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Article type : Original Article: Airway Diseases

Handling AE: Reto Crameri

Diagnostic relevance of IgE sensitization profiles to eight recombinant *Phleum pratense* molecules

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Abstract

Background – Grass pollen–related seasonal allergic rhinoconjunctivitis (SARg) is clinically heterogeneous in severity, comorbidities and response to treatment. The component-resolved diagnostics disclosed also a high heterogeneity at molecular level. Our study aimed at analyzing the characteristics of the IgE sensitization to *Phleum pratense* molecules and investigating the diagnostic relevance of such molecules in childhood.

Methods - We examined 1120 children (age 4–18y) with SARg. Standardized questionnaires on atopy were acquired through informatics platform (AllergyCARD^{TM}). Skin prick tests were performed with pollen extracts. Serum IgE to airborne allergens and eight *Phleum pratense* molecules (rPhl p 1, rPhl p 2, rPhl p 4, rPhl p 5b, rPhl p 6, rPhl p 7, rPhl p 11, rPhl p 12) were tested by ImmunoCAP FEIA.

Results - The analysis of IgE responses against eight *Phleum pratense* molecules showed 87profiles. According to the number of molecules recognized by IgE, the more complex profiles were characterized by higher serum total IgE, higher grass-specific serum IgE and higher number and degree of sensitization to pollens. The most frequent IgE sensitization profile was the monomolecular Phl p 1. Sensitization to Phl p 7 was a reliable biomarker of asthma, whereas Phl p 12 of oral allergy syndrome. Sensitization to Phl p 7 was associated with a higher severity of SAR, and complex profiles were associated with longer disease duration.

Conclusions - In a large pediatric population, the complexity of IgE sensitization profiles against *Phleum pratense* molecules is related to high atopic features although useless for predicting the clinical severity. The detection of serum IgE to Phl p 1, Phl p 7 and Phl p 12 can be used as clinical biomarkers of SARg and comorbidities. Further studies in different areas are required to test the impact of different IgE molecular profiles on AIT response.

Keywords

Allergy; children; component-resolved diagnostics; Phleum pratense; IgE sensitization profiles

Abbreviations

SAR Seasonal Allergic Rhinoconjunctivitis

SARg Grass pollen-related Seasonal Allergic Rhinoconjunctivitis

ARIA Allergic Rhinitis and its Impact on Asthma

CI Confidence Interval

GINA Global Initiative for Asthma

I-PAN Italian Pediatric Allergy Network

ISAAC International Study of Allergy and Asthma in Childhood

OAS Oral Allergy Syndrome

OR Odds Ratio

PAN-PED Panallergens in Pediatrics

PFS Pollen-Food Syndrome

SAR Seasonal Allergic Rhinitis

SD Standard Deviation

SIT Specific Immunotherapy

SPT Skin Prick Test

Conflict of interest

Disclosure of potential conflict of interest: S. Tripodi has received a lecture fee from Thermo Fisher (Phadia) and he is cofounder of TPS Production. A. Dondi has received consultancy fees from Charité University Hospital, Berlin, Germany. C. Mastrorilli has received fellowship from Italian Society of Pediatrics. P. M. Matricardi has received research support from TFS and lecture fees from TFS and Allergopharma. The rest of the authors declare that they have no relevant conflicts of interest.

Funding sources

Reagents for this study have been kindly provided by ALK-Abello' (Milan, Italy) (skin test reagents) and ThermoFisher Scientific (Uppsala, Sweden) (IgE assays); the Informatics Platform "AllergyCARD™" has been kindly provided by TPS Production (Rome, Italy).

Introduction

Grass pollen–related seasonal allergic rhinoconjunctivitis (SARg) affects millions of people worldwide with a noteworthy health (1) and economic burden.(2) Severity, comorbidities (e.g. asthma, oral allergy syndrome - OAS) and response to pharmacotherapy and to allergen immunotherapy (AIT) can vary among patients. (3) The causes of this clinical heterogeneity are unknown and serological biomarkers of disease endotypes are currently missing.(4) The analysis of IgE sensitization at molecular level (so-called component-resolved diagnostics, CRD) disclosed a high heterogeneity of the molecular IgE sensitization profiles to grass pollen.(5) This observation might explain clinical diversity (6) and raise the hypothesis that IgE to individual molecules may serve as predictive biomarkers of specific clinical phenotypes and of disease evolution.(7) (8)

In Europe, eight molecules of *Phleum pratense*, officially listed by the IUIS Allergen Nomenclature Sub-Committee, are frequently used in studies of grass pollen allergy.(9) They include grass specific molecules, such as rPhl p 1, rPhl p 2, rPhl p 5 and rPhl p 6, and cross-reactive molecules, such as nPhl p 4, rPhl p 7, rPhl p 11 and rPhl p 12.(10) Measurement of serum IgE to these molecules may be useful in the management of the grass pollen allergic patient.(11) For example, detection of IgE to Phl p 1 may be sufficient to confirm grass pollen allergy in SAR (12); detection of IgE to Phl p 12 may predict OAS (12, 13), and the simultaneous detection of IgE to Phl p 1, Phl p 5 and Phl p 12 may predict a higher risk of adverse reactions during AIT with grass extract.(14)

We have previously shown that sensitization to *Phleum pratense* molecules starts with an IgE response to Phl p 1 in early childhood and later grows quantitatively and qualitatively by involving other grass molecules.(15) We also described that this “molecular spreading” generates several different molecular IgE sensitization profiles, which may then influence the prescription of AIT.(16) The present study aims at describing the molecular profile of IgE sensitization to *Phleum pretense* and testing the hypothesis that complex profiles or IgE sensitization to single molecules are useful to predict specific clinical phenotypes of SARg. To this end, we measured the concentration of serum

IgE to the eight *Phleum pratense* molecules and assessed IgE complex sensitization profiles in a very large population of Italian children affected by SARg, whose clinical phenotype, severity and comorbidities had been carefully diagnosed.

Materials and methods

Study design and population – The study population was enrolled in a large Italian nationwide observational cross-sectional survey ("Panallergens in Pediatrics" [PAN-PED]), conducted by the Italian Pediatric Allergy Network (I-PAN) (17). Children were recruited between May 2009 and June 2011 by 16 pediatric outpatient clinics in 14 Italian cities distributed in 3 main geographic areas: Northern Italy (Milan, Verona, Genoa, Parma and Bologna), Central Italy (Empoli, Ascoli Piceno, Ostia, three centers in Rome), and Southern Italy & Major Islands (Naples, Benevento, Iglesias, Palermo and Crotone). Criteria for eligibility were: a) age 4 to 18 years; b) a history of pollen-induced allergic rhinitis and/or asthma in one of the last two pollen seasons; c) positive skin-prick tests (SPT) for the relevant pollen extracts. Exclusion criteria were: 1) previous immunotherapy for any pollen allergen; 2) other severe chronic diseases. Parents or tutors of all participants provided informed written consent to clinical investigations. The ethical committee of each participating center approved the study design and procedures. In this analysis, we included only children with SPT to *Phleum pratense* ≥3 mm who were examined for sIgE to all eight *Phleum pratense* molecules.

Questionnaire – Internationally validated questionnaires were administered to recruited children's parents: the International Study of Allergy and Asthma in Childhood (18, 19), Allergic Rhinitis and its Impact on Asthma (ARIA) classification (20), and the Global Initiative for Asthma (GINA)(21). Demographic data, history of atopic disease, occurrence of OAS and triggering foods were also recorded. SAR was classified as intermittent or persistent, mild or moderate-severe according to the "ARIA classification" (20). An informatics platform ("AllergyCARD™", TPS Production, Rome, Italy) was used for data input.

Skin-prick tests – SPT were performed with a panel of commercial extracts (ALK-Abelló, Italy), including: *Phleum pratense* (Timothy grass)*, Cynodon dactylon* (Bermuda grass)*, Chenopodium album* (white goosefoot)*, Betula verrucosa* (birch)*, Cupressus arizonica* (cypress)*, Corylus avellana* (hazel)*, Platanus orientalis* (plane tree)*, Olea europaea* (olive tree)*, Parietaria judaica* (pellitory)*, Artemisia vulgaris* (mugwort), *Plantago lanceolata* (plantago), *Salsola spp.* (Russian thistle), *Fagus spp.* (holm) and *Ambrosia artemisiifolia* (ragweed). Histamine 0.1 mg/ml and glycerol solution were the positive and negative controls, respectively. Morrow-Brown needles were used to prick the skin. Readings were taken at 15 minutes and a wheal ≥3 mm was regarded as positive.

IgE assays – All the serum samples were tested for total IgE (tIgE) and specific IgE (sIgE) to pollen extracts *(Cynodon dactylon, Betula verrucosa, Cupressus arizonica, Platanus orientalis, Olea europaea, Parietaria judaica, Artemisia vulgaris, Plantago lanceolata* and *Ambrosia artemisiifolia*) and to perennial airborne allergens listed above (ImmunoCAP FEIA; Phadia, Uppsala, Sweden). IgE to a panel of *Phleum pratense* molecules (including rPhl p 1, rPhl p 2, rPhl p 5b, rPhl p 6, rPhl p 7, rPhl p 11, rPhl p 12) (ImmunoCAP FEIA; Phadia, Uppsala, Sweden) were measured in sera of patients showing a SPT wheal reaction ≥ 3 mm elicited by the *Phleum pratense* extract. IgE reactivity to Phl p 4 was defined by a positive response on an ELISA with rPhl p 4 in the solid phase (Allergopharma, Reinbeck, Germany), as previously described (22). Results were expressed in kilounits per liter (kU_A/L) and classified as positive or negative according to the cut-off level of 0.35. We additionally calculated the Phl p 1 "specific activity" (sIgE to tIgE ratio), as previously described.(23) Patients were categorized as "monomolecular", "oligomolecular", "polymolecular" in case of IgE recognition to 1, 2-4, or 5-8 molecules of *Phleum pratense*, respectively. The IgE sensitization profiles to the eight *Phleum pratense* molecules were defined according to the Allergen-Profile-Codification-System (APCS), as previously reported.(22)

Statistics - Data were summarized as numbers (n) and frequencies (%) if they were categorical and as mean and standard deviation (SD) if quantitative. The average concentration of tIgE and sIgE levels were calculated as geometric mean, specifically for sIgE antibodies only the positive serum samples were considered (see the above definition). Comparison of the general characteristics between participants included and excluded from this study were evaluated by χ^2 test (categorical data) and by T-test, when conditions were respected, or Mann Whitney U test (quantitative data). To assess the normal distribution of quantitative data, the Shapiro–Wilk test was applied. The prevalences of IgE sensitization (≥0.35 kU_A/L) to the eight *Phleum pratense* molecules were examined and the frequencies of all different sensitization profiles were identified by APCS combinatorial analysis. Trend analysis by one-way ANOVA, when appropriate, or non-parametric Jonckheere-Terpstra test for ordered alternatives was used to compare quantitative variables between independent groups of patients with: 1) an increasing number of *Phleum pratense* molecules recognized by IgE; 2) a different APCS profile of sensitization after ordering them according to the increasing number of *Phleum pratense* molecules recognized by IgE. With the same aim, the Mantel–Haenszel linear-by-linear association χ^2 test for trend was used to compare categorical characteristics of clinical outcomes and atopic features. P value of multiple comparison was adjusted by Bonferroni's correction. Generalized estimating equation models taking into account multiple measures from the same subject, were utilized to compare patients with different IgE sensitization to the individual *Phleum pratense* molecules. More precisely a categorical variable indicating the specific-molecule sensitization for each subject (*Phleum pratense* index) was included in each model as independent factor variable and adjusting all models for the number of *Phleum pratense* molecules recognized by IgE. To estimate the effect of the number of *Phleum pratense* molecules recognized by IgE on sIgE to *Dermatophagoides pteronyssinus*, a multivariable linear regression analysis was applied. The regression model was adjusted by significant factors identified through univariate analysis. Missing values were not considered for statistical analyses. A p value

<0.05 was considered statistically significant. Statistical analysis was performed by using IBM SPSS Statistical software, version 21 (IBM Corporation, Armonk, NY, USA).

Results

Study population – The target population consisted in 1271 pediatric patients, recruited in the context of "PAN-PED" project.(24) The present study evaluated 1120 of these 1271 children, i.e. those with a SPT positive reaction to *Phleum pratense* extract (≥3 mm) and a complete dataset for sIgE to the eight *Phleum pratense* molecules **[Figure e1]**. We found no differences in age, familiar atopy, characteristics of SAR and OAS frequency between children included and excluded from the study **[Table 1]**. By contrast, a male gender, Northern Italian residency, higher atopic reactivity and absence of asthma were more common among included patients than among those excluded **[Table 1]**. Among the 1120 patients included in the study population, we found that the co-sensitization to other pollens represents a frequent event (e.g. 69.1% of patients were sensitized to Olive tree, 40.8% to Cypress and 38.9 to Birch) [**Table e1**].

Molecular profiles of IgE to *Phleum pratense* – The analysis of IgE responses against eight *Phleum pratense* molecules in the 1120 participants showed a wide spectrum of 87 different sensitization profiles **[Figure 1a]**. After exclusion of the "0" profile, the arbitrary threshold of 50% of the whole study population was covered by the eight most frequent profiles, defined by the APCS code as "128" (17.4%), "249" (6.3%), "216" (6.1%), "250" (5.2%), "248" (5.1%), "251" (4.9%), "192" (4.8%) and "152" (3.6%) **[Figure 1a]**. These eight profiles were further examined in depth (see below). Overall, 233/1120 (22%) patients presented a "monomolecular" sensitization profile: of these, only 3 (1%) were sensitized to Phl p 5, while 195 (84%) were sensitized to Phl p 1 **[Figure 1b]**. Moreover, 430/1120 (40%) were sensitized to 2-4 molecules ("oligomolecular") and 410/1120 (38%) were sensitized to >4 molecules ("polymolecular"); 8 of the 1120 patients were sensitized to all the eight

molecules **[Figure 1b]**. The prevalence and the geometric mean level of IgE antibodies to any positive reaction to each molecule (≥0.35 kU_A/L) were related **[Figure e2]**.

Characteristics of IgE sensitization - We analyzed the characteristics of atopic sensitization after stratification in relation to three different classifications of the IgE sensitization: A) to each of the eight individual molecules; B) to each of the eight most common IgE sensitization profiles; C) to the number of molecules recognized by IgE:

- **Individual molecules –** The 38/1120 (3%) patients sensitized to Phl p 7 had the highest levels of serum tIgE and of sIgE to the *Phleum pratense* extract or to other pollens, and the highest number of SPT positive reactions to the panel of the examined pollen extracts **[Table 2a]**.
- **IgE molecular sensitization profiles –** The serum level of tIgE strongly increased in parallel with the complexity of the IgE sensitization profiles, ranging from 278 kU/L of the profile "128" (monomolecular Phl p 1) to 803 kU/L of the profile "251" (seven IgE recognized molecules). A similar trend was followed by the levels of sIgE to the *Phleum pratense* extract (from 3.4 to 176 kU_A/l) and to Phl p 1 (from 4.1 to 84 kU_A/l) and by the overall sensitization to pollens extracts, expressed as number of positive SPT reactions or as sum of the serum sIgE antibodies **[Table 2b]**.
- **Number of *Phleum pratense* molecules recognized by IgE –** The serum levels of tIgE, of sIgE to *Phleum pratense* extract, of sIgE to Phl p 1 and the number or overall degree of sensitization to pollens extracts were strongly related to the number of *Phleum pratense* molecules recognized by IgE **[Figure 2]**. By contrast, the level of sIgE to *Dermatophagoides pteronyssinus* was inversely related to the number of *Phleum pratense* molecules recognized by IgE (p for trend <0.001) **[Figure e3]**. A multivariable linear regression analysis showed that the number of *Phleum pratense* molecules recognized by IgE had a negative effect on sIgE to *Dermatophagoides pteronyssinus* regardless of relevant confounders factors identified through univariate analysis (age, gender, tIgE, pollen not related AR and pollen not related asthma) **[Table e2]**.

Clinical outcomes – Sensitizations to Phl p 7 and Phl p 12 were associated with a longer duration of SAR, while sensitization to Phl p 1 with a shorter duration **[Table 3a]**. Moreover, sensitization to Phl p 7 was significantly associated to a higher severity of SAR, with symptoms persisting for >4 months/year and a higher prevalence of asthma. Finally, sensitization to Phl p 12 was associated with OAS (32.4%, p <0.001) **[Table 3a]**. By contrast, a higher complexity of the APCS profile was directly associated to a longer duration of SAR only and it was inversely associated to symptoms persisting for >4 months/year, but not to all the other clinical parameters **[Table 3b]**.

Discussion

We examined IgE sensitization to eight recombinant *Phleum pratense* allergenic molecules in the sera of 1120 children affected by grass pollen allergy and we found: 1) an extreme heterogeneity of the IgE molecular sensitization profiles; 2) a strong relationship of the intrinsic complexity of the sensitization profiles with both, the intensity and diversification of the IgE response to other pollens, but not with those of IgE response to mites or other indoor allergens; 3) an absolute dominance of the IgE sensitization to Phl p 1, which contributes to over 85% of the "monomolecularly" sensitized patients; 4) a relatively low frequency of IgE sensitization to Phl p 5, which is rarely "monomolecular"; 5) a strong association of IgE sensitization to Phl p 7 and asthma; 6) a strong association of IgE sensitization to Phl p 12 and OAS; 7) no relevant association between a given IgE sensitization profile and the clinical phenotype of SAR. To our knowledge, this is the first comprehensive analysis of the clinical relevance of IgE sensitization profiles to eight allergenic molecules of *Phleum pratense* in a large population of grass pollen allergic children.

Heterogeneity of the IgE sensitization profiles – Our molecular combinatorial analysis confirms that the qualitative homogeneity of IgE sensitization to the extract of *Phleum pratense* among grass pollen allergic patients is only apparent. The number of described grass pollen IgE sensitization

profiles, originally limited to 39 (22), is now expanded to 87, suggesting that, theoretically, all the 256 possible combinations could be observed if the study population was big enough. This high heterogeneity had been already confirmed among German (3) grass pollen allergic patients. Our study adds that some profiles are much more frequent than others, so their clinical relevance can be investigated.

Polymolecular sensitization predicts polysensitization to pollens but not to indoor allergens – Not surprisingly, the more complex profiles were characterized by a higher levels of tIgE and sIgE to *Phleum pratense* extract and by a longer history of SAR. This data confirms our previous observations in the MAS birth cohort (15, 25) and supports the concept of the atopic march. Moreover, a higher diversity of the IgE response to the individual molecules of *Phleum pratense* was strongly and linearly associated to both, a higher number and a higher degree of IgE sensitization to other pollens. **Figure 2** shows for the first time that the polymolecular IgE sensitization profiles are strongly associated with pollen extract polysensitization. A strong propensity to atopic responses might underlie the molecular spreading of IgE responses not only to an individual allergen source (i.e. *Phleum pratense*) but also to different allergen sources (i.e. other pollens). However, we did not find any correlation between the number of *Phleum pratense* molecules recognized by IgE and the prevalence or intensity of the IgE response to cat and alternaria allergens. Surprisingly, the prevalence and the levels of IgE to *Dermatophagoides pteronyssinus* were even strongly and linearly inversely correlated to the number of *Phleum pratense* molecules recognized by IgE. This intriguing observation should be examined with caution, as we cannot exclude a selection bias due to our study inclusion criteria. Nevertheless, these data suggest that the molecular spreading process is characterized by a certain degree of "selectivity", whose nature and mechanisms should be better clarified in longitudinal studies (26).

IgE to Phl p 1, Phl p 7 and Phl p 12 as diagnostic biomarkers – Our results suggest that sIgE to a few individual molecules of *Phleum pratense* can be used as diagnostic biomarkers:

- **Phl p 1 –** As already reported by previous studies (5, 27, 28), over 89% of the grass pollen sensitized patients had IgE to Phl p 1 and the most frequent profile was APCS "128", i.e. a monomolecular IgE sensitization to Phl p 1. By contrast, monomolecular IgE sensitization to Phl p 5 (APCS "16") was observed in only 3 patients, while only 39 children were sensitized to Phl p 5 but not to Phl p 1. In a Czech population (29), IgE sensitization to Phl p 5 was less frequent in 82 children than in 48 adults with grass pollen allergy (59.8% vs 79.1%), while over 90% of children and adults were sensitized to Phl p 1. This study supported the hypothesis (15) that Phl p 1 is the initiator molecule starting the sensitization process and that a longer time is necessary to develop sensitization to Phl p 5 than to Phl p 1. In a German population of 101 grass pollen allergic adults (3) a higher frequency of IgE sensitization to both Plh p 1 and Phl p 4 (92%) was observed, however in our population the frequency of IgE sensitization to Phl p 4 was lower (42.2%); the discrepancy with our study is likely due to the use of a native form of Phl p 4, which carries highly cross-reacting CCD B-cell epitopes. Altogether, these results further confirm that the IgE sensitization to Phl p 1 is sufficient in most cases to corroborate a diagnosis of grass pollen allergy (12, 30, 31). This conclusion may be of great relevance in diagnostic algorithms for grass pollen AIT.
- **Phl p 7 –** IgE sensitization to Phl p 7 (polcalcin) was relatively infrequent but strongly associated with asthma. During the IgE molecular spreading process, Phl p 7 is often the "last" molecule inducing an IgE response (15). Accordingly, our 38 Phl p 7 sensitized children were highly polymolecularly sensitized and had high levels of serum tIgE and grass-pollen sIgE. Therefore, the "asthma-biomarker" role of IgE to Phl p 7 is likely explained by a spurious association (Phl p 7 sensitization being a proxy of extreme polysensitization) rather than by a direct causal-effect link. Nevertheless, the data suggest that testing IgE for Phl p 7 may be useful not only to explain cross- reactivity (32, 33), but also as a quite specific biomarker of asthma among SARg patients.

- **Phl p 12 –** IgE sensitization to Phl p 12 (profilin) was observed in over 25% of the examined patients and it was strongly associated with Oral Allergy Syndrome. Our study therefore confirms that profilin is a known trigger of allergic reactions to plant-derived foods, mostly limited to the oral cavity (13, 34).

Impact on grass pollen immunotherapy prescription

Has the great heterogeneity of the IgE molecular sensitization profiles to grass pollen an influence on the efficacy and safety of grass pollen AIT? This question cannot be answered by our study. Interventional and prospective studies in patient cohorts are urgently needed to test the hypothesis (16,22), that the diagnostic biomarkers examined in our study may improve the efficacy of AIT and predict its safety (14). Nevertheless, our results also confirm that patients with AR symptoms during the grass pollen-season and a positive SPT/IgE assay to grass pollen extracts should be further examined to detect serum IgE antibodies to Phl p 1 and, if possible, to Phl p 2, Phl p 5, Phl p 7, Phl p 11, and Phl p 12 (10). The identification of sensitization to Phl p 1 should followed by the prescription of grass pollen AIT. Our results also suggest that the detection of IgE to Phl p 12 (profilin) should prompt further investigation of OAS. The presence of IgE to Phl p 7 will alert the doctor of a worse prognosis, a higher risk of asthma and a greater severity of SAR. Interestingly, Immunoblot inhibition experiments demonstrated that commercial pollen extracts for AIT, with the exception of Parietaria, are normally rich in profilin, while only grass pollen extracts seem to contain also in polcalcin (35). If this observation were confirmed, then grass pollen immunotherapy would be appropriated to induce a protective immune response to these two panallergens in polysensitized patients.

Clinical relevance of IgE sensitization profiles - Our study population was big enough to test the hypothesis that molecular IgE sensitization profiles are of diagnostic value. The outcome of this analysis was rather frustrating, as no profile was associated with peculiar characteristic or with the severity of allergic disease. At least two facts may have contributed to this negative outcome. On

one hand, our study population is characterized by a very high prevalence of polysensitization to other pollens (birch, cypress, pellitory, olive tree, mugwort, plane tree) **[Table e1]**, whose peak season widely overlaps with that of grass pollen. So allergic symptoms triggered by other pollens confounded the clinical relevance of grass pollen sensitization. Differently, in Northern Europe, the peak pollen season of grasses is shorter and unaccompanied by other pollens and the prevalence of polysensitization is lower (36, 37). Thus we speculate that the clinical relevance of testing IgE sensitization profiles in grass pollen allergic children may be stronger in such regions. On the other hand, the parameters used in our cross-sectional study to graduate the severity of SAR (i.e. ARIA classification based on a few simple quest ions)(20) did not allow a fine discrimination of symptoms severity (38). Therefore the clinical relevance of IgE sensitization profiles will have to be tested again in longitudinal studies, using a prospective registration of symptoms severity through symptoms-medication scores along a full pollen season.(39) Similarly, our study design did not address the question whether different IgE sensitization profiles predict efficacy (22) and adverse reactions (14, 40) to grass pollen immunotherapy.

Limitations – We have to acknowledge additional study limitations. First, we tested sensitization to one grass pollen species only and the generalizability of our conclusions in settings and countries (e.g. Australia (2)) where *Phleum pratense* is less representative of the dominating grass pollen species may be questioned. Second, the relative frequency of IgE sensitization to *Phleum pratense* and other grass pollens in patients with seasonal allergic rhinitis is higher in Northern than in Southern Italy (17). *Phleum pratense* is the most relevant grass pollen allergen in Italy, however other grasses (e.g. Bermuda grass, *Lolium perenne*) may play in patients a role in triggering seasonal allergic symptoms (32). Third, the data on the SAR characteristics were based only on a retrospective questionnaire and, therefore, exposed to potential recall bias. Fourth, asthma and OAS could not be confirmed by lung tests and food challenge, respectively, and over-reporting cannot be excluded. Fifth, we examined a pediatric population, therefore specific studies among adults should be performed to test whether the same conclusions could be applied to older groups.

However, a recent Italian study performed in 11,235 adult patients with AR showed percentages of IgE sensitization to *Phleum pratense* molecules comparable with our data (41).

Conclusions - In conclusion, this nationwide study identifies three biomarkers in children population, Phl p 1, Phl p 7 and Phl p 12 that can be useful in the diagnostic work-up of the child with grass pollen allergy. The study also shows an extreme heterogeneity of combinatorial molecular IgE sensitization profiles, whose clinical usefulness is limited to a Mediterranean country. Prospective studies in patient cohorts are now needed to expand our knowledge on the stability and predictive value of the observed associations.

Acknowledgements

We thank ALK-Abelló and TFS for providing the reagents for the study, TPS Production for the informatics platform AllergyCARD.

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Legend to figures

Figure 1 – a) Molecular profiles of IgE sensitization (*≥ 0.35 KU_A/l*) to *Phleum pratense* molecules: rPhl p 1, rPhl p 2, rPhl p 4, rPhl p 5, rPhl p 6, rPhl p 7, rPhl p 11, rPhl p 12 in 1120 children with skin reaction to *Phleum pratense (wheal ≥ 3 mm)* and tested for sIgE to all considered molecules. The Allergen Profile Codification System (APCS) code, the number of children belonging to that specific code (n), the relative frequency (%) and the cumulative frequency (%) are shown. The profiles are ordered by decreasing frequency. **b)** Prevalence of IgE response to *Phleum pratense* allergens, according to the molecular categories. "Monomolecular": IgE sensitization to 1 molecule only; "oligomolecular": 2-4 molecules; "polymolecular": 5-8 molecules.

Figure 2 – Levels of specific IgE (sIgE) to *Phleum pratense* and Phl p 1, sum of sIgE to pollens, and number of pollens recognized by SPT, according to the number of *Phleum pratense* molecules recognized by IgE. Lines show the geometric mean level of positive sIgE response (≥0.35 kU/L) and the number of pollens recognized by SPT. *p for trend <0.001. § Sensitization to pollens: skin reactions to pollens (*wheal ≥3 mm*). SPT extracts included: *Betula verrucosa, Cupressus arizonica, Platanus orientalis, Olea europaea, Parietaria judaica, Artemisia vulgaris, Plantago lanceolata.*

Table 1. Characteristics of 1255 Italian children with seasonal allergic rhinoconjunctivitis (SAR) included or excluded from the study.

	Study population†		Excluded #		p-value*
Number	1120		135		
Age (ys) (mean,SD)	10.5	*3.4*	9.7	*3.2*	**0.004**
Males (n, %)	772	*69*	82	*61*	0.052
Familiar atopy ^					
Mother (n, %)	492	*44*	62	*46*	0.659
Father (n, %)	441	*39*	48	*36*	0.390
Geographic Area (n, %)					
North	370	*33*	20	*15*	
Center	532	*48*	57	*42*	**<0.001**
South & Islands	218	*19*	58	*43*	
Characteristics of SAR					
Age at onset of SAR (ys) (mean, SD)	5.3	*2.9*	4.9	*2.5*	0.175
Duration of SAR (ys) (mean, SD)	5.2	*3.3*	4.7	*3.1*	0.157
Moderate - severe SAR (n, %)	583	*51*	59	*44*	0.067
Months with symptoms (n) (mean, SD)	4.2	*1.8*	4.4	*1.8*	0.329
Pollen season months with symptoms (n) (mean, SD)	3.9	*1.4*	3.8	*1.4*	0.875
Asthma (n, %)	415	*37*	62	*46*	0.045
Oral allergy syndrome (n, %)	265	*24*	31	*23*	0.857
Atopic reactivity					
Overall SPT reactivity to pollens (mm) (mean,SD) §	36.5	*21*	15.5	*9*	**<0.001**
Levels of total IgE (kU/L) (mean,SD)°	2.6	*0.5*	2.5	*0.5*	**0.002**

† Skin sensitization to P pratense (wheal ≥ 3 mm)

 # Subjects not sensitized to P pratense (SPT wheal <3 mm)

** Chi squared test was used to compare frequencies; T-test for normally distributed indipendent samples and Mann-Whitney U-Test for not normally distributed indipendent samples.*

^ At least one among hay fever, asthma or atopic dermatitis

°Log_{10} trasformed data of T-IgE were used.

§ SPT extracts included: Betula verrucosa, Cupressus arizonica, Platanus orientalis, Olea europaea, Parietaria judaica, Artemisia vulgaris, Plantago lanceolata

Table 2. Characteristics of IgE sensitization in 1120 grass pollen allergic children (*wheal ≥ 3 mm*) by their: a) IgE responses to individual *Phleum pratense* molecules; b) IgE molecular sensitization profile (APCS).

a)	***Phleum pratense* molecules recognized by IgE**	**rPhl p 1**	**rPhl p 5**	**rPhl p 6**	**rPhl p 2**	**rPhl p 4**	**rPhl p 11**	**rPhl p 12**	**rPhl p 7**	**adjusted p value†**
	Number of subjects	996	672	601	535	462	295	281	38	
	Total IgE (kU/L)	406	454	499	493	450	531	586	830	**<0.001**
	sIgE to *Phleum pratense* (kU_A/L)	27	57	72	64	53	61	76	83	**<0.001**
	sIgE to Phl p 1 (kU_A/L)	20	38	44	42	37	45	47	42	**<0.001**
	Phl p 1 Specific Activity (%) *	9.9	11.7	12.2	12.4	11.1	11.4	11.4	9.5	**<0.001**
	sIgE to *Phleum pratense* molecules, mean (n)°	3.8	4.8	5.1	4.9	5.0	5.2	5.4	5.2	**<0.001**
	Sensitization to pollens, mean (n) §	5.0	5.8	6.0	6.0	6.0	6.4	6.3	6.9	**<0.001**
	sIgE to pollens (kU_A/L) °	77	128	153	141	124	140	175	198	**<0.001**
	SPT to *Phleum pratense*, wheal diameter (mm)	7.6	8.2	8.3	8.3	8.2	8.2	8.4	8.2	0.660

b)	**IgE molecular sensitization profiles (APCS)**	**128**	**192**	**152**	**216**	**248**	**249**	**250**	**251**	**p value for trend‡**
	Phleum pratense molecules recognized by IgE (n)	*1*	*2*	*3*	*4*	*5*	*6*	*6*	*7*	
	Number of subjects	195	54	40	68	57	70	58	55	
	Total IgE (kU/L)	278	321	361	450	408	583	508	803	**<0.001**
	sIgE to *Phleum pratense* (kU_A/L)	3.4	9.9	23	57	83	125	106	176	**<0.001**
	sIgE to Phl p 1 (kU_A/L)	4.1	10.5	16	39	47	65	57	84	**<0.001**
	Phl p 1 Specific Activity (%) *	4.0	7.8	10.1	12.9	14.6	12.7	14.1	12.0	**<0.001**
	Sensitization to pollens, mean (n) §	2.7	3.9	3.8	5.3	5.7	6.9	7.2	7.7	**<0.001**
	sIgE to pollens (kU_A/L) °	17	36	68	127	152	226	202	306	**<0.001**
	SPT to *Phleum pratense*, wheal diameter (mm)	5.8	6.3	7.5	8.0	8.4	9.3	8.6	9.4	**<0.001**

Quantitative variables were summarized as mean and as geometric mean for IgE concentration level (values expressed in kU/L or kU_A/L). Sporadic missing values were detected for each examined variable.

* Specific Activity (SA) = sIgE*100/tIgE

° cut-off ≥ 0.35 kUA/L

§ Skin reactions to pollens (wheal ≥3 mm). SPT extracts included: *Betula verrucosa, Cupressus arizonica, Platanus orientalis, Olea europaea, Parietaria judaica, Artemisia vulgaris, Plantago lanceolata*

† Generalized estimating equation models taking into account multiple measures from the same subject, more precisely *Phleum pratense index* (a categorical variable indicating the specific-molecule sensitization for each subject) was included as independent variable; p values were adjusted for the number of *Phleum pratense molecules* recognized by sIgE (independent variable inserted in each model).

‡Trend analysis by one-way ANOVA, when appropriated, or non-parametric Jonckheere-Terpstra test for ordered alternatives.

APCS profiles: molecular sIgE detection. "128": rPhl p 1; "192": rPhl p 1, rPhl p 2; "152": rPhl p 1, rPhl p 5, rPhl p 6; "216": rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6; "248": rPhl p 1, rPhl p 2, rPhl p 4, rPhl p 5, rPhl p 6; "249": rPhl p 1, rPhl p 2, rPhl p 4, rPhl p 5, rPhl p 6, rPhl p 12; "250": rPhl p 1, rPhl p 2, rPhl p 4, rPhl p 5, rPhl p 6, rPhl p 11; "251": rPhl p 1, rPhl p 2, rPhl p 4, rPhl p 5, rPhl p 6, rPhl p 11, rPhl p 12.

Table 3. Clinical outcomes in 1120 grass pollen allergic children (*wheal ≥ 3 mm*), by their: a) IgE responses to individual *Phleum pratense* molecules; b) IgE molecular sensitization profile (APCS).

a)

***Phleum pratense* molecules recognized by IgE**	**rPhl p 1**	**rPhl p 5**	**rPhl p 6**	**rPhl p 2**	**rPhl p 4**	**rPhl p 11**	**rPhl p 12**	**rPhl p 7**	**adjusted p value†**
Number of subjects	996	672	601	535	462	295	281	38	
Duration of SAR (yrs), mean	5.2	5.4	5.4	5.4	5.4	5.4	5.8	5.8	0.723
Moderate - severe SAR (%)	52	53	52	53	54	54	53	63	0.734
Symptoms > 4 months/yr (%)	36	35	35	33	34	34	33	42	0.818
Asthma (%)	37	37	37	37	36	40	42	63	0.019
Asthma outside pollen season (%)	24	22	23	23	23	23	23	26	0.677
OAS (%)	23	25	25	26	21	22	32	24	<0.001

b)

IgE molecular sensitization profiles (APCS)	**128**	**192**	**152**	**216**	**248**	**249**	**250**	**251**	**p value for trend‡**
Phleum pratense molecules recognized by IgE (n)	*1*	*2*	*3*	*4*	*5*	*6*	*6*	*7*	
Number of subjects	195	54	40	68	57	70	58	55	
Duration of SAR (yrs), mean	4.9	4.7	5.2	5.2	5.0	6.0	5.4	6.3	**0.018**
Moderate - severe SAR (%)	54	52	45	54	53	54	50	49	0.651
Symptoms > 4 months/yr (%)	44	30	48	41	33	30	28	33	**0.011**
Asthma (%)	35	28	28	40	39	33	31	44	0.498
Asthma outside pollen season (%)	30	20	20	25	26	21	16	24	0.068
OAS (%)	15	28	33	31	14	33	14	27	0.120

Quantitative variables were summarized as mean and categorical variables as percentages (%). Sporadic missing values were detected for each examined variable.

† Generalized estimating equation models taking into account multiple measures from the same subject, more precisely *Phleum pratense index* (a categorical variable indicating the specific-molecule sensitization for each subject) was included as independent variable; p values are adjusted for the number of *Phleum pratense molecules* recognized by sIgE (independent variable inserted in each model).

‡ Trend analysis by one-way ANOVA, when appropriated, or non-parametric Jonckheere-Terpstra test for ordered alternative for quantitative variables; Mantel–Haenszel linear-by-linear association Chi-squared test for categorical data.

APCS profiles: molecular sIgE detection. "128": rPhl p 1; "192": rPhl p 1, rPhl p 2; "152": rPhl p 1, rPhl p 5, rPhl p 6; "216": rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6; "248": rPhl p 1, rPhl p 2, rPhl p 4, rPhl p 5, rPhl p 6; "249": rPhl p 1, rPhl p 2, rPhl p 4, rPhl p 5, rPhl p 6, rPhl p 12; "250": rPhl p 1, rPhl p 2, rPhl p 4, rPhl p 5, rPhl p 6, rPhl p 11; "251": rPhl p 1, rPhl p 2, rPhl p 4, rPhl p 5, rPhl p 6, rPhl p 11, rPhl p 12.

Figure 1

a)

APCS	Number of molecules	rPAP p 1	rPAP p 2	rPAP p 4	rPAP p 5	rPAP p 6	rPAP p 7	rPAP p 11	rPAP p 12	n	%	Cumulative %
105	1	•								195	17.4	17.4
145	4	•	•	•	•	•			•	70	6.3	23.7
116	2	•	•			•				66	5.9	29.7
150	4	•	•	•	•	•		•		58	5.2	34.9
135	3	•	•	•	•	•				57	5.1	40.0
151	5	•	•	•	•	•		•	•	55	4.9	44.9
152	2	•	•							54	4.8	49.7
0	0									47	4.2	53.9
130	3	•			•	•				40	3.6	57.5
115	3	•	•			•		•		38	3.4	60.8
140	2	•				•		•		31	2.8	63.7
144	4	•		•	•	•				29	2.6	66.2
146	4	•		•	•	•		•		28	2.5	68.7
117	5	•	•		•	•			•	27	2.4	71.2
145	5	•		•	•	•			•	23	2.0	73.0
144	2	•			•					20	1.8	74.8
12	1			•						15	1.3	76.1
154	4	•			•	•		•		15	1.3	77.5
106	1	•	•			•				15	1.3	78.8
107	3	•		•	•	•		•	•	13	1.2	80.0
155	3	•	•			•			•	13	1.2	81.1
224	3	•	•	•						10	0.9	82.1
119	4	•	•		•	•		•	•	10	0.9	83.0
153	4	•			•	•			•	10	0.9	84.0
130	2	•						•		10	0.9	84.9
129	2	•							•	9	0.8	86.1
2	1							•		8	0.7	86.9
1	1								•	7	0.6	87.4
24	2				•	•				7	0.6	88.0
16	1				•	•				6	0.5	88.4
140	4	•	•		•	•				6	0.5	89.1
155	6	•	•	•	•	•	•	•	•	6	0.5	89.8
130	3	•			•				•	5	0.4	90.3
126	3	•			•				•	5	0.4	90.5
104	3	•	•		•				•	5	0.4	91.0
48	2			•	•					5	0.4	91.3
132	3	•			•		•			4	0.4	91.7
146	3	•			•				•	4	0.4	92.0
178	6	•		•	•				•	4	0.4	92.4
220	5	•	•		•	•	•		•	4	0.4	92.8
125	4	•	•	•	•				•	4	0.4	93.1
16	1				•					3	0.3	93.4
14	1				•					3	0.3	93.7
64	3			•				•	•	3	0.3	93.9
48	3			•	•	•		•		3	0.3	94.2
151	4	•			•			•	•	3	0.3	94.5
142	4	•		•				•	•	3	0.3	94.7
225	7	•	•		•	•	•	•	•	3	0.3	95.0
4	1					•				2	0.2	95.1
18	1				•			•		2	0.2	95.3
45	2				•	•				2	0.2	95.5
50	2			•	•			•		2	0.2	95.7
64	3		•						•	2	0.2	95.9
68	3		•		•	•				2	0.2	96.1
155	3	•			•		•		•	2	0.2	96.2
145	3	•			•				•	2	0.2	96.4
156	3	•			•	•	•			2	0.2	96.6
177	4	•		•	•				•	2	0.2	96.8
189	4	•		•	•	•	•			2	0.2	97.0
196	4	•	•					•	•	2	0.2	97.1
210	4	•	•		•			•		2	0.2	97.3
222	6	•	•	•	•	•	•	•		2	0.2	97.5
141	5	•	•	•	•				•	2	0.2	97.7
252	8	•	•	•	•	•	•		•	2	0.2	97.8
153	5	•	•	•	•	•	•		•	2	0.2	98.0
8	1					•				1	0.1	98.2
10	1								•	1	0.1	98.2
11	1				•					1	0.1	98.3
49	2				•	•			•	1	0.1	98.4
66	2			•		•				1	0.1	98.5
88	4	•		•		•			•	1	0.1	98.6
116	3	•				•				1	0.1	98.7
110	3	•				•		•		1	0.1	98.7
156	5	•			•	•	•	•		1	0.1	98.8
143	5	•			•		•	•	•	1	0.1	98.9
180	6	•			•	•			•	1	0.1	99.0
182	5	•			•	•	•	•		1	0.1	99.1
191	7	•			•	•	•	•	•	1	0.1	99.2
198	6	•	•				•	•	•	1	0.1	99.3
200	5	•	•			•				1	0.1	99.4
209	4	•	•		•				•	1	0.1	99.5
221	6	•	•		•	•	•		•	1	0.1	99.5
224	4	•	•					•	•	1	0.1	99.6
227	5	•	•	•				•	•	1	0.1	99.7
232	4	•	•	•		•				1	0.1	99.8
241	5	•	•	•	•				•	1	0.1	99.9
254	7	•	•	•	•	•	•	•	•	1	0.1	100.0

b)

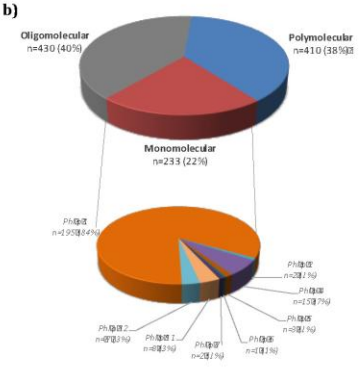

Figure 2