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Non-invasive sampling methods of inflammatory biomarkers in asthma and allergic rhinitis

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Applicability and Reproducibility of Biomarkers for the Evaluation of Anti-Inflammatory Therapy in Allergic Rhinitis

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Abstract

Background: We aimed to study the reproducibility of several biomarkers of allergic rhinitis to investigate their potential as outcome measures in clinical intervention trials. Furthermore, we investigated the kinetics of the biomarkers studied in nasal lavage and brush material following a placebo-controlled nasal allergen challenge.

Methods: We performed a skin prick test and measured serum specific immunoglobulin (Ig) E levels and inflammatory biomarkers in nasal lavage and brush material in 20 patients with allergic rhinitis on 2 separate days (washout, 14-21 days). The patients were then randomly assigned to undergo an intranasal challenge with a relevant allergen ($n = 10$) or diluent ($n = 10$) in order to assess the kinetics of several biomarkers of allergic airway inflammation in nasal lavage and brush samples.

Results: Baseline serum IgE levels and skin wheal sizes were highly reproducible measurements, with a coefficient of variation (CV) of 13.4% and 18.2%, respectively. This was not the case with the majority of inflammatory biomarkers, whose CV varied considerably (range, 6.1%-224.1%). The nasal allergen challenge induced an increase in composite symptom scores in all patients. Compared to placebo, tryptase ($p = .004$), eosinophilic cationic protein (ECP) ($p = .03$) and α 2-macroglobulin ($p = .002$) were increased in nasal lavage at 20 minutes post-allergen. Nasal lavage ECP levels and nasal brush eosinophils were still significantly increased at 7 hours ($p = .03$ and $p = .04$), but all statistical significance had been lost at 24 hours post challenge.

Conclusion: Serum specific IgE assays and skin prick tests exhibited good reproducibility in patients with clinically stable allergic rhinitis. We were also able to investigate the kinetics of allergen-induced upper airway inflammatory markers in nasal lavage and brush material. Hence, nasal allergen challenge, when used in combination with nasal lavage and brush sampling, is a suitable research tool for early drug development.

Introduction

Allergic rhinitis is a chronic inflammatory disorder of the upper airways that produces characteristic signs and symptoms in sensitized individuals exposed to relevant allergens (1). Allergic airway inflammation is characterized by immunoglobulin (Ig) E-triggered mast cells and activated eosinophils, which release proinflammatory mediators such as histamine and leukotrienes (2). Furthermore, allergic rhinitis is associated with elevated serum IgE levels and a positive skin prick test (SPT) to corresponding allergens (1, 3). In addition to their diagnostic value, serum specific IgE levels and SPT results may serve as biomarkers to monitor disease activity in response to anti-allergic therapy (4). In allergic rhinitis, interventional studies with antihistamines and immunotherapy have shown decreases in allergic symptoms and reduced wheal and flare responses to intradermal allergens (5, 6). Similarly, in patients with allergic rhinitis and asthma, treatment with the anti-IgE antibody omalizumab substantially lowered free serum IgE levels with subsequent improvements in symptoms (7, 8).

Nasal allergen challenge is a validated, reproducible clinical model for investigating the pathophysiology of allergic rhinitis that also permits the study of the kinetics of nasal inflammatory responses (9). As such, it may serve as a tool to study the effects of anti-allergic interventions targeting specific inflammatory mechanisms in relationship to upper airway response (10). Although nasal biopsies are the golden standard for studying cellular inflammatory response, there are some limitations to this invasive technique: it can only be performed by an experienced ear, nose, and throat physician; it provides information on just a limited part of the upper airways; and it does not allow repeated samplings within short time intervals (11, 12). Nasal brushing, in contrast, is a less invasive method that has emerged as a possible viable alternative for interventional trials requiring repeated sampling. In patients with allergic rhinitis, Jacobson *et al* showed that seasonal changes in the number of mast cells and eosinophils in nasal brush samples correlated well with those found in nasal biopsies (12). Likewise, intranasal fluticasone produced a similar degree of reduction in these inflammatory cells in both brush and biopsy samples. Nasal lavage is another relatively noninvasive sampling technique which allows serial assessments of the effects of anti-inflammatory therapy on soluble components of upper airway inflammation (13).

The aim of this study was to analyze the reproducibility of various biomarkers associated with allergic rhinitis to assess their potential use as outcome measures in clinical trials testing novel anti-inflammatory therapies. To this end, in a group of clinically stable patients with untreated allergic rhinitis, we combined several semi-invasive (sampling) techniques and analyzed serum

specific IgE levels, SPT results, and various cellular and soluble biomarkers of upper airway inflammation identified in nasal lavage and brush material. In addition, we tested the effect of nasal allergen challenge on the kinetics of these inflammatory biomarkers in a randomized study design.

Methods

SUBJECTS

Twenty multisensitized subjects (10 females, 10 males; age, 19-51 years) with nonsymptomatic allergic rhinitis participated in the study. All the subjects had a history of intermittent or persistent allergic rhinitis for at least 1 year prior to enrollment (1). Maintenance anti-allergy or anti-asthma medication was discontinued at least 6 weeks prior to the study. Atopy was confirmed by a positive SPT for at least 2 of 6 common airborne allergens (grass pollen, tree pollen, *Dermatophagoides pteronyssinus* [HDM], *Dermatophagoides farinae*, and cat and dog dander) (ALK Abelló, Nieuwegein, The Netherlands). Subjects with any other clinically relevant disorders were excluded. Eligible subjects were sensitized to at least 1 of the allergens used for nasal provocation (grass pollen, HDM, or cat dander extracts) (ALK Abelló). Symptomatic subjects with mite or pollen allergy were tested outside the relevant season (October-February for HDM and May-August for pollen in the Netherlands). In the case of subjects with concomitant allergy to pets, only those with no close contact with pets during the study were included. Allergic rhinitis symptoms were monitored throughout the study using a composite symptom score validated by Lebel *et al* and subjects with a baseline score of more than 2 on the allergen challenge day (visit 2) were excluded (14). Respiratory tract infections were excluded by confirming the absence of relevant symptoms in the 3 weeks prior to and during the study and each nostril was inspected for accessibility using a nasal speculum. All subjects were non-smokers or ex-smokers who had quit at least 12 months prior to the study with a smoking history of under 10 pack years. The study protocol was approved by the Leiden University Medical Centre Ethics Committee and all the subjects gave their written informed consent prior to enrollment in the study.

STUDY DESIGN

We used a combined study design (Figure 1). In the first part of the study, we tested the reproducibility of serum specific IgE measurements, SPT results, and baseline inflammatory marker measurements using nasal lavage and brush material. In the second part of the study, we tested the kinetics of the inflammatory markers analyzed in the first part of the study following a nasal

challenge with an allergen or diluent. Eligible subjects were included in the study and specific serum IgE levels, SPT, and nasal lavage and brush inflammatory markers were measured during visit 1 and repeated 14 to 21 days later during visit 2. Subsequently, subjects were allocated to an allergen group (n = 10) or a placebo (diluent) group (n = 10) in a randomized, double-blind, parallel fashion. Nasal lavage measurements were repeated 20 minutes and 7 hours post challenge (i.e., after the last placebo/allergen dose), and nasal brush measurements were repeated 7 hours post challenge. Symptom scores were recorded before the challenge, after the administration of the diluent, 10 minutes after each challenge dose, and hourly until 7 hours post challenge (14). Clinically stable subjects were dismissed from the unit 8 to 9 hours post challenge and asked to return 24 hours later (visit 3) for recording of symptom scores and nasal lavage and brush measurements and again 7 days later (visit 4) for the final lavage and brush measurements. The occurrence of adverse events was monitored throughout the study. As a safety check, airway response was measured in all patients by forced expiratory volume in 1 second (FEV₁) (Vmax Spectra; Sensor Medics, Bithoven, The Netherlands) in accordance with standardized lung function techniques. This check was performed during screening, pre allergen, and 7 hours post allergen (15).

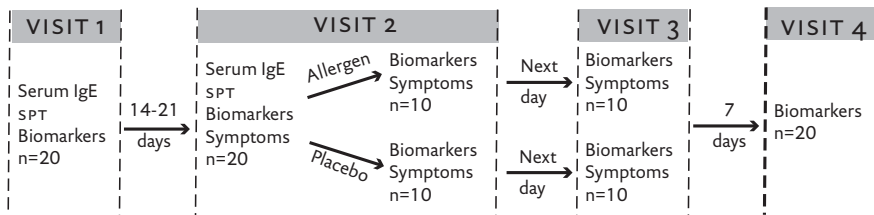


FIGURE 1 Study flowchart. The biomarkers studied were inflammatory markers in nasal brush and nasal lavage material. SPT indicates skin prick test; IgE, immunoglobulin E; n, number of subjects.

SERUM IGE LEVELS

Five mL of venous blood were collected and stored at -80°C for subsequent analysis of total and specific serum IgE levels. Total and specific IgE levels for HDM, grass pollen, tree pollen, and cat and dog dander were determined by fluorescence enzyme immunoassay (FEIA) using the Immuno CAP system (Phadia AB, Uppsala, Sweden). For the reproducibility analysis, we compared baseline specific serum IgE levels from visit 1 versus visit 2 for the allergen with the highest specific IgE level during visit 1.

SKIN PRICK TEST

SPTs were performed in duplicate at the volar site of each forearm by the same experienced technician by application of 1 drop of each allergen extract to the skin, at least 3 cm apart, according to a previously validated protocol (3). Histamine chloride (10 mg/mL) was used as a positive control and a diluent of each allergen (ALK Abelló) as a negative control. Subsequently, the dermis was punctured once with an ALK lancet (ALK Abelló); a clean lancet was used for each solution. Fifteen minutes later, the results were read by the same technician, who marked the edges of the wheal responses (excluding pseudopods) with a special thin-tipped pen. The mark was transferred to transparent tape and attached to the result form. The mean value of the 2 largest perpendicular diameters of wheal responses was included in the analysis. For the reproducibility analysis, we compared wheal sizes (visit 1 vs visit 2) for the allergen yielding the largest wheal response during visit 1.

NASAL ALLERGEN CHALLENGE

Nasal allergen challenges were performed according to a previously validated protocol (13). The procedure is briefly described as follows: to select the intranasal allergen (HDM, grass pollen, or cat dander) for each patient, account was taken of the allergen that had caused the largest wheal in the SPT in combination with the patient's history of clinical symptoms and the season. Pre challenge, subjects were acclimatized for 30 minutes in the test room and then administered 1 puff of xylometazoline 0.1% in each nostril. Approximately 20 minutes later, a puff of allergen diluent (phosphate buffered saline [PBS] containing human serum albumin 0.03% in benzoalkonium chloride 0.05%) (ALK Abelló) was sprayed into each nostril; this was followed by the administration of 1 puff of intranasal allergen (at 3 increasing concentrations of 100, 1000 and 10 000 biological units [BU]/mL) into each nostril at 10-minute intervals. Subjects in the placebo group received the allergen diluent intranasally 4 times using the same time intervals. The puffs were delivered as distally as possible into the nasal cavity using a nasal pump that delivered a fixed dose of 0.125 mL solution per puff (nasal pump 1013463; ALK Abelló). Subjects were instructed to inhale gently to prevent the solution from entering the lower airways. The nasal response at the respective time points was quantified by a validated composite symptom score system (14). Symptoms were recorded using the following scoring system: sneezes $\leq 2 = 0$, sneezes 3-4 = 1 point, sneezes $\geq 5 = 3$ points, anterior rhinorrhoea = 1 point, posterior rhinorrhoea = 1 point, difficult breathing = 1 point, 1 blocked nostril = 2 points, 2 blocked nostrils = 3 points, pruritus in the nose = 1 point, pruritus of palate or ear = 1 point, conjunctivitis = 1 point (total possible score, 0-11 points). Composite symptom scores were recorded 10 minutes after each dose and dosing was discontinued

when a total score of 6 or more was reached or after the administration of the highest dose (10 000 BU/mL or diluent).

NASAL LAVAGE

The nasal lavage was performed in the same nostril throughout the study using a modified protocol of the technique described by De Graaf-In 't Veld *et al* (13). Briefly, the subjects, in a seated position, were instructed to flex their neck approximately 30° from the vertical and to not breathe through their nose. A nasal olive was inserted into the corresponding nostril and 8 mL of PBS (37°C) was gently instilled using a 10 mL preweighed syringe with a dwelling time of approximately 10 seconds. This procedure was performed twice. The lavage fluid was then collected, kept on ice, weighed, and processed within 1 hour (centrifugation for 10 minutes at 400 g and storage of supernatant at -80°C pending analysis). The following soluble inflammatory markers, listed below with their respective assay kits and detection limits, were determined in the nasal lavage samples according to the manufacturers' directions: interleukin (IL)-8, enzyme-linked immunosorbent assay (ELISA) (CLB, Amsterdam, The Netherlands), 30 pg/mL; IL-13, ELISA (R&D systems, Minneapolis, Minnesota, USA), 600 pg/mL; eotaxin, ELISA (R&D systems), 10 pg/mL; thymus and activation-regulated chemokine (TARC), ELISA (R&D systems), 75 pg/mL; eosinophilic cationic protein (ECP), FEIA (Phadia AB), 2 µg/L; tryptase, FEIA (Phadia AB), 1 µg/L; IgE, FEIA (Phadia AB), 1 kU/L; α2-macroglobulin, ELISA (R&D systems), 10 ng/mL; mucin 5AC (MUC5AC), immunoblotting using apical secretions obtained from bronchial epithelial cells cultured at the air-liquid interface as standard (Neomarkers antibody; Lab Vision Corporation, Fremont, California, USA), 1 arbitrary unit (AU)/mL. Samples were not concentrated before analysis.

NASAL BRUSHINGS

All nasal brushings were performed in the nostril not used for the lavage throughout the study according to a modified version of the original technique described by Jacobson *et al* (12). Briefly, the brush (Buccal Swab Brush; BIOzymTC, Landgraaf, The Netherlands) was introduced between the nasal septum and the inferior turbinate and gently rotated 90° to 180°. Immediately after sampling, the brush was placed in a 3 mL plastic tube containing 2 mL of PBS at room temperature, carefully shaken, and brushed off gently against the wall of the tube. Aliquots (100 µL) containing the cell suspension were loaded into a cytocentrifuge for the preparation of slides, spun for 7 minutes at 400 g (Shandon Cytospin 4; Thermo Electron Corporation, Runcorn, UK), and dried prior to storage at 7°C. Differential cell counts of eosinophils, neutrophils, lymphocytes, macrophages, nasal epithelial cells, and squamous cells

were performed on coded May-Grünwald-Giemsa-stained cytopins. The cell differentials were expressed as a percentage of 250 nucleated nonsquamous cells; cytopins containing fewer cells were deemed unsuitable for analysis.

STATISTICAL ANALYSIS

Reproducibility of serum IgE results (highest level), SPT measurements (largest diameter), and inflammatory biomarker measurements was assessed by analyzing intrasubject variation between visits, expressed as a coefficient of variation (CV, standard deviation expressed as percentage of the mean). The necessary change in these measurements for the detection of an intervention effect in future studies was calculated using a power calculation program (nQuery advisor 3.0; Dixon Statistical Associates, Los Angeles, California, USA). The within-subject CV was used to estimate the standard deviation of the difference and the calculation was based on a crossover study of 12 patients, a power of 80%, and an α -risk of 5% in a 2-tailed comparison. The effect of each challenge was analyzed with a mixed-model analysis of covariance (ANCOVA) using intervention, time, and intervention by time as fixed factors, the subject as a random factor, and the prechallenge measurement as a covariate. Where necessary, variables were log-transformed to meet analysis requirements. Contrasts between the placebo and allergen challenge were calculated at various time points. When results fell below the detection limit, levels were set at 50% of this limit. When ANCOVA was not possible (in the placebo group, for example, due to a lack of variance), the first postchallenge measurements were analysed using the t test (null hypothesis: mean = 50% of detection limit). An intervention effect with a P value of less than .05 was considered statistically significant. Sample size estimation was based on previous work examining the reproducibility of most of the biomarkers we aimed to evaluate and their response following allergen challenge (16, 17). All the calculations were performed using SAS for Windows, version 9.1.2 (SAS Institute, Cary, North Carolina, USA).

Results

PATIENT CHARACTERISTICS AT BASELINE

None of the subjects had symptomatic allergic rhinitis on either of the 2 study visits; the maximum baseline symptom scores were 2.

REPRODUCIBILITY OF SERUM SPECIFIC IGE LEVELS AND SPT WHEAL SIZES

Both specific serum IgE levels and SPT wheal sizes were found to be reproducible parameters in patients with allergic rhinitis over an observational

period of 14 to 21 days. In all cases, total and maximal serum specific IgE levels and histamine-induced and maximal wheal sizes remained fairly constant between visits 1 and 2 (Figure 1 and Table 2).

REPRODUCIBILITY OF BASELINE INFLAMMATORY MARKERS IN NASAL LAVAGE AND BRUSH MATERIAL

The reproducibility of inflammatory markers in nasal lavage and brush material varied considerably. While nasal IgE remained fairly constant between visit 1 and 2, ECP and α 2-macroglobulin levels differed greatly (Table 1). In addition, levels of IL-8, IL-13, eotaxin, TARC, and tryptase in the nasal lavage fluid were below the detection limit in most samples and hence could not be included in the reproducibility analysis. The changes in serum IgE levels, wheal size, and inflammatory marker measurements needed to detect a significant intervention effect (eg, drug treatment) was calculated using the within-subject CV in a crossover study design with 12 patients (Table 1).

TABLE 1 INFLAMMATORY MARKER MEASUREMENTS ON VISIT 1 AND 2 WITH CORRESPONDING COEFFICIENT OF VARIATION (CV) AND REQUIRED EFFECT SIZE*

| | VISIT 1 | VISIT 2 | CV WITHIN SUBJECTS, % | REQUIRED EFFECT SIZE |
|---|---------------|--------------|-----------------------|----------------------|
| Serum Immunoglobulin (Ig) E Levels | | | | |
| Maximal specific IgE, IU/mL | 29.3 ± 5.7 | 27.5 ± 5.3 | 13.4 | 18.3%** |
| Total IgE, IU/mL | 266.2 ± 54.3 | 249.5 ± 51.4 | 18.4 | 25.9% |
| Skin Prick Test | | | | |
| Histamine wheal size, mm | 5.7 ± 0.2 | 6.3 ± 0.2 | 11.7 | 0.9 mm |
| Maximal wheal size for allergen, mm | 6.9 ± 0.5 | 7.1 ± 0.4 | 18.2 | 1.6 mm |
| Nasal Lavage | | | | |
| Nasal IgE, IU/mL | 5.4 ± 0.2 | 5.5 ± 0.1 | 6.1 | 7.9% |
| Mucin 5AC, AU/mL | 44.2 ± 6.5 | 37.9 ± 7.7 | 224.1 | 436% |
| α 2-macroglobulin, ng/mL | 377.5 ± 115.5 | 296.1 ± 91.2 | 219.7 | 436% |
| ECP, ng/mL | 3.9 ± 0.9 | 8.1 ± 4.2 | 123.3 | 234% |
| Nasal Brushing | | | | |
| Eosinophil count, % | 12.4 ± 5.5 | 12.7 ± 3.7 | 99.5 | 183.8% |
| Neutrophil count, % | 76.6 ± 6.7 | 79.8 ± 5.2 | 39.9 | 63.3% |

Abbreviations: AU, arbitrary units; ECP, eosinophil cationic protein. * Results are expressed as mean (\pm SEM) unless otherwise indicated. ** For example, in a crossover study with 12 patients, a real change in serum specific IgE of 18.3% or more can be related to the treatment effect and not the natural variation of this biomarker in 80% of experiments.

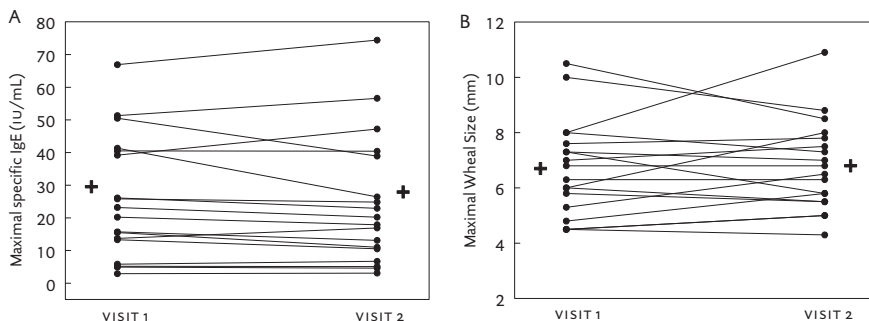


FIGURE 2 A, individual maximal serum specific immunoglobulin (IgE) levels at visits 1 and 2. B, individual maximal wheal sizes at visits 1 and 2. + shows mean levels.

NASAL ALLERGEN CHALLENGE

Intranasal interventions (allergen and diluent) were well tolerated by all subjects and no clinically significant adverse events were recorded. Furthermore, there was no significant fall in FEV₁ from baseline in any of the patients at 7 hours post challenge. The number of patients with concomitant allergic rhinitis and asthma was equal in the allergen and diluent group. As compared with placebo (P), intranasal allergen (A) induced a nasal early allergic response (EAR) in all subjects with a mean symptom score (mean \pm SEM, 7.25 ± 0.56 [A] vs 0.70 ± 0.26 [P]) at 10 minutes post challenge (Figure 3). The allergen-induced nasal EAR was accompanied by significantly increased levels of tryptase, α 2-macroglobulin and ECP (4.39 ± 2.0 [A] vs 0.5 ± 0.0 ng/mL [P]; 6630.7 ± 4240.23 [A] vs 51.7 ± 28.58 ng/mL [P]; 24.19 ± 18.33 [A] vs 1.17 ± 0.17 ng/mL [P], respectively), but not nasal IgE or MUC5AC (5.20 ± 0.14 [A] vs 5.33 ± 0.25 IU/mL [P] and 72.60 ± 23.35 [A] vs 33.8 ± 6.69 AU/mL [P], respectively) in the nasal lavage fluid at 20 minutes post challenge (Figure 3). In addition, all subjects had a nasal late allergic response (LAR), defined as a composite symptom score above baseline on 2 consecutive time points between 3 to 7 hours post allergen (1.3 ± 0.31 [A] vs 0.24 ± 0.21 [P] (Figure 3) in combination with a significant increase in nasal eosinophil count and its degranulation marker ECP (20.9 ± 4.75 [A] vs $12.1 \pm 3.79\%$ [P] and 25.19 ± 19.18 [A] vs 1.72 ± 0.39 ng/mL [P], respectively) (Figure 3) (13). Contrasts between allergen and placebo challenges were calculated at selected time points as shown in Table 2. The inflammatory markers described above had all returned to baseline levels by visit 4 (7 days post challenge). Levels of the other inflammatory markers (IL-8, IL-13, eotaxin, and TARC) remained below detection limits in most nasal lavage samples

and were not affected by either challenge, and hence could not be included in the analysis. The overall sample recovery rate for nasal lavage samples during the study was 72% (mean \pm SEM, 5.7 \pm 0.9 mL). Eighty seven percent of all the nasal brush samples were suitable for analysis.

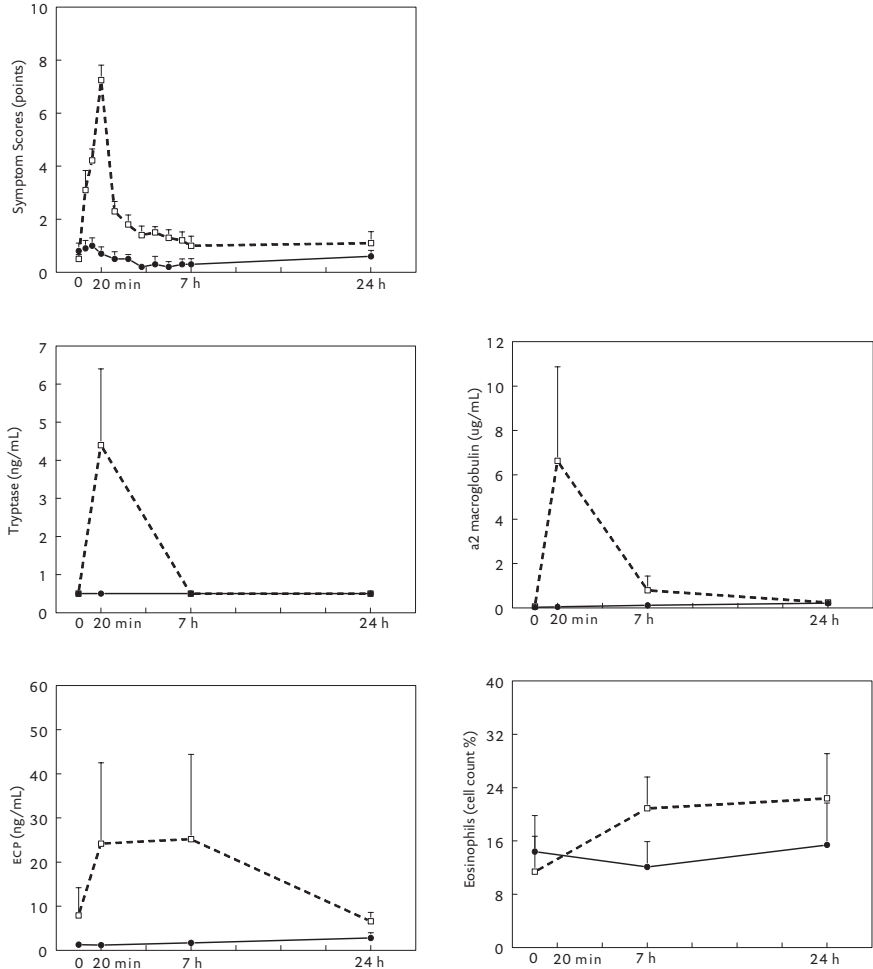


FIGURE 3 Mean change (\pm SEM) in symptom scores, tryptase, $\alpha 2$ -macro-globulin, and eosinophilic cationic protein (ECP) levels in nasal lavage and eosinophil count in nasal brush material following allergen challenge (open square) and placebo challenge (filled circle).

TABLE 2 INFLAMMATORY MARKER KINETICS FOLLOWING ALLERGEN AND PLACEBO CHALLENGES

| PARAMETER (TIME POINT) | LSM - ALLERGEN | LSM - PLACEBO | LSM DIFFERENCE, % (95% CI) | P VALUE |
|--|-------------------|------------------|----------------------------------|---------|
| Overall symptom score, points | 2.21 | 0.28 | 1.9 (2.4-1.4) | < .001 |
| IgE, IU/mL (20 min post challenge) | 5.25 | 5.22 | 0.5 (-12.5-15.5) | .93 |
| Mucin 5AC, AU/mL (20 min post challenge) | 33.52 | 39.58 | -36.2 (-82.4-131.9) | .64 |
| Tryptase, ng/mL (20 min post challenge) | NA* | NA* | 2.21 (0.9-5.3)* | .004* |
| α 2-macroglobulin, ng/mL (20 min post challenge) | 815.24 | 28.75 | 2735.2 (334-18425) | .002 |
| α 2-macroglobulin, ng/mL (7 h post challenge) | 121.4 | 50.1 | 142.2 (-30.1-741.1) | .15 |
| ECP, ng/mL (20 min post challenge) | 4.30 | 1.11 | 287.8 (11.3-1251.4) | .03 |
| ECP, ng/mL (7 h post challenge) | 5.69 | 1.44 | 295.4 (24.9-1151.9) | .02 |
| Eosinophil count, % (7 h post challenge) | 25.25 | 10.45 | 144.4 (13.8-424.8) | .04 |
| Eosinophil count, % (24 h post challenge) | 21.98 | 9.73 | 125.8 (-31.0-639.1) | .16 |

Abbreviations: CI, confidence interval; ECP, eosinophilic cationic protein; LSM, least square means; NA, not applicable. * Tryptase under challenge was tested against the null hypothesis mean = 50% of detection limit.

Discussion

In the present study of patients with allergic rhinitis, we tested the reproducibility of several inflammatory biomarkers obtained by semi-invasive sampling techniques in combination with their kinetics in nasal lavage and brush material following a nasal challenge involving allergens and placebo. We found that, in clinically stable patients with untreated allergic rhinitis, both specific serum IgE levels and SPT wheal sizes proved to be reproducible measures over a period of 14 to 21 days, indicating that these parameters may be reliable outcome measures for interventional trials on anti-allergic therapy. In agreement with previously published data, measurements of soluble and cellular inflammatory biomarkers in nasal lavage and brush samples showed marked variability at baseline (11, 13, 16). Nonetheless, on analyzing these

measurements in a nasal allergen challenge, we observed a clear increase in several upper airway inflammation biomarkers up to 24 hours post allergen, together with an increase in nasal symptoms following the allergen challenge but not the placebo challenge. Our data confirm and extend previous observations. Taken together, repeated nasal lavage and brush samplings are a useful extension of the nasal allergen challenge model and offer additional information in interventional trials with therapy targeting allergic mechanisms. Serum IgE levels showed a low CV in patients with clinically stable allergic rhinitis. Similarly, low intrasubject variation has been observed in other trials involving patients with allergic disease (7,18). Indeed, due to their correlation with symptoms and other allergic markers (nasal eosinophils) serum IgE levels may be used as outcome parameters for anti-allergic medication (7, 19). However, because there is a substantial variation in precision and accuracy between commercial IgE assays, similar assay techniques should be employed throughout a study (20). In the present study, SPT was performed according to recommendations issued by the Global Allergy and Asthma European Network (GA2LEN) with an ALK lancet (21). Employing a similar SPT technique in a study in allergic children, the CV over 1 week was found to be 24.3% for HDM and 17.3% for histamine (22). Our data thus extend earlier observations as we found a CV over 2 to 3 weeks of under 20% for the largest dermal wheal size and of 11.7% for the histamine-induced wheal size. When applying SPT serially, it is essential to use the same standardized techniques and allergen extracts and to limit confounding factors such as drug intake, changes in circadian rhythm, and an excessive number of investigators (23). Similarly to IgE levels, a decreased SPT response following anti-allergic treatment has been shown to accompany a reduction in disease-related features both in patients with allergic rhinitis and allergic asthma (5, 6, 24).

Even though the patients in our study were clinically stable, defined by an absence of symptomatic disease, all of the nasal inflammatory markers, with the exception of IgE levels, showed marked baseline variability over time. Our findings therefore, call into question the applicability of nasal lavage and brushing in longitudinal studies with a limited number of patients. In the present study, we applied a slightly modified version of the nasal lavage technique previously reported by Naclerio *et al*; nasal lavage fluid recovery was similar to that reported in other studies (65% to 82%) (13, 16, 25). Based on the low variability of nasal IgE levels throughout the study, it may be concluded that nasal lavage, in combination with other semi-invasive sampling techniques such as nasal brushing, can yield representative serial measurements in a relatively short time interval (eg, during the EAR). However, the limited dwelling time used in the present study could explain why certain soluble mediators varied while others remained below the detection limit.

Employing a similar washing technique, other researchers also found low or undetectable levels of several mediators (13, 16, 26).

How could these issues be resolved? A more sensitive assay technique, such as Luminex technology, with a lower limit of detection for a variety of inflammatory mediators, could be used (27). Alternatively, the method described by Greiff and Grundberg, which involves applying an inflated balloon into either nostril, allows an increased dwelling time, which is associated with the recovery of higher levels of soluble markers from nasal lavage fluid (28). However, we anticipated that the allergen-induced swelling in combination with multiple other procedures conducted in the present study would not be compatible with this technique. Although nasal biopsies are currently considered the most suitable tool for investigating the kinetics of inflammatory cells in nasal mucosa, nasal brushing, which is a less invasive sampling method, has been shown to yield comparable results in terms of inflammatory cell differentials (11, 12). However, comparing changes in eosinophil counts in nasal brush and biopsy samples from patients with allergic rhinitis following exposure to grass pollen for 2 weeks out of season, Godthelp *et al* found similar variability in brush cellular counts whereas biopsies yielded less variable data (11). Our overall findings show that simpler, less invasive sampling techniques such as nasal lavage and brushing allow greater flexibility and repeatability, but at the expense of increased variability, especially over longer time intervals.

In a nasal allergen challenge study by Braga *et al*, allergen-induced composite symptom scores were shown to be associated with an increase in total nasal resistance, measured by anterior active rhinomanometry (29). Based on the increase in composite symptoms scores, the nasal allergen challenge produced both an early- and late-phase response in all patients with allergic rhinitis. These events corresponded to increased levels of several upper airway inflammatory markers. In contrast to placebo, the allergen caused a significant increase in nasal lavage tryptase levels, an indicator of mast cell degranulation, at 20 minutes post challenge; these levels were undetectable during later samplings. Measuring tryptase in nasal lavage fluid without a relevant stimulus, therefore, does not seem useful. A similar, short-lived increase in nasal lavage tryptase levels has been observed in other nasal allergen challenge studies (13, 26). α 2-macroglobulin was also increased in nasal lavage fluid at 20 minutes post allergen challenge but not at 7 hours. Another nasal allergen study, however, reported increased concentrations of albumin, also a leakage marker, at both 20 minutes and 7 hours post allergen challenge (17). This different finding could be the result of a more pronounced LAR in the latter study or because the level of albumin, being a smaller molecule than α 2-macroglobulin, may also be determined by passive diffusion and secre-

tion. We specifically measured α 2-macroglobulin since it is one of the largest plasma solutes and may be more specific for the exudation process (30). In agreement with previous studies, intranasal allergen challenge also induced a rise in ECP, which reached significant levels at 20 minutes and 7 hours post challenge (13, 26). This corresponds to an increase in eosinophilic degranulation, also observed in another study following an allergic stimulus (31). In addition, we were able to demonstrate the presence of MUC5AC, the principal airway mucin, in nasal lavage fluid both at baseline and throughout the challenges (32). However, due to the large variation within and between subjects, no difference in MUC5AC levels could be found between the allergen challenge and the placebo group at any of the time points analyzed. Whether this large variation (high SD) is caused by interindividual differences in the disease process and whether levels are higher than in healthy controls remain to be established. In contrast to the placebo challenge, the allergen challenge induced a significant increase in nasal brush eosinophil counts at 7 hours; these counts were still increased at 24 hours, albeit not significantly. Hence, our data confirm and extend the results reported by Juliusson et al, who investigated the kinetics of the allergen-induced changes in nasal brush eosinophils at 2-hourly intervals until 12 hours post allergen (33). Overall, our data support the validity of repeated nasal brush samplings as a method for analyzing allergen-induced changes in nasal eosinophils. In addition, eosinophils have been shown to be responsive to anti-inflammatory therapy (12). Nonetheless, based on the large variation in eosinophil count within and between subjects, large numbers of patients need to be included in a preferentially crossover study design to ensure optimal statistical power. In conclusion, in untreated patients with clinically stable allergic rhinitis, we demonstrated that serum specific IgE levels and SPT wheal responses are reproducible parameters, characterized by low intrasubject variation ($=CV$) over a period of 14 to 21 days. Compared to well established nasal biopsies, nasal lavage and brushing are less invasive techniques that allow greater flexibility and repeatability but at the expense of greater intrasubject variation. On implementing these sampling techniques in a standardized nasal allergen challenge study, we found significantly increased levels of several inflammatory biomarkers following the allergen challenge but not the placebo challenge. We believe that nasal challenge with a relevant allergen conducted in conjunction with semi-invasive sampling techniques such as nasal lavage or brushing is a suitable method for exploring novel anti-allergic therapies. The drawbacks of large intrasubject variation could be resolved by using a crossover design and more sensitive detection techniques.

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