

Flow dependency and off-line measurement of exhaled NO in children

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Levels of exhaled nitric oxide (eNO) are flow-dependent, and the choice of an optimal flow rate for off-line and on-line eNO measurement has raised much debate. Recently, a flow rate of 50 ml/s was recommended, but children younger than 5–6 years are not capable of stabilizing their expiratory flow at low flow rates. The power of off-line eNO values to discriminate between normal and atopic children was therefore evaluated at different exhalation flow rates. At flow rates of both 8.3 ml/s and of 350 ml/s, children (8–12 years) sensitive to house dust mite have two-fold higher eNO values ($p < 0.001$) as compared with children lacking such a sensitivity. The power of eNO to discriminate between normal and atopic subjects was similar at the two flow rates (no difference in AUC of receiver operation curves, $p = 0.89$). All children from 4.5 to 5 years of age ($n = 29$) could perform a single off-line exhalation manoeuvre at high (> 350 ml/s) but not at low (8.3 ml/s) flow rates. At high exhalation flow rate, eNO was 7.1 ± 2.4 (mean $\pm$ SD) median, 6.5 p.p.b. with a mean variation coefficient of 5.5%. Depending on their developmental level, about half of the younger children (35–46 months of age) could perform an off-line exhalation manoeuvre at high flow rate with good reproducibility (mean variation coefficient of 6.6%). It is concluded that an exhalation flow rate of 350 ml/s is feasible to determine off-line eNO-values in children from 3.5 years of age, and that this high flow rate does not compromise the power of eNO to detect allergic disease.

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Introduction

Levels of exhaled nitric oxide (eNO) may be a valuable noninvasive marker for the early diagnosis and management of allergic asthma, as increased levels of eNO have been observed in patients with allergic asthma (1–5).

The level of eNO measured depends on the expiratory flow. The choice of an optimal flow rate for off-line and on-line eNO measurement has raised much debate. To allow proper comparison between different studies, the American Thoracic Society (ATS) and European Respiratory Society (ERS) (6, 7) recommended in guidelines for eNO measurements in adults and children to apply high flow rates, i.e. 50–350 ml/s, to avoid flow dependency of eNO. The ATS (7)

further specified for off-line measurement of eNO an expiratory flow rate of 350 ml/s, independent of age (the ERS does not further specify the flow rate for off-line sampling). In contrast, Silkoff et al. (8, 9) advocated the measurement of eNO at much lower flow rates (10–40 ml/s) so that higher eNO values are obtained that add to the ability to discriminate among subjects. More recently, an ATS/ERS task force (10) recommended for both off-line and on-line eNO measurement in children an intermediate flow rate, i.e. 50 ml/s. In children > 5 years (11, 12) and in adults (8, 13) eNO values obtained off-line and on-line are very comparable, provided the dead space volume is eliminated. Children under the age of 5 years, however, are not able to

perform slow exhalation manoeuvres as they are not capable of stabilizing their expiratory flow sufficiently and do not understand the biofeedback signal.

We therefore evaluated at different flow regimes the reproducibility and power to discriminate atopic from nonatopic children of eNO values obtained via off-line sampling, and the feasibility of off-line eNO measurement in children from the age of 3.5 years.

Materials and methods

Study subjects

Exhaled NO (eNO) measurements were performed in 23 adults (19–52 years) and two groups of children (3.5–12 years). One group of 46 children (21 boys, 25 girls, 8–12 years, mean age 9.9 years) was randomly selected from a primary school and tested for hypersensitivity to aeroallergens by a skin prick test as described below. Of the 21 skin prick positive children six children reported wheezing in the last year and three children reported doctor-diagnosed asthma. The 25 skin prick negative children reported neither wheezing in the last year nor doctor-diagnosed asthma. The second group of 43 younger children (3.5–5 years, mean age 4.3 years) was randomly recruited from a kindergarten. The study protocol was approved by the Medical Ethics Committee and parents had given permission to test their children. No specific inclusion or exclusion criteria were applied.

Sampling of exhaled air

Exhaled air of the adult subjects was sampled using a sampling device made up of a rigid tube connecting a mouthpiece and a sampling balloon, equipped with a calibrated narrowing in the exhalation circuit as described previously (14). The sampling device contained an upstream pressure transducer connected to a set of indicator lamps that guides the subject to exhale at the required pressure. Exhalation flow rates (8.3, 100, 120, 250 and 540 ml/s) were obtained by changing the aperture of the narrowing.

While seated, adult subjects were asked to take a deep breath and to perform immediately a single exhalation into two impermeable foil Mylar balloons (ABC ballonnen, Zeist, the Netherlands). The first 1000 ml of exhaled air putatively contaminated with ambient NO was collected at high exhalation flow rate in the first bag, connected via a wide-bore T-piece of the rigid tube of the sampling device, and discarded.

The second bag was filled over a total time of 20 s with air exhaled at 8.3–540 ml/s and an exhalation back pressure of 5–30 cm water to prevent contamination with nasal NO.

In children, exhaled air was sampled at two different flow rates only: 8.3 ml/s, as described above (at 20 cm water back pressure), and 350 ml/s (at 5 cm water back pressure for at least 2–3 s). For high exhalation flow rates a similar sampling device was used as described above, with the modification that a juggling clown was visualized when the cut-off value of 350 ml/s was attained (as detected by a pressure transducer). During all manoeuvres the first 300 ml of exhaled air was collected at high flow rate in a pre-bag and discarded. All subjects remained seated during air sampling and exhaled air was sampled twice at each flow.

Assay of exhaled NO

Content of exhaled NO in the sample bags was analysed by chemiluminescence (Sievers 280B, Boulder, USA) using a response time <200 ms. The analyser sample flow rate was kept constant at 200 ml/min. Exhaled NO-concentration in the bags was stable for at least 18 h and eNO was determined within 2 h after sampling. During sampling the ambient NO level did not exceed 21 p.p.b.

Allergy testing

Hypersensitivity to aeroallergens was assessed in 46 primary school children using a skin prick test. The five aero-allergen extracts tested were: dog dander, cat dander, *Dermatophagoides pteronyssinus*, grass pollen mixture and tree pollen mixture (Alko-Abello, Nieuwegein, the Netherlands). Negative and positive controls were included. A wheal size larger than 2 mm, and a ratio of wheal to allergen and negative control >0.4, was considered as a positive allergic reaction.

Statistical analysis

Exhaled NO-values were log-transformed before analysis. Any difference in eNO variability at low and high flow rates were evaluated with the *F*-test. The differences in eNO levels due to the presence or absence of allergy and due to the low/high flow regime were evaluated with two-way analysis of variance. A sensitivity/specificity analysis in detecting allergy was carried out using a receiver operator curve (ROC) approach. For each flow regime the area under the ROC-curve (ROC-AUC) was determined to assess its discriminating power in detecting allergy. The

AUCs were compared according to the method of Hanley and McNeill (15).

Results

Figure 1 shows the flow dependency of eNO values in 23 adult subjects. In the higher flow rate range (above 250 ml/s) eNO becomes virtually flow-independent. At a low flow rate of 8.3 ml/s the range of untransformed eNO values is much larger (17–85 p.p.b) compared with 540 ml/s (7–19 p.p.b): the variance ratio is 28.55 ($p < 0.05$). After log-transformation the variability was much more similar (variance ratio of 2.22, $p = 0.03$). Reproducibility of eNO measurement was tested in five adult subjects by repeated sampling (eight times) at an exhalation flow rate of 8.3 and 250 ml/s, respectively (cf. Table 1). At 8.3 and 250 ml/s the mean variation coefficient of the log-transformed eNO values was similar (3.3 and 2.6%, respectively).

To investigate the flow dependency of the power of eNO to discriminate between atopic and nonatopic children, eNO was determined in

atopic ($n = 21$, mean age 9.9 years) and nonatopic ($n = 25$, mean age 9.9 years) children at low (8.3 ml/s) and high (> 350 ml/s) exhalation flow rate. Figure 2 shows that at both low and high exhalation flow rates children with skin prick positivity to house dust mites have statistically significant ($p < 0.001$), two-fold higher eNO values as compared with children lacking such a sensitivity (low flow: 50.0 ± 8.3 p.p.b. vs. 26.9 ± 1.3 p.p.b.; high flow: 13.2 ± 1.7 vs. 7.8 ± 0.2 p.p.b). The differences in eNO due to the differences in flow rate were highly significant ($p < 0.001$) and the same is the case for allergy status ($p < 0.001$). The differences at low rates of flow between allergic and nonallergic subjects did not differ from those at high flow rates (p-value of the 'flow-allergy' interaction = 0.389). The ROC AUCs for, respectively, low and high flow rates were 0.766 and 0.754 ($p = 0.002$ and $p = 0.004$), but the difference in AUC did not differ from zero (95% confidence interval -0.11 to 0.12 ; $p = 0.892$).

Children between 4.5 and 5 years ($n = 29$, mean age 4.8 years) were well able to perform a

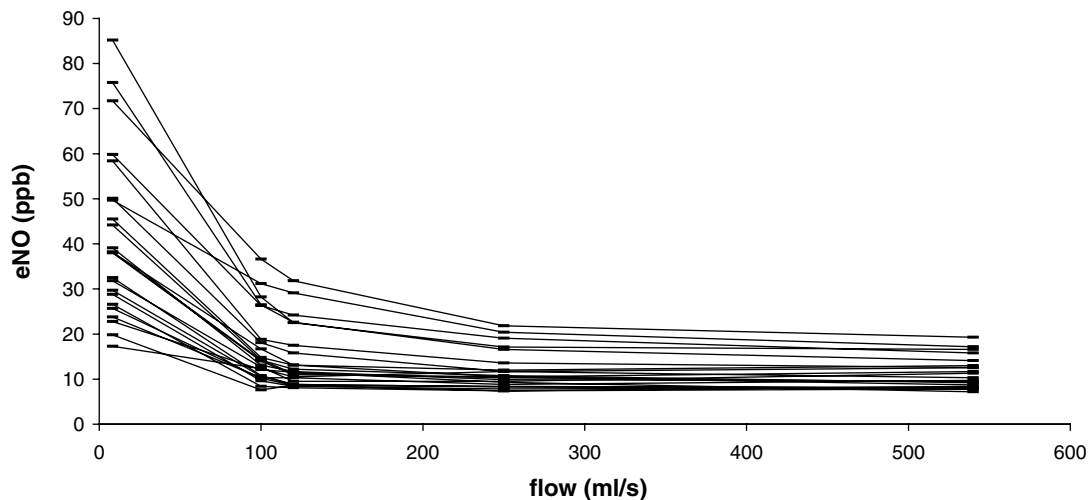


Fig. 1. Values of eNO of 23 adult subjects (19–52 years) sampled at five different exhalation flow rates between 8.3 and 540 ml/s.

Table 1. Reproducibility of eNO measurements in five adult subjects. Values are expressed as the mean eNO $\pm$ SD of eight air samples successively exhaled within 15–20 min variation coefficient

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Mean value
8.3 ml/s eNO	10.0 $\pm$ 1.4	16.3 $\pm$ 1.5	21.9 $\pm$ 1.9	34.6 $\pm$ 3.6	47.2 $\pm$ 2.5	
log eNO	1.00 $\pm$ 0.06	1.21 $\pm$ 0.04	1.34 $\pm$ 0.04	1.54 $\pm$ 0.05	1.67 $\pm$ 0.02	
var. coeff. (%)	5.8	3.6	2.8	3.0	1.4	3.3
250 ml/s eNO	5.5 $\pm$ 0.3	5.8 $\pm$ 0.5	6.2 $\pm$ 0.2	11.8 $\pm$ 0.6	14.0 $\pm$ 0.4	
log eNO	0.74 $\pm$ 0.02	0.76 $\pm$ 0.04	0.79 $\pm$ 0.01	1.07 $\pm$ 0.02	1.15 $\pm$ 0.01	
var. coeff. (%)	3.0	4.8	1.9	2.2	1.2	2.6

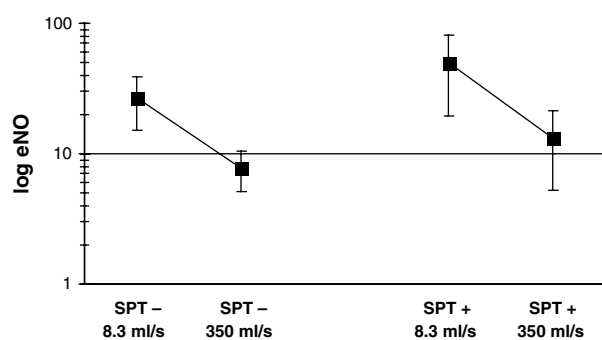


Fig. 2. Values of log-transformed eNO values of children (8.4–12.0 years) without ($n = 25$; SPT⁻) and with ($n = 21$; SPT⁺) skin prick positivity to house dust mites. Exhaled air was sampled at an exhalation flow rate of 8.3 and > 350 ml/s. Each point represents the mean $\pm$ SD of the individual eNO values measured in duplicate.

single exhalation manoeuvre at > 350 ml/s, but not at 8.3 ml/s. The mean eNO ($\pm$ SD) at high flow rate was 6.9 ± 2.4 p.p.b. (median 6.8 p.p.b.), and the mean variation coefficient of log-transformed eNO values was 5.0%. Seven younger children (37–44 months, mean age 42 months) were also capable of performing the exhalation manoeuvre at > 350 ml but not at 8.3 ml/s. At > 350 ml eNO measurements showed good reproducibility (mean variation coefficient of the log-transformed eNO values of 6.6%), and ranged from 6.3 to 10.8 p.p.b. (median 7.0 p.p.b.). However, another seven children of the same age (35–46 months, mean age 39 months) not accustomed to the kindergarten regime were either not capable of performing the manoeuvre, refused to cooperate or did not understand the instructions.

Discussion

Present results show that the use of a high exhalation flow rate (350 ml/s) in off-line air sampling results in reproducible eNO-values, does not impair the power of eNO in discriminating between atopic and nonatopic children as compared with low flow exhaled air sampling (8.3 ml/s), and is feasible in young children (> 3.5 years).

In the higher range of exhalation flow rates, i.e. above a cut-off value of 250 ml/s eNO is virtually flow-independent (cf. Figure 1). However, at lower exhalation flow rates eNO is flow dependent (9, 16, 17), such that exhaled air should be sampled at a given and constant exhalation flow to allow comparison of the data and obtain reproducible values, respectively.

Guidelines for measuring eNO have been formulated by the ERS (6, 10) and ATS (7).

The ERS recommends use of a flow rate of 150–250 ml/s, but gives no further specifications for off-line sampling. In contrast, the ATS (7) recommends an exhalation flow of 350 ml/s for off-line measurements in adults and children. Silkoff et al. (8, 9) have studied flow dependency of eNO in detail and advocate measurement of eNO at flow rates of 10–40 ml/s, which results in higher eNO values than will be obtained after sampling at high flow. In addition, asthma may be considered as a disease of the conducting airways, as in asthmatic patients steroids decrease bronchiolar but not alveolar NO output (18). This suggests that sampling of exhaled air at low flow rate, reflecting bronchial NO flux, is preferable to sampling air at high flow rate (reflecting alveolar NO flux).

Using different approaches, i.e. the comparison of eNO and carbon dioxide (19) and intrabronchiolar air sampling with a catheter (20), it has been demonstrated that eNO originates primarily from the conducting and not the alveolar airways. In addition, in single flow manoeuvres NO level rapidly reaches plateau values (9), whereas gases released in the alveolar space, such as carbon dioxide, typically show a sloping curve and never reach a constant level. At very high expiratory flows the diffusion from the bronchiolar tissue to exhaled air is negligible (21), and eNO is virtually of alveolar origin only. As in adults eNO is 'constant' at flows > 250 ml/s (cf. Figure 1), eNO values obtained in children at 350 ml/s represents NO originating mainly from the alveolar space, which is of less interest. However, using high exhalation flows several groups reported a significant difference between eNO values of healthy and asthmatic subjects (22–24). This indicates that even at high flow rates a minor amount of NO is still released from the conducting airways that is large enough to differentiate between healthy and asthmatic subjects.

The value of eNO was determined in children (8.4–12.0 years), who were skin prick positive to aeroallergens; their asthma status was not determined. Allergic but nonasthmatic subjects have been frequently reported to have increased eNO values (25–29). Indeed, both at low and high exhalation flow rate, eNO values of children hypersensitive to house dust mites were significantly higher ($p < 0.001$) as compared with those who were not allergic for this allergen. In addition, the power of eNO to discriminate hypersensitive children from controls was assessed. The discriminating power of tests depends on many factors, amongst them the magnitude and variability of the measured values.

After log-transformation of data (eNO values are log-normal distributed) it appears that the variability and the reproducibility of eNO at high and low exhalation flow rates are comparable. Moreover, we did not find a significant interaction between exhalation flow and allergy status. Therefore, skin prick positive subjects can be well distinguished from control subjects using eNO values obtained via air sampling at either low or high flow rate. This means that our goal to evaluate the discriminating power of eNO at various flow rates can be achieved in allergic children without knowing their asthma status, and implies that measurement of eNO at high exhalation flow is suitable for detection of allergy at a young age.

Pre-school children (< 5 years) are not capable of exhaling air at a low flow rate in a reproducible way, as they simply do not understand the instructions and biofeedback signal and cannot exhale over a prolonged time (10–15 s). Short-lasting exhalation at a high flow rate is therefore indicated to measure eNO (and comply with flow dependency) in such children.

In conclusion, off-line exhaled air sampling at a high flow rate does not compromise the power of eNO to detect allergic disease, can be performed with good reproducibility in children from the age of 4.5 years and is feasible in even younger children (> 37 month).

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