

Skin test with a timothy grass (*Phleum pratense*) pollen extract vs. IgE to a timothy extract vs. IgE to rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5, rPhl p 6, rPhl p 7, rPhl p 11, and rPhl p 12: epidemiological and diagnostic data

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Summary

Background The diagnostic approach to grass pollen allergy is now possible by detecting specific IgE to its allergenic components.

Objective To compare the IgE reactivity to a timothy grass pollen extract with the IgE reactivity to eight allergenic components from the same source (Phl p 1, 2, 4, 5, 6, 7, 11, 12). Both were compared with the skin test reactivity to a timothy grass extract.

Methods A population survey was carried out by means of the skin test to identify grass-allergic subjects, and to characterize them in terms of demographic and allergological parameters. Seven hundred and forty-nine sera were available for IgE detection to a timothy extract, to the recombinant Phl p 1, 2, 5, 6, 7, 11, 12, and to native Phl p 4 and bromelain. Results were stratified by means of demographic and allergy parameters.

Results Ninety-five per cent of the sera had detectable IgE to the timothy extract. Prevalence of IgE reactivity increased from 86.8% to 93.3% as the number of combined reactive molecules rose from 2 to 8. Adjusted prevalences for each allergen were: rPhl p 1 = 83%, rPhl p 2 = 55%, nPhl p 4 = 70%, rPhl p 5 = 50%, rPhl p 6 = 44%, rPhl p 7 = 7%, rPhl p 11 = 43%, rPhl p 12 = 15%. Isolated reactivity to rPhl p 1 was 6%, whereas it was negligible for the remaining molecules. IgE reactivity prevalence and mean values differed when patients were stratified on the basis of their associated pollen reactivity and their skin test reactivity grade. No differences were found when age, symptom type and duration were considered. Up to eight-fold higher IgE concentrations were found when the sum of IgE to molecules was compared with IgE to the extract. Testing for the IgE reactivity to the glycan of the native Phl p 4 allergen showed a possible interference with prevalence and value estimation. Higher prevalence values were found in previously immunotherapy-treated patients.

Conclusions The use of a complete panel of grass allergenic molecules can mimic the current use of allergenic extracts, but new relevant information, such as individual pattern of reactivity, adjusted prevalence, correct specific IgE concentration, can be achieved only by means of discrete allergenic molecules.

Keywords allergy diagnosis, grass pollen extract, glycoproteins, IgE detection, native allergens, recombinant allergens, skin tests

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Introduction

Grass pollen allergy is the most common seasonal respiratory allergy world-wide, affecting 8 to 25% of the general population and causing bronchial, nasal and conjunctival symptoms [1]. Detection of grass pollen sensitization in epidemiological and clinical studies is currently carried out by means of skin testing or by serum IgE detection using allergenic extracts from one or more grass species [1]. Several grass species have been identified

as allergenic [2], but it has been shown how one or few of them can be useful for grass pollen allergy diagnosis [3, 4].

In the last two decades several allergenic components of grass pollen have been identified, characterized and cloned [2]. Studies have been performed by means of grass allergenic molecules using both skin testing [5, 6] and IgE detection [3, 4, 7–11], to define their usefulness compared with allergenic extracts in the diagnosis of grass pollen allergy. Most of the studies used group 1, 2 and 5 allergens and profilins, though a more complete panel of allergenic molecules has been suggested [10]. Recently, Rossi and co-workers reported the first study on a larger panel of grass allergens in an Italian cohort, selected on the basis of their IgE reactivity [12]. Using Phl p 5 as a marker of sensitization to the grass pollen family, we reported differences of the IgE

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reactivity prevalence to such molecules depending on how the pollen-allergic population is stratified [13].

In the present study the IgE reactivity to the timothy grass (*Phleum pratense*) pollen extract (Phl p E), and to eight of its allergenic components (Phl p 1, 2, 4, 5, 6, 7, 11, and 12) has been comparatively evaluated within a large population of grass-allergic subjects. Selection of the subjects was carried out on the basis of the skin prick test (SPT) reactivity to a Phl p E. Data, stratified on the basis of demographic, allergological and clinical criteria, suggest that the combination of the full panel of allergenic molecules could be as sensitive as the Phl p E in the identification of grass pollen-allergic subjects. No influence on the pattern of individual reactivity to the allergenic molecules seems to be due to the age, symptom type and duration, and associated mite sensitivity. Furthermore, original data, such as correct quantification of IgE, individual pattern of reactivity, relationship between skin test reactivity and the IgE reactivity, and redefinition of grouping criteria, could be added to the identification of the triggering allergenic source exclusively by means of allergenic molecules.

Materials and methods

Allergenic extracts, purified and recombinant allergens

A Phl p E (Allergopharma, Reinbek, Germany) was used for SPT. A complete panel of allergenic extracts from pollen and non-pollen sources, already used in a previous study [13], was tested as well. Bromelain for SPT was purchased from Sigma-Aldrich (Milan, Italy). Purified *E. coli*-expressed recombinant rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6, rPhl p 7, rPhl p 11 and rPhl p 12 were used for IgE detection, whereas nPhl p 4 and bromelain (nBRO) were tested as purified native molecules (Pharmacia, Uppsala, Sweden).

Patients, skin testing and sera

Unselected subjects ($n=4606$) presenting respiratory symptoms that suggested an allergic disease, and referring to the Allergy Unit were enrolled. Demographic (age and gender) and clinical (symptoms and duration) data were recorded for each patient. Patients underwent SPT, with a standardized procedure, for Phl p E, for all the other pollen and non-pollen extracts, and for bromelain. Bromelain was used at 500 µg protein/mL as already reported [13, 14]. A SPT result was considered positive when a weal, of at least 3 mm, greater than the negative control, was recorded 15 min after the application of the test. There were 1531 grass-sensitized patients (67.8% of the pollen-allergic population), age range 2 to 70 years, and a male:female ratio of 1.25. Grass sensitized patients were grouped on the basis of their associated reactivity to other pollens. Grass monosensitized subjects were 292 (19.1%), those with an associated reactivity to one to four other pollens were 864 (56.4%), whereas patients with a positive SPT to more than five pollens were 375 (24.5%). Positive grass SPT results were scored and recorded in four classes: class 1, when the weal diameter was 1/4 to 1/2 the histamine weal; class 2, when it ranged from 1/2 to about the same size as in the positive control; class 3, from equal to twice as large as the histamine skin reaction; and class 4, greater than twice the positive control.

The patients who had previously received a specific immunotherapy course were not excluded from the study. An immunotherapy course was considered when at least 1 year of treatment was achieved. All the treated patients received alum adsorbed extract.

After the SPT, 749 sera were obtained from all the patients who consented to blood sampling for an *in vitro* diagnostic procedure. Sera matched the age and sex distribution of the original population. Sera were stored at -20°C until required.

Informed consent for SPT and blood sampling was obtained by the patients, or by the parents or caregivers during the allergy consultation.

IgE detection

Serum IgE to recombinant and native molecules were detected by means of the CAP system, following the instructions of the manufacturer (Pharmacia). Values equal to or greater than 0.4 kU_A/L were considered positive. Range values for reference classes were those indicated by the manufacturer and reported in Fig. 6. Discrepancy of the results between a positive *in vitro* test to bromelain – a glycoprotein bearing carbohydrate moieties recognized by IgE – and a negative SPT to the same glycoprotein was indicative of the presence of cross-reactive carbohydrate determinants (CCDs) [13, 14].

Data and statistics

Data were stored by means of customized databases and retrieved by means of study-tailored query programs. Statistical analyses were carried out by means of the Prism package (Graphpad Software, San Diego, CA, USA). The Fisher's exact test, the Spearman's correlation test, and the Mann-Whitney test were applied where appropriate.

Results

Prevalence data

An IgE reactivity to Phl p E was recorded in 95.8% of the subjects selected on the basis of their SPT reactivity to the same extract (Fig. 1). IgE reactivity prevalence differed if patients were grouped on the basis of their associated reactivity with other pollens. IgE reactivity to Phl p E was 90% in patients sensitized only to grasses, 95.5% in the group reacting also to one to four other pollen families, and reaching 98.7% of IgE reactive sera in the multiple pollen-sensitized group. Three subjects missing detectable IgE had class 1 SPT reactivity, 23 were SPT class 2, three were SPT class 3, and two were SPT class 4. The percentage of subjects with detectable IgE decreased to 86.8% when the combined reactivity to either rPhl p 1 or rPhl p 5 was considered (Fig. 1). The same percentage steadily increased when rPhl p 2 (88.4%), rPhl p 2 and 12 (88.6%), and rPhl p 2, 4, and 12 (90.5%) were added (88.6%), but reached 93.3% when at least one of the eight allergens tested was recorded positive (Fig. 1).

Raw prevalence of the IgE reactivity for each of the molecules compared with the Phl p E-SPT reactivity are reported in Fig. 2 (white bars). As the prevalence of reactive sera increased for all the molecules in relation to the number of other associated pollen reactivity (Fig. 3), and the distribution of the sera within the population did not match the distribution of the patients in

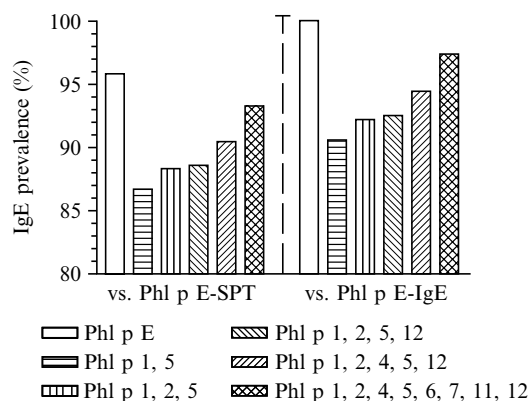


Fig. 1. Prevalence of the IgE reactivity to a *Phleum pratense* extract (Phl p E) compared with the prevalence of the reactivity to five different combinations (see graph legend) of *Phleum pratense* allergenic molecules in grass-allergic subjects (skin prick test positive to a *Phleum pratense* extract) (sera $n = 749$). Right part: different combinations vs. Phl p E SPT. Left part: different combinations vs. Phl p E IgE.

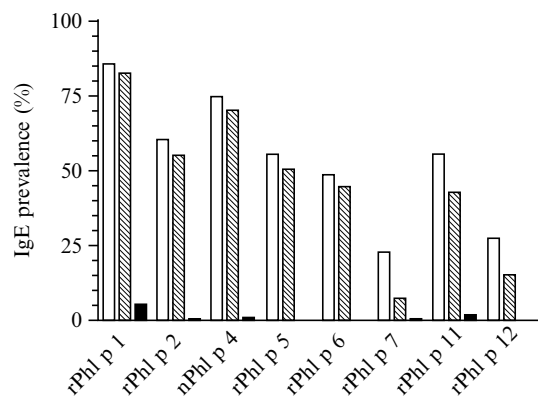


Fig. 2. Prevalence of the IgE reactivity to allergenic molecules from *Phleum pratense* (sera $n = 749$). White bars = raw prevalence. Hatched bars = adjusted prevalence for patients' distribution among pollen reactivity pattern subgroups. Black bars = prevalence of sera with IgE to a single molecule.

the three pollen SPT reactivity subgroups (data not shown), raw prevalences were reassessed (Fig. 2, hatched bars). A decrease of the raw prevalence for each molecule was recorded, more markedly for rPhl p 7 and rPhl p 12. Isolated IgE reactivity to each of the allergens was also evaluated (Fig. 2, black bars). rPhl p 1 was recorded positive without reactivity to any of the other grass molecules in 6% of the sera ($n = 43$), whereas only nPhl p 4 and rPhl p 11 among the remaining molecules had prevalence greater than 1% (eight sera, 1.1%, and 14 sera, 1.9%, respectively). When the 43 sera with an isolated reactivity to rPhl p 1 were grouped on the basis of other associated pollen reactivity, only 16 of the Phl p E-SPT monosensitized group were shown to be monosensitized on a molecular basis, whereas 27 had at least one more IgE reactivity to a molecule from another pollen source. An isolated reactivity was very rarely if ever recorded for the other molecules (rPhl p 2 = one serum, rPhl p 4 = three sera, rPhl p 5 = one serum, rPhl p 11 = four sera). An isolated IgE reactivity to rPhl p 6 was never recorded, as it was present in 87.3% of the rPhl p 5-reactive sera, with an increasing trend from grass monosensitized subjects to the multiple pollen-sensitized ones (75%, 84.3%, 90.8%).

rPhl p 1 was the molecule with the minor variation of its prevalence among patient subgroups based on their isolated SPT reactivity to grass or to grass associated with other pollens (Fig. 3, graph a). rPhl p 2, nPhl p 4, rPhl p 5, rPhl p 6 and rPhl p 11 prevalence were all below 50% when their prevalence in the monosensitized group was considered (Fig. 3, graphs b, c, d, e, g), but had a 1.5- to 2-fold increase or more in the multiple pollen-sensitized group. IgE reactivity to rPhl p 7 and rPhl p 12 was recorded only in the multiple pollen-sensitized subgroup (Fig. 3, graphs f, h). Along with the increase of the prevalence, the IgE median values increased. (Fig. 3, graphs a, b, c, d, e, g). Such an increasing trend was more evident for rPhl p 1 and rPhl p 2.

Demographic and clinical data

The male to female ratios of the patients with an IgE reactivity to the molecules were as follows: rPhl p 1 = 1.3, rPhl p 2 = 1.5, nPhl p 4 = 1.4, rPhl p 5 = 1.7, rPhl p 7 = 1.7, rPhl p 11 = 1.4, rPhl p 12 = 2. Only the rPhl p 12 ratio had a statistically significant difference (Fisher's exact test, $P > 0.05$).

Neither the mean age of the patients, and its distribution, differed among the groups (data not shown), nor the mean duration of seasonal symptoms (data not shown). Comparatively evaluating subgroups of patients with rhinitis and those having rhinitis and asthma, they differed only for the longer duration of the symptoms at the time of their allergy consultation, but no differences were recorded comparing their IgE reactivity with the eight molecules (data not shown).

IgE quantification

Grouping the grass-allergic patients on the basis of their skin test reactivity to Phl p E, the percentage of the IgE reactive molecules increased in relation to the skin reactivity to the Phl p E (Fig. 4). Though the individual values were quite dispersed in the whole range of the IgE CAP classes, mean values increased in relation to the increase of the skin reactivity (Fig. 5). Furthermore, the individual IgE values to the Phl p E were compared with the sum of the IgE reactivity to rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5, rPhl p 7, rPhl p 11 and rPhl p 12. A statistical significant difference was recorded between median values of the two groups (Mann-Whitney test: $P < 0.001$), but sera were dispersed in a wide range (data not shown). A further analysis of the data grouping the sera on the basis of their CAP classes showed no statistical significant differences between median values in classes 1 to 3 (Fig. 6, graphs a–c), whereas increasing statistical differences were recorded in CAP classes 4 to 6 (Fig. 6, graphs d–f). Summed values in the class 6 group exceeded three to eight times the IgE detected with Phl p E (Fig. 6, graph f). Subjects with detectable IgE to Phl p E and with undetectable IgE to molecules were all in the CAP classes 1 and 2 (Fig. 6, graphs a and b).

Influence of glycosylation, immunotherapy and mite sensitization

Apart from recombinant non-glycosylated molecules, glycosylated Phl p 4 was used as purified native allergen [15]. Reactivity of the glycan side chain of the molecule with CCDs-IgE was evaluated by testing 28 sera of patients with a known reactivity to CCDs [16], but with a negative SPT to the Phl p 4-containing Phl p E (Oliver Cromwell, Allergopharma, personal

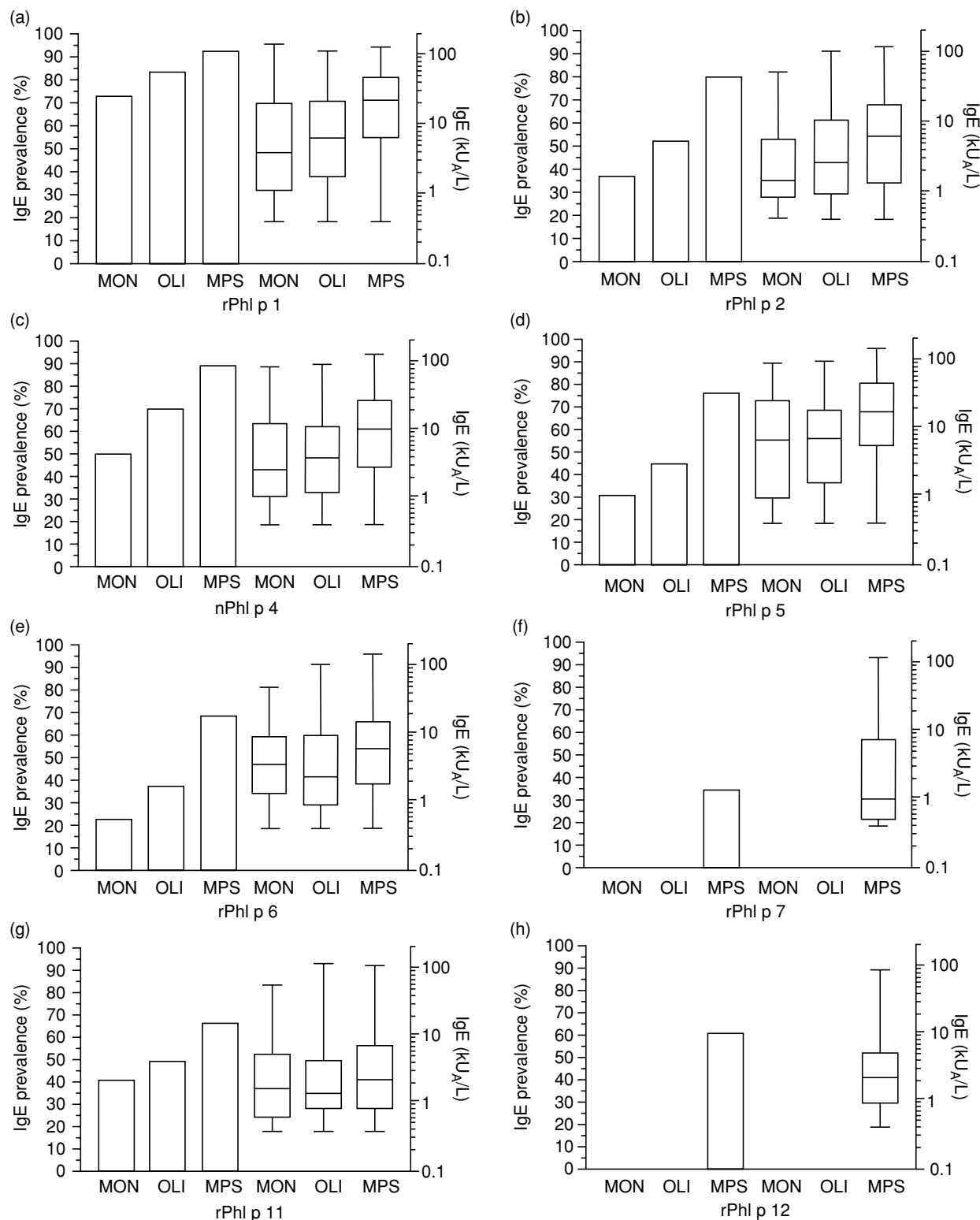


Fig. 3. Prevalence of the IgE reactivity to allergenic molecules from *Phleum pratense*. Subjects are grouped on the basis of their SPT reactivity to: MON = grasses ($n = 122$); OLI = grasses associated with one to four other pollen families ($n = 312$); MPS = more than five pollen families including grasses ($n = 315$). Right part of the graphs: prevalence of the IgE reactivity. Left part of the graphs: median and range of the IgE values.

communication) [17]. Figure 7 (graph a) shows 54% of these sera reacting with the nPhl p 4, compared with none of a group of 32 sera randomly chosen among patients lacking CCDs-IgE

reactivity. Negative results with the 28 CCDs-reactive sera were obtained when tested on non-glycosylated rPhl p 1 and rPhl p 5 (data not shown). Moreover, 15 sera carefully characterized in

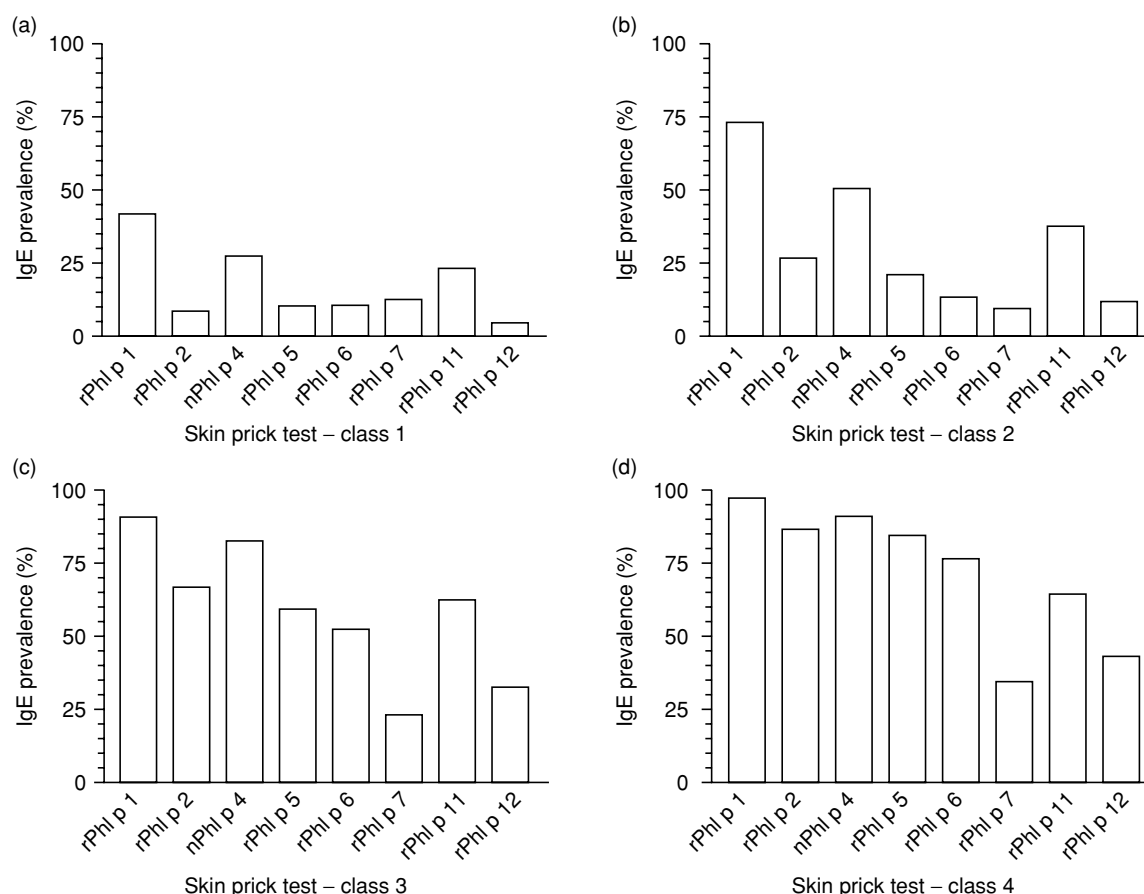


Fig. 4. Prevalence of the IgE reactivity to allergenic molecules from *Phleum pratense*. Subjects ($n = 749$) are grouped on the basis of their skin reactivity to the test with the *Phleum pratense* extract: graph a = SPT class 1 ($n = 33$); graph b = SPT class 2 ($n = 154$); graph c = SPT class 3 ($n = 324$); graph d = SPT class 4 ($n = 238$).

terms of CCDs-IgE reactivity [14], with IgE values to bromelain greater than $10 \text{ kU}_A/\text{L}$, were all positive when tested on nPhl p 4 (data not shown). Figure 7 (graph b) shows plotted paired IgE values to nBRO vs. nPhl p 4 of Phl p E SPT-reactive subjects. Although a statistical correlation was found between the two groups, 203 sera were positive to nPhl p 4 without an IgE reactivity to CCDs (range of IgE values 0.4 to 89), whereas 50 (range of IgE values 0.4 to 12.8) had IgE to CCDs but didn't bind to nPhl p 4.

Fourteen per cent of the sera were from patients who previously underwent an immunotherapy course. Comparative evaluation showed a higher prevalence of the IgE reactivity among sera of treated subjects for all the tested molecules (Fig. 8). To a different extent, values were all statistically significant.

Sixty per cent of the sera were from patients who had an associated mite sensitization. No differences were recorded between the patients with or without mite allergy for all the molecules but rPhl p 11 (data not shown).

Discussion

The present study has been carried out to examine, under a molecular perspective, several aspects of the IgE reactivity to grass pollen in subjects with SPT reactivity to a grass pollen

crude extract. Comparative studies between the IgE reactivity to a grass extract and different combination of grass allergenic molecules have been carried out on sera tested positive for a grass extract. Sera were from cohorts ranging from 98 to 276 patients. Prevalences ranging from 90.8% to 99% were reported [3, 7, 10, 11], with highest values when profilin and nPhl p 4 were added to grass group 1, 2 and 5 allergens [7, 10]. In our larger cohort of patients we found lower prevalences when identical combinations of molecules were compared with Phl p E IgE, with the highest sensitivity (97.3%) obtained when the IgE reactivity to a combination of all the eight molecules was considered. Completing the panel of grass allergens [2, 18] will certainly lead to a further increase of the sensitivity of molecule-based grass allergy diagnosis.

Several factors could lead to different results among studies. As reported by Laffer and co-authors [10], differences in the prevalence of the IgE reactivity to single grass allergenic components could be linked to geographical variation of the tested cohorts, but from our findings it seems that other factors can influence prevalence studies on molecule IgE reactivity. The preliminary observation that grouping pollen-allergic patients on the basis of their associated reactivity to other pollen sources [13] allows us to detect an increasing trend to react to several allergens was confirmed in the present study for all the tested allergens. Furthermore, a steady increase of the prevalence for all the molecules was recorded when grouping patients on the

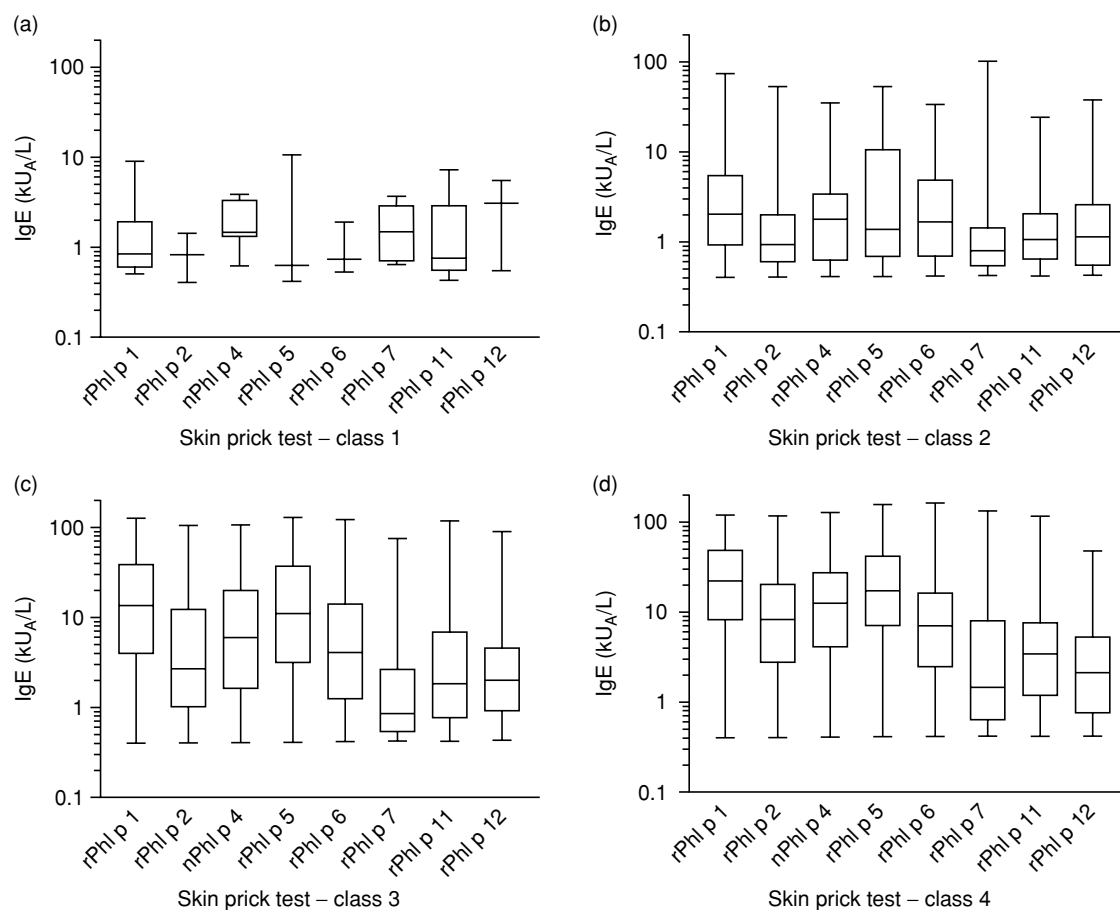


Fig. 5. Mean values of the IgE reactivity to allergenic molecules from *Phleum pratense*. Subjects ($n = 749$) are grouped on the basis of their skin prick test reactivity to the *Phleum pratense* extract: graph a = SPT class 1 ($n = 33$); graph b = SPT class 2 ($n = 154$); graph c = SPT class 3 ($n = 324$); graph D = SPT class 4 ($n = 238$).

basis of their skin test reactivity to Phl p E. Such observations suggest that discrepant results among studies could be also due to different allergological selection criteria of enrolled patients.

The use of native glycosylated allergens (nPhl p 4 in the present study) could also bias prevalence studies. In fact, the reactivity to the glycan side-chain of some native molecules by sera having IgE to CCDs [19], but lacking a reactivity to the protein backbone, could lead to an overestimation of the IgE reactivity, resembling biased results obtained with allergenic extracts [14, 16]. Our findings on the nPhl p 4 IgE reactivity with sera from patients lacking grass SPT reactivity are in favour of such an hypothesis. Comparative studies between native glycosylated and recombinant non-glycosylated allergens [3, 18–20] could be useful to define the impact of IgE to CCDs in the molecule-based diagnosis. The use of *E. coli* recombinant non-glycosylated 'glycoproteins' (like rPhl p 1, rPhl p 11 in the present study) could be useful to avoid the glycan side-chain reactivity [21], though the IgE reactivity could be negatively influenced by the absence of some relevant post-translational modifications [3, 20, 22].

Rossi and co-workers [12] carried out a study using the same panel of native and recombinant grass molecules in an Italian cohort of 77 subjects, using the CAP system. In our larger cohort of patients, adjusted values showed lower prevalence for almost all the eight molecules (rPhl p 1: 93.5 vs. 82.7;

rPhl p 2: 67.5 vs. 54.9; nPhl p 4: 88.3 vs. 70.1; rPhl p 5: 72.7 vs. 50.3; rPhl p 6: 68.8 vs. 44.3; rPhl p 7: 7.8 vs. 7.4; rPhl p 11: 53.2 vs. 42.7; rPhl p 12: 35.1 vs. 15.2). One or more of the biases reported above could prevent any correct comparison among studies, whereas some other factor seems to be less relevant in this regard (e.g. age, gender, symptoms and their duration, associated mite sensitization).

Our findings and those from Rossi and co-workers [12] show that other grass allergens like group 4 [23] and 11 [24], may be as important as groups 1, 2 and 5 for the diagnosis and therapy. Furthermore, a variable combination of several molecules characterizes the individual pattern of reactivity of grass-allergic patients [2, 11, 25]. It is important to notice that a minority of the patients is sensitized to a single molecule, and that in the grass monosensitized group reactivity to rPhl p 1 is generally high in terms of prevalence, whereas the IgE reactivity to other 'major' grass allergens seems to be less frequent. Such data would greatly influence clinical design of immunotherapy strategies with recombinant molecules.

Further helpful information comes from our stratified population survey using grass allergenic molecules. Phl p 4 has been reported to have a sequence homology with Amb a 1, the major allergen from ragweed pollen, and with other molecules with a pectate lyase activity [23]. Using monoclonal antibodies and two sera from patients with both grass and weed pollen allergy,

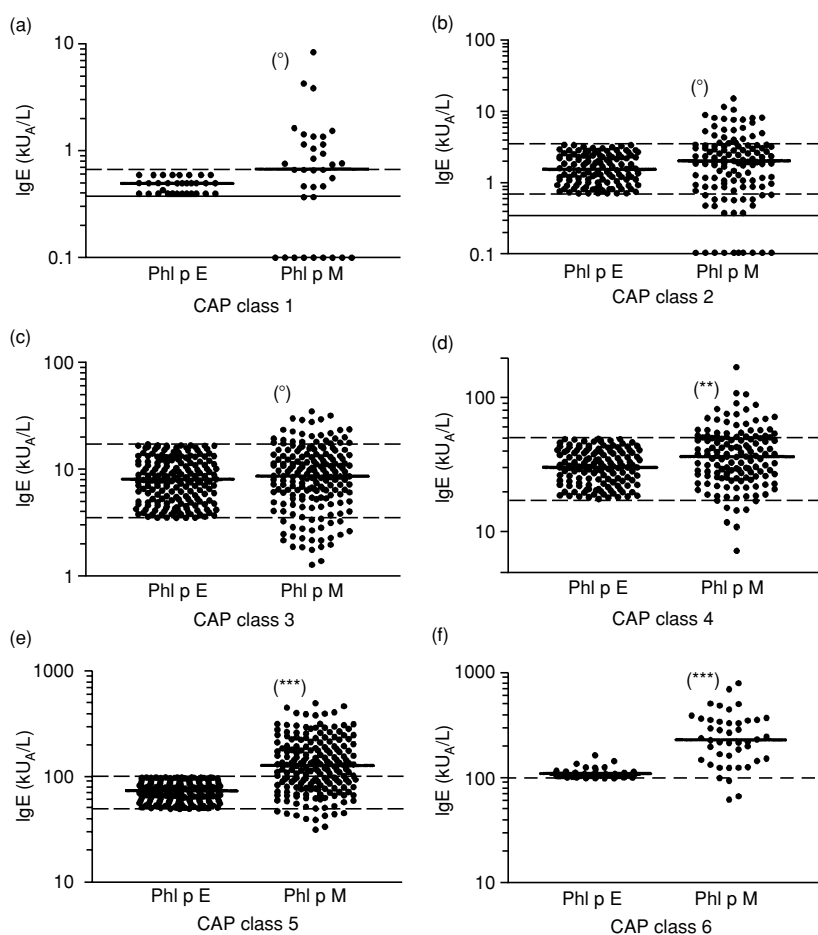


Fig. 6. Comparative evaluation of the IgE values to a *Phleum pratense* extract (Phl p E) and the sum of the IgE reactivity to rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5, rPhl p 7, rPhl p 11, and rPhl p 12 (Phl p M). Subjects ($n = 718$) are grouped on the basis of their CAP class values to the *Phleum pratense* extract. Graphs: a = CAP class 1, range values 0.35 to 0.7 ($n = 35$); b = CAP class 2, range values 0.7 to 3.5 ($n = 121$); c = CAP class 3, range values 3.5 to 17.5 ($n = 188$); d = CAP class 4, range values 17.5 to 50 ($n = 139$); e = CAP class 5, range values 50 to 100 ($n = 191$); f = CAP class 6, range values > 100 ($n = 44$). Parallel dashed lines indicate class range. Continuous line indicates negative cut-off. Statistics: Mann-Whitney test: °NS; * $P < 0.001$; ** $P < 0.0004$; *** $P < 0.0001$.

the same authors showed an immunological cross-reactivity between Phl p 4 and proteins from a ragweed extract. These findings are in contrast with our data. In the grass monosensitized group, excluding by definition patients reactive also to the ragweed extract, 49.9% of the subjects reacted to nPhl p 4, 70% lacking an IgE reactivity to CCDs (data not shown). Furthermore, a homologous allergen with a pectate lyase activity has been described in the pollen of the Cupressaceae and Taxodiaceae families [26], but the grass monosensitized patients lacked Cupressaceae reactivity as well. From an epidemiological point of view we may thus exclude that Phl p 4 is a panallergen-like molecule as its sequence homology would suggest [23]. Group 11 allergens (e.g. Lol p 11, Phl p 11) have been described as having a sequence homology with Ole e 1, the major allergen from olive pollen [24]. Because of such sequence homology, authors have suggested a possible role of Lol p 11 as a panallergen, but, as for nPhl p 4, rPhl p 11 was positive in 41.3% of grass monosensitized patients, thus excluding reactivity to the olive extract and its possible role as a panallergen. The non-glycosylated nature of the *E. coli*-expressed rPhl p 11 excluded any bias in the IgE detection caused by the CCDs present on the native molecule [24].

Nevertheless, the presence of the glycan moiety of nPhl p 4 has been useful when the comparative study between IgE to Phl p E and the sum of the IgE reactivity to the allergenic molecules has been carried out. Several studies showed that Phl p E or other grass pollen extracts bind specific IgE to CCDs

[14, 16, 19]. The presence of the carbohydrate side-chain on nPhl p 4 allowed us to detect such IgE, completing the panel of epitopes for a correct quantitative comparison between Phl p E and the sum of the IgE reactivity to the tested molecules.

Although Phl p 6 represents a distinct molecule from Phl p 5 [27], it has been shown to share some epitopes with group 5 molecules [28]. From our data it seems that IgE reactivity to Phl p 6 is most likely to be dependent on that to Phl p 5 as it was always found positive when rPhl p 5 IgE reactivity was detected. If such a close relationship will be confirmed by further inhibition studies, Phl p 6 could be thus excluded at least by the diagnostic panel of grass allergenic molecules. For the same reason, it was excluded by the comparative evaluation of the IgE concentration to the Phl p E vs. the sum of the remaining seven allergenic molecules.

Panallergens, like profilins (Phl p 12) and calcium-binding proteins (Phl p 7), have been described as relevant IgE-reactive molecules in a large number of plant sources [29, 30]. In fact, patients allergic to either profilin or calcium-binding allergens have a particular pattern of sensitivity defined as 'multiple pollen sensitization' [13]. Although in the present study the lowest IgE reactivity prevalences were recorded for rPhl p 7 and rPhl 12, data confirm that they are useful to define the pattern of sensitization rather than the reactivity to a defined pollen source *per se*. Moreover, the highest IgE reactivity prevalences for all the tested molecules were recorded within patients with the multiple pollen sensitization, suggesting that

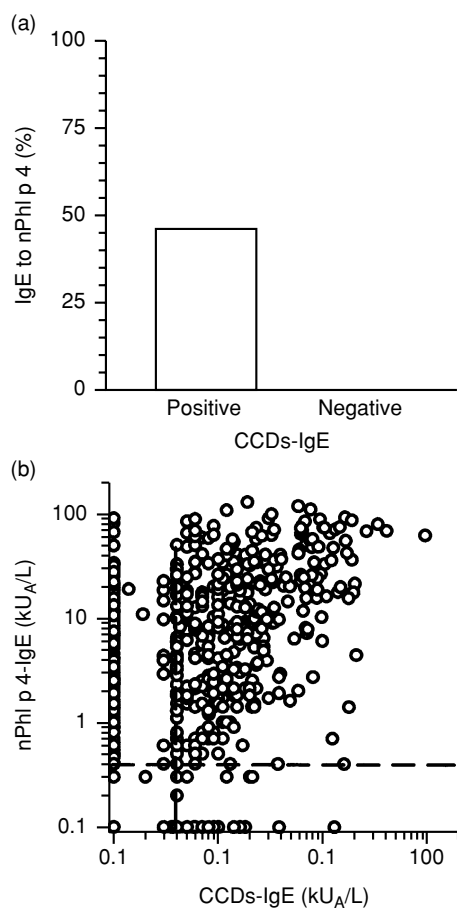


Fig. 7. Influence of IgE reactivity to cross-reactive carbohydrate determinants (CCDs) on IgE reactivity to native glycosylated molecules. Graph a = comparative test on nPhl p 4 of positive ($n = 28$) and negative ($n = 32$) cross-reactive carbohydrate determinants (CCDs) IgE sera from patients with negative SPT to Phl p E. Statistics: Fisher's exact test, $P < 0.0001$. Graph b = dot-plot graph of nPhl p 4-IgE vs. CCDs-IgE ($n = 749$); dashed lines indicate negative cut-off. Statistics: Spearman's correlation test: $r_s = 0.54$, CI 0.49 to 0.59, $P < 0.0001$.

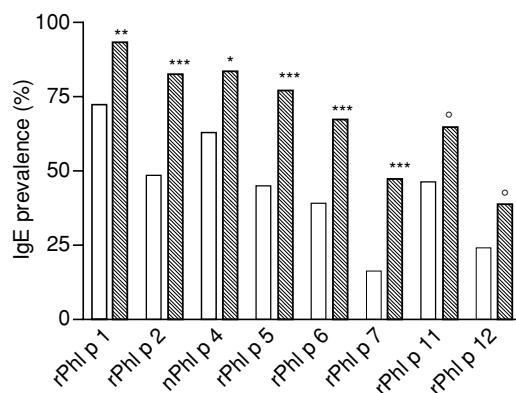


Fig. 8. Influence of grass immunotherapy on IgE reactivity to grass allergens. White bars = untreated subjects ($n = 645$). Hatched bars = immunotherapy-treated subjects ($n = 104$). Statistics: Fisher's exact test: $^{\circ}P < 0.03$; $^*P < 0.02$; $^{**}P < 0.01$; $^{***}P < 0.0001$.

such patients, due to a still unknown immunological mechanism, are at risk for developing an IgE reactivity to a huge number of allergenic molecules. This hypothesis is further confirmed by the parallel increase of the mean values of the IgE

concentration in the same group of multiple pollen-sensitized patients.

Specific IgE quantification by means of allergenic molecules showed how the use of allergenic extracts could limit their correct estimation. In fact, summing the IgE reactivity of available grass allergenic molecules and epitopes, and avoiding overlapping detections, two to eight times higher IgE values than the Phl p E were recorded. A possible explanation for this is the limiting factor represented by the amount of allergenic and non-allergenic components in an extract. Sera displaying IgE reactivity to the Phl p E in the higher CAP classes were more affected by such phenomena. More marked discrepancies could be recorded by adding other grass molecules [2, 18]. Studies showing a good correlation between IgE reactivity to a grass extract and to the sum of a limited number of molecules should be regarded as incomplete [4, 11]. Studies showing how few allergenic molecules account for a large part of the IgE reactivity to an extract should take into account the limiting effect of the amount of different components within the extract itself [2–4]. On the other hand, native allergens present in the Phl p E seem to work better in sera with lower IgE concentrations (CAP classes 1–3). The differences between natural and recombinant allergen in the post-translational modification and incorrect folding could explain the lower sensitivity of the recombinants in such a range [3, 20].

Studies have been performed to show the influence of crude grass extract immunotherapy on the allergenic molecule IgE reactivity [31, 32]. *De novo* induction of IgE to new allergens or epitopes has been reported by the same authors. In a previous study we found a higher number of patients who received an immunotherapy course within a subset of multiple pollen-sensitized patients showing an IgE reactivity to a calcium-binding protein (Jun o 4, previously Jun o 2) when compared with those reactive to birch profilin (Bet v 2) [13]. In the present study, a higher prevalence of the IgE reactivity to all the molecules has been recorded in treated patients. The major difference was recorded for the calcium-binding grass allergen (Phl p 7), confirming similarity among calcium-binding allergens [30]. As suggested by other authors [31], placebo-controlled prospective studies should be carried out in order to avoid any bias due to cross-sectional study design.

In conclusion, besides the use of allergenic molecules to mimic the current use of allergenic extracts, relevant information can be achieved only by means of the most complete panel of discrete allergens. Future work should be addressed to complete the panel of available grass molecules, to carefully define their reactivity in epidemiological and allergological terms, to try to select useful native or recombinant molecules with less interfering factors on their IgE reactivity.

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