

# Nasal Nitric Oxide Measurements in the Assessment of Nasal Allergen Challenge

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## ■ Abstract

*Background:* Several objective methods are used to assess the result of nasal allergen challenge.

*Objective:* The aim of this study was to compare the diagnostic value of nasal nitric oxide (nNO) measurements with that of peak nasal inspiratory flow (PNIF), nasal lavage fluid  $\beta$ -tryptase levels, and changes in cell count after nasal challenge with grass pollen.

*Methods:* The study population comprised 24 patients allergic to grass pollen and 24 healthy controls. All participants underwent grass allergen challenge preceded by administration of placebo. A visual analog scale was administered. nNO and PNIF were determined, and nasal lavage fluid was collected before and 30 minutes after administration of placebo and allergen. The study was performed outside the pollen season.

*Results:* Significant changes in nNO, PNIF, nasal lavage fluid  $\beta$ -tryptase level, and cell count were observed only in allergic patients after administration of the allergen. Receiver operating characteristic (ROC) curves were drawn for each determination. A change in nNO levels of  $-11.987\%$  was indicated as the best cutoff point for differentiating between allergic patients and healthy participants with a sensitivity of  $60.9\%$ , specificity of  $100\%$ , negative predictive value of  $71\%$ , and positive predictive value of  $100\%$ . Comparison of the area under the ROC curve did not show significant differences between the diagnostic value of changes in nNO levels and other objective methods of assessing the outcome of the challenge.

*Conclusion:* Changes in nNO levels do not differ significantly from other methods used to objectively assess the outcome of nasal challenge. Given their insufficient sensitivity, nNO measurements have limited value as the sole diagnostic tool when assessing the outcome of nasal challenge.

**Key words:** Nasal nitric oxide. Nasal allergen challenge. Allergic rhinitis.

## ■ Resumen

*Antecedentes:* Se utilizan varios métodos objetivos para evaluar el resultado de la provocación nasal con alérgenos.

*Objetivo:* El objetivo de este estudio fue comparar el valor diagnóstico de las mediciones del óxido nítrico nasal (NON) con el del flujo inspiratorio nasal máximo (FINM), los niveles de  $\beta$ -triptasa en el líquido de lavado nasal, y los cambios en el recuento celular tras la provocación nasal con polen de gramíneas.

*Métodos:* La población en estudio incluyó a 24 pacientes alérgicos al polen de gramíneas y a 24 controles sanos. Todos los participantes se sometieron a una provocación con alérgenos de gramíneas tras la administración previa de placebo. Se empleó una escala visual analógica. Se determinaron el NON y el FINM, y se obtuvo líquido de lavado nasal antes y 30 minutos después de la administración del placebo y el alérgeno. El estudio se realizó fuera de la estación polínica.

*Resultados:* Se observaron cambios significativos en el NON, en el FINM, en el nivel de  $\beta$ -triptasa del líquido de lavado nasal y en el recuento celular únicamente en pacientes alérgicos después de la administración del alérgeno. Para cada determinación se representaron curvas de eficacia diagnóstica (ROC). Se indicó un cambio de  $-11,987\%$  en los niveles de NON como mejor valor de corte para la diferenciación entre pacientes alérgicos y pacientes sanos, con una sensibilidad del  $60,9\%$ , una especificidad del  $100\%$ , un valor predictivo negativo del  $71\%$  y un valor predictivo positivo del  $100\%$ . La comparación del área bajo la curva ROC no mostró diferencias significativas entre el valor diagnóstico de los cambios en los niveles de NON y otros métodos objetivos de valoración del resultado de la provocación.

*Conclusión:* Los cambios en los niveles de NON no difieren significativamente de otros métodos utilizados para evaluar objetivamente los resultados de la provocación nasal. Debido a su insuficiente sensibilidad, las mediciones del NON tienen escaso valor como única herramienta diagnóstica a la hora de evaluar el resultado de la provocación nasal.

**Palabras clave:** Óxido nítrico nasal. Provocación nasal con alérgenos. Rinitis alérgica.

## Introduction

Nitric oxide (NO) was first described as an endothelium-derived relaxing factor [1]. Gustafsson et al [2] found endogenous NO to be present in the exhaled air of rabbits, guinea pigs, and humans. Since then, several studies have focused on the role of NO in the airways and the utility of measuring NO levels as a diagnostic procedure and tool for monitoring treatment of respiratory tract diseases. Alving et al [3] published data showing higher concentrations of NO in the air from the upper airways than in air from the lower airways. The paranasal sinuses are the main source of nasal nitric oxide (nNO) [4]. In contrast to the epithelial cells of the nasal cavities, the epithelium of the paranasal sinuses shows high levels of expression of NO synthase. The abundance of NO in the paranasal sinuses is probably responsible for their sterility: NO is the first line of nonspecific immunity, owing to its antiviral and antibacterial properties and its role in the regulation of mucociliary motility [5,6].

Nasal challenge is an important tool in the diagnosis of allergy and is used in cases where it is difficult to identify the culprit allergen [7]. Although several objective methods are used to determine the outcome of nasal challenge, none provide clear indicators of a positive result. Moreover, rhinomanometry and acoustic rhinometry are mainly used to measure changes in nasal blockage, a key symptom of allergic rhinitis. The utility of nNO measurement in determining the outcome of nasal allergen challenge has not been evaluated to date. Some authors have shown that nNO concentrations decrease significantly after allergen challenge [8]. This phenomenon is surprising, because nNO is a well-known marker of inflammation, and an increase in its levels is expected in response to an allergen.

The aims of this study were to determine the utility of nNO in assessing the outcome of nasal allergen challenge and to compare nNO measurement with other methods of assessment.

## Material and Methods

### Study Group

The study population comprised consecutive patients with allergic rhinoconjunctivitis due to grass pollen allergy and healthy volunteers. Patients were considered allergic when they had a history of symptoms of rhinoconjunctivitis during at least 2 grass pollen seasons (May-July) and positive results in skin prick tests only with grasses and specific immunoglobulin (Ig) E class >2. The control group consisted of healthy volunteers with similar demographic characteristics to those of the allergic patients.

Patients with a history of perennial symptoms, nasal septal deviation, nasal polyps, chronic sinusitis, smoking (current and former), and infection of the lower and/or upper respiratory tract during the 4 weeks preceding the challenge were excluded from the study.

Based on the inclusion and exclusion criteria, 24 patients with allergy to grass pollen (16 males; mean [SD] age 24.9 [7.3] years; duration of rhinitis, 4.9 [3.7] years) and 24 healthy volunteers (16 males, mean age 27.1 [11.4] years) took part in

the study. Mean serum IgE (sIgE) level in the allergic patients was 11.08 (8.84) kU<sub>A</sub>/L.

The Ethics Committee of the Medical University of Lodz approved the study protocol, and all participants gave their written informed consent.

### Skin Prick Tests

All patients underwent skin prick tests performed with common aeroallergens: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, grasses, birch, hazel, alder, mugwort, cat, dog, *Alternaria tenuis*, and *Cladosporium herbarum* (Allergopharma). Histamine 1.7 mg/mL (Allergopharma) and standard glycerol saline solution (Allergopharma) were used as a positive and negative control, respectively. A wheal diameter  $\geq 3$  mm was considered a positive result.

### Specific IgE

Serum specific IgE levels were determined using enzyme-linked immunosorbent assay (ELISA) with the commercially available TR kit (Allergopharma).

### Study Design

Before any procedure, participants rested for 30 minutes to acclimatize to room temperature. First, they completed a visual analog scale (VAS), and their nNO levels and peak nasal inspiratory flow (PNIF) were measured. Nasal lavage fluid was collected. The control solution was administered. After 30 minutes, all procedures were repeated. The allergen was administered, and the procedures were performed again 30 minutes later. Figure 1 shows the order in which the procedures were performed.

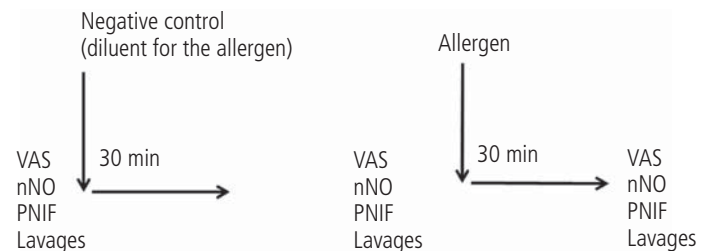


Figure 1. Order in which the study procedures were performed. nNO indicates nasal nitric oxide; PNIF, peak nasal inspiratory flow; VAS, visual analog scale.

### Nasal Allergen Challenge

Single-blind nasal allergen challenge was performed using standardized grass pollen extract (Allergopharma). The diluent for the allergen was used as the control solution.

Two puffs of allergen solution (5000 SBU/mL) were administered into 1 nostril after previous application of control solution.

The medications prohibited before the nasal challenge were as follows: nasal and systemic corticosteroids (28 days);

oral and nasal antihistamines, antileukotrienes, and oral preparations of pseudoephedrine (14 days); nasal  $\alpha$ -agonists (7 days); and nonsteroidal anti-inflammatory drugs (7 days).

Nasal challenge was performed outside the pollen season (October-March).

### Visual Analog Scale

Severity of clinical symptoms (rhinorrhea, nasal itching, ocular itching, sneezing, blockage, and postnasal drip) was described by patients before and after the challenge using the VAS [12], the results of which were expressed as a numerical value (minimum, 0; maximum, 100; maximum total, 600 points) for further analysis. A  $\geq 20\%$  change in the sum of symptoms after application of the allergen, ie, 120 points, indicated a positive result in the challenge (participants' judgment).

### nNO Measurements

Measurements of nNO were performed using an electrochemical analyzer (ExpAir, Medisoft) according to the manufacturer's instructions. Each measurement was performed in the nostril where the control solution and allergen were administered. The detection limit ranged from 1 ppb to 6000 ppb. The mean of 3 measurements was considered as the nNO level.

The patients did not perform strenuous activities and did not eat high-protein meals for 24 hours before the measurements.

### PNIF Measurements

PNIF measurements were performed under supervision using a portable nasal inspiratory flow meter (In-Check, Clement Clarke International). Participants were instructed on how to use the device correctly to obtain reliable recordings. Three measurements were recorded for each participant. The best of the 3 maneuvers was used for further calculations. The measurement was repeated when the technique was performed incorrectly.

### Nasal Lavage

Nasal lavage fluid was collected according to the method described by Greiff et al [9] at the time points specified above. Saline washings were centrifuged (10 minutes at 0.08g, 4°C) to separate the cell pellet and supernatant. The supernatant was immediately frozen (-70°C) for further determination of  $\beta$ -tryptase levels. The sediment obtained was washed in sterile phosphate buffered saline (PBS) (Sigma) and then suspended in 1.0 mL of RPMI 1640 (Sigma). Differential cell counts were determined on slides stained using the May-Grünwald-Giemsa method. A minimum of 400 cells per smear was counted to enable a differential cell count to be made for each specimen. Cells were classified according to their morphology as neutrophils, eosinophils, basophils, and squamous cells.

### $\beta$ -Tryptase

$\beta$ -tryptase concentrations in nasal lavage fluid were measured using the UniCAP Tryptase Fluoroenzymeimmunoassay

(Pharmacia) with a detection limit of 1.0  $\mu\text{g/L}$ . For the statistical analysis, values below 1.0  $\mu\text{g/L}$  were considered as 0  $\mu\text{g/L}$ .

### Statistical Analysis

The statistical analysis was performed using Statistica 8.0 (StatSoft). The results are presented as mean (SD). Data were tested for a normal distribution using the Shapiro-Wilk test. Comparisons were performed using the Wilcoxon test for paired variables and the Mann-Whitney test for unpaired variables. Receiver operating characteristic (ROC) curves were plotted for each of the methods to choose the best cutoff points. The sensitivity, specificity, positive predictive value, and negative predictive value were calculated. Statistical significance was set at  $P < .05$ .

## Results

### Rhinitis Symptoms Assessed With the VAS

The severity of nasal symptoms increased after administration of the allergen but not after administration of placebo. No changes in rhinitis symptoms were observed after administration of the control solution or the allergen in the control group. Mean symptom intensity is presented in Figure 2.

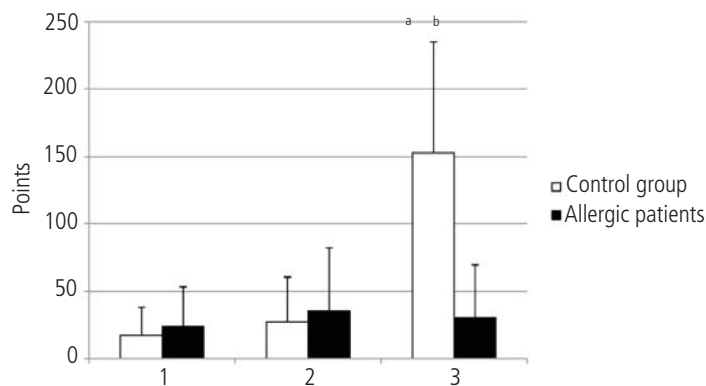


Figure 2. Total symptoms score (visual analog scale) in allergic patients (white bars) and healthy controls (black bars) before administration of the control solution (1), after administration of the control solution (2), and after administration of the allergen (3).

<sup>a</sup>Allergic patients vs healthy controls  $P < .0001$

<sup>b</sup>Allergic patients after control solution vs after allergen  $P < .0001$

Twelve allergic patients (50%) and no healthy controls showed the minimum 120-point rise in the rhinitis symptom score after administration of the allergen. The sensitivity and specificity of the assessment of nasal challenge outcome using the VAS were 50% and 100%, respectively.

### Changes in nNO During Allergen Challenge

We observed significant differences between allergic and healthy participants at baseline (2360.3 [415.9] ppb vs 1863.8 [587.7] ppb, respectively;  $P = .002527$ ) and after administration

of the negative control (2404.9 [369.1] ppb vs 1939 [661.2] ppb, respectively;  $P=.013327$ ) (Figure 3). Administration of the negative control solution did not affect nNO levels in either group. Significantly, nNO concentrations decreased 16.4% from 2404.9 (369.1) ppb to 2014.7 (522.1) ppb ( $P=.000183$ ) in the patients after administration of the allergen.

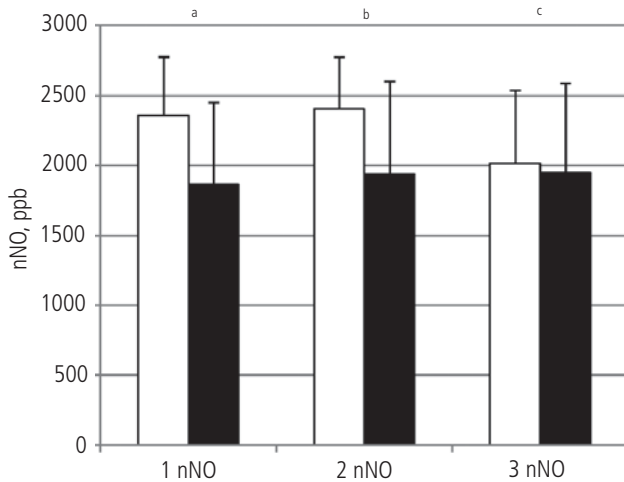


Figure 3. nNO levels in allergic patients (white bars) and healthy volunteers (black bars) before administration of the control solution (1), after administration of the control solution (2), and after administration of the allergen (3).

<sup>a</sup>Allergic vs healthy ( $P=.002527$ ).

<sup>b</sup>Allergic vs healthy ( $P=.013327$ ).

<sup>c</sup>Allergic 2nNO vs 3nNO ( $P=.000183$ ).

### PNIF and Its Association With the VAS

No differences in PNIF were observed between healthy controls and allergic patients before application of the control solution (147.2 [47.7] mL/s vs 149.5 [36.9] mL/s, respectively;  $P=.95$ ) or after application of the control solution (141.3 [46.3] mL/s vs 145.2 [39.1] mL/s, respectively,  $P=.69$ ). Moreover, administration of the control solution did not affect PNIF values in either group. Allergen challenge led to a fall in PNIF in the allergic group (44.6% [35.0] to 96.7 [42.8] mL/s,  $P<.001$ ), which was not observed in the healthy volunteers (146.4 [9.0] mL/s;  $P=.6482$ ). The percentage change in PNIF after allergen challenge correlated with an increased sensation of nasal blockage in allergic patients ( $r=-0.67$ ;  $P<.001$ ), but not with other symptoms.

### Nasal Lavage Cell Count

No significant differences were observed in the nasal lavage cell count between healthy and allergic participants before and 30 minutes after administration of the control solution. Administration of the allergen did not affect the nasal lavage cell count in the control group; however, in allergic patients, the percentage of squamous cells fell significantly (71.7% [16.5%] to 56.6% [22.7%],  $P=.0054$ ) and the percentage of neutrophils and eosinophils increased significantly (13.7% [11.8%] to

20.3% [15.0%],  $P=.034$ ; and 7.6% [9.4%] to 15.1% [14.1%],  $P=.012$ , respectively). Significant differences were observed between the groups in the percentage of squamous cells ( $P=.0075$ ), neutrophils ( $P=.042$ ), and eosinophils ( $P=.0075$ ) after allergen challenge.

### $\beta$ -Tryptase

There were no differences in  $\beta$ -tryptase concentrations in nasal lavage fluid in the control group at the different time points. A significant increase in  $\beta$ -tryptase levels was observed in allergic patients after administration of the allergen (0.067 [0.32]  $\mu\text{g/mL}$  to 3.66 [3.69]  $\mu\text{g/mL}$ ;  $P<.001$ ).

### Changes in nNO Concentrations, VAS, PNIF, and Serum sIgE Levels

We found a moderate but significant negative correlation between the percentage change in nNO and changes in nasal blockage assessed with the VAS ( $r=-0.418$ ;  $P=.046$ ). Similarly, the percentage change in nNO correlated with the absolute change and percentage decrease in PNIF after administration of the allergen ( $r=0.69$  [ $P<.001$ ] and  $r=0.716$  [ $P<.001$ ], respectively). Changes in nNO levels did not correlate with changes in severity of other VAS symptoms after allergen challenge. However, a moderate inverse correlation between serum sIgE levels and percentage of change in nNO was found ( $r=-0.64$  [ $P<.001$ ]).

### Comparison of Objective Methods of Assessing the Outcome of Nasal Allergen Challenge

The objective methods of assessing the outcome of nasal challenge showed the best diagnostic value to be a change in PNIF with a cutoff value of  $-12.5\%$ . The best cutoff value (percentage change) of nNO for discriminating between a

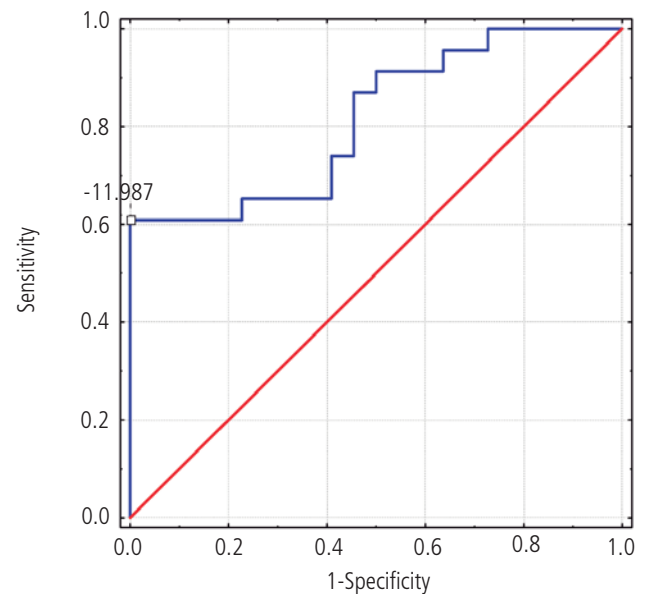


Figure 4. Receiver operating characteristic curve for the percentage change in nNO after nasal allergen challenge.

Table. Diagnostic Value of Objective Methods to Assess the Outcome of Nasal Allergen Challenge

	Cutoff Value	Sensitivity	Specificity	PPV	NPV	AUC	95% CI
$\Delta$ nNO, %	-11.987%	0.609	1.0	1.0	0.71	0.814	0.69-0.938
$\Delta$ PNIF, %	-12.5%	0.826	1.0	1.0	0.846	0.903 <sup>a,b,c</sup>	0.801-1
Squamous cells, %	69%	0.696	0.789	0.8	0.682	0.738	0.583-0.893
Neutrophils, %	21%	0.478	0.895	0.846	0.586	0.683	0.517-0.849
Eosinophils, %	9%	0.696	0.842	0.842	0.696	0.738	0.58-0.896
$\beta$ -Tryptase, $\mu$ g/L	1.09	0.783	0.955	0.947	0.808	0.878 <sup>d,e,f</sup>	0.77-0.987

Abbreviations: AUC, area under the curve; NO, nitric oxide; NPV, negative predictive value; PNIF, peak nasal inspiratory flow; PPV, positive predictive value.

<sup>a</sup> $\Delta$ PNIF vs % squamous cells ( $P=.013$ ).

<sup>b</sup> $\Delta$ PNIF vs % neutrophils ( $P=.0062$ ).

<sup>c</sup> $\Delta$ PNIF vs % eosinophils ( $P=.0174$ ).

<sup>d</sup> $\beta$ -tryptase vs % squamous cells ( $P=.0142$ ).

<sup>e</sup> $\beta$ -tryptase vs % neutrophils ( $P=.0061$ ).

<sup>f</sup> $\beta$ -tryptase vs % eosinophils ( $P=.0173$ ).

positive and a negative outcome was -11.987% (Figure 4). A comparison of the area under the ROC curve showed the superiority of the percentage change in PNIF and  $\beta$ -tryptase concentrations over the percentage of squamous cells, percentage of neutrophils, and percentage of eosinophils 30 minutes after administration of the allergen. Specificity, sensitivity, negative predictive value, positive predictive value, and comparison of the area under the curve for these methods are presented in the Table.

## Discussion

NO is an inflammatory marker in both the lower and the upper airways; however, concentrations of nNO decrease after nasal allergen challenge. Boot et al [8] provide a detailed description of this phenomenon, in which they observed that nNO levels decreased significantly 20 minutes after intranasal application of an allergen compared to placebo. After 7 hours, nNO concentrations increased, but did not differ, between the allergen and the placebo groups; after 24 hours, nNO concentrations were significantly higher in the allergen group. Korn et al [10] showed a significant fall of 19.2% in nNO levels 30 minutes after nasal challenge. During the late phase reaction (ie, after 3-8 hours), a nonsignificant rise in nNO was observed, and after 24 hours values returned to prechallenge levels. As both studies indicate that nNO levels decreased during the early phase of an allergic reaction, we decided to analyze the diagnostic value of nNO measurements 30 minutes after challenge. This time point is important from a clinical point of view, as the result of the challenge was expected to be fast and reliable. We recorded a 16.4% fall in nNO levels after nasal allergen challenge; however, sensitivity seems to be too low to use nNO measurements as the sole method for assessing the outcome of challenge.

The mechanism leading to the decrease in nNO levels after nasal provocation with an allergen remains unclear. The most probable explanation is that edema of the nasal mucosa can block the ostia of the paranasal sinuses, which are the most

prominent source of nNO [11]. The significant correlation observed between the fall in nasal flow and decrease in nNO could confirm this hypothesis.

Factors such as smoking or forced expiration during spirometry can affect exhaled NO concentrations [12]. We applied similar restrictions to avoid potential false nNO measurements. Collection of nasal lavage fluid and PNIF measurements can also affect nNO levels. This area has not been studied in the literature. Nevertheless, as we did not observe significant changes in nNO in healthy controls, we assume that these procedures do not affect nNO concentrations.

Changes in nNO levels after challenge in allergic patients correlated weakly with changes in nasal blockage assessed using the VAS. Moreover, they did not correlate with changes in any of the other symptoms according to the VAS or in the sum of symptoms. Similarly, the percentage change in PNIF only correlated with changes in nasal stuffiness. These findings illustrate the main weakness of nNO measurement as an objective method for assessing the outcome of allergen challenge, namely, changes in nNO reflect the reduction in nasal flow but not the deterioration in other allergic symptoms. The same is true of other objective methods, such as rhinomanometry and acoustic rhinometry.

We found that the best diagnostic value for the percentage change in PNIF was a cutoff point of -12.5%. Ganslmayer et al [13] observed that a 26% reduction in PNIF after allergen challenge produced the best combination of sensitivity and specificity in distinguishing allergic patients from healthy controls. The cutoff value established was twice as high as ours and can probably be explained by the different allergen challenge protocol, that is, the authors used various concentrations of allergen solution, whereas we used only one. The usefulness of PNIF measurements was compared with that of acoustic rhinometry in determining the outcome of nasal challenge. Both methods were comparable, and that of Ganslmayer et al had a sensitivity of 97% and specificity of 100% when a 29% reduction in the minimal cross-sectional area was applied as a cutoff. These results indicate that PNIF measurement may be an important tool when assessing

the outcome of nasal challenge. Other advantages are the simplicity of performing the measurement and the low cost of the equipment. The main drawback may be incorrect performance of the maneuvers leading to the false results. However, Starling-Schwanz et al [14] confirmed PNIF to be highly reliable when measured under supervision and after the patients had been properly instructed [14]. Other authors, in contrast, showed that PNIF measurement was not as useful as VAS for assessing the outcome of nasal challenge with *Dermatophagoides pteronyssinus* (AUC, 0.612 and 0.85, respectively) [15].

Concentrations of  $\beta$ -tryptase in nasal lavage fluid increase rapidly after challenge, reaching a maximum in 15–20 minutes and thus suggesting that this measurement could prove useful in assessing the outcome of nasal challenge during the early phase of an allergic reaction [16]. Indeed, we showed that this method, together with PNIF measurement, was highly sensitive and specific. Despite its good diagnostic value, measurement of  $\beta$ -tryptase cannot be widely applied in clinical practice because of its high cost and lack of an immediate result.

Nasal lavage cell count was the worst method for assessing the outcome of challenge during the early phase of an allergic response. This finding is consistent with the results of authors such as Jean et al [17], who determined the eosinophil percentage in nasal smears obtained from children undergoing nasal challenge with *Dermatophagoides pteronyssinus*. A finding  $>10\%$  was considered a positive result in the challenge. During the early phase of a reaction, the authors observed a positive result in 52% of patients and a negative result in 30%. The challenge was not performed in 18% because the eosinophil percentage was  $>50\%$ . After 24 hours, 30 of 32 children with a negative challenge outcome during the early phase of the reaction had a positive result according to the criterion of eosinophilia  $>10\%$ . These data clearly show that when the differential cell count is analyzed to determine the outcome of allergen challenge, both early and late phase reactions should be taken into account. Consistent with Boot et al [18], we observed a significant influx of eosinophils in nasal lavage fluid 30 minutes after administration of the allergen. The main obstacles in using this method are lack of immediate outcome, time necessary to perform the procedure, and the need for the patient to attend the clinic. Nevertheless, nasal lavage cell count could occasionally provide valuable information.

National guidelines have recently approached the technique of nasal allergen challenge [7,19] and suggest that it should be performed bilaterally rather than unilaterally, as was the case in our study. No clear guidelines on the procedure were available when we performed our study. Nevertheless, it is unlikely that this affected the results for nNO levels, tryptase concentrations, or nasal lavage cell counts. Even if the decrease in PNIF was underestimated by application of the allergen unilaterally, it still showed the best diagnostic value in our study.

In summary, nNO measurement has limited value as the sole diagnostic tool for assessing the outcome of nasal allergen challenge because of its insufficient sensitivity. As with other objective methods for assessing the outcome of nasal challenge, it seems that changes in nNO levels reflect edema of the nasal mucosa, which is indicated by a strong correlation between the reduction in nasal flow measured using PNIF and

the reduction in nNO levels after nasal allergen challenge. The best diagnostic value was observed for measurement of PNIF and  $\beta$ -tryptase levels in nasal lavage fluid. Changes in nNO levels do not differ significantly from those observed with other methods used for the objective assessment of the outcome of nasal challenge.

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