# Levels of nitric oxide oxidation products are increased in the epithelial lining fluid of children with persistent asthma

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**Background: Children with severe allergic asthma have persistent airway inflammation and oxidant stress.**
**Objectives: We hypothesized that children with severe allergic asthma would have increased concentrations of the nitric oxide (NO) oxidation products nitrite, nitrate, and nitrotyrosine in the proximal and distal airway epithelial lining fluid (ELF). We further hypothesized that NO oxidation products would be associated with higher exhaled NO values (fraction of exhaled nitric oxide [$F_{ENO}$]), greater allergic sensitization, and lower pulmonary function.**
**Methods: Bronchoalveolar lavage fluid was obtained from 15 children with mild-to-moderate asthma, 30 children with severe allergic asthma, 5 nonasthmatic children, and 20 nonsmoking adults. The bronchoalveolar lavage fluid was divided into proximal and distal portions and nitrite, nitrate, and nitrotyrosine values were quantified.**
**Results: Children with mild-to-moderate and severe allergic asthma had increased concentrations of nitrite (adult control subjects, 15 ± 3 μmol/L; pediatric control subjects, 23 ± 4 μmol/L; subjects with mild-to-moderate asthma, 56 ± 26 μmol/L; subjects with severe asthma, 74 ± 18 μmol/L), nitrate (37 ± 13 vs 145 ± 38 vs 711 ± 155 vs 870 ± 168 μmol/L, respectively) and nitrotyrosine (2 ± 1 vs 3 ± 1 vs 9 ± 3 vs 10 ± 4 μmol/L, respectively) in the proximal ELF. Similar results were seen in the distal ELF, although the concentrations were significantly lower ($P < .05$ for each). Although univariate analyses revealed no associations between NO oxidation products and clinical features, multivariate analyses revealed $F_{ENO}$ values to be a significant predictor of NO oxidation in asthmatic children.**
**Conclusions: NO oxidation products are increased in the ELF of asthmatic children. The relationship between $F_{ENO}$ values and airway nitrosative stress is complicated and requires further study. (J Allergy Clin Immunol 2009;124:990-6.)**

***Key words:*** *Asthma, children, nitric oxide, nitrogen oxides, nitrosation, nitrosative stress, reactive nitrogen species*

Severe allergic asthma in school-aged children is a complex disorder characterized by persistent airway inflammation, ongoing symptoms, and increased exhaled nitric oxide (NO; fraction of exhaled nitric oxide [$F_{ENO}$]) concentrations despite treatment with high doses of inhaled and oral corticosteroids.[1,2] Although airway NO is essential for epithelial signaling and host defense, excessive NO production results in NO oxidation and potential toxicity.[3] This process of excessive NO oxidation is commonly referred to as "nitrosative stress"[4] and ultimately promotes protein nitration, resulting in structural and functional protein alterations that might enhance the inflammatory response.[5] Thus excessive airway NO concentrations in children with severe allergic asthma might contribute to an ongoing cycle of airway destruction with airway injury.[6]

In the human airway, the most readily detectable NO oxidation products include nitrite ($NO_2^-$) and nitrate ($NO_3^-$), which can be derived from NO through a series of reactions involving superoxide anion ($O\cdot_2^-$) and oxygen (Fig 1). Nitrotyrosine is also easily measured in airway samples and reflects the overall degree of protein nitration.[7] Indeed, previous studies have noted increased nitrite, nitrate, and nitrotyrosine concentrations in the exhaled breath condensate of asthmatic children[8-10] and in the epithelial lining fluid (ELF) of adults with mild-to-moderate and severe asthma.[11,12] However, no study to date has examined NO oxidation products in the ELF of asthmatic children. Because children with severe asthma have profound airway oxidant stress,[13] the purpose of this study was to quantify NO oxidation products in the ELF of children with mild-to-moderate and severe allergic asthma. The secondary purpose of this study was to determine the association between increased ELF NO oxidation products and clinical features of asthma severity in children. We hypothesized that children with severe allergic asthma would have increased concentrations of the NO oxidation products nitrite, nitrate, and nitrotyrosine in the proximal and distal airway ELF. We further hypothesized that these increased NO oxidation products would be associated with increased $F_{ENO}$ values, greater allergic sensitization, and lower pulmonary function.

## METHODS

### Sample

Children 5 to 17 years of age with symptomatic asthma attending an asthma clinic at Emory University were invited to participate in this study. Asthmatic children met published criteria for persistent asthma[14] and had a history of at least a 12% change in $FEV_1$ after albuterol administration.[15] Severe asthma was diagnosed according to criteria developed by the National Institutes of Health/National Heart, Lung, and Blood Institute Severe Asthma Research Program,[1] which were adapted from the American Thoracic Society's Consensus Panel Report (see Table E1 in this article's Online Repository at www.jacionline.org).[16] Thresholds for high-dose inhaled corticosteroids

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*See the acknowledgments for a complete listing of the Severe Asthma Research Program contributors.

Supported with funds from National Institutes of Health/National Institute of Nursing Research KO1 NR010548, National Institutes of Health/National Center for Research Resources K12 RR017643, and National Institutes of Health/National Heart, Lung, and Blood Institute Severe Asthma Research Program RO1 HL69170.

Disclosure of potential conflict of interest: W. G. Teague is on the speakers' bureau for Merck and Co; receives research support from the National Institutes of Health/National Heart, Lung, and Blood Institute, the American Lung Association, and the Centers for Disease Control and Prevention; is on the review panel for the American Thoracic Society; and is on the advisory board for WESTAT–Children's Health Study. The rest of the authors have declared that they have no conflict of interest.

Received for publication March 27, 2009; revised August 5, 2009; accepted for publication August 25, 2009.

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0091-6749/$36.00

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doi:10.1016/j.jaci.2009.08.039

*Abbreviations used*
BAL: Bronchoalveolar lavage
ELF: Epithelial lining fluid
$F_{ENO}$: Fraction of exhaled nitric oxide
ICS: Inhaled corticosteroid
NO: Nitric oxide
NOS: Nitric oxide synthase

(ICSs) were defined as 440 μg or greater of fluticasone equivalent per day for children less than 12 years of age and 880 μg or greater for children 12 to 17 years of age.[14] Children with severe allergic asthma were treated with a stable dose of ICSs or oral corticosteroids for at least 6 months before recruitment. Adherence to ICS therapy was monitored by an analysis of prescription refills. Informed consent was obtained from all caregivers. Children also provided verbal and written assent.

Children who fit the criteria for severe allergic asthma underwent flexible bronchoscopy with bronchoalveolar lavage (BAL) as indicated for persistent asthma symptoms despite appropriate treatment with high-dose inhaled and systemic corticosteroids.[17] Children with mild-to-moderate asthma underwent bronchoscopy for suspected foreign body aspiration, recurrent pneumonia, persistent cough, and suspected congenital anomalies. Control subjects for this study included children with psychogenic (habit) cough or vocal cord dysfunction undergoing bronchoscopy for definitive diagnosis and healthy, nonsmoking adult volunteers. Control subjects were nonsmokers with no family history of asthma and a negative bronchodilator response.

## Procedures

Spirometry was performed before and after 2 inhalations of albuterol sulfate (90 μg per inhalation) with a portable spirometer (KoKo Legend; Ferraris, Louisville, Colo). The results fulfilled American Thoracic Society criteria for reproducibility[18] and were interpreted according to reference standards.[19] Atopic sensitization was assessed by means of skin prick testing with a standard kit (Multi-Test II; Lincoln Diagnostics, Decatur, Ill) containing tree pollen, grass pollen, ragweed pollen, weed pollen, dog hair, cat epithelium, *Alternaria* species, *Cladosporium* species, *Aspergillus* species, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cockroach, normal saline, and histamine extracts (Greer Laboratories, Lenoir, NC). The application site was examined 15 minutes after application and considered positive if both a wheal of 3 mm in diameter or larger and erythema of 10 mm in diameter or larger were present.

On the day of bronchoscopy, participants submitted $F_{ENO}$ samples and underwent venipuncture. $F_{ENO}$ was collected with a reservoir bag within 1 hour before bronchoscopy. For this procedure, subjects took 2 tidal breaths of NO-free air through a scrubbing filter, followed by a 6-second exhalation at a fixed flow rate of 0.35 L/s.[20] The first 150 mL exhaled was discarded. Subjects repeated this procedure 3 times. The resulting samples were analyzed offline by means of chemiluminescence (Sievers NOATM 280-I; Ionic Instruments, Boulder, Colo) within 1 hour of collection. The data were averaged to reflect mean $F_{ENO}$ values. Serum immunoglobulin (IgE) concentrations and plasma urea value were determined after venipuncture.

Bronchoscopy in pediatric participants was performed by pediatric pulmonologists using a laryngeal mask airway. BAL fluid was collected from the right middle lobe with three 1 mL/kg (50 mL maximum) saline lavages flushed through the suction channel of a flexible bronchoscope (Olympus BF-3C160 [3.7 mm] or BF-P160 [4.9 mm]; Olympus America, Inc, Melville, NY). Bronchoscopy was performed in adults with a flexible bronchoscope (Olympus BF-1T20D) passed transnasally into the right middle lobe. Three 50-mL saline aliquots were instilled and immediately aspirated. The first lavage sample from all participants was reserved for evaluation of proximal airway constituents.[21] The second and third lavage samples were pooled for distal airway constituent analysis. In children the BAL return volume was divided between the research and clinical laboratories.

BAL fluid was centrifuged at 1200 rpm within 1 hour of collection for 7 minutes at 4°C to separate the supernatant and cellular fractions. Given the limited number of cells present in the proximal airway lavage sample, the cell pellets from the proximal and distal airway lavage samples were pooled and resuspended in 10 mL of Dulbecco modified Eagle medium with 10% FCS for cell counting. Total cell counts were performed manually with a hemocytometer, and cellular differentials were determined from 300 consecutive cells after Wright staining.

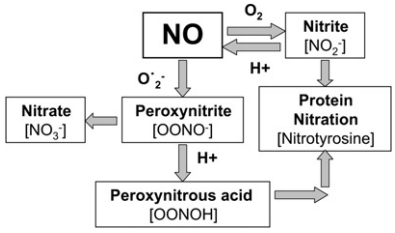


**FIG 1.** Diagram of NO metabolite formation in the airways.

The protein content of the BAL fluid supernatant was assessed by using a Coomassie (Bradford) protein assay (Pierce Biotechnology, Rockford, Ill) read at an absorbance of 595 nm with a detection limit 1 μg/mL. Urea nitrogen levels were measured in plasma and BAL supernatants by using a quantitative colorimetric assay (Pointe Scientific, Canton, Mich) with a sensitivity of 0.05 to 150 mg/dL. The dilution of the proximal and distal BAL fluid was calculated as follows: [Urea]plasma/[Urea]BAL.[22]

Nitrite and total nitrite plus nitrate concentrations were determined from the BAL supernatant by using a colorimetric assay (Cayman Chemical, Ann Arbor, Mich) analyzed at 540 nmol/L with a lower detection limit of 0.1 μmol/L. All samples were analyzed in duplicate. For this assay, nitrate was converted to nitrite with nitrite reductase, followed by the addition of Griess reagent. Nitrate concentrations were determined by subtracting the concentration of nitrite from total nitrite plus nitrate concentrations. Samples were analyzed immediately after thawing to minimize false nitrate and nitrate readings during the assay. The background nitrite and nitrate content in the saline lavage fluid was predetermined and subtracted from the final concentration values.

Nitrotyrosine concentrations were determined spectrophotometrically by using a microplate sandwich ELISA (Oxis International, Foster City, Calif) with a sensitivity of 2.0 nmol/L and interassay precision of 11%. Samples were analyzed in duplicate and corrected for the background levels of nitrotyrosine in the saline lavage fluid. Absorbance was measured at 450 nm.

## Statistical analysis

Data were analyzed with SPSS software (Version 15; SPSS, Inc, Chicago, Ill). Nitrite, nitrate, and nitrotyrosine concentrations from the proximal and distal airway lavage fluid were adjusted according to the urea dilution[22] and were logarithmically transformed. Nitrotyrosine concentrations were further adjusted for the total protein content of the BAL supernatant. Differences between groups and *post-hoc* tests were assessed by using Kruskal-Wallis tests and Mann-Whitney *U* tests, respectively. Pearson correlations were used to examine associations between NO oxidation products and clinical features. To evaluate factors that might affect NO oxidation in the ELF of asthmatic children, multivariate backward elimination linear regression was performed with total nitrite plus nitrate concentrations in the proximal and distal ELF as dependent variables and age, sex, ethnicity, ICS dose, $FEV_1$, $FEV_1$ bronchodilator reversibility, serum IgE level, history of hospitalization, $F_{ENO}$ value, and the percentage of airway eosinophils and neutrophils as predictors. Multicolinearity between predictors was assessed with tolerance statistics. Entry and removal probabilities were set at .05 and .10, respectively. Significance was defined as a 2-tailed α value of .05 or less for all tests.

## RESULTS

Initially, 49 asthmatic children (severe asthma, n = 32), 7 pediatric control subjects, and 20 healthy adult control subjects were recruited for this study. However, 6 children, including 2 pediatric control subjects, 2 subjects with mild-to-moderate asthma, and 2 subjects with severe asthma, were infected with

**TABLE I.** Features of the sample

| | Adult control subjects (n = 20) | Pediatric control subjects (n = 5) | Subjects with mild-to-moderate asthma (n = 15) | Subjects with severe asthma (n = 30) |
|---|---|---|---|---|
| Age (y) | 39 ± 10 | 11 ± 4* | 10 ± 4* | 10 ± 4* |
| Male sex | 8 (40) | 3 (60) | 10 (67) | 15 (50) |
| White | 8 (40) | 4 (80) | 14 (93)* | 9 (30)†‡ |
| African American | 11 (55) | 1 (20) | 1 (7)* | 20 (67)†‡ |
| ICS dose (μg of fluticasone/d) | 0 | 0 | 262 ± 189*† | 917 ± 236*†‡ |
| Asthma medications | | | | |
| Budesonide | 0 | 0 | 3 (20) | 7 (23) |
| Fluticasone | 0 | 0 | 1 (7) | 1 (3) |
| Fluticasone/salmeterol | 0 | 0 | 8 (53)*† | 22 (73)*†‡ |
| Montelukast | 0 | 0 | 10 (67)*† | 28 (93)*†‡ |
| Prednisone | 0 | 0 | 0 | 11 (37)*†‡ |
| Emergency department visit (previous year) | 0 | 0 | 3 (20) | 28 (93)*†‡ |
| Hospitalization (previous year) | 0 | 0 | 1 (7) | 26 (87)*†‡ |
| Intensive care unit admission (ever) | 0 | 0 | 0 | 14 (47)*†‡ |
| Intubation (ever) | 0 | 0 | 0 | 6 (20)*†‡ |
| FVC (% predicted) | 98 ± 16 | 102 ± 18 | 102 ± 15 | 87 ± 19*†‡ |
| $FEV_1$ (% predicted) | 103 ± 16 | 101 ± 15 | 100 ± 15 | 73 ± 20*†‡ |
| $FEV_1$/FVC ratio | 0.86 ± 0.07 | 0.89 ± 0.03 | 0.87 ± 0.06 | 0.74 ± 0.12*†‡ |
| $FEF_{25-75}$ (% predicted) | 121 ± 32 | 92 ± 16* | 94 ± 23* | 51 ± 25*†‡ |
| $FEV_1$ bronchodilator reversibility (%)§ | 3 ± 6 | 6 ± 5 | 9 ± 11 | 23 ± 17 |
| $F_{ENO}$ (offline, ppb) | 5 ± 3 | 7 ± 4 | 11 ± 12* | 13 ± 10*† |
| Increased baseline $F_{ENO}$ (>10 ppb) | 4 (20) | 2 (40) | 3 (20) | 20 (67)*†‡ |
| Reported allergies | Not assessed | 2 (40) | 9 (60) | 25 (83) |
| Reported atopic dermatitis | Not assessed | 0 | 5 (33) | 21 (70) |
| No. of skin prick responses | Not assessed | 0 | 2 ± 2 | 5 ± 3†‡ |
| Serum IgE (kU/L) | 100 ± 194 | 80 ± 64 | 94 ± 139 | 487 ± 730*†‡ |

Data represent means ± SDs or frequencies (percentages).

*FVC*, Forced vital capacity; $FEF_{25-75}$, forced expiratory flow.

*$P < .05$ versus adult control subjects.

†$P < .05$ versus pediatric control subjects.

‡$P < .05$ versus subjects with mild-to-moderate asthma.

§Calculated as follows: [($FEV_1$ postbronchodilator − $FEV_1$ prebronchodilator)/predicted $FEV_1$] * 100.

*Streptococcus pneumoniae*, *Haemophilus influenzae*, and/or *Moraxella catarrhalis* and were excluded from data analysis because of potential denitrification.[23] The features of the excluded children appear in the Tables E2 and E3 (available in this article's Online Repository at www.jacionline.org). Thus the final sample included in data analysis contained 30 children with severe allergic asthma, 15 children with mild-to-moderate asthma, 5 pediatric control subjects, and 20 adult control subjects.

Because bronchoscopy was performed only for clinical indications, all of the asthmatic subjects were symptomatic. None of the children with mild-to-moderate asthma had evidence of airway infection or chronic aspiration syndromes. The features of the final sample are presented in Table I. Whereas all (100%) children with severe asthma had allergic sensitization, allergic sensitization was present in only half (53%) of the children with mild-to-moderate asthma (see Table E4 in this article's Online Repository at www.jacionline.org). Children with severe allergic asthma were also treated with higher doses of ICSs but had significantly lower baseline pulmonary function and increased bronchodilator reversibility. Although $F_{ENO}$ values were increased in both groups of asthmatic subjects, there were no differences in $F_{ENO}$ values between children with mild-to-moderate and severe allergic asthma (Table I).

The characteristics of the BAL fluid are presented in Table E5 (available in this article's Online Repository at www.jacionline.org). Although larger lavage volumes were used for adult control subjects, the percentage of BAL return was similar between adult and pediatric control subjects (proximal lavage, 23% vs 27%; distal lavage, 49% vs 35%; adult vs pediatric control subjects). However, the BAL samples from adult control subjects were characterized by higher total cell counts (adult control subjects, $7.81 \pm 3.61 \times 10^6$; pediatric control subjects, $3.53 \pm 2.32 \times 10^6$; subjects with mild-to-moderate asthma, $3.81 \pm 3.06 \times 10^6$; subjects with severe asthma, $3.32 \pm 2.02 \times 10^6$; $P < .01$). Whereas subjects with severe asthma had the highest percentage of BAL eosinophils (adult control subjects, 0.4% ± 0.5%; pediatric control subjects, 0.3% ± 0.5%; subjects with mild-to-moderate asthma, 0.7% ± 0.7%; subjects with severe asthma, 1.9% ± 3.2%; $P = .03$), subjects with mild-to-moderate and severe asthma had higher percentages of neutrophils compared with both groups of control subjects (adult control subjects, 3.5% ± 3.2%; pediatric control subjects, 3.2% ± 1.4%; subjects with mild-to-moderate asthma, 5.3% ± 3.9%; subjects with severe asthma, 5.2% ± 3.2%; $P = .04$).

## NO oxidation products in the proximal and distal airway lavage fluid

Compared with control subjects, children with mild-to-moderate and severe allergic asthma had significantly higher

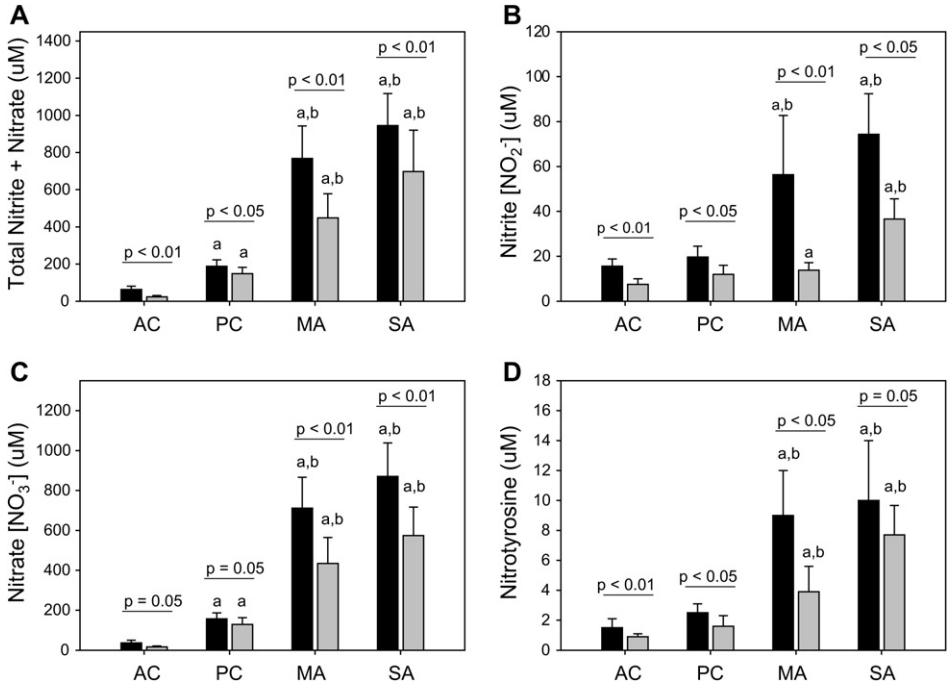


**FIG 2.** Total nitrite plus nitrate **(A)**, nitrite **(B)**, nitrate **(C)**, and nitrotyrosine **(D)** concentrations (in micromoles per liter) in the proximal *(black bars)* and distal *(gray bars)* airway ELF. Data represent means ± SEMs with adult control subjects *(AC)*, pediatric control subjects *(PC)*, subjects with mild-to-moderate asthma *(MA)*, and subjects with severe asthma *(SA)*. [a]$P < .05$ versus adult control subjects. [b]$P < .05$ versus pediatric control subjects.

concentrations of nitrite, nitrate, and nitrotyrosine in the ELF (Fig 2). However, no significant differences in concentrations of NO oxidation products were observed between children with mild-to-moderate and severe allergic asthma. In each group nitrate was the most abundant NO oxidation product measured, with concentrations nearly 10-fold higher than those of nitrite. Furthermore, nitrite, nitrate, and nitrotyrosine concentrations were also consistently higher in the proximal versus the distal airway ELF (Fig 2). Similar increases in concentrations of NO oxidation products were also apparent in the raw BAL samples without adjustment for the urea dilution (see Fig E1 in this article's Online Repository at www.jacionline.org). Analysis of the entire sample (all asthmatic and control subjects) revealed strong correlations between proximal and distal airway ELF concentrations of total nitrite plus nitrate ($r = 0.76$, $P < .01$), nitrite ($r = 0.50$, $P < .01$), nitrate ($r = 0.76$, $P < .01$), and nitrotyrosine ($r = 0.34$, $P = .02$). When this analysis was restricted only to asthmatic children, similar correlations between the proximal and distal ELF NO oxidation products were observed (total nitrite plus nitrate: $r = 0.44$, $P \leq 001$; nitrite: $r = 0.58$, $P < .01$; nitrate: $r = 0.31$, $P = .05$; nitrotyrosine: $r = 0.35$, $P = 0.05$). Within the proximal and distal airway ELF, high agreement was further observed between the measured concentrations of total nitrite plus nitrate and nitrotyrosine (Fig 3).

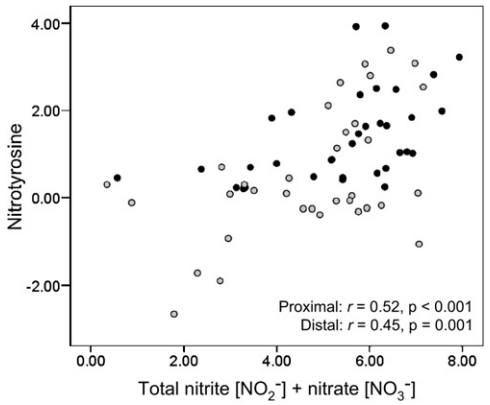


**FIG 3.** Scatterplot depicting the relationship between total nitrite plus nitrate and nitrotyrosine concentrations (in micromoles per liter) in the proximal *(black circles)* and distal *(gray circles)* airway ELF. Data were logarithmically transformed.

## Relationship of NO oxidation products to $F_{ENO}$ values and other clinical features in asthmatic children

Correlational analysis was first performed between NO oxidation products and clinical features of asthma severity, including $F_{ENO}$, serum IgE levels, the number of skin prick test responses, $FEV_1$, $FEV_1$ bronchodilator reversibility, and the percentage of BAL eosinophils and neutrophils, to determine the clinical implications of increased ELF oxidation products in children with mild-to-moderate and severe asthma. This analysis was restricted to children with mild-to-moderate and severe asthma and did not include control subjects. No significant correlations were observed between NO oxidation products and any of the clinical features measured, including $F_{ENO}$ values (see Table E6 in this article's Online Repository at www.jacionline.org). However, $F_{ENO}$ values were significantly associated with the percentage

of BAL eosinophils ($r = 0.35$, $P = .04$) and serum IgE levels ($r = 0.30$, $P = .02$).

To further evaluate factors that might affect NO oxidation in the ELF of asthmatic children, multivariate backward elimination linear regression was performed by using total nitrite plus nitrate concentrations in the proximal and distal ELF as the dependent variables and age, sex, ethnicity, ICS dose, $FEV_1$, $FEV_1$ bronchodilator reversibility, serum IgE levels, history of hospitalization, $F_{ENO}$ values, and the percentage of airway eosinophils and neutrophils as predictors. Control data were excluded. In the proximal ELF age ($P < .01$), sex ($P = .02$), and $F_{ENO}$ value ($P = .06$) were significant predictors of nitrite plus nitrate concentrations (final model: $R^2 = 0.49$, $P = .01$; see Table E7 in this article's Online Repository at www.jacionline.org). Likewise, sex ($P = .05$) and $F_{ENO}$ values ($P = .05$) were significant predictors of total nitrite plus nitrate concentrations in the distal ELF of children with mild-to-moderate and severe asthma (final model: $R^2 = 0.25$, $P = .05$; see Table E8 in this article's Online Repository at www.jacionline.org). In both the proximal and distal airway ELF, the relationship between $F_{ENO}$ values and NO oxidation was negative, such that higher $F_{ENO}$ values were associated with lower NO oxidation product formation.

## DISCUSSION

To our knowledge, this is the first study to directly measure NO oxidation products in the ELF of children with persistent asthma. Compared with control subjects, children with mild-to-moderate and severe allergic asthma had increased concentrations of nitrite, nitrate, and nitrotyrosine in the ELF that were consistently higher in the proximal versus the distal airways. Contrary to our hypothesis, we did not detect significant differences in concentrations of NO oxidation products between children with mild-to-moderate and severe asthma. Furthermore, no associations between NO oxidation products and clinical features, such as $F_{ENO}$ values, were detected by using univariate analyses. However, with multivariate modeling to control for the potential confounding effects of ICSs and atopy on NO synthesis, $F_{ENO}$ values were identified as a modest predictor of NO oxidation product formation. Although the clinical relevance of this finding is yet unclear, these data highlight the complexity of NO biology in children with asthma and suggest that the relationship between $F_{ENO}$ values and NO oxidation is not directly proportional. Thus in children with severe asthma, lower $F_{ENO}$ values might not necessarily indicate the absence of airway inflammation but instead might reflect decreased NO bioavailability from increased NO oxidation.

Airway NO biochemistry is complex, and the exact contribution of NO to the pathogenesis of asthma is not fully understood. NO is produced by nitric oxide synthases (NOSs) in a variety of cell types and serves as an important signaling molecule both within and outside of the cell.[3] NO production is also vital to the epithelial antiviral and immune defenses of the airways.[24] Although the generation of NO oxidation products from NO is important for transcription factor activation and the regulation of airway inflammation,[3] excessive airway NO production from altered NOS isoforms or lack of endogenous NOS inhibition can lead to the oxidation of NO and potential nitrogen oxide toxicity.[25] The resulting nitrosative stress might ultimately contribute to protein dysfunction and airway cellular destruction.[26] Our findings of increased nitrite, nitrate, and nitrotyrosine concentrations in the ELF of asthmatic children confirm that nitrosative stress is a distinguishing feature of the asthmatic airway. However, the underlying mechanisms responsible for this finding are unclear and warrant further study.

Although this is the first study to directly measure NO oxidation products in the ELF of children with mild-to-moderate and severe allergic asthma, our findings support previously reported observations in exhaled breath condensate. In these previous studies baseline concentrations of nitrite, nitrate, and nitrotyrosine were significantly higher in the exhaled breath condensate of asthmatic children.[8,10,27] Whereas others have shown reductions in nitrite and nitrate concentrations after 8 weeks of ICS therapy,[28] we observed NO oxidation in the ELF of children with mild-to-moderate and severe allergic asthma despite ICS treatment. This observation is intriguing and might reflect decreased sensitivity to ICSs in this population. Alternatively, NO oxidation products in the ELF might reflect complex biochemical abnormalities that are distinct from other types of airway inflammation and are not necessarily influenced by ICS treatment.[29]

Although there is increasing evidence of distal airway inflammation in human[30] and experimental[31] models of asthma, our results show that airway nitrosative stress is consistently higher in the proximal versus the distal airways. For this study, we performed sequential BAL of the right middle lobe to separate proximal and distal airway constituents.[21] Because this method of lavage was adapted for children to account for different body weights, our data might not accurately reflect nitrosative stress in the bronchial versus alveolar airspace. Thus our distal airway samples might have contained a pooling of bronchial and alveolar NO oxidation products. However, our findings are similar to those of others showing increased inflammation in the bronchial versus alveolar space in asthmatic adults[32] and lend support to the more proximal involvement of the airways in asthmatic children.

Our data do not show clear linear associations between ELF NO oxidation products and clinical features of asthma in children, which might be a function of our limited sample size or our patient selection. In addition, it is possible that our measurements of $F_{ENO}$ values and concentrations of NO oxidation products were confounded by ICSs and atopy. In steroid-naive asthmatic subjects $F_{ENO}$ values decrease in a dose-dependent manner after the initiation of ICSs.[33] Allergic sensitization is also associated with increased $F_{ENO}$ values independent of asthma,[34] a finding that might be attributable to a late-phase influx of eosinophils. In the present study all of the children with severe asthma were treated with ICSs and had objective evidence of aeroallergen sensitization. Furthermore, 80% (n = 12) of the children with mild-to-moderate asthma were taking daily ICSs, and 53% (n = 8) had positive skin prick test responses. Whereas NO metabolites were not associated with any clinical features, like others,[35] we did observe an association between $F_{ENO}$ values and airway eosinophils. This finding might explain the utility of $F_{ENO}$ values in guiding ICS reduction and evaluating asthma control.[36] Because there might also be neutrophilic or other patterns of airway inflammation in children with severe asthma, our findings also might reflect the marked heterogeneity of this group of patients. Alternatively, the differences in $F_{ENO}$ values among asthmatic subjects might be due to airway pH[37] or altered S-nitrosothiol metabolism[38] and not NO oxidation.

This study had a number of limitations. Because bronchoscopy cannot be ethically performed in healthy children, our pediatric control group was limited to nonasthmatic children with

significant respiratory symptoms. The inclusion of these children might have resulted in inadvertent selection of a group of children with significant nitrosative stress. It is also possible that some of our subjects with mild-to-moderate asthma were undertreated. Thus the concentrations of NO oxidation products measured in our group of children with mild-to-moderate asthma might not be reflective of the larger population and might have been reduced with more aggressive ICS treatment.

In summary, we have demonstrated significant increases in the formation of NO oxidation products in the proximal and distal airway ELF of children with persistent asthma. Contrary to our hypothesis, concentrations of NO oxidation products did not differ between children with mild-to-moderate and those with severe allergic asthma. Although these data highlight the magnitude of oxidant stress that is present in the airways of children with symptomatic asthma, the relationship of this nitrosative stress to asthma severity is yet unclear. Additional studies are warranted to determine the clinical utility of measuring NO oxidation products in asthmatic children, particularly given the marked heterogeneity of the disease. It might be that targeted interventions to reduce nitrosative stress are indicated in children with significant nitrosative stress, despite ICS treatment.

The Severe Asthma Research Program is a multicenter asthma research group funded by the National Heart, Lung, and Blood Institute and consists of the following contributors (*Steering Committee members): Brigham and Women's Hospital, Boston, Mass—Elliot Israel,* Bruce D. Levy, and Gautham Marigowda; Cleveland Clinic, Cleveland, Ohio—Serpil C. Erzurum,* Raed A. Dweik, Suzy A. A. Comhair, Abigail R. Lara, Sumita Khatri, Marcelle Baaklini, Daniel Laskowski, and Jacqueline Sharp; Emory University, Atlanta, Ga—W. Gerald Teague,* and Anne M. Fitzpatrick; Imperial College School of Medicine, London, United Kingdom—Kian F. Chung,* Mark Hew, and Sally Meah; National Jewish Medical and Research Center, Denver—Colo—Sally E. Wenzel*; University of Pittsburgh, Pittsburgh, Pa—William J. Calhoun,* and Bill T. Ameredes; University of Virginia, Charlottesville, Va—Benjamin Gaston*; University of Wisconsin, Madison, Wis—William W. Busse,* Nizar Jarjour, and Cheri Swenson; Wake Forest University, Winston-Salem, NC—Eugene R. Bleecker,* Deborah Meyers, Wendy Moore, Stephen Peters, Annette Hastie, and Gregory Hawkins; Washington University in St Louis, St Louis, Mo—Mario Castro,* Leonard Bacharier, Iftikhar Hussain, and Jaime Tarsi; Data Coordinating Center, Denver, Colo—Douglas Curran-Everett*; and National Heart, Lung, and Blood Institute, Bethesda, Md—Patricia Noel.

We thank Dr Benjamin Gaston and Dr Serpil Erzurum for their thoughtful assistance and careful review of this manuscript. We also thank Eric Hunter for his assistance with subject recruitment and Meredith Brown for her assistance with sample preparation and laboratory analyses.

**Clinical implications: Symptomatic children with mild-to-moderate and severe allergic asthma have significant nitrosative stress despite corticosteroid treatment. Additional therapies to decrease airway nitrosative stress might be warranted in these children.**

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## Celebrating JACI's 80th Anniversary – Francis M. Rackemann, Editorial Board Member

Francis Rackemann (1887-1973) was born in Milton, Mass, on Cape Cod, and attended Harvard University (AB, 1909; MD, 1912). After 2 years as house officer at Massachusetts General Hospital (MGH), he pursued further training in New York at Columbia University-Presbyterian Hospital (coincident with Harry Alexander) as research assistant to Warfield Longcope. Studying animal models of anaphylaxis and the relevance of immune reactions to renal and cardiovascular disease, with Longcope he published original studies on the correlation of antibodies and serum sickness.

In 1916, after World War I military service dealing with the influenza pandemic, he returned to Harvard and MGH as assistant in medicine and chief of the Medical Outpatient Department. Two years later, he published a classic report of intensive and critical studies of patients with asthma. His findings provided the original concept that all cases of asthma could not be proven to be of allergic origin and that the symptom of bronchial asthma might have multiple causes, extrinsic or intrinsic. In 1919, all work on asthma and hay fever at MGH was brought under Rackemann's direction as chief of the anaphylaxis clinic (later renamed allergy clinic), rising in Harvard faculty rank to Associate and Physician to MGH.

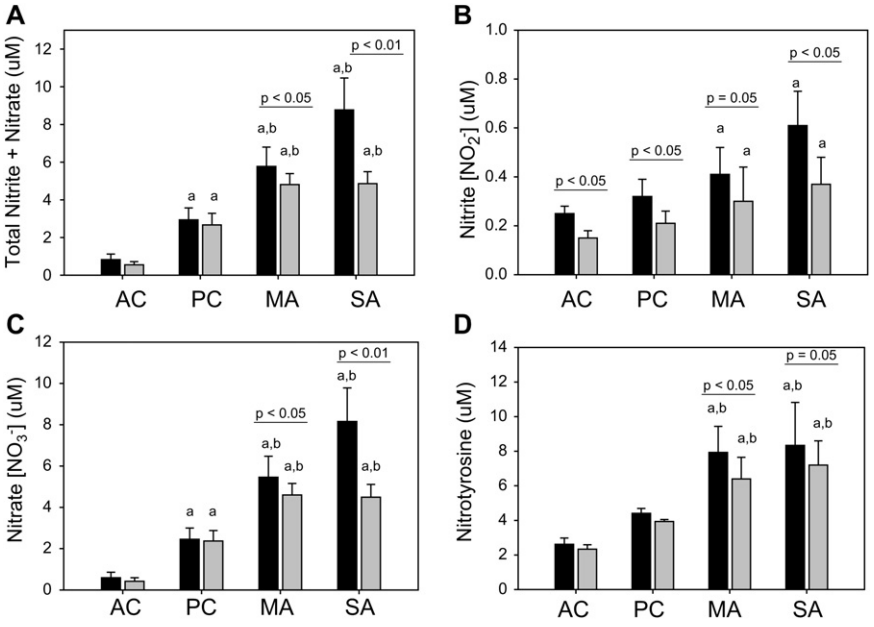


**FIG E1.** Total nitrite plus nitrate **(A)**, nitrite **(B)**, nitrate **(C)**, and nitrotyrosine **(D)** concentrations (in micromoles per liter) in the proximal *(black bars)* and distal *(gray bars)* in the raw BAL supernatant. Data were not adjusted for the urea dilution. Data represent means ± SEMs with adult control subjects *(AC)*, pediatric control subjects *(PC)*, subjects with mild-to-moderate asthma *(MA)*, and subjects with severe asthma *(SA)*. [a]$P < .05$ versus adult control subjects. [b]$P < .05$ versus pediatric control subjects.

**TABLE E1.** Criteria for severe asthma in children, as defined by the National Institutes of Health/National Heart, Lung, and Blood Institute Severe Asthma Research Program

| |
|---|
| Major criteria (must have ≥1) |
| Daily high-dose ICSs (≥6 continuous months) |
| Children <12 y: ≥440 μg of fluticasone equivalent/d |
| Children ≥12 y: ≥880 μg of fluticasone equivalent/d |
| Daily oral corticosteroid use (≥6 continuous months) |
| Minor criteria (must have ≥2) |
| Treatment with a daily controller medication in addition to ICSs |
| Daily short-acting bronchodilator use (≥5 of 7 d) |
| Airway obstruction with $FEV_1$ < 80% of predicted value at baseline |
| One or more emergency department visits in the previous 12 mo |
| Three or more oral corticosteroid bursts in the previous 12 mo |
| History of worsening symptoms with a reduction in corticosteroid dose |
| History of intubation |

**TABLE E2.** Characteristics of pediatric subjects excluded from data analysis

| Subject no. | Asthma status | Sex | Age (y) | ICS dose* | $FEV_1$ (%) | Reason for exclusion |
|---|---|---|---|---|---|---|
| 1 | No asthma | Female | 14 | 0 | 82 | *Streptococcus pneumoniae* |
| 2 | No asthma | Male | 17 | 0 | 100 | *Haemophilus influenzae* |
| 3 | Mild-to-moderate asthma | Male | 16 | 600 | 106 | *Haemophilus influenzae* |
| 4 | Mild-to-moderate asthma | Male | 10 | 200 | 95 | *Streptococcus pneumoniae* |
| 5 | Severe asthma | Male | 9 | 1000 | 54 | *Moraxella catarrhalis* |
| 6 | Severe asthma | Male | 10 | 1000 | 64 | *Streptococcus pneumoniae* |

*ICS dose is expressed as micrograms of fluticasone equivalents per day.

**TABLE E3.** Biomarkers of nitrosative stress in children excluded from data analysis

| NO metabolite (μmol/L) | Proximal airway ELF | Distal airway ELF |
|---|---|---|
| Total nitrite plus nitrate | 1394 ± 1718 | 631 ± 513 |
| Nitrite ($NO_2^-$) | 62 ± 44 | 37 ± 28 |
| Nitrate ($NO_3^-$) | 1363 ± 1674 | 593 ± 491 |
| Nitrotyrosine | 1.41 ± 0.91 | 0.92 ± 2.63 |

Data are expressed as micromoles per liter per milliliter of ELF and represent means ± SDs.

**TABLE E4.** Skin prick test responses to aeroallergens in children with mild-to-moderate and severe allergic asthma

| | Mild-to-moderate asthma (n = 15) | Severe asthma (n = 30) |
|---|---|---|
| House dust mites | | |
| *Dermatophagoides pteronyssinus* | 4 (27) | 17 (57) |
| *Dermatophagoides farinae* | 4 (27) | 20 (67)* |
| Mold species | | |
| *Alternaria* | 4 (27) | 17 (57) |
| *Cladosporium* | 3 (20) | 12 (40) |
| *Aspergillus* | 3 (20) | 12 (40) |
| Weeds | | |
| Weed mix | 0 | 9 (30)* |
| Ragweed | 1 (7) | 11 (37) |
| Animal dander | | |
| Dogs | 3 (20) | 6 (20) |
| Cats | 3 (20) | 17 (57)* |
| Cockroach | 1 (7) | 12 (40)* |
| Tree mix | 3 (20) | 9 (30) |
| Grass mix | 3 (20) | 5 (17) |

Data represent frequencies (percentages).
*$P < .05$ versus mild-to-moderate asthma.

**TABLE E5.** Characteristics of the BAL fluid from control subjects and children with asthma

| | Adult control subjects (n = 20) | Pediatric control subjects (n = 5) | Subjects with mild-to-moderate asthma (n = 15) | Subjects with severe asthma (n = 30) |
|---|---|---|---|---|
| BAL fluid recovery (% of volume instilled) | | | | |
| Proximal airway lavage | 23 ± 9 | 27 ± 9 | 38 ± 11* | 37 ± 8* |
| Distal airway lavage | 49 ± 13 | 35 ± 6 | 42 ± 16 | 38 ± 14 |
| Protein (μg/dL) | | | | |
| Proximal airway lavage | 81 ± 73 | 227 ± 190* | 194 ± 159* | 238 ± 221* |
| Distal airway lavage | 118 ± 79 | 223 ± 142* | 241 ± 113* | 219 ± 166* |
| Urea (mg/dL) | | | | |
| Proximal airway lavage | 0.36 ± 0.30 | 0.34 ± 0.24 | 0.27 ± 0.24 | 0.27 ± 0.44 |
| Distal airway lavage | 0.50 ± 0.28 | 0.44 ± 0.63 | 0.43 ± 0.53 | 0.40 ± 0.51 |
| Total leukocyte cell count ($\times 10^6$)‡ | 7.81 ± 3.61 | 3.53 ± 2.32* | 3.81 ± 3.06* | 3.32 ± 2.02* |
| Cellular differential (%)‡ | | | | |
| Macrophages/monocytes | 90.1 ± 4.3 | 90.2 ± 7.2 | 89.4 ± 7.5 | 88.0 ± 4.8 |
| Neutrophils | 3.5 ± 3.2 | 3.8 ± 1.4 | 5.3 ± 3.9*† | 5.2 ± 3.2*† |
| Eosinophils | 0.4 ± 0.5 | 0.3 ± 0.5 | 0.7 ± 0.7 | 1.9 ± 3.2*† |
| Lymphocytes | 4.9 ± 2.6 | 3.9 ± 2.8 | 3.5 ± 2.9 | 4.7 ± 3.0 |

Data represent means ± SDs.

*$P < .05$ versus adult control subjects.

†$P < .05$ versus pediatric control subjects.

‡Cells from the proximal and distal airway lavage samples were pooled for cell counting and differential analysis.

**TABLE E6.** Correlations between NO oxidation products (in micromoles per liter) and clinical features in asthmatic children*

| Proximal airway lavage | Total nitrite plus nitrate | Nitrite ($NO_2^-$) | Nitrate ($NO_3^-$) | Nitrotyrosine |
|---|---|---|---|---|
| $F_{ENO}$† | −0.12 (.53) | 0.31 (.11) | −0.15 (.43) | −0.35 (.11) |
| Serum IgE† | −0.18 (.31) | −0.09 (.63) | −0.21 (.22) | 0.09 (.66) |
| No. of skin prick test responses | −0.05 (.83) | 0.33 (.11) | −0.07 (.74) | −0.25 (.29) |
| $FEV_1$ (% predicted) | −0.06 (.73) | 0.03 (.88) | −0.03 (.88) | −0.08 (.70) |
| $FEV_1$/FVC (ratio) | −0.08 (.65) | −0.29 (.11) | 0.02 (.90) | −0.05 (.80) |
| $FEV_1$ bronchodilator reversibility (%) | 0.80 (.70) | −0.14 (.49) | 0.02 (.92) | 0.21 (.38) |
| BAL eosinophils (%)† | 0.02 (.91) | −0.18 (.32) | 0.02 (.92) | −0.17 (.41) |
| BAL neutrophils (%)† | 0.17 (.35) | 0.33 (.06) | 0.12 (.49) | −0.06 (.76) |
| Distal airway lavage | | | | |
| $F_{ENO}$† | −0.21 (.27) | 0.31 (.10) | −0.30 (.11) | −0.00 (.99) |
| Serum IgE† | 0.30 (.06) | 0.06 (.73) | 0.29 (.07) | 0.10 (.60) |
| No. of skin prick test responses | 0.02 (.91) | 0.32 (.11) | −0.02 (.92) | 0.09 (.70) |
| $FEV_1$ (% predicted) | −0.28 (.09) | 0.14 (.40) | −0.27 (.10) | −0.05 (.78) |
| $FEV_1$/FVC (ratio) | −0.09 (.59) | −0.11 (.52) | −0.07 (.66) | −0.25 (.19) |
| $FEV_1$ bronchodilator reversibility (%) | 0.03 (.89) | −0.13 (.48) | 0.04 (.83) | 0.10 (.64) |
| BAL eosinophils (%)† | 0.05 (.77) | −0.20 (.22) | 0.03 (.88) | −0.22 (.25) |
| BAL neutrophils (%)† | 0.07 (.66) | −0.06 (.70) | 0.07 (.65) | −0.11 (.59) |

Data shown represent the Pearson correlation coefficient *(r)* and the *P* value (in parentheses). NO oxidation products were logarithmically transformed due to a nonnormal distribution before analysis.

*Data are from children with mild-to-moderate and severe allergic asthma. Adult and pediatric control subjects were excluded.

†Data were logarithmically transformed before analysis.

**TABLE E7.** Results of backward linear regression of selected clinical features on the concentration of total nitrite plus nitrate in the proximal ELF

| Model | Regression coefficient | SE | *t* | *P* value | 95% CI |
|---|---|---|---|---|---|
| Included variables* | | | | | |
| Constant | 8.82 | 0.68 | 13.05 | <.001 | 7.39 to 10.26 |
| Age | −0.15 | 0.04 | −3.34 | <.01 | −0.24 to −0.05 |
| Sex | 0.71 | 0.28 | 2.54 | .02 | 0.18 to 1.31 |
| $F_{ENO}$† | −0.45 | 0.22 | −2.00 | .06 | −0.93 to 0.03 |
| Excluded variables | | | | | |
| Ethnicity | 0.02 | | 0.11 | .91 | |
| ICS dose | 0.01 | | 0.06 | .95 | |
| History of hospitalization | −0.05 | | −0.29 | .78 | |
| $FEV_1$ (% predicted) | −0.20 | | −1.05 | .31 | |
| $FEV_1$ bronchodilator reversibility (%) | 0.03 | | 0.13 | .90 | |
| Serum IgE (kU/L)† | −0.16 | | −0.85 | .41 | |
| BAL eosinophils (%)† | −0.09 | | −0.45 | .66 | |
| BAL neutrophils (%)† | 0.09 | | 0.49 | .63 | |

Total nitrite plus nitrate data were logarithmically transformed. Data are from children with mild-to-moderate and severe allergic asthma. Adult and pediatric control subjects were excluded.

*Sum of squares (model/total) = 4.908/9.944; $R^2 = 0.49$; $P = .01$.

†Data were logarithmically transformed for analysis.

**TABLE E8.** Results of backward linear regression of selected clinical features on the concentration of total nitrite plus nitrate in the distal ELF

| Model | Regression coefficient | SE | *t* | *P* value | 95% CI |
|---|---|---|---|---|---|
| Included variables* | | | | | |
| Constant | 7.27 | 0.78 | 9.39 | <.001 | 5.66 to 8.89 |
| Sex | 0.89 | 0.42 | 2.09 | .05 | 0.00 to 1.77 |
| $F_{ENO}$† | −0.74 | 0.36 | −2.07 | .05 | −1.48 to 0.01 |
| Excluded variables | | | | | |
| Age | −0.31 | | −1.69 | .11 | |
| Ethnicity | 0.14 | | 0.69 | .50 | |
| ICS dose | −0.18 | | −0.91 | .38 | |
| History of hospitalization | −0.04 | | −0.18 | .86 | |
| $FEV_1$ (% predicted) | −0.18 | | −0.88 | .39 | |
| $FEV_1$ bronchodilator reversibility (%) | −0.11 | | −0.49 | .63 | |
| Serum IgE (kU/L)† | 0.09 | | 0.46 | .65 | |
| BAL eosinophils (%)† | 0.08 | | 0.35 | .73 | |
| BAL neutrophils (%)† | −0.04 | | −0.17 | .86 | |

Total nitrite plus nitrate data were logarithmically transformed. Data are from children with mild-to-moderate and severe allergic asthma. Adult and pediatric control subjects were excluded.

*Sum of squares (model/total) = 6.173/24.708; $R^2 = 0.25$; $P = .06$.

†Data were logarithmically transformed for analysis.