

# Rhinitis symptoms caused by grass pollen are associated with elevated basophile allergen sensitivity and a larger grass-specific immunoglobulin E fraction

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### Summary

**Background** The mechanisms responsible for the difference between clinically irrelevant IgE-sensitization and allergic rhinitis are not fully understood.

**Objective** We evaluated the humoral and cellular mechanisms that may be associated with the presence of allergic rhinitis symptoms.

**Methods** We selected 26 subjects with positive grass pollen skin tests and IgE antibodies to Timothy (g6) and the major grass allergens rPhl p 1, 5b. Fourteen of those patients reported a history of allergic rhinitis. During winter, we performed a grass pollen CD63 basophile activation test using four log allergen concentrations, followed by a grass nasal provocation test (NPT). We obtained symptom scores in the subsequent pollination season. **Results** We showed that subjects with a positive NPT have significantly higher CD63 basophile grass pollen responsiveness than NPT-negative subjects, preferably at submaximal allergen concentrations, which represent cellular sensitivity. Moreover, basophile sensitivity positively correlated with the size of the grass-specific IgE fraction in relation to total IgE, and it was highly predictive of allergic rhinitis symptoms in the following pollination season.

**Conclusion and Clinical Relevance** Allergic rhinitis symptoms are significantly associated with allergen-specific basophile sensitivity. *In vitro* evaluation of basophile sensitivity should prove useful for distinguishing clinical phenotype of allergic sensitization.

**Keywords** allergic rhinitis, basophil activation, CD63, grass pollen, IgE fraction, symptom scores

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## Introduction

A considerable number of individuals with positive allergy skin tests or specific IgE antibodies are asymptomatic [1]. Population surveys show that more than one-third of individuals with positive IgE reactivity to grass pollen report no symptoms during the pollination season [2, 3]. The reasons why many sensitized and exposed persons do not develop symptoms are not completely understood. The absence of symptoms may be due to profilins and/or cross-reactive carbohydrate determinants that are capable of inducing IgE antibodies with no or very limited clinical significance [4]. These cross-reactive epitopes are also present in whole allergen extracts; hence, they may give rise to false-positive diagnostic results.

It has been postulated that, among immunologically related allergens, the allergen containing the most IgE-binding epitopes can be used as a representative marker allergen for diagnostic purposes [5]. Species-specific marker allergens indicate genuine sensitization to certain taxonomically closely related allergen sources. Patients with IgE reactivity to the recombinant allergens from *Phleum pratense* (Timothy grass), Phl p 1, Phl p 2 and Phl p 5, are identified as being allergic to grass pollen [6]. Despite the recognized diagnostic usefulness associated with these antigens [7], there are uncertainties regarding their clinical predictivity, because it has been shown that a substantial number of asymptomatic subjects also have IgE antibodies specific for major grass allergens [8, 9]. For example, an epidemiological study on a random sample in Finnish and

Russian Karelia showed that up to 50% of Timothy-sensitized asymptomatic subjects showed positive IgE reactivity to Phl p 1, 2, 5 or 6 [9]. Nevertheless, previous follow-up studies have clearly suggested that asymptomatic sensitization is a significant risk factor for later development of allergic rhinitis [10]. Obviously, beyond relevant sensitization, additional cell-related factors should significantly influence the development of symptoms.

Activation of allergic inflammation effector cells, such as mast cells and basophiles, is a key event during the development of allergic symptoms [11]. It was recently suggested that pollen immunotherapy induces allergen-specific suppression of CD63 basophile responsiveness, and this cellular-based suppression correlates with an improvement of rhinitis symptoms [12, 13]. Moreover, those studies showed that pollen immunotherapy induced changes at the level of basophile allergen sensitivity and not at the level of basophile maximal response; that is, reactivity. Therefore, evaluation of cellular allergen sensitivity may be a useful tool for mirroring clinical allergen sensitivity and also for comparing asymptomatic vs. symptomatic sensitization. With this knowledge, we selected a group of subjects with positive IgE reactivity to grass major allergens, but with an unequal retrospective history of allergic rhinitis. During winter, we evaluated grass allergen-specific basophile activation and nasal responses to allergen provocation, and then we compared these results with rhinitis symptoms during the following grass pollination season.

## Materials and methods

### Study subjects

This study was initiated during winter 2009. All subjects were recruited from the Golnik University Clinic. They were medication-free and had not been treated with allergen-specific immunotherapy. The flowchart of patient inclusion and their follow-ups are presented in Fig. 1. The first inclusion criteria included a positive grass pollen skin prick test (Mixed Grasses; HAL Allergy, Leiden, the Netherlands) and IgE antibodies to Timothy ( $>0.35$  kU/L). In total, we included 33 subjects, 15 with and 18 without a history of grass pollen allergic rhinitis in the last two pollination periods (2008 and 2009). The second criterion was the presence of IgE antibodies to the major grass allergens rPhl p 1, p 5b and/or rPhl p 2, which was fulfilled in 27 subjects. All of these subjects were also negative to cross-reactive rPhl p 7. Next, we performed CD63 basophile activation tests and quantified basophile FcεRI and IgE levels, followed by grass pollen nasal provocation tests (NPTs). One patient had a non-specific NPT and was excluded; therefore, in total, we studied 26 subjects. Their detailed

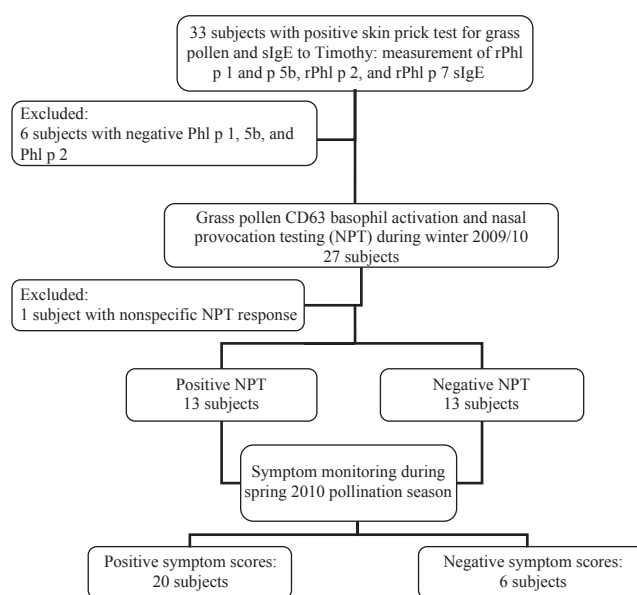


Fig. 1. Flowchart of the inclusion of study subjects.

demographical and clinical data are presented in Table 1. All 26 subjects were tested for rhinitis symptoms and medication scores during the subsequent grass pollination season of 2010. We also analysed the grass pollen concentrations in the central region of Slovenia. Throughout 2008, the sum value of grass pollen was 2074 grains/m<sup>3</sup>, during 2009 it was 3072 grains/m<sup>3</sup> and during 2010 it was 3712 grains/m<sup>3</sup>. We also included 10 healthy non-sensitized controls (Table 1). The study protocol was approved by the Slovenian National Medical Ethics Committee (KME 155/01/08).

### Nasal provocation tests

Nasal provocation tests were performed according to EAACI guidelines [14] with a nebulizer and grass pollen allergen solution (Mixed Grasses) provided by HAL Allergy. The solution was applied following inspiration when the patients held their breath. First, unspecific nasal reactivity was assessed by the application of 50 µL of saline to both nasal cavities. After 15 min, allergen provocation was performed with 50 µL of test solution containing 10 000 AU/mL of grass pollen allergen applied to both nasal cavities. Semi-quantitative recording of symptoms was assessed after 15 min, as previously described [15, 16]. We rated sneezing, rhinorrhoea and stuffiness (each up to 3 points); itching of the nose and/or ears (up to 2 points); and itching or erythema of the eye (1 point). The maximum total score was 12 points. If the sum was 5 points or more, the provocation was considered positive; otherwise, it was considered negative [15, 16]. If the symptom score sum was negative ( $<5$  points), two inhalations per each nasal cavity were applied, and the scores were assessed again.

Table 1. The characteristics of the test subjects according to NPT and rhinitis symptoms in the subsequent pollination season

|                                       | NPT ( <i>n</i> = 26; winter 2009/2010) |                           | Rhinitis symptoms ( <i>n</i> = 26; spring 2010) |                          | Controls ( <i>n</i> = 10) |
|---------------------------------------|----------------------------------------|---------------------------|-------------------------------------------------|--------------------------|---------------------------|
|                                       | Positive ( <i>n</i> = 13)              | Negative ( <i>n</i> = 13) | Positive ( <i>n</i> = 20)                       | Negative ( <i>n</i> = 6) |                           |
| Age (years)                           | 33 (20–46)                             | 32 (20–41)                | 29 (20–46)                                      | 37 (33–41)               | 29 (21–49)                |
| Sex (male/female)                     | 9/4                                    | 8/5                       | 13/7                                            | 4/2                      | 6/4                       |
| NPT (pos/neg)                         | 13/0                                   | 0/13                      | 13/7                                            | 0/6                      | 0/10                      |
| History (pos/neg) <sup>†</sup>        | 13/0                                   | 1/12                      | 14/6                                            | 0/6                      | 0/10                      |
| Symptom score <sup>††</sup>           | 43 (7–74)                              | 2 (0–57)**                | 34 (2–74)                                       | 0                        | 0                         |
| Medication score <sup>††</sup>        | 4 (0–12)                               | 0 (0–1)**                 | 1 (0–12)                                        | 0                        | 0                         |
| sIgE Timothy (kU/mL)                  | 16.9 (1.3–91.6)                        | 3.9 (0.7–100.0)           | 13 (1.3–100)                                    | 1.1 (0.7–5.1)*           | <0.35 (<0.35–0.77)        |
| sIgE rPhl p 1, p 5b (kU/mL)           | 18.2 (0.4–70.2)                        | 3.6 (0.5–100.0)           | 12.2 (0.4–100)                                  | 1.7 (0.5–3.6)*           | <0.35                     |
| sIgE rPhl p 2 (kU/mL)                 | 0.83 (<0.35–17.4)                      | <0.35 (<0.35–7.5)         | <0.35 (<0.35–17.4)                              | <0.35 (<0.35–2.9)        | <0.35                     |
| Total IgE (kU/mL)                     | 153 (34.5–855.0)                       | 197 (31.3–1616)           | 165 (34.5–1616)                                 | 164 (31.3–868)           | 60.9 (8.7–258)*           |
| sIgG4 Timothy (mgA/L)                 | 0.23 (0.01–0.45)                       | 0.2 (0.05–1.65)           | 0.26 (0.01–0.56)                                | 0.13 (0.05–1.65)         | 0.1 (0.0–1.0)             |
| Basal CD63 (%)                        | 3.2 (2.4–3.8)                          | 2.7 (2.0–5.9)             | 2.7 (2.1–4)                                     | 2.8 (2.5–5.9)            | 3.3 (2.4–3.6)             |
| fMLP CD63 (%)                         | 57.5 (12.6–85.8)                       | 34.2 (10–85.7)            | 46.5 (10–85.8)                                  | 30.4 (25.1–70.5)         | 57.8 (33.3–81.4)          |
| Anti-FcεRI CD63 (%)                   | 97.6 (51.9–100)                        | 97.3 (63–100)             | 97.3 (51.9–100)                                 | 96.2 (63–98.8)           | 95.7 (14.8–98.5)*         |
| IgE-mol per cell (×1000) <sup>‡</sup> | 165 (126–191)                          | 203 (84–214)              | 170 (96–214)                                    | 150 (84–208)             | 128 (15–155)*             |
| FcεRI per cell (×1000) <sup>‡</sup>   | 131 (93–162)                           | 149 (67–175)              | 138 (93–163)                                    | 133 (67–175)             | 105 (20–137)              |

Parameters shown include demographical and clinical data, humoral parameters, CD63 basophile responsiveness to control stimuli and surface basophile IgE and FcεRI enumeration. The results are presented as median and range.

NPT, nasal provocation test; fMLP, L-formyl-L-methionyl-L-leucyl-L-phenylalanine.

<sup>†</sup>Retrospective history of grass pollen allergic rhinitis in the 2008 and 2009 allergy seasons.

<sup>††</sup>Prospective symptom severity on a visual analogue scale and medication scores during spring 2010.

<sup>‡</sup>Data were measured in seven subjects with positive NPT scores, in nine subjects with negative NPT scores, in 11 subjects with positive rhinitis symptoms and in five subjects with negative rhinitis symptoms. We also included seven control subjects.

\*Significantly lower than in subjects with positive rhinitis symptoms or compared with grass pollen-sensitized subjects;  $P \leq 0.03$ .

\*\*Significantly lower than in subjects with positive NPT;  $P < 0.01$ .

### Symptom severity and the medication scores

Overall symptom and medication scores were monitored during the subsequent grass-flowering season of 2010. Symptom severity was graded on a visual analogue scale (VAS), ranging from 0 (no symptoms at all) to 10 (very severe symptoms). The maximum total symptom score was 80 points. Patients rated itching and erythema of the eye, lacrimation, itching of the nose, sneezing, watery discharge from the nose, stuffiness of the nose, cough and dyspnoea. A questionnaire on the medications they had used (systemic antihistamine, local antihistamine, nasal steroid, intraocular antihistamine or chromones) and the dose administered was completed according to the following scale: 0 = no treatment; 1 = seldom; 2 = frequent; 3 = almost every day; 4 = every day and 5 = every day at maximal dose. The maximal medication score was 15 points.

### Specific immunoglobulin E, total immunoglobulin E and specific immunoglobulin G4 measurements

The levels of IgE antibodies to Timothy grass (g6), rPhl p 1 and p 5b (g213), rPhl p 2 (g206) and rPhl p 7 (g210); total IgE; and IgG4 antibodies to Timothy were quantified using the CAP FEIA system (Phadia, Uppsala,

Sweden). The amount of disease-relevant antibodies was defined and calculated as the percentage (%) of IgE antibodies to Timothy (kU/L) relative to total IgE (kU/L), as previously described [17].

### Basophile activation tests

Basophile activation tests were performed, as described previously [13, 18]. Briefly, whole blood (with heparin anticoagulant) was pre-incubated in a basophile stimulation buffer with IL-3 (Buhmann Laboratories, Basel, Switzerland) containing a final concentration of 0.01, 0.1, 1 or 10 µg/mL of grass pollen allergen (AQU-AGEN® SQ, freeze-dried allergen extract of grass pollen mix L299 from ALK Abello, Madrid, Spain); 0.55 µg/mL of anti-FcεRI mAb (Buhmann Laboratories); or 2 µM L-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP; Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 15 min. Degranulation was stopped by chilling on ice, after which FITC-conjugated anti-CD63 mAb, PE-conjugated anti-CD123 mAb and PerCP-conjugated anti-HLA-DR mAb (BD Biosciences, San Jose, CA, USA) were added and incubated for 20 min on ice. Finally, samples were lysed, washed, fixed and analysed within 2 h on a FAC-SCalibur flow cytometer (BD Biosciences). Data were acquired using a FL2 threshold that eliminated most

CD123-negative cells, and at least 600 CD123-positive cells were acquired per sample. Basophiles were identified as low side scatter, CD123-positive and HLA-DR-negative cells. The percentage of degranulated basophiles was determined by FL1 (CD63). The differences in basophile allergen-specific sensitivity were evaluated by comparing basophile CD63 responses at submaximal concentrations [13]. In addition, CD-sens sensitivity analyses were performed as previously described [12, 17].

#### *Basophile FcεRI and immunoglobulin E level determination*

Basophiles (from whole blood) were first stained with FITC-conjugated anti-FcεRI (eBioscience, San Diego, CA, USA) or anti-IgE mAb (Miltenyi Biotec, Bergisch Gladbach, Germany) and PE-conjugated anti-CD123 and PerCP-conjugated anti-HLA-DR mAbs (both from BD Biosciences) at 37°C for 15 min. Then, the erythrocytes were lysed, and cells were washed, fixed and analysed with a FACSCalibur flow cytometer. The number of IgE molecules and FcεRI receptors per basophile was calculated using a standard curve of FITC-conjugated Calibration Beads (QIFIKIT; Dako Cytomation, Glostrup, Denmark) [17]. With assigned molecules of equivalent fluorochrome values for the fluorescent bead populations, arbitrary units of mean fluorescence intensity were transformed into absolute units.

#### *Statistical analyses*

The distribution of the data was calculated using the Shapiro–Wilk test. According to the distribution of the data, we used a *t*-test or a Mann–Whitney test. Correlation coefficients were calculated using Spearman's rank correlation coefficient. Probability values (*P*) of <0.05 were accepted as significant. Unless otherwise stated, the results are presented as the median and the range. Analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

### **Results**

Of the 26 subjects included, 13 subjects had a positive grass pollen NPT and 13 subjects showed a negative NPT (Fig. 1). The median symptom score in the NPT-positive group was 5 (range: 5–9), and the median symptom score in the NPT-negative group was 3 (range: 2–4). In all NPT-negative subjects, we performed two inhalations per each nasal cavity, which was done in five subjects with positive NPT scores. All NPT-positive subjects and one NPT-negative subject indicated a positive history of allergic rhinitis in the last two grass pollen seasons. Conversely, 12 of 13 subjects with neg-

ative NPT scores claimed no symptoms during the last two seasons. When we compared humoral parameters in positive vs. negative NPT subjects, we found no significant differences in the presence of IgE antibodies to Timothy, rPhl 1, 5b and rPhl 2, total IgE and IgG4 antibodies to Timothy (Table 1). Next, we evaluated the CD63 basophile response. We showed that the basal (inclusion of IL3-containing stimulation buffer alone) CD63 response and the response to fMLP and anti-FcεRI mAb stimuli were highly comparable between NPT-positive and NPT-negative subjects and also the control subjects (Table 1). The CD63 basophile response to grass pollen allergens in NPT-positive subjects was 2.4 (range: 0.4–5.5%) at 0.01 µg/mL, 7.3 (range: 0.9–30.1%) at 0.1 µg/mL, 80.2 (range: 2.0–96.3%) at 1 µg/mL and 96.5 (range: 29.4–99.3%) at 10 µg/mL (Fig. 2a). These results were significantly higher than the CD63 basophile allergen-specific response in NPT-negative subjects, which was 2.4 (range: 0.6–4.3%) at 0.01 µg/mL, 4.1 (range: 0.6–7.8%) at 0.1 µg/mL (*P* = 0.03), 7.2 (range: 2.4–89.3%) at 1 µg/mL (*P* = 0.005) and 81.3 (range: 2.3–99.2%) at 10 µg/mL (*P* = 0.005). Similar, NPT-positive subjects had significantly higher CD-sens (222, range: 6–500) than NPT-negative subjects (24 range: 1–250; *P* = 0.002). In control subjects, the median CD63 basophile response to grass pollen allergens was lower than 3% for each of the log grass pollen allergen concentrations that were used, with a maximum range of 8%. Although the IgE antibodies to Timothy or rPhl 1, 5b or rPhl 2 showed no marked differences according to the NPT results, there was a significant difference when we compared the percentage of IgE antibodies to Timothy in relation to total IgE. In the group with positive NPTs, we encountered a significantly higher percentage of disease-relevant IgE antibodies than in the group with negative NPT (Fig. 2b; *P* = 0.01).

During the subsequent grass-flowering season, we monitored overall symptom severity by VAS score evaluation. We found that 20 subjects displayed positive VAS scores and six subjects negative VAS scores (Fig. 1). Of those 20 symptomatic subjects, 13 subjects previously demonstrated positive NPTs, whereas seven had negative NPTs. Fourteen of those 20 subjects indicated a previously positive rhinitis history, whereas six subjects did not (Table 1). All six asymptomatic subjects with negative VAS scores had previously negative NPT scores and a negative retrospective history. The elevated CD63 basophile allergen-specific responsiveness was highly predictive for allergic rhinitis because symptomatic subjects showed significantly higher values at allergen concentrations of 1 µg/mL (53.4, range: 2.0–96%) and 10 µg/mL (95.5, range: 21.4–99.3%) compared with asymptomatic subjects (4.4 at 1 µg/mL; range: 2.4–9.2% and 45.1 at 10 µg/mL, range: 2.3–82.1%;

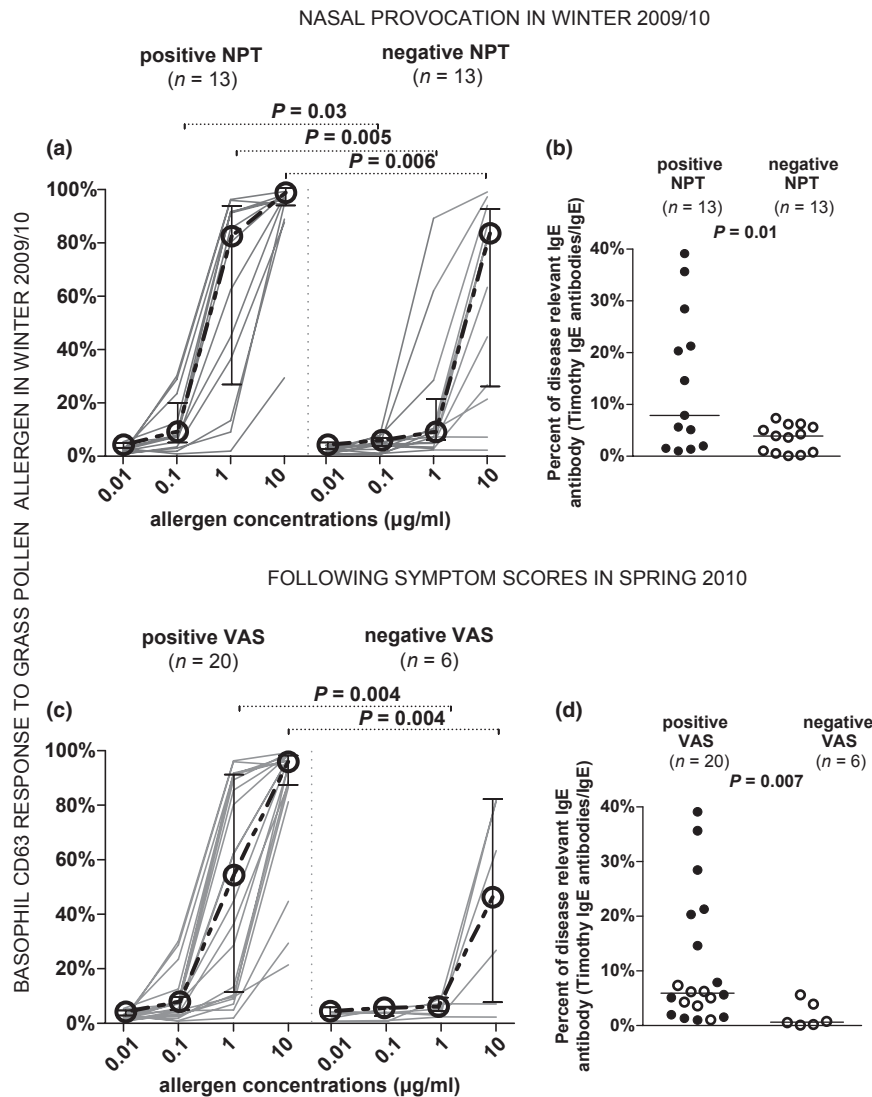


Fig. 2. Comparison of *in vitro* CD63 basophile dose-response curves to grass pollen allergen according to (a) a positive or negative nasal provocation test (NPT) with grass allergens performed during winter 2009/2010 and (c) symptoms graded on a visual analogue scale (VAS) in the subsequent flowering season in spring 2010. The dotted line indicates the median with interquartile range for 0.01, 0.1, 1 and 10 µg/mL of allergen concentration. Comparison of the percentage of disease-relevant IgE antibodies to Timothy in relation to total IgE according to the (b) provocation and (d) symptoms. The horizontal line indicates the median.

$P = 0.004$ ; Fig. 2c). Moreover, these results reflect the association between basophile allergen threshold sensitivity and rhinitis symptoms because in symptomatic subjects the concentration threshold for *in vitro* basophile activation was 1 µg/mL of allergen, and in asymptomatic subjects this threshold was 10 µg/mL (Fig. 2c). Comparably, CD-sens was about tenfold higher in symptomatic (110, range: 5–500) than in asymptomatic subjects (9, range: 1–25;  $P = 0.002$ ). Symptomatic subjects also showed higher IgE antibodies to Timothy and rPhl 1, 5b (Table 1). Even more significant differences were observed when we calculated the percentage of IgE antibodies to Timothy in relation to total IgE (Fig. 2d;  $P = 0.007$ ). We also compared

seven symptomatic subjects vs. six asymptomatic ones, both of which groups had previously negative NPT scores and/or retrospective history. Those seven subjects that developed symptoms have significantly higher basophile allergen sensitivity (10.4 at 1 µg/mL; range: 4.9–89.3%;  $P = 0.02$  and 86.7 at 10 µg/mL, range: 21.0–82.1%) and percentage of IgE antibody fraction to Timothy ( $P = 0.03$ ; Figs 2c and d).

The number of IgE molecules and FcεRI receptors per basophile were highly comparable between subjects with different NPT and/or symptoms results (Table 1). However, both the number of IgE molecules and FcεRI receptors per basophile were significantly higher in sensitized subjects than in control subjects (Table 1;

$P \leq 0.03$ ). Similarly, sensitized subjects showed significantly higher total IgE compared with control subjects (Table 1;  $P = 0.01$ ).

We quantified the correlation between major independently measured experimental variables such as specific CD63 basophile response and IgE antibodies. The closest and the most significant correlation was found between the CD63 basophile response to 1  $\mu\text{g}/\text{mL}$  of grass allergen compared with the percentage of IgE antibody fraction to Timothy in NPT-positive (Fig. 2a) and symptomatic subjects (Fig. 3b), with the  $R$  coefficient reaching 0.84 and the  $P$ -value approaching 0.0002, and with an  $R$  coefficient of 0.76 compared with all sensitized subjects (Spearman's rank correlation test). However, there was no correlation in asymptomatic subjects (Fig. 3b) and no correlation when we compared the CD63 response to 10  $\mu\text{g}/\text{mL}$  of allergen. In NPT-positive, symptomatic and all sensitized subjects, we also found a correlation between the levels of IgE antibodies to Timothy or rPhl 1, 5b (Table 1) and the basophile response to 1  $\mu\text{g}/\text{mL}$  of allergen, but it was less significant ( $R < 0.71$  and  $P < 0.002$ ; Spearman's

rank correlation test) than in comparison to the percentage of IgE antibody fraction to Timothy.

We also examined the major clinical outcomes of the study, the prospective symptom VAS and the medication scores. There was a significant correlation between basophile allergen sensitivity measured as CD63 response to 1  $\mu\text{g}/\text{mL}$  of grass allergen in comparison to both the VAS scores ( $R = 0.4$  and  $P = 0.04$ ) and medication scores ( $R = 0.43$  and  $P = 0.02$ ; Spearman's rank correlation test). In contrast, there was no correlation between those clinical outcomes and the percentage of IgE antibody fraction to Timothy, levels of IgE antibodies to Timothy or rPhl 1, 5b or basophil response at 10  $\mu\text{g}/\text{mL}$ .

## Discussion

This study showed that allergic rhinitis symptoms were associated with elevated basophile allergen sensitivity, which was evaluated by the allergen-specific CD63 response. These results suggest that the evaluation of allergen-specific basophile sensitivity could be a useful tool for distinguishing the clinical phenotype of allergic sensitization.

Basophiles and mast cells are directly responsible for the clinical symptoms of IgE-mediated reactions by releasing histamine and other mediators. At the cellular level, the responses of basophiles and mast cells correspond with two main independent variables [11]. Reactivity is the maximal response to allergen stimulation (the plateau of the dose-response curve). Cellular sensitivity represents the allergen threshold sensitivity [11]. Basophile allergen sensitivity can be evaluated by different approaches [12, 13, 17, 18]. The main approach we used in this study is to expand the dose-response curve by at least four log allergen concentrations and compare the responses between different submaximal concentrations [13]. We also included some CD-sens analysis [12, 17]. Skin tests and provocation tests are not suitable for evaluating allergen sensitivity because they cannot be measured precisely enough to permit exact quantitative evaluation. For allergen sensitivity measured by a skin test, a CV of 100–200% was reported [19]. These *in situ* variations can be overcome by the use of controlled conditions provided by an allergen challenge chamber, but this procedure is cumbersome for patients and not feasible in routine clinical practice [20]. On the other hand, for allergen sensitivity measured by the CD63 basophile *in vitro* response, a CV of 5–15% was reported [21].

This study tested the usefulness of *in vitro* basophile allergen responsiveness as a mirror of clinical allergen sensitivity. We therefore first excluded the factors that were previously described to be involved in clinically irrelevant sensitizations [4, 6, 7, 22, 23]. For example,

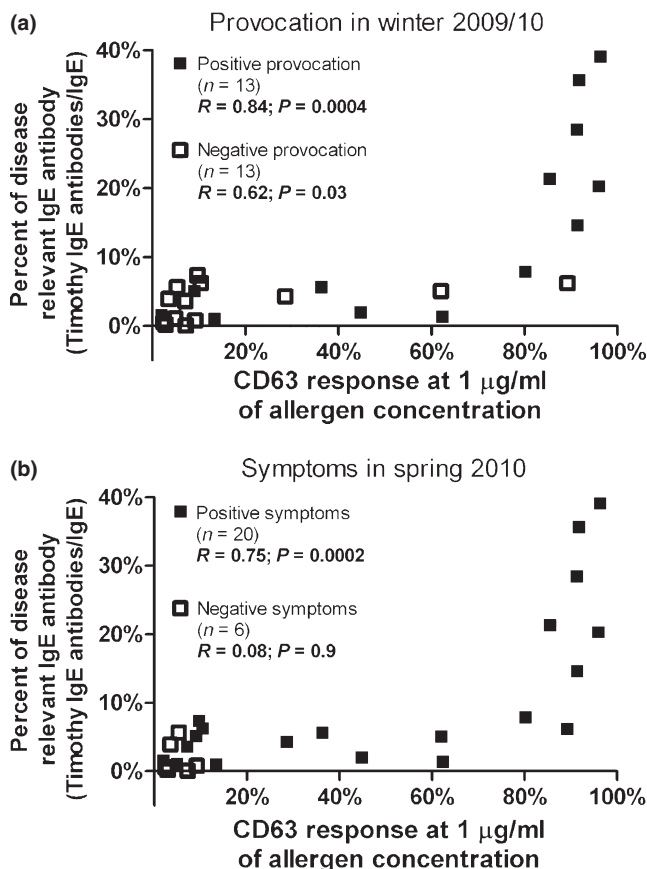


Fig. 3. Correlation between *in vitro* CD63 basophile responses to 1  $\mu\text{g}/\text{mL}$  of grass pollen allergen and the percentage of disease-relevant IgE antibodies to Timothy in subjects divided according to (a) provocation testing in winter 2009/2010 and (b) rhinitis symptoms monitored in spring 2010.

false-positive IgE reactivity can occur when total IgE is grossly elevated (usually in excess of a few thousand kU/L) [22]; thus, the highest total IgE in our subjects was 1616 kU/L, with a median of 165 kU/L. Profilins and cross-reactive carbohydrate determinants are capable of inducing cross-reactive IgE antibodies with little clinical relevance [4]. Therefore, all of our study subjects demonstrated negative IgE reactivity to the cross-reactive pollen allergen Phl p 7 and positive reactivity for major grass allergens (all were Phl p 1, 5b and/or Phl p 2 positive) [6, 7, 23]. We also determined the number of IgE molecules and FcεRI receptors per basophile, and they were highly comparable between sensitized subjects after excluding possible basophile FcεRI phenotype influence. One limitation of our study was rough semi-quantitative recording, but not the quantitative measurement of the nasal patency that was used for evaluating response after nasal provocation [15, 16]. Nevertheless, we have used this procedure in the clinical setting for many years, and we therefore have experience with interpreting and rationalizing these data. Another limitation was the use of a global evaluation of the grass pollen season rather than a diary. However, for a study that concentrated on the cellular mechanisms behind symptomatic sensitization, we considered a global evaluation to be sufficient.

Our most surprising observation was a clear correlation between elevated cellular allergen sensitivity and the manifestation of rhinitis symptoms. In particular, the median basophile response at submaximal allergen concentrations, which mirrors the cellular allergen sensitivity, was more than tenfold higher in symptomatic than in asymptomatic subjects. Moreover, basophile sensitivity also significantly correlated with symptom VAS and medication scores. We speculate that basophile allergen sensitivity not only reflects the degree of IgE-sensitization but also provides a marker of the sensitivity to allergen exposure in target organs; however, this hypothesis should be further evaluated and confirmed in a larger study group. It has been previously shown that basophiles are recruited to the airways during an allergen inhalation challenge and that, after mast cells, basophiles are effector cells that are highly associated with the local response [24].

We demonstrated that the extent of IgE-sensitization, which was presented as the percent of disease-relevant IgE antibody fraction, correlated with basophile allergen sensitivity, which was associated with the manifestation of rhinitis symptoms. Interestingly, the percent of disease-relevant IgE antibodies and decrease in basophile allergen sensitivity was also associated with the efficiency of anti-IgE therapy [17]. The clinical predictivity of the IgE antibodies is a contested issue. Previous studies have shown that in Hymenoptera venom allergy specific IgE levels are not associated with the severity of

sting reactions [25]. In contrast, another study showed that patients with higher total IgE predominantly develop lower grades of sting reactions [26]. In some food allergies, higher levels of IgE antibodies have been shown to correlate with a higher likelihood of a clinical reaction, but do not generally correlate with reaction severity [27]. It has been suggested that higher levels of IgE antibodies to inhalant allergens correlate with allergic rhinitis and asthma, and even a quantitative clinical cut-off value of 10.7 kU/L has been proposed [28]. On the other hand, other studies have shown that the levels of IgE antibodies are not sufficiently predictive, even if recombinant allergen components were used [9]. Comparably, in our six subjects with no clinical reactivity, we clearly showed positive IgE reactivity for major grass allergens. Moreover, with regard to clinical outcome, we demonstrated that evaluation of cellular basophile allergen sensitivity is significantly more predictive than IgE antibodies or ratio, even in selected patients without irrelevant IgE cross-reactivates.

It is important to note the similarities and dissimilarities between basophile allergen sensitivity in relation to symptomatic vs. asymptomatic sensitization and in relation to immunotherapy. During immunotherapy, the patients become tolerant, but the allergy tests that reflect IgE-sensitization, such as skin tests and IgE measurement, frequently remain positive [29]. However, immunotherapy induces a significant decrease in basophile allergen sensitivity, which is associated with an increase in the blocking IgG antibodies [12, 13]. These correlations were not observed in the current study because both symptomatic and asymptomatic subjects demonstrated minor and highly comparable low levels of specific IgG antibodies.

Previous follow-up studies have clearly demonstrated that asymptomatic sensitization is a significant risk factor for the later development of allergic rhinitis [10]. In our study, seven subjects that claimed a negative retrospective history and/or negative NPT demonstrated rhinitis symptoms in the 2010 season. The reason for this discrepancy may be the fact that during the 2007 and 2008 seasons, the measurements of grass pollen sum concentrations were considerably lower (2074 and 3072 grains/m<sup>3</sup>) than during the pollination season of 2010 (sum of 3712 grains/m<sup>3</sup>). Moreover, in 2010, grass pollen concentrations were on average the highest in the last few years in Slovenia. There is also the possibility that psychological factors – specifically, awareness after learning about the presence of allergic sensitization – may contribute to self-reported symptoms in the subsequent season [30]. Nevertheless, this is rather unlikely because the median VAS score of those apparently symptomatic subjects was 25 points, and these scores are too high to be the result of psychological factors. However, those subjects also demonstrated significantly

higher basophile allergen sensitivity and a larger grass-specific IgE fraction than the subject that remained asymptomatic.

In conclusion, we showed that the manifestation of allergic rhinitis symptoms is associated with cellular allergen sensitivity in a limited number of patients. We accurately measured cellular allergen sensitivity with CD63 basophile activation tests. Moreover, we showed that this allergen-specific cellular sensitivity positively

correlated with the percentage of disease-relevant IgE antibodies compared with total IgE antibodies.

### Clinical Relevance

*In vitro* evaluation of basophile sensitivity should prove useful for distinguishing clinical phenotype of allergic sensitization. Further studies are needed to confirm these promising results.

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