

ORIGINAL ARTICLE Allergens

Heterogeneity of molecular sensitization profiles in grass pollen allergy – implications for immunotherapy?

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Summary

Background Data on molecular allergy diagnostics in adults with grass pollen allergy with regard to conjunctival and nasal provocation test outcome and specific immunotherapy are lacking to date.

Objective To assess whether molecular allergy diagnostics for grass pollen allergens could help with predicting provocation test outcomes and serve as a basis for future component-resolved specific immunotherapy.

Methods Sera of 101 adults with grass pollen allergy was analysed for IgE against timothy grass pollen (*Phleum pratense*), *rPhl p 1*, *rPhl p 2*, *nPhl p 4*, *rPhl p 5b*, *rPhl p 6*, *rPhl p 7*, *rPhl p 11* and *rPhl p 12* and correlated with the individuals' outcome in the nasal and conjunctival provocation tests and investigated in regard to a potential component-resolved specific immunotherapy.

Results An increasing number of sensitizations to timothy grass allergens was correlated to a positive reaction in the conjunctival (4.9 vs. 3.6, $P = 0.003$) and nasal provocation tests (4.5 vs. 2.2, $P = 0.0175$). In molecular sensitization profiles, a substantial heterogeneity was detected, with none of the patients exactly matching the allergen composition of a previously published component-resolved specific immunotherapy containing *Phl p 1*, *Phl p 2*, *Phl p 5a/b* and *Phl p 6*. The results indicate that in 95% of the patients, a proportion of 50% of timothy-IgE would be targeted with such a specific immunotherapy, while in 50% and 10% of patients, 80% and 90% of timothy-IgE would be targeted, respectively.

Conclusion and Clinical Relevance Molecular allergy diagnostics is a prerequisite for future component-resolved specific immunotherapy due to the high heterogeneity of sensitization profiles. However, of current clinical relevance is the observed correlation between the number of sensitizations and provocation test outcome.

Keywords allergenic molecules, combinatorial analysis, component-resolved diagnosis, component-resolved therapy, hay fever, molecular allergy diagnostics, nasal/conjunctival provocation test, *Phleum pratense*, specific IgE, timothy

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Introduction

In a recent study published in this journal, we assessed whether immunoglobulin E (IgE)-measurements can replace challenge tests in allergic rhinoconjunctivitis to grass pollen and concluded that the allergen-specific IgE-concentration is not closely related to the clinical response [1]. This finding and its implications were

discussed by Hamilton in an accompanying editorial emphasizing that serologic or skin test measures of allergen-specific IgE antibodies alone are not diagnostic of allergic disease [2]. It was pointed out that due to multiple variables associated with the environment (e.g. allergen heterogeneity and exposure levels) and the host (immune response heterogeneity), IgE antibodies alone are necessary, but not sufficient for the manifestation of

allergic symptoms. Considering the multitude of humoral immune response parameters necessary to alter mast cell and basophil mediator release in an allergic patient including IgE antibody concentration, affinity, clonality and specific activity, it becomes obvious that knowledge of the IgE-concentration in the blood is not sufficient to predict symptomatic allergic disease [3]. However, new diagnostic tools providing additional insight into the molecular mechanisms of allergy have shown that molecular IgE-measurements closely correlate with clinical symptoms. This is especially true for food allergy where on the molecular level, single allergens can help to predict clinical reactivity, as have been shown in nut and peanut allergy recently [4–8].

While there is increasing evidence that IgE-measurements on the molecular level closely correlate with clinical symptoms in food allergy, there is little data on this topic in regard to aeroallergens. In order to investigate whether molecular allergy diagnostics in pollen allergy could deliver measurable biomarkers with the ability to predict the presence of symptomatic disease, as desired from the allergy community for decades, we analysed serum IgE to timothy grass (*Phleum pratense*) pollen extract (IgE-timothy) as well as to timothy grass pollen allergens on the molecular level (IgE-*Phl p* allergens) in serum samples from our above-mentioned study [1]. It was the aim of this reassessment to investigate, whether data on the molecular sensitization profile of IgE to 8 timothy grass pollen allergens could help (1) to predict provocation test outcomes better than the extract-based IgE-timothy reported previously and furthermore (2) provide the basis for future component-resolved specific immunotherapy (CRT) as has been suggested in previous years [9–11].

For this purpose, we analysed sera of 101 adult pollinosis patients for IgE against *rPhl p 1*, *rPhl p 2*, *nPhl p 4*, *rPhl p 5b*, *rPhl p 6*, *rPhl p 7*, *rPhl p 11* and *rPhl p 12* and correlated the individual levels to the outcomes of the nasal and conjunctival provocation tests. Subsequently, we grouped the patients according to sensitization profiles and analysed this data in regard to clinical responses and potential CRT as has been proposed recently [12].

Methods

Patients and study design

A total of one hundred and one adult patients with allergic rhinoconjunctivitis (ARC) and allergy to timothy grass pollen were included during two consecutive grass pollen seasons in 2006 and 2007 in a four-visit prospective monocentric study in Munich, Germany. Median age was 27 years and 59 of the participants

were female (58.4%). Inclusion criteria were a serum level of IgE-timothy ≥ 0.35 kU_A/l and a positive history of grass pollen allergy. Exclusion criteria were pregnancy, concomitant disease and allergy-related systemic medication including previous specific immunotherapy. Compared with the previously published data set of this study, 3 of 104 patients were excluded due to the lack of serum samples for the reassessment. The four study visits took place within 1 year and were scheduled during (visit 1) or outside the grass pollen season (visit 2–4). Nasal provocation tests (NPT), conjunctival provocation tests (CPT) and skin prick tests were performed during visit 2–4. These procedures have been described in detail previously [1]. In short, the provocation tests were performed with solutions of increasing timothy grass pollen allergen content and were evaluated based on clinical symptoms according to the respective scoring systems and as for the nasal challenge with respect to a significant flow reduction in anterior rhinomanometry [13]. The development of clinical symptoms in the provocation tests was rated as positive, the absence of a reaction upon allergen challenge as a negative provocation test outcome.

An informed consent was obtained from all subjects before participating in the study. The protocol of this study was approved by the local ethics committee prior to the initiation of the study.

IgE analysis

Serum samples were analysed for IgE-timothy (g6) at every study visit using the ImmunoCAP® system according to the manufacturer's guidelines (Thermo Fisher Scientific, Uppsala, Sweden). Additionally at visit 1 and 4, the following panel of IgE-*Phl p* allergens were measured: *rPhl p 1*, *rPhl p 2*, *nPhl p 4*, *rPhl p 5b*, *rPhl p 6*, *rPhl p 7*, *rPhl p 11* and *rPhl p 12*. Furthermore, IgE to the following common atopy-related allergens was determined: house dust mite, cat, dog, cow's milk, hen's egg, hazelnut, celery, *Alternaria alternata*, as well as pollen from birch, hazel, mugwort and ragweed.

Statistics

All statistical analyses were performed using the SAS® statistical software system version 9.3 (SAS Institute Inc., Cary, NC, USA). All statistical tests were two-sided and a significance level of < 0.05 was regarded as statistically significant. Spearman's rank correlation test was used to determine the dependence between the geometric mean concentration ranks of IgE-*Phl p* allergens and the corresponding prevalence ranks. Only values above the respective cut-off were included in the calculations. The Mann–Whitney nonparametric test

was used to determine whether the number of sensitizations in patients with positive provocation test was different from the number of sensitizations in patients with negative provocation test outcome.

The sensitization profiles to IgE-*Phl p* allergens were analysed through a combinatorial approach using the 8 timothy molecules listed above. A binary code was thus generated describing each sensitization profile with '1' for the presence of a sensitization and '0' for its absence. Applying the Allergen Profile Codification System (APCS), this binary code was converted into a decimal code [12].

Results

On average, each participant was sensitized to 5.5 of the 13 common atopy-related allergens analysed (SD 3.1, range 1–10). Aside from timothy pollen, sensitizations were found most frequently to birch, hazel, house dust mite and mugwort (Table S1).

The data of IgE-*Phl p* allergens $\geq$ a cut-off of 0.35 kU_A/L is presented in a box plot in Fig. 1. The *Phl p* allergens causing sensitizations most frequently were *Phl p* 1 and *Phl p* 4 (both 92%) followed by *Phl p* 5b (81%). The lowest sensitization rate was found for *Phl p* 7 which caused sensitization in only two of 101 patients and therefore was excluded from the analysis due to too few observations. The Spearman's rank correlation test showed that the geometric median

concentration ranks of IgE-*Phl p* allergens correlate closely to the prevalence of sensitization to the same molecules ($r = 0.9$, $P = 0.01$, Fig. 1).

As shown in Fig. 2, the number of *Phl p* allergen sensitizations was significantly higher in the provocation-positive group compared with provocation-negative patients for CPT and NPT in the range between 4.9 versus 3.6 ($P = 0.003$) and 4.5 versus 2.2 ($P = 0.0175$) of the eight allergens tested, respectively. Prevalence figures at four different cut-offs are demonstrated in Figure S1. Reduced sensitization profiles to six and four allergens are shown in Table S2.

Additionally, the magnitude of sensitization in regard to the different *Phl p* allergens was assessed by calculating the relative IgE-reactivity to each of the allergens in relation to the sum of IgE-timothy. This calculation indicated the following decreasing order regarding the magnitude of sensitization: *Phl p* 1, *Phl p* 5b, *Phl p* 4, *Phl p* 6, *Phl p* 2, *Phl p* 11, *Phl p* 12 and *Phl p* 7 (Fig. 3).

Performance characteristics of IgE-*Phl p* allergens

Table 1 shows that IgE to seven of the eight *Phl p* allergens predicted positive (PPV) CPT reactions between 62 and 70% at optimal cut-off points ranging from 0.1 to 4.1 kU_A/L (*Phl p* 7 was excluded due to too few observations). The accuracy of IgE to the same seven *Phl p* allergens for predicting negative (NPV) CPT reactions

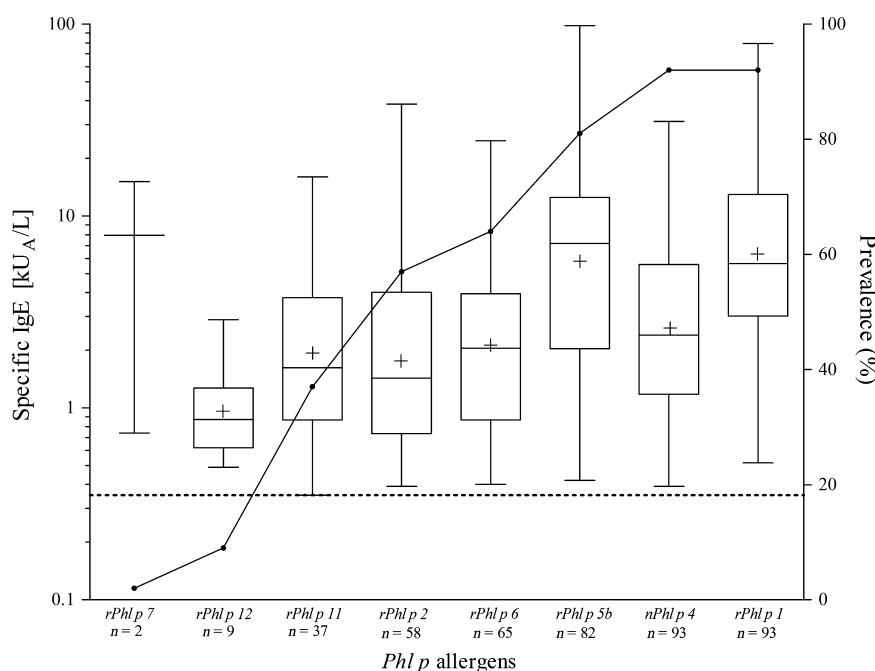


Fig. 1. sIgE-*Phl p* molecule sensitization levels of positive samples in correlation to the prevalence of sensitization. Left Y-axis: Boxes include median (line), geometric mean (+) values and interquartile range (25–75%). Whiskers extend to most extreme data points. Dotted line shows cut-off at 0.35 kU_A/L. Right Y-axis: Solid black line shows the prevalence of IgE to *Phl p* allergens.

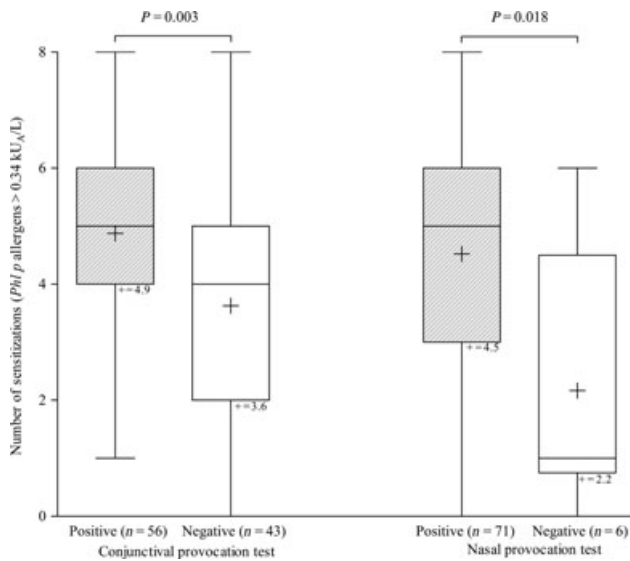


Fig. 2. Number of sensitizations to *Phl p* allergens in regard to CPT (left) and NPT (right) outcome (positive: grey, negative: white). Boxes include median (line) and mean (+) values and the interquartile range (25–75%). Whiskers extend to most extreme data points (Mann–Whitney nonparametric test).

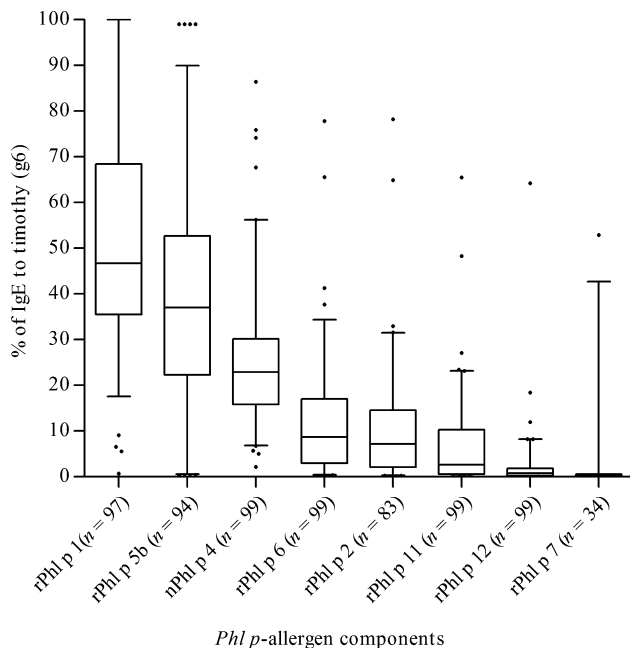


Fig. 3. Ratio of IgE-*Phl p* allergens/IgE-timothy. Boxes include median (line) and interquartile range (25–75%). Whiskers extend to 5–95 percentiles. Dots show most extreme data points. Values > 100% were truncated ($n = 12$).

was in the range of 50% to 67%. At the fixed cut-off point of 0.35 kU_A/L , the PPV and NPV were in the range between 58% to 78% and 46% to 75%, respectively. Due to too few observations, these calculations could not be performed in regard to NPT.

Phl p allergens IgE-sensitization profiles

Among the 101 participants, there were 27 different sensitization profiles identified for the eight *Phl p* allergens tested (Table 2). On average, each patient was sensitized to 4.3 of these allergens. The profile which appeared in the highest frequency (24%) was APCS 250 (Table 3). When testing 8 *Phl p* allergens, 11 of 27 profiles were needed to cover 80% of all patients. Corresponding numbers for the six and four *Phl p* allergens were seven and four profiles, respectively (Table S2).

Of 101 patients, 99 underwent the CPT (positive $n = 56$; negative $n = 43$). The number of different profiles is slightly higher in the CPT provocation-positive group (21 vs. 17) (Table S3).

Patient's profiles matched to molecular experimental CRT preparation

The allergen composition of an experimental CRT preparation containing a mixture of *Phl p* 1, *Phl p* 2, *Phl p* 5a/b and *Phl p* 6 was compared with our patient's sensitization profiles [12]. The CRT preparation profile matched none of the 101 adult patients exactly. There were 48 patients (47.5%) that matched all four allergens in the CRT profile but were also sensitized to one or more additional allergens, classified as underpowered immunization (Table 3). Another 48 patients (47.5%) were sensitized to between 1 and 3 allergens that matched with the CRT profile, corresponding to underpowered and overpowered immunization. Five patients showed a complete mismatch and were not sensitized to any of the allergens included in the CRT profile characterizing the category-unrelated immunization.

Finally, it was calculated how many of the patients and to which proportion the experimental CRT would target IgE-reactivity to the timothy extract: The results indicate that in 95%, 50% and 10% of our patients, a proportion of 50%, 80% and 90% of timothy-IgE would be targeted with the experimental CRT preparation (Fig. 4).

Discussion

This is the first study to systematically investigate molecular IgE-sensitization profiles of patients with grass pollen allergy in regard to conjunctival and nasal provocation test outcomes. The main findings of the study are (1) a significant relationship between IgE-levels to timothy grass pollen allergens and the prevalence of sensitization to these allergens, (2) a substantial degree of heterogeneity of molecular sensitization profiles, and most importantly (3) a positive correlation between the number of allergens a patient is sensitized

Table 1. Number of patients and performance characteristics for CPT outcome in *Phl p* allergens and in a combination of *Phl p 2* and *Phl p 12*. Figures are shown for both optimal and fixed cut-offs. Optimal cut-offs were determined by receiver operating characteristic curve analysis. Number of observations for each cell in a '2 × 2' table are shown in the two columns 'positive at IgE-test' and 'negative at IgE-test', respectively

Allergen Component	Cut-off*		Positive at IgE-test (n)				Negative at IgE-test (n)				PPV†		NPV‡		Sens.§		Spec.¶		LR**	
			Optimal		Fixed		Optimal		Fixed											
	Optimal	Fixed	CPT pos	CPT neg	CPT pos	CPT neg	CPT pos	CPT neg	CPT pos	CPT neg	Opt.	Fix.	Opt.	Fix.	Opt.	Fix.	Opt.	Fix.	Opt.	Fix.
<i>Phl p 1</i>	4.6	0.35	37	19	54	37	19	24	2	6	66.1	59.3	55.8	75.0	66.0	96.4	55.8	14.0	1.5	1.1
<i>Phl p 2</i>	0.39	0.35	39	17	39	17	17	26	17	26	69.6	69.6	60.5	60.5	69.6	69.6	60.5	60.5	1.8	1.8
<i>Phl p 4</i>	2.08	0.35	31	18	54	37	25	25	2	6	63.3	59.3	50.0	75.0	55.3	96.4	58.1	14.0	1.3	1.1
<i>Phl p 5</i>	1.34	0.35	43	23	49	31	13	20	7	12	65.2	61.3	60.1	63.2	76.7	87.5	46.5	27.9	1.4	1.2
<i>Phl p 6</i>	0.21	0.35	46	23	42	22	10	20	14	21	66.7	65.6	66.7	60.0	82.1	75.0	46.5	48.8	1.5	1.6
<i>Phl p 11</i>	0.15	0.35	31	13	27	9	25	30	29	34	70.5	75.0	54.6	54.0	55.3	48.2	69.8	79.1	1.8	2.3
<i>Phl p 12</i>	0.06	0.35	31	18	7	2	25	25	49	41	63.3	77.8	50.0	45.6	55.5	12.5	58.1	95.3	1.3	2.7
<i>Phl p 2 & 11</i>	–	0.35	–	–	45	19	–	–	11	24	–	70.3	–	69.6	–	80.3	–	55.8	–	1.8

Phl p 7 was not included due to only 2 results > 0.35.

*kU_A/L.

†Positive predictive value (%).

‡Negative predictive value (%).

§Sensitivity.

¶Specificity.

**Likelihood ratio.

Table 2. APCS codes* of 8 *Phl p* allergens in 99 patients: Pos% = CPT positive (n = 56), Neg% = CPT negative (n = 43)

APCS	250	248	160	184	178	186	56	96	128	146	161	176	185	187
Pos%	32	23	5	5	4	4	2	2	2	2	2	2	2	2
Neg%	12	14	5	19	–	2	–	–	5	–	–	12	–	–
APCS	224	240	241	146	249	251	255	32	16	144	162	226	0	
Pos%	2	2	2	2	2	2	2	–	–	–	–	–	–	–
Neg%	2	5	–	–	2	–	2	7	2	2	2	2	5	

*See Table 3 for APCS coding.

to and the clinical reaction to the conjunctival and nasal provocation test.

The first two findings are in line with previously published data from other patient collectives [12, 14, 15]. The novel finding of our study, however, is the correlation between the number of sensitizations on the molecular level and provocation tests outcome which might have direct clinical implications.

Molecular allergy diagnostics and provocation test outcome

For timothy grass pollen, 13 different allergens have been described so far [14]. Eight of these allergens have been investigated repeatedly in epidemiologic studies regarding their prevalence of sensitization showing *Phl p 1* and *Phl p 5b* to be the most important ones, followed by *Phl p 2*, *Phl p 4*, and *Phl p 6* while *Phl p 11*, *Phl p 12* and especially *Phl p 7* cause sensitizations less frequently [16, 17]. Our data on the prevalence of sensitization against major grass pollen allergens are in

concordance with previous studies stating sensitization rates of 90% for *Phl p 1* and up to 85% for *Phl p 5b* in grass pollen allergics [14] and also hold true for *Phl p 2* and *Phl p 6* which are responsible for sensitization rates of 40–60% and 60–70%, respectively. A *Phl p 7*-sensitization was described previously in about 10% of patients [14] as opposed to 2% in our collective. For *Phl p 4*, an especially high sensitization rate of 92% was observed in our study, which is not in concordance with other investigations in adults from different geographic regions reporting sensitizations between 54 and 74% [14, 18]. However, the high *Phl p 4* sensitization in our patients is species-specific and cannot be explained by cross-reactivity to other allergens such as *Amb a 1* or cross-reactive carbohydrates which only showed sensitizations in 3% and 16%, respectively, of all patients investigated (data not shown).

Even though sensitization to *Phl p 1* and *Phl p 5b* was highly prevalent and responsible for the highest sIgE levels, our data clearly indicates that the risk for

Table 3. Sensitization profiles to 8 *Phl p* allergens in 101 patients. Dots indicate presence of antibodies. Red dots indicate matching with the experimental CRT profile. Cut-off = 0.35 kU_A/l. The profile binary code represented by 1 and 0 indicates positive (1) or negative (0) test result for each allergen. Matching of the molecular profiles against the composition of the experimental CRT profile is shown in four different colour categories depending on type of matching

	Profile APCS code	Profiles binary code (8 mol.) Cut-off 0.35	rPhl p 1	rPhl p 2	rPhl p 4	rPhl p 5	rPhl p 6	rPhl p 7	rPhl p 11	rPhl p 12	N	%	Cumm. %
1	250	11111010	•	•	•	•	•	•	•	•	24	24	24
2	248	11111000	•	•	•	•	•	•	•	•	19	19	43
3	184	10111000	•	•	•	•	•	•	•	•	11	11	53
4	176	10110000	•	•	•	•	•	•	•	•	6	6	59
5	160	10100000	•	•	•	•	•	•	•	•	5	5	64
6	240	11110000	•	•	•	•	•	•	•	•	4	4	68
7	32	100000	•	•	•	•	•	•	•	•	3	3	71
8	128	10000000	•	•	•	•	•	•	•	•	3	3	74
9	186	10111010	•	•	•	•	•	•	•	•	3	3	77
10	178	10110010	•	•	•	•	•	•	•	•	2	2	79
11	224	11100000	•	•	•	•	•	•	•	•	2	2	81
12	249	11111001	•	•	•	•	•	•	•	•	2	2	83
13	255	11111111	•	•	•	•	•	•	•	•	2	2	85
14	16	10000	•	•	•	•	•	•	•	•	1	1	86
15	56	111000	•	•	•	•	•	•	•	•	1	1	87
16	96	1100000	•	•	•	•	•	•	•	•	1	1	88
17	144	10010000	•	•	•	•	•	•	•	•	1	1	89
18	146	10010010	•	•	•	•	•	•	•	•	1	1	90
19	161	10100001	•	•	•	•	•	•	•	•	1	1	91
20	162	10100010	•	•	•	•	•	•	•	•	1	1	92
21	185	10111001	•	•	•	•	•	•	•	•	1	1	93
22	187	10111011	•	•	•	•	•	•	•	•	1	1	94
23	226	11100010	•	•	•	•	•	•	•	•	1	1	95
24	241	11110001	•	•	•	•	•	•	•	•	1	1	96
25	242	11110010	•	•	•	•	•	•	•	•	1	1	97
26	251	11111011	•	•	•	•	•	•	•	•	1	1	98
27	0	0	•	•	•	•	•	•	•	•	2	2	100

Immunization category	Frequency n (%)
under powered	48/101 (47.50)
under/over powered	43/101 (42.60)
over powered	5/101 (4.95)
unrelated	5/101 (4.95)

clinical symptoms does not only depend on single allergens but increases with the overall number of grass pollen sensitizations: there was a significantly higher risk of a positive reaction in the CPT and NPT when the patient was sensitized to a higher number of allergens. This observation can be discussed in regard to the additive effect of the simultaneous exposure to several allergens on multisensitized basophils as has been shown by Nopp et al. [19]. In this study, the combined exposure to pairs of timothy, cat, birch and mite respectively resulted in a shift of the dose-response curve to higher CD63 percentages and higher CD-sens compared with single exposure. As a positive response in CPT and NPT ultimately is effector cell degranulation

translated into clinical symptoms, one could speculate that this concept also applies on the molecular level when challenging a patient with the multitude of different, physically unlinked *Phl p* allergens contained in a timothy extract. In addition to this, additive effect of allergen exposure molecular degranulation models [3, 20] has to be considered to impact cooperatively on provocation test outcome. Based on this approach, it was experimentally demonstrated that higher IgE antibody concentration and affinity, specific activity (ratio of specific IgE/total IgE) as well as IgE-heterogeneity all contributed to increased effector cell degranulation in regard to each individual allergen molecule-specific IgE repertoire.

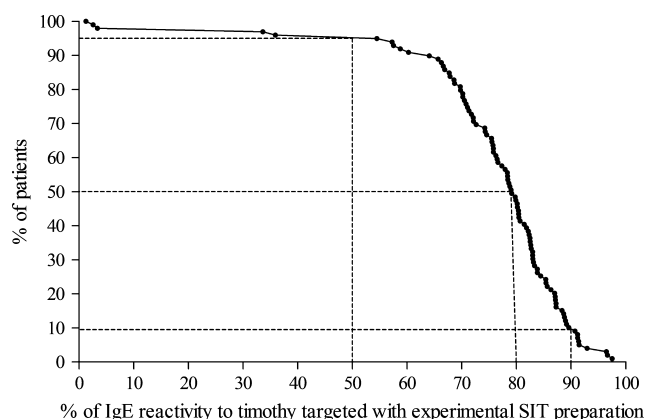


Fig. 4. Proportion of timothy-IgE that would be targeted by experimental CRT preparation (containing *rPhl p 1*, *rPhl p 2*, *rPhl p 5a/b*, *rPhl p 6*). Dotted lines indicate percentage of patients in which 50%, 80% and 90% of timothy-IgE would be targeted.

As the number of sensitizations to *Phl p* allergens influences provocation test outcome, it is important to note that also epidemiological data from the German Multicentre Allergy Study [15] suggest molecular spreading to take place during sensitization development and to have a predictive value on the IgE response to grass pollen allergens. Here, the authors describe the stages of seasonal allergic rhinitis to timothy and show that the number of *Phl p* allergens recognized by the patient's IgE antibodies increases over time until clinical symptoms occur. Along the same line but on the epitope level are the findings by Willumsen et al. [21] describing intra-molecular epitope spreading as the cause for the progression from low-complexity to full-complexity IgE repertoires by the spread from the inducing epitope to other non-cross-reactive epitopes on the same antigen [15]. Our study extends these findings in that an increasing number of sensitization to *Phl p* allergens is positively correlated with symptoms of allergic disease as confirmed by CPT and NPT.

An additional analysis targeted to discriminate between the numbers of sensitizations from the overall IgE antibody concentration to timothy in regard to provocation test outcome did not prove beneficial due to the strong correlation of these two factors.

As indicated above, high IgE antibody affinity is another factor enhancing effector cell degranulation and thus should correlate with clinical symptoms upon allergen challenge, while a low-affinity IgE-reponse would consequently correspond to a negative provocation test outcome. However, these aspects could not be assessed within the scope of our study as the ImmunoCAP[®] assay does not allow a sufficient differentiation between high- and low-affinity antibodies.

There are a number of limitations of the study: firstly, the diagnostic value of the provocation tests has

to be critically evaluated. While the NPT is well standardized [13] and the method of choice to objectively assess allergic rhinitis [22], the CPT is – even though of high reproducibility – only poorly associated with clinical symptoms [23, 24]. This explains the high number of CPT-negative patients in spite of the studies inclusion criterion of having seasonal allergic symptoms. However, statistical calculation of performance characteristics was only possible for the CPT data which was evenly distributed regarding provocation outcome, but not for the NPT due to a very low number of negative test results.

Secondly, the diagnostic value of the molecular IgE-test is limited, as it is only able to predict a positive CPT with a probability of approximately 66% at its best. This stresses the importance of the need to integrate different results (e.g. number of sensitizations, molecular IgE-levels, specific activity) and evaluate them in the context of patient history and clinical findings.

Furthermore, the topic of the allergen composition of the ImmunoCAP[®] and the issue of binding capacity represents a technical limitation of the method. This becomes evident when calculating the ratio of sIgE-*Phl p* allergens/sIgE-timothy indicating levels above 100% in 12 of 101 patients. This observation shows that in some cases, allergens are not represented in excess on the extract-based ImmunoCAP[®] in contrast to the test on a molecular level. This phenomenon can also affect test results when dilutions are performed. Nevertheless, previous investigations indicate that the composition of the extract-based timothy grass pollen ImmunoCAP[®] (g6) contains the *Phl p* allergens in sufficient concentrations and is designed to detect the relevant sIgE in most of the patients [14]. While a different grass pollen extract might lead to different results, the performance of the allergens included in g6 could be confirmed in our collective in approximately 90% of patients.

Molecular allergy diagnostics and specific immunotherapy

In a recent study, molecular allergy diagnostics was used to investigate IgE-sensitization profiles to *Phl p* allergens in a paediatric population from Rome, Italy, with allergic rhinitis and asthma [12]. Based on the concept of CRT introduced more than a decade ago by Valenta et al. [25, 26] these profiles were theoretically compared with the mixture of allergens from timothy grass pollen (*Phl p 1*, *Phl p 2*, *Phl p 5a/b*, and *Phl p 6*) previously used for grass pollen-CRT in an experimental setting [10]. Sensitization profiles were shown to be highly heterogeneous in the 176 children investigated and would have matched the profile of the experimental CRT preparation in only 4% of cases while the

remaining patients were classified in four mismatch categories [12]. In our study analysing sera from 101 adults with grass pollen allergy, 27 profiles of sensitization could be detected when measuring IgE to 8 *Phl p* allergens. This shows that a substantial heterogeneity on the molecular level in IgE-sensitization profiles is also present in adults and other geographic regions. The degree of mismatch of our patients' sensitization patterns with the molecular profile proposed for CRT is comparable to the paediatric population described above [12]. None of our patients showed a perfect match and a high level of underpowered and overpowered immunizations were detected. This finding could partly be due to the high prevalence of *Phl p* 4 sensitizations in our cohort, as this allergen is not included in the CRT preparation. Nevertheless, the results indicate that in 95% of the patients, a proportion of 50% of timothy-IgE would be targeted with such a specific immunotherapy, while in 50% and 10% of patients, 80% and 90% of timothy-IgE would be targeted, respectively. One could speculate that this constellation might lead to sufficient response rates even when applying an underpowered immunization via CRT. It means that despite not fitting perfectly, a specific immunotherapy composed of the four allergens mentioned above would target between 50 and 90% of timothy-IgE in the vast majority of patients included in the study. A negative impact of a possible overpowered CRT has to be questioned as well: even though new IgE-sensitization has been shown to occur under SIT, its clinical relevance could not be demonstrated [27]. Thus, the main finding of our study, the high level of sensitization heterogeneity, might have less therapeutic consequences in regard to a potential CRT than could be expected. However, clinical proof of an added therapeutic value of CRT compared with conventional SIT is still missing [28] and assessing this subject is beyond the scope of our study.

In regard to extract-based allergy, immunotherapy in general recent data suggests molecular diagnostics to

be of benefit by improving the identification of the disease-eliciting pollen source when prescribing SIT in polysensitized patients with heterogeneous sensitization profiles [29].

Based on our analysis, 11 different molecular profiles were needed to cover 80% of the study population whereas in the paediatric Italian patients, 15 profiles were needed to cover the same proportion. Overall, the findings of both studies in adults and children in different geographic regions yielded comparable results and clearly show the high heterogeneity in individual sensitization profiles. Previous assumptions that this heterogeneity is typical for childhood and less pronounced in adults with more homogeneous profiles are not supported by our data [12, 30].

Considering the high heterogeneity of sensitization profiles, we conclude that molecular allergy diagnostics could be helpful in regard to future CRT as well as when deciding on the indication for conventional SIT in polysensitized patients. However, most added clinical value is currently associated to the observed correlation between the number of sensitizations and provocation test outcome as it helps to estimate the patient's reaction upon allergen challenge and thus can impact on clinical decision-making.

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Conflict of interest

JHM, CJP and MB are employees of Thermo Fisher Scientific.

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Supporting Information

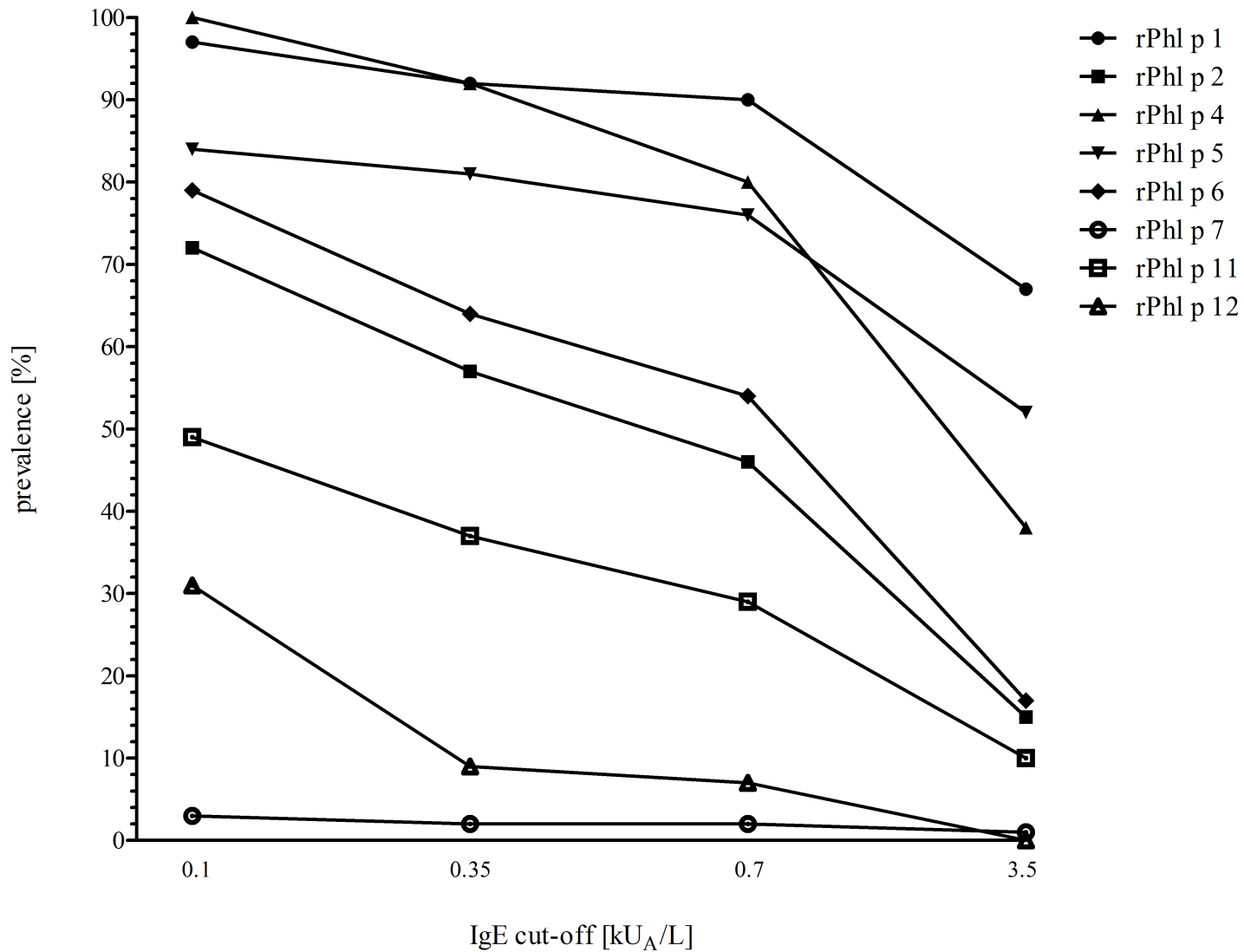
Additional Supporting Information may be found in the online version of this article:

Figure S1. Prevalence of sensitization to *Phl p* allergens at four different levels of cut-off (0.1; 0.35; 0.7 and 3.5 kU_A/L) in 101 adults with confirmed rhinoconjunctivitis to grass pollen.

Table S1. Sensitization characteristics in patients with allergic rhinoconjunctivitis.

Table S2. (a,b) Reduced sensitization profiles to (a) 6 and (b) 4 *Phl p* allergens, respectively. Dots indicate presence of antibodies at cut-off 0.35 kU_A/L.

Table S3. (a,b) Sensitization profiles to *Phl p* allergens in 99 patients with (a) positive CPT outcome ($n = 56$) and (b) with negative CPT outcome ($n = 43$).



Supporting Information for online version of the article:

Heterogeneity of molecular sensitization profiles in grass pollen allergy

– Implications for immunotherapy?

Condensed title: **Molecular sensitization profiles in grass pollen allergy**

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Formattato: Rientro: Sinistro: 0 cm, Sporgente 2,3 cm

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Tables for Supporting Information: [43](#)

Figures for Supporting Information: 1

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Conflict of interest:

JHM, CJP and MB are employees of Thermo Fisher Scientific.

Figure S1. Prevalence of sensitization to *Phl p* allergens at 4 different levels of cut-off (0.1; 0.35; 0.7 and 3.5 kU_A/L) in 101 adults with confirmed rhinoconjunctivitis to grass pollen.

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Table S1. Sensitization characteristics in patients with allergic rhinoconjunctivitis

Other than <i>Phl p</i> allergens		<i>Birch pollen</i>	<i>House dust mite</i>	<i>Hazel</i>	<i>Muggwort pollen</i>	<i>Cat epith.</i>	<i>Dog dander</i>	<i>Cow's milk</i>	<i>Hen's egg</i>	<i>Hazelnut</i>	<i>Celery</i>	<i>Alter. alternate</i>	<i>Common ragweed</i>
<i>Serum specific IgE (kU_N/l)</i>	<i>Mean (SD)</i>	16.7 (29.6)	5.7 (14.7)	10.7 (18.0)	0.7 (1.2)	1.6 (5.9)	1.7 (8.4)	0.1 (0.1)	0.1 (0.2)	4.7 (10.8)	0.6 (1.1)	0.3 (1.5)	0.9 (2.1)
	<i>Median (range)</i>	3.0 (0-181)	0.3 (0-98)	2.0 (0-106)	0.3 (0-6)	0.1 (0-44)	0.2 (0-82)	0 (0-0.9)	0 (0-1.4)	0.4 (0-61)	0.2 (0-8)	0.1 (0-13)	0.3 (0-17)
	<i>Prevalence (at ≥0.35 kU_N/L)</i>	65%	47%	62%	46%	33%	42%	4%	3%	53%	46%	5%	47%
<i>Phl p</i> allergens		<i>rPhl p 1</i>	<i>rPhl p 2</i>	<i>nPhl p 4</i>	<i>rPhl p 5b</i>	<i>rPhl p 6</i>	<i>rPhl p 7</i>	<i>rPhl p 11</i>	<i>rPhl p 12</i>	<i>Grass*</i>			
<i>Serum specific IgE (kU_N/L)</i>	<i>Mean (SD)</i>	10.5 (14.8)	1.9 (4.5)	4.1 (5.2)	9.4 (15.9)	2.3 (3.9)	0.16 (1.5)	1.2 (2.6)	0.2 (0.4)	20.0 (25.5)			
	<i>Median (range)</i>	5.1 (0-79)	0.5 (0-38)	2.1 (0.1-31)	3.9 (0-98)	0.8 (0-24)	0 (0-15)	0.1 (0.2-16)	0.1 (0-2.4)	10.5 (0.4-127)			
	<i>Prevalence (at ≥0.35 kU_N/L)</i>	92%	57%	92%	81%	64%	2%	37%	9%	-			

*Timothy pollen (g6)

Table S2 a-b. Reduced sensitization profiles to **a) 6** and **b) 4** *Phl p* allergens respectively ~~in 101 patients with allergic rhinocconjunctivits to grass pollen ordered in decreasing frequency~~. Dots indicate presence of antibodies ~~at~~ at. ~~C~~cut-off 0.35 kU_A/L. Reduced test profiles obtained by removing highly cross-reactive *Phl p* 7 and *Phl p* 12 (6 allergens tested) and subsequently removing the more weakly cross-reactive *Phl p* 4 and *Phl p* 11 (4 allergens tested).

a

	Profile APCS code	rPhl p 1	rPhl p 2	rPhl p 4	rPhl p 5b	rPhl p 6	rPhl p 7	n	%	Cum. %
1	63	•	•	•	•	•	•	27	27	27
2	62	•	•	•	•	•	•	21	21	48
3	46	•	•	•	•	•	•	12	12	59
4	40	•	•	•	•	•	•	6	6	65
5	44	•	•	•	•	•	•	6	6	71
6	60	•	•	•	•	•	•	5	5	76
7	47	•	•	•	•	•	•	4	4	80
8	8	•	•	•	•	•	•	3	3	83
9	32	•	•	•	•	•	•	3	3	86
10	45	•	•	•	•	•	•	2	2	88
11	56	•	•	•	•	•	•	2	2	90
12	4	•	•	•	•	•	•	1	1	91
13	14	•	•	•	•	•	•	1	1	92
14	24	•	•	•	•	•	•	1	1	93
15	36	•	•	•	•	•	•	1	1	94
16	37	•	•	•	•	•	•	1	1	95
17	41	•	•	•	•	•	•	1	1	96
18	57	•	•	•	•	•	•	1	1	97
19	61	•	•	•	•	•	•	1	1	98
20	0	•	•	•	•	•	•	2	2	100

b

	Profile APCS code	rPhl p 1	rPhl p 2	rPhl p 5b	rPhl p 6	n	%	Cum. %
1	15	•	•	•	•	48	48	48
2	11	•	•	•	•	16	15	63
3	8	•	•	•	•	10	10	73
4	10	•	•	•	•	10	10	83
5	14	•	•	•	•	6	6	89
6	12	•	•	•	•	3	3	92
7	2	•	•	•	•	1	1	93
8	3	•	•	•	•	1	1	94
9	4	•	•	•	•	1	1	95
10	0	•	•	•	•	5	5	100

Table S3 a-b. Sensitization profiles to *Phl p allergens* in 99 patients with **a)** positive CPT outcome (n = 56) and **b)** with negative CPT outcome (n = 43). Dots indicate presence of antibodies $\geq$ cut-off 0.35 kU_A/L.

a

	Profile APCS code	rPhl p 1	rPhl p 2	nPhl p 4	rPhl p 5b	rPhl p 6	rPhl p 7	rPhl p 11	rPhl p 12	n	%	Cumm. %
1	250	•	•	•	•	•		•		18	32	32
2	248	•	•	•	•	•				13	23	55
3	160	•		•						3	5	61
4	184	•		•	•	•				3	5	66
5	178	•		•	•	•		•		2	4	70
6	186	•		•	•	•		•		2	4	73
7	56			•	•	•				1	2	75
8	96		•	•						1	2	77
9	128	•								1	2	79
10	146	•			•			•		1	2	80
11	161	•		•					•	1	2	82
12	176	•		•	•					1	2	84
13	185	•		•	•	•		•		1	2	86
14	187	•		•	•	•		•	•	1	2	87
15	224	•	•	•						1	2	89
16	240	•	•	•	•					1	2	91
17	241	•	•	•	•				•	1	2	93
18	146	•			•			•		1	2	95
19	249	•	•	•	•	•			•	1	2	96
20	251	•	•	•	•	•		•	•	1	2	98
21	255	•	•	•	•	•	•	•	•	1	2	100

b

	Profile APCS code	rPhl p 1	rPhl p 2	nPhl p 4	rPhl p 5b	rPhl p 6	rPhl p 7	rPhl p 11	rPhl p 12	n	%	Cumm. %
1	184	•		•	•	•				8	19	19
2	248	•	•	•	•	•				6	14	33
3	176	•		•	•					5	12	44
4	250	•	•	•	•	•		•		5	12	56
5	32			•						3	7	63
6	128	•								2	5	67
7	160	•		•						2	5	72
8	240	•	•	•	•					2	5	77
9	16				•					1	2	79
10	144	•		•						1	2	81
11	162	•		•				•		1	2	84
12	186	•		•	•	•		•		1	2	86
13	224	•	•	•						1	2	88
14	226	•	•	•				•		1	2	91
15	249	•	•	•	•	•			•	1	2	93
16	255	•	•	•	•	•	•	•	•	1	2	95
17	0									2	5	100