



Specificity and reproducibility of nasal biomarkers in patients with allergic rhinitis after allergen challenge chamber exposure

Philipp Badorrek, MD^{*,†}; Meike Müller, PhD^{*}; Wolfgang Koch, PhD^{*}; Jens M. Hohlfield, MD^{*,†}; Norbert Krug, MD^{*,†}

^{*} Fraunhofer Institute of Toxicology and Experimental Medicine, Hannover, Germany

[†] German Center for Lung Research, Biomedical Research in Endstage and Obstructive Lung Research, Hannover, Germany

ARTICLE INFO

Article history:

Received for publication November 16, 2016.

Received in revised form January 5, 2017.

Accepted for publication January 18, 2017.

ABSTRACT

Background: Allergic rhinitis is an inflammatory disease that causes cellular influx and mediator release in the nose. These inflammatory changes might be used as nasal biomarkers to assess the efficacy of novel anti-allergic treatments.

Objective: To assess the specificity and reproducibility of nasal biomarkers in patients with allergic rhinitis after grass pollen exposure in an allergen challenge chamber.

Methods: In a monocenter pilot study, 15 patients with allergic rhinitis and 19 healthy individuals underwent two 4-hour *Dactylis glomerata* pollen challenges in the challenge chamber with an interval of 21 days. Before challenge, on exit, and after 2 and 22 hours, a nasal lavage was performed and nasal secretions were collected on filter paper to determine a wide panel of cells and mediators. Furthermore, total nasal symptom score, nasal flow, and nasal nitric oxide were measured.

Results: Pollen exposure significantly increased eosinophil, interleukin (IL) 5, IL-6, IL-13, and macrophage inflammatory protein 1 β levels in allergic patients but not in healthy individuals. The effect could be reproduced for eosinophils, IL-5, IL-6, and macrophage inflammatory protein 1 β after the second allergen challenge. By contrast, the IL-13 levels were higher and eotaxin levels first increased after repetitive allergen challenge. There was no correlation between total nasal symptom score and elevated cell or cytokine levels. Nasal nitric oxide levels were nonspecifically elevated in both patients with allergy and healthy controls.

Conclusion: A subset of cellular and soluble biomarkers in nasal lavage and secretion reveals specificity and reproducibility in patients with allergic rhinitis. These can be used to measure the immunologic efficacy of antiallergic treatments in an allergen challenge chamber. Carryover effects attributable to priming must be considered when designing cross-over studies.

Trial Registration: clinicaltrials.gov Identifier: NCT00297843.

© 2017 American College of Allergy, Asthma & Immunology. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Allergic rhinitis is an inflammatory disease that causes an influx of inflammatory cells and the release of mediators, such as cytokines, chemokines, and growth factors, into the nasal mucosa and nasal secretions.^{1,2} Changes in the level of these cells and mediators might be used as biomarkers to assess the efficacy of novel anti-allergic treatments.

Reprints: Philipp Badorrek, MD, Fraunhofer Institute of Toxicology and Experimental Medicine, Feodor-Lynen-Str 15, 30625 Hannover, Germany; E-mail: philipp.badorrek@item.fraunhofer.de.

Disclosures: All authors are employees of the Fraunhofer Institute for Toxicology and Experimental Medicine.

Funding Sources: This study was supported by Fraunhofer Institute of Toxicology and Experimental Medicine, Hannover, Germany.

<http://dx.doi.org/10.1016/j.anai.2017.01.018>

1081-1206/© 2017 American College of Allergy, Asthma & Immunology. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Many authors have found that cytokine levels (eg, interleukin [IL] 4, granulocyte-macrophage colony-stimulating factor [GM-CSF], and IL-5) and the total nasal symptom score (TNSS) increase in nasal secretions after antigen provocation in patients with allergic rhinitis.^{3,4} Others have found an increase in eosinophils, IL-1 β , IL-4, IL-5, IL-6, IL-13, granulocyte colony-stimulating factor (G-CSF), GM-CSF, eotaxin, RANTES (regulated on activation, normal T cell expressed and secreted), monocyte chemoattractant protein (MCP) 1, and macrophage inflammatory protein (MIP) 1 α after allergen challenge or natural in-season exposure in the nasal lavage of patients with allergic rhinitis.^{1,2} Local biomarkers have only more recently been examined with increased interest,⁵ and only limited information is available on the specificity of nasal biomarkers from studies with rhinitic and healthy participants.⁶ Furthermore, only a few studies have investigated the reproducibility of these changes.² Moreover, the allergen provocations were performed using allergen

extracts or allergen extract–soaked discs at doses expected to result in a relevant TNSS response (ie, a setting that does not reflect the exposure under natural conditions). Therefore, only limited data are available to date that demonstrate the specificity and reproducibility of nasal biomarkers after allergen provocation under more natural conditions in patients with allergic rhinitis.

The aim of our study was to determine the specificity and reproducibility of nasal biomarkers after pollen provocation in an allergen challenge chamber (ACC). The ACC is a well-established method to expose allergic participants to defined, stable airborne concentrations of pollen under defined conditions that mimic the natural exposure situation outside in a meadow on a summer day.^{7–9} In our study, the grass pollen atmosphere is created by using a validated pollen generation apparatus. The chamber is ventilated with high-efficiency particulate air–filtered air, and the air flow through the chamber is 1,500 m³/h. The pollen concentration in the chamber is continuously measured via a laser particle counter and discontinuously by taking 2 samples (1 sample after half of the challenge duration and 1 sample after the challenge is completed) on a pollen sampler and counting the pollens under the microscope.⁷ To also assess reproducibility, we challenged study participants with seasonal allergic rhinitis and healthy control participants in a 2-part repeated-measurement design in the Fraunhofer ACC and determined changes in nasal symptoms, nasal flow, cells in nasal lavage, 27 different mediators in nasal secretions, and nasal nitric oxide.

Methods

Study Participants and Study Design

This was a single-center pilot study that was conducted in a 2-part repeated-measurement design to also assess reproducibility. Fifteen participants with allergic rhinitis and 19 healthy participants (Table 1) underwent two 4-hour exposures to *Dactylis glomerata* grass pollen (4,000 pollen/m³; Biopol Laboratory Inc, Spokane, Washington) in the Fraunhofer ACC in the months of March and April (ie, before the local grass pollen season). A concentration of 4,000 grains/m³ is equivalent to the concentration of grass pollen in a meadow on a summer day in Hannover, Germany (Badorrek et al, unpublished data, 2004). Another research group found that a similar mean (SD) concentration of 3,500 (500) grains/m³ is an optimal concentration to produce symptoms similar to those experienced in the outdoor environment.¹⁰ The concentration of pollen in the ACC is closely monitored and stable over the complete duration of the challenges.⁷ The interval between the 2 challenges was 21 days. Inclusion criteria were smoking or nonsmoking with a history of less than 10 pack-years and forced expiratory volume in 1 second greater than 80% predicted. For patients with allergic rhinitis, inclusion criteria were a known history of intermittent allergic rhinitis attributable to grass pollen confirmed by a positive skin prick test

result to *Dactylis glomerata* within 12 months of enrollment if the skin efflorescence was at least 3 mm in diameter, TNSS of at least 6 during a 4-hour screening exposure in the ACC, and asymptomatic or with mild symptoms (TNSS <3 and a score <2 for each individual symptom) at the time of inclusion and before each allergen challenge. For nonallergic individuals, inclusion criteria were no history of allergic rhinitis and a negative skin prick test result to 15 major aeroallergens, including grass, rye, birch, hazel, house dust mite, *Alternaria*, cat, and dog. Exclusion criteria were structural nasal defect, nasal polyps, infection of the upper airways within 4 weeks of study entry, sinusitis, asthma (except mild intermittent asthma using β_2 -agonists only), pregnancy, breastfeeding, current immunotherapy, known hypersensitivity, and participation in another clinical trial within 30 days of study enrollment. Washout periods for drugs before screening were 4 weeks for inhaled and 8 weeks for oral, intravenous, or dermal steroids and 1 week for cromolyn, antihistamines, or any other antirhinitis or hay fever medication.

Before study participation, each participant gave written informed consent after having been informed orally and in writing. The consent obtained from the participants also included consent for publication and reporting data of the participants. The study was approved by the ethics committee of the Medical School of Hannover and performed in accordance with both the Declaration of Helsinki (Somerset West Amendment, 1996) and the ICH Guideline on Good Clinical Practice (Note for Guidance on Good Clinical Practice, January 17, 1997).

Assessment of TNSS

Nasal symptoms (obstruction, rhinorrhea, sneezing, and itching) were self-assessed before, every 30 minutes throughout the challenge in the ACC, and 2 and 22 hours after allergen exposure. For each symptom, the participant recorded scores on diary sheets according to the following 4-point severity scale: 0 indicating none; 1, mild; 2, moderate; and 3, severe (see Table 2 for the severity scales of the 4 domains of nasal congestion or obstruction, rhinorrhea, nasal itching, and sneezing). The TNSS was calculated by adding the scores of the 4 individual nasal symptoms. The last 5

Table 1
Characteristics of the Study Participants

Characteristic	Total participants	Patients with allergic rhinitis	Healthy participants
Sex, No. (%)			
Male	17 (50)	7 (21)	10 (29)
Female	17 (50)	8 (24)	9 (27)
Age, y			
Mean (SD)	28 (5)	29 (5)	28 (4)
Range	22–40	22–40	22–29
Body weight, kg			
Mean (SD)	76 (13)	75 (15)	77 (12)
Range	52–113	53–113	52–94

Table 2
Severity Scales of the 4 Study Domains

Domain	Score	Definition
Nasal congestion or obstruction	0	None
	1	Mild (slight congestion, intermittent, easily tolerated)
	2	Moderate (continuous congestion that is bothersome but tolerable)
	3	Severe (nose completely blocked, hard to breathe through the nose)
Rhinorrhea	0	None
	1	Mild (intermittent rhinorrhea, easily tolerated)
	2	Moderate (continuous rhinorrhea that is bothersome but tolerable)
	3	Severe (continuous rhinorrhea that is hard to tolerate)
Nasal itching	0	None
	1	Mild (intermittent itching, easily tolerated)
	2	Moderate (continuous itch that is bothersome but tolerable)
	3	Severe (continuous itch requiring rubbing and that is hard to tolerate)
Sneezing	0	None
	1	Mild (intermittent sneezing, easily tolerated)
	2	Moderate (occasional sneezing that is bothersome but tolerable)
	3	Severe (frequent sneezing that is hard to tolerate)

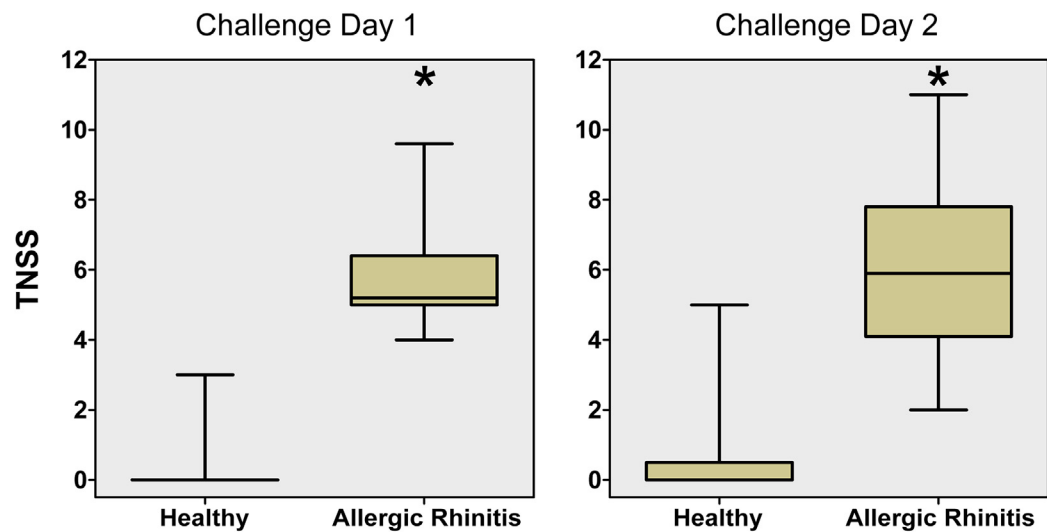


Figure 1. Mean total nasal symptom score (TNSS) of the last 5 measurements during challenge in healthy participants and patients with allergic rhinitis on challenge day 1 (left) and challenge day 2 (right). The data are displayed in a box-and-whiskers graph. The box extends from the 25th percentile to the 75th percentile, with a line at the median. The whiskers extend to the highest and lowest value. Asterisk indicates a significant ($P < .001$) difference between healthy participants and patients with allergic rhinitis.

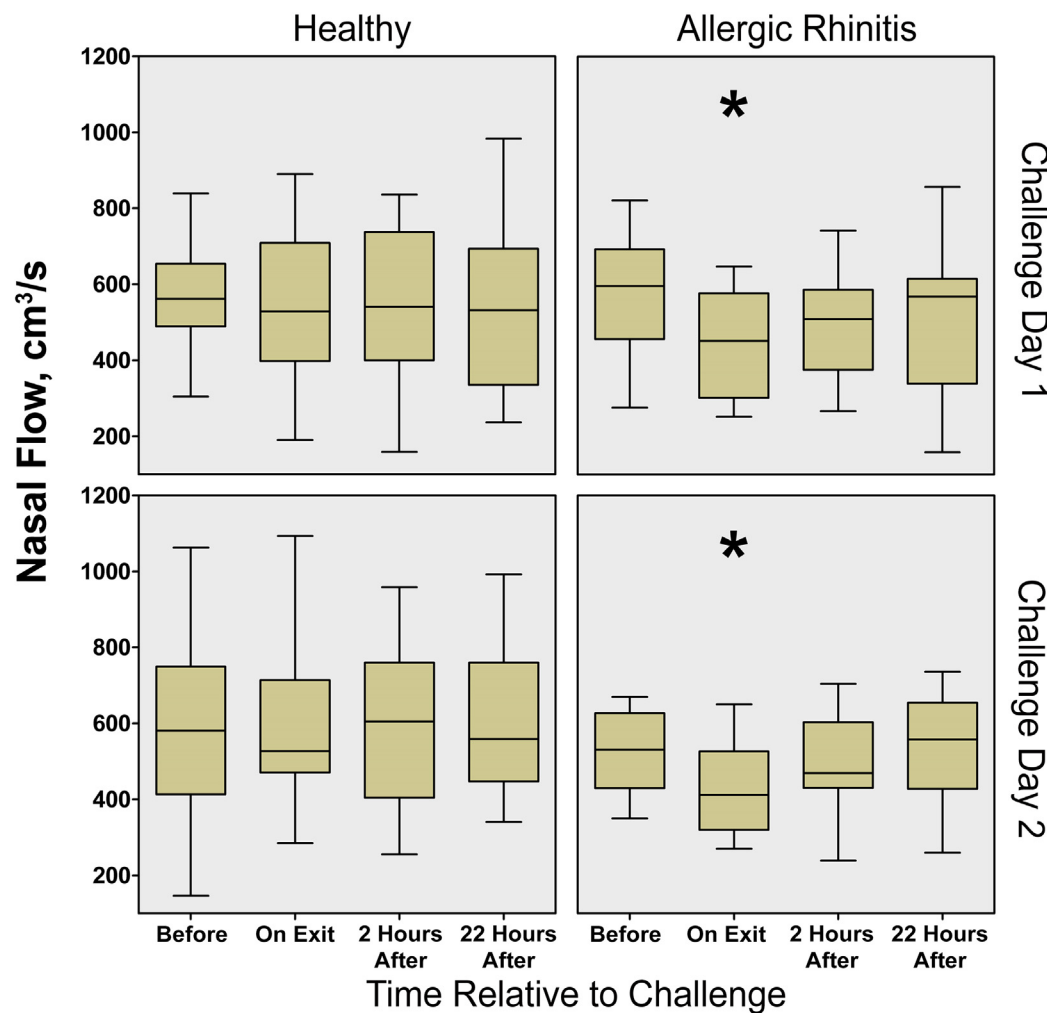


Figure 2. Nasal flow measured by rhinomanometry in healthy participants (left) and patients with allergic rhinitis (right) for challenge day 1 (top) and challenge day 2 (bottom). The data are displayed in a box-and-whiskers graph (see legend for Fig 1). Asterisk indicates a significant decrease ($P < .05$) compared with baseline (before).

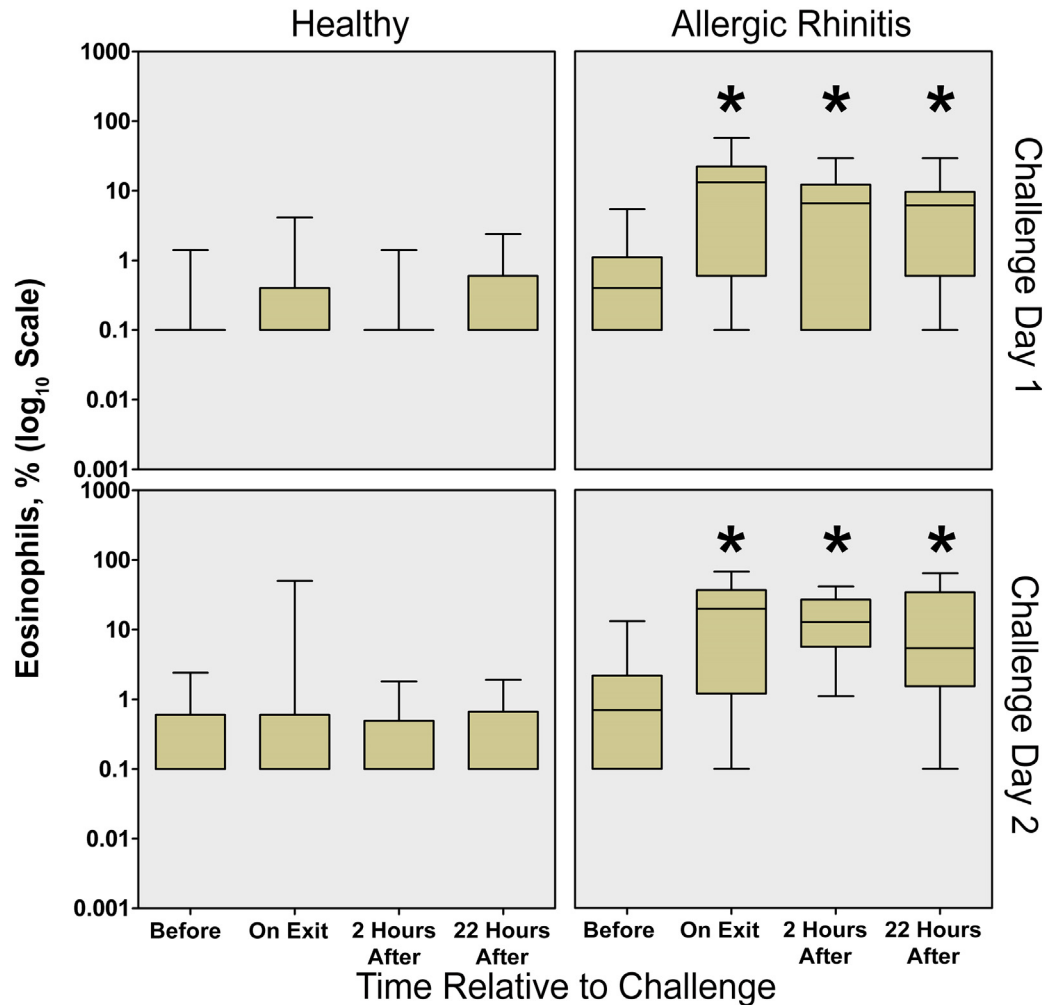


Figure 3. Relative percentage of eosinophils in healthy participants (left) and patients with allergic rhinitis (right) for challenge day 1 (top) and challenge day 2 (bottom). The data are displayed in a box-and-whiskers graph (see legend for Fig 1). Asterisk indicates a significant increase ($P < .05$) compared with baseline (before). Reproduced with permission from Krug et al¹² (copyright 2012 Wiley).

assessments during the 4-hour challenge were used to determine the mean TNSS.

Rhinomanometry

The nasal flow rate was measured using rhinomanometry (Viasys Healthcare, Höchberg, Germany) before allergen exposure, on exit from the ACC, and 2 and 22 hours after challenge. An active anterior measuring technique using a face mask was applied. Inspiratory flow rates at 150 Pa were obtained separately for the right and left nostrils, and the sum of the flow rates of both nostrils was then calculated.

Nasal Lavage and Collection of Nasal Secretion

Nasal lavages were conducted before allergen exposure, on exit from the ACC, and 2 and 22 hours after the challenge. The nasal lavages were performed with prewarmed, sterile, physiologic saline solution on the participants' left nostril using a syringe. A nasal olive was attached to the prefilled syringe and placed into the nasal opening to prevent leakage of lavage fluid. The participants were seated in a forward flexed neck position to prevent fluid from reaching the nasopharynx. Before the challenge, both nostrils were rinsed with 6 mL of saline, and the resulting fluids were discarded. For lavages, 6 mL of saline solution was flushed back and forth 10 times into the nasal cavity during a 1-minute period. The fluid was

centrifuged (10 minutes, 400g, 4°C) and the cells collected for further analysis.

To collect nasal secretions, 2 strips of filter paper (Whatman No. 42; Whatman Paper Ltd, Maidstone, United Kingdom) were placed into the right nostril on either side of the nasal cavity before the lavages (modified method described in the article by Alam et al¹¹) and left in the nostril for 2 minutes to absorb nasal secretions. To assess the amount of nasal secretion, the strips were weighed before and after this procedure. To elute the soluble molecules, the filter paper was incubated in 500 μ L of elution buffer (phosphate-buffered saline + 1% bovine serum albumin + 0.05% Tween-20 + 0.05% sodium acid) under gentle agitation on a roller for 15 minutes at room temperature. Subsequently, the filter was centrifuged (10 minutes, 400g, 4°C), and 75- μ L aliquots were stored at -80°C until measurement.

Determination of Cells and Cytokines

Cell count was determined in a Neubauer hemocytometer. Cytospin slides were produced from cell suspensions and stained with Diff-Quik stain (Dade-Behring, Deerfield, Illinois), differentiated by counting 400 leukocytes on the cytospin. Furthermore, the concentrations of 27 mediators (IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, IL-1 receptor antagonist [IL-1ra], IL-1 β , RANTES, MCP-1, MIP-1 α ,

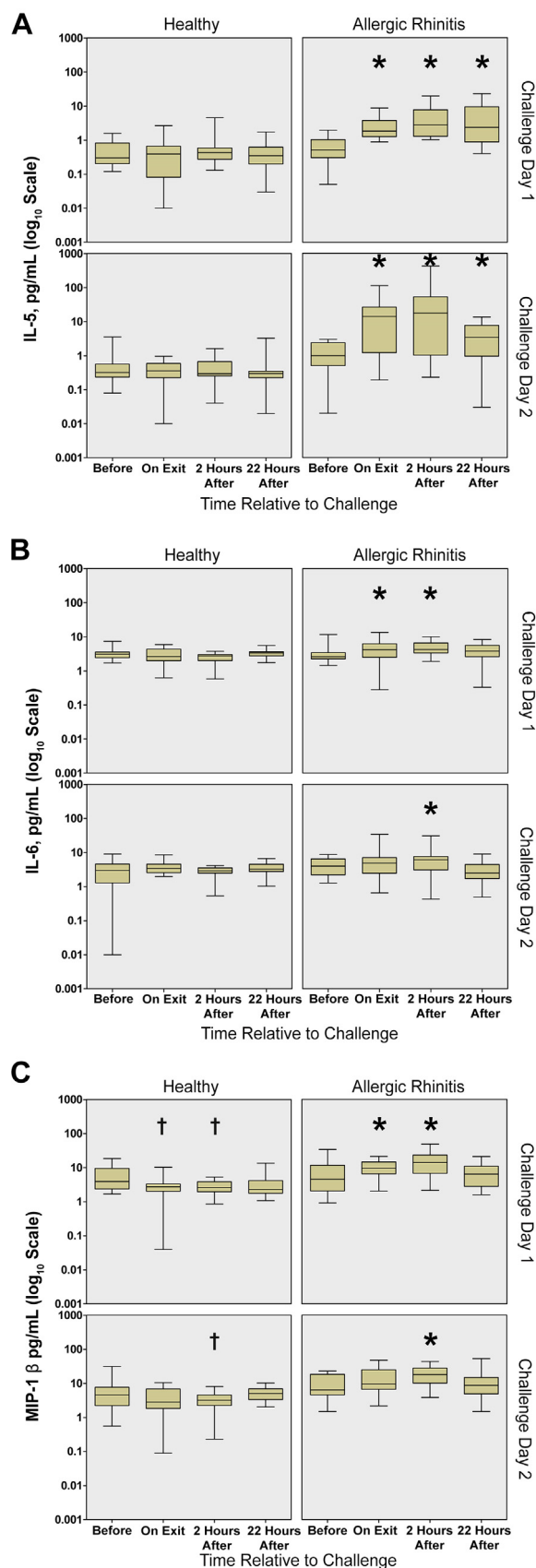


Figure 4. Interleukin (IL) 5 (A), IL-6 (B), and macrophage inflammatory protein (MIP) 1β (C) levels in healthy participants (left) and patients with allergic rhinitis (right) for challenge day 1 (top) and day 2 (bottom). Cytokines were measured in nasal secretion of patients with allergic rhinitis and healthy participants at the times

MIP-1β, platelet-derived growth factor [PDGF], inducible protein ([P]-10, fibroblast growth factor [FGF], GM-CSF, G-CSF, tumor necrosis factor [TNF]-α, interferon [IFN]-γ, and vascular endothelia growth factor [VEGF]) were determined simultaneously with a multiplex assay kit (Lincoplex, Linco Research Inc, St Charles, Missouri) containing premixed antibody-coated microsphere beads using a Luminex100 (Luminex Corporation, Hercules, California) according to the manufacturer's recommendations. The detection limits for the cytokine assays were IL-6, MIP-1β: 0.14 pg/mL; IL-4, IL-2: 0.13 pg/mL; IL-8, IFN-γ, IL-9, PDGF, RANTES: 0.17 pg/mL; IL-10, IL-5, IL-7: 0.26 pg/mL; GM-CSF: 0.11 pg/mL; TNF-α: 0.54 pg/mL; IL-1β: 0.23 pg/mL; IL-12, IL-17: 0.27 pg/mL; IL-13: 0.07 pg/mL; G-CSF: 0.28 pg/mL; MCP-1, VEGF: 0.20 pg/mL; IL-1ra: 0.24 pg/mL; IL-15: 0.22 pg/mL; eotaxin: 0.08 pg/mL; FGF: 0.16 pg/mL; and MIP-1α: 0.12 pg/mL.

Nasal Nitric Oxide

Nasal nitric oxide was measured with a stationary, chemiluminescence-based nitric oxide analyzer (Niox apparatus) according to the manufacturer's instructions before the challenge, on exit from the ACC, and 2 and 22 hours after allergen exposure. Nasal nitric oxide was recorded 3 times per nostril, and every measurement was assessed for acceptance. Measurements were accepted when there was a logistic growth of the curve with a stable plateau. The mean plateau of acceptable curves was reported.

Statistical Analysis

Comparisons of variables before and after challenge were performed separately for each group and challenge day by applying the paired *t* test. The unpaired *t* test was used to compare the groups with respect to their mean changes (Δ) from before to after challenge time points. If necessary, variables were log₁₀ transformed before analysis to yield normal distributions. To check for reproducibility and to control the multiple α error at 5 %, a linear mixed model analysis of variance was applied with the Δ values of each parameter as dependent variable and group, challenge day, time point, and group by challenge day interaction as fixed effects and the intercept of each participant as a random effect.

In a secondary analysis, the correlation between TNSS and a subset of cells and cytokines that in the primary analysis were found to be specific for patients with allergic rhinitis was calculated using the Spearman correlation coefficient. The sample size in this pilot study was not calculated a priori but decided on by feasibility.

Results

Safety and Analysis Set

Fifteen participants with intermittent allergic rhinitis and 19 healthy participants (Table 1) were exposed to grass pollen for 4 hours in the Fraunhofer ACC on 2 different days. Two participants discontinued participation before the end of the study because of adverse events, an upper respiratory tract infection in 1 participant and symptoms of a common cold in 1 participant. None of the participants had serious adverse events. The data from the intention-to-treat group (including data from the first challenge of the 2 participants who discontinued the study) were included in the analysis.

shown in the graphs. The data are displayed in a box-and-whiskers graph (see legend for Fig 1). Asterisk indicates a significant ($P < .05$) increase and dagger indicates a significant ($P < .05$) decrease compared with baseline (before).

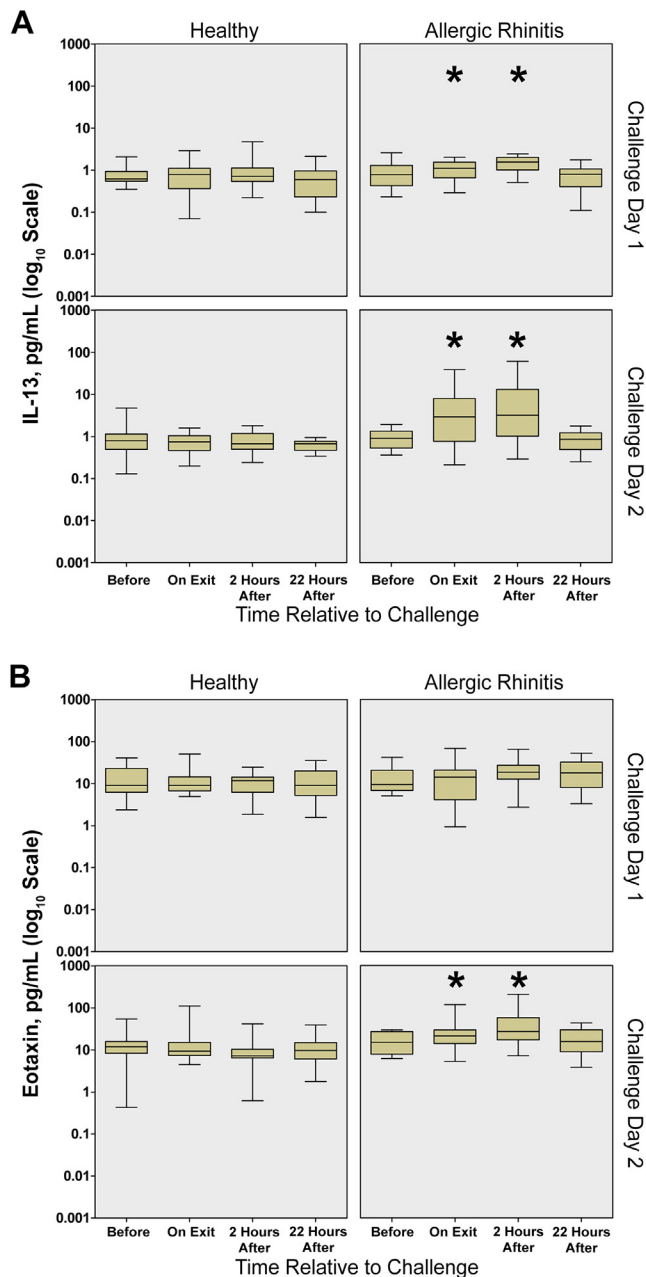


Figure 5. Interleukin (IL) 13 (A) and eotaxin (B) were measured in nasal secretion-soaked filter papers of patients with allergic rhinitis and healthy participants at the times shown in the graphs. The data are displayed in a box-and-whiskers graph (see legend for Fig 1). Asterisk indicates a significant ($P < .05$) increase in patients with allergic rhinitis.

TNSS and Nasal Flow

The TNSS increased in patients with allergic rhinitis but not in healthy participants ($P < .001$) (Fig 1). Correspondingly, nasal flow reproducibly decreased in patients with allergic rhinitis on both challenge days but not in healthy participants ($P < .05$) (Fig 2). For both parameters, there was excellent reproducibility and no difference between the 2 challenge days ($P > .10$).

Increased Eosinophil Cell Count in Patients With Allergic Rhinitis

Eosinophils, neutrophils, lymphocytes, and monocytes or macrophages were determined in the nasal lavage of patients with allergic rhinitis and healthy participants. Figure 3 shows a

significant increase in the relative number of eosinophils at all assessments after pollen exposure in patients with allergic rhinitis compared with baseline values on both challenge days. No difference was seen between the 2 days ($P = .10$). The absolute number of eosinophils was increased significantly both directly after and 2 hours after the challenge on both challenge days, with no difference between the 2 challenge days ($P = .19$). There was no difference in the number of cells before or after challenge in healthy participants on both challenge days. The cell counts of the other cells measured (neutrophils, lymphocytes, and monocytes or macrophages) did not change after pollen exposure in the 2 groups (ie, patients with allergic rhinitis and healthy participants) when comparing challenge day 1 and challenge day 2.

Increased IL-5, IL-6, MIP-1 β , IL-13, and Eotaxin Levels in Patients With Allergic Rhinitis

In secretion from filter papers, IL-5 (Fig 4A), IL-6 (Fig 4B), and MIP-1 β (Fig 4C) levels were significantly increased after pollen exposure in patients with allergic rhinitis on both challenge days but not in healthy participants. In the case of IL-5, this difference was still present at 22 hours after challenge, unlike IL-6 and MIP-1 β , for which the effect was no longer observed 22 hours after exposure. A significant difference between both days was not observed for IL-5, IL-6, or MIP-1 β ($P = .32$, $P = .43$, and $P = .54$, respectively). Healthy participants had significantly decreased MIP-1 β levels after pollen exposure ($P < .05$) (Fig 4C).

The levels of IL-13 in the allergic rhinitis group were significantly increased after pollen challenge on both days (Fig 5A) compared with baseline, whereas they did not increase in healthy participants. For eotaxin, this was true for the second challenge day (Fig 5B). In contrast to IL-5, IL-6, and MIP-1 β , the levels of IL-13 and eotaxin were higher on the second challenge day compared with the first ($P = .006$ and $P = .03$, respectively). The remaining cytokines that were tested (IL-2, IL-4, IL-7, IL-8, IL-9, IL-10, IL-12, IL-15, IL-17, IL-1 α , IL-1 β , RANTES, MCP-1, MIP-1 α , PDGF, IP10, FGF, GM-CSF, G-CSF, TNF- α , IFN- γ , and VEGF) did not increase after allergen challenge in both groups.

Correlation Between Nasal Biomarkers and TNSS

The correlation between TNSS and relative eosinophils, absolute eosinophils, IL-5, IL-6, IL-13, and MIP-1 β was determined for all 3 relevant time points (on exit from ACC, 2 hours after ACC, and 22 hours after ACC) in both periods. No correlation could be observed for relative eosinophils, absolute eosinophils, IL-5, IL-6, and IL-13 ($P > .05$). MIP-1 β revealed a significant negative correlation for the time points 2 hours after challenge in period 1 and on exit from the ACC in period 2 ($P < .05$). For all other time points, there was no correlation between MIP-1 β and TNSS ($P > .05$).

Increased Nasal Nitric Oxide in Patients With Allergic Rhinitis and Controls

In participants with allergic rhinitis, nasal nitric oxide significantly and reproducibly decreased directly after pollen exposure (Fig 6) and increased 22 hours after the challenge. However, in healthy participants, nitric oxide levels increased directly (first challenge day) and also at 22 hours after the challenge (second challenge day).

Discussion

The aim of this pilot study was to investigate the specificity and reproducibility of nasal biomarkers in patients with seasonal allergic rhinitis and healthy control participants after 2 separate controlled grass pollen exposures in an ACC. Nasal lavages were used to determine cellular changes, whereas the filter paper

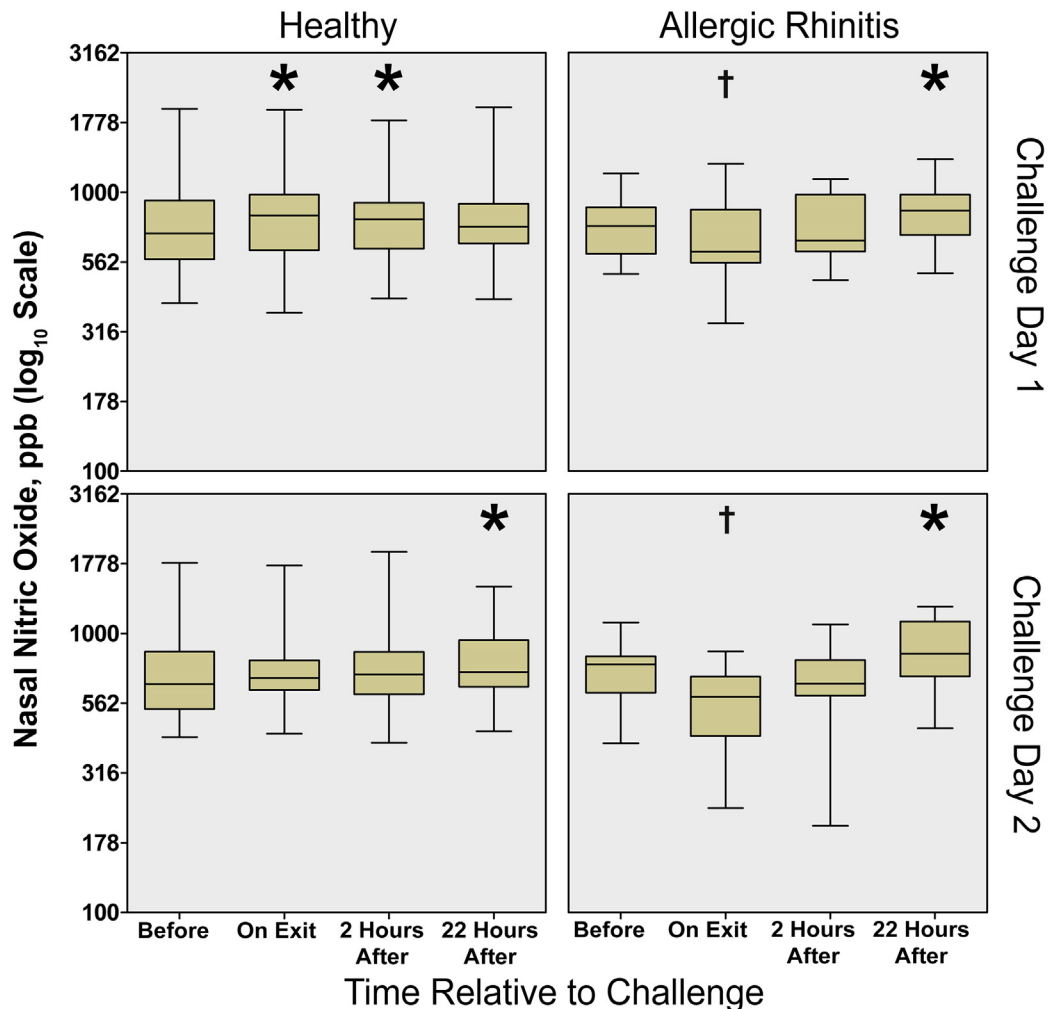


Figure 6. Nasal nitric oxide measurements in healthy participants (left) and patients with allergic rhinitis (right) for challenge day 1 (top) and challenge day 2 (bottom). The data are displayed in a box-and-whiskers graph (see legend for Fig 1). Asterisk indicates a significant increase ($P < .05$) and dagger indicates a significant decrease ($P < .05$) compared with baseline (before).

method was used to collect nasal secretions, which are less diluted. This method enabled the detection of protein signals that are below the detection limits of nasal lavages.¹³ Furthermore, nasal nitric oxide was determined together with clinical assessments. All participants tolerated the procedures well on both challenge days.

Eosinophils were the only leukocytes to specifically increase in nasal lavages from allergic participants on both challenge days. Furthermore, cytokine levels of IL-5, IL-6, IL-13, and MIP-1 β specifically increased in allergic participants. These cytokines are specific for the allergic and proinflammatory reactions that occur in the participants after challenge. Thiele et al¹⁴ also described a specific increase in eosinophils after nasal allergen challenges in participants with allergic rhinitis compared with healthy controls. An increase of IL-1 β , IL-5, MIP-1 β , and IL-13 was observed by Leaker et al¹⁵ in patients with allergic rhinitis after nasal allergen challenge by means of a grass pollen nasal spray without information regarding healthy control participants. König et al¹ found elevated levels of MCP-1, MIP-1 β , IL-5, eotaxin, MIP-1 α , RANTES, and IL-17 and decreased levels of IFN- γ , IL-12, and IL-10 in participants with seasonal allergic rhinitis, whereas values of GM-CSF, G-CSF, IL-1 β , and IL-6 did not differ from the healthy controls. Interestingly, reduced levels of IL-13 were observed in participants with seasonal allergic rhinitis; however, methodologic reasons, such as omission of a prior nasal allergen challenge in a number of participants, could not be ruled out.

Regarding reproducibility, we found a reproducible increase for eosinophils, IL-5, IL-6, and MIP-1 β , whereas levels for IL-13 were higher on the repeated challenge. This was surprising for us because we had assumed a 21-day washout period would be sufficient to prevent any carryover effects. Thiele et al² also found a reproducible upregulation of IL-5, IL-6, IL-13, MCP-1, and MIP-1 β on 2 nasal allergen challenge tests performed at an interval of 21 days, with the cytokine levels appearing slightly lower for the second nasal allergen challenge test. Eotaxin was specifically elevated only after the second challenge in our study, whereas König et al¹ observed this increase already after a single challenge. These data suggest that for some cytokines, a repetitive allergen stimulation, or so-called priming, is necessary to increase their level. Moreover, it is most likely that the local nasal allergen concentration plays a major role because the grass pollen nasal spray used in the study of Leaker et al¹⁵ may have released more allergen compared with the situation in the ACC. Given this lack in reproducibility and the carryover effect for some cytokines in repetitive challenges, the washout period between 2 challenges must be carefully selected in crossover study designs.

Unlike other studies,² our study did not find a correlation between nasal symptoms (TNSSs) and cells and cytokines that increase specifically in patients with allergic rhinitis. From our results, we conclude that nasal biomarker assessments cannot be used to estimate nasal symptoms. This is especially important

when nasal biomarkers are used to test the efficacy of novel anti-allergic treatments because a therapeutic decrease in nasal biomarkers does not necessarily lead to a decrease of nasal symptoms.

Our data on nasal nitric oxide confirms own previous findings¹⁶ and other reports^{17,18} that nasal nitric oxide is a poor outcome measure for the differentiation between allergic patients and healthy individuals, whereas others regard nitric oxide as a useful surrogate marker of rhinitis.^{19,20} Nasal nitric oxide increased in both populations 22 hours after challenge. However, we observed a specific and reproducible decrease in nasal nitric oxide directly after the allergen challenge in patients with allergic rhinitis, whereas this was not observed in healthy participants. This effect has been previously described^{4,21} and is probably attributable to the swelling of the nasal mucosa that hampers the release of nitric oxide from the paranasal sinuses.²² After a period of 22 hours, when the nose is no longer congested, the increase in nitric oxide is measurable.

In conclusion, a subset of cellular and soluble biomarkers in nasal lavage and secretion, but not in nasal nitric oxide, has specificity and reproducibility in patients with allergic rhinitis. These factors can be used as immunologic biomarkers to determine immunologic efficacy of antiallergic treatments. However, it is unclear how an immunologic effect correlates with effects related to nasal symptoms. Carryover effects attributable to priming must be considered when designing crossover studies with allergen challenge chambers.

Acknowledgments

Birgit Luettig at the Fraunhofer Institute of Toxicology and Experimental Medicine provided editorial support in the development of the manuscript draft. Hartmut Hecker at the Hannover Medical School provided the statistical support for this project.

References

- [1] König K, Klemens C, Eder K, et al. Cytokine profiles in nasal fluid of patients with seasonal or persistent allergic rhinitis. *Allergy Asthma Clin Immunol*. 2015;11:26.
- [2] Thiele J, Soliman M, Steacy LM, Adams D, Ellis AK. The Allergic Rhinitis Clinical Investigator Collaborative (AR-CIC): cytokine analysis of nasal secretions before and after nasal allergen challenges (NAC). *J Allergy Clin Immunol*. 2016;137:AB165.
- [3] Krug N, Gupta A, Badorrek P, et al. Efficacy of the oral chemoattractant receptor homologous molecule on TH2 cells antagonist BI 671800 in patients with seasonal allergic rhinitis. *J Allergy Clin Immunol*. 2014;133:414–419.
- [4] Serrano CD, Valero A, Bartra J, et al. Nasal and bronchial inflammation after nasal allergen challenge: assessment using noninvasive methods. *J Investig Allergol Clin Immunol*. 2012;22:351–356.
- [5] Zissler UM, Esser-von Bieren J, Jakwerth CA, Chaker AM, Schmidt-Weber CB. Current and future biomarkers in allergic asthma. *Allergy*. 2016;71:475–494.
- [6] Terada N, Hamano N, Kim WJ, et al. The kinetics of allergen-induced eosinophil level in nasal lavage fluid: its key role in eosinophil recruitment in nasal mucosa. *Am J Respir Crit Care Med*. 2001;164:575–579.
- [7] Krug N, Hohlfeld JM, Larbig M, et al. Validation of an environmental exposure unit for controlled human inhalation studies with grass pollen in patients with seasonal allergic rhinitis. *Clin Exp Allergy*. 2003;33:1667–1674.
- [8] Krzych-Falta E, Sowa J, Wojas O, Piekarska B, Sybilski A, Samoliński B. Allergen Challenge Chamber: an innovative solution in allergic rhinitis diagnosis. *Postep Derm Alergol*. 2015;32:421–425.
- [9] North ML, Soliman M, Walker T, et al. Controlled allergen challenge facilities and their unique contributions to allergic rhinitis research. *Curr Allergy Asthma Rep*. 2015;15:11.
- [10] Ellis AK, Steacy LM, Hobsbawn B, Conway CE, Walker TJB. Clinical validation of controlled grass pollen challenge in the Environmental Exposure Unit (EEU). *Allergy Asthma Clin Immunol*. 2015;11:5.
- [11] Alam R, Sim TC, Hilsmeier K, Grant JA. Development of a new technique for recovery of cytokines from inflammatory sites in situ. *J Immunol Methods*. 1992;155:25–29.
- [12] Krug N, Badorrek P, Hohlfeld JM. Experience with an allergen challenge chamber for clinical trials in allergic rhinitis. *Clin Exp Allergy*. 2012;12:14–19.
- [13] Erin EM, Zacharasiewicz AS, Nicholson GC, et al. Topical corticosteroid inhibits interleukin-4, -5 and -13 in nasal secretions following allergen challenge. *Clin Exp Allergy*. 2005;35:1608–1614.
- [14] Thiele J, Adams DE, Soliman M, Steacy LM, Ellis AK. Nasal allergen challenge (NAC) induced eosinophilia: the Allergic Rhinitis Clinical Investigator Collaborative (AR-CIC). *J Allergy Clin Immunol*. 2015;135:AB223.
- [15] Leaker BR, Malkov VA, Mogg R, et al. The nasal mucosal late allergic reaction to grass pollen involves type 2 inflammation (IL-5 and IL-13), the inflammasome (IL-1 β), and complement [published online September 28, 2016]. *Mucosal Immunol*. <http://dx.doi.org/10.1038/mi.2016.74>.
- [16] Hohlfeld JM, Holland-Letz T, Larbig M, et al. Diagnostic value of outcome measures following allergen exposure in an environmental challenge chamber compared with natural conditions. *Clin Exp Allergy*. 2010;40:998–1006.
- [17] Leaker BR, Scadding G, Jones CR, Barnes PJ. Using magnetic resonance imaging to quantify the inflammatory response following allergen challenge in allergic rhinitis. *Immun Inflamm Dis*. 2015;3:445–454.
- [18] Tworek D, Kuna P. Nasal nitric oxide measurements in the assessment of nasal allergen challenge. *J Investig Allergol Clin Immunol*. 2012;22:102–108.
- [19] Gupta N, Goel N, Kumar R. Correlation of exhaled nitric oxide, nasal nitric oxide and atopic status: a cross-sectional study in bronchial asthma and allergic rhinitis. *Lung India*. 2014;31:342–347.
- [20] Nesic VS, Djordjevic VZ, Tomic-Spiric V, Dudvarski ZR, Soldatovic IA, Arsovic NA. Measuring nasal nitric oxide in allergic rhinitis patients. *J Laryngol Otol*. 2016;130:1064–1071.
- [21] Okuma Y, Okamoto Y, Yonekura S, et al. Persistent nasal symptoms and mediator release after continuous pollen exposure in an environmental challenge chamber. *Ann Allergy Asthma Immunol*. 2016;117:150–157.
- [22] Suojalehto H, Vehmas T, Lindström I, et al. Nasal nitric oxide is dependent on sinus obstruction in allergic rhinitis. *Laryngoscope*. 2014;124:E213–E218.