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Additive effect of eosinophilia and atopy on exhaled nitric oxide levels in children with or without a history of respiratory symptoms

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Although atopy and blood eosinophilia both influence exhaled nitric oxide (eNO) measurements, no study has quantified their single or combined effect. We assessed the combined effect of atopy and blood eosinophilia on eNO in unselected schoolchildren. In 356 schoolchildren (boys/girls: 168/188) aged 9.0–11.5 yr, we determined eNO, total serum IgE, blood eosinophil counts and did skin prick tests (SPT) and spirometry. Parents completed a questionnaire on their children's current or past respiratory symptoms. Atopy was defined by a SPT $>$ 3 mm and eosinophilia by a blood cell count above the 80th percentile ($>$ 310 cells/ml). eNO levels were about twofold higher in atopic–eosinophilic subjects than in atopic subjects with low blood eosinophils [24.3 p.p.b. (parts per billion) vs. 14.1 p.p.b.] and than non-atopic subjects with high or low blood eosinophils (24.3 p.p.b. vs. 12.2 p.p.b. and 10.9 p.p.b.) ($p < 0.001$ for both comparisons). The additive effect of atopy and high eosinophil count on eNO levels remained unchanged when subjects were analyzed separately by sex or by a positive history of wheeze (n = 60), respiratory symptoms other than wheeze (n = 107) or without respiratory symptoms (n = 189). The frequency of sensitization to *Dermatophagoides* (Dpt or Dpf) was similar in atopic children with and without eosinophilia (66.2% and 67.4%, respectively); eosinophilia significantly increased eNO levels in Dp-sensitized children as well in children sensitized to other allergens. In a multiple linear regression analysis, eNO levels were mainly explained by the sum of positive SPT wheals and a high blood eosinophil count (t = 4.8 and 4.3, p = 0.000), but also by the presence of respiratory symptoms (especially wheeze) and male sex (t = 2.6 and 2.0, p = 0.009 and 0.045, respectively). Measuring eNO could be a simple, non-invasive method for identifying subjects at risk of asthma in unselected school populations.

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Exhaled nitric oxide (eNO) is now recognized as a non-invasive tool for monitoring various respiratory diseases. Because eNO is usually found increased in asthmatic subjects, numerous studies have focused on the correlation of eNO levels with eosinophil counts in blood, bronchial tissues and secretions in asthmatic subjects (1–7) and in those defined as atopic by positive allergen-skin test reactions or high serum IgE levels (8–15). Less is known about the possible additive effect of eosinophilia and atopy on eNO levels in asthmatic children and more important, in healthy children.

The meaning of increased eNO levels in children with a negative history of chronic respiratory symptoms is also under debate, partly

because ethical reasons preclude invasive studies of airway inflammation in healthy children. In a preceding study, whereas we found increased eNO in atopic asthmatic children, in healthy atopic children we did not, probably because few children had severe atopy alone (14). Conversely, others, in a larger healthy population reported that eNO levels increased in parallel with the number of positive allergen-skin reactions (16). Neither of these two studies included blood eosinophil counts.

Studying the combined effect of blood eosinophilia and atopy on eNO levels could provide important insights into airway disease in unselected schoolchildren populations. High blood eosinophil counts and atopic status are known risk factors of lung function impairment and respiratory symptoms (17–20). Measuring eNO therefore has the potential advantage of allowing preventive measures at a very early stage, i.e. before lung function impairment or symptoms, or both, develop.

In this study, to extend our previous findings in asthmatic children (14), we primarily investigated the relationship between eNO levels and the combined outcomes of atopy (a negative or positive skin prick test) and blood eosinophil counts (low or high) in an unselected population of children. In the same population we also compared eNO levels, serum IgE, lung function and respiratory symptoms. We analyzed the data overall and separately for sex and in children with and without a history of respiratory symptoms. We also assessed the distribution of house-dust mite sensitization and its eventual influence on our results. A multiple linear regression analysis was then used to assess the effect of all the potential explanatory variables on eNO.

Materials and methods

Population

Subjects were drawn from a non-selected population of schoolchildren from central Italy (Ronciglione, Caprarola, and Guardea) following a previously described method (14). Parents of children (approximately 9–11 yr of age) from IV and V grade courses were asked to complete a questionnaire and to give permission for their children to be tested. Permission for all measurements (blood sampling, skin prick tests, lung function and eNO measurements) was obtained for 370 of 450 (82%) subjects. None of these children were currently using inhaled or oral corticosteroids. The study was approved by the Ethics Committee of the Pediatric Clinic.

Study design

According to the questionnaire replies the total population was analyzed by the presence or absence of chronic respiratory symptoms and allocated to three groups: no respiratory symptoms, respiratory symptoms other than wheeze, and wheeze. All subjects were assessed by the same criteria (questionnaire, blood eosinophils, IgE levels, skin prick tests, and eNO measurements), between October and December 2001.

Because our study was conducted in the Autumn, we measured the distribution of house-dust mite sensitization among atopic subjects in the whole population.

Questionnaire

Parents completed a modified version of the American Thoracic Society questionnaire for respiratory symptoms (21) that sought information on the child's history of respiratory symptoms and current medication as previously described (14). An affirmative answer to the question on 'current or past wheeze' (Has your child ever had asthma?; wheezing or whistling attacks) was accepted as a questionnaire-based diagnosis of wheeze. This criteria was not applied to subjects who answered affirmatively to any other question on respiratory symptoms but negatively to 'current or past wheeze'. 'Respiratory symptoms other than wheeze' was defined by an affirmative answer to persistent cough (a 3-month history of cough on ≥4 days a week), or a physician diagnosis of bronchitis or pneumonia in the previous 12 months, or rhinitis (hay fever or runny nose, apart from colds). Detailed respiratory questions are published elsewhere (14).

Spirometry

Dynamic volumes and flows were measured with a heated pneumotachograph (Hans Rudolph, Inc., Kansas City, MO, USA) connected to a lung function device (Oscillink®, Datalink, Montpellier, France); forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1) and mid-maximal expiratory flow ($FEF_{25–75}$) were measured and accepted as recommended (22).

Blood sampling

Blood eosinophil cell counts were assessed with an automatic blood cell counter (Advia 120, Bayer®, Leverkusen, Germany). Blood

eosinophils were measured as either the percentage or the absolute cell number of the total leukocyte count (cells/ml). An absolute blood eosinophil count above the 80% percentile in the whole population was used as a cut-off for defining a 'high eosinophil count'.

Skin prick tests

Allergen sensitization was measured by means of skin prick tests on the volar aspect of the forearm. The battery of allergens comprised *Dermatophagoides pteronyssinus* (Dpt), *Dermatophagoides farinae* (Dpf), cat hair, *Alternaria tenuis*, mixed grass, mixed tree pollen and *Parietaria officinalis* (Soluprick, ALK-Abellò, Horsholm, Denmark). Histamine dihydrochloride (10 mg/ml) was used as the positive control and diluent (50% glycerol and 50% physiological saline) as the negative control. After 12 min, wheal size was recorded in mm as the long axis and its perpendicular; the mean of these two measurements was calculated. A wheal ≥3 mm in size was considered to be a positive reaction to the allergen. Atopy was defined as the presence of at least one positive skin reaction. The sum of all positive reactions (sum of wheal sizes) in a subject was termed the 'prick index' (23).

Total IgE assay

Serum samples from venous blood were stored in aliquots at −70°C until tested. Aliquots of all sera were tested for total IgE by the immuno-CAP method (Pharmacia, Uppsala, Sweden).

Exhaled NO measurements

eNO was measured with a chemiluminescence NO analyzer (Sievers NOA^{TM} 280; Sievers Instruments, Inc., Boulder, CO, USA; response-time 0.02 s, sensitivity <1 p.p.b., range of measures < 1–500.000 p.p.b., repeatability ±1 p.p.b., sampling flow 200 ml/min. The zero signal was calibrated with an air filter (Sievers ACT 01400), and the measurement scale was calibrated with a gas containing 10 parts per million (p.p.m.) of NO in nitrogen (SIAD Srl, Bergamo, Italy).

Exhaled gas was collected into 1-l mylar bags (exhaled pressure of 10 cm H_2O and flow 58 ml/s) by a single-breath technique previously described and validated in the epidemiological field (14, 24).

All subjects were asked to avoid food intake and physical exercise for at least 2 h before testing. To ensure inhalation of NO-free air, a scrubber (North part no. N7500–2, Cranston, RI, USA) was placed in the inhalation port. An effort was accepted when the expiratory pressure fluctuated less than ±2 cm H_2O (from 10 cm H_2O) during the maneuver. Ambient eNO concentrations were assessed by filling two mylar bags with room air (one at the start, the other at the end of the testing procedures). All samples were transported to the laboratory and analysed with a maximal delay of 2 h after collection; in each subject, the mean eNO was calculated from two samples.

Statistical analysis

The Kolmogorov–Smirnov goodness-of-fit test was used to analyse the normal distribution of continuous variables. Skewed variables were transformed into natural logarithms (ln) and expressed as geometric means and 95% confidence intervals (CIs). Mean ratios and 95% CI were used to quote differences between two geometric means (because ln A−ln B = ln A/B). The Spearman's rho correlation coefficient was used to assess the relationship between eNO levels and measurements of atopy and blood cell count. Contingency tables (chi-squared test) were used for comparison of proportions; the unpaired *t*-test and a one-way analysis of variance (ANOVA), and *post hoc* Scheffé test, were used to compare two or more groups, as needed. A multiple linear regression analysis (stepwise method) was performed with ln-transformed eNO values as dependent variables and several potentially-explanatory (independent) variables selected on the basis of either a significant correlation with ln eNO or 2) significant differences for ln eNO values between categories of a single variable (e.g. differences in ln eNO between sexes, females = 0 and males = 1; or between subgroups of respiratory symptoms: absence = 0, symptoms other than wheeze = 1, wheeze = 2). 'Atopy' and 'prick index' were entered into the regression analysis as a single variable termed the 'prick index categories': no atopy (=0), and prick index values below the 33 percentile (≥ 3 ≤ 6.5 mm = 1), between percentiles 33 and 66 (>6.5 ≤ 12.5 mm = 2) and above the 66 percentile (>12.5 mm = 3).

Partial correlation plots between the dependent and independent variables were used to assess the linearity of residuals. Tolerance statistics was used to assess co-linearity (i.e. strong correlations between independent variables); tolerance = $1-R_i^2$, where R_i^2 is the squared multiple correlation of a variable with the other independent variables. The statistical software SPSS+ version

10.0 (SPSS, Chicago, IL, USA) was used for calculations. p-Values of less than 0.05 were considered statistically significant.

Results

A total of 370 participants underwent blood sampling, skin tests, and eNO measurements. Of these 370 subjects, 14 (eight without chronic respiratory symptoms, five with chronic respiratory symptoms other than asthma and one asthmatic subject) failed to complete the tests (five children refused blood sampling, three refused skin tests, and six were unable to cooperate in eNO measurements). The remaining 356 subjects completed the tests (mean age 10.2 yr, range 9.0–11.5, males/females 168/188), no significant difference was found for age according to sex (data not presented). No significant difference was found for age between boys and girls (data not presented). As described in study the design, the 356 subjects were classified as having no respiratory symptoms (healthy, $n = 189$), respiratory symptoms other than wheeze ($n = 107$), and wheeze ($n = 60$).

During eNO measurements, ambient NO levels were very low and remained fairly stable in all sessions (0.1 to 1.5 p.p.b.). eNO values correlated with blood eosinophil counts and percentages (for both: $r = 0.33$, $p = 0.000$), prick index ($r = 0.37$, $p = 0.000$) and total serum IgE levels ($r = 0.22$, $p = 0.000$). eNO was also weakly related with $FEF_{25-75}\%$ ($r = -0.12$, $p = 0.035$) but not with $FEV_1\%$. Females had lower eNO levels than males (geometric means 11.6 and 14.4 p.p.b.; mean ratio 0.80; 95% CI: 0.72–0.90). Geometric mean eNO levels (95% CI) increased significantly with the children's respiratory symptoms: healthy 11.2 p.p.b. (10.5–12), respiratory symptoms other than wheeze 13.7 p.p.b. (12.3–15.2), wheeze 17.5 p.p.b. (14.5–21.2), $p < 0.05$ between all groups by ANOVA, *post hoc* Scheffé test.

In the whole population, the 80% percentile for the absolute blood eosinophil count was 0.310×10^3 cells/ml (the 80% percentile for the eosinophil percentage was 4.1%). According to the cut-off value 217 subjects had a low eosinophil count and no atopy, 25 a high eosinophil count and no atopy, 68 a low eosinophil count and atopy and 46 a high eosinophil count and atopy. The atopic groups (low eosinophil count vs. high eosinophil count) yielded a similar mean prick index (8.5 mm, 95% CI: 7.2–10.1 vs. 9.7 mm, 95% CI: 8.2–11.6). Children with a high eosinophil count and atopy had twofold higher eNO levels than the other three groups. Subjects with a low eosinophil count and atopy showed slightly but significantly higher eNO values than children with a low eosinophil count and no atopy. Total serum IgE levels gradually increased in parallel with the blood eosinophil count, and the presence of atopy, or both, whereas lung function remained unchanged (Table 1). A high eosinophil count and atopy affected eNO values similarly in both sexes (Fig. 1). These findings remained similar when we analysed the data separately in subjects with or without chronic respiratory symptoms (Table 2).

Atopic children with low blood eosinophil counts had comparable frequencies of positive skin-test reactions for at least one Dp (Dpt or Dpf) to atopic children with high eosinophil counts: 45/68 (66.2%) and 31/46 (67.4%). In these atopic children the presence of eosinophilia significantly increased eNO levels in Dp-negative (sensitized to other allergens) and in Dp-positive subjects (Table 3).

The multiple linear regression analysis (stepwise) with ln eNO as dependent variable and

Table 1. Combined effect of atopy and high blood eosinophil count (>310 cells/ml) on prick index, total IgE levels, exhaled nitric oxide (eNO) and lung function

	No atopy		Atopy	
Variables	Low eosinophil count	High eosinophil count	Low eosinophil count	High eosinophil count
n	217	25	68	46
Prick index (mm)	0	0	8.5 (7.2–10.1)	9.7 (8.2–11.6)
IgE (UI/ml)	33.4 (27.5–70.4)	76.4 (48.4–120.6)*	114.5 (84.6–155.0)**	216.0 (161.2–288.4)**
eNO (p.p.b.)	10.9 (10.3–11.6)	12.2 (10.6–14.0)	14.1 (12.2–16.4)*	24.3 (20.0–29.4)**
$FEV_1\%$	104.2 (102.3–106.1)	102.6 (98.3–107.0)	104.9 (101.9–107.9)	107.8 (104.2–111.4)
$FEF_{25-75}\%$	112.1 (108.4–115.9)	103.7 (94.4–112.9)	109.9 (103.3–116.5)	112.3 (102.9–121.8)

Values are expressed as geometric means (prick index, IgE and eNO) or arithmetic means (lung function) and 95% confidence intervals. The prick index is defined as the sum of the positive wheals to allergen skin prick tests.

Analysis of variance (*post hoc* Scheffé test): eNO: **$p < 0.001$ vs. all groups; *$p < 0.01$ vs. non-atopic subjects with low blood eosinophil counts. IgE: **$p < 0.001$ and *$p < 0.05$ vs. non-atopic subjects with low blood eosinophil counts. Atopic subjects with high eosinophil counts had also significantly higher IgE levels than non-atopic subjects with high eosinophil counts ($p < 0.02$).

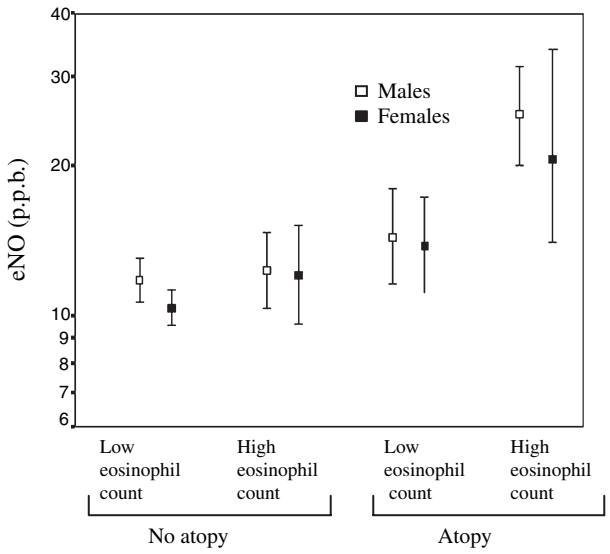

Fig. 1. Effect of high blood eosinophil count (>310 cells/ml) and atopy on exhaled nitric oxide (eNO) by sex. eNO values are expressed as geometric means and 95% CI.

logarithmic values of IgE (ln IgE), blood eosinophil counts (ln Eos), prick index categories, Dp sensitization, FEF_{25--75}%, respiratory symptoms, age, height and sex as independent variables yielded a four-variable model ($r = 0.51$, $r^2 = 0.26$). This model included prick index category ($t = 4.81$, $p = 0.000$), ln Eos ($t = 4.30$, $p = 0.000$), respiratory symptoms ($t = 2.64$, $p = 0.009$) and sex ($t = 2.01$, $p = 0.045$) as explanatory variables for ln eNO. The model is expressed by the equation:

$$\ln \text{eNO} = 1.455 + (0.149 \text{ prick index category}) + (0.174 \ln \text{Eos}) + (0.107 \text{ respiratory symptoms}) + (0.119 \text{ sex})$$

where prick index categories (0–3): no atopy (=0), prick index values below the 33 percentile (≥3 and ≤ 6.5 mm = 1), between percentiles 33 and 66 (>6.5 and ≤ 12.5 mm = 2) and above the 66 percentile (>12.5 mm = 3).

ln Eos: natural logarithm of blood eosinophil cell count (absolute cell number of the total leukocyte count expressed as ln cells/ml). respiratory symptoms (0–2): absence = 0, symptoms other than wheeze = 1, wheeze = 2). sex (0–1): female (0), male (1).

Discussion

Our main finding is that in a unselected population of schoolchildren eNO levels were highest in subjects who had a high blood eosinophil count and atopy; this finding remained unchanged among children sub-grouped either by sex or by a history of respiratory symptoms. Overall, eNO levels in our population were predicted by four variables: the main eNO-enhancing effect came from the degree of atopy and blood eosinophil number.

Table 2. Combined effect of atopy and high blood eosinophil count (>310 cells/ml) on prick index, total IgE levels, exhaled nitric oxide (eNO) and lung function in subjects grouped according to chronic respiratory symptoms

	No atopy		Atopy	
Variables	Low eosinophil count	High eosinophil count	Low eosinophil count	High eosinophil count
Healthy children				
n	139	13	25	12
Prick index (mm)	0	0	6.8 (5.0–9.2)	10.5 (7.3–15.2)
IgE (UI/ml)	34.9 (27.0–45.1)	71.9 (34.6–149.6)	87.9 (47.6–162.3)*	195.0 (126.0–301.6)**
eNO (p.p.b.)	10.2 (9.5–10.9)	13.1 (10.5–16.4)	13.4 (10.6–16.8)*	18.9 (13.0–27.6)**
FEV_1%	105.5 (102.9–108.1)	99.1 (93.3–105.0)	106.4 (101.0–111.7)	105.2 (99.1–111.4)
Respiratory symptoms other than wheeze				
n	56	10	26	15
Prick index (mm)	0	0	7.9 (6.0–10.5)	9.7 (7.2–13.1)
IgE (UI/ml)	29.7 (20.8–42.4)	87.6 (40.9–187.3)	101.2 (66.4–154.1)**	151.0 (81.3–280.2)**
eNO (p.p.b.)	12.6 (11.0–14.4)	10.8 (8.6–13.5)	13.8 (10.6–18.1)	21.4 (16.0–28.7)*
FEV_1%	103.0 (97.7–103.0)	107.3 (99.7–115.0)	102.4 (97.7.5–107.0)	111.1 (102.3–119.8)*
Wheeze				
n	22	2	17	19
Prick index (mm)	0	0	13.2 (10.1–17.3)	9.3 (6.8–12.7)
IgE (UI/ml)	34.1 (21.1–55.0)	57.5 (0.01–814.1)	204.1 (121.4–343.3)**	304.0 (195.7–472.0)**
eNO (p.p.b.)	11.8 (9.2–15.1)	13.5 (7.7–23.8)	15.8 (11.2–22.2)	31.3 (22.3–43.9)**
FEV_1%	105.8 (98.7–112.9)	100.5 (27.2–173.9)	106.2 (99.1–113.2)	107.2 (101.7–112.7)

*$p < 0.05$ and **$p < 0.005$ vs. non-atopic subjects with low blood eosinophil counts by anova and *post hoc* Scheffé test. In the group with respiratory symptoms other than wheeze, atopic subjects with high eosinophil counts had higher eNO values than non-atopic subjects with high eosinophil counts; in the wheeze group, atopic subjects with high eosinophil counts had higher eNO values than their counterparts with low eosinophil counts ($p < 0.05$ for both comparisons).

Table 3. Geometric mean eNO values (95% CI) in atopic children sensitized to house-dust mite (Dp+) or to other allergens (Dp−)

	Low eosinophil count		High eosinophil count		Mean ratios (95% CI)
	n	eNO (p.p.b.)	n	eNO (p.p.b.)	
Dp+	45	14.9 (12.6–17.7)	31	26.4 (21.1–33.1)**	0.56 (0.43–0.74)
Dp−	23	12.7 (9.4–17.3)	15	20.5 (14.0–30.1)*	0.62 (0.39–0.99)

*$p < 0.05$ and **$p < 0.001$ (unpaired *t*-test) when compared to children with low eosinophil count.

A previously unreported finding in this study is the increased eNO levels in apparently healthy schoolchildren (189 of the 356 children studied, 53%) with a high blood-eosinophil count and atopic sensitization combined. Our findings also confirm the relationship between eNO and blood eosinophilia in atopic asthmatic children (25), and in unselected schoolchildren (26).

In the whole unselected population, eNO values correlated with total IgE levels, prick index and blood eosinophilia, three markers whose levels vary widely in individuals (27). Concomitant atopy and eosinophilia was found in 40% of our atopic subjects. Hence approximately 13% of the unselected schoolchildren had markedly raised eNO levels. Overall our results suggest that increased eNO arises not merely from 'atopy' but from 'severe atopy' associated with eosinophilia. Others have also underlined the lack of correlation between eNO and atopy in healthy children (28) but without measuring blood eosinophil counts.

The probable reason for increased eNO in atopic subjects is the ability of eosinophils to provoke lung-tissue damage. Eosinophils are essential effector cells involved in the late-phase response to allergens (29). A T-helper lymphocyte (Th2-type) response allows interleukin-5 (IL-5) to stimulate bone-marrow eosinophil lineage, differentiation and their release from bone marrow into the peripheral circulation (30). IL-5 also augments the chemoattractive effect of eotaxin and, together with IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) prolongs eosinophil survival by reducing apoptosis (29). Activation of eosinophils promotes oxidative damage of proteins (31). A consequence of eosinophil-mediated airway-epithelium injury is the release of cytokines and other pro-inflammatory mediators followed by further leukocyte-cell recruiting and increased airway inflammation. Inducible NO synthase (NOs type II) is upregulated by cytokines; because IgE and eosinophil-mediated pathways both stimulate cytokine release, in subjects with marked involvement of both pathways the effect on eNO concentrations would probably be potentiated.

Blood eosinophilia has been proposed as a marker of airway inflammation (5, 19, 20, 32); although other factors can influence leukocyte levels. Theoretically, better results could be expected by combining results from airway-wall derived eosinophils (e.g. in sputum) and total IgE. For epidemiological purposes, blood eosinophil counts and skin prick tests are nevertheless easy and practical to perform. An easier and less invasive way of assessing eNO levels is the off-line method. In an earlier study we described reproducible eNO off-line measurements in school-aged children by using an expiratory flow of 58 ml/s (24); others have found reproducible off-line eNO measurements at higher expiratory flows in younger children (33).

Previously, we showed an effect of house-dust mite sensitization (as assessed by skin prick tests) on eNO levels in an unselected schoolchildren population (14). Others have also found a relationship between eNO levels and serum specific IgE for house-dust mite in asthmatic children (15). Hence, we also investigated whether our results could be biased by unequal distribution of Dp sensitization in the two atopic groups and examined data in atopic Dp-negative subjects. The comparable distribution of Dp sensitization among atopics with vs. those without eosinophilia and the similar increasing-eNO effect we observed in Dp-negative (sensitized to other allergens) as well as in Dp-positive children, imply that a 'seasonal effect' had no influence on our results.

Despite reports of decreased lung function (17–19) in subjects who have high eosinophil counts, we did not find decreased lung function in subjects with concomitantly increased blood eosinophil counts and atopy when compared with their non-eosinophilic non-atopic counterparts, independently of their history of respiratory symptoms. Neither did two recent studies find that lung function was related to blood eosinophil counts in atopic children (19, 25). More information is now needed on the 'eosinophilic-atopic' condition from studies investigating eNO and other non-invasive markers of respiratory injury. Another interesting question is whether the high eNO levels we found in atopic-eosinophilic healthy children indicate an increased risk of developing respiratory symptoms over time.

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