

Component-resolved diagnosis of house-dust mite allergy with purified natural and recombinant mite allergens

G. Pittner*, S. Vrtala*, W. R. Thomas†, M. Weghofer*, M. Kundi‡, F. Horak§, D. Kraft* and R. Valenta*

*Division of Immunopathology, Department of Pathophysiology, Vienna General Hospital, Medical University of Vienna, Austria, †Centre for Child Health Research, University of Western Australia, Telethon Institute for Child Health Research, Australia, ‡Department for Occupational and Social Hygiene, Institute of Environmental Health, Medical University of Vienna, Austria and §Department of ENT, Medical Vienna General Hospital, University of Vienna, Austria

Summary

Background Mites belong to the most frequent and potent allergen sources. Immunotherapy with mite allergen extracts is frequently performed if allergen avoidance is not possible or successful. However, highly controversial results have been reported for mite-specific immunotherapy.

Objective The aim of this study was to develop diagnostic concepts that may contribute to an improved selection of patients for immunotherapy with Der p allergen extracts and that may be used for immunological monitoring of patients undergoing this treatment.

Methods The IgE reactivity profiles to Der p extract were determined in a Middle European mite-allergic population by IgE immunoblotting and by using a panel of seven purified natural or recombinant Der p allergens (nDer p 1, nDer p 4, rDer p 2, rDer p 5, rDer p 7, rDer p 8, rDer p 10). Furthermore, we investigated the sensitization and cross-reactivity to house-dust- and storage-mite allergen extracts by CAP FEIA measurements and by IgE competition studies.

Results More than 95% of the patients could be diagnosed with a combination of nDer p 1 and rDer p 2. With the methods used, we could discriminate mite-allergic patients who were mainly sensitized to the major Der p allergens (Der p 1, Der p 2) from patients with a broad sensitization profile, including highly cross-reactive allergens (e.g. Der p 10: tropomyosin) as well as reactivity to storage mites.

Conclusions Diagnostic tests containing the major mite allergens (i.e. Der p 1, Der p 2) and highly cross-reactive mite allergens (e.g. Der p 10) may improve the diagnostic selection of patients for immunotherapy with Der p extracts. These tests may also be used for the immunological monitoring of patients undergoing immunotherapy.

Keywords cross-reactivity, diagnosis, house-dust mites, IgE reactivity profile, immunotherapy, natural and recombinant allergens

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Introduction

House-dust mites (HDMs) represent one of the most important allergen sources worldwide [1]. In temperate climates, the pyroglyphid mites *Dermatophagoides pteronyssinus* (originally known as European HDM) and *Dermatophagoides farinae* (American HDM) predominate [2] but storage mites may also be important in certain geographical locations [3]. More than 50% of allergic patients and up to 80% of asthmatic children are sensitized to mite allergens [4, 5]. When measures of allergen avoidance are not successful, symptomatic pharmacological treatment or allergen-specific immunotherapy may be considered for the treatment of allergy to HDM [5]. Allergen-specific immunotherapy represents the

only causative approach for allergy treatment, but highly conflicting results have been obtained for HDM allergy vaccination. On the one hand, it has been reported that immunotherapy with HDM allergen extracts is effective for the treatment of allergic rhinitis and asthma [6]. In addition, it was found that HDM-specific immunotherapy results in decreased bronchial hyper-reactivity [7]. Moreover, it has been described that HDM-specific immunotherapy performed in early childhood may prevent the onset of allergic sensitizations to other allergen sources [8, 9].

On the other hand, it has been demonstrated that HDM-specific immunotherapy seems less efficient than immunotherapy with pollen allergens and is associated with a high rate of side-effects [10, 11]. Furthermore, it may have a harmful effect in children suffering from combined snail and mite allergy [12] and can induce food allergy to invertebrates [13]. The latter observation has been attributed to the induction of IgE responses against tropomyosin, a highly cross-reactive allergen occurring in mites (i.e. Der p 10) and in a variety of sea foods (e.g. shrimps) [13, 14].

Correspondence: Susanne Vrtala, PhD, Division of Immunopathology, Department of Pathophysiology, Vienna General Hospital, Medical University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria.
E-mail: Susanne.vrtala@akh-wien.ac.at

In this study, we have analysed the IgE reactivity profiles of mite-allergic patients to immunoblotted HDM (Der p) allergens. Furthermore, we used a panel of seven purified natural and recombinant Der p allergens to establish the molecular sensitization profiles of these patients. In addition, IgE reactivity and cross-reactivity between HDM and storage mite allergens were analysed. More than 95% of the mite-allergic patients could be diagnosed with a combination of purified major HDM allergens (nDer p 1, rDer p 2). Perhaps more important, component-resolved diagnosis (CRD) with purified HDM allergens allowed us to discriminate patients who were mainly sensitized to the two major HDM allergens from others who exhibited reactivities also to highly cross-reactive allergens (e.g. Der p 10) and to storage mites. Since most allergen extracts used for mite-specific immunotherapy are mainly standardized with respect to their major allergens (i.e. Der p 1, Der p 2) [15], we suggest the use of diagnostic tests based on purified major and cross-reactive allergens for the refined selection of patients for mite-specific immunotherapy and for the immunological monitoring of this treatment.

Methods

Patients

The study population comprised 208 mite-allergic individuals (107 male and 101 female patients; age: 5–79 years; median age: 28.3 years). The diagnosis of allergy was based on case history and skin prick test results. Serum IgE specific for Der p extract was determined by CAP FEIA measurements (Pharmacia, Uppsala, Sweden). The total IgE levels were determined in all patients using the Pharmacia CAP system (Pharmacia).

Mite extracts and recombinant mite allergens

House-dust and storage mites (*D. pteronyssinus*, *D. farinae*, *Glycyphagus domesticus*, *Lepidoglyphus destructor*, *Tyrophagus putrescentiae*) were purchased from Allergon (Vällinge, Sweden). Extracts were prepared by aqueous extraction using protease inhibitors as described previously [16]. Protein content and quality of the lyophilized extracts were analysed by reducing SDS-PAGE and Coomassie Brilliant Blue (BioRad, Richmond, CA, USA) staining [17, 18]. In addition, the protein content of extracts was determined by the BCA assay (Pierce, Rockford, IL, USA), using bovine serum albumin (BSA) as a standard.

Natural Der p 1 was affinity purified from a Der p extract using the monoclonal antibody 4C1, and Der p 4 was purified by cyclodextrin affinity chromatography [19, 20]. The recombinant mite allergens rDer p 5, rDer p 7, rDer p 8 and rDer p 10 were expressed in the vector pGEX-2T as glutathione-S-transferase fusion proteins [21]. rDer p 2 was expressed in the vector pET 19b with an N-terminal hexahistidine tag [22].

IgE immunoblots

Mite extracts (approximately 50 µg/cm of gel) were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany) [23]. Membranes

were blocked two times for 5 min and once for 30 min with buffer A (50 mmol/L sodium phosphate [pH 7.5], 0.5% v/v Tween-20, 0.5% w/v BSA, and 0.05% w/v NaN₃) and were then exposed to 1 : 10 (in buffer A) diluted patients' sera, or, for control purposes, to serum from a non-allergic individual. Bound IgE antibodies were detected with ¹²⁵I-labelled anti-human IgE antibodies (RAST; Pharmacia) as described [24].

ELISA measurements

ELISA plates (Greiner, Austria) were coated with 100 µL (5 µg/mL in phosphate-buffered saline (PBS)) of each purified allergen (nDer p 1, nDer p 4, rDer p 2, rDer p 5, rDer p 7, rDer p 8, rDer p 10) and, for control purposes, with glutathione S-transferase. The plates were then washed two times with buffer B (PBS, 0.05% v/v Tween 20) and blocked with buffer B containing 1% w/v BSA for 3 h at room temperature. Afterwards, the plates were incubated with 100 µL/well of 1 : 10 (in buffer B containing 0.5% w/v BSA) diluted patients' sera overnight at 4 °C. Bound IgE antibodies were detected with an alkaline phosphatase-coupled mouse monoclonal anti-human IgE antibody (PharMingen, San Diego, CA, USA) as described [25]. GST reactivity was measured on the same plate and subtracted to create a baseline for non-reactivity.

IgE dot blot inhibition experiments

Two microlitre aliquots of extracts from *D. pteronyssinus*, *D. farinae*, *G. domesticus*, *L. destructor*, *T. putrescentiae* (dissolved in water at a concentration of 0.5 mg/mL) and, for control purposes, of BSA (0.5 mg/mL) were dotted onto a nitrocellulose membrane. The sera from mite-allergic patients were diluted 1 : 10 in buffer A. These serum dilutions were then individually pre-incubated with each of the above-described extracts (10 µg/mL serum dilution), or, for control purposes, with 10 µg/mL of BSA overnight at 4 °C. Next, extract-containing membranes were blocked two times for 5 min and once for 30 min with buffer A and then exposed to the preadsorbed serum dilutions. Bound IgE antibodies were detected as described [24].

Statistical analysis

The statistical significance of differences of the total IgE level and the Der p-specific IgE level between patients of groups A and B was assessed using the Mann & Whitney U-test. The Fisher's exact probability test was applied to assess statistically significant differences between the percentages of patients from groups A and B reactive with individual mite allergens. Differences were regarded as statistically significant if the *P*-value was lower than 0.05.

Results

Analysis of IgE reactivity to nitrocellulose-blotted *D. pteronyssinus* allergens reveals two highly divergent reactivity profiles in mite-allergic patients

The IgE reactivity profiles of 208 mite-allergic patients with a nitrocellulose-blotted *D. pteronyssinus* extract were determined. Two highly divergent IgE reactivity profiles were

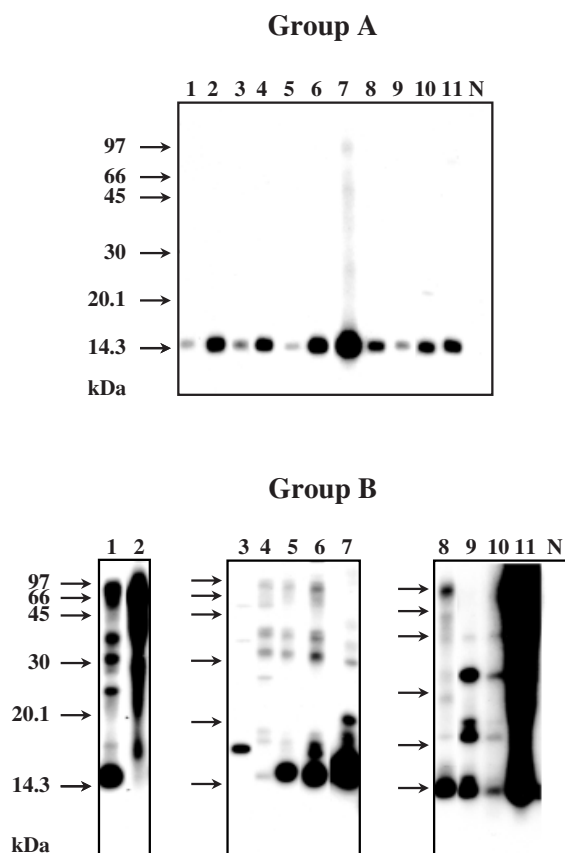


Fig. 1. Representative IgE reactivity profiles to nitrocellulose-blotted *Dermatophagoides pteronyssinus* extract. Nitrocellulose strips containing blotted Der p extract were incubated with sera from mite-allergic patients (group A: lanes 1–11; group B: lanes 1–11) and with serum from a non-allergic individual (lanes N). Bound IgE antibodies were detected with ^{125}I -labelled anti-human IgE antibodies and visualized by autoradiography. Molecular weights are displayed in kDa on the left margins.

observed among the patients, which are exemplified by the representative patients of groups A and B (Fig. 1).

Patients with a profile depicted in group A reacted almost exclusively with a 14-kDa band presumably representing Der p 2 and/or other low-molecular-weight mite allergens (e.g. Der p 5). A reactivity profile as depicted for group A was observed in 78 out of the 208 analysed patients. The reactivity profile of patients represented by group B was characterized by IgE reactivity either to a great variety of antigens with molecular weights ranging from 14 to 200 kDa or by reactivity to allergens other than the 14-kDa band (Fig. 1). Forty-one out of the 208 mite-allergic patients exhibited a reactivity profile as shown for group B.

Twenty-nine patients reacted in addition to the 14-kDa band with one more distinct band and therefore could not be strictly classified as belonging to group A or B.

Sixty patients showed no detectable IgE reactivity to nitrocellulose-blotted Der p extracts. This lack of IgE reactivity may be attributed either to low Der p-specific IgE levels (Der p-specific IgE: RAST class = 2) or to a sensitization to mite allergens, which cannot be detected by denaturing Western blotting (e.g. Der p 1). Furthermore, certain allergens are reported to occur in low amounts in Der p extracts (e.g. Der p 7) [26, 27].

Clinical and serologic features of 11 patients representative for groups A and B are listed in Table 1. As shown for representative patients, individuals belonging to group A were characterized by total IgE levels, which were generally lower than those of patients belonging to group B (Table 1). The mean total IgE level in the 78 patients of group A (304 kU) was significantly lower than the mean total IgE level in the 41 patients of group B (1010 kU/L) ($P < 0.001$). Patients of group B showed sensitizations to more different allergen sources than those of group A. No significant difference was found between the Der p-specific IgE levels of groups A and B ($P = 0.222$). A mean Der p-specific RAST class of 3.4 (19.32 kUA/L) was found for the patients of group A and a mean RAST class of 3.8 (41.15 kUA/L) for the patients of group B. In accordance with the latter finding, we did not note remarkable differences with respect to the quality and magnitude of symptoms between the two groups.

Use of purified natural and recombinant mite allergens to establish the IgE reactivity profiles of mite-allergic patients

Western blot analysis with mite allergen extracts does not provide information regarding the identity of the antibody binding mite allergens, and reactivity to certain mite allergens (e.g. Der p 1) cannot be detected by this assay [28, 29]. Therefore, we performed a detailed analysis of the IgE reactivity profile for those sera from groups A and B where sufficient volumes were available (group A: $n = 56$; group B: $n = 22$). IgE reactivity to seven different purified natural and recombinant Der p allergens (nDer p 1, nDer p 4, rDer p 2, rDer p 5, rDer p 7, rDer p 8 and rDer p 10) and GST was investigated by non-denaturing ELISA assays (Table 2). We found that 91% of the patients reacted with nDer p 1 and 86% recognized rDer p 2. Although nDer p 1 was recognized more frequently than rDer p 2, its IgE-binding capacity was considerably lower than that of rDer p 2 [average optical density (OD) level 65% of that of Der p 2]. Using a combination of nDer p 1 and rDer p 2, all mite-allergic patients, except one from group B, could be diagnosed. Patients from group A reacted significantly more frequently with Der p 2 than patients from group B ($P = 0.001$) (Table 2). Patients from group B reacted more frequently with Der p 5 and Der p 10 in a significant manner ($P = 0.018$ and $P = 0.021$, respectively) (Table 2). Only one patient reacting with the highly cross-reactive tropomyosin, Der p 10, was found in group A. Besides nDer p 1 and rDer p 2, nDer p 4 was the most frequently recognized mite allergen, but the intensity of recognition was very low (average OD level 17% of that of Der p 2). After nDer p 1, rDer p 2 and nDer p 4, rDer p 7 was the most frequently recognized mite allergen (average OD level 57% of that of Der p 2).

IgE dot blot inhibition experiments and CAP FEIA determinations discriminate patients with a predominant Der p-sensitization from patients with co-sensitization to a variety of mite species

When we analysed IgE reactivity to house-dust- (Der p, Der f) and storage-mite (Gly d, Lep d, Tyr p) extracts by CAP FEIA measurements in our mite-allergic population, the majority of

Table 1. Demographic, clinical and serological characterization of mite-allergic patients

Patient number	Sex	Age	Symptoms	Total IgE (kU/L)	Der p-specific IgE (RAST class)	Sensitization to other allergen sources
<i>Group A</i>						
1	f	21	r, c	264	2	Grasses (6)*, birch (4)
2	f	28	r, co	49	3	Cat (1)
3	f	37	r	210	3	Grasses (2), birch (4)
4	m	18	r, co	182	4	0
5	m	42	r, c, d	52	2	Grasses (1), birch (3)
6	m	28	r, c, co	32	3	Grasses (3)
7	f	19	r, c, co	180	4	Grasses, mugwort (+)†
8	f	23	r, d	55	3	0
9	m	38		23	3	0
10	m	36	r	147	3	Grasses (4), mugwort, cat, dog (+)
11	m	16	r	71	(+)†	0
<i>Group B</i>						
1	m	20	r, c	383	4	Birch (2), cat (2)
2	m	25	r, d	1109	6	Grasses, cat, dog (+)
3	f	76	r, c	>2000	(+)	Birch, mugwort, mouldmix, trees, milk, wheatflour, nuts (+)
4	m	17	d	923	(+)	Grasses, cat (+)
5	m	19	r	455	4	Grasses (2), birch (+)
6	m	24		239	4	Grasses (3), birch (3); mugwort (+)
7	f	20	r, d	727	5	Grasses (3), mugwort (2)
8	m	25	r, c, d	484	5	Grasses (2); birch, mugwort, mouldmix, cat, dog (+)
9	m	21	r	1073	5	Grasses, dog (+)
10	m	34	r, c, ad	>2000	2	Grasses (6), birch (6), mugwort (4); cladosporium, cat (+)
11	m	36	ad, as	>2000	6	Ryeflour (3); grasses, mugwort, crustaceae, birch, cat, dog, trees (+)

kU/L, kilo units/litre; m, male; f, female; r, rhinitis; c, conjunctivitis; co, cough; d, dyspnoea; as, asthma; ad, atopic dermatitis.

* () RAST class. † () Skin prick test response; +, positive reaction; 0, no reaction.

Table 2. IgE reactivity with purified *Dermatophagoides pteronyssinus* allergens

	Total		Group A		Group B		P-value
	%	n = 78	%	n = 56	%	n = 22	
nDer p 1	91	71	91	51	91	20	1*
rDer p 2	86	67	95	53	64	14	0.001†
nDer p 4	74	58	73	41	77	17	0.781*
rDer p 5	18	14	11	6	36	8	0.018†
rDer p 7	27	21	25	14	32	7	0.578*
rDer p 8	10	8	9	5	14	3	0.680*
rDer p 10	6	5	2	1	18	4	0.021†

*Not significant. †Significant.

n, number of patients with IgE reactivity; %, percentage of patients with IgE reactivity.

patients showed no or low IgE reactivity to storage mites (Table 3: patients I, II) but high reactivity to and cross-inhibition between Der p and Der f extracts (Fig. 2). Accordingly, storage mite extracts did not inhibit IgE reactivity of these sera to HDM extracts (Fig. 2: I, II).

Approximately 30% of the patients exhibited strong IgE reactivity to extracts from house dust and storage mites (Table 3: patients III and IV). These patients displayed IgE reactivity to storage mite extracts, which was not always inhibited by pre-incubation of the sera with HDM allergen extracts (Fig. 2, III, IV). Only partial cross-inhibition between the two HDM allergen extracts was noted in these sera (Fig. 2).

Discussion

HDM are major allergen sources for almost 50% of allergic patients [4, 5]. Specific immunotherapy with Der p allergen extracts is frequently performed but highly divergent results have been reported [6–14]. In this study, we sought to search for immunological parameters that could be useful for a refined selection of patients for specific immunotherapy with Der p allergen extracts. In order to establish the IgE reactivity profiles of mite-allergic patients, IgE immunoblot analysis was performed. IgE reactivity to certain heat-labile mite allergens (e.g. major HDM allergen, Der p 1) or allergens, which occur in low amounts in extracts (e.g. Der p 7), cannot be detected by IgE immunoblotting [26–29]. Two highly divergent IgE reactivity profiles were established by this method for a large proportion of patients. Patients belonging to the first group (group A) were characterized by a predominant IgE reactivity to Der p 2, whereas the second group (group B) was characterized by sensitization to mite allergens other than Der p 2. These patients frequently reacted with a great variety of different antigens when tested by immunoblotting and with purified mite allergens. Sensitization to cross-reactive mite allergens (e.g. Der p 10: tropomyosin) and to storage mite allergens was more frequently observed for these polysensitized patients. This observation is notable in view of several reports describing that sensitization to tropomyosin may be an important cause for food allergy (e.g. shrimps, molluscs, snails) in mite-allergic patients [12]. Furthermore, it has been reported that certain mite-allergic patients develop clinically manifest food allergy

Table 3. Demographic, clinical and serological characterization of patients studied by IgE inhibition experiments

Patient number	Sex	Age	Symptoms	Total IgE (kU/L)	Specific IgE									
					Der p (d1)		Der f (d2)		Gly d (d73)		Lep d (d71)		Tyr p (d72)	
					Class	kUA/L	Class	kUA/L	Class	kUA/L	Class	kUA/L	Class	kUA/L
I	m	36	r, c, d	79	4	29.9	4	17.9	0	<0.35	0	<0.35	2	0.7
II	m	25	r, c	406	4	42.2	5	51.6	0	<0.35	1	0.38	2	1.02
III	m	26	ad, p	>2000	6	>100	6	>100	5	72.6	6	>100	5	83.6
IV	m	43	ad, as, c	>2000	6	>100	6	>100	3	14.4	3	15.6	4	21.5

Der p, *Dermatophagoides pteronyssinus*; Der f, *D. farinae*; Gly d, *Glycyphagus domesticus*; Lep d, *Lepidoglyphus destructor*; Tyr p, *Tyrophagus putrescentiae*. kU/L, kilo units/litre; kUA/L, kilo units antigen/litre; r, rhinitis; c, conjunctivitis; p, pruritus; d, dyspnoea; ad, atopic dermatitis; as, asthma.

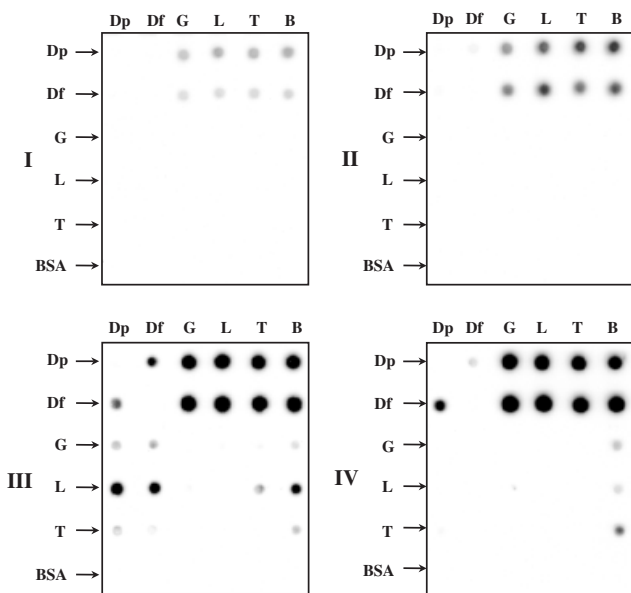


Fig. 2. Sera from four representative mite-allergic patients (I, II, III, IV) were preadsorbed with various mite extracts (Dp, *Dermatophagoides pteronyssinus*; Df, *Dermatophagoides farinae*; G, *Glycyphagus domesticus*; L, *Lepidoglyphus destructor*; T, *Tyrophagus putrescentiae*) or bovine serum albumin (BSA) (B) as indicated on the top of the figure and then exposed to nitrocellulose-dotted mite extracts and BSA (arrows on the left margins). Bound IgE antibodies were detected with ^{125}I -labelled anti-human IgE antibodies and visualized by autoradiography.

after immunotherapy with mite extracts due to *de novo* sensitization to tropomyosin [13].

The use of purified natural and recombinant HDM allergens for CRD not only confirmed the discrimination of oligo- and polysensitized mite-allergic patients obtained by immunoblotting but also allowed us to detect sensitization to mite allergens that are not present in sufficient quantities in natural mite extracts (e.g. Der p 7) [26, 27]. Using a combination of only two major mite allergens, Der p 1 and Der p 2, more than 95% of mite-allergic patients were diagnosed, thus allowing the identification of patients who are sensitized against major constituents of HDM allergen extracts. The latter finding may be of particular importance for the selection of patients for specific immunotherapy with HDM allergen extracts as depicted in Fig. 3.

According to our results, we suggest to confirm IgE-mediated allergy to HDM by IgE serology using allergen extracts of HDM (e.g. Der p, Der f), which may be spiked with allergens present in low abundance (e.g. Der p 7) as has

been demonstrated for latex extract [30]. Additional testing with storage mite allergens may be performed to identify patients with strong IgE-reactivity (i.e. RAST class >3) to storage mites that lack cross-reactivity with HDM allergens [31]. These patients will likely not be sufficiently treated with HDM allergens alone. Patients with a preferential reactivity to HDM allergens can be further analysed with purified HDM allergens to establish their detailed sensitization profile.

CRD with purified major allergens, i.e. Der p 1, Der p 2, which represent major components in most therapeutic allergen extracts [15], may allow the identification of those patients who are best suited for immunotherapy using a Der p extract. We suggest that only those patients who are sensitized to Der p 1 and/or Der p 2 should receive immunotherapy with HDM extracts. This consideration is based mainly on the fact that most therapeutic HDM extracts contain these molecules and are standardized for Der p 1 and Der p 2 [15]. Consequently, patients without sensitizations to these components are less likely to benefit from therapy with such extracts. At this time, recommendations regarding inclusion or exclusion of patients for HDM immunotherapy based on sensitization to allergens other than Der p 1 and Der p 2 cannot be made, because most therapeutic HDM extracts are not standardized for allergens other than Der p 1 and/or Der p 2. Diagnostic tests containing cross-reactive mite allergens, e.g. Der p 10, may be used to identify patients with broad cross-reactivity who may be less suitable for immunotherapy and to detect novel IgE reactivities possibly induced by the therapy [12, 13]. Support for our assumption comes from earlier observations that atopic individuals may be categorized into oligo- and polysensitized patients who also differ in terms of their IL-4 and sCD23 profiles, and that especially the polysensitized patients did not benefit from specific immunotherapy [32, 33]. However, the hypothesis that patients who are sensitized to highly cross-reactive allergens may benefit less from immunotherapy with currently available allergen extracts needs further investigation.

In addition, purified natural and recombinant mite allergens may be used for the monitoring of the immunotherapy. Although the beneficial role of allergen-specific IgG antibodies is a matter of considerable controversy [34, 35, 36] we think that the detection of therapy-induced IgG antibodies specific for purified mite allergens may be useful for two reasons. First, these IgG antibodies will indicate the immunological efficacy of immunotherapy. Second, the measurement of allergen-specific IgG with purified allergens will serve to discriminate potentially protective allergen-specific IgG

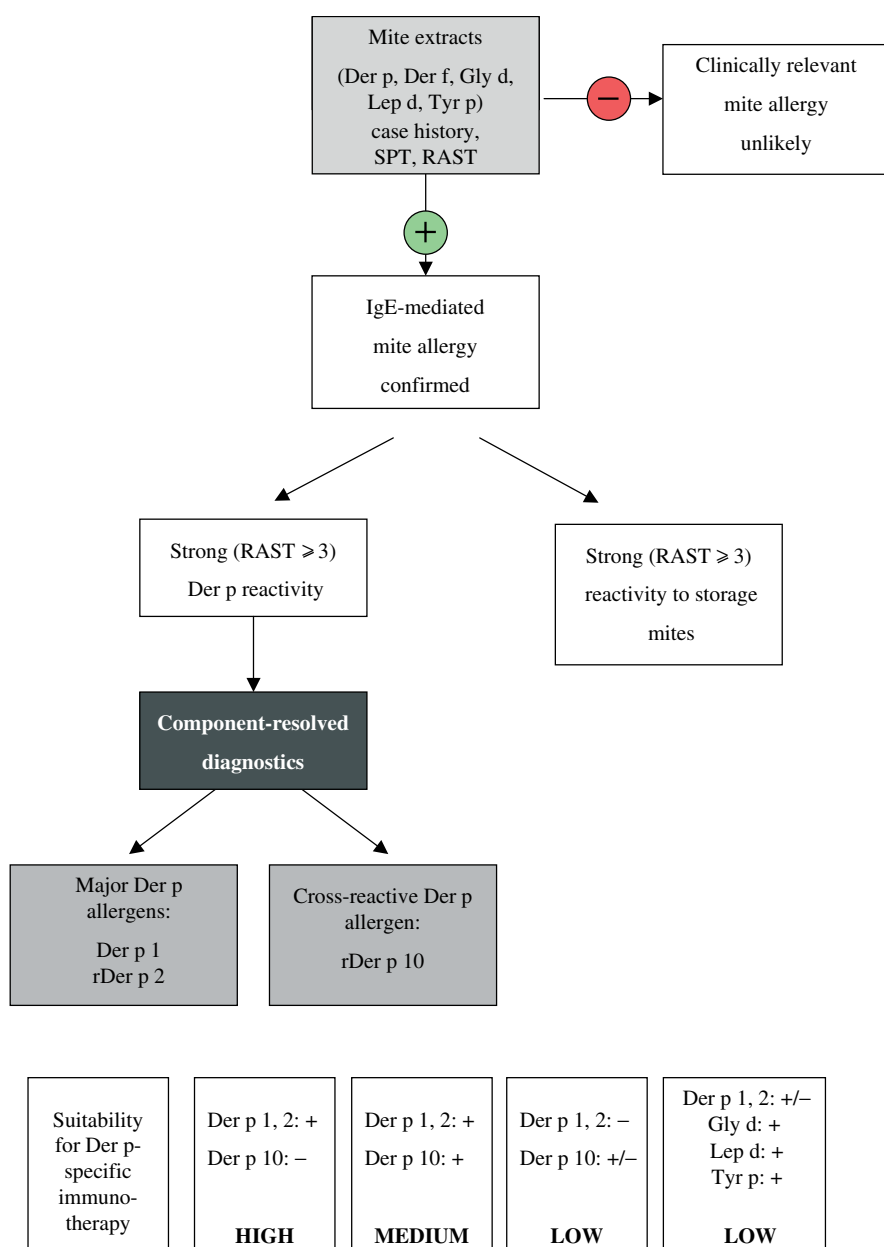


Fig. 3. A possible decision tree for Der p-immunotherapy. Serological testing with mite allergen extracts (Der p, Der f, Gly d, Lep d, Tyr p) confirms IgE-mediated allergy. In the case of strong Der p reactivity, component-resolved diagnosis with major (Der p 1, Der p 2) and cross-reactive (Der p 10) allergens may be performed to further determine suitability for Der p-specific immunotherapy.

responses from IgG responses to non-allergenic components in the allergen extracts and thus allow to reinvestigate the concept of blocking antibodies.

In conclusion, we suggest CRD with purified mite allergens for a refined selection of patients for specific immunotherapy with Der p allergen extracts and the immunological monitoring of this treatment.

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