

# Molecular profiles of IgE to *Phleum pratense* in children with grass pollen allergy: Implications for specific immunotherapy

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**Background:** The so-called component-resolved immunotherapy of allergies proposes an immunization tailored to the molecular sensitization profiles of individual patients.

**Objectives:** We sought (1) to investigate the profiles of IgE sensitization to *Phleum pratense* in children with grass pollen allergy and (2) to define the compatibility of these profiles with a mixture of recombinant allergenic molecules of *P pratense* previously proposed for specific immunotherapy.

**Methods:** We examined 200 children (age, 4-18 years; 126 boys) with allergic rhinitis, asthma, or both ascertained through validated questionnaires. Each child underwent skin prick testing (ALK-Abelló) and serum IgE assays (ImmunoCAP, Phadia) with 9 pollen extracts. Sera reacting against *P pratense* were tested for the individual molecules (rPhl p 1, rPhl p 2, rPhl p 4, nPhl p 4, rPhl p 5b, rPhl p 6, rPhl p 7, rPhl p 11, and Phl p 12). Through a combinatorial approach, the IgE individual sensitization profiles were matched against an experimental allergen-specific immunotherapy (SIT) preparation containing Phl p 1, Phl p 2, Phl p 5, and Phl p 6.

**Results:** Among the 176 of 200 children with IgE sensitization to *P pratense* extract, 39 profiles of sensitization to the 8 allergenic molecules tested (cutoff, 0.35 kU/L) were identified. This high heterogeneity was reduced by considering only 6 or 4 *P pratense* molecules but not by increasing the cutoff levels of IgE positivity. The molecular profile of the experimental SIT preparation matched that of 7 (4%) of 176 patients only; the

remaining 169 patients were classified in 4 mismatch categories: underpowered (29%), overpowered (32%), underpowered/overpowered (32%), and unrelated (3%).

**Conclusions:** IgE sensitization profiles to *P pratense* are highly heterogeneous. Molecularly designed SIT preparations tailored to patients' needs should consider this high heterogeneity and be driven by locally performed population studies. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

**Key words:** Allergenic molecules, atopy, children, classification, combinatorial analysis, component-resolved diagnosis, component-resolved therapy, epidemiology, grass pollen, hay fever, molecular profiles, *Phleum pratense*, specific IgE

Grass pollen is the most important allergen source causing allergic rhinoconjunctivitis and among the most important contributing to asthma.<sup>1</sup> Pollen from trees (eg, birch, cypress, and olive) and weeds (eg, ambrosia, *Parietaria* species, and mugwort) are also relevant at the regional level, but only grass pollen is significant at every latitude and in every continent.<sup>1</sup> Although there are no "official" estimates, at least 11% of adults in Europe have grass pollen allergy<sup>2</sup> in the form of allergic rhinitis (hay fever), allergic asthma, or both.<sup>2</sup> The burden associated with this allergenic source is therefore enormous.<sup>3,4</sup>

Avoidance of grass pollen allergens is difficult, and therefore treatment is based mostly on drugs.<sup>5</sup> Pharmacotherapy has no direct effect on the immunologic mechanisms sustaining IgE sensitization, and the efficacy of drugs is dependent on their administration.<sup>5</sup> By contrast, allergen-specific immunotherapy (SIT) is the only treatment with long-lasting effects that continue after treatment discontinuation<sup>6</sup> and can contribute to prevention of disease progression.<sup>7</sup> SIT has been traditionally based on the administration of extracts of allergenic sources, which are supposed to contain all the major allergenic proteins. The exact composition of subcutaneous immunotherapy<sup>8</sup> and sublingual immunotherapy<sup>9</sup> preparations, however, cannot be defined precisely. Moreover, grass pollen extract preparations from different companies can change in their molecular composition,<sup>10</sup> and this might reduce their efficacy.

To optimize SIT efficacy, Valenta et al<sup>11</sup> proposed the concept of component-resolved therapy (CRT). Accordingly, an SIT preparation should be characterized and standardized at a molecular level, and its composition should match the patient's molecular profile of IgE sensitization.<sup>11,12</sup> To tailor a patient's CRT to grass species (eg, *Phleum pratense*) is rather complex. Actually, to date,

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**Abbreviations used**

CRT: Component-resolved therapy  
SIT: Allergen-specific immunotherapy  
SPT: Skin prick test

at least 11 allergenic proteins have been characterized in this species,<sup>13,14</sup> which would correspond to more than 2000 theoretic sensitization profiles. By contrast, only 1 CRT preparation has been produced thus far<sup>15</sup>; it is comprised of a mix of 5 recombinant allergenic molecules of *P pratense* (namely rPhl p 1; rPhl p 2; rPhl p 5, with 2 isoforms, a and b; and rPhl p 6) chosen on the basis of their allergenic relevance in the population.<sup>13,14</sup> This experimental preparation was proved safe and effective in adult patients.<sup>15</sup> However, the authors speculated that some patients might have gained additional benefit from the inclusion of further allergens and concluded that the most prevalent sensitization profiles should be identified and allergen combinations established to treat the majority of patients allergic to grass pollen.<sup>15</sup>

Thus far, the epidemiologic studies have investigated the frequency of IgE responses to each of the 8 most important individual allergenic proteins of *P pratense* in adults and children. In general, such studies have provided relatively concordant information, showing that Phl p 1 and Phl p 5 are the most important allergenic proteins, followed by Phl p 2, Phl p 4, and Phl p 6, whereas IgE responses to Phl p 11, Phl p 12, and especially Phl p 7 are less frequent.<sup>16,17</sup> The largest of these studies has investigated in detail the prevalence of IgE sensitization against *P pratense* molecules in relation to the patient's age.<sup>18</sup> However, to our knowledge, no systematic information is available about the extended profiles of sensitization for each of the 8 major *P pratense* molecules both at an individual and a population level, so that the question raised by Jutel et al<sup>15</sup> in 2005 remains unanswered.

To answer this question, we have taken advantage of the pilot phase of a longitudinal study called Panallergens in Pediatrics that is being performed by the Italian Pediatric Allergy Network.<sup>19</sup> This large multicenter longitudinal study is prospectively aimed to investigate the clinical relevance of comprehensive sensitization profiles for allergenic proteins in childhood. We used the pilot phase of the project (1) to analyze, using a combinatorial approach, the profiles of IgE sensitization to 8 *P pratense* molecules in children affected by pollen allergy and (2) to classify these children on the basis of the different types of match/mismatch between their molecularly tailored needs of allergen-specific treatment and the molecular composition of the SIT preparation proposed by Jutel et al.<sup>15</sup>

**METHODS****Study population and study design**

We performed a cross-sectional observational study of children with seasonal allergic rhinoconjunctivitis, asthma, or both spontaneously seeking care in 3 pediatric outpatient clinics in Rome, Italy. The study represents the pilot phase of a longitudinal multicenter research project entitled Panallergens in Pediatrics that is being performed by the Italian Pediatric Allergy Network.<sup>19</sup>

Children (mean age, 11.0 ± 3.3 years; range, 4–18 years) consecutively recruited during normal routine activity between May and October 2009 were considered for inclusion in the study. Patients underwent a full diagnostic workup (questionnaire, objective examination, and skin tests) according to a

standard protocol. Seasonal allergic rhinoconjunctivitis caused by pollen hypersensitivity was diagnosed in children with nasal symptoms, eye symptoms, or both (apart from cold) for at least 3 weeks during one of the 2 last pollen seasons in accordance with positive skin prick test (SPT) responses (wheal reaction of ≥3 mm) to 1 or more pollen allergen sources. Children with a diagnosis of seasonal allergic rhinoconjunctivitis were recruited after parents provided written informed consent. Exclusion criteria were a history of pollen SIT and severe chronic disease. Recruited children underwent venipuncture, and 3 serum aliquots were stored at –70°C until use. The Panallergens in Pediatrics study design and procedures have been approved by the ethics committees of all participating centers.

**Clinical history and informatics**

A standard questionnaire was administered to all participants that was based on, among others, the following internationally validated components: (1) International Study of Allergy and Asthma in Childhood questions for respiratory allergies<sup>20</sup> and (2) diagnostic criteria to ascertain the severity of asthma (Global Initiative for Asthma)<sup>21</sup> and (3) allergic rhino-conjunctivitis (Allergic Rhinitis and its Impact on Asthma).<sup>22</sup> An informatics platform (“@llergy.card”; Technology Projects & Software Productions [TPS], Rome, Italy) was used for data input.

**SPTs**

SPTs were performed with a standard panel of extracts (ALK-Abelló, Hørsholm, Denmark), including *P pratense*, *Cynodon dactylon*, *Betula verrucosa*, *Cupressus arizonica*, *Platanus orientalis*, *Olea europaea*, *Parietaria judaica*, *Artemisia vulgaris*, *Plantago lanceolata*, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Blattella germanica*, cat, dog, *Alternaria tenuis*, and *Cladosporium herbarum*. Histamine (0.1 mg/mL) and glycerol solution were used as positive and negative controls, respectively. Morrow-Brown needles were used to perform the SPTs, and the skin reactions were read after 15 minutes. A wheal greater than or equal to 3 mm after subtraction of the negative control was regarded as positive unless otherwise specified.<sup>23</sup> Polysensitization (at the source level) to pollen or indoor allergens was defined as a positive response to at least 3 pollen or indoor allergen sources, respectively.

**IgE assays**

All sera underwent the measurement of total IgE and IgE antibody levels specific for a grass pollen extract (*P pratense*), 4 highly cross-reactive allergens (rPhl p 7, rPhl p 12, rBet v 1, and rPru p 3), and the major olive pollen allergen nOle e 1 (ImmunoCAP FEIA; Phadia, Uppsala, Sweden). IgE specific for pollen extracts (*C dactylon*, *B verrucosa*, *C arizonica*, *P orientalis*, *O europaea*, *P judaica*, *A vulgaris*, *P lanceolata*, and *Ambrosia artemisiifolia*) and for a panel of molecules (including rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5b, rPhl p 6, rPhl p 11, nCyn d 1, rBet v 2, nArt v 1, and rPar j 2) were measured in sera of patients showing a wheal reaction of 2 mm or greater elicited by the corresponding allergenic source (see the Methods section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Results were expressed in kilounits per liter and classified as positive or negative according to the cutoff levels (0.35, 0.7, and 3.5 kU/L) required by the different analyses. IgE reactivity to Phl p 4 was defined by a positive response on an ELISA with rPhl p 4 in the solid phase, as described elsewhere (see the Methods section in this article's Online Repository).<sup>24</sup> For the purposes of the present study, only results concerning *P pratense* and its molecules are presented.

**Statistics**

Atopic sensitization and disease characteristics of the study population were analyzed by using descriptive statistics. The profiles of sensitization to pollen extracts, as well as to *P pratense* allergenic molecules, also were described. These profiles were analyzed through a combinatorial approach using 8, 6, or 4 molecules (see the Results section). Briefly, a binary number representing each profile was elaborated. The decimal equivalent of this binary number was then used as a label of each profile. This code was defined by using

TABLE I. Characteristics of the population sample

|                                        | Allergic rhinitis<br>(n = 175) |         | Asthma<br>(n = 103) |         | All<br>(n = 200)* |         |
|----------------------------------------|--------------------------------|---------|---------------------|---------|-------------------|---------|
|                                        | No.                            | Percent | No.                 | Percent | No.               | Percent |
| Age (y), mean $\pm$ SD                 | 11.1                           | 3.4     | 10.7                | 3.3     | 11.0              | 3.3     |
| Male sex (no./total [%])               | 111                            | 63.4    | 65                  | 63.1    | 126               | 63.0    |
| Atopic sensitization (SPT $\geq$ 3 mm) |                                |         |                     |         |                   |         |
| <i>Phleum pratense</i>                 | 158                            | 90.3    | 90                  | 87.4    | 182               | 91.0    |
| Wheal diameter 3-5 mm                  | 44                             | 27.8    | 29                  | 32.2    | 20                | 11.0    |
| Wheal diameter 6-10 mm                 | 61                             | 38.6    | 31                  | 34.4    | 102               | 56.0    |
| Wheal diameter >10 mm                  | 70                             | 44.3    | 43                  | 47.8    | 60                | 33.0    |
| <i>Cynodon dactylon</i>                | 126                            | 72.0    | 67                  | 65.0    | 141               | 70.5    |
| <i>Betula verrucosa</i>                | 43                             | 24.6    | 26                  | 25.2    | 51                | 25.5    |
| <i>Cupressus arizonica</i>             | 88                             | 50.3    | 53                  | 51.5    | 98                | 49.0    |
| <i>Platanus orientalis</i>             | 53                             | 30.3    | 33                  | 32.0    | 63                | 31.5    |
| <i>Olea europaea</i>                   | 113                            | 64.6    | 63                  | 61.2    | 128               | 64.0    |
| <i>Parietaria judaica</i>              | 75                             | 42.9    | 44                  | 42.7    | 83                | 41.5    |
| <i>Artemisia vulgaris</i>              | 27                             | 15.4    | 18                  | 17.5    | 33                | 16.5    |
| <i>Plantago lanceolata</i>             | 67                             | 38.3    | 31                  | 30.1    | 76                | 38.0    |
| $\geq$ 3 Pollens                       | 103                            | 58.8    | 59                  | 57.3    | 117               | 58.5    |
| <i>Dermatophagoides pteronyssinus</i>  | 87                             | 49.7    | 57                  | 55.3    | 100               | 50.0    |
| <i>Dermatophagoides farinae</i>        | 92                             | 52.6    | 62                  | 60.2    | 106               | 53.0    |
| <i>Blattella germanica</i>             | 20                             | 11.4    | 15                  | 14.6    | 26                | 13.0    |
| Cat                                    | 42                             | 24.0    | 27                  | 26.2    | 48                | 24.0    |
| Dog                                    | 48                             | 27.4    | 31                  | 30.1    | 58                | 29.0    |
| <i>Alternaria tenuis</i>               | 57                             | 32.6    | 38                  | 36.9    | 63                | 31.5    |
| <i>Cladosporium herbarum</i>           | 15                             | 8.6     | 13                  | 12.6    | 17                | 8.5     |
| $\geq$ 3 Indoor allergens              | 67                             | 38.3    | 42                  | 40.8    | 77                | 38.5    |

\*Seventy-eight children reported allergic rhinitis and asthma, 97 children reported allergic rhinitis only, and 25 reported asthma only.

the Allergen Profile Codification System. The number of profiles with 8, 6, or 4 molecules required to describe 80% of the patient population, respectively, was calculated. To test for the influence of IgE antibody serum levels on the frequency distribution of the profiles, different cutoff points for positivity were used, as indicated above.

## RESULTS

### Characteristics of the study population

Most participants were boys (126/200 [63%]). The vast majority of participants (175/200 [87.5%]) were affected by allergic rhinoconjunctivitis (Table I); most of them were classified as having a persistent form of disease according to the Allergic Rhinitis and its Impact on Asthma guidelines, whereas asthma was in most cases intermittent (see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). The average duration of the reported allergic rhinoconjunctivitis from onset to the time of the visit was  $5.0 \pm 3.3$  years (range, 0-15 years).

About 90% of the participants had a positive skin reaction to *P pratense* pollen extract; polysensitization to pollen extracts was highly frequent, with 58.5% of the participants reacting to at least 3 unrelated pollen sources (Table I). A combinatorial analysis of pollen sensitization showed that 75% of the patients reacting to *P pratense* were sensitized to at least 2 pollen species with a similar pollen season (see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Moreover, 50% of the participants were sensitized to mites, and 38% were sensitized to at least 3 unrelated indoor allergenic sources.

### IgE responses to individual *P pratense* allergenic molecules

Phl p 1 was by far the allergen most frequently (>90%) recognized by patients' sera, followed by Phl p 4, Phl p 5, Phl p 6,

and Phl p 2 (about 50% each) and by Phl p 11 and Phl p 12 (about 20% each), whereas only 4 patients showed IgE against Phl p 7. The geometric mean concentrations of IgE responses against these allergens ranged from 3.5 kU/L (Phl p 12) to 26.0 kU/L (Phl p 5), and as expected, concentration ranks roughly correspond to prevalence ranks (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). IgE responses to the examined major allergenic proteins (Ole e 1, Art v 1, and Par j 2) of the other pollen sources (*Olea europaea*, *Artemisia vulgaris*, mugwort, and *P judaica*) mirrored the frequency distribution of IgE responses to the respective extracts (not shown).

### Profiles of IgE responses to *P pratense* allergenic molecules

The combinatorial analysis of IgE responses against *P pratense* molecules showed a wide spectrum of sensitization profiles. Among the 176 subjects reacting to *P pratense* pollen extract (cutoff,  $\geq 0.35$  kU/L) and having a complete dataset for each of the 8 molecules examined, we found 39 different profiles of IgE sensitization. Only about 20% of the patients were monosensitized to Phl p 1 (profile 128: 36/176 [20.8%]). Interestingly, only 8 patients were monosensitized to other molecules: Phl p 4 (profile 32, n = 5; 2.9%), Phl p 2 (profile 64, n = 2; 1.2%), and Phl p 5 (profile 16, n = 1; 0.6%). The second most frequent profile combined Phl p 1, Phl p 2, Phl p 4, Phl p 5, and Phl p 6 (profile 248, n = 21; 12.1%). After descending sorting of the 39 profiles by their frequency, the arbitrary threshold of 80% of the whole patient population was covered by 15 profiles (Fig 1).

We then examined the effect of reducing the number of molecules taken into consideration, first by ignoring highly cross-reactive allergens (rPhl p 7 and rPhl p 12, see Fig E3 in

| Profile<br>APCS<br>code | profile's<br>binary code<br>(8 molecules):<br>cut-off 0.35 kU/L | rPhl p 1 | rPhl p 2 | rPhl p 4 | rPhl p 5 | rPhl p 6 | rPhl p 7 | rPhl p 11 | rPhl p 12 | n pos. mol. | n  | %    | cum.<br>% |
|-------------------------|-----------------------------------------------------------------|----------|----------|----------|----------|----------|----------|-----------|-----------|-------------|----|------|-----------|
| 128                     | 10000000                                                        | ●        |          |          |          |          |          |           |           | 1           | 36 | 20,8 | 21        |
| 248                     | 11111000                                                        | ●        | ●        |          |          |          |          |           |           | 5           | 21 | 12,1 | 33        |
| 160                     | 10100000                                                        | ●        |          | ●        |          |          |          |           |           | 2           | 10 | 5,8  | 39        |
| 184                     | 10111000                                                        | ●        |          | ●        | ●        |          |          |           |           | 4           | 8  | 4,6  | 43        |
| 186                     | 10111010                                                        | ●        |          | ●        | ●        | ●        |          |           |           | 5           | 8  | 4,6  | 48        |
| 251                     | 11111011                                                        | ●        | ●        | ●        | ●        | ●        |          |           |           | 7           | 8  | 4,6  | 53        |
| 192                     | 11000000                                                        | ●        | ●        |          |          |          |          |           |           | 2           | 7  | 4,0  | 57        |
| 216                     | 11011000                                                        | ●        | ●        |          | ●        |          |          |           |           | 4           | 7  | 4,0  | 61        |
| 249                     | 11111001                                                        | ●        | ●        | ●        | ●        | ●        |          |           | ●         | 6           | 7  | 4,0  | 65        |
| 250                     | 11111010                                                        | ●        | ●        | ●        | ●        | ●        |          |           |           | 6           | 7  | 4,0  | 69        |
| 32                      | 1000000                                                         |          | ●        |          |          |          |          |           |           | 1           | 5  | 2,9  | 72        |
| 224                     | 11100000                                                        | ●        | ●        | ●        |          |          |          |           |           | 3           | 5  | 2,9  | 75        |
| 152                     | 10011000                                                        |          | ●        |          | ●        |          |          |           |           | 3           | 4  | 2,3  | 77        |
| 185                     | 10111001                                                        | ●        |          | ●        | ●        | ●        |          |           | ●         | 5           | 4  | 2,3  | 79        |
| 208                     | 11010000                                                        | ●        | ●        |          |          |          |          |           |           | 3           | 3  | 1,7  | 81        |
| 218                     | 11011010                                                        | ●        | ●        |          | ●        | ●        |          |           | ●         | 5           | 3  | 1,7  | 83        |
| 48                      | 1100000                                                         |          | ●        | ●        |          |          |          |           |           | 2           | 2  | 1,2  | 84        |
| 64                      | 1000000                                                         | ●        |          |          |          |          |          |           |           | 1           | 2  | 1,2  | 85        |
| 144                     | 10010000                                                        | ●        |          | ●        |          |          |          |           |           | 2           | 2  | 1,2  | 86        |
| 162                     | 10100010                                                        | ●        |          | ●        |          |          |          | ●         |           | 3           | 2  | 1,2  | 87        |
| 187                     | 10111011                                                        | ●        |          | ●        | ●        | ●        |          | ●         | ●         | 6           | 2  | 1,2  | 88        |
| 193                     | 11000001                                                        | ●        | ●        |          |          |          |          |           | ●         | 3           | 2  | 1,2  | 90        |
| 217                     | 11011001                                                        | ●        | ●        |          | ●        | ●        |          |           | ●         | 5           | 2  | 1,2  | 91        |
| 225                     | 11100001                                                        | ●        | ●        | ●        |          |          |          |           | ●         | 4           | 2  | 1,2  | 92        |
| 16                      | 10000                                                           |          |          | ●        |          |          |          |           |           | 1           | 1  | 0,6  | 92        |
| 34                      | 100010                                                          |          | ●        |          |          |          |          | ●         |           | 2           | 1  | 0,6  | 93        |
| 58                      | 111010                                                          |          | ●        | ●        | ●        |          |          | ●         |           | 4           | 1  | 0,6  | 94        |
| 96                      | 1100000                                                         |          | ●        | ●        |          |          |          |           |           | 2           | 1  | 0,6  | 94        |
| 129                     | 10000001                                                        | ●        |          |          |          |          |          |           | ●         | 2           | 1  | 0,6  | 95        |
| 130                     | 10000010                                                        | ●        |          |          |          |          |          | ●         |           | 2           | 1  | 0,6  | 95        |
| 132                     | 10000100                                                        | ●        |          |          |          |          |          | ●         |           | 2           | 1  | 0,6  | 96        |
| 156                     | 10011100                                                        | ●        |          | ●        | ●        | ●        |          |           |           | 4           | 1  | 0,6  | 97        |
| 188                     | 10111100                                                        | ●        |          | ●        | ●        | ●        |          |           |           | 5           | 1  | 0,6  | 97        |
| 194                     | 11000010                                                        | ●        | ●        |          |          |          |          | ●         |           | 3           | 1  | 0,6  | 98        |
| 232                     | 11101000                                                        | ●        | ●        |          |          | ●        |          |           |           | 4           | 1  | 0,6  | 98        |
| 240                     | 11110000                                                        | ●        | ●        | ●        | ●        |          |          |           |           | 4           | 1  | 0,6  | 99        |
| 254                     | 11111110                                                        | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 7           | 1  | 0,6  | 99        |
| 255                     | 11111111                                                        | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 8           | 1  | 0,6  | 100       |
| 0                       | 0                                                               |          |          |          |          |          |          |           |           | 0           | 3  | 1,7  |           |

**FIG 1.** Profiles of IgE sensitization to 8 *P pratense* molecules in 176 children with an IgE reaction to *P pratense* and complete dataset. The Allergen Profile Codification System (APCS) code and the absolute and cumulative frequencies are shown. The profiles are ordered by decreasing frequency, and the point at which the arbitrary threshold of 80% of the patient population has been reached is marked in red.

this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) and then also by excluding more weakly cross-reactive allergens (rPhl p 4 and Phl p 11, see Fig E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). As a consequence of the reduced number of possible combinations, the heterogeneity was greatly and progressively reduced. Actually, the number of profiles based on 6 and 4 molecules necessary to cover 80% of the patients' population decreased to 9 and 3, respectively (see Figs E3 and E4).

Lastly, we examined the effect of the cutoff point chosen for positivity on the heterogeneity and frequency distribution of the sensitization profiles. The complexity of the profiles based on all 8 molecules examined was roughly unchanged by increasing the cutoff levels of IgE positivity to 0.7 or 3.5 kU/L (see Fig E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

### Matching patients' IgE profiles with a molecularly designed SIT preparation

We examined the potential suitability of a molecularly designed SIT preparation as a CRT treatment in our study population. This preparation contained a balanced and standardized mixture of Phl p 1, Phl p 2, Phl p 5a and Phl p 5b, and Phl p 6.<sup>15</sup> Altogether, only 7 (4%) of 176 patients had a sensitization profile matching exactly the one proposed in the experimental SIT

preparation (Fig 2). In the other 169 (96%) patients there was a mismatch between the sensitization profile of each patient and the composition of the SIT preparation. Four different categories of mismatch were observed:

- *type 1 or underpowered immunization*: patients sensitized to all the molecules contained in the SIT preparation and to additional ones;
- *type 2 or overpowered immunization*: patients sensitized only to some of the molecules contained in the SIT preparation but not to molecules absent from the SIT preparation (at least among those tested for IgE sensitization);
- *type 3 or underpowered and overpowered immunization*: patients showing both type 1 and type 2 mismatch; and
- *type 4 or unrelated immunization*: patients sensitized only to molecules that are not present in the SIT preparation.

Type 1, 2, and 3 mismatch displayed roughly similar frequencies (about 30% each), whereas the fourth type of mismatch was observed only in 9 (5%) of 176 patients (Fig 2). This scenario did not change substantially on reducing to only 6 (after exclusion of Phl p 7 and Phl p 12) molecules being taken into account, and it was of course simplified by including in the analysis only the molecules contained in the SIT preparation (see Table E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

### DISCUSSION

Through a combinatorial approach, we analyzed the distribution and characteristics of the individual profiles of IgE sensitization to 8 molecules of *P pratense* in a population of children with grass pollen allergy living in Rome, Italy. We found that (1) the profiles of IgE sensitization to *P pratense* molecules were extremely heterogeneous, (2) such heterogeneity can be reduced only by excluding some molecules from the analysis and not by increasing the cutoff levels, (3) only a small minority of the profiles (7/176 [4%]) were matched to the only SIT preparation for *P pratense* recombinant allergenic molecules validated in human subjects thus far, and (4) IgE sensitization profiles can be divided into 5 categories when matched against that SIT preparation. To our knowledge, this is the first attempt to use the combinatorial analysis to classify patients' sensitization profiles in relation to the molecular composition of a recombinant SIT preparation.

In a seminal article Valenta et al<sup>11</sup> described 10 sensitization profiles based on the same 8 *P pratense* molecules. However, we found as many as 39 profiles in only 176 patients (Fig 1). This figure corresponded to only 4.5 patients per profile and prevented us from analyzing the clinical correlates of each profile; this would be a target of a future study.\* Many more profiles would likely have been found in a larger population, and their theoretic number is limited only by the maximal amount of possible combinations ( $n = 2^8$  or 256). In this perspective the Allergen Profile Codification System might be further improved to be proposed as a standard tool to compare the molecular profiles of sensitization reported by different studies.

According to the CRT approach, a very high number of molecular combinations would be needed to properly treat a population of patients with grass allergy. Unfortunately, the present norm for registering and distributing SIT preparations

\*This analysis is an objective of the extended version of our Panallergens in Pediatrics project, targeting more than 1400 children.

| patient's profile:<br>APCS code <sup>o</sup>                                         | example patient's profile <sup>o</sup> | molecular SIT profile | example molecular overlapping <sup>o</sup> | patient's immunization category                    | frequency by category n (%) |
|--------------------------------------------------------------------------------------|----------------------------------------|-----------------------|--------------------------------------------|----------------------------------------------------|-----------------------------|
| 216                                                                                  |                                        | +                     | =                                          | MATCHING                                           | 7/176 (4%)                  |
| 217,218,248<br>249,250,251<br>254,255                                                |                                        | +                     | =                                          | TYPE 1 MISMATCH<br>under powered immunization      | 50/176 (28%)                |
| 16,48,64,<br>128,144,152,<br>192,208                                                 |                                        | +                     | =                                          | TYPE 2 MISMATCH<br>over powered immunization       | 57/176 (32%)                |
| 58,96,129,130,<br>132,156,160,162,184,<br>185,186,187,188,193<br>194,224,225,232,240 |                                        | +                     | =                                          | TYPE 3 MISMATCH<br>under/over powered immunization | 53/176 (30%)                |
| 0,32,34                                                                              |                                        | +                     | =                                          | TYPE 4 MIS-MATCH<br>unrelated immunization         | 9/176 (5%)                  |

<sup>o</sup> the pattern chosen as an example is marked in italic red

**FIG 2.** Matching molecular profiles of IgE sensitization to *P pratense* against the composition of a molecularly designed SIT preparation in 176 children with grass pollen respiratory allergies. Each of the 39 profiles represented in Fig 1 is reported here. Five categories have been created with all 5 different possible typologies of matching/mismatching between sensitization profiles and composition of the SIT preparation.

makes a flexible modular approach to SIT impossible.<sup>25</sup> Only predefined mixes of allergenic molecules can be produced, and each of them must undergo an extremely complex and expensive authorization procedure.<sup>25</sup> Not surprisingly, only 1 molecular preparation for grass pollen SIT based on Phl p 1, Phl p 2, Phl p 5a and Phl p 5b, and Phl p 6 has been developed thus far.<sup>15</sup> Our analysis shows that this predefined preparation would be perfectly tailored only to 4% of our patients (Fig 2). We grouped the remaining 96% into an additional 4 categories (Fig 2) and speculated on the immunologic consequences of their administration:

- In type 1 mismatch (underpowered immunization) patients would receive immunization with only some of the molecules to which they are sensitized. The efficacy of the immunization might be insufficient in this category. On the other hand, an underpowered immunization could be still effective if the immune response would exert a bystander-protective influence on immune reactions against other allergenic molecules coexisting in the pollen grains and particles (so-called linked suppression).
- In type 2 mismatch (overpowered immunization) patients would receive all the molecules to which they are sensitized but also molecules to which they are not sensitized. Three mutually exclusive consequences could be predicted in this category: (1) a beneficial induction of IgG antibodies, prevention of new IgE sensitization, or both<sup>15</sup>; (2) an unwanted IgE sensitization to these molecules<sup>26</sup>; or (3) no effect.<sup>27</sup>
- Type 3 mismatch (underpowered and overpowered immunization) includes patients with a mixed (type 1 and 2) mismatch. In this group a combination of the effects described in the previous categories could be expected.

- In type 4 mismatch (unrelated immunization) patients would be immunized with molecules to which they are not sensitized and would not be immunized with the molecules to which they are sensitized. This category would probably be the one with the lowest (if any) benefit and the highest risk of unwanted effects.

The hypotheses generated by our classification might be tested and inspire trials aimed at comparing the immunologic and clinical effect of the same preparation in different patient categories. Such a trial would be useful as a proof-of-concept study of the CRT approach.

Our methodologic approach is indirectly validated by the results of IgE sensitization to individual *P pratense* proteins, the frequencies and magnitude of sensitization to which (see Fig E2) were similar to those previously published.<sup>9,10,16-18</sup> Nevertheless, we should also consider at least 5 potential limitations of our study.

First, it could be argued that the level of profile diversity was an artificial byproduct of too low and clinically insignificant cutoff points (0.35 kU/L). However, by increasing the cutoff point to 0.7 or 3.5 kU/L, the profile complexity at a population level remained substantially unchanged. On the other hand, this heterogeneity might be typical of childhood, whereas adults might have more homogeneous profiles.<sup>18</sup>

Second, it could also be argued that not all 8 molecules of *P pratense* are important for specific immunotherapy. Actually, Phl p 1, Phl p 2, Phl p 5, and Phl p 6 generated a distinct cluster of IgE reactivity together in a recent large epidemiologic study,<sup>18</sup> and they seem to have a higher clinical relevance,<sup>28</sup> so that they were considered for 2 molecularly designed SIT preparations produced as a mix<sup>15</sup> or as a single hybrid molecule.<sup>29</sup> However, we extended our analysis to smaller panels of 6 and 4

molecules obtained by excluding the 2 panallergens (Phl p 7 and Phl p 12) or by including only the grass pollen hallmark molecules (Phl p 1, Phl p 2, Phl p 5, and Phl p 6; see Fig E4 and Table E2).

Third, on the other hand, our results might have been affected by exclusion of some relevant allergenic molecules, such as Phl p 3, Phl p 5b, and Phl p 13. However, Phl p 5a contains all but 1 of the B-cell epitopes of Phl p 5b,<sup>13</sup> and the clinical role of Phl p 3 and Phl p 13 is still unclear.<sup>28</sup>

Fourth, we must acknowledge that a complete analysis should include many allergenic molecules from other relevant pollens sources; actually, most of our patients were sensitized to other allergenic sources (see Fig E1) and their major allergenic molecules (eg, Bet v 1, Ole e 1, Par j 2, and Art v 1; data not shown). However, by taking into consideration these IgE specificities, potential combinations would have been in the thousands (2<sup>12</sup>), and our population sample would have not been sufficiently powered for analysis.<sup>1</sup>

Finally, cross-reactivity, protein sequence homology, and isoallergen variants of the used recombinant molecules versus native extracts might have affected their potency and our results. Nevertheless, comparative studies have shown that the sensitivity and specificity of these molecules in relation to the extracts is excellent.<sup>13</sup>

In conclusion, our study might be a useful start for looking at the gap between the current possibility of using a predefined SIT preparation and the complexity of sensitization at the population level. Our results might inspire similar studies in different geographic areas, as well as trials aimed at testing whether different sensitization profiles to grass pollen underlie different clinical responses to the same SIT preparation. We hope also that our results might foster more flexible rules governing the production of molecularly based SIT preparations.

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**Clinical implications: Trials are needed to test whether different molecular sensitization profiles to grass pollen underlie different clinical responses to the same SIT preparation. Registration rules for molecularly designed SIT preparations should consider the extreme heterogeneity of sensitization profiles in populations.**

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## METHODS

### Ethics

Participants complied with the relevant Italian and European Union legislation, including the Chart of Fundamental Rights and the European Union Directive 95/46/EC on the protection of individuals with regard to processing of personal data and on the free movement of such data. In addition, the project adheres to the Helsinki Declaration and the United Nations Convention on the Rights of the Child.

### SPTs

Histamine (0.1 mg/dL) and glycerol solution were used as positive and negative controls, respectively. Morrow-Brown needles were used to perform the SPTs, and skin reactions were read after 15 minutes.

The broad SPT panel of the study Panallergens in Pediatrics is aimed at testing sensitization to airborne allergens relevant in Italy in 1400 children all over Italy. The pilot study (performed in Rome only) has therefore included all these airborne allergens. Before starting the pilot study, teams have been carefully trained to properly use both arms for SPTs and to respect the necessary distance between SPT reaction sites according to accepted standards.<sup>E1</sup>

### IgE assays: Inclusion criteria and assays for Phl p 4

According to international standards,<sup>E2</sup> we have used a cutoff of 3 mm to consider an SPT wheal reaction as positive (Fig E1), which is the best method to differentiate between sensitized and nonsensitized children. A 2-mm cutoff has been used only as a criterion for further IgE molecular testing to explore the very low concentration of IgE responses.

We sequentially used rPhl p 4 (experimental ELISA) for a qualitative assay and nPhl p 4 (ImmunoCAP, Phadia) for a quantitative assay. Briefly, we used the recombinant form of Phl p 4 to identify subjects with a positive or negative IgE response to this molecule, thus avoiding a confounding effect of its highly cross-reacting CCD moieties. Then we used the concentration of IgE antibodies against nPhl p 4 obtained with the ImmunoCAP system, thus avoiding methodologic differences with the measure of the IgE levels against the other 7 *P pratense* molecules.

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| pattern<br>positive SPT | n. of<br>positive<br>SPT | <i>Phleum p</i> | <i>Platanus o</i> | <i>Cupressus a</i> | <i>Betula v</i> | <i>Olea e</i> | <i>Parietaria j</i> | <i>Artemisia v</i> | <i>Plantago l</i> | n  | %   |
|-------------------------|--------------------------|-----------------|-------------------|--------------------|-----------------|---------------|---------------------|--------------------|-------------------|----|-----|
| 11111111                | 8                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 9  | 4,5 |
| 10101100                | 4                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 7  | 3,5 |
| 11011111                | 7                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 5  | 2,5 |
| 11101111                | 7                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 4  | 2   |
| 11111101                | 7                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 3  | 1,5 |
| 11111100                | 6                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 3  | 1,5 |
| 11101101                | 6                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 3  | 1,5 |
| 10101110                | 5                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 3  | 1,5 |
| 10101101                | 5                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 3  | 1,5 |
| 10001100                | 3                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 3  | 1,5 |
| 11101100                | 5                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 2  | 1   |
| 11011101                | 6                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 2  | 1   |
| 10001111                | 5                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 2  | 1   |
| 11101110                | 6                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 11001111                | 6                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 11001100                | 4                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 10111111                | 7                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 10111101                | 6                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 10011100                | 4                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 10001101                | 4                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 10001000                | 2                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 24 | 12  |
| 10101000                | 3                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 8  | 4   |
| 11111001                | 6                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 5  | 2,5 |
| 10101001                | 4                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 5  | 2,5 |
| 11111011                | 7                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 4  | 2   |
| 10001001                | 3                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 4  | 2   |
| 11101001                | 5                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 3  | 1,5 |
| 11101000                | 4                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 2  | 1   |
| 10111001                | 5                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 2  | 1   |
| 10111000                | 4                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 2  | 1   |
| 11111010                | 6                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 11011011                | 6                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 11011001                | 5                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 11011000                | 4                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 11001001                | 4                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 11001000                | 3                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 10100100                | 3                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 5  | 2,5 |
| 10000100                | 2                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 5  | 2,5 |
| 10110100                | 4                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 2  | 1   |
| 11010101                | 5                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 10010100                | 3                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 10000101                | 3                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 10000000                | 1                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 26 | 13  |
| 10100000                | 2                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 7  | 3,5 |
| 10000001                | 2                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 4  | 2   |
| 11110001                | 5                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 2  | 1   |
| 11000000                | 2                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 2  | 1   |
| 10100001                | 3                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 2  | 1   |
| 11100001                | 4                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 10010000                | 2                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 10000010                | 2                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 1011100                 | 3                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 2  | 1   |
| 1100                    | 2                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 1011001                 | 4                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 1001001                 | 3                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 101000                  | 2                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 1001                    | 2                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 100                     | 1                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 5  | 2,5 |
| 100100                  | 2                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 3  | 1,5 |
| 10100                   | 2                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 1100001                 | 3                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |
| 0                       | 0                        | ●               | ●                 | ●                  | ●               | ●             | ●                   | ●                  | ●                 | 1  | 0,5 |

**FIG E1.** Profiles of sensitization to pollens characterized at the source level with extracts. The pollen species (*P pratense*, olive, and pellitory) sharing the same pollen season (April-June) with grasses in Rome are reported in red. The binary code expressing the profile of sensitization is reported in the first column. Other columns report the number of positive results for each profile. Frequency distribution is also shown.

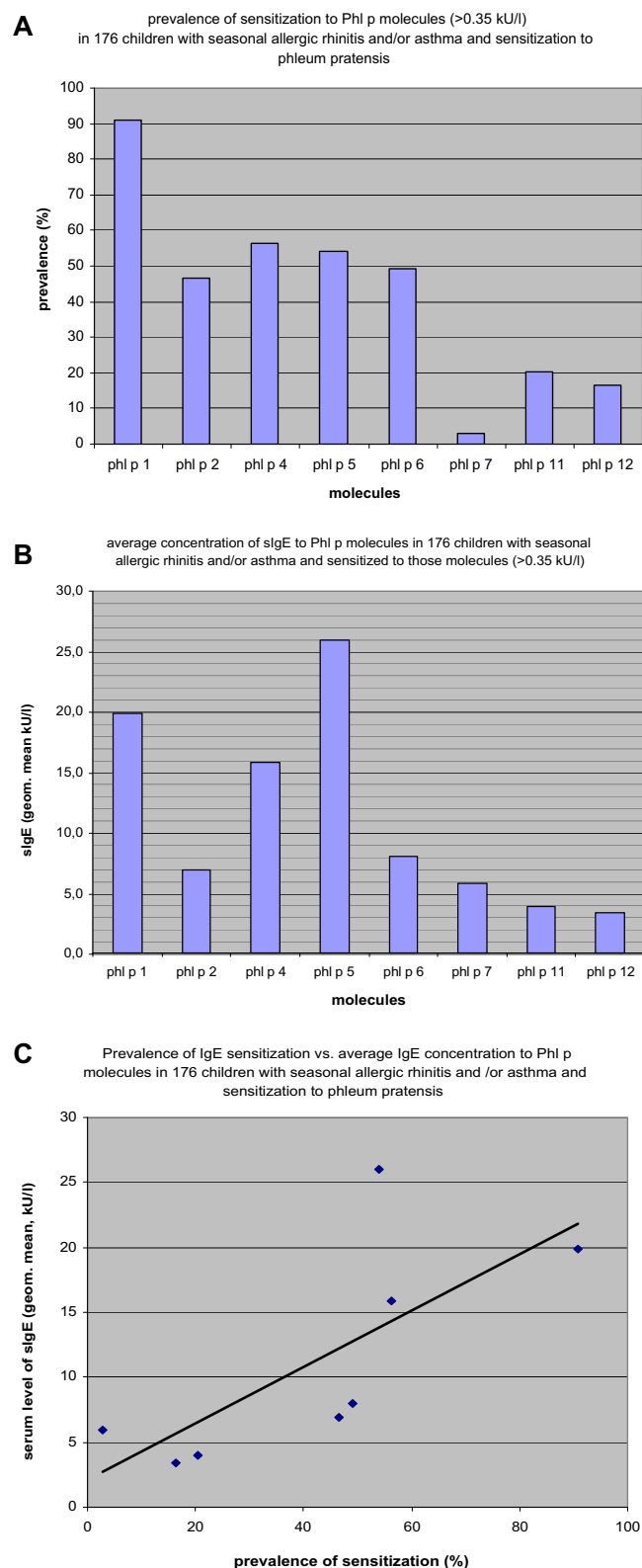


**FIG E2.** Serum IgE responses to individual allergenic molecules of *P. pratense*. The frequency distribution of positive reactions (cutoff, 0.35 kU/L; **A**), the geometric mean concentration of positive samples (**B**), and the relationship between these 2 measures (**C**) are shown.

| profile's<br>binary code<br>(6 molecules):<br>cut-off 0.35 kU/L | rPhl p 1 | rPhl p 2 | rPhl p 4 | rPhl p 5 | rPhl p 6 | rPhl p 11 | n pos. mol. | n  | %    | cum.<br>% |
|-----------------------------------------------------------------|----------|----------|----------|----------|----------|-----------|-------------|----|------|-----------|
| 100000                                                          | ●        |          |          |          |          |           | 1           | 38 | 22,0 | 22,0      |
| 111110                                                          | ●        | ●        | ●        | ●        | ●        |           | 5           | 28 | 16,2 | 38,2      |
| 111111                                                          | ●        | ●        | ●        | ●        | ●        | ●         | 6           | 17 | 9,8  | 48,0      |
| 101110                                                          | ●        |          | ●        | ●        | ●        |           | 4           | 13 | 7,5  | 55,5      |
| 101000                                                          | ●        |          | ●        |          |          |           | 2           | 10 | 5,8  | 61,3      |
| 101111                                                          | ●        |          | ●        | ●        | ●        | ●         | 5           | 10 | 5,8  | 67,1      |
| 110000                                                          | ●        | ●        |          |          |          |           | 2           | 9  | 5,2  | 72,3      |
| 110110                                                          | ●        | ●        |          | ●        | ●        |           | 4           | 9  | 5,2  | 77,5      |
| 111000                                                          | ●        | ●        | ●        |          |          |           | 3           | 7  | 4,0  | 81,5      |
| 1000                                                            |          |          | ●        |          |          |           | 1           | 5  | 2,9  | 84,4      |
| 100110                                                          | ●        |          |          | ●        | ●        |           | 3           | 5  | 2,9  | 87,3      |
| 110100                                                          | ●        | ●        |          | ●        |          |           | 3           | 3  | 1,7  | 89,0      |
| 110111                                                          | ●        | ●        |          | ●        | ●        | ●         | 5           | 3  | 1,7  | 90,8      |
| 1100                                                            |          |          | ●        | ●        |          |           | 2           | 2  | 1,2  | 91,9      |
| 10000                                                           |          | ●        |          |          |          |           | 1           | 2  | 1,2  | 93,1      |
| 100100                                                          | ●        |          |          | ●        |          |           | 2           | 2  | 1,2  | 94,2      |
| 101001                                                          | ●        |          | ●        |          |          | ●         | 3           | 2  | 1,2  | 95,4      |
| 100                                                             |          |          |          | ●        |          |           | 1           | 1  | 0,6  | 96,0      |
| 1001                                                            |          |          | ●        |          |          | ●         | 2           | 1  | 0,6  | 96,5      |
| 1111                                                            |          |          | ●        | ●        | ●        | ●         | 4           | 1  | 0,6  | 97,1      |
| 11000                                                           |          | ●        | ●        |          |          |           | 2           | 1  | 0,6  | 97,7      |
| 100001                                                          | ●        |          |          |          |          | ●         | 2           | 1  | 0,6  | 98,3      |
| 110001                                                          | ●        | ●        |          |          |          | ●         | 3           | 1  | 0,6  | 98,8      |
| 111010                                                          | ●        | ●        | ●        |          | ●        |           | 4           | 1  | 0,6  | 99,4      |
| 111100                                                          | ●        | ●        | ●        | ●        |          |           | 4           | 1  | 0,6  | 100,0     |
| 0                                                               |          |          |          |          |          |           | 0           | 3  | 1,7  |           |

**FIG E3.** Molecular profiles of IgE sensitization to 6 *P pratense* molecules: Phl p 1, Phl p 2, Phl p 4, Phl p 5, Phl p 6, and Phl p 11. *n pos mol.*, Number of positive molecules.

| profile's<br>binary code<br>(4 molecules);<br>cut-off 0.35 kU/L | rPhl p 1 | rPhl p 2 | rPhl p 5 | rPhl p 6 | n<br>pos. mol. | n  | %    | cum.<br>% |
|-----------------------------------------------------------------|----------|----------|----------|----------|----------------|----|------|-----------|
| 1111                                                            | ●        | ●        | ●        | ●        | 4              | 57 | 34,1 | 34,1      |
| 1000                                                            | ●        |          |          |          | 1              | 51 | 30,5 | 64,7      |
| 1011                                                            | ●        |          | ●        | ●        | 3              | 28 | 16,8 | 81,4      |
| 1100                                                            | ●        | ●        |          |          | 2              | 17 | 10,2 | 91,6      |
| 1110                                                            | ●        | ●        | ●        |          | 3              | 4  | 2,4  | 94,0      |
| 10                                                              |          |          | ●        |          | 1              | 3  | 1,8  | 95,8      |
| 100                                                             |          | ●        |          |          | 1              | 3  | 1,8  | 97,6      |
| 1010                                                            | ●        |          | ●        |          | 2              | 2  | 1,2  | 98,8      |
| 1101                                                            | ●        | ●        |          | ●        | 3              | 1  | 0,6  | 99,4      |
| 11                                                              |          |          | ●        | ●        | 2              | 1  | 0,6  | 100,0     |
| 0                                                               |          |          |          |          | 0              | 9  | 5,1  |           |

**FIG E4.** Molecular profiles of IgE sensitization to *P pratense* molecules: Phl p 1, Phl p 2, Phl p 5, and Phl p 6. *n pos mol.*, Number of positive molecules.

| APCS code | profile's binary code (8 molecules): cut-off 0.35 KU/L | rPhl p 1 | rPhl p 2 | rPhl p 4 | rPhl p 5 | rPhl p 6 | rPhl p 7 | rPhl p 11 | rPhl p 12 | n pos. mol. | n  | %    | cum. % |
|-----------|--------------------------------------------------------|----------|----------|----------|----------|----------|----------|-----------|-----------|-------------|----|------|--------|
| 128       | 10000000                                               | ●        |          |          |          |          |          |           |           | 1           | 36 | 20,8 | 21     |
| 248       | 11111000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 21 | 12,1 | 33     |
| 160       | 10100000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 10 | 5,8  | 39     |
| 184       | 10111000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 8  | 4,6  | 43     |
| 186       | 10111010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 8  | 4,6  | 48     |
| 251       | 11111011                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 7           | 8  | 4,6  | 53     |
| 192       | 11000000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 7  | 4,0  | 57     |
| 216       | 11011000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 7  | 4,0  | 61     |
| 249       | 11111001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 6           | 7  | 4,0  | 65     |
| 250       | 11111010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 6           | 7  | 4,0  | 69     |
| 32        | 10000000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 1           | 5  | 2,9  | 72     |
| 224       | 11100000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 5  | 2,9  | 75     |
| 152       | 10011000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 4  | 2,3  | 77     |
| 185       | 10111001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 4  | 2,3  | 79     |
| 208       | 11011000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 3  | 1,7  | 81     |
| 218       | 11011010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 3  | 1,7  | 83     |
| 48        | 11000000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 2  | 1,2  | 84     |
| 64        | 10000000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 1           | 2  | 1,2  | 85     |
| 144       | 10010000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 2  | 1,2  | 86     |
| 162       | 10100010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 2  | 1,2  | 87     |
| 187       | 10111011                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 6           | 2  | 1,2  | 88     |
| 193       | 11000001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 2  | 1,2  | 90     |
| 217       | 11011001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 2  | 1,2  | 91     |
| 225       | 11100001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 2  | 1,2  | 92     |
| 16        | 10000                                                  | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 1           | 1  | 0,6  | 92     |
| 34        | 100010                                                 | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 1  | 0,6  | 93     |
| 58        | 111010                                                 | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 1  | 0,6  | 94     |
| 96        | 1100000                                                | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 1  | 0,6  | 94     |
| 129       | 10000001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 1  | 0,6  | 95     |
| 130       | 10000010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 1  | 0,6  | 95     |
| 132       | 10000100                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 1  | 0,6  | 96     |
| 156       | 10011100                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 1  | 0,6  | 97     |
| 188       | 10111100                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 1  | 0,6  | 97     |
| 194       | 11000010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 1  | 0,6  | 98     |
| 232       | 11101000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 1  | 0,6  | 98     |
| 240       | 11110000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 1  | 0,6  | 99     |
| 254       | 11111110                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 7           | 1  | 0,6  | 99     |
| 255       | 11111111                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 8           | 1  | 0,6  | 100    |
| 0         | 0                                                      |          |          |          |          |          |          |           |           | 0           | 3  | 1,7  |        |

| APCS code | profile's binary code (8 molecules): cut-off 0.70 KU/L | rPhl p 1 | rPhl p 2 | rPhl p 4 | rPhl p 5 | rPhl p 6 | rPhl p 7 | rPhl p 11 | rPhl p 12 | n pos. mol. | n  | %    | cum. % |
|-----------|--------------------------------------------------------|----------|----------|----------|----------|----------|----------|-----------|-----------|-------------|----|------|--------|
| 128       | 10000000                                               | ●        |          |          |          |          |          |           |           | 1           | 41 | 25,2 | 25     |
| 216       | 11011000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 14 | 8,6  | 34     |
| 248       | 11111000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 13 | 8,0  | 42     |
| 192       | 11000000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 9  | 5,5  | 47     |
| 248       | 11111000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 8  | 4,9  | 52     |
| 249       | 11111001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 6           | 7  | 4,3  | 56     |
| 152       | 10011000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 7  | 4,3  | 61     |
| 160       | 10100000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 7  | 4,3  | 61     |
| 184       | 10111000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 6  | 3,7  | 64     |
| 250       | 11111010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 6           | 5  | 3,1  | 67     |
| 186       | 10111010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 4  | 2,5  | 70     |
| 251       | 11111011                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 7           | 4  | 2,5  | 72     |
| 32        | 10000000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 1           | 3  | 1,8  | 74     |
| 136       | 10010000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 3  | 1,8  | 76     |
| 185       | 10111001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 3  | 1,8  | 78     |
| 208       | 11010000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 3  | 1,8  | 80     |
| 217       | 11011001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 3  | 1,8  | 82     |
| 218       | 11011010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 3  | 1,8  | 83     |
| 224       | 11100000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 3  | 1,8  | 85     |
| 48        | 110000                                                 | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 2  | 1,2  | 87     |
| 58        | 111010                                                 | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 2  | 1,2  | 88     |
| 187       | 10111011                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 6           | 2  | 1,2  | 89     |
| 16        | 10000                                                  | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 1           | 1  | 0,6  | 90     |
| 34        | 100010                                                 | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 1  | 0,6  | 90     |
| 64        | 10000000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 1           | 1  | 0,6  | 91     |
| 129       | 10000001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 1  | 0,6  | 91     |
| 153       | 10011001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 1  | 0,6  | 92     |
| 154       | 10011010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 1  | 0,6  | 93     |
| 156       | 10011100                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 1  | 0,6  | 93     |
| 162       | 10100010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 1  | 0,6  | 94     |
| 176       | 10110000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 1  | 0,6  | 94     |
| 188       | 10111100                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 1  | 0,6  | 95     |
| 193       | 11000001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 1  | 0,6  | 96     |
| 194       | 11000010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 1  | 0,6  | 96     |
| 219       | 11011011                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 6           | 1  | 0,6  | 97     |
| 225       | 11100001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 1  | 0,6  | 98     |
| 232       | 11101000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 1  | 0,6  | 98     |
| 240       | 11110000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 7           | 1  | 0,6  | 99     |
| 254       | 11111110                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 7           | 1  | 0,6  | 99     |
| 255       | 11111111                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 8           | 1  | 0,6  | 100    |
| 0         | 0                                                      |          |          |          |          |          |          |           |           | 0           | 13 | 7,4  |        |

| APCS code | profile's binary code (8 molecules): cut-off 3.50 KU/L | rPhl p 1 | rPhl p 2 | rPhl p 4 | rPhl p 5 | rPhl p 6 | rPhl p 7 | rPhl p 11 | rPhl p 12 | n pos. mol. | n  | %    | cum. % |
|-----------|--------------------------------------------------------|----------|----------|----------|----------|----------|----------|-----------|-----------|-------------|----|------|--------|
| 128       | 10000000                                               | ●        |          |          |          |          |          |           |           | 1           | 33 | 23,4 | 23     |
| 216       | 11011000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 11 | 7,8  | 31     |
| 192       | 11000000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 9  | 6,4  | 38     |
| 248       | 11111000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 9  | 6,4  | 44     |
| 152       | 10011000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 8  | 5,7  | 50     |
| 144       | 10010000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 7  | 5,0  | 55     |
| 184       | 10111000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 7  | 5,0  | 60     |
| 160       | 10100000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 5  | 3,5  | 63     |
| 208       | 11010000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 5  | 3,5  | 67     |
| 249       | 11111001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 6           | 5  | 3,5  | 70     |
| 250       | 11111010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 6           | 5  | 3,5  | 74     |
| 176       | 10110000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 4  | 2,8  | 77     |
| 186       | 10111010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 4  | 2,8  | 79     |
| 218       | 11011010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 3  | 2,1  | 82     |
| 24        | 11000                                                  | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 2  | 1,4  | 83     |
| 32        | 100000                                                 | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 1           | 2  | 1,4  | 84     |
| 185       | 10111001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 2  | 1,4  | 86     |
| 217       | 11011001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 5           | 2  | 1,4  | 87     |
| 16        | 10010                                                  | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 1  | 0,7  | 88     |
| 50        | 110010                                                 | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 1  | 0,7  | 89     |
| 57        | 111001                                                 | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 1  | 0,7  | 89     |
| 64        | 1000000                                                | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 1           | 1  | 0,7  | 90     |
| 129       | 10000001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 2           | 1  | 0,7  | 91     |
| 137       | 10001001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 1  | 0,7  | 91     |
| 154       | 10011010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 1  | 0,7  | 92     |
| 162       | 10100010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 1  | 0,7  | 93     |
| 168       | 10101000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 1  | 0,7  | 94     |
| 193       | 11000001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 1  | 0,7  | 94     |
| 194       | 11000010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 1  | 0,7  | 95     |
| 210       | 11010010                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 1  | 0,7  | 96     |
| 224       | 11100000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 3           | 1  | 0,7  | 96     |
| 225       | 11100001                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 4           | 1  | 0,7  | 97     |
| 240       | 11110000                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 7           | 1  | 0,7  | 98     |
| 251       | 11111011                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 7           | 1  | 0,7  | 99     |
| 254       | 11111110                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 7           | 1  | 0,7  | 99     |
| 255       | 11111111                                               | ●        | ●        | ●        | ●        | ●        | ●        | ●         | ●         | 8           | 1  | 0,7  | 100    |
| 0         | 0                                                      |          |          |          |          |          |          |           |           | 0           | 35 | 19,9 |        |

**FIG E5.** Molecular profiles of IgE sensitization to *P. pratense* by cutoff. Each panel represents the whole population of 176 children with a positive IgE reaction to *P. pratense* and with a complete dataset. Cutoff points for positivity increase

**TABLE E1.** Characteristics of the population sample

|                                 |         |      |
|---------------------------------|---------|------|
| Age (y), mean $\pm$ SD          | 11.0    | 3.3  |
| Male sex (no./total [%])        | 126/200 | 63.0 |
| Allergic rhinitis               | 175/200 | 87.5 |
| Mild intermittent               | 45/175  | 25.7 |
| Moderate-to-severe intermittent | 11/175  | 6.3  |
| Mild persistent                 | 49/175  | 28.0 |
| Moderate-to-severe persistent   | 66/175  | 37.7 |
| Not specified                   | 4/175   | 2.3  |
| With asthma                     | 78/175  | 50.0 |
| Allergic asthma                 | 103/200 | 51.5 |
| Intermittent                    | 72/103  | 69.9 |
| Mild persistent                 | 16/103  | 15.5 |
| Moderate persistent             | 12/103  | 11.7 |
| Severe persistent               | 2/103   | 1.9  |
| Not specified                   | 1/103   | 1.0  |
| With rhinitis                   | 78/103  | 75.7 |

**TABLE E2.** Frequency distribution of the patient's immunization categories, by the number of *P pratense* molecules taken into account

| Patient's immunization category                        | Eight molecules<br>(Phl p 1, phl p 2, Phl p 4, Phl p 5,<br>Phl p 6, Phl p 7, Phl p 11, Phl p 12) |                                | Six molecules<br>(Phl p 1, Phl p 2, Phl p 4, Phl p 5,<br>Phl p 6, Phl p 11) |                                | Four molecules<br>(Phl p 1, Phl p 2, Phl p 5, Phl p 6) |                                |
|--------------------------------------------------------|--------------------------------------------------------------------------------------------------|--------------------------------|-----------------------------------------------------------------------------|--------------------------------|--------------------------------------------------------|--------------------------------|
|                                                        | Patient profiles                                                                                 | Frequency by category, no. (%) | Patient profiles                                                            | Frequency by category, no. (%) | Patient profiles                                       | Frequency by category, no. (%) |
| Matching                                               | 216                                                                                              | 7/176 (4%)                     | 54                                                                          | 9/176 (5%)                     | 15                                                     | 57/176 (32%)                   |
| Type 1 mismatch: underpowered immunization             | 217,218,248 249,250,251 254,255                                                                  | 50/176 (28%)                   | 55,62,63                                                                    | 48/176 (27%)                   | 2,3,4,8,10,11,12,13,14                                 | 110/176 (62%)                  |
| Type 2 mismatch: overpowered immunization              | 16,48,64,128,144,152,192,208                                                                     | 57/176 (32%)                   | 4,16,32,36,38,48,52                                                         | 60/176 (34%)                   | NA                                                     | NA                             |
| Type 3 Mismatch: underpowered/overpowered immunization | 58,96,129,130,132,156,160,162,184, 185,186,187,188,193,194,224,225,232,240                       | 53/176 (30%)                   | 12,15,24,33,40,41, 46,47,49,56,58,60                                        | 50/176 (28%)                   | NA                                                     | NA                             |
| Type 4 mismatch: unrelated immunization                | 0,32,34                                                                                          | 9/176 (5%)                     | 0,8,9                                                                       | 9/176 (5%)                     | 0                                                      | 9/176 (5%)                     |