# AIRWAY BIOLOGY

# Relationship between exhaled NO, respiratory symptoms, lung function, bronchial hyperresponsiveness, and blood eosinophilia in school children

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**Background:** Exhaled nitric oxide (eNO) may serve as a non-invasive marker of airway inflammation but its relationship with other commonly used measures has not been evaluated.

**Methods:** Levels of eNO in a sample of 450 children aged 7–12 years out of a total sample of 2504 school children living in different urban areas near motorways were determined. The aim of this cross-sectional study was to explore the relationship between eNO, impairment of lung function (PEF, FVC, $FEV_1$ and MMEF), bronchial hyperresponsiveness (BHR), and blood eosinophilia in children with and without atopy as assessed by skin prick testing.

**Results:** Regression analysis showed that wheezing and nasal discharge and conjunctivitis that had occurred during the previous 12 months were positively associated with eNO levels in atopic children (relative increase of 1.48 and 1.41, respectively; $p < 0.05$) but not in non-atopic children. Similarly, BHR and the number of blood eosinophils per ml were positively associated with eNO levels in atopic children (relative increase of 1.55 and 2.29, respectively; $p < 0.05$) but not in non-atopic children. The lung function indices PEF, FVC, $FEV_1$ and MMEF were not associated with eNO levels.

**Conclusions:** In addition to conventional lung function tests and symptom questionnaires, eNO is a suitable measure of airway inflammation and its application may reinforce the power of epidemiological surveys on respiratory health.

L ung function and respiratory symptoms are routinely measured in studies on asthma and airway allergy. Recently, exhaled NO (eNO) has been shown to be a useful marker of the inflammatory response in allergic airway disease. Asthmatic and atopic subjects[1–9] have increased eNO levels which subsequently decrease in response to anti-inflammatory treatment.[10] The function of NO in the lungs is not yet known in detail. For example, inhibition of NO synthase does not affect the baseline value of forced expiratory volume in 1 second ($FEV_1$)[11 12] and inhalation of methacholine or salbutamol does not change eNO.[13] It has, however, been clearly established that inducible NO synthase is expressed in the lung epithelium of asthmatic subjects[12] for which proinflammatory cytokines are thought to be responsible.

Airway inflammation is involved in asthma and respiratory allergy.[14–16] The degree of impairment of lung function is related to airway inflammation but is not a measure of inflammation as such. Subclinical inflammation may precede actual functional impairment so that conventional lung function measures may be insufficient to detect the inflammatory component of adverse health effects.

We previously applied eNO as a marker of inflammation to study the inflammatory responses involved in the adverse health effects of air pollution and observed positive associations between increased exposure to air pollution, eNO levels, and proinflammatory nasal lavage markers in healthy[17] and allergic children.[6] In addition, in a relatively small cohort of 68 children aged 10–11 years studied for a period of 7 weeks we found positive associations between respiratory symptoms and eNO levels (p<0.05), but not between respiratory symptoms and pulmonary function measures (peak expiratory flow (PEF), forced vital capacity (FVC), $FEV_1$, and maximal mid expiratory flow (MMEF)).[18]

To explore further whether eNO is related to impairment of lung function and reported respiratory symptoms, a cross sectional study was performed within the framework of a Dutch study of children living near motorways.

## METHODS
### Study subjects
The study sample consisted of a convenience sample of 450 children aged 7–12 years out of a total sample of 2504 children living in different urban areas near motorways in The Netherlands. Children were recruited from seven public schools situated near motorways and permission was obtained to assess lung function. The schools came from a larger sample of 24 schools which were studied in random sequence. For logistical reasons the eNO measurements could only be performed near the end of the study period in the last seven schools to be studied. These were unselected with respect to the total sample of 24 schools. The medical ethics committee of Wageningen University approved the study protocol and parents of all subjects gave written consent for their participation in the study. No specific inclusion or exclusion criteria were applied. The study protocol comprised the ISAAC questionnaire and additional questions on potential confounders and lung function testing. For children aged 8 years or older a bronchial challenge with 4.5% NaCl, skin prick tests (SPT), and blood withdrawal was added for the determination of eosinophil counting and serum IgE.

### Skin prick tests
The skin prick tests were performed according to the standardised ISAAC phase II protocol.[19] The ISAAC panel of six aeroallergens was used: *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, cat, *Alternaria tenuis*, mixed tree pollen (*Betula verrucosa*, *Alnus glutinosa*, *Corylus avellana*) and mixed

grass pollen (*Dactylis glomerata*, *Lolium perennae*, *Festuca pratensis*, *Poa pratensis*, *Phleum pratense*, *Avena eliator*). In addition, dog allergen was used. Standardised allergen extracts (all but *Alternaria*) and positive and negative controls (saline and histamine 10 mg/ml) were provided by ALK (Copenhagen, Denmark). The test was performed on the volar side of the left forearm using ALK lancets. After 15 minutes the outside contour of the wheal was measured and the mean of the longest diameter and length of the perpendicular line through its middle was calculated. Children with a reaction (>0 mm) to the negative control or no reaction to histamine were set missing. A mean wheal diameter of 3 mm or more was regarded as a positive reaction according to the position paper on skin tests of the European Academy of Allergy and Clinical Immunology.[20]

### Spirometric tests and bronchial challenge
Lung function indices (FVC, $FEV_1$, PEF, MMEF) were measured according to the ERS guidelines using a Jaeger pneumotachograph.[21] Bronchial hyperresponsiveness (BHR) was assessed according to the ISAAC protocol[22] using hypertonic saline. The result of the bronchial challenge test was expressed as BHR defined by a fall in $FEV_1$ of ⩾15% compared with the reference value after inhalation of a minimum of 23 ml.

### Sampling of exhaled air and assay of exhaled NO
Exhaled air of the children was sampled in balloons using a sampling device equipped with a "pre-balloon" of 500 ml to exclude dead space volume from the sample, as previously described.[23] In short, after exhalation at 4–6 l/min into a "pre-balloon", exhaled air was collected at a low flow rate of 500 ml/min at 20 cm $H_2O$ back pressure in a foil bag of 1000 ml (Mylar balloon, ABC ballonnen, Zeist, The Netherlands). A low flow rate was chosen to obtain relatively high eNO concentrations. Although this method is different from ERS and ATS recommended methods,[24 25] our group and others have shown previously that it correlates well with on line measurements made in the laboratory which have followed ERS and ATS recommendations.[26 27]

Outdoor air was sampled by an air sampling pump (Dupont de Nemours & Company Inc, Model P-200, Wilmington, Delaware, USA) in aluminium foil air bags and ambient temperature was measured continuously. A rapid response chemiluminescence analyser (Sievers 280B, Boulder, USA) with a response time of <200 ms was used to determine the concentration of eNO. The analyser sample flow rate was kept constant at 200 ml/min. The eNO concentration was measured within 4 hours after sampling. In the aluminium foil bags the NO concentration remains constant for at least 48 hours. Repeatability of eNO measurement was good (standard deviation <5%).

### Questionnaire
The questionnaire consisted of the "core questionnaire module" and the "additional respiratory questions module" of the ISAAC questionnaire[22] which asked questions about the child's home, family composition, education level, and smoking habits of the residents. The following symptoms as reported by the respondents were analysed: wheezing, waking up with a wheeze, itchy rash, dry cough, nasal discharge and conjunctivitis, phlegm not due to a cold (all within the last 12 months), hay fever ever, asthma ever, eczema ever, and bronchitis ever. In addition, respondents were asked whether bronchitis or allergy had been assessed by a physician—that is, doctor diagnosed (DD).

### Blood eosinophils
Peripheral blood eosinophils were determined in a venous blood sample by Coulter counter autoanalyser (AML, Antwerp, Belgium) and expressed as the number of cells per ml.

### Statistical analysis
Associations between eNO levels, lung function, respiratory symptoms, and other variables were analysed with a mixed model (SAS proc MIXED) taking into account that children attending the same school may share certain characteristics that may influence the relation between eNO and individual determinants of eNO. The eNO values were log transformed before analysis to normalise residuals. The analyses were adjusted for the following potential confounders: age, sex, gas cooking, unvented water heater, environmental tobacco smoke exposure, and having a cold during sampling. The results were expressed as the relative increase in eNO in children with compared with children without symptoms, eosinophilia or BHR. For continuous variables, relative increases were expressed per litre (per second) for the lung function variables, and per interquartile range (that is, the difference between the 25th and 75th percentiles of the distribution) for the number of eosinophils per ml peripheral blood. As atopy may alter the relationship between eNO and its other determinants, we have performed the analysis stratifying for atopy as assessed by skin prick testing. A mean wheal diameter of 3 mm or more was used to establish the presence of atopy.[20] A more detailed analysis of the association between eNO levels and allergic sensitisation will be published elsewhere.[28]

## RESULTS
Table 1 shows that the children in our study sample did not differ from those of the initial study population from which they were drawn.

Regression analysis showed that a number of symptoms were associated with a significant increase in eNO levels after adjustment for relevant confounders. Table 2 shows that wheezing, waking up with wheeze, and nasal discharge combined with conjunctivitis were significantly associated with eNO levels (relative increase of 1.26, 1.33, and 1.55, respectively). In addition, asthma ever, hay fever ever, eczema ever, and recent bronchitis were also significantly associated with eNO levels (relative increase of 1.29, 1.46, 1.18, and 1.25, respectively). Reported exposure to tobacco smoke did not affect the level of eNO. Following stratification for the presence of atopy as assessed by skin prick test (compared with tables 3 and 4), it is of interest to note that, in non-atopic children, no positive associations were observed (significant negative associations were observed for wheezing and history of hay fever). In contrast, even stronger positive associations were observed in atopic children between eNO levels and wheezing and nasal discharge and conjunctivitis. The presence of BHR and the number of blood eosinophils were also closely

**Table 1** Characteristics (%) of the study population and the sample population

|  | Study population (n=450) | Sample population (n=2504) |
|---|---|---|
| Mean age (years) | 10.0 | 9.5 |
| Sex (boys) | 50.0 | 48.9 |
| Itchy rash in last 12 months | 19.0 | 15.1 |
| Wheeze in last 12 months | 10.0 | 9.5 |
| Dry cough in last 12 months | 21.9 | 21.7 |
| Nasal discharge in last 12 months | 25.6 | 22.6 |
| Conjunctivitis in last 12 months | 10.9 | 7.9 |
| Phlegm in last 12 months | 11.3 | 9.7 |
| Eczema ever | 29.1 | 29.9 |
| Asthma ever | 9.0 | 8.1 |
| Hay fever ever | 8.2 | 7.3 |
| Doctor diagnosed bronchitis | 8.4 | 7.8 |

**Table 2** Adjusted relative increase in eNO levels in children with symptoms compared with children without symptoms; in children with and without bronchial hyperresponsiveness; and per unit increase in eosinophils and lung function

| Variable | Relative increase | 95% confidence interval |
|---|---|---|
| Wheeze in last 12 months | 1.26 | 1.07 to 1.48 |
| Woken up with wheeze in last 12 months | 1.33 | 1.10 to 1.62 |
| Itchy rash in last 12 months | 1.08 | 0.95 to 1.22 |
| Asthma ever | 1.29 | 1.09 to 1.52 |
| Hay fever ever | 1.46 | 1.22 to 1.74 |
| Eczema ever | 1.18 | 1.06 to 1.32 |
| Bronchitis in last 12 months | 1.25 | 1.05 to 1.48 |
| Dry cough in last 12 months | 0.99 | 0.88 to 1.11 |
| Nasal discharge and conjunctivitis in last 12 months | 1.55 | 1.33 to 1.80 |
| Having a cold during exhaled breath collection | 1.30 | 1.14 to 1.47 |
| Bronchial hyperrresponsiveness | 1.30 | 1.13 to 1.49 |
| Eosinophils (per 1000) | 1.89 | 1.51 to 2.38 |
| Increase in eNO/l change in $FEV_1$ | 0.95 | 0.77 to 1.17 |
| Increase in eNO/l change in FVC | 1.04 | 0.85 to 1.26 |
| Increase in eNO/l/s change in PEF | 1.02 | 0.96 to 1.09 |
| Increase in eNO/l/s change in MMEF | 1.00 | 0.91 to 1.10 |

Adjusted for age, sex, gas cooking, unvented water heater, environmental tobacco smoke exposure, and having a cold during the eNO sampling; lung function analyses were adjusted also for height and weight.

**Table 3** Adjusted relative increase in eNO for children with symptoms compared with children without symptoms; for children with and without bronchial hyperresponsiveness; and per unit increase in eosinophils; children without atopy (n=205)

| Variable | Relative increase | 95% confidence interval |
|---|---|---|
| Wheeze in last 12 months | 0.76 | 0.60 to 0.96 |
| Woken up with wheeze in last 12 months | 0.66 | 0.46 to 0.93 |
| Itchy rash in last 12 months | 0.93 | 0.81 to 1.06 |
| Asthma ever | 0.90 | 0.70 to 1.16 |
| Hay fever ever | 0.68 | 0.48 to 0.98 |
| Eczema ever | 1.03 | 0.91 to 1.17 |
| Bronchitis in last 12 months | 1.11 | 0.88 to 1.41 |
| Dry cough in last 12 months | 0.89 | 0.78 to 1.01 |
| Nasal discharge and conjunctivitis in last 12 months | 1.04 | 0.81 to 1.32 |
| Bronchial hyperrresponsiveness | 0.94 | 0.82 to 1.09 |
| Eosinophils (per 1000) | 1.13 | 0.88 to 1.46 |

Adjusted for age, sex, gas cooking, unvented water heater, environmental tobacco smoke exposure, and having a cold during the eNO sampling.

**Table 4** Adjusted relative increase in eNO for children with symptoms compared with children without symptoms; for children with and without bronchial hyperresponsiveness; and per unit increase in eosinophils; children with atopy (n=106).

| Variable | Relative increase | 95% confidence interval |
|---|---|---|
| Wheeze in last 12 months | 1.48 | 1.09 to 2.02 |
| Woken up with wheeze in last 12 months | 1.61 | 1.10 to 2.36 |
| Itchy rash in last 12 months | 1.01 | 0.76 to 1.33 |
| Asthma ever | 1.36 | 0.98 to 1.89 |
| Hay fever ever | 1.18 | 0.87 to 1.61 |
| Eczema ever | 1.26 | 0.98 to 1.62 |
| Bronchitis in last 12 months | 1.15 | 0.80 to 1.65 |
| Dry cough in last 12 months | 0.98 | 0.74 to 1.30 |
| Nasal discharge and conjunctivitis in last 12 months | 1.41 | 1.08 to 1.84 |
| Bronchial hyperrresponsiveness | 1.55 | 1.14 to 2.10 |
| Eosinophils (per 1000) | 2.29 | 1.32 to 3.44 |

Adjusted for age, sex, gas cooking, unvented water heater, environmental tobacco smoke exposure, and having a cold during the eNO sampling.

associated with increased eNO levels (table 2; relative increases of 1.30 and 1.89, respectively). This relationship again was only significant in the atopic children. Surprisingly, commonly used lung function indices such as PEF, FVC, $FEV_1$ and MMEF were not associated with increased eNO levels.

## DISCUSSION
This study has shown that, in a random sample of school children, eNO was significantly associated with respiratory symptoms, BHR, and blood eosinophilia but not with spirometric indices of lung function.

Strong positive associations were observed in atopic but not in non-atopic children for recent wheeze (+48%) and rhinoconjunctivitis (+41%). These results are in agreement with the previously reported increase in eNO in asthmatic and atopic subjects.[1-9] Sensitisation to seasonal outdoor allergens is related to allergic rhinitis,[29] whereas sensitisation to indoor allergens appears to be more closely related to asthma.[30 31]

The present data show that the lung function measures PEF, $FEV_1$, FVC, and MMEF were not associated with eNO, indicating that these functional measures either fail or have too low sensitivity to detect airway inflammation as assessed by eNO measurements. This is also in line with the results of Silvestri et al[32] who considered that the airway inflammation may not be strictly related to the reduction in lung volumes or to the degree of airflow limitation. In contrast, in atopic children the presence of BHR was positively associated with eNO (relative increase of 1.55). This is in agreement with previous results[33] and suggests that BHR may be associated with airway inflammation.[14-16]

Finally, it has been reported that the number of blood eosinophils and the level of eosinophil derived proteins correlate well with asthma severity[15 16] and bronchial hyperresponsiveness.[34-36] The present study shows a strong association between eNO levels and the number of blood eosinophils in atopic children (relative increase of 2.29 for an increase of 1000 eosinophils per ml, which is approximately equivalent to one interquartile range). This finding is in agreement with previous results obtained in asthmatic but not in control subjects.[32 33 37 38] Indeed, in our study a significant association was also found between eNO and blood eosinophilia only in atopic subjects. Eosinophilia is a feature of allergy and asthma. Eosinophils generate reactive oxidative intermediates including NO[39] which may cause tissue damage.

Spirometric lung function testing and measurement of respiratory symptoms by questionnaire have a proven track record in determining adverse respiratory effects in epidemiological studies. Measurement of eNO may add another dimension because it enables inflammatory responses to be detected without the presence of functional impairment. In addition, eNO appears to be a reliable tool for confirming asthmatic, allergic, and bronchitis-like symptoms.

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