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The allergenic activity and clinical impact of individual IgE-antibody binding molecules from indoor allergen sources

Luis Caraballo[a]*, Rudolf Valenta[b,c,l], Leonardo Puerta[a], Anna Pomés[d], Josefina Zakzuk[a], Enrique Fernandez-Caldas[e], Nathalie Acevedo[a], Mario Sanchez-Borges[f], Ignacio Ansotegui[g], Luo Zhang[h], Marianne van Hage[i], Eva Abel-Fernández[e], L. Karla Arruda[j], Susanne Vrtala[b], Mirela Curin[b], Hans Gronlund[k], Antonina Karsonova[l], Jonathan Kilimajer[e], Ksenja Riabova[l], Daria Trifonova[l] and Alexander Karaulov[l]

ABSTRACT

A large number of allergens have been discovered but we know little about their potential to induce inflammation (allergenic activity) and symptoms. Nowadays, the clinical importance of allergens is determined by the frequency and intensity of their IgE antibody binding (allergenicity). This is a rather limited parameter considering the development of experimental allergology in the last 20 years and the criteria that support personalized medicine. Now it is known that some allergens, in addition to their IgE antibody binding properties, can induce inflammation through non IgE mediated pathways, which can increase their allergenic activity. There are several ways to evaluate the allergenic activity, among them the provocation tests, the demonstration of non-IgE mediated pathways of inflammation, case control studies of IgE-binding frequencies, and animal models of respiratory allergy. In this review we have explored the current status of basic and clinical research on allergenic activity of indoor allergens and confirm that, for most of them, this important property has not been investigated. However, during recent years important advances have been made in the field, and we conclude that for at least the following, allergenic activity has been demonstrated: Der p 1, Der p 2, Der p 5 and Blo t 5 from HDMs; Per a 10 from *P. americana*; Asp f 1, Asp f 2, Asp f 3, Asp f 4 and Asp f 6 from *A. fumigatus*; Mala s 8 and Mala s 13 from *M. sympodialis*; Alt a 1 from *A. alternata*; Pen c 13 from *P. chrysogenum*; Fel d 1 from cats; Can f 1, Can f 2, Can f 3, Can f 4 and Can f 5 from dogs; Mus m 1 from mice and Bos d 2 from cows. Defining the allergenic activity of other indoor IgE antibody binding molecules is necessary for a precision-medicine-oriented management of allergic diseases.

INTRODUCTION

The characterization of an allergen involves from the analysis of its IgE antibody binding capacity to the demonstration of clinical relevance. This long process, that also includes physicochemical properties, biological function, and structure determination, is challenging to achieve for all allergens. The clinical impact of indoor allergenic sources has been extensively demonstrated using whole extracts but has not been evaluated for most of the individual IgE antibody binding molecules. Recent advances in experimental allergology show that

*Corresponding author. Institute for Immunological Research, University of Cartagena, Cartagena de Indias, Colombia.
E-mail: lcaraballog@unicartagena.edu.co
Full list of author information is available at the end of the article

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the **allergenic activity** (the capacity to induce tissue inflammation) of indoor allergens, including house dust mite (HDM), cockroach, fungi, and animal allergens, can be exerted through several mechanisms, such as the adaptive and innate Th2/IgE responses and other lesser known pathways. However, allergenic activity has traditionally been linked only to the IgE response, ignoring other of the allergen intrinsic proinflammatory properties. It is currently assumed that molecules with high IgE antibody binding frequencies (>50%) are relevant, whereas those with low frequencies (<50%) are considered "minor" allergens, even though some of them can induce a non-IgE mediated inflammation, and in some patients a strong specific IgE response. In general, allergens with high IgE antibody binding frequency also induce strong IgE responses, which makes the IgE antibody binding frequency the main criterium for deciding the clinical impact of allergens. However, exceptions have been described, such as the major timothy grass pollen allergen Phl p 4, a glycoprotein which although recognized by approximately 70% of grass pollen allergic patients has low or no allergenic activity as demonstrated by skin testing, basophil activation testing, and lack of symptoms in mono-sensitized subjects.[1–3] Besides, Blo t 13 from *Blomia tropicalis* has low IgE antibody binding frequency and in some patients is the main allergen inducing symptoms.[4]

Therefore, and to be more in line with precision and personalized allergology, it is necessary to consider additional properties of allergens to better define their allergenic activity and clinical relevance for specific patients. Most allergenic components from the main HDMs, *Dermatophagoides pteronyssinus* and *B. tropicalis*, as well as those from cockroaches, fungi, and pets, remain to be fully analyzed in terms of their allergenic activity, which, at the end, underlies their clinical relevance. In this review we present an updated description of the demonstrated allergenic activity of individual IgE antibody binding molecules of several indoor allergen sources. An attempt towards the assessment of the allergenic activity and clinical impact of the individual allergens was performed by taking into consideration the following lines of evidence. First, we considered results from *in vivo* provocation tests (such as skin testing, nasal, conjunctival, and bronchial provocation) or *in vitro* surrogate assays such as basophil and/or mast cell activation tests and induction of T cell proliferation and cytokine releases. Second, we examined the experimental evidence for a given allergen molecule to induce inflammation through non-adaptive immune pathways. Third, we investigated publications showing the induction of symptoms in animal models and those reporting IgE sensitization in association with symptoms in patients, not only at certain ages but also for prediction. The assays employed to explore these lines of evidence are discussed in the next section and presented in Table 1.

Assays to evaluate allergenic activity

During recent years great advances have been made in the field of experimental allergy. The availability of recombinant allergenic molecules with similar physical and biological properties to the native counterparts, as well as the use of animal and *ex vivo* experimentation supported by robust biomedical technology, have provided important information to believe that the IgE antibody binding demonstration is just the initial step for studying allergenic activity. Among the procedures employed by researchers in this field, we have chosen some that can be used to define the allergenic activity and clinical relevance of IgE antibody binding molecules, among them the intrinsic proinflammatory mechanism of action, provocation tests, animal models of experimental allergy, case control studies, avoidance studies, and immunotherapy trials (Table 1).

The list is not complete because other potentially useful strategies, such as immunogenetic or epigenetic studies with individual molecules, are not commonly used. Although avoidance and immunotherapy trials using components are also very limited, they are included in this review to highlight their great potential for confirming clinical relevance. Considering that IgE antibody binding is a requisite to be an allergen (in fact, nowadays, it is the only way used to detect potential allergens), this review will focus on other criteria that could also be valuable to define the clinical impact of allergens. Since not all the assays have the same relevance in the pathophysiology of allergic diseases (for example, bronchial provocation versus basophil activation test) and not all the

Assay	Comment
IgE-binding capacity	Evaluates allergenicity, an important component of allergenic activity.
In vivo provocation tests: skin, nasal, conjunctival, bronchial	The most representative test of clinical relevance.
In vitro provocation tests: histamine release, basophil activation	Evaluate IgE-dependent cellular activation.
Case control studies	Statistical support of clinical relevance
Mechanisms of action	Evaluate additional, non-IgE mediated, inflammatory pathways.
Animal model of allergy	Supports the hypothesis of clinical relevance
Passive cutaneous anaphylaxis	Induction of a specific IgE response
Avoidance studies	An indirect way to confirm clinical effects
Component specific immunotherapy	Supports causality and confirms clinical relevance

Table 1. Assays to evaluate allergenic activity and clinical relevance of allergens

patients react with the same intensity to an allergenic stimulus, it is not possible to make a formal classification about which allergens have high, low or intermediate allergenic activity. In addition, we must keep in mind that in the real world the exposure is not to purified allergens but to mixtures of several allergens and other environmental elements that could modify the effects of individual components. However, it seems rational to suggest that those IgE antibody binding components with positive *in vivo* provocation tests in humans, plus positive association in case-control studies and defined proinflammatory mechanisms of action, have a demonstrated allergenic activity.

General allergen mechanisms of action

Adaptive immunity pathways

In genetically susceptible individuals, the immune response to allergens is based mainly on specific IgE and type 2 cytokines, such as IL-4, IL-5 and IL-13. The specific IgE primary response to allergens is exclusively developed through the adaptive pathways of immunity. After sensitization, the re-exposure to allergens initiates the IgE-dependent inflammatory cascade, which involves additional cells and cytokines that actively participate in the inflammatory reaction. Under shared environments and similar levels of exposure only a small proportion of people are sensitized; therefore, genetic factors have been extensively investigated, to conclude that they play an important role in the Th2 immunity. It is well known that environmental conditions determine not only the adaptive Th2 response to allergens but also the inception of allergic diseases.[5,6] Although the association between HDM allergen concentration and IgE sensitization is not always positive,[7,8] theoretically high environmental levels of these allergens will increase the probabilities that genetically susceptible individuals inhale them and become sensitized. Several factors, sometimes acting concomitantly, modulate the IgE primary response and sensitization process, including the levels of allergen exposure (which in turn depends on protein production by the source, permanence in the house dust and the air, effects of other enzymes upon the allergen), persistence of exposure, age of exposed individuals, boosting of the IgE responses by conditions such as air pollution or helminth infections, and stimulation of Th1 responses by bacterial and other products.

Innate immunity pathways

Historically, the word "allergen" has been associated with a specific inducer of either IgE sensitization or an allergic response by IgE-mediated

release of cell mediators such as histamine. In addition, allergens have diverse pro-inflammatory properties that can increase their allergenic activity. In fact, several innate non-IgE mediated inflammatory mechanisms have been reported, including the ability to bind adjuvants or to stimulate the innate immunity via toll-like receptors and other receptors in the bronchial epithelium.[9–13] Der p 1 and Der p 2 are good examples of molecules with high allergenic activity that could be due in part to their ability to stimulate both innate and adaptive pathways of the allergic response.[14,15] However, since not all individuals exposed to these allergens have bronchial inflammation, innate mechanisms seem to be also under genetic control.

Other pathways that could be activated by HDM exposure are the stimulation of airway cells to secrete vascular endothelial growth factor (VEGF) which contributes to edema observed in airway remodeling;[16,17] the enhancing of cysteinyl leukotriene synthesis from dendritic cells and mast cells through a glycan-dectin 2 interaction;[18] a reprogramming of proinflammatory eicosanoids in alveolar-like monocyte-derived macrophages with suppression of 5-LOX expression and product formation and triggering of prostanoid production,[19] and the induction of epigenetic changes, among them the expression of miRNA-16, miRNA-21, and miRNA-126 through the activation of TLR4.[20] Other studies reveal that the epigenome might influence the susceptibility to mite sensitization by modifying DNA methylation in B cells,[21] and the hypomethylation of the interleukin 13 gene.[22] More interestingly, HDM can induce epigenetic modifications in mice experimental models of airways inflammation, changing the methylation pattern of important genes such as *Phosphodiesterase 4 D*,[23] and *tgfb1*.[24] In addition, using an *ex vivo* model of inflammation in human bronchial epithelial cells, HDM induces the same epigenetic modifications as does diesel exhaust.[25] These works suggest that HDM, in addition to inducing IgE-mediated bronchial inflammation, can alter the epigenetic patterns of cells involved in bronchial homeostasis, inducing inflammation. In contrast, allergen specific immunotherapy can change DNA methylation levels at the forkhead box P3 gene (FOXP3) and, by improving the function of regulatory T cells, modify the IgE response to mites.[26] Hence, environmental exposures affecting the epigenome or polymorphisms influencing the interaction between the genome and the epigenetic machinery may play a role in modulating the gene-environment signals that lead to mite sensitization.

House dust mite allergens

HDMs are the most important elicitors of allergic reactions indoors and can be found almost worldwide.[27] In areas where mites are prevalent, up to 20% of the population is sensitized, constituting about 50% of all allergic patients.[28] HDMs can cause severe and chronic allergic symptoms such as asthma, allergic rhinoconjunctivitis, and atopic dermatitis.[29,30] In temperate climates, the two house dust mite species, *D. pteronyssinus* and *D. farinae,* are prevalent. Most of their allergens show a high sequence homology (80–85%) and are highly cross-reactive.[31] In tropical climates, *B. tropicalis* is an important source of sensitizing allergens associated with asthma and rhinitis;[32] therefore, this section will focus on the allergens of *D. pteronyssinus* and *B. tropicalis*.

Dermatophagoides pteronyssinus

Der p 1. So far, 38 groups of allergens have been identified in HDMs, 24 of them were identified in *D. pteronyssinus*. Der p 1 was the first allergen identified in HDMs and shows IgE antibody-binding frequencies of more than 80%.[33] A high allergenic activity has been shown for this component using different assays, including *in vivo* and *in vitro* provocation tests;[34–37] and its sensitization is associated with asthma.[38] IgE levels to Der p 1 are significantly higher in asthmatic children than in children without asthma and IgE antibodies to Der p 1 at an age of <5 years are a risk factor for the development of asthma.[39,40] Avoidance studies suggest that diminishing levels of Der p 1 and Der p 2 are associated with improvement of asthma symptoms in patients allergic to *D. pteronyssinus*.[41] The mechanisms of action of Der p 1 have been extensively investigated,[42] showing that it can induce inflammation through both innate and adaptive pathways. Der p 1 has

cysteine protease activity, which seems to enhance selectively the IgE response, conditions T cells to produce more IL-4 and less IFN-gamma, and can destroy the barrier function of the bronchial epithelium, by disrupting the transmembrane molecules occludin and claudin and activating the protease-activated receptor PAR-2 which induces the release of pro-inflammatory cytokines.[43,44] Der p 1 cleaves and inactivates surfactant protein-A (SP-A) and SP-D, lung collectins that have protective roles in allergy, which could contribute to the potent allergenic activity of this allergen. Studies in animal models suggest that the cysteine protease activity of Der p 1 contributes to the *in vivo* immune responses, including the production, not only of IgE, but also of IgG. Intranasal administration of proteolytically active Der p 1 to sensitized mice leads to an enhanced inflammatory cellular infiltration of the lungs and systemic production of IgE in comparison to inactive Der p 1, which has no effect.[45] Der p 1 acts as the activator of the precursors of Der p 3 and Der p 6 according to an uncommon activation cascade.[46]

Der p 2. Another important allergen of *D. pteronyssinus* is Der p 2, which shows IgE antibody-binding frequencies of more than 90%.[47] Mite allergic patients, in particular patients suffering from asthma, have high levels of Der p 2-specific IgE-antibodies[39] and Der p 2 allergenic activity has been demonstrated *in vitro* and *in vivo,* by means of methods such as skin tests and histamine release.[11,12,48,49] Der p 2 represents a lipopolysaccharide (LPS)-binding protein with structural homology to the toll-like receptor (TLR) 4 co-receptor, MD-2.[14] Because of this, Der p 2 can activate the toll-like receptor 4 (TLR4) and promote airway epithelial inflammation.[50] For long time, Der p 1 and Der p 2 have been considered as the two important HDM allergens necessary for diagnosis and immunotherapy, but several studies have indicated that these two allergens are not enough to diagnose all HDM-sensitized patients.[51,52] Additionally, immunotherapy with a HDM extract-based vaccine, which contained mainly Der p 1 and Der p 2, was not successful for all HDM allergic patients, but only for those with exclusive sensitization to these two allergens.[53] *In vitro* and animal model studies show that Der p 2 induces airway inflammation and elevated nerve growth factor (NGF) release through increasing reactive oxygen species (ROS) production and the mitogen-activated protein kinases (MAPK) dependent pathway.[54] Complexed with LPS it also induces Th2 responses in MD-2- but not TLR4-deficient animals,[14] and specifically up-regulates mitogen-activated protein kinase phosphatase-1 (MKP-1) expression and activity in human B cells, which in turn, results in p38 mitogen-activated protein kinase (p38/MAPK) dephosphorylation, triggering TLR4 induction.[55]

Der p 3. This allergen is a trypsin-like serine protease with structural similarity with other serine proteases, including chymotrypsin A. Hales et al., using natural Der p 3 in a dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) assay, found in Australian population a prevalence of IgE binding of 9% in non-hospitalized and 14,3% in hospitalized mite allergic subjects.[56] The allergenic activity of Der p 3 evaluated in a murine model of Der p 3 sensitization showed a typical Th2 cytokine profile, characterized by the high production of IL-5 and low or no secretion of IFN-γ. Mice sensitized with rDer p 3 exhibited a typical Th2-biased immune response, characterized by high titers of Der p 3-specific IgG1 and IgE and the absence of IgG2a production. Natural and recombinant Der p 3 induced a similar release of beta-hexosaminidase by humanized basophils in RBL assays, and both protein forms induced a similar upregulation of CD203c expression on the surface of blood basophils from allergic donors, suggesting that the recombinant allergen and its natural counterpart have the same structural and allergenic properties.[37]

Der p 5. The IgE reactivity of rDer p 5 was 31% in sera from 117 mite-allergic patients, with no relevant cross-reactivity to group 5 allergens from storage mites.[57] rDer p 5-specific rabbit IgG antibodies and immunogold electron microscopy localize the allergen to secretory granules of midgut epithelial cells of HDM. The structure of Der p 5 is a dimer with a large hydrophobic cavity that could be involved in the binding of ligands like LPS, with the ability of shifting the immune response from tolerance to allergic inflammation.[58] Recombinant Der p 5 produced in *Pichia pastoris* binds lipid ligands, only under acidic conditions. Asthmatic patients reacted more frequently to Der p 5 and Der p 5-specific IgE

levels were higher in asthmatic compared to non-asthmatic patients.[39,59,60] rDer p 5 induced basophil histamine release in an allergic patient;[57] in addition, it induced degranulation of basophils pre-loaded with IgE from Der p 5-sensitive individuals and stimulated the production of IL-6 and IL-8 in human airway epithelial cells.[61] The biological function of Der p 5 is so far unknown.

Der p 10. Der p 10 has significant homology with tropomyosins from different species. Sera from allergic patients with strong IgE reactivity showed relevant rDer p 10-IgE induced degranulation of basophils in rat basophil leukemia cells.[62] Additionally, IgE sensitization to Der p 10 was associated with asthma in the tropics but its allergenic activity in temperate zones seems to be lower.[62,63]

Der p 13. Der p 13 belongs to the fatty acid binding protein family; it may, through its lipid-binding capacity, play a role in the initiation of the HDM-allergic response through TLR2 activation. Der p 13 showed 7% of IgE-binding frequency in Thai HDM-allergic patients as well as limited propensity to activate basophil degranulation in a rat basophil degranulation assay using the rat basophil leukemia cells expressing the human FcεRI receptor (RBL SX-38 cells). Nevertheless, the protein with its presumptively associated lipid(s) triggers the production of IL-8 and GM-CSF in respiratory epithelial cells through a TLR2, MyD88, NF-kB, and MAPK-dependent signaling pathway.[11]

Der p 18. Der p 18 is mainly localized in the peritrophic matrix of the HDM gut and to a lower extent in fecal pellets; it binds weakly to chitin[12]. Specific IgE to this allergen is reported in 17 out of 27 (63%) of allergic sera from Australia,[64] and in 10% of mite allergic patients from Austria by non-denaturing dot blot assay.[12] Using the basophil activation test with samples from allergic patients, rDer p 18 induced basophil activation in an IgE-dependent manner.

Der p 21. By immunogold electron microscopy Der p 21 was localized in the midgut (epithelium, lumen and feces) of *D. pteronyssinus*. Using dot blot analysis in a population of 117 *D. pteronyssinus* allergic patients from Austria, Der p 21 showed 26% frequency of IgE reactivity. The allergenic activity of Der p 21 has been demonstrated by specific histamine release, upregulation of CD203c expression and induction of beta-hexosaminidase release from humanized RBL cells sensitized with patient's sera.[65]

Der p 23. This allergen is found mainly in peritrophic membranes surrounding mite fecal pellets, binds IgE in around 70% of mite allergic patients and shows high allergenic activity.[66] Der p 23 is a small, globular protein stabilized by two disulphide bonds, and contains chitin-binding domains, although functional assays failed to confirm chitin binding.[67] Approximately 5% of HDM allergic patients are exclusively sensitized to Der p 23.[52] Specific IgE levels to Der p 23 detected in the Multicentre Asthma Genetics in Childhood study also recognized it as a major allergen.[39] Its frequent recognition as a respiratory allergen may be explained by the fact that it becomes airborne and respirable through its association with mite feces.[66] Fifty-four per cent of Thai HDM-allergic patients displayed Der p 23-specific IgE responses, and rDer p 23 induces basophil degranulation of rat basophil leukemia cells.[68]

Der p 24. This allergen is an ubiquitinol cytochrome C reductase binding (UQCRB) protein homolog. In serum samples from HDM allergic from Guangzhou, China, serum IgE reactivity and positive SPT to rDer p 24 was demonstrated in 5 out of 10 HDM allergic patients (50%). The immunodominant IgE epitope of Der p 24 was localized to the N-terminal 32-residue region, which produces a high specific IgE antibody titer in immunized Balb/c mice and promotes mast cell β-hexosaminidase release from mast cell line RBL-2H3.[69]

Other known *D. pteronyssinus* allergens are recognized by IgE from allergic patients but do not have other assays of allergenic activity suggesting clinical relevance. A detailed information of the search for allergenic activity of this mite is presented in Supplementary Table 1. In summary, as shown in Table 2, not all the IgE binding molecules from this HDM have been tested for other properties related to allergenic activity.

D. pteronyssinus	Provocation test *in vivo*	Provocation test *in vitro*	Non-IgE induced inflammation	Case-control study	Respiratory Animal model	PCA animal model	Avoidance	AIT trials
Der p 1	ST, B	BAT, HR	Yes	Asthma	Asthma	Yes	Yes	Yes
Der p 2	ST, B	BAT, HR	Yes	Asthma	Asthma		Yes	Yes
Der p 5	ST	HR	Yes	Asthma	Asthma			
Der p 3		BAT, RBL	Yes		Asthma			
Der p 10	ST			Asthma		Yes		
Der p 21	ST	BAT		Asthma				
Der p 13		BAT, HR	Yes					
Der p 23		BAT		Asthma				
Der p 18		BAT						
Der p 24		HR						
B. tropicalis								
Blo t 5	ST	BAT, HR	Yes	Asthma	Asthma	Yes		
Blo t 8	ST				Asthma	Yes		
Blo t 10	ST			Asthma				
Blo t 12	ST				Asthma			
Blo t 13	ST	HR						
Blo t 2		RBL, HR						
Blo t 7			Yes					
Blo t 11	ST							

Table 2. Allergenic activity assays accomplished by house dust mite allergens (In addition to IgE-binding) *PCA: Passive cutaneous anaphilaxis; AIT: Allergen Immunotherapy; ST: Skin test; BAT: Basophil Activation test; HR: Histamine release; RBL: Rat Basophil Leukemia cells test*

Blomia tropicalis

Blo t 2: In Latin America, 54.5% (24/44) of Brazilian patients allergic to *B. tropicalis* reacted to rBlo t 2. Lopez et al. tested IgE reactivity to rBlo t 2 in Colombian children under age 5-years with recurrent wheezing and found a sensitization rate of 35%. Blo t 2 induces beta-hexosaminidase release in RBL cells. It would be also interesting to confirm its lipopolysaccharide (LPS) binding activity, because in Der p 2 it is involved in allergenicity. Amino acid replacement in Blo t 2 isoforms (compared to *Dermatophagoides spp*. homologous proteins) could affect its interaction with lipids. Sequence analyses showed that all the variants of Blo t 2 lack the key basic TLR-4-binding residue K77. In addition, an aromatic residue at position 75 was shown to be important for TLR-4 binding (Tyr102 of MD-2).

Blo t 5. This is one of the most studied allergens of *B. tropicalis*.[70] Frequency of sensitization up to 90% has been reported in *B. tropicalis* allergic patients living in tropical and subtropical countries[71]; although in Colombia it does not surpass 50%.[8,72] Two-dimensional analysis of natural Blo t 5 shows that it is composed by different isoforms[73] whose differences in geographic distribution impact on allergenic activity have not been sufficiently explored. However, the lack of recognition of natural Blo t 5 in Singapore by monoclonal antibodies produced in mice immunized with the American isolated isoform[73] suggests that this issue is relevant. In Colombia, the presence of the Brazilian isolated isoform was confirmed by its identification in a cDNA library built with locally reared mites.[72] Also, Jimenez et al. evaluated a C-terminal truncated recombinant version of Blo t 5, known as BtM, which was reactive in 14 out of 26 *B. tropicalis* allergic and asthmatic patients from Cartagena.[74] This indicates that most of the allergenic potential remains in the C-terminal portion where several linear epitopes have been mapped.[75]

Yi et al. evaluated the allergenic activity of recombinant and native Blo t 5 by skin test responses and histamine release in 38 *B. tropicalis* sensitized patients, finding that both allergens elicit positive responses. Nine patients reacted higher to native Blo t 5, while 11 developed a greater wheal to the recombinant. There was no significant difference in histamine release between native and recombinant protein.[76] In addition, basophil activation tests indicate that rBlo t 5 highly upregulate CD203c expression.[77] Using transgenic mice, Chua et al. generated *in vitro*, under polarizing conditions, Th2 cells specific for a Blo t 5 peptide. Adoptive transfer of these Blo t 5 specific Th2 cells followed by repeated intranasal (i.n.) exposure to recombinant Blo t 5 induced severe allergic asthma. In the second weekly cycle of i.n. challenges, most mice exhibited lack of movement, a reduction in specific airway compliance and a fall in body temperature. This study indicates that the presence of a Th2 cell response to Blo t 5 may have substantial clinical effects on asthma phenotype.[78] In contrast to Der p 5, Blot 5 lacks lipid binding activity, as suggested by *in silico* analysis. Blo t 5 was identified as a monomer,[75,79] but Der p 5 may organize as a dimer, creating a large hydrophobic cavity with potential ligand binding activity.[58] Due to its monomeric state, it is less probable that Blo t 5 behaves as a lipid binding protein; however, this must be experimentally defined.

Blo t 7. *B. tropicalis* has several potential lipid binding proteins, but this has only been confirmed for Blo t 7 and Blo t 13.[10,80] Blo t 7 binds the naturally fluorescent lipid probe cis-parinaric (cPNA) and induces the TLR2-, NF-kB- and MAPK-dependent production of IL-8 and GM-CSF in respiratory epithelial cells. Based on indirect assays, some authors affirm that this inflammatory activity of rBlo t 7 is related to the lipid cargo originated during the protein expression process in the yeast *Pichia pastoris*.[10]

Blo t 8: Two isoforms of Blo t 8 have been isolated from Singapore and Colombia. The 3D structure of the isoform published by the Colombian group has been experimentally defined by Mueller et al. and matches with a typically mu-GST.[81] Sensitization to Blo t 8 has been shown to be more frequent in Colombia than in Singapore.[82] This can be explained by higher helminth exposure in developing countries, an effect observed for the GST of Ascaris.[8] Blo t 8 can induce positive skin test reactions.[83] Also, positive passive cutaneous anaphylaxis tests have been reported with the recombinant molecule.[84]

Blo t 10. Sensitization to the tropomyosin of *B. tropicalis* is about 30% in different settings of the tropics, including Singapore and Colombia. Blo t 10 has also been tested by SPT, finding a similar rate of sensitization (20%) to that reported by ELISA-IgE immunoassay in a Singaporean pediatric population.[85] In Colombia, it was reported that sensitization to Blo t 10 was associated with asthma. If this is a side effect from the highly cross reactive *A. lumbricoides* sensitization should be defined.[86,87]

Blo t 11. By dot blot immunoassay 33 out of 63 (52%) patients allergic to *B. tropicalis* reacted to Blo t 11. Epitope mapping of rBlo t 11 indicated that it has various antigenic regions; however, there is an immunodominant peptide denominated as fD.[88] Although in terms of frequency, the full length Blo t 11 and natural counterpart had similar rates, the intensity of the response with the natural allergen and its capacity to induce a positive result in the skin prick test were higher. Comparative *in vitro* and *in vivo* allergenicity tests and the cross-inhibition studies between the native and recombinant Blo t 11 showed that recombinant fD, but not the rFL-Blo t 11, has comparable IgE reactivity with the native counterpart.[89]

Blo t 12: There are two isoforms with 97% amino acid sequence identity that were isolated in Cartagena, Colombia (Blo t 12.0101)[90] and Singapore (Blo t 12.0102), respectively.[91] IgE reactivity to Blo t 12.0101 ranges from 25 to 50%. Colombian patients showed a higher IgE response to the isoform isolated locally. No homologous allergen in *Dermatophagoides spp*. has been identified. Even in genomic data mining, no genes coding for a group 12 allergen have been found in *Dermatophagoides genus*. Zakzuk et al. tested the pro-inflammatory capacity of Blo t 12 isoforms under different adjuvant conditions. Blo t 12 was confirmed as a chitin binding allergen that induces allergic inflammation when tested alone, with chitin or alum. A neutrophil enriched inflammatory infiltrate was found in exposed mice.[13]

Blo t 13: Among asthmatic patients allergic to *B. tropicalis* in Colombia only 11% showed IgE reactivity to Blo t 13,[4] but a frequency of IgE antibody binding of 53% was found in Cuba.[92] Jimenez found 15% of positive responses to Blo t 13 by skin prick test.[74] It is remarkable that in spite of a low frequency the mean wheal diameters of positive responses were higher than those to Blo t 5.[74] This in agreement with similar observations of this group reporting that Blo t 13 was able to inhibit more than half of IgE antibody binding to the complete extract.[4] Blo t 13 is an example of an allergen that causes important clinical reactions in few sensitized individuals. In summary, as shown in Table 2, not all the IgE antibody binding molecules from this HDM have been tested for other properties related to allergenic activity.

Cockroach allergens

More than 4000 cockroach species are known to live in a wide variety of habitats around the world, but only two are recognized as the main domiciliary species associated with allergic disease: the German (*Blattella germanica*) and American (*Periplaneta americana*) cockroach. The first reports of cockroach sensitization date back to the 1960s when Bernton and Brown showed positive skin tests to cockroach extract in 44% of 755 patients living in New York.[93] Since the 1970s, significant progress in understanding cockroach allergy was possible thanks to the identification of cockroach allergens.[94–97] These studies led to the molecular cloning of the first allergens from groups 1, 2 and 3 in the 1990s.[98–103] The list of cockroach allergens has been extended up to 13 groups currently listed in the official database maintained by the World Health Organization and the International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-Committee (http://www.allergen.org/). The function and structure of cockroach allergens determine exposure, and therefore sensitization. This section describes intrinsic properties of cockroach allergens, grouped according to similar origin or function.

The relative importance of cockroach allergens was originally studied for the only few allergens that were known. Satinover et al. analyzed IgE reactivity to 5 allergens from groups 1, 2, 4, 5 and 7 in a US population, and reported that Bla g 2 and Bla g 5 were immunodominant with IgE sensitization rates (in 118 patients) of 54% and 37%, respectively.[104] In a sub-group of highly allergic

patients (IgE 3.5–100 IU/mL) this prevalence was up to 71% and 58%, respectively. In a study carried out in Taiwan, prevalence of IgE recognition was the highest for Bla g 2 (63%), followed by Bla g 4 (53%), vitellogenin (47%), Bla g 1 and arginine kinase (34%), Bla g 5 and Bla g 7 (31%), and enolase (25%), on an IgE dot-blot immunoassay using recombinant allergens.[105] On the other hand, among 1255 patients diagnosed with allergic diseases from the Czech Republic, studied by ImmunoCAP-ISAC, sensitization to cockroach allergens Bla g 1, Bla g 2, and Bla g 5 was very rare, observed in 0.6% of cases, and to Bla g 7 in 1.5%.[106] Therefore, the IgE prevalence of sensitization varies according to the population, and none of the cockroach allergens reached high levels of IgE reactivity. Recently, an analysis of the IgE reactivity to an expanded set of 8 cockroach allergens in a US population also showed that there were no immunodominant allergens, and that additional allergens were major in a highly allergic population (Bla g 6, Bla g 9 and Bla g 11).[107] A recent component analysis using the same set of eight cockroach allergens revealed significant differences in IgE reactivity associated with asthma and rhinitis in a group of 10-year old children from the URECA cohort. Recognition of more cockroach allergens, with higher allergen specific IgE, was associated with disease.[108] These studies highlight the complexity of cockroach allergy.[109]

Groups 1 and 2

Groups 1 and 2 cockroach allergens are excreted in the feces of the cockroach, from where they become airborne and can induce sensitization after inhalation of particles that carry the allergen. Bla g 1 and Bla g 2 have been used as markers of environmental exposure to cockroach. Although the exact function is unknown, Bla g 1 is secreted in the midgut especially by females and after a blood meal.[110,111] Its primary structure is homologous to: 1) the ANG12 protein secreted by adult female mosquitos after a blood meal, and 2) microvillar membrane-associated proteins from other insects.[112,113] Molecular cloning showed evidence that group 1 cockroach allergens contain ~100 amino acid repeats, that originated from gene duplication.[99] This primary structure has also been found only in other insect species.[114] The basic structural unit of this allergen is formed by two consecutive repeats, each containing a 6-helices fold, which encapsulate a large and hydrophobic cavity that contains lipids such as palmitic, oleic and stearic acids.[115] Therefore, these allergens could have a digestive function associated with lipid transport. In contrast, Bla g 2 is homologous to aspartic proteases, but amino acid substitutions in the area corresponding to the catalytic site make this allergen enzymatically inactive.[116,117] The antigenic structure of Bla g 2 has been analyzed by determining the structures of the allergen in complex with each of two IgG monoclonal antibodies that overlap with IgE, and by performing detailed site-directed mutagenesis analyses of the epitopes.[118,119] A triple epitope mutant was produced that folds like the wild type allergen and has T-cell modulatory capacity, supporting it as a potential candidate for immunotherapy.[120] The role of a surface carbohydrate moiety present in Bla g 2 has been investigated, and results from crystallographic and site-directed mutagenesis analyses revealed a positive contribution of a carbohydrate in antibody binding.[119] Using glycomic studies, Bla g 2 was shown to contain N-glycans that are complex hybrid-types that are terminated with mannose-, galactose, and/or N-acetyl glucosamine.[121] In keeping with previous observations, deglycosylated Bla g 2 showed significant reduction in binding to IgE from sera of cockroach allergic patients, and induced significantly less histamine release from basophils, compared to glycosylated Bla g 2. These data indicate that N-glycan may be a critical component involved in IgE antibody binding to Bla g 2.[121]

Bla g 1 and Bla g 2 levels have been measured to assess: a) exposure to cockroach in homes of patients with asthma, and b) outcomes of interventions to decrease cockroach infestation in the homes.[122] Clinical trials in children with asthma revealed a link between reduction in cockroach exposure and improvement in asthma morbidity. However, clinical benefit has been observed only with reductions $\geq$50–90% in cockroach allergen concentrations (reviewed by Ahluwalia & Matsui).[123] In a study from Taiwan, in a mouse model of asthma, oral treatment with

Per a 2 expressed in Chinese cabbage, from an hypoallergenic partial clone Per a 2–372, blunt the development of allergen-specific IgE and IL-4 mRNA expression after challenge with intranasal Per a 2, and had preventive effects on airway hyperresponsiveness and inflammation.[124]

Group 3 and 4

These are hexameric proteins homologous to arylphorins or insect storage proteins, insect hormone-suppressible proteins and arthropod hemocyanins.[103,125,126] Several isoallergens and isoforms (variants) have been described for Per a 3, that have a wide range of skin test reactivity (26–95%).[127] Cockroach allergens from group 4 are lipocalins that are involved in reproduction.[128] They are expressed only in males and transport small ligands that might act as insect pheromones.[129] Bla g 4 was shown to be transferred to females in the spermatophore during copulation.[130]

Groups 5, 9, 10, 11 and 12

Allergens belonging to these four groups are enzymes: glutathione *S*-transferases (GST) (group 5), arginine kinases (group 9), serine proteases (group 10), α-amylases (group 11) and chitinases (group 12).[131–136] Some of these allergens are excreted from the gut (i.e. serine proteases, α-amylase and chitinases are digestive enzymes), whereas others are released from the body after death of the cockroach. Comparative analysis of the structure and IgE reactivity of Bla g 5 with other GST (mite Der p 8 and Blo t 8 and *Ascaris* Asc s 13) revealed that despite sharing a similar molecular fold, large differences at the level of the molecular surface existed, which reflected on lack of significant cross-reactivity among the four GST homologs. The antigenically distinct nature of these allergens suggests that each GST should be used as a different antigen for diagnosis.[81]

The effects of exposure to proteolytically active Per a 10 on a human derived airway epithelial cell line cultured at air liquid interface (Calu-3 cells) was investigated, and compared to effects of recombinant Per a 10 and heat inactivated natural Per a 10 which lacks proteolytic activity.[137] The results demonstrated that only proteolytically active Per a 10 increased epithelial permeability by disruption of tight junction proteins, ZO-1 and occludin, enhanced the migration of dendritic cell precursors towards epithelial layer, and induced secretion of IL-33 and TSLP. It was also demonstrated that Per a 10 was able to cleave CD23 and CD25 from PBMCs and purified B and T cells from healthy individuals, which might favour IgE production and Th2 responses.[137] Per a 10 has also been shown to activate airway epithelial cells to secrete IL-6, IL-8 and GMCSF in an activity dependent manner via PAR-2 receptors.[138] PAR-2 activation induced by Per a 10 in murine bone marrow derived dendritic cells resulted in downstream activation of STAT3 to regulate the balance between IL-12/IL-23 subunits, causing a cytokine milieu rich in IL-23 to favour Th2 polarization.[139] These data indicated that protease activity of Per a 10 plays a significant role in initiating and promoting allergic airway inflammation.

In India, the serine protease allergen Per a 10 has been identified as a major allergen, inducing positive reactions on intradermal skin tests in 37/45 (82.2%) of cockroach allergic patients with asthma.[134] Purified natural Per a 10 was also shown to bind IgE on ELISA and immunoblotting in >80% of patients, and it could induce significant histamine release in blood and production of IL-4 and IL-5 in culture supernatant of PBMCs from cockroach sensitized patients. Recombinant Per a 10 showed IgE reactivity on ELISA and immunoblotting and induced histamine release from basophils; however, it did not show proteolytic activity.[140]

A study from China reported that purified Per a 11 and Per a 12 midgut cockroach allergens induced positive skin prick tests in 12 (80%) and 9 (60%) of 15 cockroach-allergic patients, respectively.[136] These allergens were also capable of binding IgE on immunoblotting, and to cause histamine release from basophils in cockroach allergic patients.[136]

Animal models have been used to evaluate the efficacy of component-based cockroach allergen vaccines. A model of airway allergic disease was generated by sensitization of mice with cockroach extract and challenge with purified Per a 10.[141] Within this model, an immunotherapy protocol was developed wherein mice were treated with Per a 10-derived T cell peptide. Treatment with peptides T-P8 and T-P10 reduced recruitment of

inflammatory cells to airways, lowered specific IgE, caused induction of IgG2a antibodies, immune deviation towards a Th1 cytokine milieu, suppression of Th2 cytokines in BALF, resolution of lung inflammation and induction of T regulatory cell mediated peripheral tolerance.[141]

Another study in mice investigated the therapeutic effects of an intranasal cockroach allergy vaccine made of purified natural Per a 9 (arginine kinase, AK), entrapped in liposome.[142] After provocation with cockroach extract, mice treated with liposome-entrapped purified natural Per a 7 showed attenuated airway inflammation and a shift of allergic Th2 to Th1 and Treg responses.[142] As a development of this strategy, mice treated intranasally with a liposome-entrapped vaccine made of mouse Tregitope289-Per a 9 and Tregitope167-Per a 9, exhibited reduced Th2 responses, decreased lung inflammation and low respiratory tissue remodeling, and increased expression of immunosuppressive cytokine genes (IL-10, TGF-β, and IL-35). These results indicate that vaccines constructed with major allergens linked with Tregitopes are effective for reducing allergen-mediated respiratory tissue inflammation and remodeling, via generation of regulatory lymphocytes.[143]

Groups 6, 7, 8 and 13

Allergens from these groups are associated with muscle contraction and include troponin C (group 6), tropomyosin (group 7) and myosin light chain (group 8).[144–146] A new cockroach allergen group 13 has been recently been reported by Dr. Ji-Fu Wei in China (unpublished) and listed in the official WHO/IUIS allergen database. This group contains glyceraldehyde-3-phosphate dehydrogenases, enzymes that are known to be in the muscle of the cockroach since 1962.[147] The availability of these allergens in the environment requires death of the cockroach and release from muscle into the environment.

In Brazil, skin tests with a panel of 5 recombinant allergens (Per a 1, Per a 7, Bla g 2, Bla g 4 and Bla g 5) revealed that 24/57 (42%) cockroach allergic patients with asthma and/or rhinitis had positive IgE binding to Per a 7, but showed a surprisingly remarkable low reactivity to the other allergens tested (≤7%).[148] The high prevalence of IgE reactivity to Per a 7 (cockroach tropomyosin) in Brazil could reflect cross-reactivity to tropomyosins derived from other invertebrates including mites and intestinal parasites, particularly *A. lumbricoides*.[149]

Fungal allergens

It is considered that fungi are the third most frequent cause of respiratory diseases after pollens and mites, being patients allergic to fungi those with more severe asthma and a worse prognosis.[150–152] The first case of fungal asthma was described in 1726, when asthmatic symptoms were observed in a patient after visiting a winery.[153] Since then, several authors have linked fungi with respiratory allergic symptoms and due to its prevalence, fungal allergens may also be responsible for occupational diseases, such as contact dermatitis and occupational asthma, which may affect farmers, gardeners, foresters and other professions.[154] The role of fungi in the development of allergic diseases has not been fully evaluated. The main reason why fungi have not always been considered as a possible causative agent of respiratory allergy, including asthma, is that fungal allergens can be found in the atmosphere throughout the year in both the outdoor and the indoor environment. While the link between outdoor fungi exposure and allergic symptoms is clear, there is limited documented evidence suggesting a relationship between cultivable fungi levels in homes and asthma.[155] However, evidence suggests that fungi levels in household dust could increase asthma symptoms in children.[156,157] Epidemiological studies support that IgE sensitization to fungal genera such as, *Cladosporium*, *Alternaria*, *Aspergillus* and *Penicillium* has been linked to an increased risk of developing asthmatic respiratory symptoms[158] and asthma exacerbations.[159]

Approximately 80 genera of all the species of pathogenic fungi described so far produce Type I hypersensitivity, which usually manifests as allergic rhinitis, rhinosinusitis, allergic asthma or atopic dermatitis.[160] The most important allergy-inducing fungi belong to the Phylum Ascomycota: *Alternaria*, *Cladosporium*, *Aspergillus*, *Penicillium*, *Curvularia*, *Fusarium*, *Stachybotrys*, *Candida*, *Epicoccum,* and *Trichophyton*; to the Phylum Basidiomycota: *Malassezia*, *Coprinus*, *Schizophyllum*, *Psilocibe,* and *Rhodotula*; and to the

Phylum Zygomycota: *Rhizopus*.[161-163] A summary of the purified allergens in clinically relevant sources is reviewed in Fukutomi and Taniguchi.[164]

According to the WHO/IUIS allergen database, 112 allergenic proteins have been identified in 30 species of fungi (http://www.allergen.org/index.php, accessed August 2019). In the AllFam database there are reported 123 fungi allergens included in 42 allergenic protein families (http://www.meduniwien.ac.at/allfam/, August 2019). Fungal allergens can be classified into different groups, according to their biological function (Supplementary Table 2).

The molecular mechanisms involved in allergic responses upon fungal exposure are not clear. Recent studies support that in addition to Type I hypersensitivity reactions mediated by IgE, fungal allergens promote airway inflammation by several mechanisms including: disruption and irritation of mucosal surfaces; direct effects on the extracellular matrix (ECM) structure and the airway smooth muscle; and interactions with innate immune sensors and subsequent induction of proinflammatory cytokines.[165-168] However, most of these effects have been studied using fungi extracts; therefore, in this section we will describe the general effect of the extracts and those specific for individual components when available.

Alternaria alternata

Alternaria-derived serine protease activity induced a rapid IL-33 secretion via protease activated receptor-2 (PAR-2) and adenosine triphosphate signaling.[169] This effect involves initial ATP release as a cellular damage signal and the purinoceptor-dependent activation of DUOX1, an epithelial NADPH oxidase;[170] explaining the typical and prominent IL-33 release upon *Alternaria* exposure and the severe exacerbations induced by fungi.[169] The increases in IL-5 and IL-13 that follow IL-33[171] upregulation by *Alternaria* are independent of adaptive immunity, and regarded as an innate mechanism mediated by group 2 innate lymphoid cells (ILC2).[172,173] *A. alternata* also activates autophagy in airway epithelial cells and induces them to secrete IL-18.[174] Increased expression of cytokines (TSLP) and chemokines (CCL2) has been also reported upon exposure of lung epithelial cells to the extract of *A. alternata* via activation of Toll Like Receptor 2 (TLR-2) signaling.[175] Single intranasal exposure to *Alternaria* induced rapid production of cysteinyl leukotrienes that activate ILC2s through CysLT1R by an IL-33 independent mechanism.[176] Moreover, eosinophils isolated from the lungs of *Alternaria* challenged mice are cytokine enriched and can survive *ex vivo* in the absence of exogenous cytokine support.[177]

Alt a 1. Most of the inflammatory effects of *Alternaria* have been observed using extracts that contain a complex mixture of proteins, mycotoxins, cell wall fragments, chitin, mannans, and β-1,3-glucans, which altogether make it very challenging to define the specific components underlying the proinflammatory effects. Nevertheless, recent studies using the purified Alt a 1, have revealed that this allergen has potent innate immunostimulatory activities in bronchial epithelial cells. Alt a 1 in its tetrameric form interacts with the solute carrier family 22 member 17 (SLC22A17) receptor on the airway epithelium, undergoes receptor-mediated endocytosis and without evident alterations of the epithelial integrity, induces the expression of proinflammatory cytokines such as IL-8, IL-33 and IL-25.[178] Moreover, Alt a 1 has been found to induce several cytokines and chemokines in bronchial epithelial cells, including CXCL1, CXCL2, CXCL3, IL-8 and CCL2 and this effect was dependent and mediated by pattern recognition receptors and adaptor molecules such as TLR2, TLR4, MyD88, and TIRAP.[179] Alt a 1, either in its natural or recombinant form is a reliable tool for the diagnosis of *A. alternata* sensitization and induces skin prick reactivity comparable with that produced by the *A. alternata* extract.[180] In addition, histamine release has also been tested and reported for Alt a 1.[181] Its clinical impact is best exemplified by the results of the allergen specific immunotherapy with the purified Alt a 1. This is a 30 kDa glycoprotein composed of two protein subunits (15.3 kDa and 16.4 kDa), joined by disulfide bridges, and a 20% of carbohydrates. Despite Alt a 1 is the main allergen of *A. alternata* and the most studied mold allergen so far, its biological function is still unknown; although it is believed that it could be involved in the germination of spores and plant infection ion.[182,183] Up to 98% of IgE sensitization to *A. alternata* can be detected towards Alt a 1.[184] Fig. 1 shows the IgE antibody

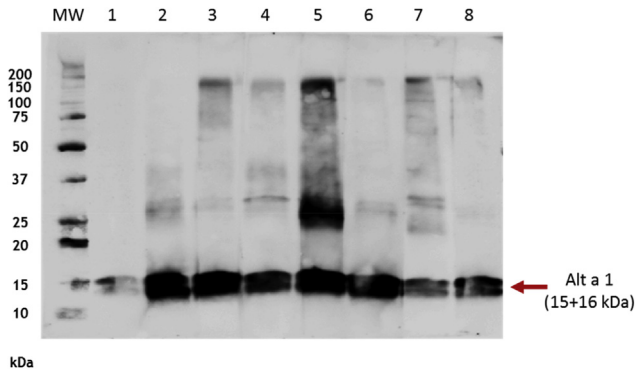

Fig. 1 The immunoblotting shows the IgE antibody binding profile obtained using sera form 8 Spanish patients with allergy to *A. alternata*. All patients show IgE antibody binding to the Alt a 1 allergen with high intensity

binding profile obtained using sera form 8 Spanish patients with allergy to *A. alternata*. All patients showed IgE antibody binding to the Alt a 1 allergen with high intensity.

There are two clinical studies using purified Alt a 1 in immunotherapy. The first clinical trial was published in 2010 and demonstrated the safety of the treatment in patients with rhinitis and asthma.[185] The second study published in 2019 was a multicenter, randomized, double blind, placebo-controlled trial that demonstrated the efficacy and safety of this treatment. The study demonstrated a reduction in symptoms and medication scores, reduced skin test reactivity, a decrease in specific IgE as well as an increase in IgG_4 levels after one year of treatment.[186] The recombinant mannitol dehydrogenases from *A. alternaria* (Alt a 8) and *Cladosporium herbarum* (Cla h 8) also induce positive skin tests in sensitized subjects.[187,188]

Aspergillus fumigatus

After inhalational challenge with *A. fumigatus* there is upregulation of HYAL1 and HYAL2, two hyaluronidases that degrade hyaluronic acid to its pro-inflammatory form of low molecular weight which is known to promote eosinophilia and is co-localized with the areas of pulmonary inflammation and collagen deposition.[189] Exposure to *Aspergillus versicolor* induced a strong Th17 response with IL-17A, IL-17F and IL-22 promoting severe asthma and steroid resistance. This effect is independent of fungal sensitization and characterized by a mixed Th2/Th17 response.[190] Recent studies suggest that fungal exposure may regulate TLR signaling and macrophage polarization by affecting microRNA levels.[191] For instance, inhalation of *A. fumigatus* induces the expression of mir-132 in human monocytes and dendritic cells and downregulates the expression of microRNAs that regulate the expression of dectin-1 and mannose receptor.[192]

The clinical relevance of individual allergens is very well exemplified by the role of purified fungal allergens in the diagnosis of allergic broncho-pulmonary aspergillosis (ABPA),[193–195] in which IgE reactivity to purified *Aspergillus* allergens such as rAsp f 2, rAsp f 4 and rAsp f 6 was significantly more frequent in the sera of patients with clinically relevant ABPA compared with asthmatics or healthy controls.[196] Indeed, the secreted ribotoxins rAsp f 1 and rAsp f 3 are recognized by serum IgE of *A. fumigatus*-sensitized asthmatics with or without ABPA, whereas the non-secreted manganese superoxide dismutase rAsp f 6 and the rAsp f 4 are exclusively recognized by serum IgE of ABPA patients. The dissection of IgE-mediated immune responses to single recombinant *A. fumigatus* allergens in asthmatic patients allows a discrimination between ABPA and *A. fumigatus* sensitization with high specificity (100%) and sensitivity (90%).[197] Since mold extracts are difficult to standardize and there is a significant level of allergenic cross-reactivity among diverse fungal sources, component resolved diagnosis holds a great value as diagnostic tool and the alternative to replace extract-based tests. The rAsp f 1 also elicits positive immediate type I skin reactions in individuals allergic to *A. fumigatus* but not in healthy control individuals.[198] Moreover, the recombinant *A. fumigatus* allergens rAsp f 4, a protein with unknown biologic function, and rAsp f 6 (manganese superoxide dismutase) can provoke immediate skin reactions exclusively in patients with ABPA. The reactions, which are elicited by a few nanograms of the allergens, strictly depend on the presence of allergen-specific serum IgE.[199]

Purified *Aspergillus* allergens (*e.g.* Asp f 1) also induce basophil activation,[200] and the release of sulfidoleukotrienes by basophils upon stimulation as reported for rAsp f 4 and rAsp f 6.[201] Thereby the basophil activating activities of these allergens are being considered as reliable biomarkers of allergic bronchopulmonary

aspergillosis.[200,202] Regarding animal models, Asp f 5 and Asp f 13, two purified proteases from *A. fumigatus*, induced the recruitment of inflammatory cells and airway remodeling in a murine model.[203] Also, a reproducible mouse model of Asp f 1 sensitization has been established.[204] Mice exposed to Asp f 1, Asp f 3, and Asp f 4 also showed inflammatory changes in the lungs and airway hyperreactivity. The immune responses elicited, including elevated serum IgE, enhanced eosinophils, recruitment in the peripheral blood and lungs, and expression of regulatory cytokines are characteristic of a Th2 response.

Interestingly, Asp f 6 has demonstrated only a reduced response in these animals, suggesting that individual responses to fungal allergens vary considerably with different purified antigens.[205] Indeed, Asp f 13 induced stronger peribronchiolar inflammation, higher IgE levels and higher eosinophilia compared to mice immunized and challenged with Asp f 2, a "major" allergen from *A. fumigatus* that albeit having strong IgE antibody binding capacity, only produced a minimal degree of inflammation and immune response when administered alone. In contrast, a mice model of Asp f 2 sensitization revealed that high-affinity IgG antibodies are also produced after exposure and have the ability to retard allergic responses upon lung challenge.[206] Overall, these results suggest that immunopathology induced by the crude *A. fumigatus* extract results from the cumulative effects of its allergens.[207]

Penicillium spp.

Two other fungal alkaline serine proteases have been purified in *Penicillium citrinum* (Pen c 13) and *Penicillium chrysogenum* (Pen ch 13). The latter with demonstrated ability to induce histamine release from basophils,[208] degradation of occluding, CD44 cleavage in bronchial epithelial cells and production of prostaglandin-E2, IL-8 and transforming growth factor (TGF)-beta1.[209,210] The homologous Pen c 13 also led to increased airway hyperresponsiveness, significant inflammatory cell infiltration, mucus overproduction, collagen deposition in mice lungs; dramatically elevated serum total and specific IgE and IgG1 levels, as well as increased production of IL-4, IL-5, and IL-13 by splenocytes stimulated *in vitro*. Pen c 13 exposure also causes junctional structure alterations and actin cytoskeletal rearrangements, resulting in increased permeability and airway structural changes.[211]

Malassezia spp.

Malassezia extracts induce mediator release from mast cells by IgE-mediated and non-IgE mediated pathways,[212] and although many more studies are needed to understand the underlying pathogenic mechanisms,[213] the significant association between IgE sensitization to purified *Malassezia* allergens and the severity of atopic dermatitis/eczema it is quite clear from case control studies as described for Mala s 7[214] and other allergens.[215,216]

Mala s 13. This allergen induces IL-1β and IL-23 production in monocyte derived dendritic cells upon direct interaction with Dectin-1 and Dectin-2 which in turn results in a Th2/Th17 polarizing milieu.[217] Cross-reactivity between fungal and human proteins (for instance Mala s 13 with human thioredoxin, or Mala s 11 with human manganese superoxide dismutase) may also mediate IgE and T cell cross reactivity leading to skin inflammation.[218,219] However, dendritic cells can differentiate fungal allergens from human homologs. For instance, whereas human manganese superoxide dismutase did not affect the phenotype of monocyte derived dendritic cells, rMala s 11 up-regulated the maturation marker CD83, the co-stimulatory molecules CD40, CD80, CD86 and HLA-DR to a similar extent as lipopolysaccharide, and induced high levels of TNF-alpha, IL-6, IL-8, IL-10 and IL-12p70.[220]

Mal f 1. This allergen also induces a hitherto unknown dendritic cell activation profile whereby TNF-alpha, IL-6, and IL-10 are significantly increased and IL-12 is not affected, suggesting that rMal f 1 can predispose a dendritic cell bias toward the Th22/Th17 pathway beyond the IgE-dependent Th2 pathway.[221]

Group 8 allergens. In vitro tests for measuring mediator release have shown that MGL_1304 produced by *Malassezia globosa* is the major histamine releasing antigen from this source. Indeed, MGL_1304 and Mala r 8 showed higher histamine release activity in basophils from patients with

atopic dermatitis compared to that induced by Mala s 8. It is an excellent example of how an allergen with low IgE antibody binding frequency might display potent *in vivo* effects in sensitized subjects.[222,223]

The sequences of an increasing number of fungal allergens are now available, mainly from the most important allergenic species; as well as the knowledge about IgE and T-cell epitopes that will help in developing new hypoallergenic derivates and more effective means to treat and diagnose mold allergies. However, as presented in Table 3 very few mold allergens are being fully evaluated to elucidate their allergenic activity and clinical relevance beyond IgE antibody binding frequencies. Therefore, many more mechanistical studies are urgently needed.

Animal derived allergens

In this section we provide an overview of animal-derived allergen molecules which have been officially recognized by the WHO/IUIS Allergen Nomenclature Sub-Committee (http://www.allergen.org/). We have grouped the allergen molecules according to common allergen sources, among them cat, dog, horse, cow, mouse, rat, guinea pig, hamster and rabbit. Allergic patients may exhibit symptoms either upon direct exposure to these animals, for instance by animal ownership or by occupational activity. In addition, indirect exposure to animal allergens may also occur via indoor environment in public buildings, where dust settle and allergens accumulate or by contact with persons carrying allergens in their clothes.[224]

Cats and dogs are the most favored pets in households (http://www.gfk.com/global-studies/global-studiespet-ownership/). In certain countries such as in Russia and the Scandinavian countries cat allergy is extremely frequent, affecting up to 20% of the population.[225,226] As indicated above, allergen exposure does not depend directly on presence of pets. Cat as well as dog allergens were found even in households without dogs, in day care centers, schools, public buildings and public transportation.[224] Moreover, two recent studies from USA and The Netherlands found higher cat and dog allergen levels in classrooms compared to student's homes, especially those without pets.[227,228] Furthermore, it has been reported that many of the cat sensitized individuals with current asthma do not live in a home with a cat and 48% have no cat or dog in the house. This observation is in agreement with the fact that animal dander allergens are present throughout the community, including schools and homes without a cat.[229]

Cat allergens

Cat allergens occur not only in dander but also in hair, skin, saliva, serum, and urine[230] and sensitization represents a strong risk factor for the development of asthma.[231]

Fel d 1. This major allergen is a member of the secretoglobin protein family and one of the best studied animal allergens.[232,233] It is recognized by more than 90% of cat allergic patients and accounts for 50–90% of the total allergenic activity in cat dander.[231] Due to the high species specificity and prevalence of IgE to Fel d 1 it is considered to be a reliable marker allergen for cat allergy.[234] Grönlund et al. have shown that asthmatic children with cat allergy had higher Fel d 1-specific IgE levels compared to children with rhinoconjunctivitis only, thus elevated IgE antibody levels to Fel d 1 could be a possible risk factor for asthma development in children.[235] Furthermore, in the Bamse birth cohort sensitization to Fel d 1 in childhood was found to be significantly associated with allergic symptoms to cat at age 16 years.[226] The allergenic activity of Fel d 1 has been demonstrated *in vitro* by basophil activation assays,[236] but no *in vivo* provocation tests have been performed with Fel d 1. However, cat allergic patients with Fel d 1-specific IgE showed allergic symptoms when exposed to cat allergens in challenge chambers.[237,238]

Fel d 2 is serum albumin which is abundant in saliva and dander. It is a highly cross-reactive molecule,[239,240] thought to be associated with pork-cat syndrome,[241,242] but only a relatively small number (10–20%) of patients is sensitized to Fel d 2. Sensitization to Fel d 2 may be associated with more severe respiratory symptoms[226,243] and is recognized preferentially by cat-allergic patients suffering from severe manifestations of atopy (e.g., atopic dermatitis, allergic asthma).[244,245]

Allergen	Provocation test *in vivo*	Provocation test *in vitro*	Non-IgE induced inflammation	Case-control study	Respiratory Animal model	PCA animal model	Avoidance	AIT trials
Asp f 2	ST	HR	Yes	ABPA, CF + ABPA	Yes			Mouse model IT
Alt a 1	ST	HR	Yes	Yes				Human IT
Pen c 13	ST	HR	Yes	Asthma/Rhintis	Yes			
Asp f 4	ST	HR	Yes	ABPA, asthma + bronchiectasis, CF + ABPA	Yes			
Asp f 6	ST	HR	Yes	ABPA, CF + ABPA	Yes			
Asp f 1	ST	BAT	Yes	Asthma	Yes			
Asp f 3	ST		Yes	Asthma, asthma + bronchiectasis	Yes			
Mala s 8	ST	HR		Atopic eczema				
Mala s 13	ST		Yes	Atopic eczema				
Asp f 13			Yes		Yes			
Asp f 5			Yes		Yes			
Alt a 8	ST							
Cla h 8	ST							

Table 3. Allergenic activity assays accomplished by some purified fungal allergens (In addition to IgE-binding) *PCA: Passive cutaneous anaphilaxis; AIT: Allergen Immunotherapy; ABPA: Allergic Bronchopulmonary aspergillosis; CF: Cystic fibrosis; ST: Skin test; BAT: Basophil Activation test; HR: Histamine release*

Fel d 3 is a cystatin (i.e., a cysteine protease inhibitor) of approximately 11 kDa which was reported to be recognized by 10%–90% of cat allergic patients in different studies.[246] The cDNA coding for Fel d 3 was isolated by IgE immunoscreening of a skin cDNA library with sera from cat allergic patients. Fel d 3 showed more than 75% sequence homology with bovine and human cystatin but cross-reactivity has not been studied in detail. The allergenic activity and clinical relevance of Fel d 3 has not been investigated so far.

Fel d 4 is a lipocalin-like cat allergen which was identified as IgE-reactive cDNA clone in a submandibular salivary gland cDNA expression library and showed high sequence similarity with the horse allergen Equ c 1.[247] Reports indicate that Fel d 4 is recognized by more than 50% of cat allergic patients and could be a major allergen. The dog allergens Can f 2 and Can f 6 also show structural similarity with Fel d 4 and potential cross-reactivity.[248–250] There is evidence that IgE sensitization to Fel d 4 is linked with respiratory and skin symptoms and Fel d 4 has been shown to induce basophil activation. The IgE level against Fel d 4 has recently been reported to be associated with the blood eosinophil count in young asthmatics. Moreover, co-sensitization to Fel d 1 has been related to current asthma and asthma symptoms.[251]

Fel d 5[252] and **Fel d 6**[253] represent cat IgA and IgM, respectively. These allergens were reported to be recognized by IgE antibodies in 38% of cat allergic patients. The patient's IgE antibodies seemed to be directed against carbohydrate epitopes of these allergens which appear to lack relevance for cat allergy.[254] The carbohydrate epitope has been shown to be galactose-alpha-1,3-galactose, a major IgE antibody binding epitope on cat IgA which is thought to play an important role in mammalian meat allergy.[231,255,256] There are no studies regarding the clinical relevance of IgE sensitization to Fel d 5 and 6 and the allergenic activity of the two molecules has not been studied in detail.

Fel d 7 is a lipocalin-like protein which shares sequence homology and IgE cross-reactivity with the dog allergen Can f 1.[257,258] The allergenic activity of Fel d 7 has been demonstrated in basophil activation experiments and its IgE recognition frequency may be in the range of 30–40% but there are no data regarding the association of IgE reactivity to Fel d 7 and allergic symptoms.

Fel d 8 is a latherin-like protein which seems to show IgE reactivity in approximately 20% of cat allergic patients. There are no data regarding the association of Fel d 8 with symptoms and regarding its allergenic activity.[257]

Dog allergens

Dog dander, fur and skin are traditionally considered sources for allergen extract preparation[259] but approximately one-fifth of patients with symptoms induced by dog lacked IgE reactivity to dander and were IgE positive to saliva.[260] Thus it does not come as a surprise that allergen extracts prepared from dogs, in particular skin prick test solutions used for diagnosis, are highly heterogeneous regarding the contents of the individual dog allergens[261,262] confirming the assumption that, for diagnosis, natural allergen extracts need to be replaced by defined allergen molecules as soon as possible.[263]

Can f 1 is a lipocalin[264] with IgE reactivity prevalence ranging from 49 to 75% depending on the studied population.[234,264,265] IgE sensitization to Can f 1 early in childhood seems to be a predictor for the development of allergy in adolescence and is associated with respiratory and ocular symptoms.[226] Allergenic activity of Can f 1 has been demonstrated in basophil activation experiments and there is cross-reactivity with the cat allergen Fel d 7 and possibly with Can f 2.

Can f 2, like Can f 1, is a saliva-derived lipocalin-like protein which is recognized by IgE antibodies in 10–40% of dog allergic patients depending on the tested populations.[264] Can f 2 may show IgE cross-reactivity with Can f 1 and Fel d 4 which is also a lipocalin-like protein. IgE reactivity with Can f 2 may be associated with respiratory symptoms. Furthermore, IgE sensitization to Can f 2 and Equ c 1 is more common in children with severe asthma than in those with controlled asthma.[266]

Can f 4 is another lipocalin-like protein that shows IgE reactivity with sera from approximately 35% of dog allergic patients and displays IgE

cross-reactivity with a 23 kDa protein present in cow dander extract, which is related to a family of odorant-binding proteins (Supplementary Table 3).[267] IgE sensitization to Can f 4 and Can f 6 was found to be associated with a positive nasal provocation test with dog dander extract.[268]

Can f 5, a prostatic kallikrein-like protein derived from male dogs is a frequently recognized allergen among dog sensitized and dog allergic patients (approx. 70% of patients).[265] However, monosensitization to Can f 5 has recently been shown to lack association with a positive nasal provocation test with dog dander extract.[268] Nonetheless, other studies found a relation between respiratory symptoms and IgE reactivity to Can f 5.[269] In multivariable analysis of specific IgE assessment in sera from the OLIN cohort, Perzanowski et al. demonstrated that IgE to Fel d 1 and Can f 5 were each associated with current asthma.[229] Can f 1, Can f 2 and Can f 5 may be regarded as relatively specific markers for sensitization to dogs but it is suggested to measure IgE reactivity against all three allergens, as neither of them reaches a sensitization rate above 90%. Can f 1 also displays some cross-reactivity with Fel d 7 and it is therefore recommended to exclude sensitization to cat by testing for IgE reactivity to Fel d 1.[270]

Can f 7, a NPC2, lipid-binding protein, was identified in aqueous dog extracts by 2-dimensional polyacrylamide gel electrophoresis followed by IgE immunoblotting. The allergen was expressed as a recombinant protein and found to react with IgE antibodies from 10 to 20% of dog allergic patients. Cross-reactivity with house dust mite allergen Der p 2 was reported.[271] No information regarding the allergenic activity and IgE reactivity associated with symptoms is available.

Horse allergens

In Germany the IgE-sensitization prevalence to horse dander was found to be 4.4% in the KiGGS cohort.[272] The major horse allergen, **Equ c 1**, is a member of the lipocalin family[273] with a high level of IgE cross-reactivity with the cat allergen Fel d 4 and the dog allergen Can f 6.[249,274,275] Sensitization to Equ c 1 has been associated with severe asthma in Swedish children.[266] Equ c 1 was regarded as marker allergen, but this may be reconsidered taking into account its high cross-reactivity with Fel d 4 and Can f 6.[270] In this context it should be mentioned that in an urban atopic population 5% of patients displayed a positive skin prick test to horse dander extract although more than half of the sensitized patients denied ever having contact with horses and therefore may be attributed to cross-reactivity with cat and dog.[276]

Allergens from small furry animals and cow

For rabbit the most potent allergen sources are saliva, fur and urine.[277,278] Several rabbit allergens have been described of which **Ory c 1**, **Ory c 2** and **Ory c 4** are lipocalin-like proteins whereas **Ory c 3** is a secretoglobin.[278,279] **Mus m 1**[280] and **Rat n 1** from mouse and rat respectively, seem to be cross-reactive and have sequence homology with Ory c 4 (51–54%) and Fel d 4 (55% for Rat n 1, 49% for Mus m 1).[270] Ory c 4 probably also cross-reacts with Can f 6 and Equ c 1.[281] Other aspect of allergenic activity of individual allergens from rabbit have not been determined.

The major mouse allergen Mus m 1 and the major rat allergen Rat n 1 belong to the lipocalin family and may cross-react. They may cause severe respiratory symptoms and are important for persons working with small animals. Allergens from different species of hamster seem to be different from each other because it was reported that patients allergic to the Siberian hamster did not react to commercial skin prick test solutions made from the European or Golden hamster. The major allergen, Mes a 1, of the Golden hamster has been identified recently as male-specific submaxillary gland protein and was found to be different from the major allergen of Phodopus hamsters, **Phod s 1**.[282]

Cav p 1, Cav p 2 and **Cav p 3** are guinea pig lipocalins which seem to lack cross-reactivity with cat and dog extracts.[283,284] **Cav p 4** is a cross-reactive serum albumin, **Cav p 6** is a lipocalin with high sequence similarity with Fel d 4, Equ c 1, and Can f 6 and hence may be cross-reactive. For small furry animals Cav p 2, Cav p 3, Ory c 3, Phod s 1 and Mesm1 may be considered as specific marker allergens for sensitization to guinea-pig, rabbit, dwarf and golden hamster, respectively.[270] Other aspects of allergenic activity of

Allergen	Provocation test *in vivo*	Provocation test *in vitro*	Non-IgE induced inflammation	Case-control study	Respiratory Animal model	PCA animal model	Avoidance	AIT trials
Fel d 1	ST	BAT, RBL	Yes	Asthma, Rhinitis, Conjunctivitis	Yes	Yes		
Can f 1	ST	BAT		Asthma, Rhinitis, Conjunctivitis				Yes
Can f 2	ST	BAT		Asthma, Rhinitis				
Can f 3	ST, NPT	BAT		Rhinitis, Asthma, Skin symptoms				
Can f 4	ST, NPT	BAT						
Can f 5	ST, NPT	BAT		Rhinitis, Asthma, Rhinoconjunctivitis				
Can f 6	ST	BAT						
Mus m 1	NPT, ST	BAT	Yes	Asthma				
Bos d 2	NPT, ST		Yes	Asthma				
Equ c 1		BAT		Asthma, Rhinitis				
Ory c 3		BAT		Asthma, Rhinitis, Conjunctivitis				
Rat n 1		BAT		Asthma				
Cav p 2		BAT, RBL						
Cav p 3		BAT, RBL						

Table 4. Allergenic activity assays accomplished by some purified pets allergens (In addition to IgE-binding). *PCA: Passive cutaneous anaphylaxis; AIT: Allergen Immunotherapy; ST: Skin test; NPT: Nasal provocation test; BAT: Basophil Activation test; RBL: Rat Basophil Leukemia test*

individual allergens from guinea-pig have not been determined.

Bos d 2, a cow lipocalin, is considered as major respiratory allergen from cow[285] and has been tested for allergen activity by nasal provocation and skin tests.

In summary, the allergenic activity and clinical relevance have so far only been studied for few animal allergens, mainly for cat and dog allergens (Table 4). Identification of the clinically relevant animal allergens will be an important step towards the engineering of innovative molecular allergy vaccines.

CONCLUSIONS

Despite the large number of discovered and registered allergens, we know little about the allergenic activity and clinical importance for most of them. In fact, the current field of allergology is based on the criterium that the clinical importance of the allergenic molecules is determined by the frequency and intensity of their IgE antibody binding. This is a rather limited parameter considering the development of experimental allergology in the last 20 years and the criteria that support personalized medicine. This review confirms that allergenic activity of most indoor IgE antibody binding components has not been investigated; however, during the last years important advances have been made in the field. If we consider those allergens with positive results from provocation tests, case-control studies and animal models of allergic inflammation, and having demonstrated innate or adaptive inflammatory mechanisms, we can say that at least the following have demonstrated allergenic activity: Der p 1, Der p 2, Der p 5 and Blo t 5 from HDMs; Per a 10 from *Periplaneta americana*; Asp f 1, Asp f 2, Asp f 3, Asp f 4 and Asp f 6 from *Aspergillus fumigatus*; Mala s 8 and Mala s 13 from *Malassezia sympodialis*; Alt a 1 from *Alternaria alternata*; Pen c 13 from *Penicillium chrysogenum*; Fel d 1 from cats; Can f 1, Can f 2, Can f 3, Can f 4 and Can f 5 from dogs; Mus m 1 from mice and Bos d 2 from cows.

The reasons why there are so few well characterized allergens are diverse, including difficulties with *in vitro* tests for allergenic activity such as basophil activation, case control studies; as well as restrictions for performing *in vivo* tests with recombinant molecules. An additional important reason is the traditional way of thinking, just around major and minor allergens, based only on the frequency of IgE antibody binding. This limited perspective, that excludes other mechanisms of allergenicity, has restricted the scope of allergy research for a long time. One of the objectives of this review was to support, based on an extensive literature review, the idea that the characterization, in terms of clinical impact, of an allergen, just starts with detecting IgE antibody binding properties. There are more properties that can influence the whole spectrum of allergenic activity, and these properties will certainly be important for selecting individual allergens for component-based immunotherapy and preventive vaccines.

Consent for publication

All the authors agree with the publication of this manuscript.

Ethics approval

It does not apply.

Declaration of competing Interest

The authors declare that there are not competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.waojou.2020.100118.

Author details

[a]Institute for Immunological Research, University of Cartagena, Cartagena, Colombia. [b]Division of Immunopathology, Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria. [c]NRC Institute of Immunology FMBA of Russia,

Moscow, Russian Federation. [d]Indoor Biotechnologies, Inc. Charlottesville, VA, USA. [e]Inmunotek, Madrid, Spain and University of South Florida College of Medicine, Tampa, USA. [f]Allergy and Clinical Immunology Department, Centro Médico Docente La Trinidad, Caracas, Venezuela. [g]Department of Allergy & Immunology Hospital Quironsalud Bizkaia, Bilbao, Spain. [h]Department of Otolaryngology Head and Neck Surgery, Beijing Tongren Hospital, Capital Medical University, Beijing, 100730, China. [i]Department of Medicine Solna, Division of Immunology and Allergy, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden. [j]Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, SP, Brazil. [k]Department of Clinical Neuroscience, Karolinska Institute, Stockholm, Sweden. [l]Department of Clinical Immunology and Allergy, Laboratory of Immunopathology, Sechenov First Moscow State Medical University, Moscow, Russia.

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