with intensive molecular spreading to 3 different allergen sources. In clinical practice AIT would be normally prescribed at an advanced stage after some years of symptoms. An interesting hypothesis is that AIT would be more efficient if started much earlier, ideally at disease onset (early AIT [*e-AIT*]).<sup>39</sup> Moreover, it could be investigated whether an immune intervention at the earliest preclinical stages of disease could even better change the natural history of the sensitization and prevent or delay disease onset (AIP).<sup>45</sup> Finally, in patients with extreme polysensitization and comorbidities, AIT might have less chance of success, and experimental use anti-IgE therapy, addressing simultaneously multiple IgE responses and comorbidities, could be considered.<sup>88</sup>

evaluate the level of analytic precision of the assays. Moreover, the decision on diagnostic method remains the doctor's and depends also on the test's availability and cost versus reimbursement conditions.

## APPROACHES TO IMMUNOLOGIC INTERVENTION INSPIRED BY MOLECULAR ALLERGOLOGY

As discussed in previous sections, allergic patients are very different in their propensity to produce weak or strong IgE responses to a given allergen source. We can observe a minority of patients with persistent monomolecular sensitization (not undergoing molecular spreading); patients with modest molecular spreading, reaching an oligomolecular sensitization to 1 allergen source only and patients with intensive molecular spreading to many different allergen sources (Fig 5).<sup>39,88</sup> We and others have proposed that the timing, typology, and objectives of an immunologic intervention can be adapted to the stage and expansion of the IgE response of the individual patient. Accordingly, we can distinguish in preventative, early therapeutic, allergen-specific, and non-allergen-specific approaches, as indicated below (Fig 5).

### AIP

AIP (ie, allergy prevention through controlled administration of allergens in healthy subjects) is not a synonymous with AIT. The schedule, administration route, and preparations that can be used for AIP (ie, to prevent the onset of allergic inflammation and the onset of symptoms) might be quite different (shorter or less invasive) than those used to suppress an already established allergic inflammation and the corresponding symptoms.<sup>39</sup> Patrick Holt's proposal of "immunoprophylaxis of atopy" can today be expanded from a "primary" prevention concept for nonsensitized patients<sup>89,90</sup> to a "secondary" AIP for patients (mainly children) already sensitized to grass pollen but still healthy.<sup>91</sup> The

Preventive Allergy Treatment (PAT) study proved the disease-modifying potential of AIT when applied early enough.<sup>92</sup> We can also postulate that the immune response is more susceptible for modification in the weaker, preclinical, monomolecular stages than in advanced, stronger, polymolecular conditions.<sup>39</sup> Therefore early monitoring of the IgE response might be helpful to start an allergen-specific intervention as soon as possible. In regard to the risk of a first allergic disease, a recent randomized controlled trial proved the disease-modifying potential of AIT when applied early enough in previously healthy subjects.<sup>93</sup> Moreover, the simplicity of a preclinical IgE response (monomolecular or oligomolecular stage) suggests that a tailored prophylactic at early stages would be more feasible<sup>94</sup> than a therapeutic at later stages.<sup>54</sup> Such an intervention has been also defined as component-resolved prophylaxis,<sup>39</sup> similar to the concept of component-resolved therapy.<sup>16</sup>

Healthy preschool children with IgE to grass pollen and parents affected by hay fever are at high risk of seasonal allergic rhinitis.<sup>95</sup> These children might also be eligible for AIP during their preclinical window of opportunity for immunologic intervention. A prophylactic intervention with grass pollen extract would be targeted to prevent the onset of seasonal allergic rhinoconjunctivitis or to delay disease onset and make symptoms milder. From a biological perspective, AIP might prevent molecular spreading and block an increase in the concentration of IgE to grass pollen (Fig 5). Considering the breeding role of IgE-driven inflammation on later IgE responses,<sup>96</sup> a secondary outcome would also be prevention of new sensitizations to additional pollens or other airborne allergenic sources.

### Early AIT

Similarly, it might be expected that allergen-specific immunotherapy would have greater efficacy if started immediately after

disease onset instead of years later, as usually done. This concept of early AIT deserves to be tested in properly designed clinical trials.<sup>39</sup> A recent systematic review and meta-analysis from the EAACI AIT Task Force has assessed the current evidence of AIT for the prevention of allergy<sup>97</sup> and found an AIT-related reduction in the short-term risk of asthma in patients with allergic rhinitis, as well as a diminution of new sensitizations in allergic patients (Fig 5).<sup>93,98,99</sup>

## Anti-IgE treatment

In patients with multimorbidities (eg, allergic rhinitis, conjunctivitis, asthma, and food allergies) and extreme genuine polysensitization to several non-cross-reacting major allergenic proteins from different allergenic sources, AIT might have a lower chance of success given the high amount of antigens that should be administered and protective immune responses that should be induced. At this very advanced stage of the IgE response, experimental use of anti-IgE therapy (ie, omalizumab) could be considered.<sup>97</sup> Anti-IgE is addressing simultaneously multiple IgE responses and comorbidities (eg, allergic rhinoconjunctivitis, OAS, and asthma) and might be cost-effective in patients with extreme IgE polymolecular sensitization and multiple comorbidities. These patients can be well identified by using IgE microarrays<sup>100</sup> and macroarrays<sup>85</sup> (Fig 5). This hypothesis, although attractive, can be balanced by health-economic issues but deserves to be tested in experimental trials.<sup>101</sup>

## Molecular biomarkers of AIT efficacy

Several clinical studies on the efficacy of AIT have successfully used allergenic molecules to monitor changes in the specific antibody repertoire of treated patients.<sup>88,102</sup> An EAACI Task Force on “Biomarkers for monitoring the clinical efficacy of allergen Immunotherapy” concluded that those biomarkers should be grouped in 7 domains. The first 2 domains are IgE (total IgE, specific IgE, and specific IgE/total IgE ratio) and IgG (sIgG<sub>1</sub> and sIgG<sub>4</sub>, including the specific IgE/IgG<sub>4</sub> ratio) subclasses.<sup>103</sup> A very recent study has shown that the efficacy of SLIT in patients with mite allergy is highly influenced by the IgE sensitization profile before AIT starts. The negative outcome of a trial of SLIT in patients with mite-persistent allergic rhinitis would have been converted to be positive if only patients with IgE sensitization limited to Der p 1 or Der p 2 had been included. By contrast, SLIT had no efficacy in the group of patients also sensitized to other *D pteronyssinus* molecules.<sup>104</sup> This study seems to confirm that the efficacy of AIT might be lower in patients with stronger molecular spreading, as previously proposed (see “mismatch type 1, underpowered immunization”; Fig 3).<sup>27,38-40</sup> This further reinforces the hope that the use of molecular assays in predicting and monitoring AIT efficacy is a promising approach, needing more investigation to be considered a model of predictive biomarker of efficacy.

## THE COMING ERA OF PRECISION ALLERGOLOGY

The use of defined molecules for allergy diagnostics and therapy was organized for the first time in a formal theory and program by Rudolf Valenta and colleagues in 1990s.<sup>16</sup> Most allergists and clinicians, who were initially skeptical, are now

accepting the evidence that molecules add value to allergen extracts in the management of the allergic patient in a cost-effective fashion, according to the principles of the Choosing Wisely initiative.<sup>29</sup> Hence molecular allergology has outgrown infancy and adolescence and has reached adulthood, opening at last the era of precision allergology.<sup>17,18,85,105</sup>

## REFERENCES

- Noon L. Prophylactic inoculation against hay fever. *Lancet* 1911;1:1572-3.
- Calderón M, Cardona V, Demoly P. EAACI 100 Years of Immunotherapy Experts Panel. One hundred years of allergen immunotherapy European Academy of Allergy and Clinical Immunology celebration: review of unanswered questions. *Allergy* 2012;67:462-76.
- Muraro A, Roberts G, Halken S, Agache I, Angier E, Fernandez-Rivas M, et al. EAACI guidelines on allergen immunotherapy: Executive statement. *Allergy* 2018;73:739-43.
- Pajno GB, Fernandez-Rivas M, Arasi S, Roberts G, Akdis CA, Alvaro-Lozano M, et al. EAACI Guidelines on allergen immunotherapy: IgE-mediated food allergy. *Allergy* 2018;73:799-815.
- Lockey RF. The importance of knowing how allergen extracts are manufactured. *Ann Allergy Asthma Immunol* 2017;118:2-3.
- Scala E, Alessandri C, Bernardi ML, Ferrara R, Palazzo P, Pomponi D, et al. Cross-sectional survey on immunoglobulin E reactivity in 23 077 subjects using an allergenic molecule-based microarray detection system. *Clin Exp Allergy* 2010;40:911-21.
- Pfaar O, Demoly P, Gerth van Wijk R, Bonini S, Bousquet J, Canonica GW, et al. Recommendations for the standardization of clinical outcomes used in allergen immunotherapy trials for allergic rhinoconjunctivitis: an EAACI Position Paper. *Allergy* 2014;69:854-67.
- Kaul S, May S, Lüttkopf D, Vieths S. Regulatory environment for allergen-specific immunotherapy. *Allergy* 2011;66:753-64.
- Chapman MD, Ferreira F, Villalba M, Cromwell O, Bryan D, Becker WM, et al. The European Union CREATE project: a model for international standardization of allergy diagnostics and vaccines. *J Allergy Clin Immunol* 2008;122:882-9.e2.
- Vieths S, Barber D, Chapman M, Costanzo A, Daas A, Fiebig H, et al. Establishment of recombinant major allergens Bet v 1 and Phl p 5a as Ph. Eur. reference standards and validation of ELISA methods for their measurement. Results from feasibility studies. *Pharmazie Bio Sci Notes* 2012;23:118-34.
- Jutel M, Agache I, Bonini S, Burks AW, Calderon M, Canonica W, et al. International Consensus on Allergen Immunotherapy II: mechanisms, standardization, and pharmacoeconomics. *J Allergy Clin Immunol* 2016;137:358-68.
- Ishizaka K, Ishizaka T. Identification of gamma-E-antibodies as a carrier of reagent activity. *J Immunol* 1967;99:1187-98.
- Wide L, Bennich H, Johansson SGO. Diagnosis of allergy by an in vitro test for allergen antibodies. *Lancet* 1967;2:1105-7.
- Hamilton RG. Clinical laboratories worldwide need to report IgE antibody results on clinical specimens as analytical results and not use differential positive thresholds. *J Allergy Clin Immunol* 2015;136:811-2.
- Curin M, Garib V, Valenta R. Single recombinant and purified major allergens and peptides: how they are made and how they change allergy diagnosis and treatment. *Ann Allergy Asthma Immunol* 2017;119:201-9.
- Valenta R, Lidholm J, Niederberger V, Hayek B, Kraft D, Gronlund H. The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). *Clin Exp Allergy* 1999;29:896-904.
- Matricardi PM, Kleine-Tebbe J. Molecular allergology between precision medicine and the Choosing Wisely initiative. *Clin Exp Allergy* 2016;46:664-7.
- Canonica GW, Bachert C, Hellings P, Ryan D, Valovirta E, Wickman M, et al. Allergen Immunotherapy (AIT): a prototype of precision medicine. *World Allergy Organ J* 2015;8:31.
- Casset A, Mari A, Purohit A, Resch Y, Weghofer M, Ferrara R, et al. Varying allergen composition and content affects the in vivo allergenic activity of commercial *Dermatophagoides pteronyssinus* extracts. *Int Arch Allergy Immunol* 2012;159:253-62.
- Brunetto B, Tinghino R, Braschi MC, Antonicelli L, Pini C, Iacovacci P. Characterization and comparison of commercially available mite extracts for in vivo diagnosis. *Allergy* 2010;65:184-90.
- Mari A, Scala E, Alessandri C. The IgE-microarray testing in atopic dermatitis: a suitable modern tool for the immunological and clinical phenotyping of the disease. *Curr Opin Allergy Clin Immunol* 2011;11:438-44.
- Sastre J, Landivar ME, Ruiz-Garcia M, Andregnette-Rosigno MV, Mahillo I. How molecular diagnosis can change allergen-specific immunotherapy prescription in a complex pollen area. *Allergy* 2012;67:709-11.

23. Westman M, Asarnoj A, Hamsten C, Wickman M, van Hage M. Window of opportunity for tolerance induction for allergy by studying the evolution of allergic sensitization in birth cohorts. *Semin Immunol* 2017;30:61-6.
24. Valenta R, Karaulov A, Niederberger V, Gattlinger P, van Hage M, Flicker S, et al. Molecular Aspects of Allergens and Allergy. *Adv Immunol* 2018;138:195-256.
25. Kleine-Tebbe J, Jakob T. Molecular allergy diagnostics using IgE singleplex determinations: methodological and practical consideration for use in clinical routine—part 18 of the Series Molecular Allergology. *Allergo J Int* 2015;24:185-97.
26. Van Hage M, Hamsten C, Valenta R. ImmunoCAP assays: pros and cons in allergology. *J Allergy Clin Immunol* 2017;140:974-7.
27. Tripodi S, Frediani T, Lucarelli S, Macri F, Pingitore G, Di Rienzo Businco A, et al. Molecular profiles of IgE to *Phleum pratense* in children with grass pollen allergy: implications for specific immunotherapy. *J Allergy Clin Immunol* 2012;129:834-9.
28. Alessandri C, Ferrara R, Bernardi ML, Zennaro D, Tuppo L, Giangrieco I, et al. Diagnosing allergic sensitizations in the third millennium: why clinicians should know allergen molecule structures. *Clin Transl Allergy* 2017;7:21.
29. Matricardi PM, Kleine-Tebbe J, Hoffmann HJ, Valenta R, Hilger C, Hofmaier S, et al. EAACI molecular allergology user's guide. *Pediatr Allergy Immunol* 2016;27(suppl 23):1-250.
30. Weghofer M, Grote M, Resch Y, Casset A, Kneidinger M, Kopeck J, et al. Identification of Der p 23, a peritrophin-like protein, as a new major *Dermatophagoides pteronyssinus* allergen associated with the peritrophic matrix of mite fecal pellets. *J Immunol* 2013;190:3059-67.
31. Tuppo L, Alessandri C, Pomponi D, Picone D, Tamburrini M, Ferrara R, et al. Peamaclein—a new peach allergenic protein: similarities, differences and misleading features compared to Pru p 3. *Clin Exp Allergy* 2013;43:128-40.
32. Jensen-Jarolim E, Jensen AN, Canonica GW. Debates in allergy medicine: molecular allergy diagnosis with ISAC will replace screenings by skin prick test in the future. *World Allergy Organ J* 2017;10:33.
33. Morden NE, Colla CH, Sequist TD, Rosenthal MB. Choosing Wisely—the politics and economics of labeling low-value services. *N Engl J Med* 2014;370:589-92.
34. Melioli G, Marcomini L, Agazzi A, Bazurro G, Tosca M, Rossi GA, et al. The IgE repertoire in children and adolescents resolved at component level: a cross-sectional study. *Pediatr Allergy Immunol* 2012;23:433-40.
35. Schwarz A, Panetta V, Cappella A, Hofmaier S, Hatzler L, Rohrbach A, et al. IgG and IgG4 to 91 allergenic molecules in early childhood by route of exposure and current and future IgE sensitization: results from the Multicentre Allergy Study birth cohort. *J Allergy Clin Immunol* 2016;138:1426-33.
36. Hofmaier S, Hatzler L, Rohrbach A, Panetta V, Hakimeh D, Bauer CP, et al. "Default" versus "pre-atopic" IgG responses to foodborne and airborne pathogenesis-related group 10 protein molecules in birch-sensitized and non-atopic children. *J Allergy Clin Immunol* 2015;135:1367-74.
37. Huang X, Tsiolochristou O, Perna S, Hofmaier S, Cappella A, Bauer CP, et al. Evolution of the IgE and IgG repertoire to a comprehensive array of allergen molecules in the first decade of life. *Allergy* 2018;73:421-30.
38. Hatzler L, Panetta V, Lau S, Wagner P, Bergmann RL, Illi S, et al. Molecular spreading and predictive value of preclinical IgE response to *Phleum pratense* in children with hay fever. *J Allergy Clin Immunol* 2012;130:894-901.e5.
39. Matricardi PM. Allergen-specific immunoprophylaxis: toward secondary prevention of allergic rhinitis? *Pediatr Allergy Immunol* 2014;25:15-8.
40. Posa D, Perna S, Resch Y, Lupinek C, Panetta V, Hofmaier S, et al. Evolution and predictive value of IgE responses toward a comprehensive panel of house dust mite allergens during the first 2 decades of life. *J Allergy Clin Immunol* 2017;2:541-9.
41. Posa D, Hofmaier S, Arasi S, Matricardi PM. Natural evolution of IgE responses to mite allergens and relationship to progression of allergic disease: a review. *Curr Allergy Asthma Rep* 2017;17:28.
42. Custovic A, Sonntag HJ, Buchan IE, Belgrave D, Simpson A, Prosperi MCF. Evolution pathways to IgE responses to grass and mite allergens throughout childhood. *J Allergy Clin Immunol* 2015;136:1645-52.
43. Asarnoj A, Hamsten C, Waden K, Lupinek C, Andersson N, Kull I, et al. Sensitization to cat and dog allergen molecules in childhood and prediction of symptoms of cat and dog allergy in adolescence: a BAMSE/MeDALL study. *J Allergy Clin Immunol* 2016;137:813-21.
44. Asarnoj A, Hamsten C, Lupinek C, Melen E, Andersson N, Anto JM, et al. Prediction of peanut allergy in adolescence by early childhood storage protein-specific IgE signatures: the BAMSE population-based birth cohort. *J Allergy Clin Immunol* 2017;140:587-90.
45. Wickman M, Lupinek C, Andersson N, Belgrave D, Asarnoj A, Benet M, et al. Detection of IgE reactivity to a handful of allergen molecules in early childhood predicts respiratory allergy in adolescence. *EBioMedicine* 2017;26:91-9.
46. Cipriani F, Mastorilli C, Tripodi S, Ricci G, Perna S, Panetta V, et al. Diagnostic relevance of IgE sensitization profiles to eight recombinant *Phleum pratense* molecules. *Allergy* 2018;73:673-82.
47. Darsow U, Brockow K, Pfab F, Jakob T, Petersson CJ, Borres MP, et al. Heterogeneity of molecular sensitization profiles in grass pollen allergy—implications for immunotherapy? *Clin Exp Allergy* 2014;44:778-86.
48. Weghofer M, Thomas WR, Kronqvist M, Mari A, Purohit A, Pauli G, et al. Variability of IgE reactivity profiles among European mite allergic patients. *Eur J Clin Invest* 2008;38:959-65.
49. Becker S, Schleeder T, Kramer MF, Haack M, Vrtala S, Resch Y, et al. Real-life study for the diagnosis of house dust mite allergy—the value of recombinant allergen-based IgE serology. *Int Arch Allergy Immunol* 2016;170:132-7.
50. Käck U, Asarnoj A, Grönlund H, Borres MP, van Hage M, Lilja G, et al. Molecular allergy diagnostics refine characterization of children sensitized to dog dander. *J Allergy Clin Immunol* 2018;142:1113-20.e9.
51. European Directorate for the Quality of Medicines (EDQM). Allergen products—pro-ducta allergenica. In: European Pharma-copoeia. 6th ed. Strasbourg: Council of Europe; 2010. pp. 679-80.
52. Løwenstein H. Characterization and standardization of allergen extracts. *Chem Immunol Allergy* 2014;100:323-32.
53. Focke M, Marth K, Flicker S, Valenta R. Heterogeneity of commercial timothy grass pollen extracts. *Clin Exp Allergy* 2008;38:1400-8.
54. Focke M, Marth K, Valenta R. Molecular composition and biological activity of commercial birch pollen allergen extracts. *Eur J Clin Invest* 2009;39:429-36.
55. Wojtalewicz N, Goseberg S, Kabrodt K, Schellenberg I. Six years of INSTAND e.V. sIgE proficiency testing. An evaluation of in vitro allergy diagnostics. *Allergo J Int* 2017;26:43-52.
56. Klimek L. Zulassungen von Testallergenen: Diagnostika in Not. *Allergo J* 2013;22:522.
57. Klimek L, Hoffmann HJ, Renz H, Demoly P, Werfel T, Matricardi PM, et al. Diagnostic test allergens used for in vivo diagnosis of allergic diseases are at risk: a European Perspective. *Allergy* 2015;70:1329-31.
58. Cromwell O, Matricardi PM, Fiebig H. Patient-tailored recombinant allergen products—mission impossible? *Arb Paul Ehrlich Inst Bundesinstitut Impfstoffe Biomed Arzneim Langen Hess* 2009;96:210-7.
59. Cromwell O, Häfner D, Nandy A. Recombinant allergens for specific immunotherapy. *J Allergy Clin Immunol* 2011;127:865-72.
60. Jutel M, Jaeger L, Suck R, Meyer H, Fiebig H, Cromwell O. Allergen-specific immunotherapy with recombinant grass pollen allergens. *J Allergy Clin Immunol* 2005;116:608-13.
61. Moverare R, Elfman L, Vesterinen E, Metso T, Haahtela T. Development of new IgE reactivities to allergenic components in pollen extracts during specific immunotherapy studied with immunoblotting and the Pharmacia CAP system. *Allergy* 2002;57:423-30.
62. Asero R. Lack of de novo sensitization to tropomyosin in a group of mite allergic patients treated by house dust mite-specific immunotherapy. *Int Arch Allergy Immunol* 2005;137:62-5.
63. Asero R. Pollen specific immunotherapy is not a risk factor for de novo sensitization to cross-reacting allergens in monosensitized subjects. *J Investig Allergol Clin Immunol* 2006;16:253-7.
64. Calderon MA, Cox L, Casale TB, Moingeon P, Demoly P. Multiple-allergen and single-allergen immunotherapy strategies in polysensitized patients: looking at the published evidence. *J Allergy Clin Immunol* 2012;129:929-34.
65. Bahceciler NN, Galip N, Cobanoglu N. Multiallergen-specific immunotherapy in polysensitized patients: where are we? *Immunotherapy* 2013;5:183-90.
66. Ciprandi G, Melioli G, Passalacqua G, Canonica GW. Immunotherapy in polysensitized patients: new chances for the allergists? *Ann Allergy Asthma Immunol* 2012;109:392-4.
67. Lee JE, Choi YS, Kim MS, Han DH, Rhee CS, Lee CH, et al. Efficacy of sublingual immunotherapy with house dust mite extract in polyallergen sensitized patients with allergic rhinitis. *Ann Allergy Asthma Immunol* 2011;107:79-84.
68. Patellis A, Gunnbjornsdottir M, Alving K, Borres MP, Högman M, Janson C, et al. Allergen extract vs. component sensitization and airway inflammation, responsiveness and new-onset respiratory disease. *Clin Exp Allergy* 2016;46:730-40.
69. Kohler J, Blank S, Muller S, Bantleon F, Frick M, Huss-Marp J, et al. Component resolution reveals additional major allergens in patients with honeybee venom allergy. *J Allergy Clin Immunol* 2014;133:1383-9, e1-6.
70. Stringari G, Tripodi S, Caffarelli C, Dondi A, Asero R, Di Rienzo Businco A, et al. The effect of component-resolved diagnosis on specific immunotherapy prescription in children with hay fever. *J Allergy Clin Immunol* 2014;134:75-81.
71. Mari A, Iacovacci P, Afferni C, Barletta B, Tinghino R, Di Felice G, et al. Specific IgE to cross-reactive carbohydrate determinants strongly affect the in vitro diagnosis of allergic diseases. *J Allergy Clin Immunol* 1999;103:1005-11.

72. Erwin EA, Custis NJ, Satinover SM, Perzanowski MS, Woodfolk JA, Crane J, et al. Quantitative measurement of IgE antibodies to purified allergens using streptavidin linked to a high-capacity solid phase. *J Allergy Clin Immunol* 2005;115:1029-35.
73. Kleine-Tebbe J, Matricardi PM, Hamilton RG. Allergy work-up including component-resolved diagnosis: how to make allergen-specific immunotherapy more specific. *Immunol Allergy Clin North Am* 2016;36:191-203.
74. Canonica GW, Ansotegui IJ, Pawankar R, Schmid-Grendelmeier P, van Hage M, Baena-Cagnani CE, et al. A WAO-ARIA-GA(2)LEN consensus document on molecular-based allergy diagnostics. *World Allergy Organ J* 2013;6:17.
75. Sastre J, Rodriguez F, Campo P, Laffond E, Marin A, Alonso MD. Adverse reactions to immunotherapy are associated with different patterns of sensitization to grass allergens. *Allergy* 2015;70:598-600.
76. Mastrorilli C, Tripodi S, Caffarelli C, Perna S, Di Renzo-Busino A, Sfika I, et al. Endotypes of pollen-food syndrome in children with seasonal allergic rhinoconjunctivitis: a molecular classification. *Allergy* 2016;71:1181-91.
77. Douladiris N, Savvatis N, Roumpedaki I, Skevaki C, Mitsias D, Papadopoulos NG. A molecular diagnostic algorithm to guide pollen immunotherapy in southern Europe: towards component-resolved management of allergic diseases. *Int Arch Allergy Immunol* 2013;162:163-72.
78. Lyons JJ, Milner JD. Primary atopic disorders. *J Exp Med* 2018;215:1009-22.
79. Valenta R, Twaroch T, Swoboda I. Component-resolved diagnosis to optimize allergen-specific immunotherapy in the Mediterranean area. *J Invest Allergol Clin Immunol* 2007;17:88-92.
80. Moreno C, Justicia JL, Quirarte J, Moreno-Ancillo A, Iglesias-Cadarso A, Torrecillas M, et al. Olive, grass or both? Molecular diagnosis for the allergen immunotherapy selection in polysensitized pollinic patients. *Allergy* 2014;69:1357-63.
81. Mothes-Luksch N, Jordakieva G, Hinterhölzl L, Jensen AN, Hallmann PK, Kundi M, et al. Allergy diagnosis from symptoms to molecules, or from molecules to symptoms: a comparative clinical study. *World Allergy Organ J* 2018;11:22.
82. Saltabayeva U, Garib V, Morenko M, Rosenson R, Ispayeva Z, Gatauova M, et al. Greater real-life diagnosis efficacy of allergen molecule-based diagnosis for prescription of immunotherapy in an area with multiple pollen exposure. *Int Arch Allergy Immunol* 2017;173:93-8.
83. Lupinek C, Wollmann E, Baar A, Banerjee S, Breiteneder H, Broecker BM, et al. Advances in allergen-microarray technology for diagnosis and monitoring of allergy: the MeDALL allergen-chip. *Methods* 2014;66:106-19.
84. Anto JM, Bousquet J, Akdis M, Auffray C, Keil T, Momas I, et al. Mechanisms of the development of allergy (MeDALL): introducing novel concepts in allergy phenotypes. *J Allergy Clin Immunol* 2017;139:388-99.
85. Skrinde I, Lupinek C, Valenta R, Hovland V, Pahr S, Baar A, et al. The use of the MeDALL-chip to assess IgE sensitization: a new diagnostic tool for allergic disease? *Pediatr Allergy Immunol* 2015;26:239-46.
86. Heffler E, Puggioni F, Peveri S, Montagni M, Canonica GW, Melioli G. Extended IgE profile based on an allergen microarray: a novel tool for precision medicine in allergy diagnosis. *World Allergy Organ J* 2018;11:7.
87. Di Fraia M, Arasi S, Castelli S, Dramburg S, Potapova E, Villalta D, et al. A new molecular multiplex IgE assay for the diagnosis of pollen allergy in Mediterranean countries: a validation study. *Clin Exp Allergy* 2018;1-9.
88. Gadermaier E, Staikuniene J, Scheiblhofer S, Thalhammer J, Kundi M, Westritschnig K, et al. Recombinant allergen-based monitoring of antibody responses during injection grass pollen immunotherapy and after 5 years of discontinuation. *Allergy* 2011;66:1174-82.
89. Holt PG. Immunoprophylaxis of atopy: light at the end of the tunnel? *Immunol Today* 1994;15:484-9.
90. Holt PG, Sly PD, Sampson HA, Robinson P, Loh R, Lowenstein H, et al. Prophylactic use of sublingual allergen immunotherapy in high risk children: a pilot study. *J Allergy Clin Immunol* 2013;132:991-3.
91. Arasi S, Corsello G, Villani A, Pajno GB. The future outlook on allergen immunotherapy in children: 2018 and beyond. *Ital J Pediatr* 2018;44:80.
92. Niggemann B, Jacobsen L, Dreborg S, Ferdousi HA, Halken S, Host A, et al. Five-year follow-up on the PAT study: specific immunotherapy and long-term prevention of asthma in children. *Allergy* 2006;61:855-9.
93. Zolkipli Z, Roberts G, Cornelius V, Clayton B, Pearson S, Michaelis L, et al. Randomized controlled trial of primary prevention of atopy using house dust mite allergen oral immunotherapy in early childhood. *J Allergy Clin Immunol* 2015;136:1541-7, e1-11.
94. Valenta R. The future of antigen-specific immunotherapy of allergy. *Nat Rev Immunol* 2002;2:446-53.
95. Hatzler L, Panetta V, Illi S, Hofmaier S, Rohrbach A, Hakimeh D, et al. Parental hay fever reinforces IgE to pollen as pre-clinical biomarker of hay fever in childhood. *Pediatr Allergy Immunol* 2014;25:366-73.
96. Aalberse RC. The IgE response and atopy. *Eur Respir J Suppl* 1991;13:78s-84s.
97. Kristiansen M, Dhami S, Netuveli G, Halken S, Muraro A, Roberts G, et al. Allergen immunotherapy for the prevention of allergy: a systematic review and meta-analysis. *Pediatr Allergy Immunol* 2017;28:18-29.
98. Pifferi M, Baldini G, Marrazzini G, Baldini M, Ragazzo V, Pietrobelli A, et al. Benefits of immunotherapy with a standardized *Dermatophagoides pteronyssinus* extract in asthmatic children: a three-year prospective study. *Allergy* 2002;57:785-90.
99. Szepefalusi Z, Bannert C, Ronceray L, Mayer E, Hassler M, Wissmann E, et al. Preventive sublingual immunotherapy in preschool children: first evidence for safety and pro-tolerogenic effects. *Pediatr Allergy Immunol* 2014;25:788-95.
100. Siroux V, Ballardini N, Soler M, Lupinek C, Boudier A, Pin I, et al. The asthma-rhinitis multimorbidity is associated with IgE polysensitization in adolescents and adults. *Allergy* 2018;73:1447-58.
101. Papadopoulos NG, Custovic A, Cabana MD, Lee PR, Dell SD, Deschildre A, et al. Pediatric asthma: an unmet need for more effective treatments. *Pediatr Allergy Immunol* 2019;30:7-16.
102. Rossi REI, Monasterolo G, Coco G, Silvestro L, Operti D. Evaluation of serum IgG4 antibodies specific to grass pollen allergen components in the follow up of allergic patients undergoing subcutaneous and sublingual immunotherapy. *Vaccine* 2007;25:957-64.
103. Shamji MH, Kappen JH, Akdis M, Jensen-Jarolim E, Knol EF, Kleine-Tebbe J, et al. Biomarkers for monitoring clinical efficacy of allergen immunotherapy for allergic rhinoconjunctivitis and allergic asthma: an EAACI Position Paper. *Allergy* 2017;72:1156-73.
104. Chen KW, Zieglmayer P, Zieglmayer R, Lemell P, Horak F, Bunu CP, et al. Selection of house dust mite allergic patients by molecular diagnosis may enhance success of specific immunotherapy. *J Allergy Clin Immunol* 2019;143:1248-52.e12.
105. Galli SJ. Toward precision medicine and health: opportunities and challenges in allergic diseases. *J Allergy Clin Immunol* 2016;137:1289-300.