

### Measurements of exhaled nitric oxide in healthy subjects age 4 to 17 years

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**Background:** Fractional exhaled nitric oxide (FE<sub>NO</sub>) is used in monitoring of asthma.

**Objectives:** The aim of this multicenter study was to establish normal values of FE<sub>NO</sub> and assess feasibility in children with a standardized method and equipment approved for clinical use.

**Methods:** FE<sub>NO</sub> was measured in healthy subjects of 4 to 17 years according to American Thoracic Society guidelines (single breath online, exhalation flow 50 mL/s) with a chemiluminescence analyzer (NIOX Exhaled Nitric Oxide Monitoring System, Aerocrine, Sweden) in 3 European and 2 US centers. Each child performed 3 acceptable nitric oxide measurements within 6 attempts and completed an extended International Study of Asthma and Allergy in Children questionnaire.

**Results:** Measurement of FE<sub>NO</sub> was attempted in 522 children. Four hundred five children completed the study according to the protocol. Geometric mean FE<sub>NO</sub> in 405 children was 9.7 ppb, and the upper 95% confidence limit was 25.2 ppb. FE<sub>NO</sub> increased significantly with age, and higher FE<sub>NO</sub> was seen in children with self-reported rhinitis/conjunctivitis or hay fever.

The success rate was age-dependent and improved from 40% in the children 4 years old to almost 100% from the age of 10 years. The repeatability of 3 approved measurements was 1.6 ppb (95% CI, 1.49-1.64 ppb).

**Conclusion:** FE<sub>NO</sub> in healthy children is below 15 to 25 ppb depending on age and self-reported atopy. Measurement of FE<sub>NO</sub> by NIOX<sup>®</sup> is simple and safe and has a good repeatability. Feasibility depends on age and may be difficult in the preschool child. (J Allergy Clin Immunol 2005;115:1130-6)

**Key words:** Exhaled nitric oxide, children, normal values

The fractional concentration of nitric oxide (NO) in exhaled air (FE<sub>NO</sub>) is generally higher in individuals with asthma than in healthy subjects,<sup>1</sup> although reported values of FE<sub>NO</sub> in healthy subjects vary from 3 ppb to 88 ppb.<sup>2-7</sup> These variations may partly be attributed to different measurement techniques but also to low numbers studied. In addition, confounding factors such as age, sex, and atopic symptoms have not always been taken into account.

Increasing use of FE<sub>NO</sub> measurement in the diagnosis and monitoring of asthma has urged the need for reference values of FE<sub>NO</sub> measured with commercially available equipment.

In 1999, guidelines on the measurement of FE<sub>NO</sub> were issued by the American Thoracic Society (ATS),<sup>8</sup> updated for children in 2001 by an European Respiratory Society/ATS task force.<sup>9</sup> Recently commercial equipment (NIOX, Aerocrine, Sweden) obtained Food and Drug Administration clearance, allowing dissemination of its clinical application.

The primary objective of this study was to establish reference values of FE<sub>NO</sub> according to international guidelines in children of 4 to 17 years. Secondary objectives were to determine the short-term repeatability of FE<sub>NO</sub> levels; to investigate the effect of possible confounders (ambient NO level, age, sex, ethnicity, height, weight, and self-reported information on passive smoking, eczema, and atopic symptoms in the children and family); to determine the success rate for the procedure for each age group; and to report any possible discomfort or adverse events during the measurements.

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#### Abbreviations used

ATS: American Thoracic Society  
FE<sub>NO</sub>: Fractional concentration of exhaled nitric oxide  
ISAAC: International Study of Asthma and Allergy in Children  
NO: Nitric oxide

## METHODS

### Study subjects and protocol

Five centers, 3 in Europe (Copenhagen, Denmark, Rotterdam, The Netherlands, and Padova, Italy) and 2 centers in the US (Denver, Colo, and Charlottesville, Va) participated in this open, multicenter study. We planned to recruit 450 children equally distributed between boys and girls and divided into 9 age groups (4, 5, 6, 7, 8-9, 10-11, 12-13, 14-15, 16-17 years).

The children were recruited from kindergartens and public schools. Written informed consent was provided by parents and the child (if 12 years or older). Exclusion criteria were a history of asthma or related respiratory symptoms defined by the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire<sup>10</sup>; history of treatment with asthma-specific medication ( $\beta$ -agonists, steroids, leukotriene receptor antagonists); other chronic pulmonary diseases or recent airway infection (cold, flu, sore throat within the last 7 days), active smoking, or inability to comply with the study procedure.

The study was approved by the local ethics committee in each country and local health authorities where needed.

### FE<sub>NO</sub> measurements

FE<sub>NO</sub> was measured by using the online single breath method with NIOX (Nitric Oxide Monitoring System; Aerocrine, Sweden) according to ATS guidelines.<sup>8</sup> The subject was seated comfortably, with the instrument at a convenient position in front of the subject. No nose clip was used.

The subject inserted the mouthpiece and inhaled NO-free air to total lung capacity over a period of 2 to 3 seconds through the mouthpiece of the NIOX instrument. The subject then started exhalation against a positive mouthpiece counter pressure of 10 to 20 cm H<sub>2</sub>O to ensure that the soft palate was closed against the nasal cavity, thus preventing contamination of exhaled NO with NO of nasal origin. Nose clip was not used.<sup>11</sup> Exhalation flow was 50 mL/s. Children younger than 10 years performed a 6-second exhalation, and FE<sub>NO</sub> was calculated during the last 2 seconds of the exhalation. Children  $\geq 10$  years performed an exhalation of 10 seconds, and FE<sub>NO</sub> was calculated during the last 3 seconds of the exhalation. A measurement was accepted if (1) the mean flow was 0.045 to 0.055 L/s, (2) the instant flow was 0.0375 to 0.0625 L/s, and (3) the instantaneous mouth pressure was 5 to 20 cm H<sub>2</sub>O. Exhalations were approved if they did not deviate more than 2.5 ppb or 10% and were completed within a 15-minute period. The interval between exhalations was at least 30 seconds. Each subject performed no more than a total of 6 exhalations, and the total number of exhalations performed to obtain 3 acceptable FE<sub>NO</sub> values was recorded. FE<sub>NO</sub> was calculated as the mean of 3 correct exhalations.

All measurements were performed between 8:00 AM and 5:00 PM. Information on eating or any strenuous physical activity during the last 60 minutes before testing was obtained. The subject rested in the sitting position for 5 minutes before the measurement procedure.

Nitric oxide-free air was used in all measurements to minimize the influence of ambient NO on the results.

After the measurements, children were asked if they had any adverse event in association with the procedure.

### Questionnaire

Each subject completed the ISAAC core questionnaires for asthma, rhinitis, and eczema.<sup>10</sup> Atopy was defined as any positive answer regarding rhinitis/conjunctivitis and/or hay fever. Additional information was obtained on recent infection, passive smoking, parental disposition to asthma or atopy, and ethnicity. The questionnaire was completed by the parents. When data were missing, these were filled in by a telephone interview afterward. The parents were asked to contact the investigator in case a subject developed a respiratory tract infection within 3 days after the FE<sub>NO</sub> measurement.

### Data analyses

Analyses were performed by using data from all subjects who completed the study according to the protocol ( $n = 405$ ), except feasibility analyses, which were performed on a data set consisting of the total number of subjects having performed or having tried to perform FE<sub>NO</sub> measurements ( $n = 522$ ).

Because the distribution of FE<sub>NO</sub> was skewed, all analyses were performed with log-transformed data ( $e^{\ln}$ ), and results are presented as point estimates and 1-sided CIs with back-transformed values. Reference values of FE<sub>NO</sub> are presented as geometric means and upper 95% 1-sided confidence limits for individuals. Individual upper 95% confidence limits were calculated as  $e^{(\text{LN}(\text{mean}) + t \times \text{LN}(\text{SD}))}$ , where  $t$  is the Student  $t$  with the appropriate number of degrees of freedom ( $n - 1$ ) and  $n$  the number of observations in a group.

All analyses were performed with and without outliers, and both results were presented if different. Outliers were defined as FE<sub>NO</sub> values above arithmetic mean + 2 SD. Factors possibly affecting FE<sub>NO</sub> were analyzed by linear regression multivariate analyses and Pearson correlation analysis. Group comparisons were analyzed by independent  $t$  tests and 4-field tables by the Fisher exact test.

Short-term repeatability (intraindividual SD) was calculated as the SD of 3 measurements within a subject. The CIs for repeatability were calculated by using the  $\chi^2$  distribution, because the variance of repeatability was distributed according to this distribution.

Success rates were calculated as the frequencies of children of different ages that were able to perform the measurement procedure according to the protocol. Multivariate regression and partial correlation analyses were performed to investigate possible influence on FE<sub>NO</sub> from confounding factors such as age, sex, and other factors.

## RESULTS

### Subject flow chart

Five hundred thirty-four children were recruited for the study. Four hundred five children (191 boys) completed the study with approved measurements. Subject flow diagram and demographic data are presented in Fig 1 and Table I.

Within 1 hour before the FE<sub>NO</sub> measurement, 16% of the subjects had food or liquid intake (other than water), and 1.5% had performed strenuous physical activity.

### Reference FE<sub>NO</sub> values for age and sex

Table II presents geometric means and upper 95% limits of FE<sub>NO</sub> reference values according to age for all children ( $n = 405$ ). Sixteen children had extreme FE<sub>NO</sub> values ( $>34.9$  ppb) and were considered outliers. Data are also presented without these 16 outliers ( $n = 389$ ), and

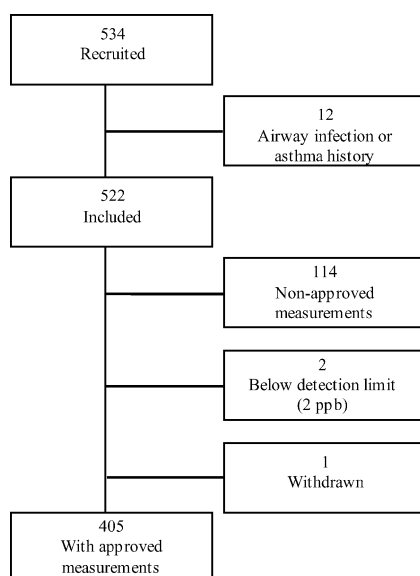


FIG 1. Subject flow diagram.

without outliers and 57 atopic children (rhinitis/conjunctivitis and hay fever;  $n = 332$ ).

There was no difference in  $FE_{NO}$  between boys and girls (geometric mean = 10.0 ppb vs 9.4 ppb, respectively;  $P = .27$ ). Individual values are presented in Fig 2 (without outliers).  $FE_{NO}$  was significantly and positively related to age in both sexes with an increase (slope) of  $FE_{NO}$  of 5% (95% CI, 3% to 6%) per annum.

On the basis of simple linear regression analysis, estimates of geometric means and upper 1-sided 95% limits for the reference values according to age with and without outliers are presented in Fig 3.

## Outliers

Extremely high  $FE_{NO}$  levels ( $>34.9$  ppb) were found in 16 subjects (10 boys), predominately older children (88%  $>10$  years). The outliers were characterized by having more reports of rhinitis symptoms ever (37.5% vs 11.9%) or within the last 12 months (31.3% vs 8.2%) or itchy-watery eyes (25.0% vs 5.1%; Fisher exact test,  $P < .01$ ), whereas reporting of hay fever was similar (6.3% vs 6.7%) to the rest of the sample ( $n = 389$ ). Among non-Caucasian children, 22% were outliers, in contrast with only 3% of Caucasian children. The outliers were older ( $P < .002$ ), and in a multivariate regression analysis controlling for age, they were also significantly heavier ( $P < .005$ ) and taller ( $P < .037$ ) but did not have a significantly higher BMI ( $P < .24$ ).

## Height and weight

In partial correlation analyses, adjusted for age, anthropometric variables height ( $r = 0.115$ ;  $P < .02$ ), weight ( $r = 0.139$ ;  $P < .005$ ), and body surface area ( $r = 0.145$ ;  $P < .004$ ) were all significantly positively related to natural logarithm  $FE_{NO}$  when including all children ( $n = 405$ ). After removing the outliers, the correlations

TABLE I. Anthropometric and demographic characteristics

| N                    | 405                   |
|----------------------|-----------------------|
| Male                 | 191 (47 %)            |
| Height, mean (range) | 140.9 cm (91.0-193.0) |
| Weight, mean (range) | 37.4 kg (14.0-115.0)  |
| Ethnic background    |                       |
| Caucasian            | 374 (92.3%)           |
| Black                | 23 (5.7%)             |
| Asian                | 5 (1.2%)              |
| Hispanic             | 3 (0.8%)              |

were no longer statistically significant, with the exception of body surface area ( $r = 0.102$ ;  $P < .05$ ). Body mass indexes were not significant correlated to  $LN FE_{NO}$ .

## ISAAC questionnaire

Fourteen percent ( $n = 57$ ) of the children reported 1 or more of the following symptoms: running nose/conjunctivitis ( $n = 52$ ), itching eyes ( $n = 24$ ), and hay fever ( $n = 27$ ); they were defined as atopics. Children with symptoms of rhinitis had significantly higher  $FE_{NO}$  values than children without (12.7 ppb vs 9.3 ppb;  $P < .004$ ). The same was true for children reporting itching eyes (13.5 ppb vs 9.5 ppb;  $P < .019$ ) or hay fever (13.9 ppb vs 9.5 ppb;  $P < .001$ ). These self-reported manifestations were all significantly positively related to  $FE_{NO}$  ( $P < .01$ ), even when controlling for age.

History of atopy in the family was significant more frequent in atopic children compared with nonatopic children (24% vs 12%;  $P < .003$ ).

A history of eczema (11%), ambient NO level, time of day, atopy in family (34%), and passive smoking within the last 6 months (32%) had no significant influence on  $FE_{NO}$  level.

## Ethnic background

Non-Caucasian subjects ( $n = 31$ : African American subjects, Asian subjects, and others) had significantly higher  $FE_{NO}$  ( $P < .002$ ) mean values (mean difference = 9.1 ppb [95% CI, 3.6-14.7 ppb]) compared with Caucasian subjects. However, when controlled for age, sex, and self-reported atopy in a stepwise forward linear regression analysis, ethnic origin had only a borderline significant effect on  $FE_{NO}$  ( $P = .076$ ).

## Country difference

When controlling for age, sex, ethnicity, and self-reported atopy, there was a significant effect of geographic location: the site in Virginia had significantly higher arithmetic  $FE_{NO}$  levels (mean  $FE_{NO}$  difference = 6.5 ppb; 95% CI, 2.7-10.3 ppb;  $P < .001$ ) compared with the other centers, also after excluding outliers.

## Diurnal variation

There was a trend of increased  $FE_{NO}$  in the afternoon ( $r = 0.11$ ;  $P < .03$ ), but when performing a regression analysis including time point and age as independent variables, the variation of  $FE_{NO}$  was explained by the

**TABLE II.** Reference values of FE<sub>NO</sub>

| Age, y | Geometric mean, Individual 95%<br>N ppb upper limit |      |      | Geometric mean, Individual 95%<br>N ppb upper limit |      |      | Geometric mean, Individual 95%<br>N ppb upper limit |      |      |
|--------|-----------------------------------------------------|------|------|-----------------------------------------------------|------|------|-----------------------------------------------------|------|------|
|        | With outliers                                       |      |      | Without outliers                                    |      |      | Without outliers and atopic*                        |      |      |
| 4      | 29                                                  | 7.1  | 15.7 | 29                                                  | 7.1  | 15.7 | 27                                                  | 7.0  | 15.0 |
| 5      | 35                                                  | 7.9  | 16.6 | 35                                                  | 7.9  | 16.6 | 33                                                  | 8.0  | 17.1 |
| 6      | 49                                                  | 8.2  | 19.3 | 48                                                  | 8.0  | 17.1 | 42                                                  | 7.5  | 15.5 |
| 7-9    | 107                                                 | 8.1  | 18.4 | 106                                                 | 8.0  | 17.2 | 89                                                  | 7.8  | 17.1 |
| 10-13  | 105                                                 | 11.2 | 28.2 | 98                                                  | 10.1 | 19.2 | 85                                                  | 9.8  | 18.0 |
| 14-17  | 80                                                  | 13.7 | 39.2 | 73                                                  | 11.9 | 24.2 | 56                                                  | 11.6 | 22.4 |
| Total  | 405                                                 | 9.7  | 25.2 | 389                                                 | 9.0  | 19.4 | 332                                                 | 8.8  | 18.5 |

\*Positive answer to rhinitis/conjunctivitis or hay fever symptoms.

age rather than the time point. This was because older children more often performed their measurement in the afternoon.

### Success rate

One hundred forty-three children violated the protocol because they could not cooperate or needed more than 6 attempts to obtain 3 approved measurements. Twenty-nine of these were still included in the analyses because they had apparently been interrupted by other children during the procedure. Success rates improved with age and increased from 40% at 4 years and 60% at 5 years to 100% at school age.

### Repeatability

The within-subject SD estimated from individuals with 3 approved measurements was 1.6 ppb (95% CI, 1.49-1.64).

### Safety

One 5-year-old boy was withdrawn after the first measurement because he started crying. No serious or nonserious adverse events were reported.

## DISCUSSION

This is the first report on normal values of exhaled NO in healthy children from preschool age to adolescence performed according to current ATS guidelines by using commercially available equipment and the online single breath technique.

We report that the upper normal level of FE<sub>NO</sub> in children from 4 to 17 years ranges from 15 ppb in the young children to 25 ppb in adolescents, with a mean increase of 1 ppb per year. We established this age dependency, which has been suggested in other, smaller studies.<sup>3,12,13</sup> The reason FE<sub>NO</sub> increases with age is not known, but there is no age-related FE<sub>NO</sub> trend in adulthood.<sup>14</sup> The age dependency in children may be related to the developmental and maturational changes; increased lung volume and airway surface area<sup>12</sup>; changes in airway NO diffusion coefficients, which may be dependent on

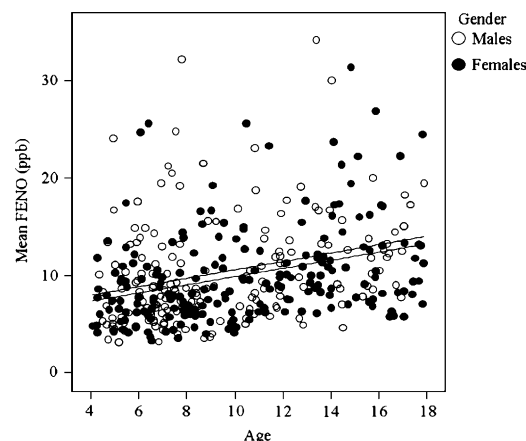


**FIG 2.** Individual FE<sub>NO</sub> values broken down by sex and age for all included children except outliers (>34.9 ppb).

surface area<sup>3</sup>; or age-dependent induction of inducible nitric oxide synthase secondary to recurrent immunological stimulation.<sup>15</sup> The use of a constant exhalation flow rate (50 mL/s) in children of different airway sizes may also be a factor explaining the age dependency of exhaled NO, because the same flow is relatively higher for young children.<sup>16</sup>

Age per se may not influence FE<sub>NO</sub>, because variables like body weight, height, and body surface area all were related to FE<sub>NO</sub> when controlling for age. However, the partial correlation coefficients were rather low (0.115-0.139). Studies in children have not previously reported any correlation between FE<sub>NO</sub> and height or weight, contrary to healthy adults, in whom FE<sub>NO</sub> has been reported to depend on height, weight, body mass index, and body surface area.<sup>14</sup> Interestingly, when outliers were excluded, the correlations were not significant. Whether differences in body size explain the FE<sub>NO</sub> difference between US and Europe is not clear, but the US subjects were significantly taller and heavier when controlled for age, atopy, and ethnicity compared with the European children. In addition, 50% of the outliers came from the US site (Virginia), which to some extent could explain the increased FE<sub>NO</sub> in this group.

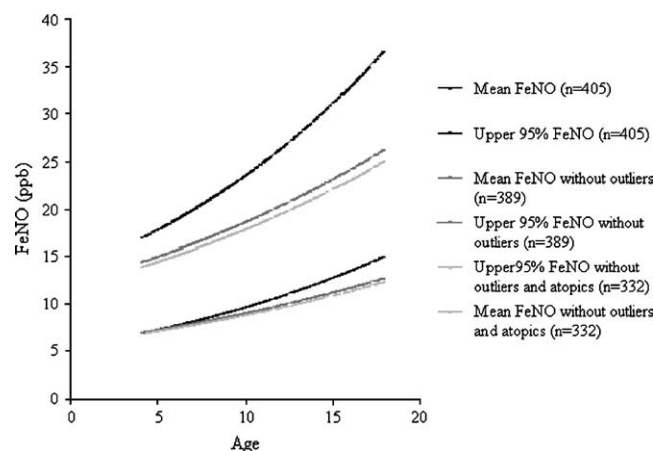


FIG 3. Individual values of  $FE_{NO}$  in healthy children.

Upper limits of normal  $FE_{NO}$  values between 15 and 25 ppb in the current study are comparable with findings of the few earlier studies in children<sup>11,12,17,18</sup> using the same exhalation flow of 50 mL/s as recommended by the ATS.<sup>8</sup> However, children younger than 6 to 7 years have not been included in any of the previous studies with similar methodology. An earlier study in children between 4 and 7 years measured  $FE_{NO}$  by using a single breath and 50 mL/s exhalation flow reported normal values of 4.9 ppb and 7.6 ppb for boys and girls, respectively.<sup>7</sup>

A significantly higher  $FE_{NO}$  has been reported in healthy men compared with women<sup>11</sup> and in girls compared with boys.<sup>7</sup> We found no sex effect in children, nor has that been found in other previous studies in children.<sup>3,12,15</sup>

Children who reported allergic symptoms in the nose or eyes or hay fever had higher  $FE_{NO}$  levels than children without. Previous studies have reported that adolescents and adults with allergic rhinitis have increased  $FE_{NO}$ ,<sup>19,20</sup> and that sensitized but asymptomatic children and adults have higher  $FE_{NO}$  than asymptomatic nonsensitized individuals.<sup>12,21,22</sup> Although data are still conflicting, sensitization, rhinitis, and conjunctivitis may be independent determinants of  $FE_{NO}$  in subjects without asthma of all ages.<sup>3,23</sup>

Excluding subjects with a suspected history of allergic rhinitis causes only a minor change of the upper 95%  $FE_{NO}$  levels (Table II). Still, suspicion of allergic rhinitis should be considered when measuring  $FE_{NO}$ , because acute allergen exposure might influence  $FE_{NO}$ .<sup>19,24</sup> Because this study included healthy children from public institutions, unfortunately it was not feasible to measure atopy objectively by skin prick tests or total specific IgE, so we cannot rule out that atopic subjects have higher  $FE_{NO}$  levels. However, because the ISAAC questionnaire is simple and validated, we believe that these reports are reliable.

A history of eczema or passive smoking was not correlated to  $FE_{NO}$ , and previous reports have been conflicting.<sup>12,25,26</sup>

Our study was a cross-sectional study with 1 visit during the daytime; hence, diurnal variation could not be

investigated. In 6 nonatopic school children, a circadian rhythm of the inflammatory markers ( $FE_{NO}$  and urine eosinophilic protein X) has been demonstrated with a peak in the early morning.<sup>27</sup> However, the majority of the children in our study were measured within a rather narrow period of time during the day (9 AM to 3 PM). Indeed, other studies have not been able to prove a significant variation during the day.<sup>3,11,28</sup>

Most subjects were Caucasian, and this study was not powered to detect any effect from ethnic background on  $FE_{NO}$ . Indeed, 43% of the extreme outliers were non-Caucasian subjects, but they accounted for only 7% of all included children. It can be speculated whether genetic differences might explain the high prevalence of non-Caucasian outliers. Second, the significantly increased mean  $FE_{NO}$  level in 1 US center (Virginia) compared with the other participating centers may partly reflect genetic differences, because this US center contributed 67% of the non-Caucasian subjects. Significant differences in allele frequencies between Caucasian subjects and African American subjects in the neuronal nitric oxide synthase (NOS1) gene have been described, and this enzyme is involved in the endogenous NO production.<sup>29</sup> Further studies may reveal the need for ethnicity-specific reference  $FE_{NO}$  values.

Even though  $FE_{NO}$  is assumed to be unaffected by ambient NO when using the single breath technique,<sup>16,30</sup> it is generally recommended to use NO-free air if ambient NO is in excess of 50 ppb.<sup>8</sup> We therefore used NO-free air for all measurements. Exposure during the morning hours to high levels of outdoor pollution has been associated with increased  $FE_{NO}$ , which persisted as long as 5 hours,<sup>31</sup> but because ambient NO levels in general were higher in Europe compared with the United States, such a phenomenon cannot explain the difference in mean  $FE_{NO}$  between the United States and Europe.

Food intake or drinking (not water) and extreme physical activity within an hour before the measurement may confound the  $FE_{NO}$  measurement. Previous studies have shown that nitrate-rich food before measurement

might increase  $FE_{NO}$  by denitrifying microbiological organisms in the mouth,<sup>32,33</sup> and intake of caffeine, which is found in coffee, tea, and cola, has been associated with a brief reduction of  $FE_{NO}$  in healthy adults over a period of 4 hours.<sup>34</sup> Sixteen percent of our children had been eating or drinking shortly before measurements, but any effect of this consumption on  $FE_{NO}$  could not be demonstrated, because their  $FE_{NO}$  level was measured only once. Exercise might increase<sup>35</sup> or decrease<sup>36</sup>  $FE_{NO}$  in healthy subjects, but very few subjects had performed physical activity within 60 minutes before the measurement procedure without any significant effect on the results.  $FE_{NO}$  values were similar to the other children when controlled for confounders such as age and atopic symptoms.

Nitric oxide measurement was possible in 40% of 4-year-old children. The success rate increased to 80% to 100% in late school age. The NIOX has a built-in dynamic flow restrictor, which helps the child maintain constant flow despite small variations in mouth pressure. To increase feasibility in the youngest age groups, we reduced exhalation time to 6 seconds for children younger than 10 years, which deviates from the current guidelines.<sup>8</sup> However, in the ATS guidelines of 1999, a plateau of 2 seconds and minimal exhalation of 4 seconds are recommended for children younger than 12 years.

Short-term repeatability (intrasubject variation) of 3 approved measurements was 1.6 ppb. This is even better than in the study by Kharitonov et al<sup>11</sup> using the same NO analyzer, in which the mean pooled SD of all measurements from 59 children and adults was 2.1 ppb.

In conclusion,  $FE_{NO}$  measurement with NIOX is safe, has excellent short-term repeatability and is feasible in school-age children. The upper limit of normal  $FE_{NO}$  level in healthy children significantly increases with age from 15 ppb at the age of 4 years to 25 ppb in adolescence.  $FE_{NO}$  was higher in children with self-reported atopic manifestations.

All involved centers thank the children and their parents for participating, as well as the involved kindergartens and public schools.

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