

Determination of sIgE to rPhl p 1 is sufficient to diagnose grass pollen allergy

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Abstract

Background: New diagnostic tools such as the basophil activation test (BAT) and component-resolved diagnosis (CRD) are promising for hymenoptera venom or food allergy. A clear benefit for inhalant allergens has not yet been shown. Our aim was to compare new and established tests for grass pollen allergy.

Methods: Forty-nine patients with grass pollen allergy and 47 controls were prospectively enrolled in the study. A symptom score was calculated for each patient. Conjunctival provocation tests (CPT), skin prick tests (SPT), BAT, and sIgE determination including CRD were performed. Sensitivity and specificity were compared and results were correlated with the symptom score.

Results: Single determination of sIgE to rPhl p 1 showed the best balance between sensitivity (98%) and specificity (92%). Use of additional components, such as rPhl p 2 and 5, did not increase sensitivity. Generally, sensitivity of tests was high: SPT 100%, ISAC-112 100%, sIgE to timothy grass 98%, BAT 98%, ISAC-103 84%, and CPT 83%. Specificity ranged from 79% (SPT) to 96% (CPT). All test results and calculated values (e.g. ratio sIgE/tIgE) did not correlate with symptom severity. Asymptomatic sensitization to timothy grass in controls was rare in the CAP (11%) and predominantly due to Phl p 1 sensitization.

Conclusion: rPhl p 1 was sufficient to diagnose grass pollen allergy, and sIgE patterns were the same in symptomatically and asymptotically sensitized subjects. The testing of multiple components was of minor importance, and no test correlated with symptom severity.

During the last decade, significant progress in allergy diagnosis has been made. Recombinant technology as the basis of component-resolved diagnosis (CRD) was introduced into clinical routine and is about to improve the diagnosis of allergies caused by cross-reactive sIgE. Recombinant allergens have the advantage of lacking cross-reactive carbohydrate determinants (CCD), thus increasing the specificity of allergy diagnosis. For the same reason, allergen immunotherapy can be more accurately monitored. There is also potential to determine individual immunotherapy-induced IgG responses to different allergens (1).

Recently, biochip technology has been introduced that allows the simultaneous measurement of sIgE of multiple allergen components in a single test, using only a few µl of serum. Although the screening of sIgE is not recommended,

allergen chips may help to detect cross-reactivity. A major drawback of this method is the partial use of natural, glycosylated allergens; however, the commercially available immuno solid-phase allergen chip (ISAC; Thermo Fisher Scientific, Waltham, MA, USA) showed a high sensitivity of 97.7% and specificity of 92.3% in well-selected patients and controls (2).

Several studies also confirmed the usefulness of the CD63-based basophil activation test (BAT) as a routine diagnostic tool particularly for hymenoptera venom allergy (3–5) and as a valuable test in patients with inconclusive tests and history (6, 7). Compared with the IgE determination in the serum, the BAT has the advantage of demonstrating functional responses: positive test results will only occur after the successful cross-linking of two Fcε-receptor bound IgE

antibodies and not by monovalent binding as in IgE assays. Usually BAT results should not be affected by the presence of CCDs, but positive results may occur if multiple CCD epitopes are present.

In theory, both CRD and the BAT should have a higher specificity and at least the BAT as a functional test could correlate with symptom severity. However, new diagnostic tests are either sophisticated to perform or are more expensive that makes it important to evaluate the benefit of these tests.

The primary objective of this study was to evaluate whether CRD (biochip or conventional sIgE determination) and the BAT showed higher sensitivity and specificity in the diagnosis of grass pollen allergy. Furthermore, we aimed to evaluate whether the new techniques had higher potential in predicting symptom severity in allergic subjects when compared with established methods, and to identify sIgE patterns that might be representative for grass pollen allergic patients and asymptotically sensitized controls.

Methods

Patients and controls

Forty-nine patients with grass pollen allergy and 47 controls were prospectively enrolled after they signed a written informed consent (characteristics Table S1). Patient and control groups did not differ in gender distribution, but slightly in age. Although there was a statistically significant difference in age, it was not considered clinically relevant for the study.

Patients were recruited outside pollen seasons 2010 and 2011. Inclusion criterion was a conclusive history of grass pollen allergy (symptoms of allergic rhinitis and/or conjunctivitis during grass pollen season confirmed by skin prick test (SPT) and/or sIgE testing). Exclusion criteria were ongoing or completed immunotherapy with grass pollen allergens, and pregnancy or breast-feeding in females, but not sensitization to other inhalant allergens. The severity of symptoms was assessed by a simple rhinoconjunctivitis symptom score (RSS), using a verbal descriptor scale consisting of four levels of increasing symptom severity of the nose and eyes (VDS-4) (8).

Controls were defined by personal history; only subjects without symptoms in grass pollen season were included. 20 of 47 controls were positive for at least one allergen in the standard prick test panel and therefore considered to be atopic.

Skin prick tests, IgE determination (whole extract and components), and the BAT were performed in all patients and controls. Conjunctival provocation test (CPT) was performed in all but three patients who refused the test.

The study protocol was approved by the Institutional Review Board of the Medical University of Graz (23-025 ex 10/11).

IgE determination

Determination of sIgE was performed by ImmunoCAP (timothy grass, rPhl p 1, 2, 5, 7, 12) and ISAC (rPhl p 1, 2, n Phl p 4, rPhl p 5, 6, 7, 11, 12) (Thermo Fisher Scientific) according to the manufacturer's instructions. Levels

>0.35 kU/l were defined as positive test results. For multiplex analysis, the ISAC with 103 components (ISAC-103) was primarily used. After the introduction of an improved version with 112 components (ISAC-112), all patients were tested again to re-evaluate sensitivity and specificity. In singleplex assays, we additionally calculated the ratio of sIgE to total IgE, the so-called specific activity as described by Hamilton et al. (9).

Basophil activation test

Basophil CD63 up-regulation in response to stimulants was determined as previously described (4). Cell responsiveness to allergens was measured using five serial dilutions of purified six grass mixes (1 : 25, 1 : 50, 1 : 500, 1 : 5.000, 1 : 50.000–100.000 SQ-E/ml; ALK-Abello, Horsholm, Denmark). Cell samples were analyzed by three-color flow cytometry (FC 500, Beckman Coulter, Brea, CA, USA). For re-evaluation of the optimal threshold, receiver operating characteristic (ROC) analysis was performed. An approximately 2.6-fold increase in the number of activated basophils (>25.75%) as compared with the negative control (10%) at any of the test concentrations of the allergen (1 : 25–1:50.000) was considered to be a positive response. In addition, basophils were analyzed for allergen threshold sensitivity by measuring CD-sens. CD-sens is defined as the inverted value for the allergen concentration giving 50% of the maximum CD63% upregulation multiplied by 100 and used to describe a patient's allergen-specific sensitivity (10, 11).

Skin prick test

Skin prick tests were carried out in all individuals according to standard practice. The sensitization spectrum was determined by using extracts of 10 allergens: six grass mixes, *Dermatophagoides pteronyssinus*; *Dermatophagoides farinae*, cat and dog hair, ragweed, birch, mugwort, *Alternaria* and *Cladosporium* (ALK-Abello). Furthermore, we tested extracts of apple, peach, kiwi, peanuts, tomato, celery, carrot, soya, latex, and additionally profilin, polcalcin, and peach lipid transfer protein (LTP) as markers for cross-reactivity (all ALK-Abello, except kiwi extract from Stallergenes; Hauts-de-Seine, France). Prick tests were considered to be positive if a wheal of ≥ 3 mm in diameter with erythema was observed.

Conjunctival provocation (CPT)

Conjunctival provocation was carried out with a 5-grass pollen mix according to the manufacturer's instructions (ALK-Abello) and to general recommendations (12). A single drop of diluent placed in the conjunctival sac of one eye served as negative control. Afterward, single drops of two increasing concentrations of solutions (2 BU/ml and 10 BU/ml) were consecutively placed in the conjunctival sac of the other eye at intervals of 10 min. A positive reaction was reached when eye redness, weeping, itching, or burning were observed. A reaction exceeding the negative control was obligatory.

Statistical methods

Sample size calculation was conducted with the Z-test with pooled variance assuming specificities of 50% and 80% for the detection of sIgE with the CAP and the BAT, respectively. Continuous variables were described with median values, and lower and upper quartiles unless otherwise mentioned. Chi-square tests were performed for comparison of frequencies of categorical data. Two independent groups with nonparametric data were compared with the Mann-Whitney *U* test. McNemar's test was used for comparison of paired categorical variables. Bivariate correlations of nonparametric or ordinal scaled data were evaluated by calculating the Spearman rho. *P*-levels <0.05 were regarded as statistically significant. Statistical analysis was performed with IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL, USA).

Results

Sensitivity and specificity of diagnostic tests

Single determination of sIgE to Phl p 1 showed the best balance between sensitivity (98%) and specificity (92%; Table 1). Sensitivity of the BAT, CRD with rPhl p 1 (CAP), SPT, and sIgE determination to the whole extract of timothy grass was extensively consistent. Importantly, adding sIgE determination against recombinant allergens rPhl p 2 and 5 to rPhl p 1 in the CAP did not improve sensitivity because there was no sensitization profile to recombinant allergens without rPhl p 1. A significantly lower sensitivity of 83% was

calculated for the CPT and also for the ISAC-103 (84%; $P < 0.0001$); however, the improved version of the ISAC with 112 components reached a sensitivity of 100%. Having found that the sensitivity of the BAT and CRD was equal to established tests, we examined whether specificity was superior. Specificity of rPhl p 1 was similar to sIgE to timothy grass, but superior to that of the SPT. Sensitivity of the ISAC-112 was increased at the cost of specificity: specificity decreased from 92% to 83%. BAT specificity was as low as that of the SPT. Atopic status influenced specificity of most diagnostic tests.

Determination of sIgE to rPhl p 1 showed the best balance between positive predictive value (92%) and negative predictive value (NPV) (98%). A negative result in the SPT and the ISAC-112 was most reliable. Slightly lower NPV were obtained with the determination of sIgE to timothy grass and rPhl p 1 in CAP as well as with the BAT. Details on positive and NPVs are summarized in Table S2.

Correlation between test outcomes and symptom score

Median RSS was 10 (range 2–16). No correlation was observed between symptom score values and the maximum activation of the BAT (BAT max.) as well as the half-maximum activation (CD-sens) (Table 2). Neither levels of sIgE to timothy grass, rPhl p 1, 2, and 5 nor specific activities (ratio sIgE/tIgE) correlated with symptom severity during pollen season. We hypothesized that the total number of sensitizations to grass pollen allergens in the ISAC-112 may be correlated with the RSS. However, we did not find a

Table 1 Sensitivity and specificity of diagnostic tests

	Sensitivity (%), no. of patients			Specificity (%), no. of controls				
	Total	tIgE≤100	tIgE>100	Total	tIgE≤100	tIgE>100	Nonatopic	Atopic [†]
SPT	100	100	100	79	83	50	100***	50***
	49/49	21/21	28/28	37/47	34/41	3/6	27/27	10/20
CAP Timothy grass	98	95	100	89	93	67	100**	75**
	48/49	20/21	28/28	42/47	38/41	4/6	27/27	15/20
CAP rPhl p 1	98	95	100	92	93	83	100*	80*
	48/49	20/21	28/28	43/47	38/41	5/6	27/27	16/20
CAP rPhl p 5	55	43	46	100	100	100	100	100
	27/49	9/21	18/28	47/47	41/41	6/6	27/27	20/20
CAP rPhl p 1 + 2 + 5	98	95	100	92	93	83	100*	80*
	48/49	20/21	28/28	43/47	38/41	5/6	27/27	16/20
ISAC-112	100	100	100	85	90	50	100***	65***
	49/49	21/21	28/28	40/47	37/41	3/6	27/27	13/20
ISAC-103	84	76	89	92	95	67	100*	80*
	41/49	16/21	25/28	43/47	39/41	4/6	27/27	16/20
BAT	98	100	96	82	84	67	100***	58***
	45/46	20/20	25/26	36/44	32/38	4/6	25/25	11/19
	(3 NR excl.)	(1 NR excl.)	(2 NR excl.)	(3 NR excl.)	(3 NR excl.)		(2 NR excl.)	(1 NR excl.)
CPT	83	72	89	96	98	83	100	90
	38/46	13/18	25/28	45/47	40/41	5/6	27/27	18/20

NR, nonresponder.

* $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

[†]Positive for at least one allergen in the standard prick test panel.

Table 2 Correlations between diagnostic tests and between tests and symptom severity

A										
	tlgE	slgE TG	slgE rPhl p 1	slgE rPhl p 2	slgE rPhl p 5	BAT max.	CD-sens	CPT	SPT	RSS
tlgE	–	$r_s = 0.683$	$r_s = 0.514$	$r_s = 0.532$	$r_s = 0.464$	n.s.	n.s.	n.s.	n.s.	$r_s = 0.329$
slgE TG	$r_s = 0.683$	–	$r_s = 0.857$	$r_s = 0.666$	$r_s = 0.808$	n.s.	n.s.	$r_s = 0.504$	$r_s = 0.290$	n.s.
slgE rPhl p 1	$r_s = 0.514$	$r_s = 0.857$	–	$r_s = 0.475$	$r_s = 0.575$	n.s.	n.s.	$r_s = 0.321$	n.s.	n.s.
slgE rPhl p 2	$r_s = 0.532$	$r_s = 0.666$	$r_s = 0.475$	–	$r_s = 0.454$	n.s.	n.s.	$r_s = 0.474$	n.s.	n.s.
slgE rPhl p 5	$r_s = 0.464$	$r_s = 0.808$	$r_s = 0.575$	$r_s = 0.454$	–	n.s.	$r_s = 0.309$	$r_s = 0.478$	$r_s = 0.321$	n.s.
BAT max.	n.s.	n.s.	n.s.	n.s.	n.s.	–	$r_s = 0.456$	$r_s = 0.365$	n.s.	n.s.
CD-sens	n.s.	n.s.	n.s.	n.s.	$r_s = 0.309$	$r_s = 0.456$	–	$r_s = 0.364$	n.s.	n.s.
CPT	n.s.	$r_s = 0.504$	$r_s = 0.321$	$r_s = 0.474$	$r_s = 0.478$	$r_s = 0.365$	$r_s = 0.364$	–	n.s.	n.s.
SPT	n.s.	$r_s = 0.290$	n.s.	n.s.	$r_s = 0.321$	n.s.	n.s.	n.s.	–	n.s.
RSS	$r_s = 0.329$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	–
B										
	sA TG	sA rPhl p 1	sA rPhl p 2	sA rPhl p 5						
BAT max.	$r_s = 0.401$	$r_s = 0.337$	n.s.	$r_s = 0.343$						
CD-sens	$r_s = 0.397$	n.s.	$r_s = 0.456$	$r_s = 0.416$						
CPT	$r_s = 0.476$	$r_s = 0.299$	$r_s = 0.515$	$r_s = 0.484$						
SPT	n.s.	n.s.	n.s.	$r_s = 0.342$						
RSS	n.s.	n.s.	n.s.	n.s.						

n.s., statistically not significant; sA, specific activity; $r_s \leq 0.35$: weak correlation, 0.36–0.67: moderate correlation, 0.68–1.0: strong correlation.

statistically significant correlation ($r_s = 0.212$; $P = 0.163$). Additionally, there was no correlation between wheal diameters in the SPT and symptom severity, and patients reacting to different allergen concentrations in the CPT did not differ in symptom score. Only tlgE was weakly correlated with the RSS ($r_s = 0.329$). For test results of allergic subjects in detail, see Table S3.

Conjunctival provocation test

CPT was positive in 83% (38/46) among allergic subjects. Interestingly, all three patients who denied symptoms of allergic conjunctivitis were positively tested. On the other hand, 19% (8/43) were tested negative although they had a history of conjunctival symptoms.

Levels of sIgE to timothy grass were higher in subjects with positive CPT (13.10 kU/l (2.82; 23.70) vs 1.22 kU/l (0.58; 4.98); $P = 0.003$) than in patients with negative CPT. This observation was also made with respect to rPhl p 1 levels (7.50 kU/l (2.61; 17.00) vs 0.84 kU/l (0.63; 2.09); $P = 0.008$). Concerning rPhl p 5, a statistical trend was found (3.82 kU/l (0.00; 9.77) vs 0.01 kU/l (0.00; 1.48); $P = 0.052$). Subjects with positive CPT had significantly more sensitizations to recombinant grass pollen allergens in the CAP compared with subjects with negative CPT (3 vs 1; $P = 0.002$).

Sensitization patterns obtained by CRD

Interestingly, all patients positive for timothy grass in the CAP were sensitized to rPhl p 1 and monosensitization to rPhl p 1 was most frequently observed. Generally, the frequency of sensitizations to single components was consistent

with the literature (Fig. 1A) and did not differ between CAP and ISAC-112 (kappa 0.790–0.959) (Fig. 1A,B). Sensitization to Phl p 7 and 12 was rarely observed and always associated with sensitizations to the major allergens (Fig. 2). There was a significant lack of sensitivity of the ISAC-103 particularly in detecting sIgE to Phl p 1. Interestingly, total IgE of 16 Phl p 1 negative patients was significantly lower with 75.2 kU/l (35.3; 155.5) compared with 160.0 kU/l (74.7; 563.0) in 33 Phl p 1 positive patients ($P = 0.02$).

Asymptomatic sensitization

Among established tests, asymptomatic sensitization ranged from 11% (CAP timothy grass) to 21% in the prick test (Table S4). In the CAP, sensitization to Phl p 1 was again predominant, indicating that sensitization patterns do not differ between patients allergic to grass pollen and grass pollen sensitized controls.

Discussion

Determination of sIgE with rPhl p 1 or timothy grass showed the best balance between sensitivity and specificity among the evaluated tests. This is consistent with a previous report where a combination of Phl p 1, Phl p 5, and natural timothy extract identified grass pollen allergic patients with a sensitivity of 99.3% (13). It is somewhat surprising that the detection of circulating IgE antibodies, which are considered to be irrelevant for the allergic reaction, is better with respect to specificity than functional tests like the SPT and the BAT, where cross-linking of two identical cell-bound sIgE antibodies is required. The low sensitivity of the CPT, however, may

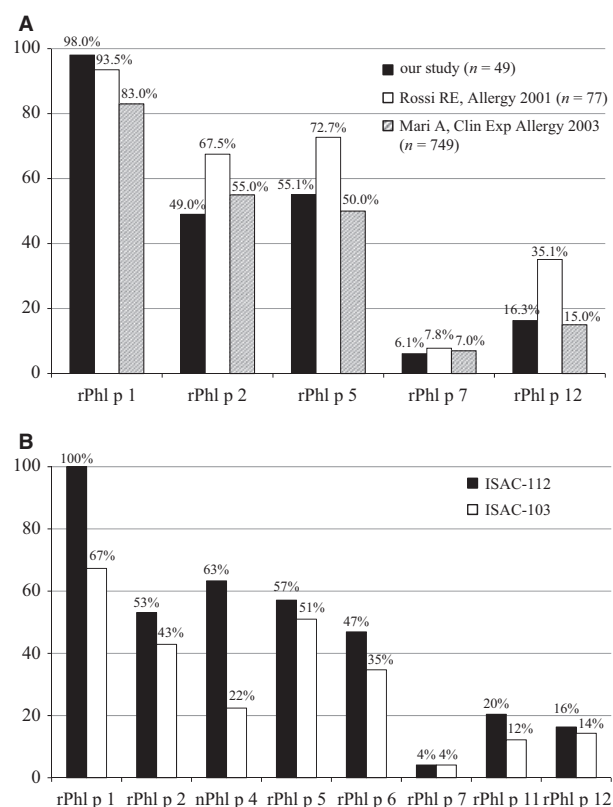


Figure 1 Frequency of sensitizations to grass pollen allergens in CAP and ISAC-112. (A) Frequency of sensitizations to single recombinant grass pollen allergens obtained by CAP compared with published data by Rossi et al. (30) and Mari et al. (31). (B) Frequency of sensitizations to single recombinant grass pollen allergens with the ISAC-112 and the ISAC-103.

be explained by the predominance of other symptoms such as rhinitis or asthma. Moreover, conjunctivitis can occur without direct ocular exposure to the allergen. Approximately 20% of seasonal allergic rhinitis patients have been reported to have ocular symptoms after allergen provocation only to the nasal mucosa mediated by a nasal ocular reflex (14). That could be the reason why the CPT was only positive in 83% of patients while 94% reported conjunctivitis during grass pollen season.

In contrast to hymenoptera venom allergy (15, 16), additional components (Phl p 2 and 5) did not increase the sensitivity of IgE determination. Interestingly, we did not observe any monosensitization to the pan-allergens Phl p 7 and Phl p 12 or to the combination of both. Therefore, the data do not support the recommendation to determine Phl p 7 and Phl p 12 in order to assess a patient's suitability for specific immunotherapy (17). Furthermore, this also suggests that profilins or polcalcins, although causing positive skin tests to many different types of pollen in sensitized subjects, rarely provoke respiratory symptoms during pollen exposure. However, this may be helpful in patients with suspected cross-reactivity to food due to profilin.

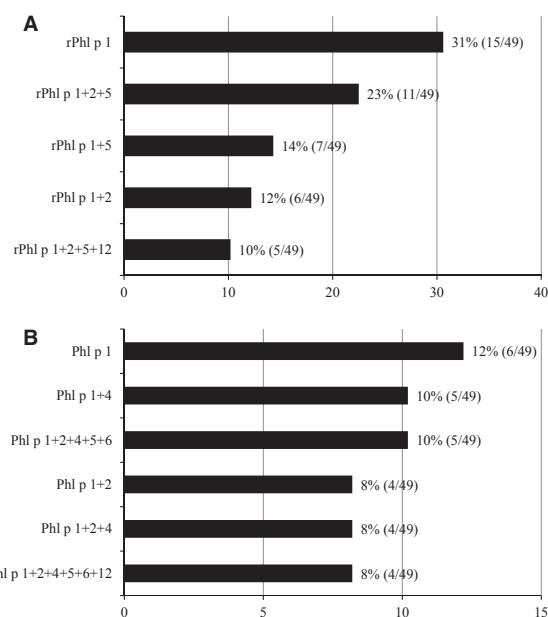


Figure 2 Sensitization patterns obtained by CAP and ISAC-112. (A) Five most frequent sensitization patterns to recombinant allergens with the CAP. (B) Six most frequent sensitization patterns to recombinant allergens with the ISAC-112.

We could not confirm the recently reported high sensitivity of 97.7% of the older version of ISAC (ISAC-103) for grass pollen allergy (2). Gadisseur et al. compared CRD with CAP and ISAC, and observed that 82 of 384 positive results in the CAP were negative in the ISAC (18) that endorses our results and a lack of sensitivity of the ISAC-103. However, ISAC has been improved and results of the new version are now well comparable with CAP results. According to the manufacturer's information, preparations of the components were optimized using new buffers and new concentrations resulting in higher sensitivities. Additionally, a new calibration procedure was established and the surface of the chip was modified.

Phl p 1, Phl p 4, Phl p 11, and Phl p 13 are known to be glycosylated (19, 20), so if antibodies against CCDs are present in the serum, patients should be false positive for the natural extract of timothy grass, but not for the CCD-free recombinant Phl p 1. Interestingly, this was only the case in one control; therefore, specificity of sIgE against rPhl p 1 and timothy grass was almost the same with 92% and 89%, respectively. Contrary to hymenoptera venom allergy, where CCDs are the major cause for clinically irrelevant (double) sensitization (21), specificity was virtually not affected. It has previously been shown that in a nonatopic population only 5% had sIgE to CCDs (22). Although controls were randomly selected, 43% were classified atopic (at least one positive allergen in the standard prick test panel). Nevertheless, prevalence of CCD-IgE (with the ISAC) was low, two allergic subjects and one of the controls were positive.

The BAT has the advantage of exerting functional responses upon allergen contact, but there are a few draw-

backs: It is time-consuming, expensive, and fresh blood is preferably needed. Recently, it has proven as a useful test in hymenoptera venom and food allergy (4, 21, 23). In the present study, the BAT did not add value when compared with other tests. Sensitivity was similar to that of SPT and sIgE determination to timothy grass or the recombinant allergen rPhl p 1, but specificity was even lower with 75% as previously mentioned. No data could be found concerning the BAT and timothy grass pollen allergy. In regard to cypress pollen allergy, a similar sensitivity but a higher specificity of 100% was found (24). The higher specificity may be a result of a highly selected control group where only healthy nonatopic subjects were included. In this study, we aimed to investigate all patients and atopic controls were not excluded.

Whether diagnostic tests can predict symptom severity is still a debated issue; in one study, nasal challenges and SPT were found to correlate with symptom scores (25), whereas more recently Radcliffe and colleagues could not find any correlation between seasonal symptom scores and preseasonal quantitative SPT or CPT (26). The BAT was found to correlate better with skin reactivity and IgE titers than with symptom score (27). In our study, a variety of tests were evaluated and it was attempted to correlate levels of sIgE, wheal diameter of the SPT, maximum activation in the BAT, as well as calculated results like specific activity (ratio sIgE/IgE) and CD-sens (half-maximum activation in BAT) with symptom severity. No test nor any calculated value was correlated with the RSS. Apparently, the situation is very similar to hymenoptera venom allergy, where no test is able to predict symptom severity (28). Maybe functional tests like assays of inhibitory IgG4 and IgE-blocking factor might add information as shown for the assessment of clinical response to grass pollen immunotherapy (29).

In conclusion, detection of sIgE to rPhl p 1 had the advantage of showing genuine sensitization and was sufficient to diagnose grass pollen allergy in our Central European study population. As cross-reactivity among different grass species is high (20), this finding may also apply to grass pollen allergic patients in other regions of the world. Specificity of the natural extract may be comparable with that of rPhl p 1; however, determination of rPhl p 1 should be preferably used as it cannot be influenced by the presence of sIgE to CCDs,

polcalcin or profilin. Virtually, all patients were sensitized to Phl p 1 and the frequency of positive sIgE to Ph p 1 was comparable to reported data in the literature. Sophisticated and costly approaches like multiplex assays, determination of multiple components, the BAT, and calculation of specific activity or CD-sens did not provide relevant additional information. However, multiplex testing may be useful in polysensitized patients to help in determining actual sensitizations and the presence of cross-reacting antibodies.

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Author contributions

DB and GS participated in all stages of the study and were the main authors of the manuscript. WA and AH contributed to the design of the study. All authors contributed with interpretation of the data, revised the article critically, and gave final approval.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Supporting Information

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

Table S1. Characteristics of patients and controls.

Table S2. Positive (PPV) and negative predictive values (NPV) of diagnostic tests.

Table S3. Test results in allergic subjects.

Table S4. Diagnostic characteristics in 10 subjects with putative asymptomatic sensitization.

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	Patients	Controls	p-value
Total (n)	49	47	-
Gender (n, %)			
Male	16 (33)	12 (26)	0.443
Female	33 (67)	35 (75)	
Age (median, quartiles)	28 (24; 34)	35 (27; 49)	0.013
Atopics (n, %)	49 (100)	20 (43)	-
Allergic symptoms (n, %)		-	-
Conjunctivitis only	2 (4)		
Rhinitis only	3 (6)		
Rhinoconjunctivitis	44 (90)		
Asthma bronchiale	11 (22)		
Sensitizations to inhalant allergens in standard SPT (n, %)			-
D. pt.	28 (57)	7 (15)	
D. f.	28 (57)	8 (17)	
Dog hair	13 (27)	6 (13)	
Cat hair	28 (75)	4 (9)	
Birch	25 (51)	8 (17)	
6 grass mix	49 (100)	10 (21)	
Ragweed	5 (10)	1 (2)	
Mugwort	14 (29)	0 (0)	
Alternaria	4 (8)	1 (2)	
Cladosporium	1 (2)	0 (0)	

	Positive predictive value	Negative predictive value
CPT	95%	85%
CAP rPhl p 1	92%	98%
ISAC-103	91%	84%
CAP Timothy grass	91%	98%
ISAC-112	88%	100%
BAT	85%	97%
SPT	83%	100%

ID	tIgE (kU/L)	sIgE TG (kU/L)	sIgE rPhl p 1 (kU/L)	sIgE rPhl p 2 (kU/L)	sIgE rPhl p 5 (kU/L)	BAT max. (% act.)	SPT – wheal diameter (mm)	CPT	RSS
1	428.00	13.10	17.00	4.35	0.00	85.6	6.0	Pos. at 2 BU/mL	9
2	65.00	2.99	1.44	0.00	1.65	85.2	8.5	-	7
3	45.40	13.10	4.79	0.00	3.97	98.0	10.0	Pos. at 10 BU/mL	9
4	7.16	1.69	1.22	0.00	0.94	98.5	9.5	Pos. at 2 BU/mL	12
5	85.30	2.01	3.91	0.00	0.00	98.0	7.5	-	16
6	396.00	74.70	16.00	5.52	43.30	99.5	14.0	Pos. at 2 BU/mL	11
7	170.00	2.04	0.54	1.17	0.00	65.7	6.0	Pos. at 10 BU/mL	9
8	923.00	19.60	32.40	0.02	0.02	72.1	8.5	Pos. at 10 BU/mL	15
9	531.00	80.10	40.90	9.96	58.90	74.8	9.5	Pos. at 10 BU/mL	14
11	72.20	4.28	11.60	0.55	0.00	28.0	5.0	Pos. at 10 BU/mL	12
14	37.70	0.56	0.18	0.00	0.00	78.0	7.5	Neg.	7
15	139.00	7.30	3.38	0.50	3.67	96.5	7.5	Pos. at 2 BU/mL	12
16	32.80	3.35	1.33	1.49	1.54	97.0	4.5	-	10
17	32.80	1.29	0.66	0.47	0.00	93.5	5.5	Pos. at 10 BU/mL	13
18	1378.00	26.50	5.64	11.10	5.42	99.0	12.0	Pos. at 2 BU/mL	6
19	605.00	23.70	7.51	0.21	10.70	88.0	6.5	Pos. at 2 BU/mL	13
20	1039.00	100.00	50.20	23.30	84.30	99.0	8.5	Pos. at 2 BU/mL	15
22	115.00	4.49	2.61	2.15	0.00	91.5	12.0	Pos. at 2 BU/mL	3
23	18.70	1.06	0.87	0.08	0.28	60.4	4.0	Pos. at 2 BU/mL	10
25	36.00	5.85	3.44	0.00	7.59	84.0	6.0	Pos. at 2 BU/mL	11
26	293.00	2.82	0.82	1.06	0.59	73.4	13.0	Pos. at 10 BU/mL	14
29	164.00	0.60	0.70	0.00	0.01	26.3	6.0	Neg.	5
30	45.60	9.84	3.89	2.01	5.49	89.0	14.0	Pos. at 2 BU/mL	9
32	131.00	8.84	9.45	1.00	5.02	83.5	12.5	Pos. at 2 BU/mL	2
35	321.00	22.10	11.00	2.10	11.20	97.5	4.0	Pos. at 2 BU/mL	10
38	147.00	2.91	0.93	0.00	3.35	22.9	6.5	Neg.	7
39	96.90	7.02	12.80	0.00	2.77	47.8	8.0	Neg.	14
40	966.00	35.20	8.17	0.07	10.60	40.5	9.5	Pos. at 2 BU/mL	10

41	107.00	8.62	19.70	0.00	0.00	NR	6.5	Pos. at 10 BU/mL	7
42	126.00	1.38	1.58	0.29	0.00	NR	5.5	Pos. at 2 BU/mL	8
43	33.10	6.55	10.60	0.06	0.00	NR	6.0	Pos. at 10 BU/mL	3
44	5000.00	37.70	24.40	16.10	5.78	91.0	8.0	Pos. at 10 BU/mL	7
45	58.50	0.89	0.71	0.20	0.00	34.2	6.5	Pos. at 10 BU/mL	7
48	166.00	70.50	57.90	7.80	19.20	100.0	6.0	Pos. at 2 BU/mL	10
52	160.00	17.60	6.24	2.75	5.78	64.8	7.0	Pos. at 10 BU/mL	13
53	1953.00	84.30	58.10	12.30	76.60	54.6	5.5	Pos. at 10 BU/mL	6
57	352.00	15.60	7.49	1.57	1.64	34.4	9.0	Pos. at 2 BU/mL	14
58	123.00	15.90	8.48	0.00	9.77	85.5	11.0	Pos. at 2 BU/mL	5
63	1027.00	100.00	100.00	100.00	56.10	89.2	10.5	Pos. at 2 BU/mL	14
64	563.00	21.40	24.20	2.34	0.01	69.5	5.0	Pos. at 2 BU/mL	15
66	302.00	7.26	0.74	0.10	0.18	47.0	6.5	Neg.	10
67	27.30	0.35	0.55	0.01	0.00	91.0	8.0	Neg.	11
72	42.40	1.08	2.91	0.01	0.00	83.0	6.5	Pos. at 10 BU/mL	4
74	74.70	0.70	1.89	0.00	0.00	90.0	5.0	Neg.	4
77	81.00	1.74	2.28	0.00	0.00	99.0	4.5	Neg.	9
79	29.80	1.25	1.42	0.00	0.00	66.3	8.5	Pos. at 10 BU/mL	12
83	35.30	15.90	9.49	1.53	7.61	99.7	9.0	Pos. at 2 BU/mL	12
92	95.00	14.50	6.17	2.98	6.21	95.3	5.5	Pos. at 2 BU/mL	10
96	167.00	0.51	0.38	0.13	0.00	43.0	7.0	Pos. at 10 BU/mL	13
Mean values, lower and upper quartiles	126.0 (45.6; 352.0)	7.3 (1.7; 19.6)	4.8 (1.3; 11.6)	0.3 (0.0; 2.2)	1.5 (0.0; 6.2)	85.4 (64.8; 96.5)	7.0 (6.0; 9.0)	-	10 (7; 13)

Controls (ID)	tIgE (kU/L)	sIgE TG (kU/L)	sIgE rPhl p 1 (kU/L)	sIgE rPhl p 2 (kU/L)	sIgE rPhl p 5 (kU/L)	sIgE rPhl p 7 (kU/L)	sIgE rPhl p 12 (kU/L)	ISAC- 112 [#]	BAT (BAT max.)	SPT	CPT
10	19.20	0.17	0.14	0.00	0.00	0.00	0.00	Neg.	NR	Pos.	Neg.
24	138	0.30	0.25	0.00	0.00	0.00	0.01	Pos.	Pos. (63.9 %)	Pos.	Neg.
46	88.50	19.00	38.90	0.01	0.00	0.00	0.01	Pos.	Pos. (91.9 %)	Pos.	Pos. at 2 BU/ml
56	19.40	0.55	0.71	0.00	0.00	0.00	0.00	Pos.	Pos. (90.5 %)	Pos.	Neg.
62	225.00	3.43	0.35	0.15	0.20	0.10	0.20	Pos.	Neg. (25.2 %)	Pos.	Neg.
65	74.90	0.33	0.02	0.19	0.00	0.00	0.01	Neg.	Pos. (33.9 %)	Pos.	Neg.
78	899.00	13.40	7.81	7.01	0.02	0.02	0.06	Pos.	Pos. (98.0 %)	Pos.	Pos. at 10 BU/ml
80	24.80	0.21	0.19	0.00	0.00	0.00	0.00	Pos.	Pos. (48.7 %)	Pos.	Neg.
84	40.30	0.44	0.68	0.02	0.00	0.00	0.01	Pos.	Pos. (43.8 %)	Pos.	Neg.
88	40.40	0.05	0.03	0.00	0.00	0.00	0.01	Neg.	Pos. (52.2 %)	Pos.	Neg.
Positive (n)	-	5	4	1	0	0	0	7	8	10	2

Table S1. Characteristics of patients and controls.

	Patients	Controls	p-value
Total (n)	49	47	-
Gender (n, %)			
Male	16 (33)	12 (26)	0.443
Female	33 (67)	35 (75)	
Age (median, quartiles)	28 (24; 34)	35 (27; 49)	0.013
Atopics (n, %)	49 (100)	20 (43)	-
Allergic symptoms (n, %)		-	-
Conjunctivitis only	2 (4)		
Rhinitis only	3 (6)		
Rhinoconjunctivitis	44 (90)		
Asthma bronchiale	11 (22)		
Sensitizations to inhalant allergens in standard SPT (n, %)			-
D. pt.	28 (57)	7 (15)	
D. f.	28 (57)	8 (17)	
Dog hair	13 (27)	6 (13)	
Cat hair	28 (75)	4 (9)	
Birch	25 (51)	8 (17)	
6 grass mix	49 (100)	10 (21)	
Ragweed	5 (10)	1 (2)	
Mugwort	14 (29)	0 (0)	
Alternaria	4 (8)	1 (2)	
Cladosporium			

	1 (2)	0 (0)	
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“Conjunctivitis only” and “Rhinitis only” means one without the other irrespective of the presence of asthma.

D. pt. *Dermatophagoides pteronyssinus*; D. f. *Dermatophagoides farinae*

Table S2. Positive (PPV) and negative predictive values (NPV) of diagnostic tests.

	Positive predictive value	Negative predictive value
CPT	95%	85%
CAP rPhl p 1	92%	98%
ISAC-103	91%	84%
CAP Timothy grass	91%	98%
ISAC-112	88%	100%
BAT	85%	97%
SPT	83%	100%

3 non-responders in the BAT were excluded.

Table S3. Test results in allergic subjects

ID	tIgE (kU/L)	sIgE TG (kU/L)	sIgE rPhl p 1 (kU/L)	sIgE rPhl p 2 (kU/L)	sIgE rPhl p 5 (kU/L)	BAT max. (% act.)	SPT – wheal diameter (mm)	CPT	RSS
1	428.00	13.10	17.00	4.35	0.00	85.6	6.0	Pos. at 2 BU/mL	9
2	65.00	2.99	1.44	0.00	1.65	85.2	8.5	-	7
3	45.40	13.10	4.79	0.00	3.97	98.0	10.0	Pos. at 10 BU/mL	9
4	7.16	1.69	1.22	0.00	0.94	98.5	9.5	Pos. at 2 BU/mL	12
5	85.30	2.01	3.91	0.00	0.00	98.0	7.5	-	16
6	396.00	74.70	16.00	5.52	43.30	99.5	14.0	Pos. at 2 BU/mL	11
7	170.00	2.04	0.54	1.17	0.00	65.7	6.0	Pos. at 10 BU/mL	9
8	923.00	19.60	32.40	0.02	0.02	72.1	8.5	Pos. at 10 BU/mL	15
9	531.00	80.10	40.90	9.96	58.90	74.8	9.5	Pos. at 10 BU/mL	14
11	72.20	4.28	11.60	0.55	0.00	28.0	5.0	Pos. at 10 BU/mL	12
14	37.70	0.56	0.18	0.00	0.00	78.0	7.5	Neg.	7
15	139.00	7.30	3.38	0.50	3.67	96.5	7.5	Pos. at 2 BU/mL	12
16	32.80	3.35	1.33	1.49	1.54	97.0	4.5	-	10
17	32.80	1.29	0.66	0.47	0.00	93.5	5.5	Pos. at 10 BU/mL	13

18	1378.00	26.50	5.64	11.10	5.42	99.0	12.0	Pos. at 2 BU/mL	6
19	605.00	23.70	7.51	0.21	10.70	88.0	6.5	Pos. at 2 BU/mL	13
20	1039.00	100.00	50.20	23.30	84.30	99.0	8.5	Pos. at 2 BU/mL	15
22	115.00	4.49	2.61	2.15	0.00	91.5	12.0	Pos. at 2 BU/mL	3
23	18.70	1.06	0.87	0.08	0.28	60.4	4.0	Pos. at 2 BU/mL	10
25	36.00	5.85	3.44	0.00	7.59	84.0	6.0	Pos. at 2 BU/mL	11
26	293.00	2.82	0.82	1.06	0.59	73.4	13.0	Pos. at 10 BU/mL	14
29	164.00	0.60	0.70	0.00	0.01	26.3	6.0	Neg.	5
30	45.60	9.84	3.89	2.01	5.49	89.0	14.0	Pos. at 2 BU/mL	9
32	131.00	8.84	9.45	1.00	5.02	83.5	12.5	Pos. at 2 BU/mL	2
35	321.00	22.10	11.00	2.10	11.20	97.5	4.0	Pos. at 2 BU/mL	10
38	147.00	2.91	0.93	0.00	3.35	22.9	6.5	Neg.	7
39	96.90	7.02	12.80	0.00	2.77	47.8	8.0	Neg.	14
40	966.00	35.20	8.17	0.07	10.60	40.5	9.5	Pos. at 2 BU/mL	10
41	107.00	8.62	19.70	0.00	0.00	NR	6.5	Pos. at 10 BU/mL	7
42	126.00	1.38	1.58	0.29	0.00	NR	5.5	Pos. at 2 BU/mL	8
43	33.10	6.55	10.60	0.06	0.00	NR	6.0	Pos. at 10 BU/mL	3

44	5000.00	37.70	24.40	16.10	5.78	91.0	8.0	Pos. at 10 BU/mL	7
45	58.50	0.89	0.71	0.20	0.00	34.2	6.5	Pos. at 10 BU/mL	7
48	166.00	70.50	57.90	7.80	19.20	100.0	6.0	Pos. at 2 BU/mL	10
52	160.00	17.60	6.24	2.75	5.78	64.8	7.0	Pos. at 10 BU/mL	13
53	1953.00	84.30	58.10	12.30	76.60	54.6	5.5	Pos. at 10 BU/mL	6
57	352.00	15.60	7.49	1.57	1.64	34.4	9.0	Pos. at 2 BU/mL	14
58	123.00	15.90	8.48	0.00	9.77	85.5	11.0	Pos. at 2 BU/mL	5
63	1027.00	100.00	100.00	100.00	56.10	89.2	10.5	Pos. at 2 BU/mL	14
64	563.00	21.40	24.20	2.34	0.01	69.5	5.0	Pos. at 2 BU/mL	15
66	302.00	7.26	0.74	0.10	0.18	47.0	6.5	Neg.	10
67	27.30	0.35	0.55	0.01	0.00	91.0	8.0	Neg.	11
72	42.40	1.08	2.91	0.01	0.00	83.0	6.5	Pos. at 10 BU/mL	4
74	74.70	0.70	1.89	0.00	0.00	90.0	5.0	Neg.	4
77	81.00	1.74	2.28	0.00	0.00	99.0	4.5	Neg.	9
79	29.80	1.25	1.42	0.00	0.00	66.3	8.5	Pos. at 10 BU/mL	12
83	35.30	15.90	9.49	1.53	7.61	99.7	9.0	Pos. at 2 BU/mL	12
92	95.00	14.50	6.17	2.98	6.21	95.3	5.5	Pos. at 2	10

								BU/mL	
96	167.00	0.51	0.38	0.13	0.00	43.0	7.0	Pos. at 10 BU/mL	13
Mean values, lower and upper quartiles	126.0 (45.6; 352.0)	7.3 (1.7; 19.6)	4.8 (1.3; 11.6)	0.3 (0.0; 2.2)	1.5 (0.0; 6.2)	85.4 (64.8; 96.5)	7.0 (6.0; 9.0)	-	10 (7; 13)

Table S4. Diagnostic characteristics in 10 subjects with putative asymptomatic sensitization.

Controls (ID)	tIgE (kU/L)	sIgE TG (kU/L)	sIgE rPhl p 1 (kU/L)	sIgE rPhl p 2 (kU/L)	sIgE rPhl p 5 (kU/L)	sIgE rPhl p 7 (kU/L)	sIgE rPhl p 12 (kU/L)	ISAC- 112 [#]	BAT (BAT max.)	SPT	CPT
10	19.20	0.17	0.14	0.00	0.00	0.00	0.00	Neg.	NR	Pos.	Neg.
24	138	0.30	0.25	0.00	0.00	0.00	0.01	Pos.	Pos. (63.9 %)	Pos.	Neg.
46	88.50	19.00	38.90	0.01	0.00	0.00	0.01	Pos.	Pos. (91.9 %)	Pos.	Pos. at 2 BU/ml
56	19.40	0.55	0.71	0.00	0.00	0.00	0.00	Pos.	Pos. (90.5 %)	Pos.	Neg.
62	225.00	3.43	0.35	0.15	0.20	0.10	0.20	Pos.	Neg. (25.2 %)	Pos.	Neg.
65	74.90	0.33	0.02	0.19	0.00	0.00	0.01	Neg.	Pos. (33.9 %)	Pos.	Neg.
78	899.00	13.40	7.81	7.01	0.02	0.02	0.06	Pos.	Pos. (98.0 %)	Pos.	Pos. at 10 BU/ml
80	24.80	0.21	0.19	0.00	0.00	0.00	0.00	Pos.	Pos. (48.7 %)	Pos.	Neg.
84	40.30	0.44	0.68	0.02	0.00	0.00	0.01	Pos.	Pos. (43.8 %)	Pos.	Neg.
88	40.40	0.05	0.03	0.00	0.00	0.00	0.01	Neg.	Pos. (52.2 %)	Pos.	Neg.
Positive (n)	-	5	4	1	0	0	0	7	8	10	2

Positive for at least one timothy grass pollen allergen.

NR: Non-responder

