Differences in Exhaled Nitric Oxide in Non- and Mono- or Polysensitised Allergic Children with Asthma Bronchiale

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Abstract: *Rationale*: Exhaled nitric oxide (eNO) is reported to be increased in the airways of patients with asthma bronchiale.

Objectives: To investigate eNO concentration and lung function in non-, mono- and polysensitised children with mild asthma bronchiale before and after exercise challenge.

Findings: Investigation of a total of 59 children (49 mono- or polysensitised, 10 nonsensitised). No significant differences regarding age, gender, height and asthma medication. Significant difference between the groups regarding weight and subsequently BMI. ENO levels were only elevated in allergic asthmatic children (mean 37.0 ppb) compared to the non allergic children (mean 10.0 ppb, $p < 0.0001$). A correlation between eNO concentrations and the residual volume was established. ENO levels after exercise challenge were only slightly lower than before exercise.

Conclusion: Elevated levels of eNO were only found in the group of asthmatic children with an allergic background. Therefore eNO can be used as a marker to differentiate between allergic and non allergic asthma. Asthmatic severity or therapy did not have a significant influence on the eNO levels. In patients with allergic asthma bronchiale eNO may be used as a helpful indicator in the adapting anti-inflammatory treatment. Although statistically significant in allergic children, the influence of exercise challenge on eNO levels is minimal.

INTRODUCTION

Asthma bronchiale is defined as a chronic inflammatory disorder of the bronchial airways. In susceptible individuals, this inflammation causes recurrent episodes with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. This inflammation causes an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli [1].

Inflammation is the basic process of the asthma disease. This process is found in all patients independent of asthma severity and is characterised by local infiltration and activation of a variety of inflammatory and immunoeffector cells [2]. This results in epithelial damage, swelling, mucus secretion and airway smooth muscle contraction, thus causing the asthmatic symptoms. Moreover these cells maintain the allergic inflammation and play an important role in airway remodelling, which may result in increased thickness of the airways, no response to treatment and irreversible loss of pulmonary function [3, 4]. Thus, even in mild asthma, monitoring of airway inflammation is necessary for measuring the severity of the disease and the efficacy of anti-inflammatory asthma medication [1]. Much effort had been made to find a non-invasive marker to assess the presence and the intensity of airway inflammation. Measurements of several blood markers of inflammation turned out to be insufficiently sensitive. Analysing bronchoalveolar fluids or bronchial tissue for inflammatory cells or their products does not present an alternative in children, as it can only be obtained invasively by an endoscopy of the lung [5].

It has been shown that patients with asthma exhibit increased amounts of NO in the airway epithelium, compared to healthy controls [6]. Because NO is generated from L-arginine by various cells in the airway including airway and alveolar epithelial cells, vascular endothelial cells, smooth muscle cells, and alveolar macrophages in consequence of the inflammatory process, the concentration of exhaled NO (eNO) is proposed to be a non-invasive and facile test or marker to assess eosinophilic airway inflammation in asthma bronchiale, even in children [7, 8]. ENO concentrations was found to be increased in allergic children with asthma and correlate with the degree of blood and airway eosinophilia, another marker of chronic allergic inflammation [6, 9].

The aim of this study was to investigate eNO concentrations in non-allergic, mono- or polysensitised allergic children with asthma bronchiale before and after exercise challenge.

METHODOLOGY

Subjects and Study Protocol

Within a period of four weeks in spring time (birch pollen season), measurements were taken from 59 consecutively in the paediatric clinic of the children's hospital of the city of Cologne referred children (37 male and 22 female, age between 6 and 17 years) with the diagnosis of asthma bronchiale according to the diagnostic criteria of the German Society of Paediatric Pneumonology [10] based on the evidence of a bronchial hyperresponsiveness. All Children were able to perform a lung function test. Because the study was

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1874-3099/08

conducted prospectively in a continuous time interval limited by the beech pollen season, an exercise challenge was only performed when clinically necessary i.e. when bronchial hyperresponsiveness had to be quantified to adjust the anti-inflammatory asthma therapy. Exercise challenge was conducted on a treadmill for 6 min with a subsequent eNO measurement and lung function testing.

Allergy Test Procedure

A serum-specific IgE > 0.34 kU/L (CAP-RAST) or a positive prick skin test (wheal > 2mm larger than negative control) to at least one of the tested allergens (house dust mite (*D. pteronyssimus*, *D. farinae),* cat, dog, horse, grass or birch pollen) was used to define the subjects being allergic.

Pulmonary Function Testing

Lung function was measured by spirometry and whole body plethysmography (Masterlab, Jaeger, Würzburg, Germany) and performed pre-exercise according to the criteria of the European Respiratory Society [11]. Patients were asked to withhold β2-agonists (Salbutamol) for at least 12 hours, inhaled steroids were not withdrawn prior to testing. After measuring specific airway resistance (sR_{aw}) and residual volume (RV) by body plethysmography, baseline spirometry with forced expiration was performed. Forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), forced expiratory flow at 50% of vital capacity ($MEF_{50\%}$) were measured, and the FEV_1/VC ratio was calculated. The upper limit of normal for SR_{aw} was defined as 1 kPa*sec. All other lung function values were expressed as percentage of predicted pediatric reference values [12].

ENO Measurements

The amount of NO exhaled was measured according to the American Thoracic Society (ATS) and the European Respiratory Society (ERS) recommendations [13, 14] by using the online single breath method with the NIOX MINO system (Aerocrine AB, Stockholm, Sweden), which works with an electrochemistry method for valid NO-measurement. The performing data given by the NIOX MINO are according to 25 °C standard temperature, 50% relative humidity and 1013 kPa pressure. The subject was seated comfortably with the instrument at a convenient position in front of the subject. No nose clip was used. The subject inserted the mouthpiece and inhaled NO-free air to total lung capacity over a period of 2 to 3 seconds through the mouthpiece of the NIOX instrument. The subject then started exhalation against a positive mouthpiece counter pressure of 10 to 20 cm H_2O to ensure that the soft palate was closed against the nasal cavity thus preventing contamination of eNO with NO of nasal origin. Exhalation flow was 50 ml/s. A measurement was accepted if -1- the mean flow was 0.045 to 0.055 l/s, -2- the instant flow was 0.0375 to 0.0625 l/s, and -3- the instantaneous mouth pressure was 5 to 20 cm H_2O. NO concentrations of 20 parts per billion (ppb) and higher are supposed to be predictive of the diagnosis of asthma bronchiale [15, 16].

Statistical Analysis

Descriptive data were expressed as mean with standard deviation scores or median when appropriate. The Mann-Whitney U-Test was used to compare two non-parametric, non-paired groups of small sizes (patients clinical characteristic data of non-allergic and allergic children), the Wilcoxon rank sum test for two non-parametric, paired groups of small sizes (eNO before and after exercise test), the Kruskal-Wallis-Test for multiple non-parametric, non-paired groups of small sizes (eNO with multiple sensitizations and allergens) and the Spearman rank correlation coefficient for determining correlations (between eNO, patients characteristics, lung function parameters and asthma medication) with a *p*-value of < 0.05 considered to be statistically significant. The calculation of the statistic tests were done with the SPSS software.

RESULTS

Clinical Characteristics of Patients

Of the 59 children, 10 were non-allergic while 49 were sensitised at least to one allergen. There was no significant difference between the non-allergic and allergic group regarding age, gender, height and asthma medication with a median inhaled dose of cortisone of 107 µg budenosid-equivalent per day (range 0-1000 µg/day) (Table **1**). However, the non-allergic children showed a higher average weight (65.3 kg *vs* 42.0 kg, p = 0.03) and thus a higher BMI (22.8 kg/m² *vs* 18.7 kg/m^2, p = 0.03). This difference of allergic and non-allergic children was only due to a higher weight and BMI of the non-allergic girls (weight 62.7 kg *vs* 39.6 kg, p = 0.003; BMI 24.1 kg/m² *vs* 18.3 kg/m^2, p = 0.006).

Table 1. Clinical Characteristics of Study Patients

	Allergic	Non-Allergic	*p*-Value
Total Number	49	10	-
Age (years)	12 (7-17)	13.5 (6-16)	0.26
Male (%)	65.3	50	0.37
Height (cm)	151.5 (125-179)	160.6 (120-190)	0.08
Weight (kg)	42.0 (23.0-88.4)	65.3 (23.3-95.8)	0.03[a]
BMI (kg/m^2)	18.7 (12.7-32.1)	22.8 (16.8-26.8)	0.03[a]
Inhaled Steroids	87 (0-400)	204 (0-1000)	0.71

Data expressed as median (range), inhaled steroids (in µg budesonide equivalent per day) as mean (range); [a]significant with p<0.05, Mann-Whitney U-test.

Allergic Sensitivities

Out of the 49 sensitised patients, 20 children had one (8 to pollen, 10 to house dust mites and 2 to pets) sensitivity, another 20 children had two sensitivities (12 to pollen and house dust mites, 7 to pollen and pets and 1 to house dust mites and pets), and 9 children showed sensitivities to all types of allergens.

Pulmonary Lung Function Parameters

There were no significant differences between the allergic and the non-allergic group in lung function parameters (sRaw, RV, FEV1, FVC, FEV1/FVC, MEF50 before and sRaw and MEF50 after exercise test (*p*-value > 0.4 for each comparison).

ENO Measurements

The eNO values of the whole patient group showed a non-parametric distribution with a median of 20 ppb, a mean of 35.4 ppb and a standard deviation of 38.3 ppb.

The mean value of eNO in allergic children was 40.7 ppb, with a minimum of 5 ppb and a maximum of 199 ppb before exercise test. Children without an allergic sensibilisation exhibited remarkably lower values of eNO, with a mean value of 9.3 ppb, a minimum of 5 ppb and a maximum of 13 ppb before exercise test. Thus, the mean difference between the eNO values in the allergic children and non-allergic children was 301.4 ppb ($p < 0.0001$) (Table **2**).

Table 2. Exhaled NO Levels (eNO) in Parts Per Billion (ppb) in Alergic and Non-Allergic Children with Asthma Bronchiale

eNO	Allergic	Non-Allergic	Total
N	49	10	59
Mean ± SD (ppb)	40.7[a] ± 40.0	9.3 ± 2.8	35.4 ± 38.3
Minimum (ppb)	5	5	5
Maximum (ppb)	199	13	199

[a]Significant with $p < 0.0001$, Mann-Whitney U-test.

21 of the 49 allergic children (43 %) had raised eNO levels (higher than 20 ppb in healthy subjects); whereas none of the non-allergic children had an increased eNO concentration with the highest eNO level was only 13 ppb. In contrast, 36 out of 49 allergic children (74 %) had an eNO level above 13 ppm. Additionally, asthmatic children with only one sensibilisation had a mean eNO concentration of 48.6 ppb, with two sensibilisations of 30.9 ppb and with three sensibilisations of 45.0 ppb demonstrating no significant correlation between eNO concentrations and the number of sensibilisations ($p = 0.59$). There was also no significant difference in the eNO concentrations regarding the different types of allergens. 73.5% of the allergic children were at least sensitized to pollen alone or in combination to another allergen and eNO values in pollen sensibilisation (mean 47.5 ppb) were higher compared to children without pollen sensibilisation (mean 30.9 ppb), however this difference was also not statistically significant ($p = 0.35$), even when birch pollen sensibilisation (birch pollen season) was analysed separately ($p = 0.06$).

Analysing patients data for children with an eNO level over the 90th percentile, there were six children with an eNO level over 97 ppb, ranging from 97 ppb to 190 ppb. All of them had only a mild asthma according to the analysis of their lung function tests. They all, except one patient (eNO value of 97 ppb), did not have any inflammatory asthma therapy (inhaled corticosteroids), and all of them, except one (with eNO 113 ppb), who had an allergy against house dust mites, were sensitised to birch pollen.

Exercise challenges could be done with 31 allergic and 7 non-allergic children with asthma bronchiale. Allergic children had a mean eNO level of 37.0 ppb before and 32.7 ppb after exercise challenge, while non-allergic children showed a mean level of 10.0 ppb before and 9.3 ppb after exercise challenge. Thus, with all children of both groups, the level of eNO after exercise challenge was lower than before, being only significant for the allergic ($p < 0.001$) and not for the non allergic group ($p = 0.23$).

There was no correlation between eNO levels and gender, age, height, weight and BMI (all p-values > 0.26).

Evaluating the correlation between eNO and lung function parameters, there was no significance between eNo levels and sRaw, FEV1, FVC, FEV1/FVC, MEF50 before and sRaw and MEF50 after exercise test (all p-values > 0.12) (Table **3**), even when the allergic patients were analysed separately. However, a significant correlation could be established between eNO and RV ($p = 0.04$). This was even more significant, when analysis only included the group of allergic children ($p= 0.02$).

Table 3. Correlations Between eNO and Lung Function Parameters

Correlation of eNO to	N	r	*p*-Value
sRaw (kPa·s)	59	-0.20	0.12
RV (%)	55	0.28	[a]0.04
FEV1 (%)	59	0.08	0.54
FVC (%)	59	-0.005	0.97
FEV1/FVC	59	-0.02	0.86
MEF50 (%)	59	0.10	0.46
sRAW after ET	38	-0.04	0.82
MEF50 a. ET (%)	38	0.05	0.78

[a]Significant with $p<0.05$, r = Spearman rank correlation coefficient.

The analysis of the correlation between eNO values and anti-inflammatory asthma medication showed no significance ($p = 0.13$), also if the group of asthmatics receiving any asthma medications was analysed separately ($p = 0.34$).

DISCUSSION

Aim of the study was to investigate whether there was a difference in eNO concentrations and lung function parameters in non-, mono- and poly-sensitised children with mild asthma bronchiale before and after exercise challenge.

59 children were tested, of which 49 were allergic and 10 were not. The small amount of non allergic asthmatics in the given period represents the low relation of non-allergic to allergic childhood asthma. Among the allergic and non-allergic asthmatic children, there was no significant difference in clinical characteristics, except non-allergic children had a significantly higher weight and hence BMI, what was caused only by the non-allergic girls. This finding has recently been described in population-based studies in adults [17, 18], but not yet in children. The allergic and non-allergic children, who had a mild and stable asthma, did neither differ in their lung function before or after exercise challenge, nor in the medication received. However, according to the literature [6], allergic patients exhaled a remarkably higher concentration of NO compared to non-allergic children. In our collective, all non-allergic children had eNO concentrations below 13 ppb, whereas 73% of the allergic

patients had values above this level and 43% had eNO concentrations above the supposed normal value of 20 ppb.

No correlation was found between eNO levels and age, gender, weight, height or BMI. Similar to Silvestri *et al.* [6], we also found no difference in the eNO levels between mono- and poly-sensitized allergic asthmatics, indicating that the number of allergens had no effect on NO-exhalation. Concentrations of RAST values or severity of reaction of the skin-tests (PRICK) had not been investigated. We found no significant correlation between eNO and the type of allergen, but it appears that children, who were sensitised to pollen (and here especially birch-pollen), exhale the highest levels of NO. But this trend was not statistically significant. The rather high levels of eNO in birch-pollen sensitised children, who mostly exhibit polliniotic symptoms additionally, were probably due to the fact that the data had been collected during birch pollen season. In this context Welsh *et al*. [19] cautioned about eNO not being a valuable tool for the detection of asthma as it is confounded by other atopic conditions as allergic rhinitis or atopic dermatitis.

No difference was found between the eNO levels and lung function values representing asthma severity. In agreement to Mappa *et al.* [20], there was a correlation between eNO levels and the RV especially in the allergic group. Other investigators however, who did not find any correlation between lung function and eNO, conducted only a spirometry [6] or they did not analyse RV in their body plethysmographic data (8, personal communication). This correlation of eNO and RV may be a relevant finding, for RV is an indicator for a chronic airway inflammation and trapping of air within the lungs, a specific finding in the asthma disease, and has demonstrated to function as a predictive value for asthma exacerbations even in patients with normal or near normal flow-volume values [20].

Another indicator of asthma severity, the amount of medication the patients receive, did not correlate with the eNO levels. This may indicate that the intake of the anti-inflammatory medication keeps the eNO values down, suggesting that most of the children had well adjusted medication. This is exemplarily emphasized by the data of those 6 patients, who had the highest eNO levels (> 97 ppb). None of them, except one, had a severe asthma according to their lung function values, and none were on anti-inflammatory medication. All of them were allergic to birch pollen, which may bee a possible confounder of the eNO values exhaled by asthmatics

As a further aspect we found that all eNO levels after exercise challenge were lower than prior exercise challenge. This finding proves that the nature of this bronchial provocation by the exercise challenge does not induce an inflammatory process which can be measured by eNO levels. These lower eNO levels after exercise challenge can be well explained by the lungs being cleared of NO due to the increased gas exchange; however this effect was rather small. Similar findings are also mentioned regarding free exercise and the spirometry maneuver [21], in which patients ventilate an increased lung volume.

As a conclusion, the measurement of eNO can differentiate between allergic and non-allergic children with asthma bronchiale as a marker of the allergic- inflammation in the airways.

However, the eNO concentration does not reflect asthma severity. It may be helpful in adapting the anti-inflammatory treatment, but only for patients with allergic asthma bronchiale. The influence of exercise challenge on eNO levels is minimal.

ACKNOWLEDGEMENTS

Many thanks to Mrs. R. Sindermann from the Allergy Department of the Children's Hospital of Cologne who took the lung function measurements and to Mr. T. Walsh from St., Clare´s College, Oxford for critical review.

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Received: April 7, 2008 Revised: April 30, 2008 Accepted: June 7, 2008