

Exhaled nitric oxide in healthy children: Variability and a lack of correlation with atopy

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Nitric oxide (NO) is a free radical produced by several lung cells via the enzyme nitric oxide synthetase (NOS) and can be easily measured in exhaled air by chemiluminescence analysis. As the iso-enzyme iNOS may be induced by cytokines and endotoxin, NO is elevated in several chronic inflammatory airway diseases. Prior to using exhaled nitric oxide (eNO) as a non-invasive marker of airway inflammation in daily routine, the role of possibly influencing factors such as age, time of the day, smoking exposure and intra-individual variability have to be clarified. NO concentrations were measured in 107 healthy children aged 4–18 years at an expiratory flow of 184 ml/s. Spirometry and a skin-prick test were performed and a questionnaire on family history of atopy, personal symptoms of atopic disease and smoke exposure was completed. For intra-individual variability nitric oxide was measured in six children three times daily on 6 consecutive days. Median eNO concentration was 5.7 p.p.b., and increased significantly with age but did not vary with gender. No correlation was found between eNO and smoke exposure, positive skin-prick test, FEV_1 , MEF_{25} and time of the day. There was no circadian rhythm found in the six children measured on 6 consecutive days, but the eNO showed an intra-individual coefficient of variation of 25.9%. With the help of a two-compartment model of the lung the alveolar NO concentration was estimated to be 4.1 p.p.b and was shown to be constant with age, whereas the airway part of NO steadily increased with age. When comparing eNO values with standardized measurement techniques, the age of the children and the large intra-subject coefficient of variation have to be taken into account, whereas in healthy children subject-specific factors such as atopic history, gender and skin test reactivity did not affect eNO measurement.

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Exhaled nitric oxide (eNO) may represent a non-invasive marker of airway inflammation. In exhaled air of asthmatic adults (1–3) and children (4–8) increased NO concentrations have been measured and indicate possibly airway inflammation. NO is produced from the amino acid l-arginine by different iso-enzymes of NO synthetase (NOS). The expression of one of the iso-enzymes, the inducible NO synthetase

(iNOS) is upregulated by inflammatory cytokines (9,10). This leads to an increase of eNO in inflammatory airway disease, whereas an inhibition of the iNOS via corticosteroids reduces eNO in asthmatics but not in healthy subjects (1,8,11).

In several studies it was shown, that both technical factors (4,12–14) and subject specific factors like smoking (1,2), age (15,16), skin-prick test (16), family history of atopy (17) and the time of the day when the measurement is performed (18) may influence eNO. The role of these factors has to be clear before eNO can be used in daily clinical practice with comparison between differ-

ent laboratories. Technical factors may be controlled by strict definition of the measurement technique. Currently, the single breath technique is the method of choice (12). A constantly maintained exhalation flow in a range from 10 to 250 ml/s is recommended (4,13,14), with a flow of 50 ml/s being the flow of choice in the latest recommendations (19).

In this study, we used this technique to establish reference eNO values in children at one defined expiratory flow and to examine the influence of subject-specific factors on the eNO concentration. Measurements at different expiratory rates were performed to investigate in children the influence of expiratory flow on the eNO concentration as previously described (13,14,16,20). We used a previously established model to calculate alveolar and airway NO concentration from the NO elimination rate and the exhalation flow for these data sets (21–24). The effect of the above-mentioned subject-specific factors on these variables was assessed. Finally, in order to define the intra-subject variation of NO values over several days and a possible circadian rhythm in normal subjects, NO measurements were performed with the same subjects three times daily for 6 consecutive days.

Methods

Subjects

One hundred and seven healthy children (57 girls) aged between 4 and 18 (11.1; interquartile range IQR 8.8–13.5) years were recruited for this study from relatives, friends and a local sports club. Children with a history of chronic respiratory tract disease as well as children with acute respiratory symptoms or symptoms for 3 weeks prior to the test were not included in the study. All children had written agreements of their parents to undergo a medical assessment at the Department of Pulmonology and Allergy at the Kinderpoliklinik im Dr von Haunerschen Kinderspital der LMU München. The study was performed in the time between September and November and was approved by the local ethics committee.

All children answered a questionnaire with the help of their parents concerning possible clinical signs of atopic disease; time, duration and exact specification of these symptoms and current or regular medication of the subjects themselves. A family history of atopic disease was specified with questions to the subjects' parents, concerning their history of asthma, hay fever, atopic dermatitis, allergic rhinitis or food allergy.

According to the results of this questionnaire the children were grouped into those with a family history of atopy and those without. Another section concerned the smoke habits of the subjects as well as possible smoking of a family member at home, leading to classify the children into a group with smoke exposure and a group without smoke exposure.

Design

The assessment that was performed on all children consisted of first a skin-prick test followed by a spirometric lung function test and finally measurements of eNO. The subjects were asked not to eat for 1 h prior to the test, otherwise neither other food restrictions were made nor a special food intake history was taken.

Measurement of eNO

The measurements of eNO were made according to recently published recommendations (13,14) with a chemiluminescence analyzer (LR 2000, Logan Research, Rochester, UK). The analyzer was calibrated daily using certified NO mixtures (100 p.p.b) in nitrogen (BOC Gases, Guildford, UK). Briefly, the children were asked to perform a slow vital capacity manoeuvre into wide-bore Teflon tubing without wearing a nose clip. To isolate the nasopharynx from the oropharynx by a closed soft palate, the subjects exhaled against a positive pressure of 5 cm H₂O. As the exhalation flow was displayed on a computer screen, the children were able to maintain the defined exhalation flow of 184 ml/s. In order to obtain an end-expiratory NO value, exhalation manoeuvres were repeated three or four times. The eNO-plateau was only considered acceptable if it lasted for 2–3 s, if the simultaneously recorded CO₂ concentration was >4.5% and if the exhalation pressure was constant as monitored online on the computer screen. The final eNO value recorded was the median of the three or four plateaus obtained in each subject. The intra-measurement coefficient of variation for 45 subjects at 141 measurements was 6.5 (4.2–8.6)%. Environmental NO was measured prior to each eNO study and was 7.6 (0–18) p.p.b., with no correlation between the two variables.

Measurement of lung function

Forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV₁) as well as mean expiratory flow at 25% (MEF₂₅) were measured spirometrically (Master-Laboratory, Jaeger, Würzburg, Germany) according to standard

Table 1. Influence of various factors on eNO

p-Value of multivariate regression analysis		Number and median age (yr) of subjects studied	
Age	0.0006	age	107; 11.1
Symptoms	0.03	no symptoms	90; 10.9
		symptoms	17; 11.8
Skin-prick test	0.30	negative skin-prick test	58; 11.1
		positive skin-prick test	49; 11.2
Gender	0.35	girls	57; 11.8
		boys	50; 10.5
Smoke exposure	0.63	no smoke exposure	92; 11.0
		smoke exposure	15; 12.5
Family history	0.97	no family history	70; 12.1
		family history	37; 10.8

techniques. The results are presented as percentage of predicted (25).

Skin-prick test

Allergic sensitization was determined by skin-prick test reaction to 16 common allergens on the volar surface of the forearm of the children. The allergens were egg, milk, nut mixture, grass mixture, birch, alder, willow, rye, hazel, herbs mixture, epithelia from dog, cat, horse, rabbit and goose, *Alternaria alternata*, *Aspergillus niger*, *Dermatophagoides pteronyssinus* and *D. farinae* (Bencard, Smithkline Beecham, UK). Positive control was histamine (1 mg/ml) and negative control glycerol-buffer diluent. The reaction after 15 min was recorded and judged positive when the weal diameter was 3 mm larger than the negative control. The children were grouped depending on the result of the skin-prick test in

a group with only negative reactions and a group with one or more positive reactions.

Flow dependence and elimination rate of eNO

The dependence of the oral NO values on the expiratory flow was previously shown in adults (13,20). This was now investigated by measurements of eNO with different exhalation rates. Thirty-two children used a flow of 45 ml/s, 86 ml/s, 184 ml/s and 237 ml/s. Thirteen subjects performed measurements at a flow of 20 ml/s, 45 ml/s, 86 ml/s, 184 ml/s and 237 ml/s, whereas further 18 children did this using a flow of 10 ml/s, 20 ml/s, 45 ml/s, 86 ml/s and 184 ml/s.

Based on recently published work on the two-compartment model of NO gas exchange and diffusion dynamics (21–24), we calculated the relationship of the NO elimination rate (or NO output) and the exhalation rate for all the measurements with different exhalation flows

Table 2. Slopes (estimated 'alveolar' NO) and γ -intercepts ('airway' NO) of the regression line calculated with the elimination rate model for the different subgroups

	Number of subjects studied	Slope (estimated 'alveolar' NO)	γ -intercept (estimated 'airway' NO)	p-Value
All	63	4.1 (3.1–5.0)	330 (210–460)	NA
Symptoms				
Negative	53	3.8 (2.7–5.0)	330 (180–480)	
Positive	10	4.3 (2.1–6.6)	310 (18–600)	0.36
Skin-prick test				
Negative	37	4.3 (3.2–5.3)	310 (180–450)	
Positive	26	3.7 (2.5–4.8)	350 (210–500)	0.43
Family history				
Negative	42	3.7 (2.5–4.9)	350 (200–510)	
Positive	21	4.5 (3.5–5.5)	290 (160–430)	0.39
Smoke exposure				
Negative	57	3.9 (2.6–5.1)	340 (180–500)	
Positive	6	4.1 (2.7–5.6)	280 (88–460)	0.35

Slope and γ -intercept of the linear regression line from the elimination rate model for all children and for the children sorted according to personal symptoms, skin-prick test results, smoke exposure and family history. The measurements were performed with 63 children as described in the methods section. Data are given as medians and 95% confidence interval. Comparisons between the corresponding groups were made by Mann–Whitney *U*-test. The two-tailed p-value is given. NA=not applicable.

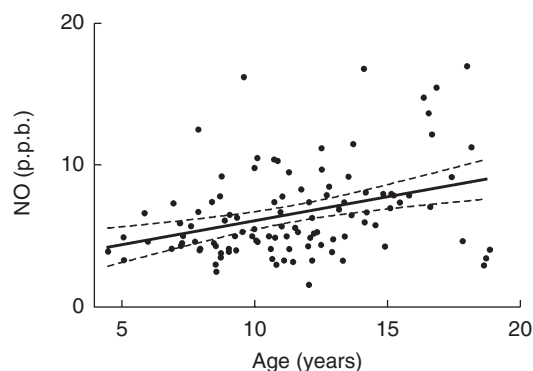
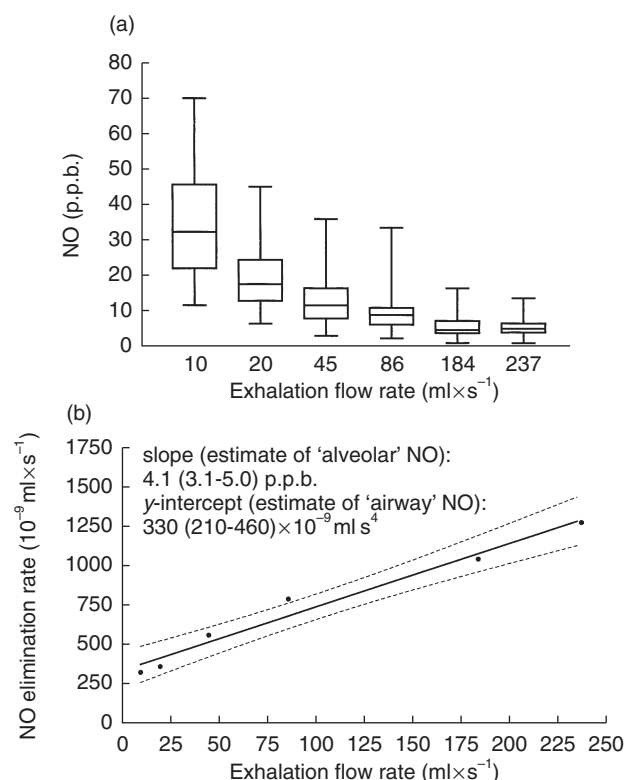


Fig. 1. Dependence of eNO on age. Individual values of eNO concentrations in 107 healthy children at an expiratory flow of 184 ml/s. A significant correlation with age was found ($r=0.33$; $p=0.0003$; Spearman test) with a slope of 0.34 and a y-intercept of 2.66.

according to the equation:

$$\text{elimination rate (10}^{-9}\times\text{ml/s)} = \text{eNO (p.p.b.)} \times \text{exhalation rate (ml/s)}.$$

This model allows an estimate of the 'alveolar' NO concentration from the slope and of the 'airway' NO concentration from the y-intercept



of the regression line between the NO elimination rate (ml/s, y-axis) and exhalation flow (ml/s, x-axis). This model was used to assess if any of the above-mentioned subject-specific factors has pronounced effects at any of the different anatomical sites (airway vs. alveoli). To this end, we calculated for each child the individual regression line and then compared these lines' slopes and y-intercepts of the same subgroups as listed in detail above. As eNO was shown to be dependent on age (16), we also looked for a possible relationship between age and individual slope and y-intercept, respectively.

Variability

To determine whether eNO shows a circadian rhythm in healthy subjects and to examine the intra-subject variability of several measurements on different days, six children were asked to perform NO measurements three times a day over a period of 6 consecutive days. The subjects (three girls, aged 9, 12 and 14 years and three boys aged 9, 12 and 15 years) were all non-smokers, had no personal or family history of atopic or chronic respiratory disease and none had had acute

Fig. 2. Exhaled NO and dependence on the exhalation flow. (a) Box plot for the eNO values of the measurements for the different exhalation flows. Median (IQR) values are 32.5 (22.3–45.8) p.p.b. with a flow of 10 ml/s in 13 subjects, 17.9 (13.2–24.7) p.p.b. at a flow of 20 ml/s in 32 subjects, 11.9 (8.2–16.8) p.p.b. with a flow of 45 ml/s, 9.2 (6.5–11.2) p.p.b. with a flow of 86 ml/s, 5.0 (4.1–7.6) p.p.b. with a flow of 184 ml/s in 63 subjects each and 5.4 (4.3–6.9) p.p.b. with a flow of 237 ml/s in 45 subjects. (b) Model-predicted relationship between elimination rate of NO and exhalation flow. The elimination rate is calculated as the product of eNO and exhalation flow. The slope of the linear regression line is defined as the alveolar concentration of NO, the y-intercept is defined as the airway part of NO. Median (IQR) values are 325 (223–458) 10^{-9} ml/s with a flow of 10 ml/s in 13 subjects, 362 (263–494) 10^{-9} ml/s with a flow of 20 ml/s in 32 subjects, 562 (365–754) 10^{-9} ml/s with a flow of 45 ml/s, 791 (555–963) 10^{-9} ml/s with a flow of 86 ml/s, 1048 (754–1380) 10^{-9} ml/s with a flow of 184 ml/s in 63 subjects each and 1280 (1019–1635) 10^{-9} ml/s with a flow of 237 ml/s in 45 subjects.

Table 3. Comparison of reference values for healthy children

	Number and median age (years) of subjects studied	eNO (p.p.b)	Expiratory flow (ml/s)	NO elimination rate (eNO $\times$ flow; 10 ⁻⁹ ml.s ⁻¹)
Silvestri <i>et al.</i> 1999	22; 11.9 (mean)	4.0	not given	NA
Dötsch <i>et al.</i> 1996	37; not given	3.0	not given	NA
Artlich <i>et al.</i> 1996	17; 8	7.2	not given	NA
Baraldi <i>et al.</i> 1999	159; not given	8.7	not given	NA
Franklin <i>et al.</i> 1999	157; 9.7 (mean)	10.3	50	515
		8.5	75	638
		7.4	100	740
This study	63; 12.2	11.9	45	536
	63; 12.2	9.2	86	791
	107; 11.1	5.7	184	1049

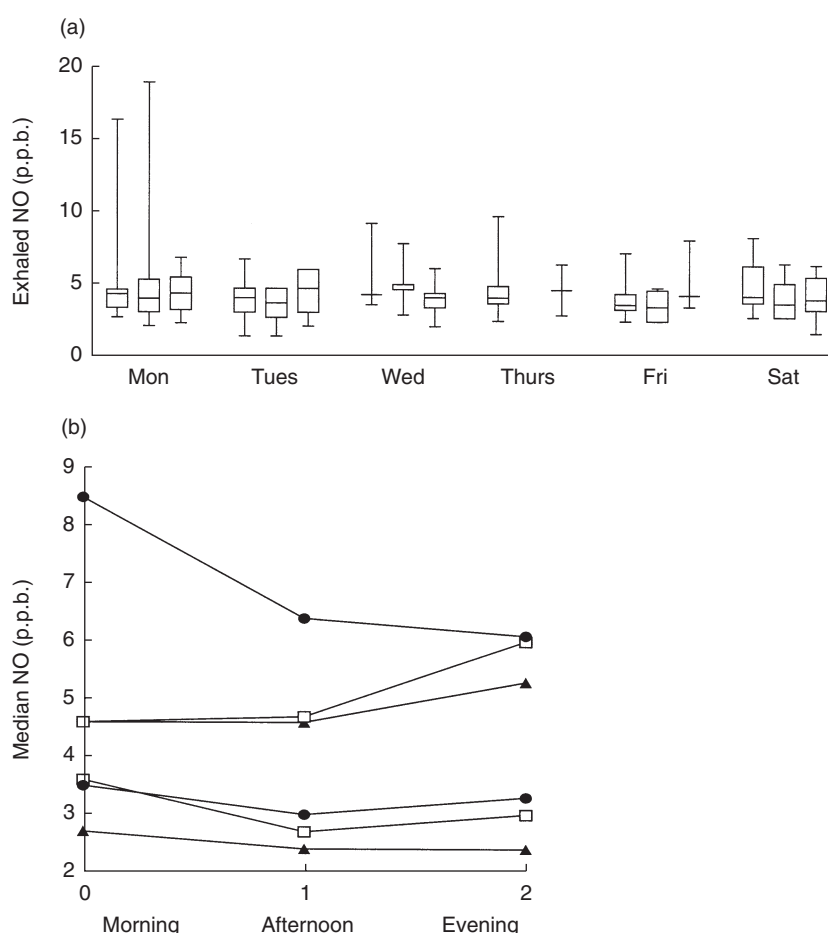
respiratory tract infection three weeks prior to the test. The children all had negative skin-prick test results and their lung function was normal (FVC and $FEV_1 > 95\%$). Expiratory NO was measured at an expiratory flow of 184 ml/s as described above. The measurements were performed in the morning (8.30–9.45 a.m.), in the afternoon (5.00–6.45 p.m.) and in the evening (10.30–11.30 p.m.) on 6 days in a row (Monday to Saturday).

Fig. 4. Variability of eNO on 6 consecutive days. (a) eNO concentrations were determined at an expiratory flow of 184 ml/s. Each box represents the median, 25–75 percentile, minimum and maximum eNO concentrations of six healthy children. The Thursday and Friday afternoon boxes stand for only four subjects and the Wednesday morning box represents five children. On Thursday afternoon no measurements were performed. No circadian rhythm was found. (b) Median eNO concentrations of all the values of the individual subjects depending on the time of the day. The six different subjects are marked with different symbols and a line connects the median values of the morning, afternoon and evening concentrations of the same subject. No statistical significance was found between morning, afternoon and evening values for any of the subjects. eNO concentrations were measured at an expiratory flow of 184 ml/s.

On Thursday afternoon it was not possible to make any measurements and on Thursday and Friday evening measurements on only four subjects were performed. One subject missed another measurement on Wednesday morning.

Statistical analysis

Statistical results are reported as medians [inter-quartile range, e.g. 25.0 and 75.0 percentile



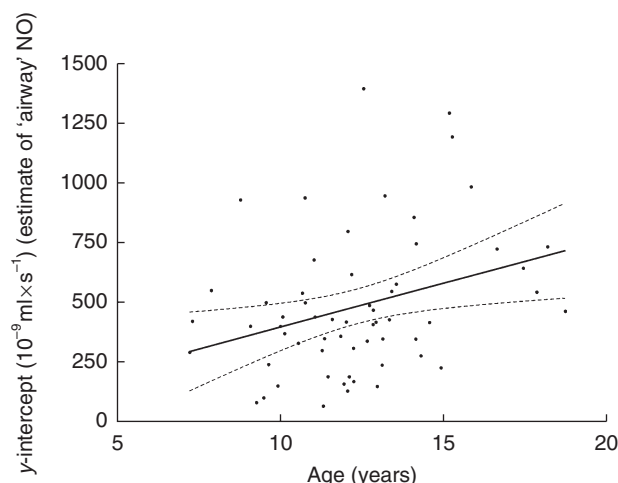


Fig. 3. Dependence of the y-intercept on age. Each point represents the y-intercept derived from the individually calculated linear regression line for 63 children with the help of the elimination rate model. The y-intercept gives an estimate of the 'airway' NO. A significant correlation with age was found ($r=0.33$; $p=0.009$; Spearman test). These results are consistent with an increasing flux of NO from the airways with age.

(IQR)] in n subjects. The possible effect of all subject-specific variables on eNO was determined by simple linear regression and Spearman's rank correlation test. As eNO was shown to be dependent on age, a multivariate linear regression model was performed with the other variables being skin-prick test reactivity, gender, smoke exposure, personal symptoms and family history. For all variables of the regression analysis 95% confidence intervals were given as a measure of variability. The morning, afternoon and evening measurements for the variability analysis were compared with the Friedmann test. Exact p -values are given, and a two-tailed $p < 0.5$ was considered significant. The statistical analysis was carried out using Graph Pad Prism software (San Diego, CA, USA); the multivariate linear regression model was performed on SPSS (SPSS Inc., Chicago, IL, USA).

Results

One hundred and seven currently healthy children (57 girls) aged between 4 and 18 years were evaluated. None of the children had a history of asthma. The questionnaire showed that 17 children had had symptoms of atopic disease at other times of the year. Five of these had hay fever in spring, three had hay fever in summer, two conjunctivitis in spring, three in spring and summer, two had a blocked nose possibly due to house dust mite and two others due to animal hair exposure. In the last four children symptoms did disappear with appropriate allergen avoidance. None of the subjects took any current or regular medication.

Lung function tests were normal with FEV_1 at 105 (99–113)% and MEF_{25} at 94 (79–122)% of predicted. All children completed the eNO measurement easily. The median eNO level at

the expiratory flow of 184 ml/s was 5.7 (4.3–7.9) p.p.b.; $n=107$ with a minimum of 1.6 p.p.b. and a maximum of 17 p.p.b., which corresponds to a NO output of 1049 (791–1454) $\times 10^{-9}$ ml/s. ENO increased significantly with age (Spearman $r=0.33$; $p=0.0003$; Fig. 1). There was no correlation found between eNO and FEV_1 (Spearman $r=0.017$; $p=0.43$), or eNO and MEF_{25} (Spearman $r=-0.002$; $p=0.49$). The time of the day at which the measurement was performed had no influence on eNO values (data not shown).

Of the variables included in the multivariate linear regression model, age ($p = 0.0006$) and symptoms ($p = 0.030$) showed a significant relation with eNO. All the other variables did not influence the NO concentration (Table 1). For smoke exposure and symptoms it must be noted that the subgroup and thus the power to detect differences was small.

Flow dependence and elimination rate

The eNO concentration was significantly dependent on the flow. A lower flow was associated with a higher eNO concentration and a larger coefficient of variation (Fig. 2a).

These data were used to calculate the elimination rate model for all children. The linear regression line had a slope of 4.1 (3.1–5) p.p.b. (estimated 'alveolar' NO) and a y-intercept of 330 (210–460) $\times 10^{-9}$ ml/s (estimated 'airway' NO; Fig. 2b).

Slope (estimated 'alveolar' NO) and y-intercept (estimated 'airway' NO) of the individually calculated regression lines for the 63 children were assessed for correlations with the subject-specific variables. A significant correlation between the y-intercept and the age of the subjects was found (Spearman $r=0.33$;

$p=0.009$; Fig. 3), whereas slope and age did not correlate (Spearman $r=-0.15$; $p=0.23$). None of the various other dichotomous subgroups correlated with the slopes or with the y -intercepts calculated with the elimination rate model (Table 2).

Variability

A group of six children was measured three times daily on 6 consecutive days. No circadian rhythm of eNO was found (Fig. 4a). The median eNO concentration of all the measurements throughout the whole period was 4.0 (3.8–5.7) p.p.b. in the morning ($n=35$), 4.2 (3.2–5.4) p.p.b. in the afternoon ($n=30$) and 4.1 (3.7–4.9) p.p.b. in the evening ($n=32$). The personal median values of each subject's measurements on the 6 days did not show any significant differences between morning, afternoon and evening eNO concentration (Fig. 4b).

As shown in the methods section, the intra-measurement coefficient of variation was 6.5 (4.2–8.6)% for 43 subjects at 137 measurements. The median intra-subject coefficient of variation of the three daily measurements on 6 consecutive days was 25.9%, with the lowest CV of 21% and one subject showing a CV of 51%. The mean of the subjects coefficients of variation for all the morning measurements was 26%, for the afternoon measurements 35% and for the evening measurements 22%.

Discussion

The concentration of eNO is dependent on the exhalation flow as already shown for adults (13,14,20) and children (16). Thus the flow must always be taken into account when comparing different studies. An overview of all the reference values available so far for healthy children is given in Table 3. Of these six studies only Franklin *et al.* (16) and this study used a constant flow during the expiration phase with a flow diagram for visual control on the computer screen. The other studies recorded the NO value between the 3rd and 6th minute of normal breathing [Baraldi *et al.* (26)], measured the mean NO value from five breaths [Dötsch *et al.* (7)] or 3 min of breathing [Artlich *et al.* (6)].

Although this study shows very similar eNO concentrations at the various exhalation flows with the study of Franklin *et al.* (16), other studies' values vary considerably. Besides the above-mentioned differences in the measurement technique, other factors might be of importance. These may include a small, but significant

dependence of eNO on nutritional intake (27,28), domestic formaldehyde levels (29), and the exact composition of the sample population.

Compared with the other studies we used a relatively high expiratory flow of 184 ml/s. The first 63 subjects performed the eNO measurements using different expiratory flows (results shown in Fig. 2a). When analysing the eNO data measured at a flow of 45 ml/s – a flow recently recommended by the ATS (19) – we also did not find any differences between the atopic and non-atopic groups. Besides the fact that it was easier for the children to maintain an expiratory flow plateau at the higher flow, the variation of the results was not as large as it was when using lower flows. A smaller variability with higher flows (Fig. 2a) and thus lower p -values have been observed in our (4) and other previous studies (30) comparing eNO at various flows in children with different diseases. A potential solution of these problems and the generation of additional information may come from the utilization of the two-compartment model introduced by Tsoukias *et al.* (23) and Silkoff *et al.* (22). If the measurements are performed with several exhalation flows, one can easily calculate the theoretical eNO values at another exhalation flow with the help of the linear regression and then compare them with other studies. In addition, the slope of the regression lines gives an estimated 'alveolar' NO and the y -intercept the contribution of 'airway' NO (22–24). With these data one might be even able to localize the level of inflammation to some extent, e.g. patients with alveolitis show an increased slope ('alveolar' NO), whereas asthmatic patients show a much higher y -intercept ('airway' NO) but the same slope as healthy controls (21).

Over the age range investigated (5–18 years), eNO clearly correlated with the age of the subjects. Similar observations have been made previously (15,16). Compared with the other groups, who did not study children older than 11 and 13, respectively, we found a further increase of eNO beyond the age of 13 years up to the age of 18 years. The correlation between y -intercept ('airway' NO) and age (Fig. 3) and the unchanged slope ('alveolar' NO) of the regression line over age shows, that the increasing contribution of the airways to the total eNO is the main reason for the increase of eNO with age, whereas the alveolar NO concentration does not change with age.

The larger airway contribution with age may be either related to a change in the diffusion coefficient of the airway wall or – as suspected by others before (16) – to a larger airway wall surface

area with growth. The constant alveolar concentration is in agreement with the terminated multiplication and remodelling of the alveoli and their septa by the age of 3–5 years (31,32).

An alternative approach to determine the alveolar concentration of NO is to use high flow rates that eliminate the airway contribution to eNO. We used relatively high flows in the subgroup of the 63 children and their eNO values asymptotized to the level of about 5.0 p.p.b. (5.0 p.p.b. at 184 ml/s and 5.4 p.p.b. at 237 ml/s), possibly building out a plateau and thus determining the alveolar concentration of NO. This level achieved in practice [5.0 (4.1–7.6) p.p.b.] and the calculated alveolar concentration of 4.1 (3.1–5.0) p.p.b. (defined as the slope in the elimination rate model) did not differ significantly. Such a small difference indeed is reassuring, as it is practically not possible to exhale so fast, that the transit time in the airways and thus the time during which the airway part of the eNO is added to the alveolar concentration equals zero (22). The alveolar NO concentration we have obtained in children is similar to that published for adults so far. Silkoff *et al.* measured a concentration of 5.3 ± 0.7 p.p.b. (22), Tsoukias *et al.* 5.6 ± 3.1 p.p.b. (24) and 8.12 p.p.b. (23), respectively, and Lehtimäki *et al.* measured 1.3 ± 0.2 p.p.b. (21). Yet no other results of children are available precluding a comparison with the same age group. However, as shown above, the slope of the regression line remained constant with age and therefore the alveolar NO concentration should not differ between adults and children.

We did not find any correlation between eNO and spirometric lung function measurements. Although in one study of asthmatics a relatively weak correlation between eNO and FEV_1 has been found (6), the other published studies did not find any correlation between eNO and FVC, FEV_1 and MEF_{25} , respectively, in asthmatics as well as in healthy controls (5,7).

There are several studies that were able to demonstrate a difference in eNO between atopic and non-atopic subjects (16,17,33,34). In a study of 30 infants under the age of 2 years, Wildhaber *et al.* reported significantly higher eNO values in infants with a positive family history of atopy (17). The study of Moody *et al.* showed an association of the eNO concentrations with skin-prick reactivity to house dust mite (33) and in the publication of Horvath *et al.* 15 atopic subjects had elevated eNO values compared with 40 asymptomatic non-atopic subjects [18.3 p.p.b. vs. 6.3 p.p.b. (34)]. Similarly, Franklin *et al.* reported higher eNO concentrations in children

with two or more positive skin-prick results (20.1 p.p.b., $n=17$) compared with children with one (10.9 p.p.b., $n=24$) or no (7.2 p.p.b., $n=116$) positive skin-prick results (16). In our study we had a more homogenous distribution between children with positive skin test results (5.3 p.p.b., $n=50$, age 11.4 years) and negative skin test results (5.9 p.p.b., $n=57$, age 11.6 years) and did not find any differences in eNO. The same lack of correlation was found when the children with none, one or two and more than two positive skin-prick test results were considered separately. Our study was sufficiently powered to detect differences in eNO between atopic and non-atopic children outside the range of the 95% confidence interval and thus we should have easily ($\alpha < 0.01$) detected the almost three-fold difference in eNO observed in the study of Franklin *et al.* and Horvath *et al.* if present in our sample.

Further possible explanations for this contrast include the different proportions of atopic subjects (26% in the study of Franklin *et al.*, 27% at Horvath *et al.*, 59% in the study of Moody *et al.* and 46% in our study) or the ethnic differences in the study populations (Moody *et al.* examined Pacific Islanders, Franklin *et al.* observed Australian children, whereas we only had European subjects). The relatively high percentage of atopic subjects in our study group may be related to the relatively high socio-economic level in the south of Munich where most of our children were recruited from, but it is similar to other epidemiological studies carried out in Munich (35). An additional cause for the different results concerning skin-prick positive children might be the different skin-prick tests and allergens used.

There is only few data available about the circadian variation of the eNO concentration. Similarly to the findings in this study, ten Hacken *et al.* (18) did not find a circadian rhythm in healthy subjects.

Of interest is the relatively large intra-subject coefficient of variation (CV) that we found in healthy children (25.9%) and that is in agreement with data in healthy adults – ranging from 19% inter-day CV (13) up to 42% CV (18). This and a circadian variation of eNO has to be taken into account when assessing asthmatic children (18) and suggesting eNO as a control marker of the patients compliance with steroid medication (36).

To summarize, in this sample of healthy children we did not find any correlation of eNO with spirometric lung function measurements, skin-prick test results, gender or family history of atopic disease. The significant increase of eNO

with age was due to a higher contribution of airway NO to the total eNO, whereas the alveolar NO concentration remained stable over age. This and the relatively large intra-subject variability we found must be considered when comparing eNO values of children between different clinics and using them as a reference. Before using eNO as a new lung function test (37), the different measurement techniques need to be further standardized. Based on the different studies on the elimination rate model and on our results, we suggest that it is desirable to measure eNO with different exhalation flows in order to differentiate between alveolar and airway part of NO and to allow better comparisons between different study groups.

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