Molecular allergology and its impact in specific allergy diagnosis and therapy

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Abstract

Progressive knowledge of allergenic structures resulted in a broad availability of allergenic molecules for diagnosis. Component resolved diagnosis allowed a better understanding of patient sensitization patterns, facilitating allergen immunotherapy decisions. In parallel to the discovery of allergenic molecules, there was a progressive development of a regulation framework that affected both in vitro diagnostics and Allergen Immunotherapy products. With a progressive understanding of underlying mechanisms associated to Allergen immunotherapy and an increasing experience of application of molecular diagnosis in daily life, we focus in analyzing the evidences of the value provided by molecular allergology in daily clinical practice, with a focus on Allergen Immunotherapy decissions.

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Abbreviations:

EMA: European Medicine Agency

EDQM: European Directorate for the Quality of Medicines

AIT: Allergen Immunotherapy

CRD: Component-resolved diagnosis

LTP: Lipid transfer protein

HBV: Honeybee venom

YJV: Yellow Jacket venom

PDV: Polistes Dominula venom

POIT: Peanut Oral Immunotherapy

Abstract:

Progressive knowledge of allergenic structures resulted in a broad availability of allergenic molecules for diagnosis. Component resolved diagnosis allowed a better understanding of patient sensitization patterns, facilitating allergen immunotherapy decisions. In parallel to the discovery of allergenic molecules, there was a progressive development of a regulation framework that affected both in vitro diagnostics and Allergen Immunotherapy products. With a progressive understanding of underlying mechanisms associated to Allergen immunotherapy and an increasing experience of application of molecular diagnosis in daily life, we focus in analyzing the evidences of the value provided by molecular allergology in daily clinical practice, with a focus on Allergen Immunotherapy decissions.

Introduction:

The progressive advance in the knowledge and characterization of allergenic molecules responsible for allergic sensitization to most sources have had a profound impact in the etiological management of allergic disease. This impact can be summarized in three main aspects. Firstly, it has profoundly transformed the way allergenic extracts are characterized and standardized which is legally reflected in the regulatory framework for allergens, especially on the Note for Guidance on Allergenic Extracts, issued by the European Medicine Agency (EMA) and on the allergen monograph of the European Pharmacopoeia, issued by EDQM (Council of Europe), that is adopted by European Directives for Medicinal products [1-3]. Secondly, it improved the accuracy of allergy diagnosis. Single or multiplexed, allergenic molecules are routinely used and have changed the way we diagnose [4]. Lastly, but not least, some sensitization profiles are linked to different clinical phenotypes, and can be used to stratify allergic patients, to predict intervention outcomes, and to perform system biology studies that open new avenues for allergy disease management[5-11].

The implementation of a new regulatory framework led to the commercialization of allergy vaccines with registration clinical trials performed during five years. Based on these studies, a better understanding of AIT mechanisms is now possible. From allergen-specific effector cell desensitization[5,12-14]to a progressive onset of T and B cell-mediated regulatory mechanisms[15-19] responsible for the disease-modifying effect, their temporal alignment is critical to establish best AIT practices. In this context, the quality of the extract used - and its standardization -, the knowledge of patient's sensitization profiles, and the link to the AIT product used are pivotal to maximize the odds of AIT success. In this review, we discuss the different aspects of molecular allergology, from an overview of allergenic molecules and diagnostic methods to the quality of allergen preparation and the clinical approaches to AIT. We aim to offer a practical document to support the allergy specialist in the daily clinical management of allergic patients.

Overview of allergenic molecules

Currently, more than one thousand allergens from various allergenic sources have been described. Most of these molecules, that are relevant for AIT decisions, belong to a limited number of protein families and have been extensively revised[4,20,21]. More than 40 % of the relevant plant allergens belong to six protein families: 2S albumins ,non-specific lipid transfer proteins (nsLTP)), legumins, vicilins , profilins and pathogenesis-related (PR)-10 proteins[22]. In contrast, there are other families with only a few but very relevant members, as they include major pollen allergens such as expansins, polcalcins, pectate lyases and defensin-like proteins. Concerning animal allergen families, four of them (cysteine proteases, lipocalins, tropomyosins and parvalbumins), account for over 70% of the relevant allergens (Table 1) [23]. A separate category is formed by the allergens from hymenoptera venom (Table 2).

Diagnosis methods and European regulation

There are different methods available for sIgE determinations of single allergenic components. These components might be available as individual diagnostics or multiplexed in arrays. These technologies are commercialized by Thermo Fisher, MADx, Hycor, Euroline and Siemens. The list of available allergens in each platform has recently been described in detail[20] . Most of the existing methods for CRD (Component-resolved diagnosis) and multiplexed platforms, have been commercialized for research purposes and will need an adaptation and upgrade to comply with new regulatory EU framework as required by the regulation affecting in vitro diagnostics and medical devices, with a transition implementation phase ending by 2025

(REGULATION (EU) 2017/746). This regulation implementation will secure the adequate performance of commercialized diagnostics

The best diagnostic strategies - using a combination of clinical evaluation, extract-based diagnosis and CRD - that set the basis for the present document has also been extensively discussed by Matricardi and cols.[4]

AIT regulations, biological potency, and link to clinical effect.

The characterization of the main allergenic molecules led to the development of quantification methods and their progressive incorporation into regulations of extract preparation of AIT. In parallel, Create International project ended in the first available reference methods for major allergen content determination[3,24-26]. Today, for any AIT product intended for human use, a proper measurement of biological potency and major allergen content is required. Major allergens use to be the most abundant proteins of allergenic sources and their content in AIT products is closely related to total biological potency. Moreover, most of the patients included in clinical trials are sensitized to major allergens and in a significant percentage monosensitized to them[27]. In consequence, as a general criterium, patients elected for AIT must be sensitized to major allergens as there is no evidence of the clinical effect of AIT products in patients sensitized only to minor allergens. Unfortunately, the content for minor allergens in AIT preparations is, in the vast majority of cases, unknown and variable. In areas where pollen exposure is very intense, and sensitization to minor allergens is very frequent, side reactions to AIT preparations containing assigned to minor allergens variability have been described[28,29].

Main AIT mechanisms involve early effector cells de-sensitization and progressive onset of tolerance through a regulatory response that needs at least three years to be consolidated[5,13]. Dose-finding studies are usually performed during relatively short times, and thus effective dose is adjusted to target desensitization mechanism and to major allergen doses

CRD: Monitoring of allergy progression

Birth cohort studies have shown a sequential broadening of the IgE response to complex allergen sources. The IgE response starts in general with a monomolecular stage and then, through an oligomolecular sensitization pattern becomes polymolecular[30]. This "molecular spreading" has been observed in many participants in the MAS birth cohort allergic to Timothy grass. In this case, the "initiator molecule" was Phl p 1. However, only a few patients develop an extremely polymolecular response, producing IgE antibodies to all the best known allergenic molecules of *Phleum pratense* (Phl p 1, Phl p 2, Phl p 4, Phl p 5, Phl p 6, Phl p 7, Phl p 11 and Phl p 12)[30]. Similarly, molecular spreading has been observed in the MAS cohort among children developing IgE sensitization against *Dermatophagoides pteronyssinus* [31]. In this case, multiple "initiator molecules" have been observed (Der p 1, Der p 2, Der p 23). In these children, sensitization started with 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 was completed with IgE to Der p 11, Der p 14, Der p 15, Der p 18, and clone 16 (group "C" molecules). This expansion of the IgE response has also been defined as "the ABC march" of mite allergy[31]. Children with a broader polymolecular IgE sensitization pattern were also more frequently affected by asthma when compared with those who remained in the "A" stage of IgE sensitization[31]. A similar trend was observed among grass pollen allergic children in the Manchester Allergy and Asthma Study (MAAS)[32]. In this case, children with broader molecular patterns of IgE sensitization had also a significantly increased risk for asthma, eczema, and rhinitis[32]. It has also been proposed that earlier administration of AIT, i.e. at a mono- or oligomolecular stage of sensitization, may be more effective than at later stages [33].

CRD and selection of preparations for AIT:

Grass pollen allergy

Grass pollen allergy is probably the most studied allergic pathology. There is a broad allergen panel for CRD of grass allergy, and a SLIT AIT product that is the only one with a complete clinical program, including

several five-years studies,[12] that provides the best available information to understand the link between sensitization profiles and AIT.

Allergen composition studies of a Phleum pollen extract[34] allowed to establish that Phl p 5 and its related allergen Phl p 6 are the most abundant proteins, accounting for more of 50% of protein content. Accumulated content of Phl p 1 represents less than 10%. The rest of allergen components are in a range from 1-10% of total protein. Despite the relatively low abundance, Phl p 1 is the most relevant single allergen sensitizer of grass allergic patients. Epidemiological studies performed in Spain[35,36]and CRD analysis of 1905 patients included in AIT clinical trials of grass pollen allergy in North America[27] demonstrated the preponderance of Phl p1 as primary sensitizer to grass pollen. In fact, a significant proportion of patients were mono sensitized to this allergen. Similar results in pediatric grass allergen patients have been reported [37].

Recently a potential explanation for this has been published[38]. Group 1 grass allergens, belonging to the beta expansins family, are present in other plant parts. In Autumn, upon plant death, plant particles are aerosolized and might be presented together with Alternaria spores, initiating sensitization process. No loss of effect in AIT is observed in patients mono-sensitized to either Phl p 1 or Phl p 5[27]; however, patients with low (first tercile) sIgE to any of the two major allergens presented no clinical benefit in the first pollen treatment season. As we have already mentioned, early effect is governed by desensitization in a dose-dependent manner. Patients with low sIgE levels might need a higher allergen dose to get desensitized; however, in a five-year mechanistic study [9], an impaired regulatory response for this type of patients was not observed, and thus they should not be excluded for AIT if they meet clinical inclusion criteria.

The role of pan-allergens

An interesting subgroup of grass pollen allergic patients are sensitized to profilin, a pan-allergen present in all vegetable tissues, with a highly conserved structure. Usually, profilin is a confounding diagnostic factor, which is minimized by CRD diagnosis either "in vitro" or even "in vivo"[39]. Profilin sensitization strongly associates with grass pollen allergy[35] in base to a relatively high profilin content in grass pollen compared to other pollens [40]. Usually, sensitized profilin patients present no food-mediated reaction or only local (oral) ones. However, in areas of very high grass pollen exposure, some allergic patients do not respond to AIT and present severe food profilin-mediated reactions[41,42]. In fact, T cell reactivity to profilin increases along with grass pollen intensity exposure. Moreover, in patients from central Spain, T cell proliferation induced by profilin is similar to that induced by the major allergen Phl p 1[34]. These peculiar patients, as well as others exposed to very high pollen levels, can be identified by combining CRD with clinical evaluation and provide invaluable models to understand allergic inflammation and AIT mechanisms and its limitations.

Finally, patients sensitized simultaneously to the pollen pan-allergens polcalcin and profilin have been shown to have twice as many primary sensitizations and to duplicate as well allergy disease evolution time[36], suggesting that AIT might not be effective. Similar results supporting clinical value for Phl p 7 and Phl p 12 have been described in pediatric cohorts[37].Moreover, this specific double sensitization profile to pan-allergens is not linked to any particular pollen[36]. At this moment other grass allergens present a limited diagnostic value for AIT decisions and some of them provide confounding information due to lack of specificity [43].

Figure 1 represents an algorithm to support decision-making in the selection of AIT for grass pollen allergy.

Olive pollen allergy and nsLTP food allergy:

One of the best models to understand the link between pollen exposure, sensitization profiles, and clinical impact is the olive pollen allergy model. Minor allergen sensitization is frequent in areas of very high exposure. Interestingly, sensitization to Ole e 7 - the nsLTP from olive pollen - is associated with a unique clinical phenotype[29,35]. Patients living in areas with high olive pollen exposure and sensitized to Ole e 7 develop a severe respiratory allergic phenotype. These patients do not respond to AIT and constitute another model to understand evolution to severe allergic phenotypes. In Figure 2 an algorithm to support the selection of olive pollen AIT is shown.

There are other pollens where nsLTPs play a preponderant role, as*Parietaria judaica* , whose main allergen

is a LTPs, or*Artemisia artimisifolia* and *Platanus orientalis* , whose Art v 3 or Pla a 3 are LTPs. Art v 3 and Pla a 3 cross-react with Pru p 3 which complicates the differential diagnosis of the primary sensitization (to pollen or peach).

LTP-mediated allergy is the predominant food allergy in adults in the Mediterranean Area and Southern European countries[44,45]. Pru p 3 - the peach LTP - is the best marker for LTP allergy. Sublingual AIT has proved to be effective for the treatment of LTP allergy, with clinical effect not only against allergy to closely related food species such as *Rosaceae* fruits[46], but also against allergy to species with about only 60% sequence identity, such as the Peanut LTP, Ara h 9[47,48]. Evaluating the spectrum of recognition to multiple LTPs is needed to make a potential AIT therapeutic decision. Patients sensitized to LTPs distant to Pru p 3 such as wheat Tri a 14 might not benefit completely from AIT based on Pru p 3. Unfortunately, SLIT AIT for LTP allergy is only available in a limited number of Countries. Figure 3 summarizes decision trees for LTP mediated AIT

Birch and Oak tree pollen allergy and PR-10 proteins

PR10 allergens are present in multiple species of deciduous trees pollen. Among them, birch in Europe[49] and oak in North America are regarded as primary sensitizers. There are products, registered worldwide for the treatment of birch pollen allergy[50]. Recent data suggest that a birch-based vaccine has clinical benefit during oak pollen season in North America [51]. Interestingly, a study analyzed the effect of birch-based AIT in cross-reactive T cell response to homologous PR10 allergens from other trees [52]. This study demonstrated that down-regulation of T cell reactivity can be achieved against multiple, T cell cross-reactive PR-10 molecules. The control of T cell proliferation based on T cell cross-reactivity might be the most relevant endpoint for successful AIT. As in the case of profilins, PR10 proteins are also present in multiple vegetable species, leading to the concept of a PR10 allergy syndrome. There is very limited evidence on the clinical benefit of PR10 based pollen AIT in the amelioration of pollen-related food allergy syndrome (FPAS)[53],[54]. However, immunotherapy with Mal d 1 has been associated with clinical improvement [55]. More studies will be needed to clarify potential use of PR10 pollen vaccines in this particular type of food allergy.

Cypress/cedar allergy

Japanese cedar pollen is the main cause for seasonal respiratory allergy in Japan and Cupressaceae are relevant allergens in the US and southern Europe as well. Major allergens belonging to the pectate lyase family (Cup a 1, Cup s 1, Jun a 1 and Cry j 1) show a very high cross-reactivity.

There is only one AIT product[56,57]with clinically documented efficacy. The recent registration of a Japanese cedar vaccine in Japan opens the possibility - as in the case of birch/oak vaccines - to explore its value for treating cedar/cypress allergy worldwide.

Ragweed pollen allergy

Ragweed allergy dominates weed pollen allergy in North America and, after the accidental introduction of ragweed in Europe about one hundred years ago, is in continuous progression in the continent.

Major allergen Amb a 1, a pectate lyase, is a true marker of ragweed allergy and presents low cross-reactivity with related proteins from other pollen sources[58]. However, there is an extensive cross-reactivity among different *Ambrosia* species[59]. Currently, there are AIT products extensively documented in adults[60] both in American and European patients as well as in children[61] and these products are available worldwide for AIT practice.

Other pollen allergies:

There is a limited clinical evidence of AIT benefit in the treatment of other pollen allergies. However, data on major allergens can be extrapolated from studies on existing registered pollen vaccines. Major specific allergens available for diagnosis include: Art v 1 (*Artemisia artimisifolia*), Salk k 1 (*Salsola Kali*), Pla l 1 (*Plantago lanceolata*), and Pla a 1 (*Platanus acerifolia*)[58].

Cat and dog allergies

In general, the severity and progression of cat and dog allergies involve IgE recognition of a progressively increasing number of components from the sensitizing allergen source (molecular spreading)[30,62-65].

The availability of CRD for different cat and dog allergens has also raised the possibility of more precisely targeted AIT, mainly because it may distinguish primary sensitization from cross-sensitization, thereby enabling selection of the primary sensitizing allergen source for therapy.

In a recent, comprehensive study[66], including most of the components available, the pattern of IgE sensitization to cat allergens showed that 92% of cat-allergic patients had positive IgE antibodies results to Fel d 1. Other allergens also seem important, such as Fel d 4 and Fel d 7. Previous studies reported similar results[67]. The content of Feld d 1 in allergenic extracts varies substantially. Nevertheless, it has been demonstrated that the maintenance efficacy dose of Fel d 1 is 15 mg/ml[68].

In the same study, in patients with dog allergy, 52.4% were positive to Can f 1, and 57.2% to Can f 5. The most frequent monomolecular sensitization was to Can f 5.

The content of different dog allergen molecules in European AIT extracts has been recently studied[69]. These authors demonstrated great variability in extracts from five companies and scarce content of major allergens[70].

In general, AIT with cat extracts yield better clinical results than with dog ones. The higher complexity of dog allergy sensitization patterns, the lack of preparations with an adequate balance of major allergens is likely to explain this divergence[66,71,72].

Further studies are needed to determine whether CRD could be used to identify patients who are most likely to respond to AIT specially in dog allergy.

Figure 5 summarizes decision-making algorithms in cat and dog allergy AIT selection.

Mite allergy

Mite major allergens Der p 1/Der f 1 and Der p 2/Der f 2 sensitize the vast majority of mite allergic patients, being the double sensitization to groups 1 and 2 strongly associated with asthma[32]. Group 1 sensitization is more prevalent in children than in adults, suggesting an initiation role probably associated with its proteolytic properties[73]. Recently Der p 23 has been described to be associated with increased asthma risk[74]. Other mite species, known as minor or storage mites, have a limited allergenic cross-reactivity with major mites. Interestingly, they display a significant cross-reactivity between them. Lep d 2 could be a good marker for minor storage allergy sensitization[75]. Some patients exposed to high mite allergen levels, and sensitized to storage mites, develop anaphylactic reactions when exposed to foods contaminated with mites [76]. Interestingly, these patients present NSAIDs intolerance and recently they have been described to present extensive oral mucosa barrier damage[8].

A post-hoc analysis of a study with AIT with *D .pteronnysinus* and *D. farinae* found no association between the clinical efficacy and sensitization to different mite allergens[77].

Recently, the antibody response to a SCIT preparation for mite allergy has been examined at the molecular level in 24 mite-allergic patients[78]. A protective IgG response has been observed for Der p 1 and Der p 2 and, to a lesser extent, Der p 23. By contrast, patients did not develop a strong response to other mite allergens. Interestingly, a better clinical response to the AIT was observed in patients sensitized only to Der p 1 and/or Der p 2, when compared to patients with a broader IgE response[78]. This suggest that the molecular profile of IgE sensitization may be useful in selecting patients that may benefit from subcutaneous AIT[78], as previously hypothesized [79]. However, no studies are available so far to ascertain whether this conclusion can be applied also to sublingual immunotherapy.

Hymenoptera venom allergy

Apis mellifera or honeybee venom (HBV) is the best-characterized Hymenoptera venom due to the outstanding importance of this species as elicitor of venom allergy worldwide. Detailed genomic information and proteomic data of the pure venom is available[8081-83]. So far 12 allergens of HBV are identified[84]. Only two of them present substantial amounts in the venom, the major allergen phospholipase A2 (Api m 1)(12%) and the minor peptidic allergen melittin (Api m 4)(50%)[85]. Despite their lower abundance in HBV compared to Api m 1, a role as relevant major allergen was also confirmed for Api m 2 (hyaluronidase), Api m 3 (acid phosphatase), Api m 5 (dipeptidylpeptidase IV) and Api m 10 (icarapin) with sIgE reactivity in the range of 47.9-52.2%, 49.6-50%, 58.3-61.7% and 61.8-72.2% of allergic patients' sera, respectively[86,87]. For the other HBV allergens less information about sensitization rates is available [88-90]. Some HBV allergens have been identified as risk markers of more severe clinical phenotypes such as Api m 4 or of venom immunotherapy (VIT) failure such as Api m 10[86,91].

Prominent *Vespula* spp. (known as yellow jackets in the USA) allergens include phospholipase A1 (Ves v 1), hyaluronidase (Ves v 2.0101) and antigen 5 (Ves v 5)[92,93]. A second hyaluronidase (Ves v 2.0201), carrying an inactivation mutation in the active site of the enzyme, was identified, which seems to be the predominant isoform[94,95]. Yellow-jacket venom (YJV) also contains a dipeptidylpeptidase IV (Ves v 3) which shows high homology to HBV Api m 5[96]. The sensitization rates of YJV-allergic patients to Ves v 1, Ves v 3 and Ves v 5 are 33.3-54% [97-102], 50-62.8%[96,97]and 84.5-100%, respectively[97,99,100,102-104]. In contrast to HBV hyaluronidase Api m 2, which is a major allergen, the YJV homologue Ves v 2 seems to be of restricted relevance and sensitization was reported in 5-25% of YJV-allergic patients[105].

The allergen composition of *Polistes dominula* venom (PDV) is very similar to that of YJV and the most important allergens are phospholipase A1 (Pol d 1), dipeptidylpeptidase IV (Pol d 3) and antigen 5 (Pol d 5) with sensitization rates of 87%[106], 66.7%[107] and 69-72%[106], respectively.

CRD is helpful to discriminate between genuine double sensitization and cross-reactivity, allowing physicians to optimize patient selection for VIT. The potential of CRD becomes evident by the fact that HBV and YJV in addition to homologous allergens also contain differentiating marker allergens that are present in either HBV or YJV. This is not the case when differential diagnosis between *Vespula spp* . and*Polistes spp* . is required (Figure 6). CRD is also helpful in identifying patients with Hymenoptera venom-induced anaphylaxis having negative test results to whole venom extracts, as it can be the case in hymenoptera venom-allergic patients with mast cell disorders[108]. Currently available allergens of honeybee and vespid venoms (see Table 2) allow a molecular diagnosis in the vast majority of patients, but not in 100% of them. Moreover, not all allergens are available for one assay system. New recombinant molecules are needed to improve the diagnosis of*Polistes spp* .-allergic patients, especially in the case of double-positivity to both *Polistes spp* . and *Vespula spp* . venom, in order to prevent unnecessary double VIT (Figure 7A). Although diagnostic sensitivity of the currently available allergen panel, particularly of HBV, is not 100%, CRD has clearly improved discrimination of primary allergy and cross-reactivity in YJV and HBV allergy, thus facilitating correct prescription of VIT. A suggested diagnostic algorithm to discriminate between HBV and YJV allergy using CRD is given in Figure 7B. Of note, the same algorithm using the corresponding PDV allergens can also be applied to discriminate between HBV and PDV allergy.

Peanut allergy

A product for oral immunotherapy (OIT) to peanut has been recently licensed by FDA and EMA and will soon become widely available[109,110]. Other products and routes of administration have been explored[111].

Peanut allergic reactions can be triggered by storage proteins, such as Ara h 1,2,3,6[112], which have been associated to life-threatening symptoms, as well as by cross-reactive allergens, such as Ara h 5[113], a profilin associated to grass pollen allergy, Ara h 8, belonging to PR10 family and associated to birch pollinosis and Ara h 9, a nsLTP, associated to the LTP syndrome discussed earlier(Table 1). Symptoms elicited by Ara h 5 and Ara h 8 are usually mild and limited to the oral cavity, the oropharynx, known as oral allergy syndrome. Ara h 2 sIgE levels are associated to both severity and threshold of allergic reactions during oral provocation challenges, thus being a good biomarker for severity.[114,115]. In fact, Ara h 2 is the dominant 2S globulin

allergen[116]. As storage proteins can trigger systemic food allergic reactions and anaphylaxis, peanut allergy with confirmed sensitization to seed storage proteins like Ara h 1,2,6 can constitute an indication for POIT to peanut.

A summary of the decision process to select POIT is shown in Figure 8.

CRD and AIT safety:

There are different heterogeneous evidence showing an association of risk factors for adverse events during AIT associated to particular IgE sensitization profiles. The most studied model is grass pollen allergy. Adverse reactions with subcutaneous immunotherapy, both local and systemic, correlate with sensitization progression (Phl p 1+5+12 >Phl p 1+5 > Phl p 1/5)[117]. In sublingual AIT, adverse events were also related with highest levels of Phl p 5 or Phl p 1 [2727] and with allergen sensitivity [118]. Under extreme exposure to grass pollen, patients are frequently sensitized to profilin and present severe adverse reactions to foods caused by profilin. Interestingly, this type of these reactions to profilin-containing foods are similar to the infrequent reactions observed during SLIT. This rare phenotype constitutes therefore a unique model to understand disease severity and limits for AIT. Profilin severe reactors undergo extensive oral mucosa damage and present a unique systemic metabolic status that points to T cell proliferation, sustained inflammation and altered repair function. The fact that profilin-sensitized patients in Spain present an enhanced T cell proliferation compared with similar patients from Denmark suggests that progression to severe phenotypes might be linked to an uncontrolled inflammation and T cell proliferation. Interestingly, in the other severity models previously mentioned systemic barrier damage, uncontrolled effector cell response, and altered repair-associated biomarkers have been described. In all cases, might be explained by T cell cross-reactive allergens: Profilin, Ole e 7 (a nsLTP from pollen) and Lep d 2 (a frequent food contaminant inducing reactions in patients with storage mite sensitization).

Future directions:

Accurate diagnosis is essential before AIT can be considered. The routine use of molecular diagnosis for allergic diseases and AIT, theorized in the late 1990s by Rudolf Valenta and colleagues[119], is relatively recent and is still evolving. Constraints include the perceived complexity of this diagnostic approach and the slightly higher costs of test execution[120-122]. The recent development of a novel multiplex test containing both extracts and molecules relevant for pollen allergy (a sort of "molecular pollen test") may help doctors in the prescription of the appropriate AIT products [123]. The development of algorithms and clinical decision support systems integrated into apps for smartphones[124] will facilitate the clinical interpretation of the outcome of IgE molecular assays, as shown in a recent pilot experience (Arasi, S et al. Clin Exp Allergy, in press). Different estimates confirm that CRD has a significant impact on AIT formula selection[125-127].

To rely on correctly standardized and clinically documented AIT products, as well as to understand their limitations, is as important as correctly diagnosing patients.

With an increasing number of new intervention possibilities, it is essential to optimize the use of AIT[58,128,129]. Major allergen sensitization and the use of up to two different clinically-documented allergen preparation is a must. In fact, even in complex exposure regions, most of the patients are sensitized to a limited number of allergens[130] and thus potentially eligible for AIT. An adequate dose of allergen as defined in dose-finding clinical trials will guarantee an adequate safety/efficacy balance. In the last years, an increasing number of allergen preparations developed with the highest pharmacological standards are available. This trend will continue and is expected to have a profound impact on AIT practice and to position it in the center of etiological management of allergic patients. In spite of correct diagnosis and the use of high-quality AIT products, a fraction of treated patients will not improve or will lose clinical benefit upon discontinuation after three years of intervention[13]. The need to advance on personalized medicine approaches to predict intervention outcome and safety - and to monitor AIT effect - is imperative[131]. System biology approaches are being explored to understand AIT response[5]. These new approaches help to understand biological effect kinetics and provide new tools to evaluate new AIT product approaches. The desensitization of allergen-specific effector cells plays a determinant role for AIT effect during the first two years of intervention, while

regulatory response, which is initiated shortly after AIT initiation, will only have a meaningful clinical benefit after at least two/three years of administration. We need better formulas, including adjuvants and tolerogenic signaling molecules to reduce AIT duration[6]. At the same time, understanding severe phenotypes is needed. Recent data obtained by system biology approaches identify T cell proliferation, inadequate regulatory function, and collapse of repair homeostasis as main causes for non-response to intervention and evolution to severe phenotypes[7-9,132]. There are new possibilities of using these systemic signatures to explore the value of new biologics to stabilize severe phenotypes allowing ulterior AIT intervention.

While CRD has proved the value for correct patient inclusion and AIT standardization, we cannot expect that it will be of great value to predict intervention outcome or safety[77]. AIT is the only treatment in allergy able to modify the course of the disease, but for achieving this goal, the correct patients, with the correct product, with adequate treatment duration and compliance are needed. Even so, about 30% of patients will not benefit from this disease-modifying effect. This 70% effect, however, ranks at the top of successful pharmacological interventions. In 2003, Dr. Allen Roses, by that time vice president of the genetics division for GSK, commented that "The vast majority of medicines, more than 90 percent, only work in 30 or 50 percent of people". Since then, massive efforts on personalized medicine are progressively changing the landscape. AIT has the potential of being the reference intervention in allergy, but for that, we need a commitment and upgrade of its clinical practice. The use of molecular allergology for diagnostic purposes will rapidly evolve in the next years and will be more linked to the identification of patients phenotypes and endotypes for an improved therapeutic approach, which implies the opening of the era of "precision allergology"[121,122]. CRD, today, provides a good starting point.

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Figure Legends

Figure 1: Diagnostic algorithm and decision tree for AIT using CRD in Grass pollen allergy. Grass pollen allergy is one of the most studied allergy models. Major allergen sensitization is required before considering AIT. The combination of major allergens and pan-allergens provides the necessary tools for AIT decisions. Profilin allergy, might be a contraindication only in severe food allergic patients, while double sensitization to both pan-allergens is associated to many years of disease evolution, poly-sensitization and poor intervention outcome

Figure 2: Diagnostic algorithm and decision tree for AIT using CRD in Olive pollen allergy. Olive pollen allergies is one of the most complex allergy models. Usually olive cultivars cover homogeneous areas and present acute differences in allergen pollen content. In dense pollen areas, patients are exposed to the highest pollen counts recorded. Th existing of complex profiles, marked by minor allergens sensitization, makes CRD a fundamental tool for patient management and AIT decissions.

Figure 3: Diagnostic algorithm and decision tree for AIT using CRD in nsLTPs mediated allergies. The existence of cross-reactivity between nsLPTs from *Artemisia* and *Platanus* pollens, makes CRD necessary. LTP immunotherapy with an enriched Pru p 3 can be considered in Countries with availability of this type of therapy

Figure 4: Diagnostic algorithm for in vitro diagnostics and Decision tree for AIT using CRD in pollen allergies. Discrimination of primary sensitizers and pan-allergens positivity is needed. AIT guidelines do not recommend AIT for poly-sensitized patients

Figure 5: Diagnostic algorithm for in vitro diagnostics in epithelia allergy and associated decision tree for AIT using CRD. The proposed algorithm discriminates between primary sensitization and cross-reactive sIgE response. Only patients sensitized to major allergens should be eligible for AIT.

Figure 6: Established discriminating and cross-reactive allergens of HBV, YJV and PDV. While allergens are identified that enable a differentiation between HBV (Api m 1, Api m 3, Api m 4 and Api m 10) and

YJV/PDV (Ves v 1/Pold 1 and Ves v 5/Pol d 5) sensitization, the so far known allergens of YJV and PDV exhibit cross-reactivity.

Figure 7: Diagnostic algorithm for in vitro diagnostics of (A) HBV and YJV allergy and (B) YJV and PDV allergy. The diagnostic algorithm shown in (A), with the corresponding PDV allergens Pol d 1 and Pol d 5 can also be used to discriminate between HBV and PDV allergy. In addition, to discriminate in the case of double-positive test results, CRD might also be useful in cases of negative results despite a convincing history of venom allergy or in cases of discrepancies between history, skin tests and venom extract-based testing. A plus indicates a positive and a minus a negative test result. Of note, the allergens Api m 4 and Pol d 1 (in brackets) are only available for selected multiplex sIgE test platforms. *The allergens Api m 2 and Api m 5 are potentially cross-reactive with their homologues from YJV and PDV (not available for CRD) and, hence, a positive test results does not necessarily exclude allergy to vespid venom.

Figure 8: Diagnostic algorithm for in vitro diagnostics in peanut allergy oriented to make POIT decision. Discriminant analysis between storage allergens and cross-reactive to pollen and nsLTPs is proposed. Only patients with positive sIgE to any of the Ara h 1,2,3,6 should be eligible for POIT.

BOXES:

MAJOR MILESTONES:

- IgE Discovery (1968)
- First commercial reagents for specific IgE (1973)
- Knowledge and Characterization of the most relevant allergens (1985-2005)
- Development of multiplexed allergens: 1999
- Handbook of Molecular Allergology: 2016
- Progressive use in clinical practice of CRD: 2010-2021
- Progressive understanding of AIT mechanisms: 2010-2021
- First AIT product registered following Pharmaceutical development guidelines: 2007
- First OIT for peanut allergy registered: 2020

FUTURE RESEARCH PERSPECTIVES:

- Affinity and avidity need to be explored in IgE response to allergens
- T-cell reactivity to allergens is a critical parameter
- Combination of new biologics and AIT and associated diagnosis will open new intervention strategies
- New regulation of in vitro diagnostics will increase the quality of CRD , but might limmit innovation and available molecules

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Table 1: Characteristics and significance of pollen, vegetables, mites and epithelia allergens available for CRD and relevant for AIT decisions

SOURCE		Allergen	Sensitization rate	Available for routine CRD	Significance
POLLEN	Grasses	Phl p 1	>90%	S,M	Expansin. Present in different plant parts. Major allergen and usually initiates sensitization
		Phl p 5	>60%	S,M	Unknown function. Most abundant pollen protein
		Phl p 7	<10%	S,M	Pan-allergen in pollens. Calcium -binding
		Phl p 12	5-50%	S,M	Pan-allergen (food/pollen). Structural protein
	Birch	Bet v 1	>90%	S,M	Pan-allergen (food/pollen) Regulatory protein. Highly expressed in case of biotic stress.
	Olive tree	Ole e 1	>90%	S,M	Unknown function
	Olive	Ole e 7	0-50%	S,M	nsLTP, minor allergen. Disease severity marker
	Olive	Ole e 9	0-50%	S,M	Glucanase, minor allergen. Highly vairable in different olive cultivars
	Russian thistle	Sal k 1	>90%	S,M	Pectin methylesterase
	Plain tree	Pla a 1	>90	S,M	Invertase inhibitor

SOURCE		Allergen	Sensitization rate	Available for routine CRD	Significance
	Arizona cedar	Cup a 1	>90%	S,M	Pectate lyase. Homologous and cross-reactive to Cry j 1 from Japanesse cedar and Jun a 1 from mountain cedar
	English plantain	Pla l 1	>70	S,M	Ole e 1 like. Non-cross reactive
	Artemisia	Art v 1	>70	S,M	Defensin like
	Ragweed	Amb a 1			Pectate lyase
	Parietaria	Parj 2	>90%	S,M	ns LTP
FOODS	Peach	Pru p 3		S,M	ns LTP. Very stable. Frequent initiator in LTP syndrome
	Peanut	Ara h 1			11S Albumin. Storage protein. Highly abundant
		Ara h 2			2S albumin. Storage protein. >Highly abundant. Risk marker for severe reactions
		Ara h 3			11S Albumin. Storage protein. Highly abundant
		Ara h 5			Profilin
		Ara h 8			PR10. Bet v 1 like
		Ara h 9			nsLTP (Pru p 3 related)
MITES	*Dermatophagoides pteronyssinus*	Der p 1	>90%	S,M	Cystein protease. Associated to mite fecal particles

SOURCE		Allergen	Sensitization rate	Available for routine CRD	Significance
		Der p 2	>90%	S,M	NCP2. Lipid binding protein. MD2 link. Associated to mite bodies
		Der p 10	<10%	S,M	Pan-allergen in arthropods.
		Der p 23			Peritrophin-like protein
	Lepidoglyphus destructor	Lep d 2	>90%	M	Related to Der p 2, but without IgE cross-reactivity. Cross-reactive with Group 2 proteins from other minor/storage mites.
EPITHELIA	Cat	Fel d 1	>90%	S,M	Secretoglobulin.
		Fel d 4,7	<50%	S,M	Lipocalins. Cross-reactivity with Can f 1
	Dog	Can f 1	>50%	S,M	Lipocalin
		Can f 5	>50%	S,M	Kalikrein. Serin protease. Regulating semen liquefaction. Present in male dogs

*Estimated from publications. S: Available in singlepex assays. M Available in Multiplex assays.

Prevalence on food allergens depends on geographical locations and age. In general, storage protein allergy is frequent in pediatric cohorts, while LTP and PR10 linked allergy is of a later onset.

Table 2. Characteristics and significance of Hymenoptera venom allergens available for routine CRD (modified according to[133]).

Allergen	**Sensitization rate***	**Available for routine CRD**	**Significance**
Honeybee venom (*Apis mellifera*)	**Honeybee venom** (*Apis mellifera*)	**Honeybee venom** (*Apis mellifera*)	**Honeybee venom** (*Apis mellifera*)

Allergen	Sensitization rate*	Available for routine CRD	Significance
Api m 1 Phospholipase A2	57-97%	S[1,2,3], M[4,5,6]	Marker allergen for HBV sensitization; Allows discrimination between HBV and YJV/PDV sensitization
Api m 2 Hyaluronidase	28-60%	S[1,2,3], M[4]	Due to limited cross-reactivity with Ves v 2 and Pol d 2 in the absence of CCDs, potential marker for HBV sensitization**
Api m 3 Acid phosphatase	28-63%	S[1]	Marker allergen for HBV sensitization; Allows discrimination between HBV and YJV/PDV sensitization; Valuable marker allergen to diagnose HBV allergy in Api m 1-negative patients
Api m 4 Melittin	17-54%	M[6]	Marker allergen for HBV sensitization; Allows discrimination between HBV and YJV/PDV sensitization; Putative marker allergen for severe VIT side-effects
Api m 5 Dipeptidylpeptidase IV	16-70%	S[1]	High cross-reactivity with Ves v 3 and Pol d 3 prevents its use as marker allergen***
Api m 10 Icarapin	35-73%	S[1,3], M[4,5]	Marker allergen for HBV sensitization; Allows discrimination between HBV and YJV/PDV sensitization; Valuable marker allergen to diagnose HBV allergy in Api m 1-negative patients; Dominant Api m 10 sensitization is a putative marker for risk of VIT failure
Yellow jacket venom (*Vespula vulgaris*)	**Yellow jacket venom** (*Vespula vulgaris*)	**Yellow jacket venom** (*Vespula vulgaris*)	**Yellow jacket venom** (*Vespula vulgaris*)

Allergen	Sensitization rate*	Available for routine CRD	Significance
Ves v 1 Phospholipase A1	39-66%	S[1], M[4,5]	Marker allergen for YJV sensitization; Allows discrimination between YJV and HBV sensitization; High cross-reactivity with Pol d 1prevents its use as marker allergen to discriminate between YJV and PDV sensitization
Ves v 5 Antigen 5	82-98%	S[1,2,3], M[4,5]	Marker allergen for YJV sensitization; Allows discrimination between YJV and HBV sensitization; High cross-reactivity with Pol d 5 prevents its use as marker allergen to discriminate between YJV and PDV sensitization
European paper wasp venom (*Polistes dominula*)	**European paper wasp venom** (*Polistes dominula*)	**European paper wasp venom** (*Polistes dominula*)	**European paper wasp venom** (*Polistes dominula*)
Pol d 1 Phospholipase A1	87%	M[4]	Marker allergen for PDV sensitization; Allows discrimination between PDV and HBV sensitization; High cross-reactivity with Ves v 1 prevents its use as marker allergen to discriminate between PDV and YJV sensitization
Pol d 5 Antigen 5	72%	S[1], M[4,5]	Marker allergen for PDV sensitization; Allows discrimination between PDV and HBV sensitization; High cross-reactivity with Ves v 5 prevents its use as marker allergen to discriminate between PDV and YJV sensitization

*Defined by different immunoassays and in different patient populations. Sensitization rates are referenced in the main text. **Cross-reactivity with Ves v 2 and Pol d 2 is possible. ***Api m 5 monosensitization may occur in HBV-allergic patients. CCDs, cross-reactive carbohydrate determinants; CRD, component-resolved diagnostics; HBV, honeybee venom; M, multiplex sIgE assay system; PDV, *Polistes dominula* venom; S, singleplex sIgE assay system; YJV, yellow jacket venom. [1]Thermo Fisher Scientific, [2]Siemens Healthcare Diagnostics,[3]Dr. Fooke Laboratories, [4]Euroimmun,[5]Macro Array Diagnostics, [6]Faber test (different suppliers).