Defining biomarkers to predict symptoms in subjects with and without allergy under natural pollen exposure

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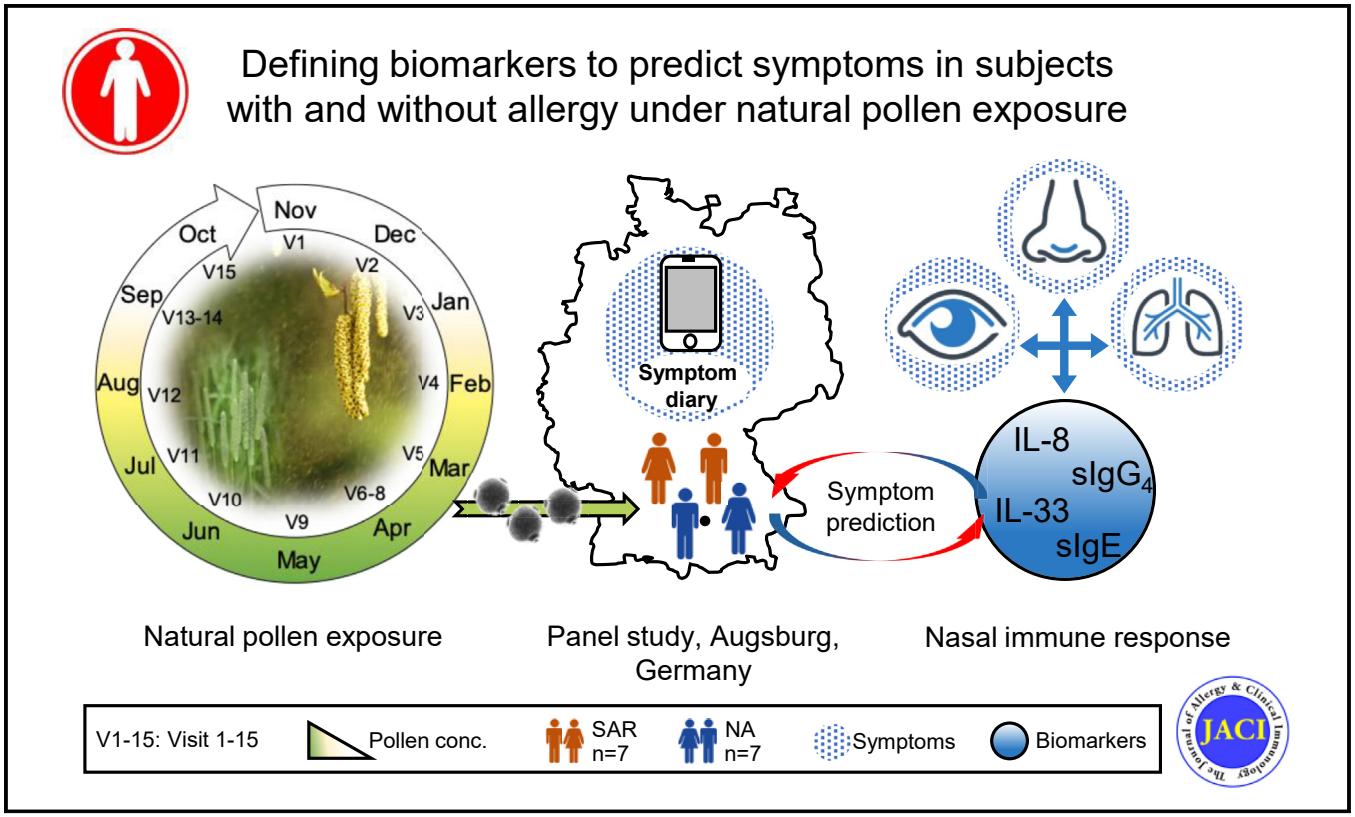
GRAPHICAL ABSTRACT



Background: Pollen exposure induces local and systemic allergic immune responses in sensitized individuals, but nonsensitized individuals also are exposed to pollen. The kinetics of symptom expression under natural pollen exposure have never been systematically studied, especially in subjects without allergy.

Objective: We monitored the humoral immune response under natural pollen exposure to potentially uncover nasal

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biomarkers for in-season symptom severity and identify protective factors.

Methods: We compared humoral immune response kinetics in a panel study of subjects with seasonal allergic rhinitis (SAR) and subjects without allergy and tested for cross-sectional and interseasonal differences in levels of serum and nasal, total, and *Betula verrucosa* 1–specific immunoglobulin isotypes; immunoglobulin free light chains; cytokines; and chemokines. Nonsupervised principal component analysis was performed for all nasal immune variables, and single immune variables were correlated with in-season symptom severity by Spearman test.

Results: Symptoms followed airborne pollen concentrations in subjects with SAR, with a time lag between 0 and 13 days depending on the pollen type. Of the 7 subjects with nonallergy, 4 also exhibited in-season symptoms whereas 3 did not. Cumulative symptoms in those without allergy were lower than in those with SAR but followed the pollen exposure with similar kinetics. Nasal eotaxin-2, CCL22/MDC, and monocyte chemoattactant protein-1 (MCP-1) levels were higher in subjects with SAR, whereas IL-8 levels were higher in subjects without allergy. Principal component analysis and Spearman correlations identified nasal levels of IL-8, IL-33, and *Betula verrucosa* 1–specific IgG_4 ($sIgG_4$) and *Betula verrucosa* 1–specific IgE (sIgE) antibodies as predictive for seasonal symptom severity.

Conclusions: Nasal pollen–specific IgA and IgG isotypes are potentially protective within the humoral compartment. Nasal levels of IL-8, IL-33, $sIgG_4$ and sIgE could be predictive biomarkers for pollen-specific symptom expression, irrespective of atopy. (J Allergy Clin Immunol 2020;■■■:■■■-■■■.)

Key words: *Allergic rhinitis, biomarkers, chemokines, cytokines, immunoglobulins, nasal symptoms, pollen*

Abbreviations used

AR:	Allergic rhinitis
Bet v:	*Betula verrucosa*
FLC:	Free light chain
ISAC:	Immuno-solid-phase Allergen Chip
PCA:	Principal component analysis
SAR:	Seasonal allergic rhinitis
sIg:	*Betula verrucosa* 1–specific immunoglobulin
SPT:	Skin prick test
TNSS:	Total nasal symptom score

The main cause of seasonal allergic rhinitis (SAR) is exposure to airborne pollen. The most relevant allergenic pollen types across the world are pollen of the large Poaceae family (grasses)[1] and in Northern and Central Europe of the families of Betulaceae (ie, alder and birch) and Corylaceae (ie, hazel).[2-4] Allergy to birch pollen goes along with cosensitization to alder and hazel pollen[5,6] because of high interspecies homology between their major allergens, all of which belong to the pathogenesis-related 10 (PR10) protein family. The pollen season of these species typically starts in late winter (ie, in mid-February) and usually ends in mid-to-late spring (ie, in late April, but sometimes extending up to May and June, depending on spring temperatures).

A positive relationship between pollen exposure and allergic symptoms in sensitized individuals has been demonstrated in experimental and natural exposure settings (reviewed in Damialis et al[7]) and therefore may appear trivial. However, this relationship is dynamic and certainly not linear, especially because pollen seasons can be short and highly peaked and are therefore hardly predictable, especially for pollen of the winter-spring flowering species of Betulaceae and Corylaceae families.[8] Moreover, under real-life pollen exposure conditions, different allergenic pollen species typically co-occur, frequently with overlapping and consecutive seasons. How the nasal immune response to pollen develops over time within each season under such complex exposure conditions is still unclear, as is how this correlates with symptoms. Also, symptom severity may change under continuous exposure on account of changes in perception or lag effects caused by the underlying immunologic mechanism. Notably, individuals without allergy are exposed to airborne pollen as well, and they mount an immune response that is thought to be dominated by local, allergen-unspecific mechanisms (mainly by the induction of granulocyte-chemotactic factors such as IL-8 and CXCL-2).[9] Why and how some individuals who were originally without allergy develop pollen allergies later in life whereas others are protected lifelong is still incompletely understood.

We therefore set up a natural pollen exposure panel study in Augsburg, Germany; the study population consisted of subjects with SAR and control subjects without allergy who underwent daily symptom recording and repeated biosampling throughout a whole year. Immune variables in sera and nasal fluid were analyzed across cohorts and seasons, and in-season and off-season immune variables were analyzed by multivariate exploratory techniques.

METHODS

Airborne pollen monitoring

Airborne pollen in the Augsburg region were monitored daily by using an automatic Bioaerosol Analyzer BAA500 (Hund, Wetzlar, Germany), as described in Oteros et al.[10] In brief, this device samples ambient air intermittently by use of a 3-stage virtual impactor at 60 m^3 per hour, with particles (among which pollen) deposited on a sticky-surface probe. Each probe is sampled and analyzed every 3 hours under a microscope equipped with a camera. With use of specially developed image recognition algorithms, images of the pollen were constructed and compared with a library of known samples. So as to ensure the highest possible identification accuracy, daily pollen samples were manually classified by expert scientists, who also validated the original, automatically acquired pollen concentrations. To determine the seasonality of the pollen distribution period, we defined as the main pollen season for each taxon the 2.5% to 97.5% of the total pollen sum per year.[11]

Study design and human cohort characteristics

The study lasted from November 2015 to October 2016. Immune monitoring was performed at intervals of 4 weeks out of season and biweekly within the main pollen season. Healthy, volunteers with and without birch and birch pollen allergy were recruited in the Augsburg region. Therefore, a total of 50 candidates underwent an initial screening procedure to confirm eligibility. The screening included questions on self-reported symptoms, a skin prick test (SPT) and a serum IgE test (ImmunoCAP, Thermo Fisher Scientific/Phadia, Uppsala, Sweden). To exclude nonallergic rhinitis, perennial AR, nasal polyps, and chronic rhinosinusitis and to minimize the possibility of idiotypic or local AR, an otolaryngologist performed detailed anamnesis, including anterior and posterior rhinoscopy and laryngoscopy. None of the subjects included had shown any obvious pathologic

TABLE I. Aeroallergen sensitization profiles of subjects with SAR and without allergy

Subject ID	Sex (M/F)	Age (y)	Total IgE (IU/mL)	SPT	Birch	Hazel	Grasses	Mugwort	House dust mite	Cat
B1	M	25	63.7	++++	1.3	1.0	2.1	0.1	0.2	0.1
B2	F	26	24.8	++	9.5	4.5	0.4	0	0	0
B4	F	53	29.0	+++	4.7	2.4	0	0	0	0
B5*	F	54	71.2	+++	4.0	3.4	2.3	0	0	1.4
B6†	F	25	52.4	++++	7.3	3.6	0.2	0	0	0.5
B7	F	39	37.2	+	5.5	3.9	0.1	0	0.1	0.1
B8	M	31	159.0	++++	41.6	29.0	4.8	0.5	0	
NA1	F	36	46.8	0	0	0	0	0	0	0
NA3	F	56	7.4	0	0	0	0	0	0	0
NA5†	M	29	5.6	0	0	0	0.1	0	0	0
NA6	F	21	37.8	0	0	0	0	0	0.1	0
NA8	F	51	17.9	0	0	0	0	0	0	0
NA9	F	27	12.2	0	0	0	0	0	0	0
NA10‡	F	23	152.0	0	0	0	0	0	0	0

Total and specific IgE levels in serum (IU/ml) were analyzed by ImmunoCAP.
*Subjects B5 and B6 did not accommodate any pets at their houses.
†Subject NA5 had low-level sensitization (0.4 U/mL) to *Phleum pratense* 11, as determined by using the ISAC test (see Table E1), whereas the result of ImmunoCAP to timothy grass pollen extract was negative (0.1 kU/L).
‡In the ISAC test, subject NA10 showed no relevant sensitization to explain the high total serum IgE level (see also Table E1).

abnormalities or signs of inflammation in the nasal cavity or nasopharynx. Cohorts of 8 patients with SAR and 10 subjects without allergy were enrolled on the basis of screening and examination. Of the 8 patients with SAR, 1 was retrospectively excluded. This patient had a positive SPT and had reported springtime allergic symptoms in the screening visit but was asymptomatic during the birch pollen season of the study. Testing using the Immuno-solid-phase Allergen Chip (ISAC, Thermo Fisher/Phadia, Uppsala, Sweden) revealed a sensitization to grass pollen and profilin, but not to *Betula verrucosa* 1 (Bet v 1)/PR10 group allergens.

Of the 10 volunteers without allergy, 1 dropped out and 2 had to be excluded from analysis on account of noncompliance (traveling during the pollen season and missing more than 20% of the diary entries within the main pollen season). Analyses were therefore performed on 7 patients with SAR and 7 subjects without allergy. Sensitization profiles of the participants were confirmed by an additional molecular allergy test (ISAC) (see Table E1 and Fig E1 in this article's Online Repository at www.jacionline.org). The volunteers without allergy had a total mean serum IgE level of 40.0 plus or minus 18.1 IU/mL, were nonreactive to birch pollen in the SPT, and had no specific IgE against aeroallergens, as tested by ImmunoCAP (Table I). The patients with SAR had a positive SPT result to birch pollen, total mean serum IgE level of 62.5 plus or minus 16.1 IU/mL, and elevated birch pollen–specific IgE levels (CAP class ≥2) without cosensitization against perennial allergens (Table I). The study was approved by the local ethics committee (internal code 19/15) and conformed to the guidelines of the Declaration of Helsinki. Study participants were enrolled after written informed consent.

Serum and nasal biosampling

Blood samples were obtained at 6 time points throughout the study. Nasal fluid was collected at 15 time points as described by Gilles-Stein et al.[12] Briefly, a strip of absorbent filter paper (Leucosorb, Pall GmbH, Dreieich, Germany) was inserted into the nostril and kept there for 90 seconds. The filter paper strip was then placed into the insert of a 1.5-mL spinning filter tube (Costar, Corning Inc, Corning, NY). Secretion fluid was extracted by adding 100 μL of double-distilled water to the paper strip and spinning it down in a precooled centrifuge (4°C) for 5 minutes at 10,000 *g*. Samples were kept at –80°C until processed.

Measurement of cytokines, chemokines, and immunoglobulins

Cytokines and chemokines were measured in nasal fluid, and immunoglobulins were analyzed in nasal samples and sera by using multiplex magnetic bead–based detection kits (the Bio-Plex Pro Human Isotyping Panel 6-plex multiplex assay for IgA, IgM, IgG_1, IgG_2, IgG_3, and IgG_4 and a human IgE Isotyping assay for IgE and a custom 9-plex multiplex assay for IL-33, CCL24/eotaxin-2, CCL4/MIP-1β, CCL2/MCP-1, CCL22/MDC, CXCL8/IL-8, IL-16, G-CSF, and IL-1β level were performed according to the manufacturer's instructions - Bio-Rad Laboratories, Inc, Hercules, Calif). The samples were acquired on a Bio-Plex 200 System, and the results were analyzed by using Bio-Plex Manager 6.1 software (Bio-Rad Laboratories, Inc). Free light chains (FLCs) were analyzed as previously described.[13]

Measurement of Bet v 1–specific immunoglobulins

The Bet v 1–specific immunoglobulins IgE, IgG_4, and IgA were measured in serum and nasal fluid via ELISA. Briefly, plates were coated with 1μg/mL of Bet v 1.0101, recombinantly expressed as previously described.[14] As detection antibodies we used alkaline phosphatase–conjugated mouse anti-human IgE, mouse anti-human IgG_4 (both by BD Pharmingen, Heidelberg, Germany), or mouse anti-human IgA (Sigma-Aldrich, St Louis, Mo).

Definition of the subjects' relevant pollen seasons

When cross-seasonal differences in immune mediator expression were compared, in-season and out-of-season were defined per subject, depending on the individual sensitization profile: for subjects with SAR who were sensitized to hazel, alder, birch, *and* grasses, visits 3 to 12, which spanned the seasons of all relevant pollen taxa, were classified as in-season visits. Because the subjects without allergy had no sensitization to any pollen types, in-season was defined in the same way. For subjects with SAR who were sensitized to hazel, alder, and birch but *not* to grasses, visits 3 to 8 were classified as in-season visits. During these visits, only the allergy-relevant taxa were detected in the air. For all comparisons of Bet v 1–specific immunoglobulins, only those visits during the main birch pollen season (visits 6-8) were classified as in-season visits.

Monitoring of symptoms

Throughout the study, participants entered their symptoms into a pollen diary (https://www.pollendiary.com/Phd/en/start) on their smartphones or computers on a daily basis. The diary covered questions on general well-being (scored from 1 to 10, with 1 meaning very good and 10 meaning very bad), medication use, remarks, and allergic symptoms. Symptoms included nasal, ocular, and pulmonary symptoms, with severity ranging from 0 to 3 (with 0 meaning none, 1 meaning mild, 2 menaing moderate, and 3 meaning severe). Total nasal symptom score (TNSS) was calculated by using a published algorithm.[15]

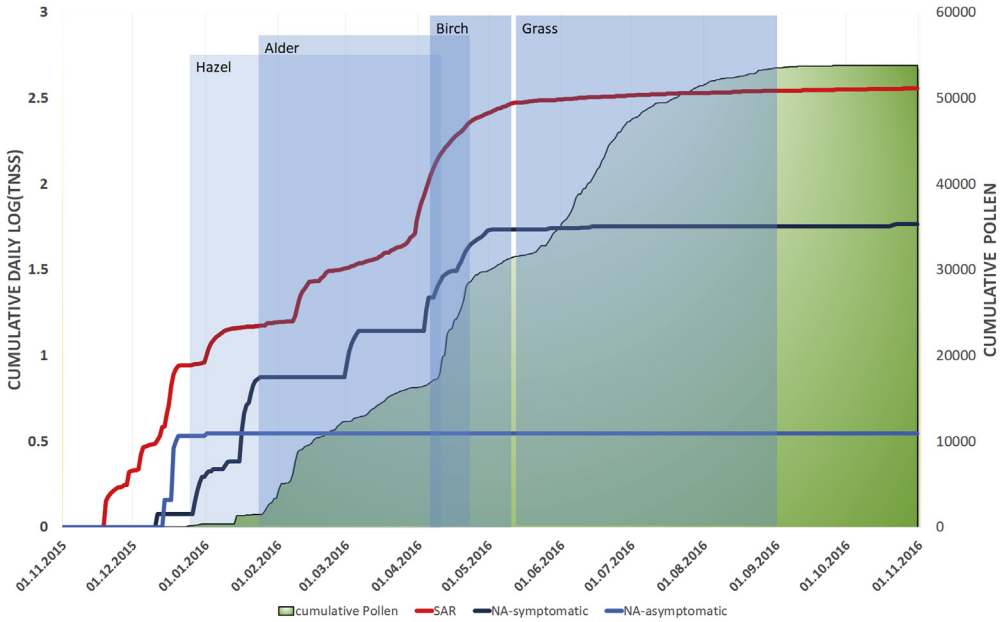

FIG 1. Total nasal symptom scores in relation to airborne pollen. Cumulative normalized TNSS (*left y axis*) is plotted against cumulative airborne pollen concentrations (*right y axis*) over time (*x axis*). The red line indicates the mean cumulative TNSSs of the patients with SAR; blue lines indicate the mean cumulative TNSSs of the subjects without allergy (light blue indicates asymptomatic subjects and dark blue indicates symptomatic subjects). Shaded areas represent the main pollen season (2.5%-97.5% of total cumulative pollen) per taxon. *NA,* Subjects without allergy.

Statistical data analysis

Statistical analyses were performed using R software (RStudio Inc, Boston, Mass). For cross-sectional kinetics, data were normalized per patient to their SD over the entire study time. For cross-sectional and cross-seasonal comparisons, data were normalized per subject by dividing each individual measurement by the SD over all measurements. From the values thus normalized, medians were calculated for the visits that took place outside and within the (individually relevant) pollen season. Visits were defined as occurring outside or inside the pollen season depending on the subjectś sensitization pattern (see earlier). The differences between the 2 cohorts were tested by using a nonparametric Mann-Whitney *U* test. The alpha level of 0.05 was adjusted by Bonferroni correction for cross-sectional analysis of immune variables. For principal component analysis (PCA), raw data were transformed to log data (0 values were set to 0.01). Single immune variables, measured as either preseason or in-season values, were correlated with in-season symptom severity by using Spearman correlation.

RESULTS

TNSS follows airborne pollen in patients with SAR and subjects without allergy

Cumulative TNSSs were normalized per individual, averaged, and plotted against cumulative airborne pollen concentrations. In the cohort of patients with SAR, symptoms followed pollen with a lag of 3 days (hazel), 13 days (alder), and 0 days (birch) (Fig 1 [*red line*]). The magnitude of the increase in TNSS was almost double during the birch pollen season, whereas during the grass pollen season it was comparatively modest.

Regarding the cohort of subjects without allergy, 3 of 7 cohort members (NA6, NA8, and NA9) did not exhibit any symptoms during the pollen season (Fig 1 [*light blue line*]). Nonetheless, 4 subjects without allergy (NA1, NA3, NA5, and NA10) were symptomatic, and their TNSS increased stepwise, in a manner similar to that in the cohort of patients with SAR, albeit on a lower scale (Fig 1 [*dark blue line*]). When compared against recordings of symptoms in the cohort of those with SAR, the bulk of symptom recordings within the cohort of those without allergy occurred in late winter and spring and followed the onset of the Betulaceae-Corylaceae pollen seasons, with a slightly longer time lag. Later, within the grass pollen season, only 2 of 7 subjects without allergy recorded any symptoms (see the individual plots for subjects with allergy in Fig E2, *A* and without allergy in Fig E2, *B* in this article's Online Repository at www.jacionline.org).

Patients with SAR and subjects without allergy show distinct kinetics of nasal and serum humoral immune responses to natural pollen exposure

Next, we studied the kinetics of the humoral immune response under natural exposure (see Fig 2, *A*). Therefore, raw values for all nasal (see Fig 2, *C*) and serum (see Fig 2, *D*) variables were compared across cohorts for each of the visits (see Fig 2, *B*). Strikingly, there were many nasal variables that showed cross-sectional differences throughout all visits but did not reach statistical significance; that is, levels of IL-1, CCL24/eotaxin-2, CCL4/MIP-1β, IL-33, CCL22/MDC, and G-CSF, as well as total IgE, IL-8, and IL-16 levels were significantly increased in patients with SAR versus in subjects without allergy. Differences in IL-8 level were significant at the onset of birch pollen season (March to the start of April 2016, $P < .05$); differences in IL-16 level during winter (November-December 2016, $P < .05$) and after the onset of birch pollen season (March 2016, $P < .05$). Nasal level of Bet v 1–specific IgE was significantly increased in SAR as

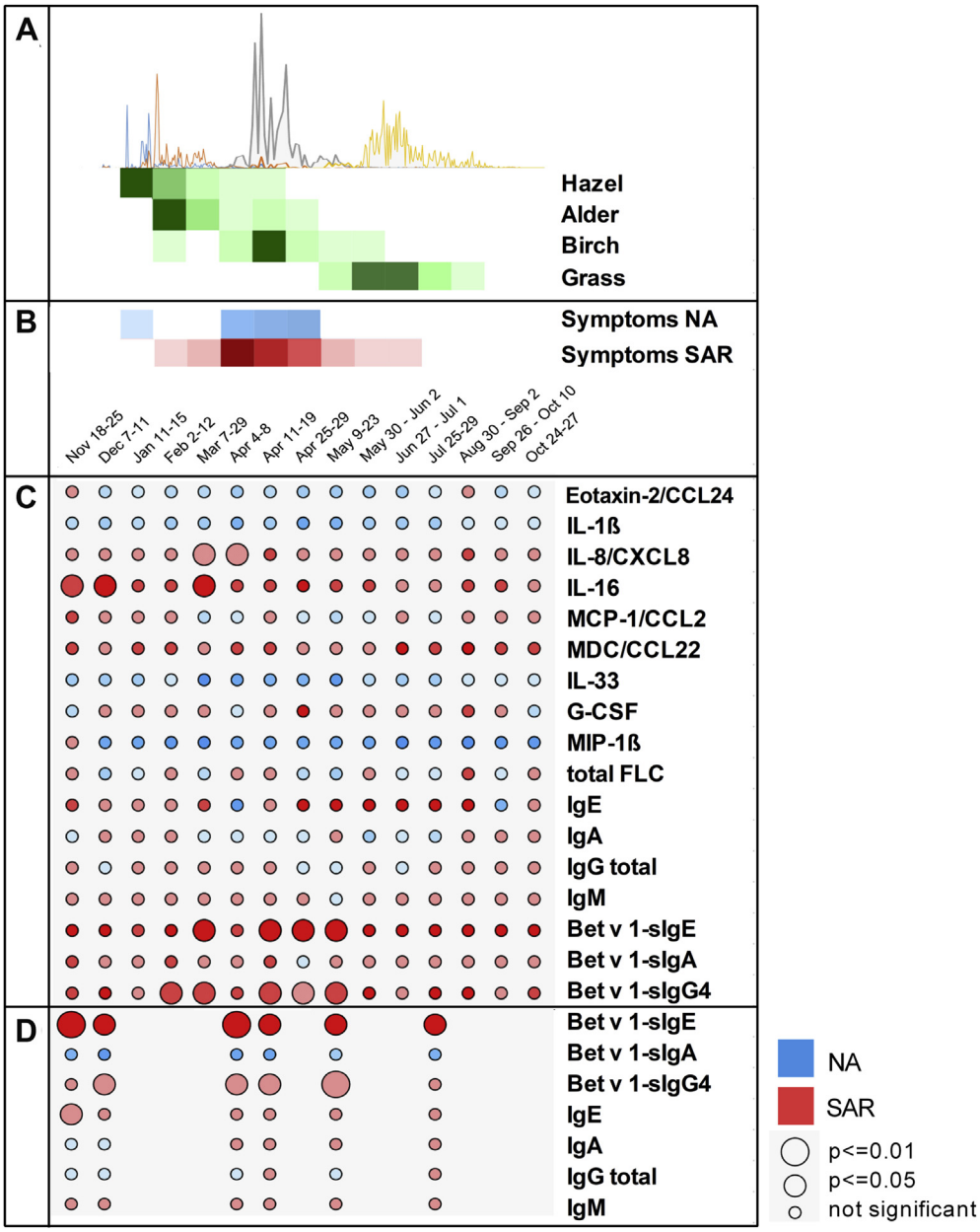

FIG 2. Cross-sectional comparison of all humoral immune variables resolved per visit. **A,** Cumulative pollen concentration per visit. Pollen concentrations per taxon are normalized to total cumulative pollen over the study period. **B,** Cumulative symptoms, normalized per cohort, in patients with SAR and subjects without allergy. **C,** Cross-sectional comparison of nasal humoral immune variables per visit. **D,** Cross-sectional comparison of serum humoral immune variables per visit. Small dots indicate no statistically significant difference between cohorts. Larger dots indicate statistical significance, with larger dots used for lower *P* values. Blue indicates that a parameter is more highly expressed in subjects without allergy (NA) than in patients with SAR, whereas red indicates that a parameter is more highly expressed in patients with SAR than in subjects without allergy. Darker color means a larger cross-sectional difference. Differences were tested by the Mann-Whitney *U* test. *P* values were adjusted for multiple testing by Bonferroni correction.

compared with that in the subjects without allergy throughout the birch pollen season (March to April, $P < .05$), as well as the following grass pollen season (start of May 2016, $P < .05$). A similar pattern (February to March and April to the start of May 2016, $P < .05$) was observed for nasal $sIgG_4$ level.

In serum, Bet v 1–specific IgE levels were significantly increased in subjects with SAR at all time points (in November 2015 and the start of April 2016, $P < .01$; at all other visits, $P < .05$). The same was observed for serum level of Bet v 1–specific IgG_4, only with less pronounced cross-sectional differences ($P < .01$ in May 2016 and $P < .05$ in December 2015 and April 2016). Serum level of Bet v 1–specific IgA was consistently higher in the cohort of those without allergy, but the difference was not statistically significant.

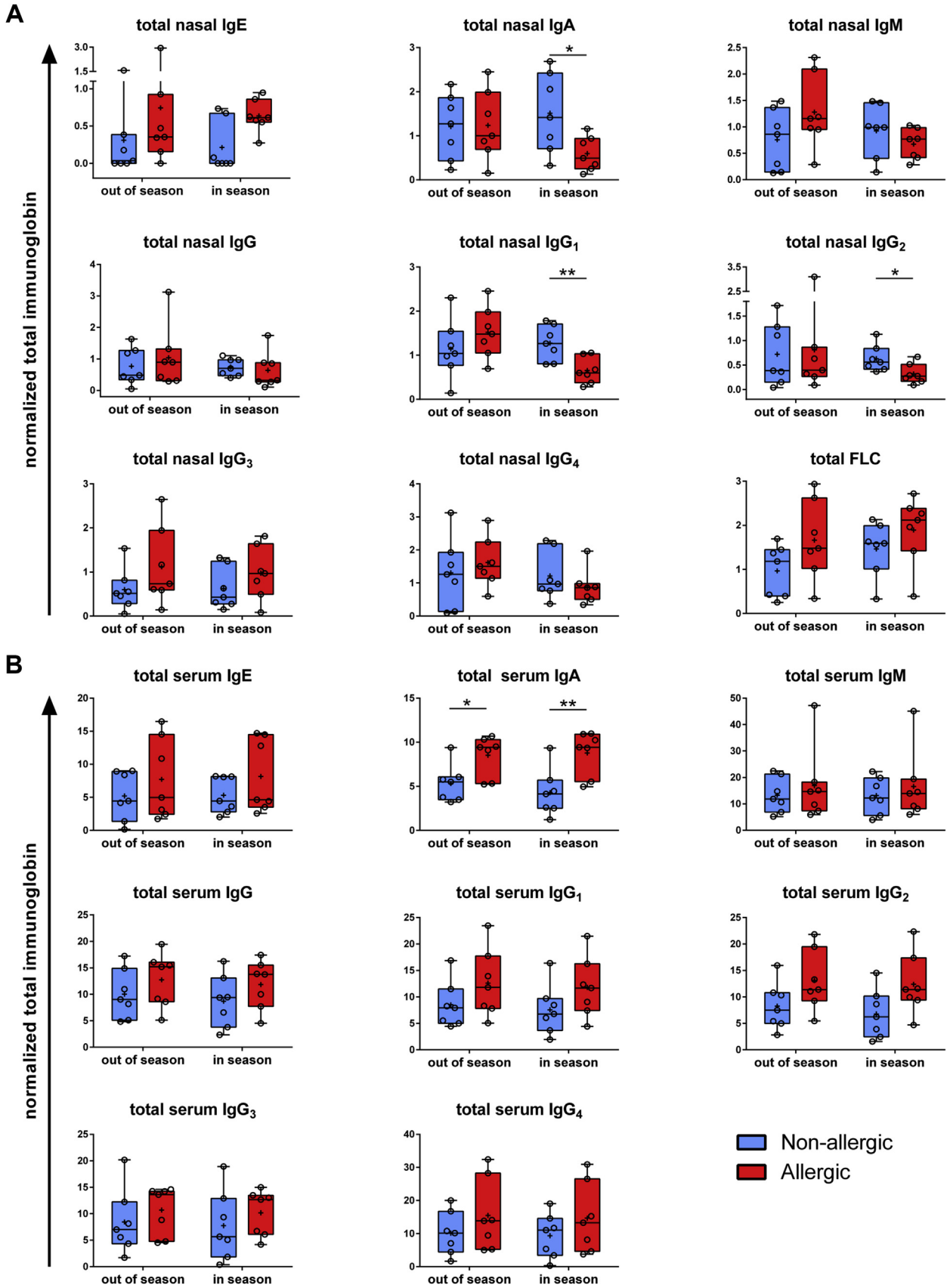

FIG 3. Cross-sectional and cross-seasonal comparison of total immunoglobulin isotype levels. **A,** Median total immunoglobulin (Ig) isotype levels in nasal fluid outside and inside the whole pollen season. **B,** Median immunoglobulin isotype and FLC levels in serum outside and inside the whole pollen season. Raw values, as measured by multiplex assay, were normalized per patient/subject by their overall SD and plotted on the *y* axis. Plus sign indicates the geometric mean. $^{*}P \leq .05$; $^{**}P < .01$; $^{***}P < .005$; Mann-Whitney *U* test.

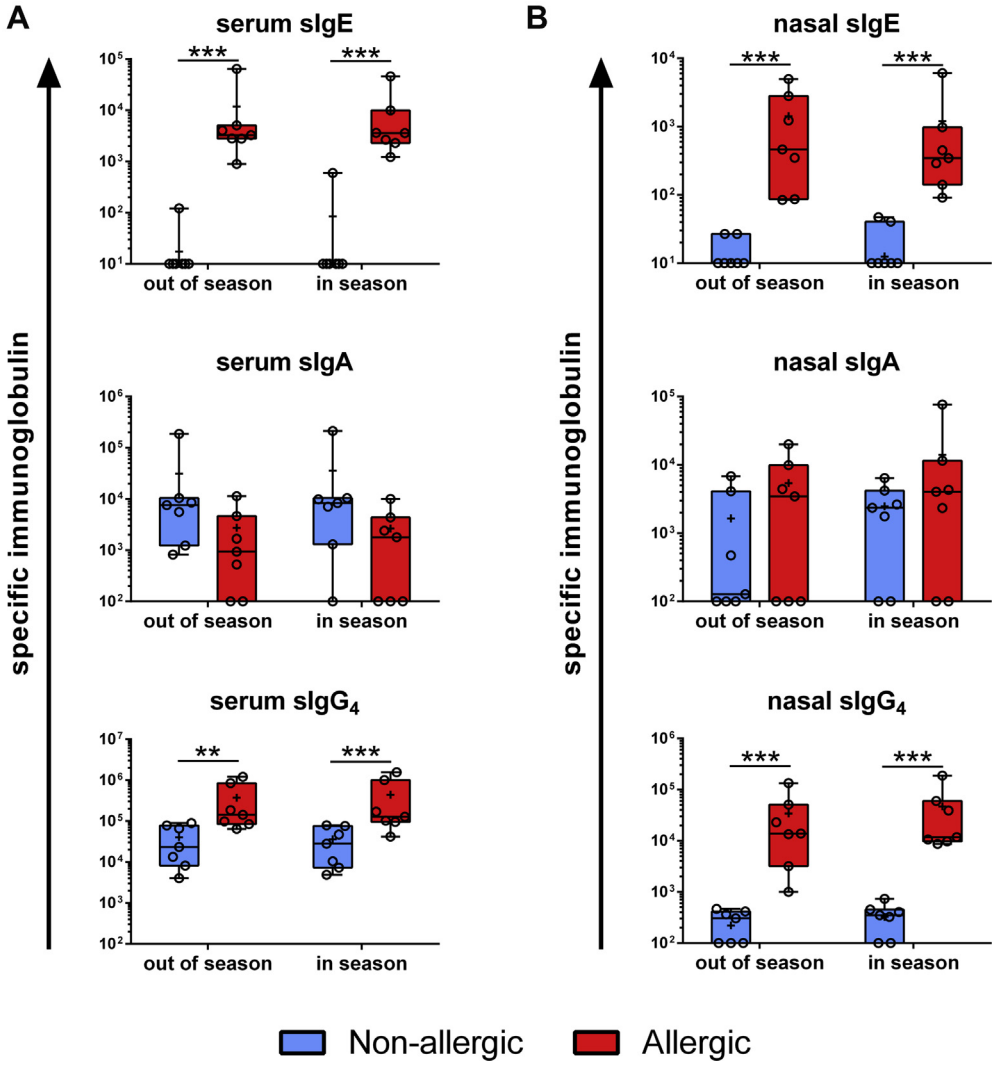

FIG 4. Cross-sectional and cross-seasonal comparison of Bet v 1–specific immunoglobulin isotype levels. Shown are median Bet v 1–specific immunoglobulin isotype levels in serum (**A**) and nasal fluid (**B**) outside and inside the main birch pollen season. Plotted are raw values (in U/mL) as measured by isotype-specific ELISA. Plus sign indicates the geometric mean. $^{*}P \leq 05$; $^{**}P < .01$; *** $P < .005$; Mann-Whitney *U* test.

Total immunoglobulin responses in serum and nasal fluid differ mainly between cohorts, less between seasons

To get a more comprehensive view of the humoral immune response to pollen, we compared in-season and out-of-season serum and nasal levels of immunoglobulin isotypes for both cohorts. We first normalized immunoglobulin levels per subject by dividing each value by the SD over all measurements. From the thus-normalized immunoglobulin levels, we calculated medians for all the visits that took place outside and within the (individually relevant) pollen season.

In serum, total immunoglobulin isotype levels were similar overall in both cohorts, the only exception being IgA, which was higher in patients with SAR than in subjects without allergy; this was true both outside ($P < .05$) and within the pollen season ($P = .007$) (Fig 3, *B*).

In nasal fluid, cross-sectional differences in immunoglobulin isotypes were observed during the pollen season, when nasal levels of IgA ($P < .05$), IgG_1, and IgG_2 ($P = .007$) were significantly higher in subjects without allergy than in patients with SAR. Nasal total immunoglobulin FLCs (κ plus λ) exhibited a tendency toward higher values in patients with SAR than in subjects without allergy; however, this tendency was not statistically significant (Fig 3, *A*).

Bet v 1–specific immunoglobulin responses differ between cohorts mainly within the birch pollen season

To examine the birch pollen–specific immunoglobulin isotype response, median values of Bet v 1–specific immunoglobulin levels were calculated for all visits that took place outside and within the main birch pollen season (from the start of April to start of May 2016).

Bet v 1–specific IgE antibodies were almost absent in subjects without allergy but were detectable in sera and nasal fluids of patients with SAR (Fig 4, *A* and *B* [*top panels*]), without seasonal changes. The same pattern was observed for $sIgG_4$ (Fig 4, *A* and *B*

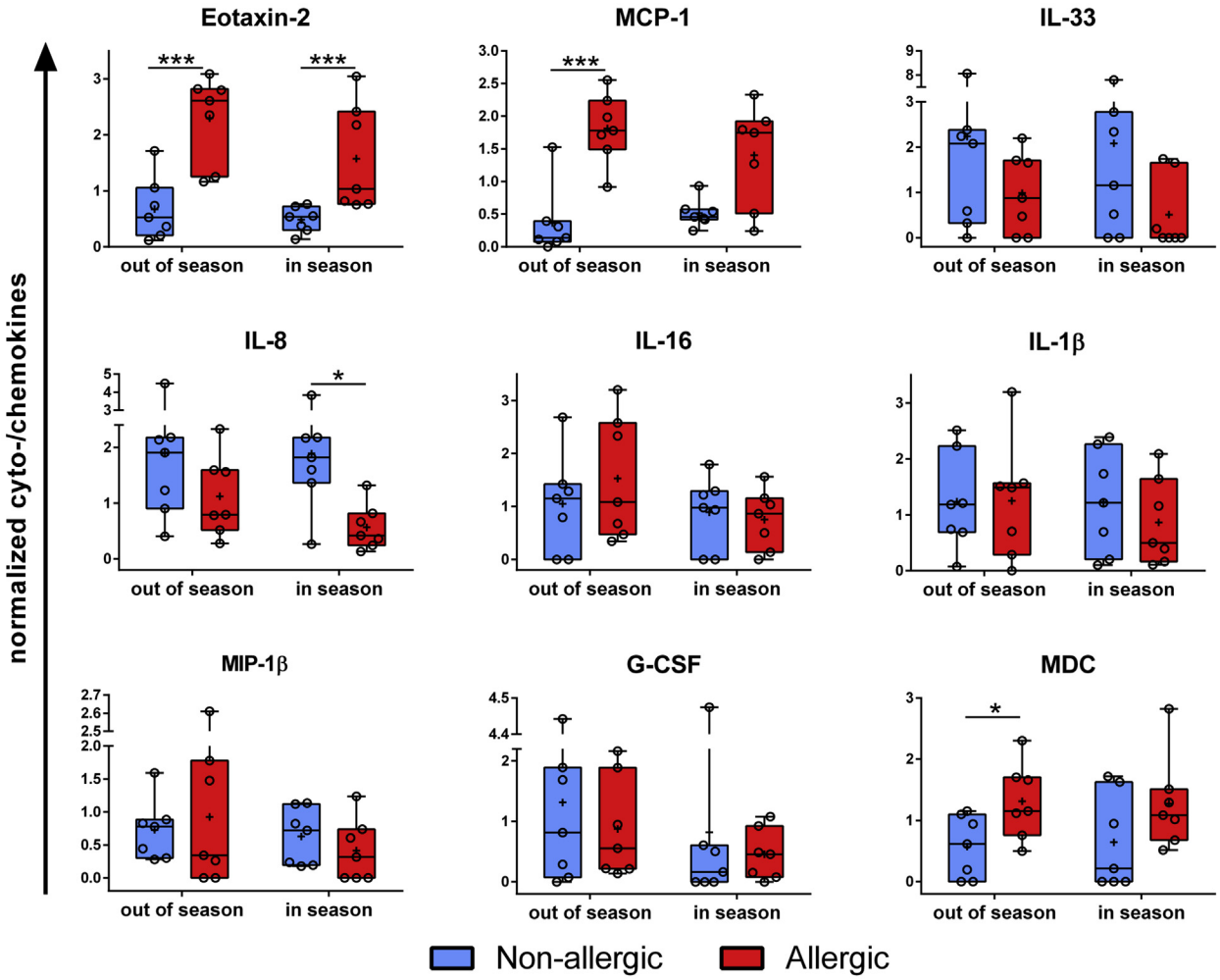

FIG 5. Cross-sectional and cross-seasonal comparison of nasal cytokines and chemokines. Shown are median cytokine and chemokine levels outside and inside the whole pollen season. Raw values, as measured by multiplex assay, were normalized per patient/subject by their overall SD and plotted on the *y* axis. Plus sign indicates the geometric mean. $^{*}P \leq .05$; $^{**}P < .01$; $^{***}P < .005$; Mann-Whitney *U* test.

[*bottom panels*]). Bet v 1–specific IgA antibodies were detected in both cohorts (Fig 4, *A* and *B* [*middle panels*]). In serum, sIgA was by trend elevated in subjects without allergy as compared with in patients with SAR (Fig 4, *A* [*middle panel*]). In nasal fluids, sIgA levels were similar in both cohorts.

Nasal cytokine and chemokine expression patterns differ mainly between cohorts

Nasal cytokine and chemokine levels were normalized per , and median levels of the in-season and out-of-season visits were compared across cohorts and seasons (Fig 5). Overall, normalized levels of nasal eotaxin-2, MCP-1, and MDC were higher in patients with SAR than in subjects without allergy, whereas the normalized nasal level IL-8 was higher in subjects without allergy. Cross-sectional differences in levels of MCP-1 ($P = .002$) and MDC ($P = .04$) were apparent only out of season; differences in IL-8 level were apparent only in season ($P = .01$). Significant cross-sectional differences in the levels of eotaxin-2 were apparent throughout the seasons (out of season, $P = .002$; in season, $P = .001$).

Nasal biomarkers for in-season symptom severity in subjects without allergy and patients with SAR

Nonsupervised PCA of all nasal immune mediators resulted in a separation of high- and low-symptomatic patients with AR, as well as in subjects without allergy, along Principal Component 1 (Fig 6, *A*). The nasal immune variables (Fig 6, *B*) explaining the largest of the overall variance were nasal levels of Bet v 1–specific IgG_4, IgA, and IgE as well as nasal levels of IL-8, IL-16, and IL-33 (see Fig E3 in this article's Online Repository at www.jacionline.org). This result was consistent irrespective of whether the variables were measured before or during the season (Fig 6 and see Fig E3). To test the robustness of the PCA results, we performed the same analysis with all the individual values of the in-season and preseason visits, thus increasing the number of data points in the PCA from 14 to 67 (see Fig E4, *A* in this article's Online Repository at www.jacionline.org). We further tested the robustness of the PCA results by using the 1-leave-out method, in which the PCA was performed 20 times, each with 1 other immune variable left out (see Fig E4, *B*). We finally tested for single Spearman correlations between in-season symptom severity and values of those immune variables that had come up as most

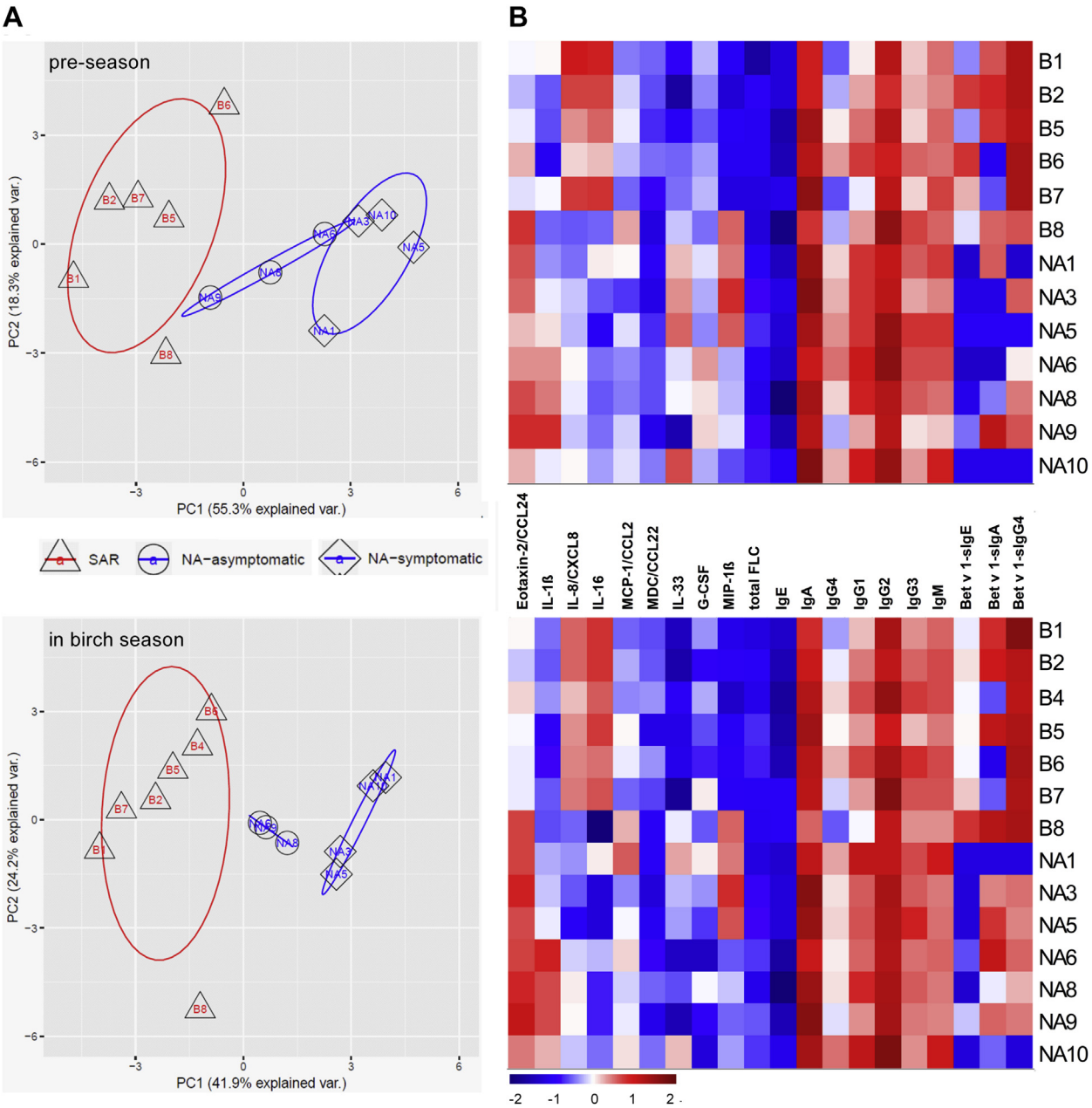

FIG 6. Nonsupervised PCA of nasal immune variables measured in patients with SAR and subjects without allergy. **A,** PCA plots for preseason immune variables (*top panel*) and in birch pollen season immune variables (*bottom panel*). **B,** Heatmaps of all nasal immune variables in patients with SAR and subjects without allergy, separated into preseason (*top panel*) and in-season (*bottom panel*) variables. Log data of all nasal immune variables were used for PCA, and true 0 values were set to 0.001.

predictive in the PCA. To identify possible predictive biomarkers, we correlated not only in-season but also preseason values of the immune variables against in-season symptoms for both cohorts (Fig 7). In the cohort of patients with SAR, preseason but not in-season level of nasal Bet v 1–specific IgE showed a significant positive correlation with in-season symptom severity. In the cohort of those without allergy, preseason nasal IL-8 and specific IgG_4 levels were negatively correlated ($P < .05$); preseason nasal IL-33 level was positively correlated with in-season symptom severity ($P < .05$). Negative correlations of IL-8 level with in-season symptom severity in the cohort of those without allergy were significant, irrespective of whether they were measured preseasonally or within or before the season.

DISCUSSION

This study compares systemic and local humoral immune responses and symptoms over time between pollen-sensitized patients with SAR and subjects without allergy under natural pollen exposure.

Among the most upregulated entities in nasal fluid of patients with SAR as compared with in the nasal fluid of subjects without allergy

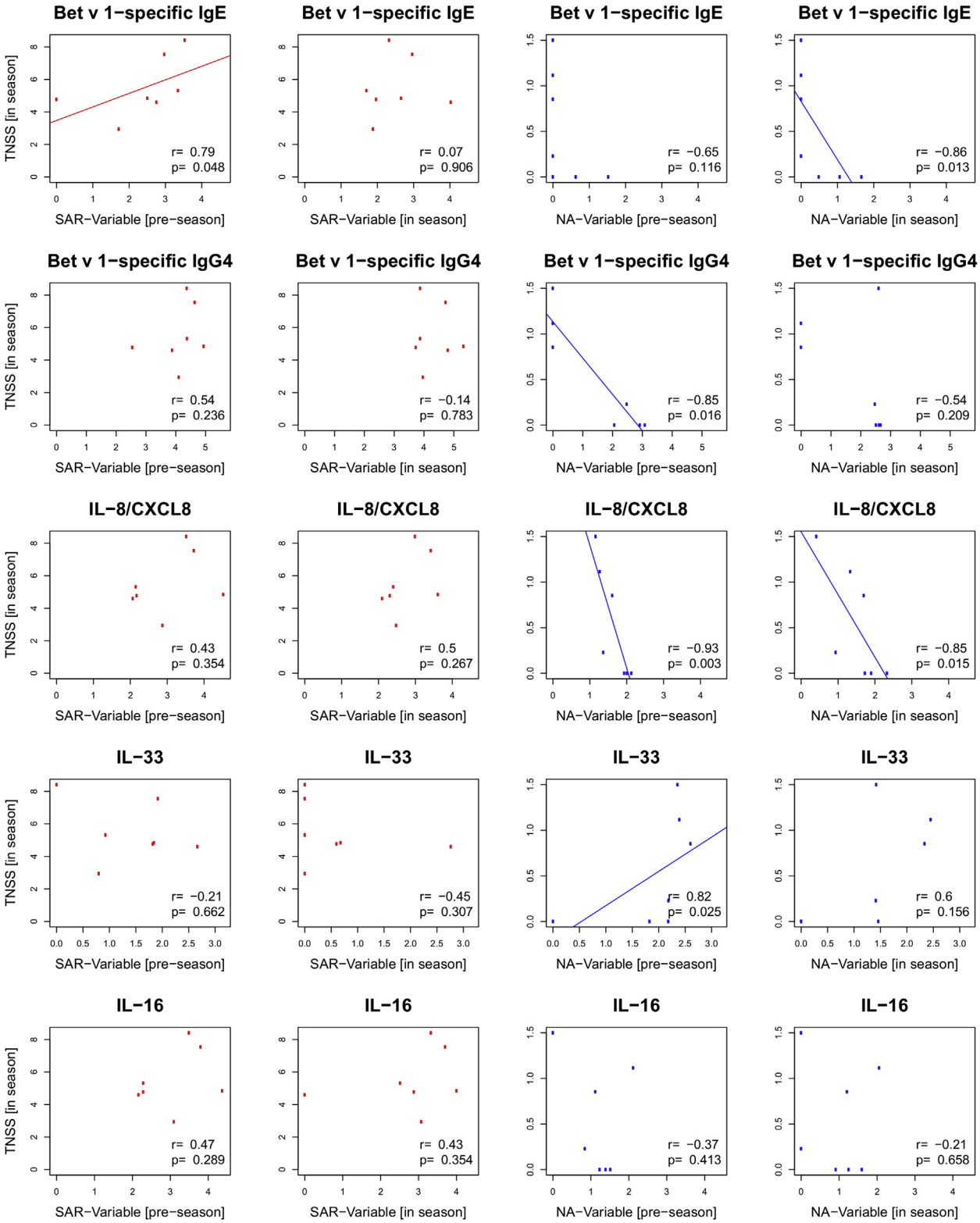

FIG 7. Correlation of single nasal immune variables with in-season symptom severity. Immune variables with the highest positive or negative PCA scores were correlated with in-season symptom severity, both for patients with SAR (*red dots*) and for subjects without allergy (*blue dots*). The *y* axis indicates in-season TNSS, and the *x* axis indicates expression level (log value) of immune variables as measured before and within the birch pollen season. $*P < .05$ (Spearman correlation). Correlation lines in the plots indicate significant results.

were the chemokines CCL2/MCP-1, CCL22/MDC, and CCL24/eotaxin-2, all of which were mainly associated with late-phase responses after experimental nasal allergen challenge.[16] Levels of MCP-1[17] and eotaxins, including eotaxin-2,[18-20] were found to be increased in the nasal fluid of patients with SAR by other studies as well. A gene polymorphism in eotaxin-3 was linked with susceptibility to AR in a Korean population–based cohort.[18]

Bet v 1–specific IgA antibodies showed a tendency toward higher values in serum of subjects without allergy than in patients with SAR, and total IgA appeared to be up-regulated in nasal secretions of subjects without allergy but not in subjects with SAR subjects within the pollen season. The discrepant finding on serum versus nasal levels of total IgA in patients with SAR could reflect inefficient transepithelial antibody transport or an impaired capacity for IgA-mediated immune exclusion. Our finding on nasal IgA agrees with the findings of a previous study[21] and raises the question of antigen specificity of pollen-binding IgA. A protective role for allergen-specific IgA in allergic conjunctivitis was suggested previously in a study comparing Bet v 1–specific IgE and IgA responses in sera and tear fluid and demonstrating distinct specificities for both isotypes.[22] In contrast, the ratio of Bet v 1–specific IgA to total IgA was found to be increased in the nasal fluid of children with SAR but not in allergy-free children under natural pollen exposure, implying an active role of allergen-specific nasal IgA in allergic inflammation.[23] Vaccination with genetically modified Bet v 1–derived proteins was shown to induce specific IgG_1, IgG_2, IgG_4, and IgA antibodies in serum and nasal secretions, but only specific IgG_4 was associated with symptom amelioration in this trial,[24] supporting the assumed beneficial role of IgG_4 responses during allergen-specific immunotherapy.[25-30] A recent study on patients undergoing birch pollen allergen–specific immunotherapy demonstrated clinical relevance of IgG antibodies, which block mast cell mediator release and have specificities to Bet v 1 epitopes distinct from those recognized by IgE antibodies.[31] Our nonsupervised PCA identified nasal Bet v 1–specific IgG_4 as the main factor within Principal Component 1 that separated the subjects according to symptom severity. Strikingly, separate Spearman correlation of preseason nasal $sIgG_4$ level with in-season symptom severity was negative in subjects without allergy and positive in patients with SAR, and similar patterns were observed for nasal levels of *Betula verrucosa* 1–specific (sIgE), FLCs, and IL-8. This could indicate pollen-specific humoral immune responses in subjects without allergy and patients with SAR, which could have opposing clinical outcomes. Our finding that nasal level of IL-33 was positively correlated with in-season symptom severity in subjects without allergy could point toward a role for a mechanism mediated by type 2 innate lymphoid cells.[32] To our surprise, IL-33 was readily detectable in the nasal fluid of subjects without allergy but absent in most of the SAR nasal samples. This is in contrast to the findings of previous articles in which IL-33 level was elevated in AR and asthma.[33] However, there is also evidence for lack of IL-33 expression or upregulation in pollen-exposed patients with SAR.[34] In the same study, the level of the IL-33 receptor ST2 in nasal fluid was positively correlated with symptom severity.

Total nasal symptom recordings followed airborne pollen concentrations in all patients with SAR (7 of 7) and, surprisingly, in a subset of subjects without allergy (4 of 7), albeit on a lower scale. To our knowledge, the time course of symptom expression under natural pollen exposure has never been systematically studied before, especially in a study including subjects without allergy. Most of the symptom registries within the cohort of subjects without allergy occurred in late winter and spring and followed the onset of hazel, alder, and birch pollen seasons, with an average time lag of up to 13 days. Within the grass pollen season, only 2 of 7 subjects without allergy recorded symptoms. Notably, the subjects without allergy who were nonsymptomatic under grass pollen exposure also included subject NA5 (see Fig E2), who demonstrated low-level reactivity to grass pollen by ImmunoCAP and low-level positivity to recombinant *Phleum pratense* 11 by testing using the ISAC (Table E1), indicating a beginning, genuine grass pollen sensitization. The other 3 subjects without allergy who expressed pollen-related symptoms in spring were nonreactive to either of the aeroallergens, as tested by using the SPT, ImmunoCAP, and ISAC (see Fig E5 in this article's Online Repository at www.jacionline.org). The concept of local AR, as defined by the occurrence of allergic symptoms and nasal sIgE in the absence of systemic sIgE,[35] cannot readily explain our symptomatic subjects without allergy either, as the only subjects without allergy but with sIgE signals in nasal fluid were subjects NA6 and NA9, who did not record any pollen-associated symptoms throughout the study.

Nasal immune responses to pollen in individuals without allergy have been demonstrated before under both natural[9,36] and experimental exposure settings[12] and have consisted of local induction of neutrophil attracting chemokines. In the present study, we observed pollen-related kinetics of symptom expression in 4 of 7 subjects without allergy that resembled those of the patients with SAR. To our knowledge, such a finding has never been reported before. Seasonal, pollen-related symptoms, as well as the biomarker signature, could be indicative of the potential of nonatopic individuals to develop SAR at some later stage in life, by piecemeal sensitization due to recurrent episodes of low-level nasal inflammation that are finally no longer controlled by pollen- and Bet v 1–specific IgG_4 and IgA antibodies. As shown recently, pollen exposure interferes with innate antiviral immune responses in the airways.[37] In addition, nasal IL-8 release and neutrophil infiltration have been found to correlate with symptom severity in patients with acute respiratory viral infection.[38] In our current study, IL-8 was among the main predictors for symptoms in those without allergy, which could reflect subclinical viral infection under coexposure to immune suppressive substances from pollen. A biomarker signature indicative of a high level of symptoms could therefore also identify individuals at high risk for viral exacerbations during the pollen season.

The current limitations of the current study, such as the comparatively small sample size due to drop outs, certainly highlight the necessity for a follow-up, cross-validation cohort. Nevertheless, our present study design with repeated biosamples per subject has already enabled us to unmask some clear response patterns despite the statistical noise that is usually present under real-life exposure conditions. Distinguishing between high- and low-symptomatic responders on the basis of a set of biomarkers could help to reveal the symptom-relevant pathophysiology, which could lead to the development of new symptomatic treatment. Because biomarkers for symptoms are indicative of disease burden, they could identify patients who profit particularly from causative treatment or the use of pollen information services. Biomarkers for symptom severity in subjects without allergy, on the other hand, could be a new piece in the puzzle of allergic sensitization and could therefore provide a key to prevention.

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Clinical implications: Individuals with allergy, as well as individuals without allergy, show pollen-associated nasal symptoms in springtime. The strength of the seasonal allergic symptoms can be predicted by nasal biomarkers.

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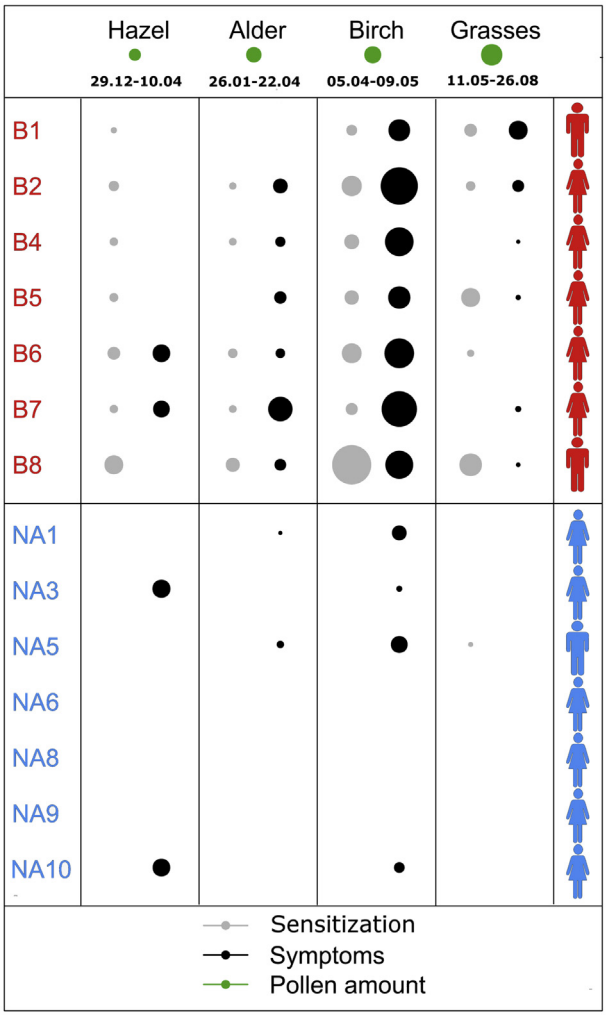

FIG E1. Overview over sensitization and symptom patterns in the cohorts of patients with SAR and subjects without allergy (NA). Patients with SAR are depicted in red, and subjects without allergy are depicted in blue. Black dots indicate specific IgE levels as determined by using the ISAC; gray dots cumulative symptoms within the respective pollen season (hazel, alder, birch, grasses); and green dots cumulative pollen concentrations for each taxon. The magnitude of the green dots reflects the amount of each pollen taxon relative to the total pollen amount per year. For each patient/subject-related variable, values were normalized to the global maximum measured across cohorts. Cumulative pollen concentrations for each taxon were normalized to the cumulative total pollen concentration at the end of the study period.

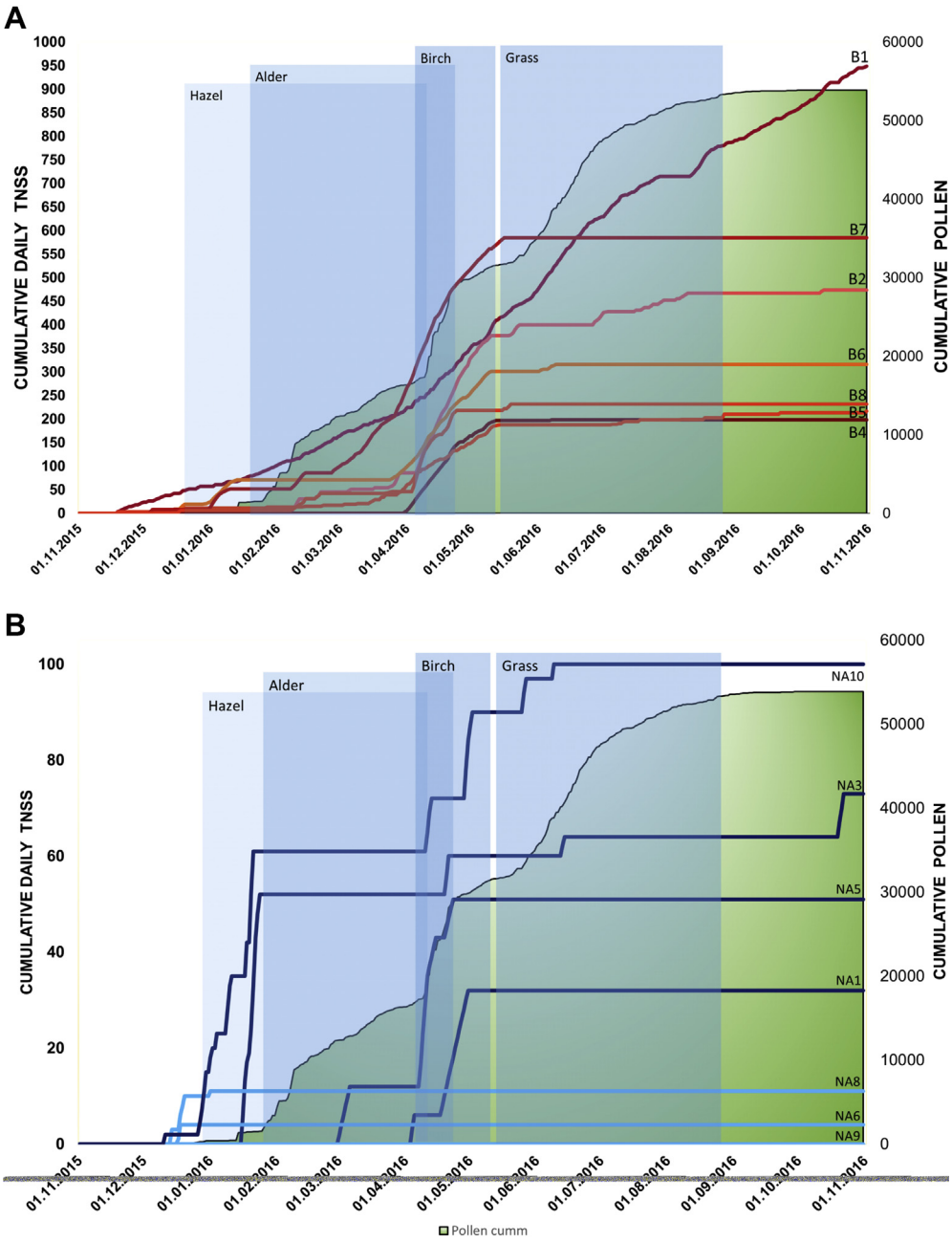

FIG E2. Single plots of cumulative symptoms versus cumulative pollen. Cumulative TNSSs (*left y axis*) are plotted against cumulative airborne pollen concentrations (*right y axis*). Shaded areas indicate main pollen seasons for each taxon. **A,** Symptoms of patients with SAR (*red lines*). **B,** Symptoms of subjects without allergy. Light blue lines represent asymptomatic subjects without allergy; dark blue lines represent subjects without allergy (NA) who display symptoms during the pollen seasons.

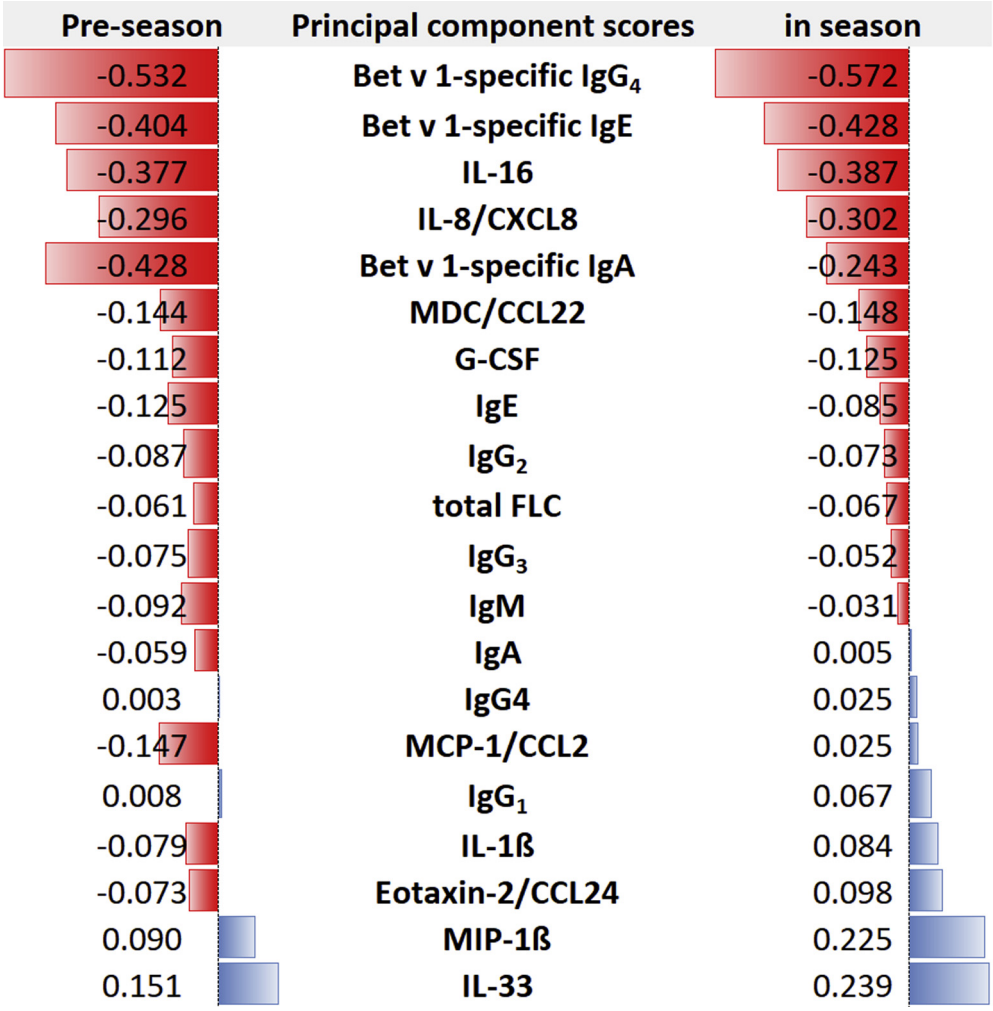

FIG E3. Principal component scores for nasal immune variables within Principal Component 1. Log data were subjected to nonsupervised PCA. The patients with SAR and subjects without allergy were clustered along Principal Component 1 according to their in-season symptom severity. Left column indicates component scores of preseason immune variables; right column indicates component scores of in-season immune variables. PCA was performed in R script.

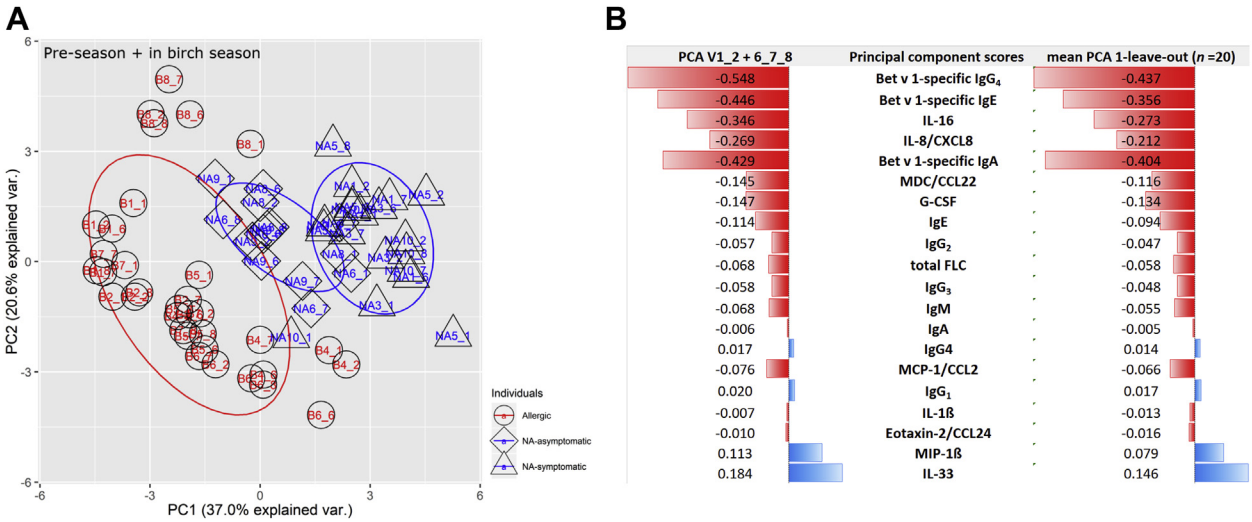

FIG E4. Testing of the robustness of the PCA results. **A**, Nonsupervised PCA analysis with all the individual values of the in-season and preseason visits. The results from the original PCA (see Fig 6, *A* and this figure) still hold for the PCA with 67 individual visit data points in terms of clustering of the groups. **B,** The robustness of the PCA was tested by using the 1-leave-out method, in which the PCA was performed 20 times, each time with 1 other variable left out and again keeping the ranking and the values of the principal components highly similar to the original PCA results. PCA was performed in R script.

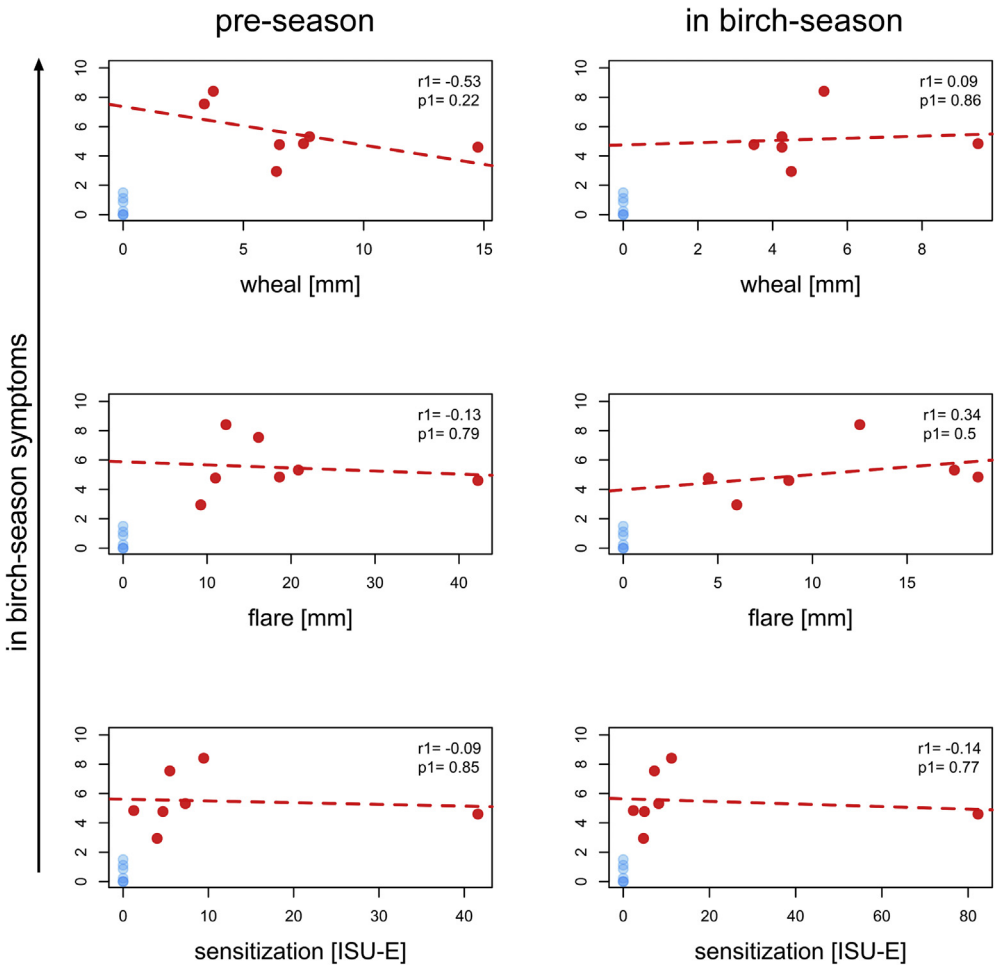

FIG E5. Correlation of clinical test results with in-season symptom severity. Spearman correlation of clinical allergy test results with symptom severity within the birch pollen season. Red dots represent test results for the patients with SAR, and blue dots represent results for subjects without allergy. Top panel shows wheal size, and middle panel shows flare size as measured by standard SPT. Bottom panel shows *Betula verrucosa* 1–specific (sIgE) result, as determined by ImmunoCAP (Phadia/Thermo Fisher). Correlations with in-season symptoms are shown for preseason (*left*) and in-season (*right side*) measured test results.

TABLE E1. Detailed overview over the sensitization profile of the study participants

Allergen	B1	B2	B4	B5	B6	B7	B8	NA1	NA3	NA5	NA6	NA8	NA9	NA10
Act d 8	0	0	0.37	0	0	0	0.66	0	0	0	0	0	0	0
Aln g 1	**0**	**1.11**	**1.25**	**0**	**2.08**	**1.27**	**5.14**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Alt a 1	0	0	0	0	1.27	0	0	0	0	0	0	0	0	0
Api g 1	0	0	0	0	0.89	0	0.88	0	0	0	0	0	0	0
Api m 1	0	0	0	0	0	0	0.49	0	0	0	0	0	0	0
Ara h 8	0	1.44	0.84	0.73	3.42	0.63	4.03	0	0	0	0	0	0	0
Bet v 1	**2.81**	**11.34**	**5.82**	**5.32**	**10.80**	**3.58**	**46.03**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Cor a 1.0101	**0.72**	**2.52**	**1.56**	**1.78**	**4.08**	**1.53**	**9.76**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Cor a 1.0401	0.34	3.96	1.41	1.21	2.98	1.36	4.93	0	0	0	0	0	0	0
Cyn d 1	1.03	0.25	0	0.78	0	0	1.33	0	0	0	0	0	0	0
Equ c 1	0	0	0	1.10	0	0	0	0	0	0	0	0	0	0
Fel d 1	0	0	0	2.64	1.27	0	0	0	0	0	0	0	0	0
Fel d 4	0	0	0	0.38	0	0	0	0	0	0	0	0	0	0
Gly m 4	0	0	0.32	0	1.94	0	0.74	0	0	0	0	0	0	0
Hev b 5	0	0	0	0.52	0	0	0	0	0	0	0	0	0	0
Hev b 8	0.82	0	0	0	0	0	0	0	0	0	0	0	0	0
Jug r 3	0	0	0	0	0	0	0.39	0	0	0	0	0	0	0
Mal d 1	0.61	2.56	1.13	0.82	2.48	1.04	15.34	0	0	0	0	0	0	0
Mer a 1	0.69	0	0	0	0	0	0	0	0	0	0	0	0	0
MUXF3	0	0	0	0.47	0	0	0	0	0	0	0	0	0	0
Ole e 1	0.3	0	0	0	0	1.49	3.94	0	0	0	0	0	0	0
Phl p 1	3.03	1.76	0	9.06	1.06	0	12.97	0	0	0	0	0	0	0
Phl p 11	0	0	0	0	0	0	0	0	0	0.41	0	0	0	0
Phl p 5	0	0.33	0	0	0	0	0	0	0	0	0	0	0	0
Pol d 5	0	0	0	0.83	0	0	0	0	0	0	0	0	0	0
Pru p 1	0	2.86	1.54	0.29	4.77	1.44	5.60	0	0	0	0	0	0	0
Ves v 5	0	0	0	3.31	0	0	0	0	0	0	0	0	0	0

Act d, Actinidia deliciosa; Aln g, Alnus glutinosa; Alt a, Alterneria alternata; Api g, Apium graveolens; Api m, Apis mellifera; Ara h, Arapis hypogaea; Bet v, Betula verrucosa; Cor a, Coryus avellana; Cyn d, Cynodon dactylon; Ecu c, Equus caballus; Fel d, Felinus domesticus; Gly m, Glycine max; Hev b, Hevea brasiliensis; Jug r, Juglans regia; Mal d, Malus domestica; Mer a, Mercurialis annua; Ole e, Olea europaea; Phl p, Phleum pratense; Pol d, Polistes dominulus; Pru p, Prunus persica; Ves v, Vespula vulgaris.

Serum IgE specific for various airborne allergens was determined by a component-resolved allergen diagnostic test (ISAC, Thermo Fisher). The cutoff for positivity of the test result, as specified by the manufacturer, is 0.4. Identifiers NA1 to NA10 refer to subjects without allergy. Identifiers B1 to B8 refer to subjects with SAR. None of the subjects without allergy (NA1-NA10) showed any sensitization against Bet v 1 or related PR-10 proteins (highlighted in boldface).