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Variations in Exhaled Nitric Oxide in Children with Asthma During a 1-Week Stay in a Mountain Village Sanatorium

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INTRODUCTION

Airway inflammation, especially in asthmatic children, is now widely monitored by measuring the fractional concentration of exhaled nitric oxide (FENO). Several studies in patients with asthma report that FENO variations probably reflect either loss of disease-control or response to corticosteroid therapy (1–9). In addition, several cut-off values of FENO have been proposed as predictors of asthma relapse after corticosteroid withdrawal or exacerbations during therapy (1, 3, 10–12). How large an increase in FENO (using similar on-line measures) is necessary to discriminate changes due to asthma relapse (or exacerbations) from the random variation in this marker remains unclear. A first step towards answering this question is to calculate the spontaneous variations in FENO measurements over time.

Previous studies investigating spontaneous variations in FENO measurements in corticosteroid-naïve or corticosteroid-treated children were mostly conducted in outpatients (13–17). FENO measurements in outpatients could nevertheless be influenced by between-individual differences in the ambient where patients live. FENO measurements in healthy children could also be influenced by environmental differences, although previous studies found them reproducible (17–20). No study has yet confirmed reproducible FENO measurements at the currently recommended expiratory flow of 50 mL/sec (21) in children maintained in similar environmental conditions, for example in a health care center or a countryside sanatorium. Knowing more about spontaneous FENO variations over time is important to help improve monitoring of patients with asthma.

We compared spontaneous FENO variations over time in asthmatic children (with vs. without inhaled corticosteroid therapy) and healthy children living in comparable ambient conditions. To this purpose, we tested lung function (spirometry) and FENO in symptom-free asthmatic children during a 1-week stay in a countryside sanatorium and in their healthy relatives staying in the immediate neighborhood on summer holiday. In these groups we also investigated whether random variations in FENO levels could nevertheless be influenced by between-individual differences in the ambient where patients live. FENO measurements in healthy children could also be influenced by environmental differences, although previous studies found them reproducible (17–20). No study has yet confirmed reproducible FENO measurements at the currently recommended expiratory flow of 50 mL/sec (21) in children maintained in similar environmental conditions, for example in a health care center or a countryside sanatorium. Knowing more about spontaneous FENO variations over time is important to help improve monitoring of patients with asthma.

METHODS

Subjects

From consecutive children with asthma hospitalized at the Srobar Institute for Respiratory Diseases (Dolny Smokovec,
Tatra, Slovak Republic) and their healthy relatives staying nearby we invited 32 children (12 healthy subjects and 20 children with asthma) to participate in a 1-week study. The Srobar sanatorium is situated in a wooded area near the Tatra mountains at an altitude of 970 m. The ambient temperature in the summer ranges from 18° to 20°C at night, and from 22° to 24°C in the morning, humidity ranges from 70% to 80%. In this Institute, children with chronic respiratory conditions follow brief diagnostic-therapeutic programs, consisting of medication, physical exercise (walking, gymnastics, games), physiokinesotherapy, and training for asthma self-management. Healthy children were patient’s relatives staying close to the sanatorium during the same school-holiday. Asthmatic and healthy children were diagnosed as described elsewhere (22). The Asthma Control Test (ACT) was used to select subjects with good disease control; ACT scores were monitored during the study (23). Inclusion criteria were good asthma control in the past 4 weeks (negative for limitation of activities, daytime symptoms, nocturnal symptoms/awakening, and need for rescue medication) and absence of respiratory infections in the preceding 4 weeks. Of the 20 asthmatic children who participated, 13 had a history of atopy and were sensitized to multiple inhaled allergens (mainly house dust mites but also cat or dog fur, grass or tree pollens, and molds). Ten asthmatic children received continuous inhaled corticosteroid therapy (fluticasone 125 µg or budesonide 200 µg twice a day) for at least 1 month. Informed consent was obtained from parents and the study was approved by the Hospital Ethics Committee.

**Study Design**

On arrival at the sanatorium all patients underwent a clinical examination, and a history was recorded of symptoms and therapy. At 8:00 pm and every 12 hours for 2 days and again on day 7 at 8:00 AM subjects underwent lung function testing and FE\textsubscript{NO} measurements. Participants were excluded from the study if their cooperation with the maneuvers was considered unsatisfactory (unable to provide acceptable and reproducible duplicate lung function or FE\textsubscript{NO} maneuvers as recommended) (21, 24) or respiratory symptoms developed during the weekly follow-up. Healthy participants living near the sanatorium underwent the same assessments.

**Lung Function Measurements**

**Spirometry.** Forced vital capacity (FVC) maneuvers were recorded with a portable spirometer (heated Fleisch pneumotachograph ESS-γ, Biomedin s.r.l., Padova, Italy) with subjects in the standing position. Duplicate measurements from at least three acceptable maneuvers were obtained and recorded as recommended (24). Values were expressed as percent of predicted (25).

**FE\textsubscript{NO} measurements.** FE\textsubscript{NO} was measured by chemiluminescence (Sievers NOA 280; Sievers Instruments Inc., Boulder, CO, USA) as previously reported (26). The single-breath on-line technique with inhalation of NO-free air was used (27). All participants were asked to avoid food intake and physical exercise for at least 2 hours before testing. Subjects did single breath maneuvers in duplicate at a constant expiratory pressure and flow (10 cm H\textsubscript{2}O and 50 mL/s) as described in detail elsewhere (26). An effort was accepted when the expiratory pressure fluctuated less than ±2 cm H\textsubscript{2}O (from 10 cm H\textsubscript{2}O) during the maneuver (14). Subjects maintained a single exhalation until they did a FE\textsubscript{NO} plateau of at least 4 seconds. Duplicate exhalations were accepted when measurements agreed within 10% (21). In all cases FE\textsubscript{NO} measurements preceded FVC maneuvers.

Low ambient averaged-NO levels were recorded during sessions (range 1.5 to 9 ppb).

**Statistical analysis.** FE\textsubscript{NO} values were non-normally distributed (Kolmogorov-Smirnov test) and were transformed to natural logarithms (Ln) and expressed as geometric means and 95% confidence intervals (CI). Differences between geometric means were expressed as mean ratios and 95% CI. Lung function variables were normally distributed and expressed as arithmetic means and 95% CI. The Kruskal-Wallis test was used to assess between-group measurement differences for each session. Nonparametric ANOVA (Friedman test) with post hoc Dunnett test was used to assess significant within-group differences between measurements obtained at each step against measurements at baseline. The within-subject coefficient of variation (CV = SD/mean) was used to assess variability in FE\textsubscript{NO} measurements across five sessions. The Bland–Altman method was used to assess the coefficient of repeatability (CR=2 SD from the mean difference) between two within-session or between-session FE\textsubscript{NO} measurements (28). Reproducibility was assessed by means of the ICC; this coefficient estimates the average correlation between all possible ordering of pairs of measures (two within-session or between-session FE\textsubscript{NO} measurements), where an ICC value of 1 denotes perfect reproducibility and a value of 0 denotes no different reproducibility than expected by chance (29). An ICC target of at least 0.6 for repeated measures was accepted as clinically useful (30).

All FE\textsubscript{NO} measurements (from five sessions) were used for calculations of within-session CR and ICC. The number of FE\textsubscript{NO} measurements used for calculations of between sessions CR and ICC was drawn by multiplying the number of subjects (n) by the number of session intervals as follows: n × 2 for 12-hour (two night-to-morning intervals) and 24-hour intervals (first vs. second night and first vs. second morning), n × 1 for 6 days interval (first vs seventh morning).

A statistical software (SPSS 10, Chicago, Ill) was used for calculations. p values less than 0.05 were considered statistically significant.

**RESULTS**

A total 29 children (M/F:14/15, mean age 10.7 ± 2.6 years, range 5.8–16.8) completed the tests (12 healthy, 9 corticosteroid-naïve asthmatics, 8 corticosteroid-treated asthmatics) (Table 1); all 17 asthmatic children remained in good disease control. Two children, 1 corticosteroid-naïve and 1 corticosteroid-treated child, were unable to cooperate. A third child (steroid-treated) had fever and chills on the second day of the study and was excluded.

Between-group comparisons at baseline (Kruskal-Wallis test) showed the lowest lung function values and the highest FE\textsubscript{NO} levels in corticosteroid-treated asthmatics; between-group differences on lung function remained unchanged at each session, whereas differences on FE\textsubscript{NO} lost statistical significance at the last session (day 7) (Table 2).
TABLE 1.—Demographic and clinical characteristics in the study sample of 29 children.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Healthy (n = 12)</th>
<th>Untreated (n = 9)</th>
<th>Treated (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>10.1 (8.5–11.7)</td>
<td>10.9 (9.0–12.8)</td>
<td>10.3 (8.1–12.4)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>5/7</td>
<td>4/5</td>
<td>5/3</td>
</tr>
<tr>
<td>Atopy (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-House dust mites</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-Cat hair</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>-Molds</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>-Grass pollen</td>
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<td></td>
<td></td>
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<tr>
<td>-Tree pollen</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>-Positive for at least one allergen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of asthma (yrs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma in the past 12 months (n)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Exercise-induced asthma (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>5/7</td>
<td>4/5</td>
<td>5/3</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>10.1 (8.5–11.7)</td>
<td>10.9 (9.0–12.8)</td>
<td>10.3 (8.1–12.4)</td>
</tr>
<tr>
<td>FEV₁ (%)</td>
<td>101.3 (95.1–107.5)</td>
<td>94.6 (87.8–101.4)</td>
<td>87.5* (78.7–96.4)</td>
</tr>
<tr>
<td>FVC (%)</td>
<td>97.0 (81.6–112.3)</td>
<td>92.9 (76.6–109.4)</td>
<td>80.4 (53.1–107.8)</td>
</tr>
<tr>
<td>FENO (ppb)</td>
<td>6.9 (5.5–8.6)</td>
<td>13.9* (12.2–19.1)</td>
<td>7.1 (5.3–9.5)</td>
</tr>
</tbody>
</table>

* p < 0.05 and ** p < 0.005 vs. healthy subjects by Kruskal-Wallis test.

Spirometric variables are expressed as percent of predicted. Values are geometric (FENO) or arithmetic means (FEV₁) and 95% confidence intervals.

TABLE 2.—Measurements of exhaled nitric oxide (FENO) and lung function.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>1st night</th>
<th>1st morning</th>
<th>2nd night</th>
<th>2nd morning</th>
<th>7th morning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy (n = 12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FENO (ppb)</td>
<td>6.9 (5.5–8.6)</td>
<td>5.9 (4.5–7.8)</td>
<td>6.6 (5.1–8.6)</td>
<td>7.0 (5.3–9.2)</td>
<td>7.1 (5.6–9.1)</td>
</tr>
<tr>
<td>FEV₁ (%)</td>
<td>101.3 (95.1–107.5)</td>
<td>100.2 (95.4–105.0)</td>
<td>100.0 (94.3–105.7)</td>
<td>96.4 (91.5–101.2)</td>
<td>98.1 (93.1–103.2)</td>
</tr>
<tr>
<td>Untreated asthmatics (n = 9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FENO (ppb)</td>
<td>13.9** (12.2–19.1)</td>
<td>11.4** (7.6–17.1)</td>
<td>11.0* (7.5–15.9)</td>
<td>11.4* (8.1–16.0)</td>
<td>9.2 (5.8–15.0)</td>
</tr>
<tr>
<td>FEV₁ (%)</td>
<td>94.6 (87.8–101.4)</td>
<td>97.4 (88.9–106.0)</td>
<td>96.5 (87.9–105.0)</td>
<td>98.4 (88.4–108.3)</td>
<td>97.9 (89.6–106.4)</td>
</tr>
<tr>
<td>Treated asthmatics (n=8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FENO (ppb)</td>
<td>7.1 (5.3–9.5)</td>
<td>6.8 (4.8–9.7)</td>
<td>6.2 (4.6–8.4)</td>
<td>6.2 (4.4–9.0)</td>
<td>6.0 (4.5–8.1)</td>
</tr>
<tr>
<td>FEV₁ (%)</td>
<td>87.5* (78.7–96.4)</td>
<td>86.1* (76.4–95.9)</td>
<td>89.5* (84.9–94.2)</td>
<td>84.3* (74.2–94.5)</td>
<td>87.8* (78.8–96.8)</td>
</tr>
</tbody>
</table>

* p < 0.05 and ** p < 0.005 vs. healthy subjects by Kruskal-Wallis test.

FEV₁ is expressed as percent of predicted. Values are geometric (FENO) or arithmetic means (FEV₁) and 95% confidence intervals.

Among-group comparisons (analysis of variance [ANOVA] Friedman with post hoc Dunnett test) showed constant lung function values throughout the week, whereas FENO levels tended to decrease among corticosteroid-naive asthmatic children, although the decrease reached only borderline statistical significance (p = 0.057) (Table 2). FENO and lung function measurements were found unrelated (data not presented).

No differences were found between nocturnal and diurnal FENO, although levels appeared higher at the first session (first night) than at the second session (first morning) (8.6 vs. 7.5 ppb, mean ratio 1.1, p = 0.068).

The mean FENO coefficient of variation for five sessions was similar in the three groups (27.6% for steroid-naive asthmatics, 21.3% for corticosteroid-treated asthmatics, and 24.5% for healthy children).

Within-session reproducibility and repeatability for two FENO measurements were high in the 3 groups (ICC = 0.99 in all groups and CR 0.9 to 1.3 ppb) (Figure 1). High reproducibility, i.e., an ICC close to 1.0 meant an almost perfect averaged correlation between two within-session FENO measures, whereas high repeatability, i.e., low CR values (0.9 to 1.3 ppb depending on the group) meant narrow deviation from mean difference between two within-session FENO measures. Between-session FENO repeatability at 12 hours was still high for each group and did not differ from that estimated at 24 hours. After 6 days, FENO repeatability decreased markedly (low ICC and high CR values), particularly in corticosteroid-naive asthmatic children (Table 3). In these untreated asthmatic children, the percentage CR (expressed as 2 SD from the percent mean difference) for FENO measurements between the first and the second morning was 69.2% and increased to 128% between the first and the seventh morning. In contrast, percentage FENO CRs for the same morning intervals increased slightly in both groups (in corticosteroid-treated asthmatics from 44.8% to 48.0% and healthy children from 60.6% to 78.4%) (Figures 2A and 2B).

DISCUSSION

This study show distinct FENO variations over time in symptom-free asthmatic and healthy children living under comparable ambient conditions (countryside sanatorium and in the immediate neighborhood on summer holiday). At the end of the 1-week study, unlike corticosteroid-treated asthmatics and healthy children, corticosteroid-naive asthmatic children failed to achieve the target of reproducibility, namely an ICC of more than 0.6. Corticosteroid-naive asthmatic children can therefore be expected to have poorly reproducible FENO levels over a week even if their lung function remains unchanged.

The high within-session reproducibility we found in symptom-free asthmatic children fits in with previous reports in healthy and asthmatic children regardless of whether they received inhaled corticosteroid therapy (13–20). Repeated FENO measurements in a single session agree with ICC values above 0.98 (14, 20, 26).

In this study, to investigate within-day and between-day FENO reproducibility we measured FENO 12 and 24 hours...
Despite the high \( FE_{NO} \) variation of first-versus-seventh morning found in our corticosteroid-naive subjects (CR 20.8 ppb), all of them remained under the risk level for asthma relapse (49 ppb) reported by Pijnenburg et al. (10). In this pediatric study, after corticosteroid withdrawal, 9 of the 40 children relapsed. In these children, geometric mean \( FE_{NO} \) values (expiratory flow of 50 mL/s) increased from 14.8 ppb to 35.3 ppb at 2 weeks and to 40.8 ppb at 4 weeks (versus 10.5, 15.7, and 15.9 ppb for the same intervals in those who did not relapse). Studying asthmatic adults, at an expiratory flow of 250 mL/s, Jones et al. found that a weekly variation in \( FE_{NO} \) levels over 10 ppb or 60% from baseline predicted loss of disease control after corticosteroid withdrawal (1). Low \( FE_{NO} \) levels and steadily low ambient NO concentrations could also explain why no subjects relapsed in our study. For instance, in a corticosteroid-naive child with a baseline \( FE_{NO} \) of 15.7 ppb the calculated weekly increase (128%) achieved 35.8 ppb, a value under the mentioned relapse-risk level of 49 ppb (10). We cannot exclude the possibility that \( FE_{NO} \) could increase further over 4 weeks and asthma symptoms could develop.

Our corticosteroid treated children had steadily low and highly reproducible \( FE_{NO} \) levels between sessions 1 week apart (ICC 0.88). Lower ICC values (0.52) have been reported in asthmatic children treated with inhaled budesonide between sessions 2 weeks apart (10). Values of 22 to 28 ppb have been reported as predictors of exacerbations of asthma in subjects under therapy with inhaled corticosteroids (11, 12). None of our corticosteroid-treated children reached these reported levels.

Unlike previous studies we explicitly chose to study inpatients rather than outpatients because we thought that staying in a similar ambient could reduce the potential effect of the ambient where the patients live. This precaution is especially important given the substantial changes in \( FE_{NO} \) measurements related to indoor allergens (e.g., house dust mites) and outdoor contaminants (ozone, particulate matter) (35–39).

Because \( FE_{NO} \) levels tended to decrease during the 1-week study period (especially after the first 12 hours) in untreated asthmatic children, we cannot exclude the possibility that these children were “challenged” by various ambient stimuli before arriving at the sanatorium. In contrast, corticosteroid-treated asthmatic children could be “protected” against the pro-inflammatory effect of these factors. Among these stimuli (whose effects respond to inhaled corticosteroids) are allergen exposure (40, 41) or ambient outdoor pollution (37, 38). The slightly decreasing \( FE_{NO} \) levels during the 1-week study period in untreated asthmatic children presumably affected their \( FE_{NO} \) reproducibility during this interval.

### Table 3

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Within session</th>
<th>Between session</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>12 hours</td>
</tr>
<tr>
<td>No. measurements</td>
<td>154</td>
<td>58</td>
</tr>
<tr>
<td>Healthy children (n = 12) MD (CR) ICC</td>
<td>0.01 (0.86) 0.99</td>
<td>0.17 (4.7) 0.80</td>
</tr>
<tr>
<td>Untreated asthmatics (n = 9) MD (CR) ICC</td>
<td>0.06 (1.30) 0.99</td>
<td>0.83 (9.8) 0.79</td>
</tr>
<tr>
<td>Treated asthmatics (n = 8) MD (CR) ICC</td>
<td>−0.08 (0.88) 0.99</td>
<td>0.03 (5.0) 0.70</td>
</tr>
</tbody>
</table>

Mean differences (MD) and coefficients of repeatability (CR = 2 SD) are expressed in ppb units.

ICC: intraclass correlation coefficient. Within session CR and ICC were calculated from all \( FE_{NO} \) measurements (5 sessions). Between session CR and ICC were calculated by multiplying the number of \( FE_{NO} \) measurements by the number of subjects as described in the Methods section.
To exclude a possible cause of bias that might have induced an opposite effect (increased rather than decreased \(FENO\)) we ascertained that sensitization to perennial and seasonal allergens was similar in both of our asthmatic groups (corticosteroid treated or not) during the 1-week stay. This effect is especially important for exposure to perennial allergens such as house dust mites, molds, and cat dander (14, 35) insofar as exposure to seasonal allergens leaves \(FENO\) levels unchanged (35). The possible influence of pollens therefore seems unlikely to account for \(FENO\) variations in sensitized-untreated asthmatic children during the season in which we undertook the study (summer time).

A possible limitation of our study is that its duration was too short to assess a relationship between \(FENO\) changes and respiratory symptoms. Indeed, none of the corticosteroid-naïve asthmatic patients experienced symptoms requiring therapy despite their apparently large \(FENO\) variations nor did corticosteroid-naïve patients require additional increases in their inhaled dose of corticosteroids. Longer periods are therefore required to assess \(FENO\) variations in asthmatic children with good disease control.

In conclusion, in healthy subjects and in asthmatic children receiving therapy with inhaled corticosteroids, \(FENO\) measurements are reproducible across a week. In corticosteroid-naïve asthmatic children \(FENO\) levels are less reproducible. Possible long-term \(FENO\) variations (over weeks or months) in asthmatic children remain an interesting question for further research.

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