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Exhaled nitric oxide in asthmatic and non-asthmatic children: Influence of type of allergen sensitization and exposure to tobacco smoke

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Asthmatic bronchial inflammation is associated with increased nitric oxide concentrations in exhaled air (eNO). Recent data suggest that this effect arises from atopy. Our aim in this study was to find out whether atopy and sensitization to particular allergens influences eNO levels. A total of 213 subjects (41 asthmatics and 172 controls) (96 boys and 117 girls, 7.3–14 years of age) were studied. Parents completed a questionnaire that sought information on their children's respiratory symptoms and exposure to tobacco smoke. Subjects underwent skin-prick tests for the following common allergens: *Dermatophagoides pteronyssinus* (*Dpt*), cat fur, *Aspergillus fumigatus*, *Alternaria tenuis*, mixed grass, mixed tree pollen, *Parietaria officinalis*, egg, and cow's milk. eNO was collected in 1-l mylar bags (exhaled pressure 10 cmH_2O, flow 58 ml/s) and analyzed by using chemiluminescence. Atopic and non-atopic children without a history of chronic respiratory symptoms had a similar geometric mean eNO (atopics, n=28, 11.2 p.p.b.; non-atopics, n=96, 10.0 p.p.b.; mean ratio 1.1, 95% confidence interval [CI]: 0.7–1.6). Conversely, atopic asthmatic subjects had significantly higher eNO values than non-atopic asthmatic subjects (atopics, n=25, 24.8 p.p.b.; non-atopics, n=16, 11.4 p.p.b.; mean ratio 2.2, 95% CI: 1.2–3.9, p= 0.000). In children with rhinitis alone (n=15) and those with lower respiratory symptoms other than asthma (n=33), eNO increased slightly, but not significantly, with atopy. eNO levels correlated significantly with *Dpt* wheal size (r=0.51) as well with the wheal size for cat, mixed grass, and *Parietaria officinalis* (r=0.30–0.29), and with the sum of all wheals (r=0.47) (p= 0.000). Subjects sensitized only for *Dpt* (but not those subjects sensitized only for grass pollen or other allergens) showed significantly higher eNO levels than non-atopic subjects (16.4 p.p.b. vs. 10.2 p.p.b., mean ratio 1.6, 95% CI: 1.1–2.3, p= 0.002). In asthmatic subjects, *Dpt* sensitization markedly increased eNO levels (*Dpt*-sensitized subjects: 28.0 p.p.b.; *Dpt*-unsensitized subjects: 12.2 p.p.b.; mean ratio 2.3, 95% CI: 1.5–3.5, p= 0.000). Non-asthmatic *Dpt*-sensitized subjects also had significantly higher eNO values than non-asthmatic, non-*Dpt*-sensitized subjects (14.2 p.p.b. vs. 10.1 p.p.b.; mean ratio 1.4, 95% CI: 1.1–1.9, p= 0.008). No difference was found between eNO levels in asthmatic subjects and control subjects exposed or unexposed to tobacco smoke. In conclusion, eNO concentrations are high in atopic asthmatic children and particularly high in atopic asthmatics who are sensitized to house-dust mite allergen.

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Measurement of exhaled nitric oxide (eNO) has gained increasing interest as a non-invasive method for assessing asthmatic airway inflammation (1). Increased eNO levels are believed to reflect the action of proinflammatory cytokines on inducible nitric oxide synthase (iNOS) (2). This enzyme catalyzes NO conversion from l-arginine and is expressed by the airway and alveolar epithelium, alveolar macrophages, vascular endothelium, and fibroblasts (3). Bronchial biopsies from asthmatic patients show enhanced immunoreactivity for iNOS (4). eNO levels are consistently higher in asthmatic patients than in normal subjects (5–7) and decrease after administration of specific iNOS inhibitors and after corticosteroid therapy (8).

The determinants of increased eNO production in asthma remain poorly understood. Current research is investigating the role of atopy. An intriguing question is to what extent the atopic status by itself can account for elevated eNO concentrations.

eNO concentrations increase during late-phase reactions to allergens (9). Recent reports have also found higher eNO concentrations in patients with atopic asthma and rhinitis than in comparable non-atopic subjects (10–13). The influence of atopy on eNO levels in subjects with other respiratory diseases or in healthy subjects is more controversial (11,14–16). Particularly in healthy subjects, some reports have found atopy to be related to increased eNO levels (14,15) while others have found no such relationship (11,16). Whether the strength of this relationship depends on the type of allergen to which the subject becomes sensitized remains unclear.

Chronic tobacco smoking is associated with decreased eNO concentrations (17). Although passive exposure to tobacco smoke could therefore influence eNO measurements, this relationship receives no support from the limited data available in children (14).

In this study, we therefore aimed to assess the influence of atopy, the type of allergen sensitization, and respiratory symptoms, on eNO levels in children. We also compared eNO levels in asthmatic and non-asthmatic subjects grouped according to passive exposure to tobacco smoke.

Subjects and methods

Study population

Subjects were drawn from two populations. The first was a non-selected population of schoolchildren from central Italy (Ronciglione, Viterbo). The population was recruited by sending a letter to all parents of children ($\approx$ 9–11 years of age) from IV and V grade courses. A total of 280 parents were contacted and 240 attended the school for an interview. Parents were asked to complete a questionnaire and to give permission for their children to be tested. Of the 240 parents interviewed, 13 refused permission for skin testing. An additional 20 children did not participate in the tests (absence for sickness, n=8; absence for other reasons, n=6; current use of inhaled corticosteroids, n=6). The remaining 207 subjects (73.9%) underwent skin tests and eNO measurements. To increase the number of patients with atopic or non-atopic asthma, using the same parental questionnaire we also enrolled 15 steroid-naive patients who regularly attended our Pediatric Respiratory Service. These patients were in clinical remission, the only asthma medication was inhaled β_2-agonists or chromones, none of them had received corticosteroid therapy for at least 1 month before the tests, nor had they suffered acute respiratory infections in the previous month. The study was approved by the Ethics Committee of the Paediatric Clinic.

Study design

According to the questionnaire replies, the total population was divided in two groups: asthmatic subjects; and a non-asthmatic control group. All subjects were assessed using the same criteria (questionnaire, skin-prick tests, and eNO measurements) between February and May 1999.

Questionnaire

Parents completed a modified version of the American Thoracic Society questionnaire for respiratory symptoms (18) that sought information on family history (health and cigarette consumption) and child's history of respiratory symptoms (ever and in the past 12 months) (See Appendix I). Reported respiratory symptoms included 'current or past asthma' (Has your child ever had asthma?; wheezing or whistling attacks), 'asthma in the last 12 months' (wheezing or whistling in an asthmatic subject), and 'exercise-induced asthma' (wheezing or cough during or after exercise). 'Lower respiratory tract symptoms in the past 12 months' was defined by report of a 'persistent cough' (a 3-month history of cough on ≥ 4 days per week) or a physician diagnosis of 'bronchitis' or 'pneumonia' in the previous 12 months. 'Rhinitis' was defined as reported hay fever or runny nose, apart from colds. The presence of 'upper or lower respiratory tract symptoms in the past 4 weeks' was defined as at least one positive report of

Table 1. Characteristics, positive skin prick for common allergens, reported respiratory symptoms, and exposure to tobacco smoke in the 213 children studied

Characteristics and outcomes	Control group (n=172)	Asthmatic subjects (n=41)	
		Out-patients (n=15)	Schoolchildren (n=26)
Age, years (mean ± SD)	10.2±0.6	10.3±1.8	10.2±0.6
Gender, M/F (%)	69/103 (40.1)	10/5 (66.6)	17/9 (65.4)
Atopy, n (%)			
Dermatophagoides pteronyssinus	28 (16.3)	8 (53.3)	12 (46.2)
Mixed grass pollen	17 (9.9)	7 (46.7)	7 (26.9)
Parietaria officinalis	11 (6.4)	6 (40.0)*	2 (7.7)
Cat fur	7 (4.1)	1 (6.7)	4 (15.4)
Mixed tree pollen	8 (4.6)	1 (6.7)	1 (3.8)
Molds (*Alternaria tenuis, Aspergillus fumigatus*)	4 (2.3)	0 (0)	3 (11.5)
Foods (egg, milk)	0 (0.0)	1 (6.7)	1 (3.8)
At least one positive allergen reaction	46 (26.7)	12 (80.0)	13 (50.0)
Reported respiratory symptoms, n (%)			
Current or past asthma	0 (0.0)	15	26
Asthma in the past 12 months	0 (0.0)	10 (66.7)	11 (42.3)
Exercise-induced asthma	0 (0.0)	5 (33.3)	7 (26.9)
Lower respiratory tract symptoms in the past 12 months	33 (19.2)	14 (93.3)*	14 (53.8)
Rhinitis alone	15 (8.7)	0	0 (0.0)
Upper or lower respiratory tract symptoms in the past 4 weeks	56 (32.5)	9 (60.0)	13 (50.0)
Current exposure to tobacco smoke, n (%)	91 (52.9)	6 (40.0)	17 (65.4)

*$p<0.05$ (as evaluated by using the chi-square test) vs. asthmatic schoolchildren.

wheeze, cough, blocked or runny nose during that period. The questionnaire also sought information on current medication. An affirmative answer to the question on 'current or past asthma' (question 1 in Appendix I) was accepted as a questionnaire-based diagnosis of asthma. This criteria was not applied to subjects who answered affirmatively to 'lower respiratory symptoms in the past 12 months' but negatively to question 1.

Current environmental exposure to tobacco smoke was defined as an affirmative answer to questions about daily cigarette consumption at home by the parents or other household members.

Allergy assessment

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*), cat hair, *Aspergillus fumigatus*, *Alternaria tenuis*, mixed grass, mixed tree pollen, *Parietaria officinalis*, egg, and cow's milk (Soluprick; ALK-Abellò, Horsholm, Denmark). As positive and negative controls, histamine dihydrochloride (10 mg/ml) and diluent (50% glycerol and 50% physiological saline), respectively, were used. 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 of ≥ 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 in a subject was termed 'prick index' (19).

Exhaled NO measurements

eNO was measured by using a chemiluminescence NO analyzer (Sievers NOA™ 280; Sievers Instruments Inc., Boulder, CO, USA; response time 0.02 s, sensitivity <1 part per billion (p.p.b.), range of measures <1–500.000 p.p.b. repeatability±1 p.p.b., sampling flow 200 ml/s). 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 (20). A 250-ml bag connected to the expiratory line by means of a one-way valve allowed the first 250 ml of exhaled gas to be discarded (dead space discarding); under visual feedback, subjects exhaled through a calibrated needle restrictor (internal diameter 1.55 mm) keeping a constant pressure of 10 cmH_2O monitored in a lateral port by means of a low-range (0–100 cmH_2O) manometer (Magnehelic, Dwyer Instruments Inc., Michigan City, IN, USA). At a constant controlled pressure of 10 cmH_2O, the restrictor generated a flow of 58 ml/s.

All subjects were asked to fast overnight and avoid physical exercise on the morning of testing. Subjects at rest, sitting down and wearing a mouthpiece, performed a single exhalation from total lung capacity, without breath-holding, and

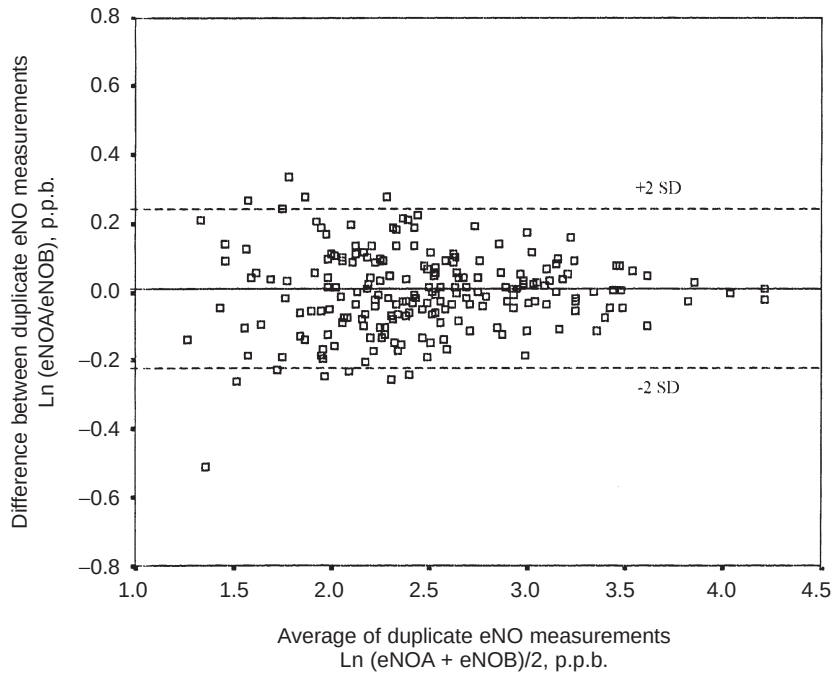

Fig. 1. Difference between logarithms of duplicate exhaled NO (eNO) measurements (i.e. Ln A/B) in 213 children, plotted against their averaged value. Duplicate measurements were obtained in the same session.

were encouraged to maintain a constant expiratory pressure throughout the maneuver. An effort was accepted when the expiratory pressure fluctuated less than ± 2 cm H_2O (from 10 cm H_2O) during the maneuver. The maneuver was stopped at 8 s of collection into the reservoir (out of dead space discarding, less than 1 s). Each patient repeated the exhalation maneuver after a 1-min interval, so that two samples for each subject were collected. Ambient eNO concentrations were assessed by filling two mylar bags with room air (one at the start and the other at the end of the testing procedures). All samples were transported to the laboratory and analyzed with a maximal delay of 2 h after collection; in each subject, the mean eNO was calculated from two samples.

Statistics

Repeatability of eNO measurements was determined by calculating two indices: the coefficient of repeatability (CR), calculated as twice the standard deviation (SD) of the differences between duplicate samples (21); and the intraclass coefficient correlation (ICC) (22). The normal distribution of between-subject eNO values was analyzed by using the Kolmogorov-Smirnov goodness-of-fit test. Because the data were significantly skewed (Z: 2.1, $p = 0.000$), they were transformed into natural logarithms (Ln) and expressed as geometric means and 95% confidence intervals (CIs). Mean ratios and 95% CI values were used to quote differences between two geometric means (because Ln A–Ln B=Ln A/B). Contingency tables (chi-square test) were used for comparison of proportions; the unpaired t-test for comparisons between groups; and one-way analysis of variance (ANOVA), Post-hoc Scheffè's test, for multiple-groups comparison. The statistical software, SPSS 9.0 for Windows, was used for calculations. p-values of <0.05 were considered statistically significant.

Table 2. Exhaled nitric oxide (eNO) levels by chronic respiratory symptoms and atopy

	Non-atopic subjects		Atopic subjects		Mean eNO ratios (95% CI)
	n	eNO (95% CI)	n	eNO (95% CI)	
Control group					
No respiratory symptoms	96	10.0 (9.2–10.9)	28	11.2 (9.4–13.3)	0.9 (0.6–1.3)
Persistent rhinitis alone, ever	7	7.8 (5.9–10.3)	8	15.0 (10.4–21.8)	0.5 (0.2–1.3)
Lower respiratory tract symptoms other than asthma	23	11.0 (9.0–13.5)	10	15.2 (8.7–26–4)	0.7 (0.4–1.5)
Asthmatic subjects	16	11.4 (8.1–15.9)	25	24.8 (19.8–30.9)*	0.5 (0.3–0.8)

*$p<0.002$ (as evaluated by one-way analysis of variance [ANOVA [Post-hoc Scheffè's test]) vs. non-atopic groups and from atopic-asymptomatic subjects.
eNO levels are expressed as geometric means in p.p.b.

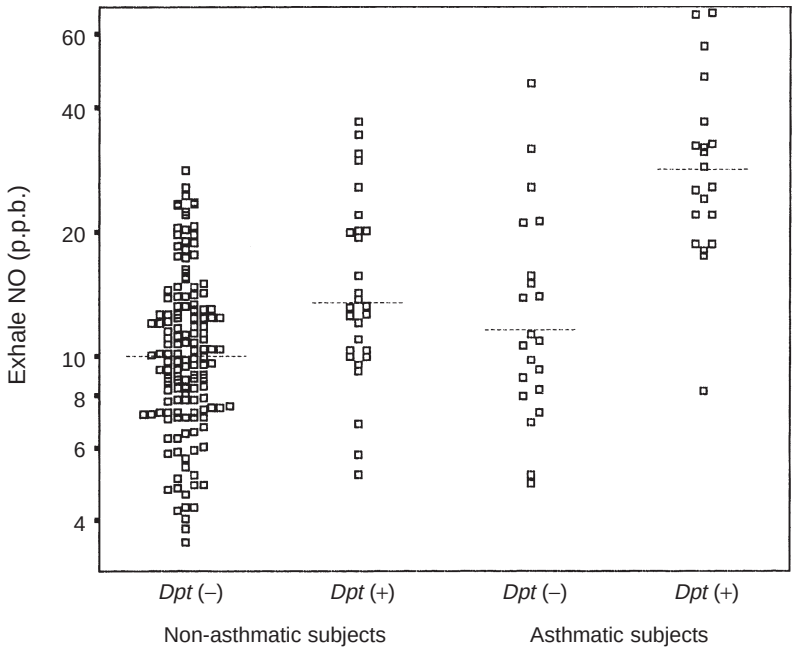

Fig. 2. Exhaled nitric oxide (eNO) concentrations (logarithmic scale) according to the presence of asthma and sensitization to Dermatophagoides pteronyssinus (Dpt). Dpt(–), non-sensitized; Dpt (+), sensitized. Dotted lines represent geometric means. Mean ratios (95% confidence intervals [CI]) were as follows: asthmatic Dpt (+) children: 2.3 (1.5–3.5) vs. asthmatic Dpt (–), 2.0 (1.3–2.9) vs. non-asthmatic Dpt (+), 2.8 (2.0–3.8) vs. non-asthmatic Dpt (–) children; p=0.000 for all comparisons. Mean ratios (95% CI) between non-asthmatic Dpt (+) and non-asthmatic Dpt (–) children: 1.4 (1.1–1.9), p=0.008.

Results

A total of 220 subjects underwent skin-prick tests and eNO measurements. Nine subjects (one asthmatic and eight control subjects) did not complete the tests (five refused skin tests; one refused eNO measurements; three were unable to co-operate in eNO measurements). The remaining 213 subjects completed the tests.

During nearly all the eNO measurements, ambient NO levels were low (0.1–1.7 p.p.b.). On only two occasions were the levels increased: one day, 3 subjects underwent eNO measurement at an ambient NO of 15 p.p.b.; on another day, 10 subjects were measured at an ambient NO of 22 p.p.b.. Although these 13 subjects reported a lower proportion of asthma and atopy than the other 200 subjects (asthma: two of 13 vs. 39/200, atopy: three of 13 vs. 68/200), their geometric mean eNO was significantly higher (23.3 p.p.b. vs. 11.3 p.p.b., mean ratio 2.1 [95% CI: 1.5–2.8], p=0.000). The 13 subjects were measured again in a single session, at an ambient NO of 0.5 p.p.b. The following results refer only to eNO measurements recorded at an ambient NO of <1.7 p.p.b.

Duplicate eNO measurements were highly replicable. For log-transformed values, the mean Ln eNO difference was –0.002 (95% CI: –0.018 to 0.014), CR: 0.236 Ln units, and ICC: 0.98 (Fig. 1).

The total population consisted of 172 control and 41 asthmatic children. Of the 213 subjects in the pooled population, 71 (33.3%) showed at least one positive skin-test reaction, and 48 subjects (68%) were positive for *Dpt*. Compared with the 26 asthmatic schoolchildren, the 15 asthmatic out-patients had a higher (although not significantly higher) frequency of positive skin-test reactions, a similar frequency of *Dpt* sensitization, and a significantly greater number of lower respiratory symptoms in the past 12 months (Table 1).

The geometric mean eNO concentration was significantly higher in the asthmatic group than in the control group (18.3 p.p.b. vs. 10.7 p.p.b.; mean ratio 1.7 [95% CI: 1.4–2.0], p= 0.000). Bet-ween-gender comparisons in each group showed no significant differences for eNO concentrations. In the asthmatic group, the 15 out-patients had higher (but not significantly higher) eNO levels than their 26 school-aged counterparts (21.4 p.p.b. vs. 16.7 p.p.b.; mean ratio 1.3 [95% CI: 0.8–2.0], p= 0.2491).

The presence of upper or lower respiratory tract symptoms in the past 4 weeks had no influence on eNO levels in controls (with symptoms, n=56, 9.8 p.p.b.; without symptoms, n=116, 11.1 p.p.b.; mean ratio 0.9 [95% CI: 0.8–1.0], p= 0.507) or asthmatic subjects (with symptoms, n=22, 19.5 p.p.b.; without symptoms, n=19, 17.0 p.p.b.; mean ratio 1.1 [95% CI: 0.7–1.8], p= 0.877).

eNO values were significantly higher in atopic than in non-atopic asthmatic subjects or control subjects with or without other chronic respiratory symptoms (Table 2). eNO levels were similarly increased in atopic-asthmatic out-patients and atopic-asthmatic schoolchildren (out-patients, n=12, 24.6 p.p.b.; schoolchildren, n=13, 24.9 p.p.b.; mean ratio 1.0 [95% CI: 0.5–1.9], p=1.000). Conversely, eNO levels were similarly decreased in non-atopic-asthmatic out-patients and non-

Table 3. Exhaled nitric oxide (eNO) levels by wheeze categories and atopy in the 41 asthmatic subjects

	Non-atopic subjects		Atopic subjects		Mean eNO ratios (95% CI)
	n	eNO (95% CI)	n	eNO (95% CI)	
Wheezing with colds	5	12.4 (4.7–33.0)	4	33.3 (22.1–50.3)*	0.4 (0.1–1.2)
Wheezing apart from colds	11	10.9 (7.4–16.2)	21	23.4 (18.1–30.2)*	0.5 (0.3–0.9)

*$p<0.05$ (as evaluated by one-way analysis of variance [ANOVA [Post-hoc Scheffè's test]) vs. non-atopic subjects who wheezed apart from colds. eNO levels are expressed as geometric means in p.p.b.

atopic-asthmatic schoolchildren (out-patients, n=3, 12.4 p.p.b.; schoolchildren, n=13, 11.2 p.p.b.; mean ratio 1.1 [95% CI: 0.4–3.3], p= 0.994.

The 41 asthmatic subjects (out-patients plus schoolchildren) belonged to two wheeze categories: nine subjects wheezing only with colds (all schoolchildren: five non-atopic and four atopic); and 32 subjects wheezing apart from colds (11 non-atopic and 21 atopic). In both wheeze categories, atopy was associated with increased eNO levels (Table 3).

Type of allergen sensitization and eNO levels

In the whole population (control and asthmatic subjects), logarithmic eNO values (Ln eNO) correlated significantly with wheal size for *Dpt* (r=0.51, p= 0.000), cat allergen (r=0.30, p= 0.000), mixed grass pollen (r=0.30, p= 0.000), and *Parietaria officinalis* (r=0.29, p= 0.000). Ln eNO was also related to the sum of all positive wheals, i.e. prick index (r=0.47, p= 0.000).

Although multiple sensitization (including *Dpt*) yielded the highest eNO levels, subjects sensitized only for *Dpt* (but not those sensitized only for grass pollen or those sensitized for one or more of the other allergens) still had significantly higher eNO levels than non-atopic subjects (Table 4).

eNO concentrations were significantly higher in asthmatic *Dpt*-sensitized children than in asthmatic non-*Dpt*-sensitized children or non-asthmatic controls who were or were not *Dpt* sensitized (28.0 [95% CI: 22.1–35.5]p.p.b.; 12.2 [9.3–15.8] p.p.b.; 14.2 [11.6–17.4] p.p.b.; 10.1 [9.4–10.9] p.p.b.; respectively, p= 0.000); eNO was also significantly higher in non-asthmatic *Dpt*-sensitized children than in non-asthmatic non-*Dpt*-sensitized children (14.2 p.p.b. vs. 10.1 p.p.b., mean ratio 1.4 [95% CI: 1.1–1.9], p= 0.008). (Fig. 2).

Environmental tobacco smoke exposure

No significant difference was found in eNO levels in subjects divided into groups according to current exposure to environmental tobacco smoke (controls: non-exposed, n=81, 11.3 p.p.b.; exposed, n=91, 10.1 p.p.b.; mean ratio 1.1 [95% CI: 0.9–1.4], p= 0.566) (asthmatic subjects: non-exposed, n=18, 23.0 p.p.b. exposed, n=23, 15.3 p.p.b.; mean ratio 1.5 [95% CI: 0.9–2.4], p=0.097).

Discussion

In this study, using an off-line method to measure eNO, we have shown that atopy increases eNO concentrations in schoolchildren, especially in asthmatic subjects sensitized to the house-dust mite (*Dpt*) allergen. Neither in asthmatic nor in non-asthmatic subjects did exposure to tobacco smoke seem to affect eNO.

In an epidemiologic setting we were unable to use more objective criteria for defining asthma (23). Nonetheless, this study confirms previous observations of significantly higher eNO values in atopic asthmatic subjects than in non-atopic asthmatics (10,11,13). Insofar as patients and schoolchildren had equally high eNO levels, the

Table 4. Exhaled nitric oxide (eNO) levels and sensitization to *Dermatophagoides pteronyssinus* (*Dpt*)

	Subjects (n)	Prick index	eNO
Non-atopic	142	0	10.2 (9.4–11.0)
Atopic			
Both *Dpt* and grass-pollen negative	13	4.7 (3.3–6.9)	10.4 (8.2–13.1)
Sensitized only to grass pollen	7	4.3 (3.0–6,3)	9.5 (5.3–17.0)
Sensitized only to *Dpt*	22	4.1 (3.4–4.8)	16.4 (12.8–21.1)*
Sensitized to *Dpt* and other allergens	29	12.3 (11.3–15.9)	20.9 (16.8–26.4)**

Prick index and eNO levels are given as mean ratios (95% confidence intervals) and significance:
*1.6 (1.1–2.3), p = 0.002 vs. non-atopic subjects;
**2.1 (1.5–2.8), p = 0.000 vs. non-atopic subjects; 1.9 (1.2–3.2), p = 0.004 vs. atopic subjects unsensitized to both *Dpt* and grass pollen; 2.2 (1.2–4.3), p = 0.007 vs. subjects sensitized only to grass pollen.
The prick index is defined as the sum of the positive wheals to allergen skin-prick tests. Data are expressed as geometric means (95% confidence intervals).

increased eNO in our atopic asthmatic subjects appeared not to be influenced by their source of recruitment. Also in our small subgroup of schoolchildren reporting asthma attacks with colds only, atopic individuals had higher eNO levels than their non-atopic counterparts. The small size of this subgroup precludes us from asserting that in atopic individuals high eNO levels were related to asthma-like symptoms triggered as a result of viral infection. Yet, this possibility receives support from the increasing evidence on synergy between allergen- and virus-induced airway inflammation (24).

Although others have shown a significant effect of atopy on eNO levels in rhinitic subjects (11,12,25), in the present study we did not, possibly because few children in our study had rhinitis alone. Neither did we find that atopic children who reportedly had lower respiratory tract symptoms other than asthma (mainly chronic cough without a history of wheeze) had substantially higher eNO than non-atopic children. A limitation inherent to all epidemiological studies involving asthma is the problem of case definition (23): do atopic children with airway symptoms and without airway hyper-reactivity have asthma? Interestingly, young atopic adults with a history of wheeze in the previous 12 months but without airway hyper-responsiveness reportedly have significantly higher eNO levels than non-atopic subjects (26). In contrast with other studies in adults (15,26) and in children (14), our atopic schoolchildren without a history of chronic respiratory symptoms had eNO levels no higher than those of their non-atopic counterparts. Only two studies have reported finding no difference in eNO between atopic healthy and non-atopic healthy subjects: one study was performed in adults (11) and the other in a small group of children (16). Our findings argue against an ongoing allergen-mediated airway inflammation in these apparently healthy children.

Our finding that eNO concentrations distinctly differed in atopic and non-atopic asthmatic subjects, raises the question of whether concomitant atopy and airway inflammation is a prerequisite for increased eNO production by the lower airways. Although mechanisms of eNO production may differ in atopic and non-atopic asthma, exactly how they differ remains unclear. Atopic and non-atopic asthmatics have similar expression of T helper 2 (Th2)-type cytokines, chemokines, and high-affinity immunoglobulin E (IgE) receptors (24). Th2-type cytokines, especially interleukin 5 (IL-5), and chemokines play an important role in the selective accumulation of eosinophils in the bronchial wall. Indeed, atopic and non-atopic asthma share a pattern of bronchial eosinophilic inflammation (27,28). Although some studies found an association of the sputum eosinophil count with eNO levels (29,30), and with respiratory symptoms in the previous 2 weeks (31), the majority of asthmatic subjects studied were atopic. We found that, regardless of being asthmatic or healthy, non-atopic subjects had comparable, low eNO levels, a result that eosinophilic infiltration could not explain.

Our findings now strongly suggest that the degree of disturbance could depend on allergen sensitization and the type of allergen. To our knowledge, no study has yet reported allergy to *Dpt* as a main determinant of increased eNO concentrations. *In vitro* challenge of bronchoalveolar lavage mononuclear cells with house-dust mite extracts provokes a shift in IL-5 in atopic asthmatic subjects but not in atopic non-asthmatic subjects, non-atopic asthmatics or healthy subjects, even though non-atopic asthmatics show baseline IL-5 production comparable to that of atopic asthmatic subjects (32). Humanized allergic mice with severe combined immunodeficiency (hu-SCID) reconstituted with peripheral blood mononuclear cells from *Dpt*-sensitized patients and exposed to *Dpt* aerosol, develop airway hyper-responsiveness, *Dpt*-specific IgE and increased IL-5 levels in bronchoalveolar lavage fluid (despite the absence of eosinophils). These effects do not develop in non-allergic hu-SCID mice (33).

The amount of allergen appears to modify eNO levels. Simpson et al. have recently reported that sensitized subjects exposed to *Dpt*, cat and dog allergens (levels of *Der* p 1, >2 p.p.m. *Fel* d 1, >8 p.p.m.; *Can* f 1, >10 p.p.m.), have significantly higher eNO concentrations than sensitized but unexposed subjects (34). Increased seasonal exposure does not explain the relationship we found between eNO and *Dpt* because we conducted our study mainly during the spring, the season when grass-pollen allergens would be expected to predominate. Moreover, subjects mono-sensitized to grass pollen had lower eNO levels than subjects mono-sensitized to *Dpt*. We consider it highly unlikely that spring-time *Dpt* exposure could decrease sufficiently for other allergens to overwhelm its ability to stimulate eNO production.

About 50% of our asthmatic out-patients – as well as 50% of our asthmatic schoolchildren – showed sensitization to *Dpt*. The similar frequency of *Dpt* sensitization could also explain why these two subgroups had comparably

increased eNO levels, even though out-patients were more frequently sensitized to other allergens and had a greater number of lower respiratory symptoms in the previous 12 months than the asthmatic schoolchildren.

Chronic tobacco smoking decreases eNO concentrations in healthy and asthmatic subjects (17,35). However, this effect has not been demonstrated in subjects exposed to passive smoke. Franklin et al. found no significant difference between eNO levels in healthy children who lived with a smoker and those who did not (14). Similarly, we found no difference among eNO levels in healthy or asthmatic children exposed or not exposed to passive tobacco smoke. School-age children spend most of their time outdoors (hence they are exposed less to tobacco smoke). Also, homes in central Italy are usually well ventilated during the spring-time. We used an approximate estimate of tobacco smoke exposure; more accurate estimates of exposure are probably needed to clarify the role of passive smoking on eNO levels.

The off-line eNO collection method is considered to be suitable for children (36). Measurements obtained off-line correlate well with those obtained on-line (20,36–38). In this study, we used a validated off-line eNO collection method (discarding air from dead space, controlling expiratory flow, pressure and exhalation duration) (20). The same method gave highly repeatable measurements when tested outside the laboratory.

Whether reliable eNO measurement necessitates prolonged fasting, and for how long, remains open to question. Some guidelines recommend withholding food and drink for 1 h beforehand (39). Yet, after a normal nitrate-containing food intake, eNO increases steadily, reaching a maximum at 2 h (40). To avoid possible eNO variations, we therefore tested our subjects after an overnight fast. Future research might prove that fasting before testing is unnecessary and children could merely avoid certain nitrate-containing foods.

In a small group of subjects with no higher frequency of reported asthma or atopy than subjects tested at ambient NO under 2 p.p.b., we found increased eNO levels only on the two occasions when ambient NO levels rose to almost 20 p.p.b. The effect of the high inhaled volume of contaminated ambient NO/air during the total lung capacity maneuver cannot be counteracted by discarding the initial 250 ml of exhaled air before collecting into the reservoir; on the other hand, discarding higher volumes would compromise the child's ability to maintain a constant exhalation pressure. Controlled inhaled NO levels are necessary to ensure reliable results when using the off-line technique (39).

In summary, we have shown that atopy substantially alters eNO levels in asthmatic subjects. Sensitization to house-dust mite seems to be a major determinant of high eNO concentrations. The role of specific allergens, environmental factors, and respiratory disease on eNO measurements is an interesting question calling for further epidemiological studies.

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Appendix 1

Children's questionnaire on respiratory symptoms (to be completed by the parent during the interview)

Question		
1. Has your child ever had asthma? (wheezing or whistling attacks noted by others?)	NO	YES
If yes, did this happen only with colds?	NO	YES
2. Has your child had wheezing or whistling in the chest in the last 12 months?	NO	YES
3. In the last 12 months has your child had wheezing or cough during or after exercise?	NO	YES
4. In the past 12 months has your child had cough on most days (4 or more days per week) for as long as 3 months?	NO	YES
If Yes, did this happen only with colds?	NO	YES
5. In the past 12 months did the doctor say that your child had any of the following illnesses:		
Bronchitis	NO	YES
Pneumonia	NO	YES
6. Has your child ever had a problem with sneezing, or a runny, or a blocked nose without a cold or flu?	NO	YES
7. In the past 4 weeks has your child had any of the following symptoms:		
Wheeze	NO	YES
Cough	NO	YES
Blocked or runny nose	NO	YES