

Biomatrix for upper and lower airway biomarkers in patients with allergic asthma



To the Editor:
Although many biomarkers have been proposed for asthma monitoring, only the noninvasively accessible fraction of exhaled nitric oxide has been established into clinical routine,

whereas implementation into guidelines is pending.¹ The upper and lower airways are considered a morphologic and functional unit and share air transport, physical barrier, mucociliary clearance, and immune interface as common features.² Furthermore, not only do allergic rhinitis and asthma share mechanisms of allergic inflammation, but also, transition of allergic rhinitis into asthma can represent a steady continuum depending on endotype. Therefore nasal secretions

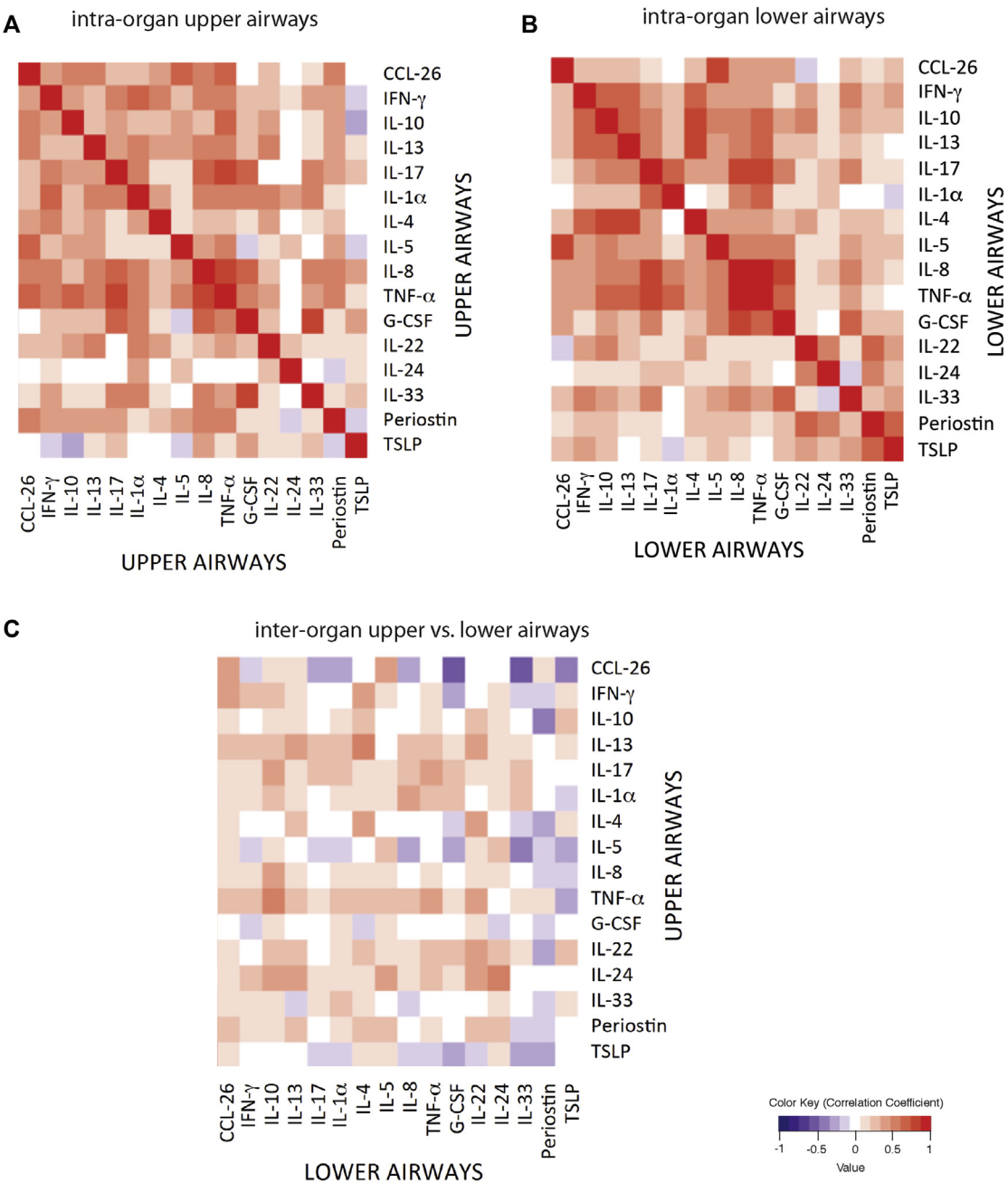


FIG 1. Biomarker grids reflecting correlations between upper and lower airways cytokine levels. Intraorgan correlations for upper (A) and lower (B) airways and interorgan correlations (C) were depicted by using respective correlation coefficients (*r*) from negative (dark blue) to positive (red) values. Intraorgan correlations seemed to be predominantly strong, whereas interorgan relations were most visible by using nasal and sputum IL-24 measurements. G-CSF, Granulocyte colony-stimulating factor; TSLP, thymic stromal lymphopoietin.

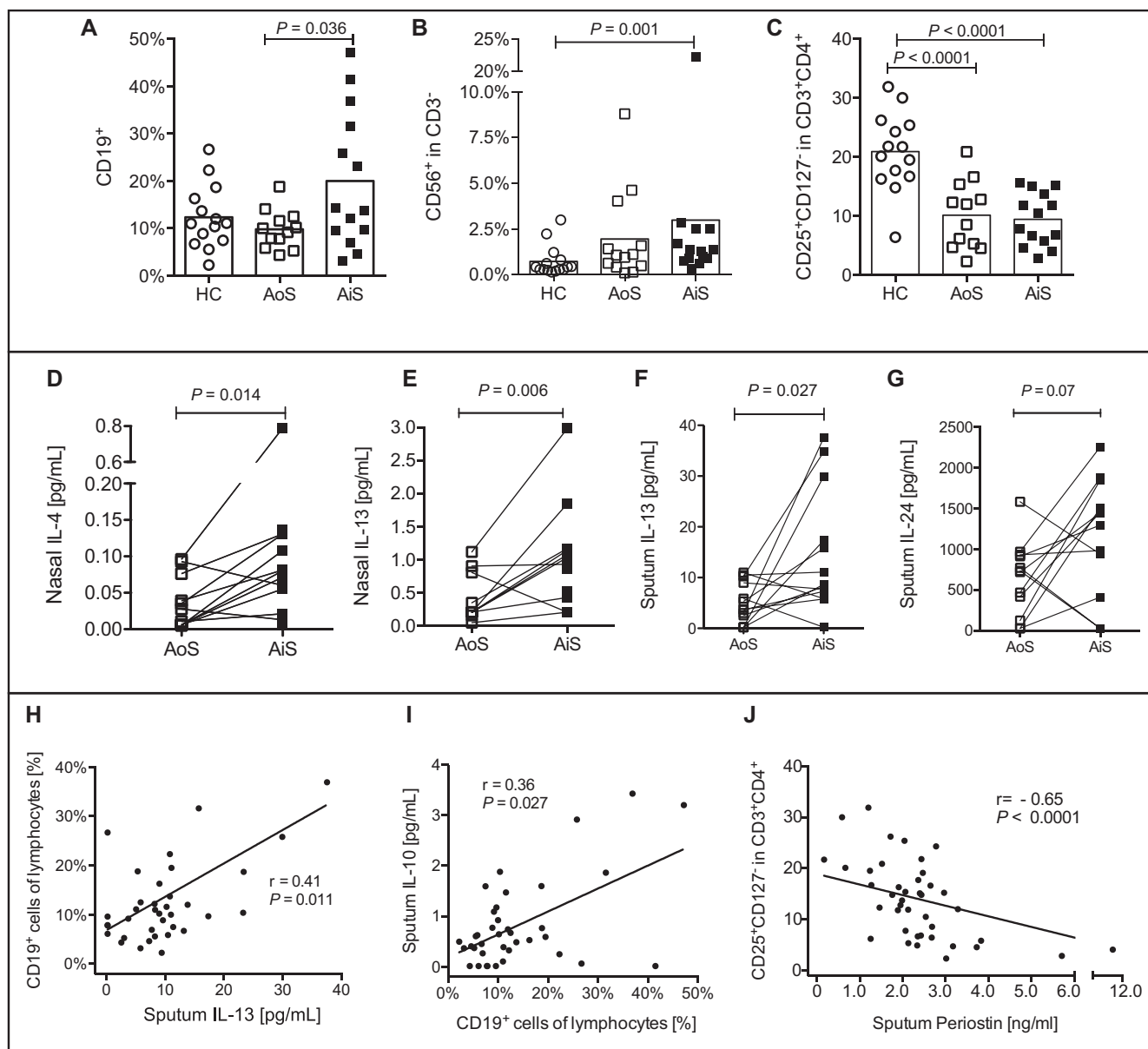


FIG 2. Airway cytokines and cell populations. **A-C**, Although numbers of B lymphocytes and natural killer cells were upregulated in asthmatic patients during the grass pollen season, in Treg cells there was only a difference between patients and healthy control subjects. **D-G**, Type 2 cytokines were differentially regulated in asthmatic patients out of the grass pollen season, as well as in the grass pollen season. **H-J**, Positive correlations were visible for B-cell counts and sputum IL-13 and IL-10 levels, respectively, whereas a strong negative correlation was seen for sputum periostin levels and Treg cell counts. AiS, Asthmatic patients in the grass pollen season; AoS, asthmatic patients out of the grass pollen season; HC, healthy control subjects.

of the upper airways can prove useful as noninvasive and easily accessible proxies mirroring lower airway inflammation.³ The objective of the current study was to visualize the relationship of elementary protein biomarkers of the upper against the lower airways to identify potential nasal proxy candidates for the lower airways.

Nasal lining fluids and induced sputum were collected to find practical solutions to assess airway inflammation by using noninvasive methods because sputum opens another window to assess lower airway inflammation.⁴ We included 15 patients with seasonal allergic asthma and 16 healthy

participants, all of them nonsmokers, into the study (see Table E1 in this article's Online Repository at www.jacionline.org) and analyzed sputum supernatants and nasal secretions by using multiarray technology (see the Methods section in this article's Online Repository at www.jacionline.org). Furthermore, we assessed clinical scores as follows: Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ), Asthma Control Score (Global Initiative for Asthma [GINA]), and Perceived Stress Questionnaire (PSQ). Standard sputum differential cell counts (see Table E2 in this article's Online Repository at www.jacionline.org) and additional

sputum flow cytometry (fluorescence-activated cell sorting) were included.

To identify potential proxy biomarkers, we displayed the strength of the interaction of the biomarker signal of the upper and lower airways in a chessboard pattern (2-sided Spearman rank correlation; Fig 1). Known pathomechanisms, such as cellular source of biomarkers and sputum cell counts, were used to augment these patterns and enrich for best biomarker selection.

Levels of many cytokines and chemokines in sputum correlated moderately to strongly ($r > 0.5$) with other mediators of sputum (intraorgan comparison; Fig 1, A and B), whereas the correlation of these mediators between sputum and nasal secretions showed a low to moderate relationship (interorgan comparison, $r < 0.5$). These correlations are mainly seen diagonally (Fig 1, C), which displays the correlation of one cytokine in the lower airways to the same cytokine in the upper airways. IL-24 seemed to be of special interest as a proxy because it showed moderate correlations between the upper and lower airways ($r = 0.53$, $P = .001$; Fig 1, C, and see Table E3 in this article's Online Repository at www.jacionline.org). Interestingly, the GINA score showed a strong negative correlation to nasal IL-24 levels ($r = -0.85$, $P = .001$). Furthermore, sputum IL-24 levels were increased in asthmatic patients in season (1084.0 ± 199.6 pg/mL) compared with out of season (641.2 ± 131.7 pg/mL; $P = .07$; Fig 2, G). Flow cytometric analysis of cellular components in sputum revealed a moderate negative correlation of CD25⁺/CD127⁺ regulatory T (Treg) cell counts to sputum IL-24 levels ($r = -0.50$, $P = .002$). We speculate that this decrease in numbers of Treg cells could be caused by T_H2-mediated suppression at the site of inflammation. This interpretation is supported by the finding that numbers of Treg cells in asthmatic patients were diminished independently of season (Fig 2, C). From a mechanistic point of view, this result supports our previous findings showing that type 2 immune responses inhibit induction of regulatory cell.⁵

As expected, correlation of levels of one cytokine with those of one of the other mediators was observed frequently in the intraorgan correlation grid (Fig 1, A and B); for example, levels of the type 2 cytokine IL-5 correlated with levels of the type 2 IL-4–inducible CCL-26 (eotaxin-3; nasal: $r = 0.726$, $P < .0001$; bronchial: $r = 0.771$, $P < .0001$). A moderate correlation was also observed for nasal CCL-26 and nasal IL-13 levels ($r = 0.57$, $P < .0001$). It is well known that IL-13 is able to seasonally promote IgE and IgG₄ production.⁶ In fact, a weak correlation of sputum IL-13 levels and sputum CD19⁺ B-cell counts was observed ($r = 0.41$, $P = .011$; Fig 2, H). In general, the proportion of total B cells in sputum within asthmatic patients showed a significant increase during the grass pollen season ($20.03\% \pm 3.85\%$) compared with off season ($9.80\% \pm 1.18\%$; $P = .036$; Fig 2, A).

Levels of the regulatory cytokine IL-10 moderately correlated with TNF- α expression levels in both the upper and lower airways ($r = 0.38$, $P = .024$; Fig 1, C) and even more so in the intraorgan comparison (lower airways: $r = 0.61$, $P < .0001$; upper airways: $r = 0.61$, $P < .0001$; Fig 1, A and B). Sputum IL-10 levels correlated weakly with the presence of CD19⁺ B cells ($r = 0.36$, $P = .027$) in sputum (Fig 2, I). IL-10 in airway lining fluid can originate from previously described IL-10–producing regulatory B cells, which are capable of regulating type 2 inflammation or promoting the

IgE to IgG₄ switch.⁶ Furthermore, regulatory B cells are known to play a pivotal role in allergen-specific immunotherapy.⁷ Surprisingly, we observed weak to moderate correlation for nasal IL-10 expression with the perceived stress burden of asthma patients assessed by PSQ score ($r = 0.42$, $P = .01$; see Fig E1 in this article's Online Repository at www.jacionline.org), which showed a strong correlation with GINA score ($r = 0.78$, $P = .003$) and moderate correlation with RQLQ score ($r = 0.58$, $P < .0001$). Nasal IL-10 levels also negatively correlated with total sputum leukocyte count ($r = -0.65$, $P < .0001$), which is considered a marker for inflammation in the lower airways (see Table E2). Although IL-10 levels in the upper airways can display clinically relevant marker functions, it shows only few or even negative (eg, periostin) correlations between the upper and lower airways.

Periostin has been proposed as a biomarker for comorbid chronic rhinosinusitis in patients with asthma, revealing its relevance in T_H2-driven diseases also in patients with local tissue inflammation.⁸ Expression pattern differed from markers because it was mostly correlated negatively with other mediators, including itself, when compared with the upper airways. This negative interaction was not observed in intraorgan comparison with one exception, namely IL-1 α . The important role of periostin in the lower airways is implied by the finding that periostin expression in sputum negatively correlated with CD25⁺CD127⁺ Treg cell ($r = -0.65$, $P < .0001$; Fig 2, J) and positively with sputum eosinophil ($r = 0.36$, $P < .034$) counts, whereas RQLQ scores seemed to be associated to levels of lower airway periostin ($r = 0.38$, $P = .021$; see Fig E1). Taken together, local periostin might prove useful to assess disease progression in the lower airways, but our data suggest periostin to be a less favorable proxy in the upper airways because of its negative relationship to the lower airways.

In conclusion, the concept of united airways and consequently representative nasal proxy biomarker analytes holds particularly true for type 2 cytokines, specifically IL-24, which we have previously shown to be an epithelial type 2 cytokine.¹ Periostin and IL-10 levels correlate moderately with clinical symptoms and might be of limited use as proxy biomarkers because of the conflicting correlations between mediators of the upper and lower airways. These findings provide not only promising biomarker candidates but also correlation matrices, which might be reflective of underlying asthma endotypes. Larger clinical studies are needed to fully assess the validity and practicability of cytokine measurements in nasal lining fluid to characterize asthmatic patients.

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Genetically engineered cell factories produce glycoengineered vaccines that target antigen-presenting cells and reduce antigen-specific T-cell reactivity



To the Editor:

Allergen-specific immunotherapy is a promising approach to reduce or remove allergic symptoms by inducing tolerance.¹ As an alternative to current allergy vaccines derived from extracts of natural allergens, recombinant vaccines offer the opportunity for *de novo* antigen design,² with enhanced targeting to antigen-presenting cells (APCs) and potentially increased ability to induce tolerance.^{3,4}

Using the type 1 allergen Bet v 1 as our model antigen, we evaluated the effects of combining a nonglycosylated antigen to a

glycomodule⁵ to target the APC-expressed family of C-type lectin receptors (CLRs). CLRs are known to recognize glycan structures and promote rapid internalization of antigens on binding (see Fig E1, A, in this article's Online Repository at www.jacionline.org).^{6,7} We used our recently developed glycoengineered cellular platform⁸ to produce Bet v 1 with defined carbohydrates and evaluated APC uptake, efficacy using patient-derived T cells, and induction of tolerance in a murine model.

Currently, allergen-specific immunotherapy is primarily administered subcutaneously or sublingually. Focusing on human skin, we examined the CLR expression pattern in skin-localized APCs. We observed expression of the major CLRs, including mannose receptor (MR), dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), and macrophage galactose-type lectin (MGL), in the dermal compartment of skin biopsy specimens (see Fig E1, B). Next, we examined expression of MR, DC-SIGN, and MGL in *in vitro*-differentiated human monocyte-derived dendritic cells (moDCs) and macrophages (M1 and M2) by means of transcriptomics and flow cytometry (see Fig E1, C-E). Expression of CLRs peaked after 3 days of differentiation, with simultaneous and significant expression of DC-SIGN, MR, and MGL in moDCs (see Fig E1, D and E). Cytokine measurements confirmed the nature of the APCs (see Fig E1, F) and their relevance as a cellular model.

To identify the optimal carbohydrate structure or structures for specific APC targeting, we evaluated uptake of chemically synthesized carbohydrate derivatives (N-acetylgalactosamine polyacrylamide [GalNAc-PAA], N-acetylglucosamine-PAA, Lewis X-PAA, and mannose-PAA) and a GalNAc glycosylated molecule with varying numbers of GalNAc residues (see Fig E2, A and B, in this article's Online Repository at www.jacionline.org). Both mannosylated and high-density GalNAc glycosylated structures were readily taken up by the APCs, and therefore GalNAc and mannose structures were selected for further studies.

We next designed Bet v 1 allergen constructs for the expression of full-length Bet v 1 fused to glycomodules, with consensus glycosylation motifs ensuring the incorporation of either O- or N-linked glycans. The fusion constructs were expressed in glycoengineered Chinese hamster ovary (CHO) cell lines and *Pichia pastoris* (Fig 1, A, and see Fig E2, C-G, and Table E1 in this article's Online Repository at www.jacionline.org). This strategy allowed us to generate fusion proteins decorated with high-mannose N-linked glycans from MGAT1 knockout CHO cells, linear α 1-2 O-linked mannose residues from *P. pastoris* (Pichia), or O-GalNAc residues from COSMC knockout CHO cells for efficient targeting of MR, DC-SIGN, and/or MGL on APCs (Fig 1, A, and see Fig E2, C-G).

Additionally, we expressed the O-glycan fusion construct in *Escherichia coli* and inserted GalNAc residues by means of *in vitro* glycosylation. Glycosylation profiles were verified by using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) of released glycans supplemented with lectin profiling (see Fig E2, C-G). As a control for glycan-mediated effects, we treated fractions of the glycoallergens with periodate, a treatment known to effectively destroy lectin recognition (see Fig E2, E).

Because moDCs were the only cell type to express high levels of DC-SIGN, as well as MR and MGL, we used moDCs to evaluate uptake of glycan-modified Bet v 1 fusion proteins. Internalization of all glycan-modified Bet v 1 fusion proteins was enhanced 1.5- to 6-fold compared with that of their

1 Supplemental methods

2 ***Study subjects***

3 Healthy subjects (n=16) and mild, well-controlled, allergic asthma patients along the
4 GINA definition in season (AiS; n=15) / off season (AoS; n=13) at the age between 20
5 and 65 years in good health (FEV1% >70%) with a history of clinically significant hay
6 fever during the grass-pollen season since more than two years were included. Two
7 healthy controls, who showed sensitization, and one subject with asthmatic symptoms
8 but missing sensitization (in and off season) were excluded. The median age of the 16
9 control subjects was 36 years (range = 23–55 years), matched for age with the patients
10 with allergic asthma (median age = 39, range = 21–63 years). For asthmatic subjects, a
11 GINA classification was performed. All subjects completed a Rhinoconjunctivitis Quality
12 of Life Questionnaire (RQLQ), Perceived Stress Questionnaire (PSQ), a lung function test,
13 and a hypertonic saline sputum induction, performed in the Allergy Section, Department
14 of Otolaryngology, TUM School of Medicine. In addition, nasal secretions from healthy
15 controls (n=14) and allergic and asthmatic patients in (n=14) and out of season (n=12)
16 were collected as previously described.¹ Each participant provided written informed
17 consent. The study was approved by the local ethics committee. Further clinical
18 characteristics of the two study populations are summarized in Table 1. Six of the 15
19 asthmatic patients had tried AIT before participating in the study, but were not free of
20 symptoms and therefore included into analysis. One of the patients had very little
21 symptoms (0 points in GINA-Score), hence did not meet the study criteria of
22 sensitization and was therefore excluded.

23 ***Skin prick tests***

24 Skin tests were performed in every patient using standardized european panel²
25 including environmental aeroallergens, such as house dust mites (*D.farinae* and

D.pteronyssimus), grass pollen, birch pollen, and animal dander of cats (ALK-Abello, Madrid, Spain). Histamine (10 mg/mL) and saline solution (0.9%) controls were taken along. Atopy was defined as the presence of at least one skin prick test to common allergens, with a wheal diameter of 3 mm or greater than negative control.²

Lung function testing

Baseline lung function was evaluated using a calibrated handheld pulmonary function testing device (Jaeger SpiroPro; Würzburg, Germany). The following parameters were recorded: VC, FEV1, FEV1/VC, and maximum expiratory flow 25% (MEF 25%). Bronchodilator reversibility was tested after 400 µg of salbutamol.

Sputum Collection and Processing

Participants first inhaled salbutamol and consecutively nebulized hypertonic saline at increasing concentrations of 3%, 4%, and 5% NaCl every 7 min according to previous publications.^{3,4} During this procedure, participants cleaned their noses and rinsed their mouth to reduce squamous epithelium cells in the samples. Sputum was processed within one hour of collection. The selected sputum plugs, which contained as little saliva as possible were placed in a weighed Eppendorf tube and processed with 4x weight/volume of sputolysin working solution (Merck, Darmstadt, Germany). Afterwards, 2x weight/volume of phosphate-buffered saline (PBS) was added. Samples were filtered through a 70-µm mesh and centrifuged for 10 min at 790xg without break to remove the cells. Supernatants were stored at -80 °C for additional analysis using a protein assay. Appropriate sputum samples were obtained from 14 healthy controls, 14 asthma patients during the grass-pollen season and 12 asthma patients off season.

Identification of Cell Populations by Differential Cell Count

Cell suspension was centrifuged in a Labofuge 400 cytopsin column (Heraeus, Germany) at 44xg for 6 min. The slides produced were air-dried, fixed with methanol for 5 min and

stained using the Pappenheim method.⁵ Specimens with a content of less than 15% of squamous epithelial cells were considered adequate. At least 400 nonsquamous cells on each sputum slide were read by two investigators blinded to the subject classification. Neutrophils, lymphocytes, eosinophils, basophils, and macrophages were calculated as proportions of the total cell count.

Identification of Cell Populations by Flow Cytometry

Sputum cells were stained for flow cytometric analysis with antibodies to surface markers CD45, CD3, CD4, CD19, CD25, CD127, and CD56 to distinguish enumeration T and B cell subsets as well as NK cells. B cells (CD45⁺CD3⁺CD19⁺) and NK cells (CD45⁺CD3⁺CD56⁺) were expressed as percentage of total living lymphocytes, whereas previously described Tregs (CD45⁺CD3⁺CD4⁺CD25⁺CD127⁻) were expressed as a percentage of the CD3⁺CD4⁺ Th cells.⁶ Cell suspensions, containing 1×10^6 cells in 100 μ L were used for each flow cytometry stain. Cells were washed in buffer (phosphate-buffered saline (PBS) containing 5% FCS, and 0.01% sodium azide), incubated with TrueStain FX (Biolegend, USA) at 5 μ L per 100 μ L for 15 min at 4°C to block non-specific binding, washed twice times, then labeled in a total volume of 50 μ L for 30 min at 4°C in dark. Subsequently, cells were washed three times with 200 μ L FACS buffer. The following antibodies were used: CD45-APC-H7, CD3-HV500, CD4-BrilliantViolet 450, CD19-AlexaFluor 700, CD25-FITC, CD56-BV605, and CD127-BV711 (all BioLegend, USA). Controls were used for gating strategies, using unstained samples, single stain and fluorescence-minus-one (FMO) controls. Cells were washed again three times and resuspended in 200 μ L FACS buffer. Afterwards, 1.5 μ L of 7AAD (BD Biosciences) were added to cells immediately before data acquisition. All stained cells were acquired with a BD LSRII Fortessa flow cytometer (BD Biosciences, Heidelberg, Germany) and analyzed with FlowJo V10.0.8 software (TreeStar, Ashland, OR). Instrument voltage settings were kept the same throughout the study. Instrument

compensation was set up using fluorescence-labeled antibodies and Compbeads (BD Biosciences).

Protein measurement by multi-Array technology

Sputum supernatants as well as nasal secretions were stored at -80 °C for analysis of protein levels. Total amount of proteins in sputum supernatants and nasal secretions were determined using the Pierce™ Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific, USA) according to manufacturers instructions. Protein levels of Eotaxin-3, IFN- γ , IL-10, IL-13, IL-17, IL-1 α , IL-4, IL-5, IL-8, TNF- α , G-CSF, IL-22, IL-24, IL-33, POSTN, and TSLP in nasal secretions were detected in duplicates by the highly-sensitive MesoScaleDiscovery's (MSD) Multi-Array technology (MSD Mesoscale, USA) as described before.⁷ The lower limits of detection of the cytokines/chemokines are: CCL-26 (Eotaxin-3): 0.68pg/mL; IFN- γ : 0.33pg/mL; IL-10: 0.02pg/mL; IL-13: 0.32pg/mL; IL-17: 0.18pg/mL; IL-1 α : 0.26pg/mL; IL-4: 0.01pg/mL; IL-5: 0.18pg/mL; IL-8: 0.06pg/mL; TNF- α : 0.07pg/mL; G-CSF: 0.08pg/mL; IL-22: 51.50pg/mL; IL-24: 6.35pg/mL; IL-33: 0.53pg/mL; POSTN: 62.50pg/mL; TSLP: 0.04pg/mL.

Statistical analysis

All experimental procedures and analyses were conducted by blinded research staff. Data are included in parenthesis throughout the figures 1 and 2 as mean $\pm$ s.e.m. Non-parametric two-sided Wilcoxon-rank sum tests were used to test for significant differences comparing sequential data points of the same patient. Non-parametric Mann-Whitney U tests were used to determine significant differences between patient groups. In order to identify coherences between the secretion of asthma-related biomarkers in the upper and lower airways, we used Spearman's rank correlation coefficient analysis to compare protein abundances in nasal secretions and sputum

supernatants. A significance level of 5% was used. All statistical tests were performed two-sided.

Supplemental References

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Supplemental Figure E1.

Correlation of clinical scores (Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ), GINA, and Preceived Stress Questionnaire (PSQ)) to laboratory parameters and cytokines of the upper and lower airways. To calculate the relation between parameters, a two-sided Spearman's rank correlation was performed and Spearman's rank correlation coefficients (r) were depicted as forest plots with 95% confidence interval.

Supplemental Table E1. Patients characteristics

Two-sided Mann-Whitney T-tests were used to test for significant differences between patient groups.

Supplemental Table E2. Sputum cell differentiation.

Two-sided Mann-Whitney T-tests were used to test for significant differences between patient groups. Statistically significant differences were defined as p values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$. Asterisks indicate significantly differences to Healthy Controls, cross indicate significantly differences compared to asthmatics off season.

Supplemental Table E3. Correlation matrix.

* statistically significant differences were defined as p values below 0.05 (two-sided),

** statistically significant differences were defined as p values below 0.01 (two-sided).

Supplemental Figure 1

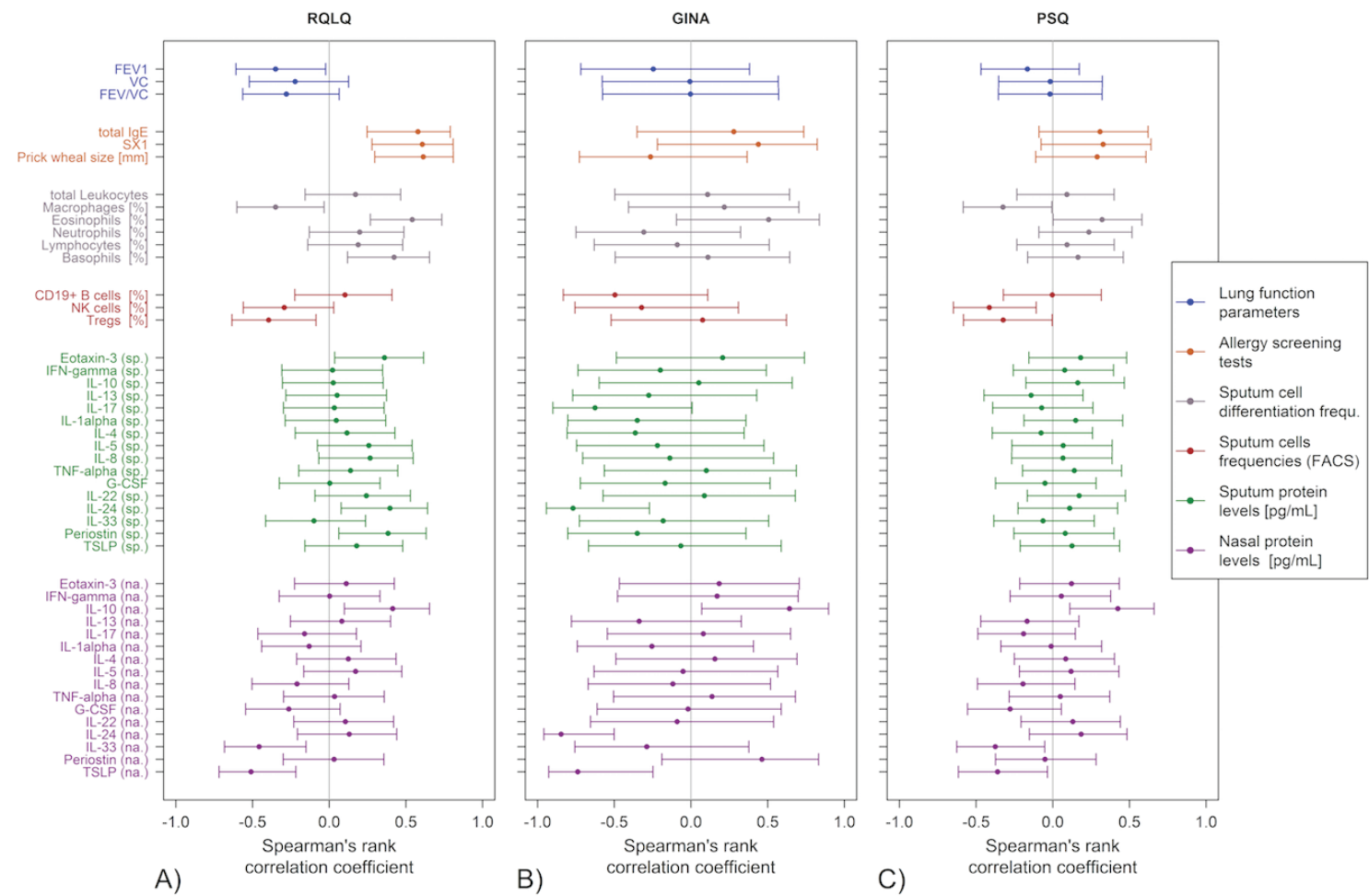


Table E1. Patients characteristics.

Characteristics	Healthy Controls (n=16)	Asthmatics (n=15)	P-value
Age	36.3 ± 3.2	39.0 ± 3.4	n.s.
Female/Male ratio	7 / 9	5 / 10	n.s.
BMI [kg/m ²]	24.05 ± 0.77	25.47 ± 0.91	n.s.
SIT-therapy	n.d.	6 of 15	
GINA-score	n.d.	1.64 ± 0.36	
Total IgE [kU/l]	54.51 ± 15.02	406.50 ± 102.69	0.0003
Sx1 [kUA/l]	2.03 ± 0.92	34.57 ± 7.88	< 0.0001
Birch prick [mm]	0.75 ± 0.49	6.07 ± 0.94	< 0.0001
Grass prick [mm]	0.62 ± 0.40	5.62 ± 0.93	< 0.0001
Sensitization (%)			
Grass	0	87.00	
Birch	6.00	74.00	
House dust mite	6.00	40.00	
Allergic Asthma (%)	0	87.00	

Two-sided Mann-Whitney T-tests were used to test for significant differences between patient groups.

Table E2. Sputum cell differentiation.

	Healthy Controls (n=16)	Astmatocs off season (n=13)	Asthmatics in season (n=14)
Total cells x10⁴/mL			
Mean (±SEM)	40.29 (±8.38)	54.04 (±6.56)	82.29 (±13.65)**
Makrophages [%]			
Mean (±SEM)	92.50 (±1.50)	81.00 (±3.09)**	75.43 (±4.42)**
Neutrophils [%]			
Mean (±SEM)	6.55 (±1.44)	10.92 (±2.42)	19.39 (±4.42)**
Eosinophils [%]			
Mean (±SEM)	0.41 (±0.15)	3.35 (±0.90)*	3.95 (±0.99)***
Basophils [%]			
Mean (±SEM)	0.04 (±0.04)	1.85 (±0.58)**	0.41 (±0.16)**
Lymphocytes [%]			
Mean (±SEM)	0.50 (±0.16)	0.79 (±0.29)	0.84 (±0.19)

Two-sided Mann-Whitney T-tests were used to test for significant differences between patient groups. Statistically significant differences were defined as p values * p<0.05, ** p<0.01, *** p<0.0001 in relation to Healthy Controls.