Increase in Alveolar Nitric Oxide in the Presence of Symptoms in Childhood Asthma*

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Study objectives: To determine respective contributions of alveolar and proximal airway compartments in exhaled nitric oxide (NO) output (QNO) in pediatric patients with asthma and to correlate their variations with mild symptoms or bronchial obstruction. 

Patients and design: In 15 asthmatic children with recent mild symptoms, 30 asymptomatic asthmatic children, and 15 healthy children, exhaled NO concentration was measured at multiple expiratory flow (V) rates allowing the calculation of alveolar and proximal airway contributions in QNO, using two approaches, ie, linear and nonlinear models.

Measurements and results: Asymptomatic and recently symptomatic patients were not significantly different regarding FEV1 and maximum V between 25% and 75% of FVC (MEF25–75): FEV1, 93.3 ± 13.4% vs 90 ± 7.5%; MEF25–75, 70 ± 22% vs 68 ± 28% of predicted values, respectively (mean ± SD). Maximal airway QNO output was significantly higher in recently symptomatic vs asymptomatic patients (p < 0.0001), and in asymptomatic patients vs healthy children (p < 0.02): 134 ± 7 nL/min, 55 ± 43 nL/min, and 19 ± 8 nL/min, respectively. In a multiple regression analysis, variables that influenced airflow QNO output were symptoms (p < 0.0001) and distal airway obstruction as assessed by MEF25–75 (p < 0.05). Alveolar NO concentration (FA NO) was significantly higher in recently symptomatic vs patients without symptoms, whereas it was not significantly different between asymptomatic patients and healthy children: 7.2 ± 2.4 parts per billion (ppb), 5.5 ± 2.7 ppb, and 4.2 ± 2.0 ppb, respectively. 

Conclusions: An increase in FA NO was observed in the presence of symptoms, and proximal airway NO output was correlated with distal obstruction during asthma. 

Key words: exhaled nitric oxide; multiple flow analysis; nitric oxide

Abbreviations: ATS = American Thoracic Society; CN; NO = nitric oxide concentration in the airway wall; DNO = proximal airway nitric oxide diffusing capacity; FANO = alveolar nitric oxide concentration; FENO = exhaled nitric oxide fraction; FENO,50 = exhaled NO fraction calculated at an expiratory flow of 50 mL/s; MEF25–75 = maximal expiratory flow between 25% and 75% of FVC; NO = nitric oxide; ppb = parts per billion; Qbr,maxNO = maximal proximal airway nitric oxide output; QNO = nitric oxide output; V = expiratory flow

Asthma is characterized by an inflammation of both the proximal and the distal lung airways. Exhaled nitric oxide (NO) is considered a noninvasive marker of lung airway and alveolar inflammation. Increased NO output (QNO) in asthma has been extensively documented. Cellular inflammation located in the very small airways and alveoli could contribute to this increased QNO output. Along this line, increased numbers of total eosinophils, activated eosinophils, and lymphocytes have been found in the distal asthmatic lung. Furthermore, there is compelling evidence linking proinflammatory cyto- kine expression, eosinophil recruitment, and NO synthase expression or reactive nitrogen species in distal airspaces as evaluated by BAL. Consequently, we hypothesized that an increased QNO output could occur from distal airways and/or alveoli of asthmatic subjects. 

The flow dependency of exhaled NO fraction...
(FENO) has been extensively reported. Convincing mathematical models have related this experimental observation to a dual origin of NO, which is produced both by the proximal airway compartment and by the expansible compartment comprising the alveolar space and very small airways.\textsuperscript{9,11} More recently, Törnberg and colleagues\textsuperscript{12} demonstrated that oral cavity contributes significantly to exhaled NO; consequently, the nonexpansible compartment would better be named proximal or conducting airways (orotracheal plus bronchial parts). The ability of multiple-flow analysis to differentiate increase in FENO due to either distal (alveolitis or hepatopulmonary syndrome) or to bronchial (asthma) origin has been verified.\textsuperscript{13,14}

We previously demonstrated,\textsuperscript{13} as others,\textsuperscript{9,15} that the increased QNO observed in asthma is due to an increased maximal proximal airway QNO (Qbr,maxNO), the alveolar NO concentration (FANO) being normal. However, these studies were not designed to assess whether FANO could be elevated owing to the presence of symptoms or bronchial obstruction. Consequently, our objectives were to evaluate the respective contributions of Qbr,maxNO and FANO to FENO in asthmatic children using multiple-flow analysis of QNO, and to evaluate whether recent symptoms and/or airway obstruction in these children were related to significant increases in Qbr,maxNO or FANO. To ensure the validity of FANO calculation, two approaches of its determination were used using multiple-flow analysis, both of them based on the two-compartment model of the NO exchange dynamic.\textsuperscript{9,10}

**Materials and Methods**

**Patients**

Informed consent for the participation to the study was obtained from the parents in every case. Asthma was defined as a history of recurrent wheezing episodes, daily use of asthma medication, and absence of evidence for an alternative diagnosis. Consecutive asthmatic children referred for pulmonary function testing were enrolled. The aim of this study was to assess whether FANO could be a marker of distal obstruction and/or mild symptoms. Thus, we first defined a detailed structured questionnaire (symptom and medications) that was prospectively recorded in our population. Recorded symptoms were part of those included in the Asthma Quality of Life Questionnaire (chest tightness, 6 questions; cough, 12 questions; chest heaviness, 14 questions; woken at night by asthma, 24 questions; lack of a good night’s sleep, 29 questions).\textsuperscript{16} Symptomatic children were defined as having at least one symptom within 72 h before testing. Since the evaluation was planed to enroll mildly symptomatic children, in order to assess whether exhaled NO measures could reflect the onset of exacerbation (before the occurrence of significant airflow limitation as decrease in FEV\textsubscript{1}), wheezing patients were not included. For the same reason daily peak expiratory flow (V\textsubscript{pex}) was not recorded in this young population. Consequently, only mild asthma symptoms were evaluated. Only one investigator (B.M.) obtained all clinical histories. This study reports the results obtained in consecutive children or adolescents who were evaluated by functional respiratory tests in the follow-up of asthma. A group of healthy children without history of atopy (relatives of medical staff) was also evaluated.

**NO Measurements**

The breathing circuit consisted of a mouthpiece with a bacterial filter connected to a one-way valve, through which the children exhaled into an expiratory resistance while targeting a fixed mouth pressure of 16 cm H\textsubscript{2}O displayed on a water column to prevent contamination from nose and sinuses. Each NO measurement was done as recommended by the American Thoracic Society (ATS).\textsuperscript{17}

Children exhaled via separate resistances in turn while maintaining the same expiratory pressure, thus producing multiple flows that were measured by a downstream pneumotachograph (Fleisch #1; Fleisch; Lausanne, Switzerland, connected to Valin- dyne ± 2 cm H\textsubscript{2}O; Validyne Engineering; Northridge, CA). Each child was asked to perform at least one maneuver in five different ranges: very low flow (< 40 mL/s), low flow (40 to 60 mL/s), intermediate flow (60 to 100 mL/s), high flow (100 to 150 mL/s), and very high flow (> 150 mL/s). The criteria used for FENO interpretation were those in the ATS guidelines,\textsuperscript{17} the expiratory time being at least 6 s with a plateau of at least 3 s.

FENO was measured using a chemiluminescence NO analyzer (EVA4000; Seres; Aix en Provence, France), as previously described.\textsuperscript{13} NO concentration, V, and expired volume were displayed on a computer (Biopac Systems; Santa Barbara, CA).

**Modeling the QNO/Flow Rate Relationship**

We used two different approaches using multiple-flow analysis. One approach, described by Tsoukias and George,\textsuperscript{10} takes advantage of the fact that the relationship between QNO and V appears to be linear above a threshold of 50 mL/s. The second approach, described by Silkoff and colleagues,\textsuperscript{9} is based on the nonlinear relationship observed between QNO and V (< 50 mL/s), which gives additional information on the proximal airway characteristics of the QNO.

Both the linear and the nonlinear models allow characterizing distal (18th generation and beyond) and proximal airway QNO by lower airways. It can be assumed that such models can be used in children due to similar physiologic characteristics as in adults; along this line, a study\textsuperscript{10} has demonstrated similar value for FANO in healthy children than previously reported in healthy adults.

**Linear Model for Flow Rates > 50 mL/s**

Simultaneously measured FENO and V values were used to calculate QNO as follows:

\[
\text{QNO} = \text{FENO} \times V \times 0.06
\]

where QNO is expressed in nanoliters per minute, FENO in parts per billion (ppb), and V in milliliters per second (0.06 is a unit correcting factor).

The calculated QNO was represented as a function of flow rate. Least-square linear regression was performed for flow rates ≥ 50 mL/s. In this range of flows, inhaled NO concentration can be considered as negligible compared to airway wall concentration, so that the proximal airway QNO is maximal and constant, whatever the flow rate. Tsoukias and George\textsuperscript{10} have shown that the slope of this linear relationship is representative of the constant FANO, and the intercept at zero flow of the Qbr,maxNO. At least two measurements at different ranges of V are necessary.

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to determine FANO and Qbr,maxNO by this way. The knowledge of the relationship allows to calculate FENO at different V rates, which are computed as follows:

$$\text{FENO (V)} = \text{FANO} + \text{Qbr,maxNO} / (V \times 0.06)$$

where V is the chosen V rate.

We have previously shown that the height of the subject influences Qbr,maxNO, and consequently exhaled NO, probably by affecting the size of airways. Consequently, it was important to ensure that the three groups of patients were comparable regarding their height. Moreover, the healthy group of children allowed to test whether their height similarly affected Qbr,maxNO than demonstrated in healthy adults.

**Nonlinear Model With Flow Rates < 60 mL/s**

When at least one low flow (40 to 60 mL/s) and one very low flow (< 40 mL/s) were computed, FANO, NO concentration in the airway wall (CW,NO), and proximal airway NO diffusing capacity (DNO) can be computed as described by Silkoff and colleagues:

$$\text{FENO} = \text{CW,NO} \times (1 - e^{-\text{DNO}V}) + \text{FANO} \times e^{-\text{DNO}V}$$

where e is exponential, and V is expiratory flow rate. The parameters of the model were calculated by using the solver function in Excel 97 software (Microsoft; Redmond, WA) to minimize the sum of the square residual values for FENO. All available measurements of FENO and V were used to compute these parameters. Several measurements at very low flow (< 40 mL/s) enhance the validity of the results concerning DNO and CW,NO.

In this model, the product (DNO × CW,NO) is the largest amount of NO that can be delivered by the nonexpansible compartment. The product (DNO × CW,NO) obtained by the nonlinear method reflects the same proximal airway ability to produce NO than Qbr,maxNO calculated by the linear method.

**Lung Function Tests**

Spirometry measurements and flow-volume curves were obtained using a spirometer (PF/DX 108S; SensorMedics; St. Paul, MN) after NO measurements. Pulmonary function tests were performed at least 12 h after discontinuation of long-acting β2-agonists (if possible). The highest values of three technically satisfactory forced expirations were taken and expressed as the percentages of predicted normal values. Maximal expiratory flow between 25% and 75% of FVC (MEF25-75) was used as an index of small-airway caliber. The children were classified into two subgroups according to whether their MEF25-75 value was < 50% of predicted or ≥ 50% of predicted.

**Statistical Analysis**

Data are expressed as means ± SD. Comparison of the three groups (asymptomatic, recently symptomatic asthmatic children, and healthy children) was done using analysis of variance. When a significant difference was found, individual means were compared using the modified t test. Correlations between variables were analyzed using least-square regression techniques, and multiple regression analysis was also performed using NO measurement as the dependent variable and symptoms and/or peripheral obstruction as independent variables. For all comparisons, p < 0.05 was considered significant.

**Results**

Forty-five consecutive children or adolescents with asthma were enrolled (mean age, 12.3 ± 2.7 years; mean height, 147 ± 15 cm). Thirty-eight participants (84%) had positive skin test results for one or more airborne allergens. All participants used inhaled corticosteroid therapy (beclomethasone, n = 23; budesonide, n = 8; fluticasone, n = 14). Fifteen patients had mild and recent symptoms; the others (n = 30) were asymptomatic. The average dose of inhaled steroids and their mean height were similar in these two groups of asthmatic patients (data not shown). Fifteen healthy children, with no significantly different mean height as compared to asthmatic groups (143.7 ± 10.1 cm), were also enrolled.

**Technical Aspects of Multiple Flow Analysis of FENO in Children**

All 60 subjects were able to perform at least one stable prolonged V rate maneuver in low, intermediate, and high V rate, allowing the determination of the parameters of the linear model. As expected, we observed in the 60 children a marked flow dependency of FENO.

By contrast, both very low-flow and very high-flow rates were not obtained in all children. FENO at a “very” high flow (> 150 mL/s) was obtained in only 20 participants, whereas measurements did not satisfy the criteria of stability in the remaining patients. Most frequent aspect in these cases was a progressive decrease in FENO without a stable plateau. FENO was only interpretable at a “very” low flow (< 40 mL/s) in 25 asthmatic children. At this very low flow, the time needed to obtain a stable NO plateau was > 12 s, and stable V was difficult to sustain for younger patients in this condition close to apnea. The mean values of the very low flows and the low flows allowing resolution of the equation of Silkoff et al were 25 ± 9 mL/s and 39 ± 10 mL/s, respectively. We applied the equation in these 25 children. Thus, modeling was achieved in all 60 participants using the linear method and in 25 asthmatic participants using the nonlinear method.

In the 25 asthmatic participants for whom both models were used, DNO × CW,NO, which reflected Qbr,maxNO with the nonlinear method, was significantly correlated to Qbr,maxNO calculated with the linear method (Fig 1, top, A); the mean values of these two parameters were 77 ± 68 nL/min and 81 ± 73 nL/min, respectively. Similarly, FANO computed with the linear method was significantly correlated to FANO calculated by the analysis of Silkoff (Fig 1, bottom, B); their mean values were 5.8 ± 2.5 ppb and 6.8 ± 4.6 ppb, respectively. Because the linear model could be applied to the entire popula-
tion only FANO and Qbr,maxNO calculated using the linear model are reported hereafter.

**Comparison of NO Indexes Between Symptomatic and Nonsymptomatic Asthmatic Patients and Healthy Children**

The results of Qbr,maxNO, FANO, and FENO calculated at a V of 50 mL/s (FENO,50) are summarized in Figure 2. Symptomatic children were characterized by an increase in both Qbr,maxNO and FANO as compared to asymptomatic asthmatic patients and healthy children (Table 1). For healthy children, the observed values of Qbr,maxNO were not significantly different from predicted values that were calculated based on the relationship established for healthy nonsmoker adults:

\[
Q_{br,maxNO} \text{ (nanoliters per minute)} = 0.666 \times \text{height(centimeters)} - 78 \quad \text{(see Materials and Methods)}.
\]

FANO contributed substantially to FENO, the proportion of FENO due to FANO increased with the flow rate: respective contribution of FANO in FENO in symptomatic, asymptomatic, and healthy children is illustrated in Figure 3. No correlation was found between alveolar and proximal airway NO, namely FANO and Qbr,maxNO.

**Correlation With Functional Respiratory Tests in Asthmatic Children**

Pulmonary function tests demonstrated no significant difference between recently mildly symptom-
Table 1—Characteristics of the Children*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Healthy Children (n = 15)</th>
<th>Asymptomatic Asthmatic Children (n = 30)</th>
<th>Symptomatic Asthmatic Children (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>10.7 ± 1.1</td>
<td>12.1 ± 2.9</td>
<td>12.3 ± 2.6</td>
</tr>
<tr>
<td>Beclomethasone, µg/d</td>
<td></td>
<td>626 ± 253</td>
<td>636 ± 214</td>
</tr>
<tr>
<td>FEV₁</td>
<td>93 ± 13.5</td>
<td>70 ± 22</td>
<td>90 ± 16</td>
</tr>
<tr>
<td>MEF₂₅–₇₅</td>
<td>10.4 ± 3.1†‡</td>
<td>24 ± 14‡†</td>
<td>32 ± 24</td>
</tr>
<tr>
<td>FENO, 50, ppb</td>
<td>18.7 ± 8.3‡†</td>
<td>54.7 ± 43†‡</td>
<td>134 ± 72</td>
</tr>
<tr>
<td>Qbr, maxNO, nL/min</td>
<td>4.1 ± 2.3†</td>
<td>5.5 ± 2.4†</td>
<td>7.2 ± 2.7</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD.
†p < 0.05 between healthy and asymptomatic asthmatic children.
‡p < 0.05 between healthy and symptomatic children.
§p < 0.05 between asymptomatic and symptomatic children.

We sought to determine respective contribution of alveolar and proximal airway compartment in NO output in asthmatic children with recent mild symptoms as compared to asymptomatic asthmatic and healthy children. To our knowledge, this is the first study demonstrating that symptoms are associated with an increased NO output from distal lung (bronchiolar tree and alveoli) during asthma. Interestingly, a study²⁰ suggests quite similar findings. Moreover, the relationship between proximal airway QNO and peripheral obstruction, independently from the presence of symptoms, could suggest a causality relationship between QNO and both airway inflammation and remodeling.

Discussion

Our study shows that multiple-flow analysis is reliable in children and adolescents and differentiates the contributions of both proximal airway and alveolar NO. Multiple-flow analysis is reliable in children who are able to perform spirometry, i.e., 8 to 9 years old. The approach described by Tsoukas and George¹⁰ assumes nonlimited NO transfer from the airway wall to the lumen. Theoretically, solving the equation of Silkoff et al⁹ can provide a more precise analysis of airway status, including determination of DNO and CWNO.⁹ Since our goal was to compare values obtained by both linear and nonlinear models, namely FANO and Qbr,maxNO, DNO and CW,NO values were not provided. Moreover, only 25 asthmatic children were able to perform low and very low flows. Nevertheless, the good correlation between the values of alveolar NO and proximal airway NO obtained from both linear and nonlinear regressions supports that a nonlimited NO transfer exists at flow rates > 50 mL/s in these patients receiving inhaled corticosteroids.

Firstly, it was essential to ensure that alveolar NO increase was not related to a methodologic bias.
Indeed, FANO calculated by linear regression would be overestimated if a limited transfer of NO from proximal airway wall exists at low flows, between 50 mL/s and 100 mL/s, if the relation was nonlinear in this flow rate range. However, the good correlation observed in our patients between values obtained by the linear and nonlinear methods supports the existence of the FANO increase.

We and others\(^9,13,14\) have reported that the concentration of NO in alveoli is normal and that Q\(\text{br, max NO}\) is increased in asthmatic patients. However, these studies were not designed to assess whether distal NO could be increased during asthma exacerbation. A further increase in FENO in the presence of asthma exacerbation has been largely documented.\(^21\) However, no study has evaluated the origin of such an increase. Inflammatory processes have been described in distal lung during asthma. For instance, in wheezing asthmatic children, BAL studies demonstrated that alveolar macrophages have an increased production of inflammatory cytokines such as tumor necrosis factor-\(\alpha\),\(^25\) and that eosinophil cationic protein levels are increased, even during relatively quiescent periods.\(^23\) Moreover, an experimental study\(^6\) has suggested that recruited eosinophils in distal lung may contribute to NO production. Consequently, our finding of the association between an increased FANO with mild symptoms seems logical since both allergenic exposure and viral infection are responsible for distal inflammation.\(^24\) However, another likely hypothesis could be a decrease in blood flow to alveoli, and thus consumption of NO by hemoglobin, in symptomatic patients, accounting for ventilation/perfusion mismatching.\(^25\)

From a clinical point of view, evidence that FENO is elevated even in stable condition\(^13,15,26\) limits the interpretation of a single measurement of FENO. By contrast, multiple-flow analysis allowing the distinction between proximal airway and alveolar compartments provides a more useful appreciation of Q\(\text{NO}\). It is not surprising that proximal airway NO is increased in children presenting mild symptoms as compared to asymptomatic asthmatic children. Conversely, progression from normal to increased alveolar concentration is an original finding. Although the purpose of our study was not to appreciate prospectively the predictive value for asthma exacerbation of an elevated FANO, we can hypothesize that modification in deep lung NO production reflects distal inflammatory cell recruitment that may enhance the risk of acute asthma.\(^27\) The increased Q\(\text{NO}\) in recently symptomatic children cannot be ascribed to lower dose of inhaled steroids since both groups received a similar dosage.

In summary, the Q\(\text{NO}\) increase in mildly symptomatic children with asthma was mainly related to an increase in Q\(\text{br, max NO}\). Alveolar concentration is normal in stable asthmatic children but increases in case of mild recent symptoms, possibly reflecting deep lung inflammatory cell recruitment. Q\(\text{br, max NO}\) is associated with distal obstruction in these children treated with inhaled steroids. Further studies are warranted to assess whether the increase in FANO, associated with recent symptoms, indicates a loss of asthma control.

### References

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