Pediatric Pulmonology 45:182–191 (2010)

Impact of Analysis Interval on the Multiple Exhalation Flow Technique to Partition Exhaled Nitric Oxide

James L. Puckett, PhD,[1] Richard W.E. Taylor, BS,[1] Stanley P. Galant, MD,[2] and Steven C. George, MD, PhD[1,3]*

Summary. Exhaled nitric oxide (eNO) is elevated in asthmatics and is a purported marker of airway inflammation. By measuring eNO at multiple flows and applying models of eNO exchange dynamics, the signal can be partitioned into its proximal airway [$J'_{aw_{NO}}$ (nl/sec)] and distal airway/alveolar contributions [CA_{NO} (ppb)]. Several studies have demonstrated the potential significance of such an approach in children with asthma. However, techniques to partition eNO are variable, limiting comparisons among studies. The objective of this study is to examine the impact of the analysis interval (time or volume) on eNO plateau concentrations and the estimation of $J'_{aw_{NO}}$ and CA_{NO}. In 30 children with mild to moderate asthma, spirometry and eNO at multiple flows (50, 100, and 200 ml/sec) were measured. The plateau concentration of eNO at each flow was determined using two different methods of analysis: (1) constant time interval and (2) constant volume interval. For both methods of analysis, a two-compartment model with axial diffusion was used to characterize $J'_{aw_{NO}}$ and CA_{NO}. At a flow of 200 ml/sec, the time interval analysis predicts values for eNO that are smaller than the volume interval analysis. As a result, there are significant differences in CA_{NO} between the methods of analysis (volume $>$ time). When using the multiple flow technique to partition eNO, the method of analysis (constant time vs. constant volume interval) significantly affects the estimation of CA_{NO}, and thus potentially the assessment and interpretation of distal lung inflammation. **Pediatr Pulmonol. 2010; 45:182–191.** © 2010 Wiley-Liss, Inc.

Key words: asthma; NO; inflammation; children.

INTRODUCTION

Nitric oxide (NO) was first measured in the exhaled breath of humans in 1991.[1] Since its discovery there have been significant efforts to develop methods to accurately and reliably characterize the concentration of NO in the exhaled breath.[2] Research has shown that the shape and magnitude of the NO exhalation profile depends strongly on the exhalation flow,[3,4] presence of inflammation,[5,6] and lung volume.[7] These unique features are a consequence of the significant proximal airway source relative to the small concentration in the distal airway/alveolar region,[8–11] and creates new challenges to develop methodologies that effectively characterize the exhaled NO signal.

Guidelines for the online measurement of exhaled NO were initially presented in 1997,[12] and later updated in 2005 by the American Thoracic Society (ATS) and the European Respiratory Society (ERS).[13] Three major features of the current guidelines include: (1) exclusion of the nasal sinuses by exhaling at a pressure >5 cmH_2O and subsequently closing the velopharangeal aperture, (2) a constant exhalation flow of 50 ml/sec, and (3) prior to analysis, exhalation should occur for at least 4 sec in subjects <12 years old or 6 sec in subjects ≥ 12 years old. If these conditions are satisfied, the guidelines suggest that a plateau concentration of NO can be recorded. The plateau concentration is defined as a time-averaged value over a 3 sec window (signal does not vary by $>10\%$), which is denoted as the fractional concentration of exhaled NO in exhaled breath (FE_{NO}).

The current guidelines have only been established for a single exhalation flow of 50 ml/sec ($FE_{NO,50}$). At an exhalation flow of 50 ml/sec, FE_{NO} is predominately of proximal airway origin[14] and the much smaller concen-

[1]Department of Biomedical Engineering, University of California at Irvine, Irvine, California 92697.

[2]Children's Hospital of Orange County, Orange, California 92697.

[3]Department of Chemical Engineering and Materials Science, University of California at Irvine, Irvine, California 92697.

Grant sponsor: National Institutes of Health; Grant number: R01 HL070645; Grant sponsor: Children's Hospital of Orange County.

*Correspondence to: Steven C. George, MD, PhD, Department of Biomedical Engineering, 2420 Engineering Hall, University of California at Irvine, Irvine, CA 92697-2730. E-mail: scgeorge@uci.edu

Received 16 August 2009; Revised 13 October 2009; Accepted 13 October 2009.

DOI 10.1002/ppul.21182
Published online 13 January 2010 in Wiley InterScience (www.interscience.wiley.com).

tration from the distal airways cannot be ascertained. However, our group, as well as others, have presented numerous techniques in which the exhalation flow is varied (either within or between consecutive single exhalations) from 50 ml/sec to as high as 300 ml/sec in an effort to partition the exhaled NO signal into its proximal airway [$J'_{aw_{NO}}$ (nl/sec), maximum airway flux] and distal airway/alveolar contributions [CA_{NO} (ppb), alveolar NO concentration].[4,7,15–17] Several studies have demonstrated the potential significance of such an approach,[18–24] particularly in children with asthma.[14,25–27] However, if the interval of analysis to determine FE_{NO} is a fixed time (i.e., 3 sec) then incommensurate volumes of exhaled breath will be analyzed at different flows (e.g., 150 ml at 50 ml/sec and 900 ml at 300 ml/sec) and at different lung volumes. This could affect the calculated value of FE_{NO} at each flow (and hence the estimation of $J'_{aw_{NO}}$ and CA_{NO}), since the slope of the exhaled NO profile is statistically negative in healthy adults, decreasing by approximately 6% per second of exhalation.[7] The objective of our study was to examine the impact of the analysis interval (time or volume) on NO plateau concentrations and partitioning the exhaled NO signal into proximal airway and distal airway/alveolar contributions in children with asthma.

MATERIALS AND METHODS

Subjects

Thirty pediatric patients between the ages of 6–17 years with mild to moderate asthma who presented to the Children's Hospital of Orange County (CHOC) Breathmobile® for a routine asthma evaluation participated in the study. Criteria for the diagnosis of asthma included a previous history of recurrent coughing, wheezing, shortness of breath (at rest or following exercise), and symptomatic improvement following short acting bronchodilator.[28] Patients were excluded from the study if they had any other heart or lung disease or any smoking within the past 5 years. Short and long acting β_2 agonists were withheld for 12 hr prior to the study. Each subject and their guardian began their visit by reading and completing the requirements stated in the informed consent documents; the consent form had been approved by the University of California, Irvine and CHOC Institutional Review Boards.

Measurements

Due to the potential confounding effect of lung function tests on the exhaled NO signal,[29] the exhaled NO measurements at multiple flows (NIOX Flex, Aerocrine Ltd, Stockholm, Sweden) were performed prior to spirometry. Briefly, the patients inhaled through an NO-scrubbing filter (inspired NO-free air) via a mouthpiece to total lung capacity. This was followed immediately by full exhalation at a constant flow (50, 100, or 200 ml/sec) and pressure (>5 cmH_2O) through the mouthpiece into the NO measuring device. We chose 200 ml/sec as the fastest flow since the total exhalation time at flows >200 ml/sec is not always long enough in children to achieve a stable plateau concentration. We chose 50 ml/sec as the lowest flow as this should be high enough to maintain a constant airway NO flux during a single exhalation in children,[2] and it is the recommended flow for a single breath exhaled NO measurement. The exhaled NO measurements at multiple flows (50, 100, and 200 ml/sec) were randomized and performed in triplicate for a total of nine single exhalations. To ensure an adequate plateau region for analysis in the NO exhalation profile, and account for progressively larger lung volumes as children's age, the exhalation times for the 50, 100, and 200 ml/sec maneuvers were set to 10, 8, and 6, or 15, 10, and 8, or 20, 15, and 10 sec for ages 6–9, 10–13, and 14–17 years, respectively. The subjects were allowed to rest for at least 30 sec between attempts. Standard spirometry was performed (WinDx Spirometer, Creative Biomedics International, CA) in accordance with ATS criteria.[30] The best spirometric measure of at least three maneuvers was recorded for analysis.

Data Analysis

The plateau concentrations of NO at multiple flows (50, 100, and 200 ml/sec) were determined using two different methods: (1) time interval analysis and (2) volume interval analysis. First, in accordance with current guidelines, the exhaled NO plateau concentration was determined as a time-averaged value over a 3 sec window. We analyzed the same time interval for all three flows within each subject, and chose the time interval to be the final 3 sec from the highest flow, since the lower two flows have longer exhalation times. Hence, the time interval for analysis was 4–6, 6–8, and 8–10 sec for ages 6–9, 10–13, and 14–17, respectively. A profile was removed from the analysis is the variation of NO concentration in the window varied by more than 10% (consistent with ATS/ERS guidelines). This is consistent with ATS and ERS guidelines, and allowed for a progressively longer exhalation time for older (and hence larger) children.

Second, the exhaled NO signal was analyzed based on the volume of exhaled breath. In addition, we also sought to determine the exhaled NO concentration at equivalent lung volumes across all subjects. Hence, we normalized the exhaled volume by an estimate of conducting airway volume, or exhaled airway volume turnovers (V_{ex}/V_{aw}; where V_{ex} is the exhaled volume and V_{aw} is an estimate of the subject's conducting airway volume). The subject's conducting airway volume was estimated in milliliters using the following previously reported relationship: $V_{aw} = 1.018 \times \text{Height (cm)} - 76.2$.[31]

For each exhalation, we examined both the NO and flow tracings over one to 10 airway volume turnovers in 15 sequentially increasing increments: 1–3, 1.5–3.5, 2–4, 2.5–4.5, 3–5, 3.5–4.5, 4–6, 4.5–6.5, 5–7, 5.5–7.5, 6–8, 6.5–8.5, 7–9, 7.5–9.5, and 8–10. Over each airway volume turnover increment, with respect to both flow and exhaled NO, we measured the mean, the standard deviation, and the slope by linear regression. We normalized the slope and standard deviation by the mean. The criteria to determine the ideal airway volume turnover were consistent with current ATS and ERS guidelines: (1) the normalized slope of the flow and exhaled NO profile was approximately equal to zero (i.e., a plateau had been achieved), (2) the coefficient of variation of the flow was $\leq$5%, and (3) the coefficient of variation of exhaled NO was $\leq$10%.

Calculation of $J'_{aw_{NO}}$ and CA_{NO}

At flows $\geq$50 ml/sec in children, the elimination rate, V_{NO} (pl/sec, product of mean exhaled NO concentration and flow), of NO can be approximated by the following linear equation: $V_{NO} = CA_{NO} \times V_E + J'_{aw_{NO}}$, where CA_{NO} is the distal airway/alveolar NO concentration (ppb), $J'_{aw_{NO}}$ is the maximum airway NO flux (pl/sec) and V_E is the exhalation flow (ml/sec).[2] Thus, to estimate CA_{NO} and $J'_{aw_{NO}}$, one can plot V_{NO} against V_E and apply a linear least squares analysis to determine the slope, S, and the intercept, I. The two-compartment model with axial diffusion can then be applied to estimate CA_{NO} and $J'_{aw_{NO}}$ using the following simple relationships: $CA_{NO} = S - I/a$ and $J'_{aw_{NO}} = I \times b$, where "a" and "b" are constants determined from the mathematical model which account for axial (or "back") diffusion of NO.[15] These relationships, to estimate $J'_{aw_{NO}}$ and CA_{NO}, do not consider variability in airway volume, which impacts the size and shape of the trumpet, and thus the cross-sectional area for axial diffusion. If both children (>4 years) and adults are considered, V_{aw} can range broadly from 25 to 300 ml. Details of the mathematical model have been presented previously;[15] thus, we will present only the salient features here to account for a variable V_{aw}.

Briefly, to account for changes in V_{aw}, we scaled the size of the trumpet (length and cross-sectional area) based on the bifurcating structure of the Weibel lung model A,[32] which has a volume of 217 ml through generation 17, the end of the airway compartment, and beginning of the alveolar compartment. In other words, the lengths and diameters of the symmetric bifurcating Weibel model were each scaled by $(V_{aw}/217)^{1/3}$, then the resulting dimensions were fit to the previously described trumpet shape: $A = A_1(z/z_1)^{-2}$, where A is the cross-sectional area of the trumpet, z is the axial position along the trumpet, and the subscript "1" refers to the axial position at generation 17.[15] The constants "a" and "b" are then determined using the governing equations for the model as previously described[15] for discrete values of V_{aw} ranging from 25 to 300 ml. The resulting relationship fits a power law extremely well ($R^2 = 0.998$) resulting in the following equations to estimate $J'_{aw_{NO}}$ and CA_{NO}:

$$J'_{aw_{NO}}\,(pl/sec) = I(1.2(V_{aw}^{0.087})) \quad (1)$$

$$CA_{NO}(ppb) = S - \left(\frac{I}{840V_{aw}^{-0.012}}\right) \quad (2)$$

where V_{aw} is expressed in milliliters. Equations (1) and (2) were used in both methods of analysis to determine the region specific $J'_{aw_{NO}}$ and CA_{NO}.

Statistical Analysis

Statistical analysis was performed using Sigma Stat (Systat Software, San Jose, CA). Kolmogorov–Smirnov test was used to determine normality. Differences between time interval and volume interval analysis were determined using a paired Student's *t*-test if the data set passed the Kolmogorov–Smirnov test, otherwise the Wilcoxon Signed Rank Test was used. Differences between endpoints evaluated over different time or exhaled volume intervals were assessed with analysis of variance (ANOVA). A value $P < 0.05$ was considered statistically significant.

RESULTS

Patient Characteristics

Thirty asthmatic subjects between the ages of 6–17 years were enrolled into the study. All of the enrolled subjects were able to perform the exhaled NO (none of the subjects had previous experience using the NIOX Flex) and spirometric maneuvers. The general patient characteristics are shown in Table 1.

TABLE 1—Demographics of Subjects and Pre-Bronchodilator Spirometry

Subjects, n	30
Age, years	11 ± 3
Male/female gender, n	14 (47)/16 (53)
Atopy, n (%)	85%
ICS treated/ICS naïve, n (%)	15 (50)/15 (50)
FEV_1, % predicted	108 ± 15
FVC, % predicted	105 ± 16
FEV_1/FVC	86 ± 7
FEF_{25-75}, % predicted	102 ± 30

Data are presented as mean ± standard deviation.
Inhaled corticosteroid (ICS) naïve was defined as no oral or ICS within the last 8 weeks and ICS treated was defined as prescribed ICS treatment for at least 8 weeks.

Determining the Ideal Airway Volume Turnover Interval

At all three flows, the subjects initially exhaled at a rate greater than the target; however, the flow quickly reached a steady value (Figs. 1A, 2A, and 3A). At a flow of 50 ml/sec, the normalized slope of the flow was approximately equal to zero and the coefficient of variation of the flow was $\leq$5% at 2.5–4.5 airway volume turnovers (Fig. 1B,C). There is an inverse relationship between exhalation flow and exhaled NO concentration (Figs. 1D, 2D, and 3D). The normalized slope of exhaled NO was approximately equal to zero and the coefficient of variation of the exhaled NO signal was $\leq$10% at 4.5–6.5 airway volume turnovers (Fig. 1E,F).

At a flow of 100 ml/sec, the normalized slope of the flow was approximately equal to zero and the coefficient of variation of the flow was $\leq$5% at 3–5 airway volume turnovers (Fig. 2B,C). The normalized slope of exhaled NO was approximately equal to zero and the coefficient of variation of the exhaled NO signal was $\leq$10% at 5–7 airway volume turnovers (Fig. 2E,F).

At a flow of 200 ml/sec, the normalized slope of the flow was approximately equal to zero and the coefficient of variation of the flow was $\leq$5% at 4–6 airway volume turnovers (Fig. 3B,C). The normalized slope of exhaled NO was approximately equal to zero and the coefficient of variation of the exhaled NO signal was $\leq$10% at 5–7 airway volume turnovers (Fig. 3E,F). At each flow, the criteria of having normalized slopes of the flow and

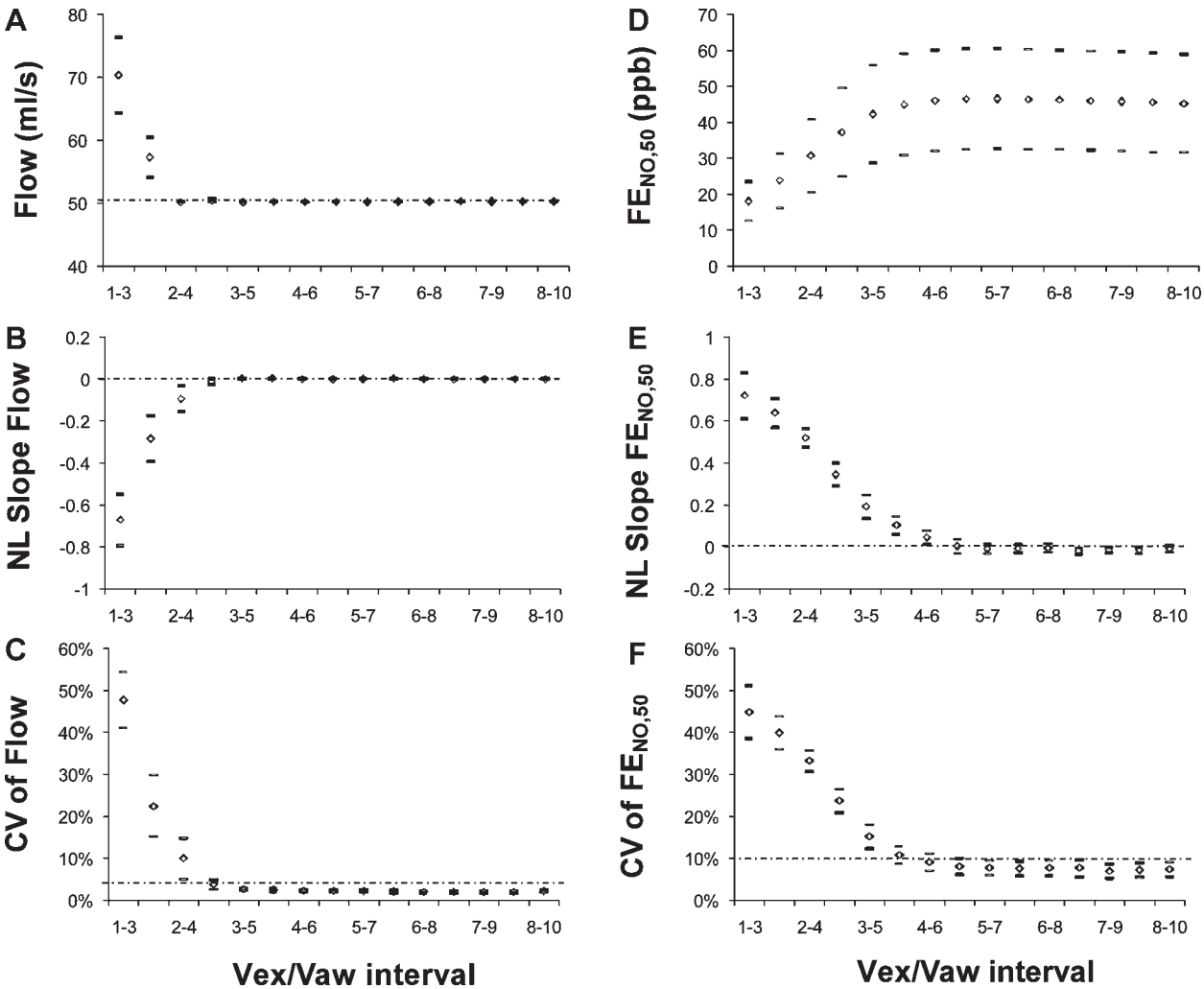

Fig. 1. Critical analysis of exhaled nitric oxide at a flow of 50 ml/sec. A: Flow, (B) normalized (NL) slope of flow, (C) coefficient of variation (CV) of flow, (D) exhaled nitric oxide at flow of 50 ml/sec ($FE_{NO,50}$), (E) NL slope of $FE_{NO,50}$ and (F) CV of $FE_{NO,50}$. All variables are plotted as a function of the analysis interval for V_{ex}/V_{aw}. Region between each consecutive pair of hash marks represents an increment of 0.5 units on the lower and upper bound of V_{ex}/V_{aw} interval. Criteria for determination of the ideal airway volume turnover were met at 4.5–6.5 airway volume turnovers. Data presented as mean, upper and lower 95th confidence intervals.

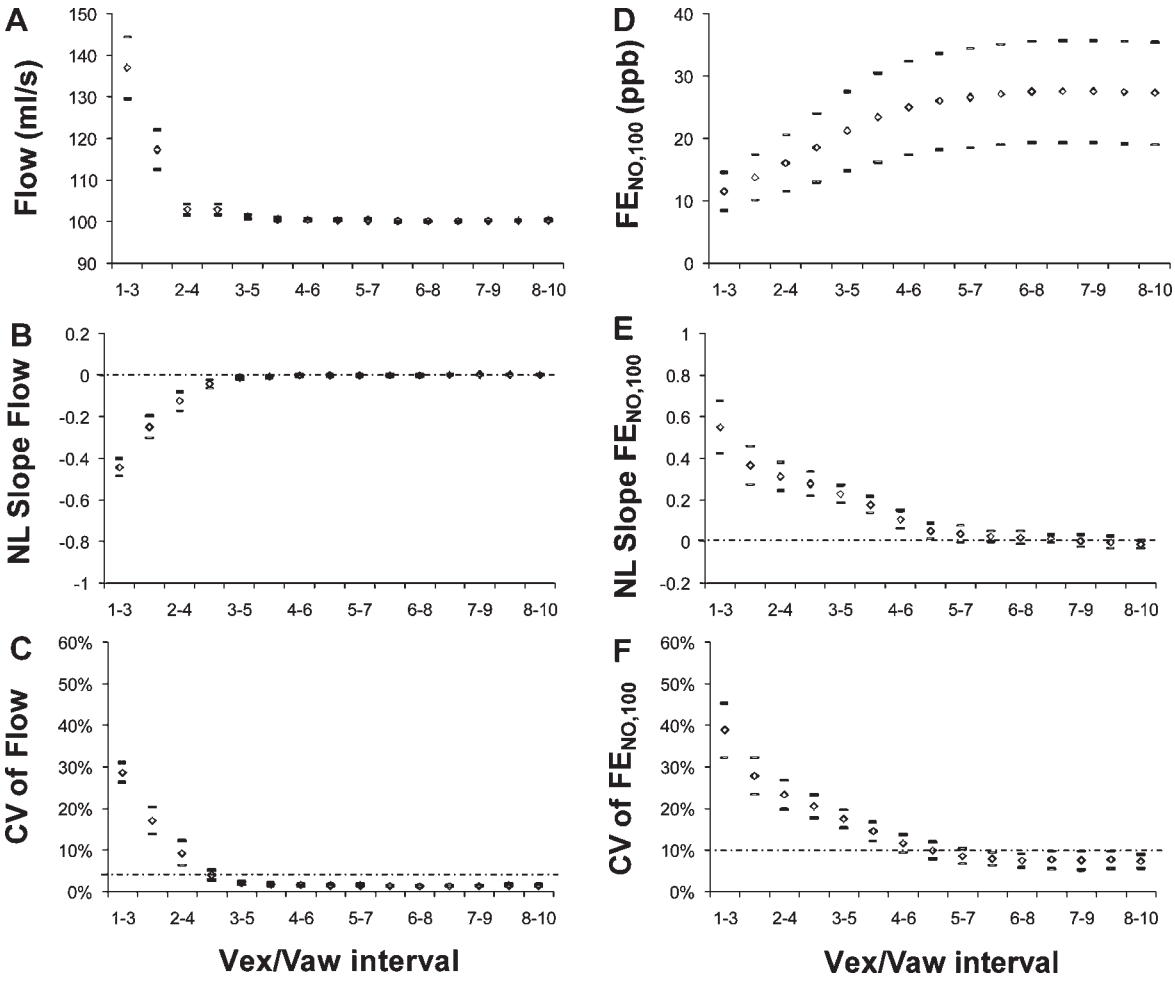

Fig. 2. Critical analysis of exhaled nitric oxide at a flow of 100 ml/sec. A: Flow, (B) normalized (NL) slope of flow, (C) coefficient of variation (CV) of flow, (D) exhaled nitric oxide at flow of 100 ml/sec ($FE_{NO,100}$), (E) NL slope of $FE_{NO,100}$ and (F) CV of $FE_{NO,100}$. Region between each consecutive pair of hash marks represents an increment of 0.5 units on the lower and upper bound of V_{ex}/V_{aw} interval. Criteria for determination of the ideal airway volume turnover were met at 5–7 airway volume turnovers. Data presented as mean, upper and lower 95th confidence intervals.

exhaled NO profile equal to zero and coefficients of variation of the flow and slope $\leq$5% and $\leq$10%, respectively were satisfied for the remaining airway volume turnovers (i.e., $7 < V_{ew}/V_{aw} < 10$).

The first airway volume turnover which fulfilled our ideal analysis criteria for all three flows was 5–7 airway volume turnovers. Therefore, we determined $J'_{aw_{NO}}$ and CA_{NO} for each of seven sequentially increasing volume turnover increments of analysis: 5–7, 5.5–7.5, 6–8, 6.5–8.5, 7–9, 7.5–9.5, and 8–10. There were no differences in the estimation of $J'_{aw_{NO}}$ ($P = 1.0$) or CA_{NO} ($P = 0.4$) across these airway volume intervals. An interval of 5–10 airway volume turnovers also allows for analysis of approximately a minimum of 3 sec, even at the highest flow (i.e., 200 ml/sec). Hence, we chose $5 < V_{ex}/V_{aw} < 10$ as the ideal volume interval window to analyze and apply the model of NO exchange dynamics.

Comparison of Time Interval and Volume Interval Analysis

Next, we compared the effect of time interval analysis (i.e., 3 sec) and volume interval analysis (i.e., 5–10 airway volume turnovers) on the measurement of the flow dependent $FE_{NO,50}$, $FE_{NO,100}$, and $FE_{NO,200}$ and calculation of the flow independent $J'_{aw_{NO}}$ and CA_{NO} (Fig. 4). All data sets passed the Kolmogorov–Smirnov test with the exception of $FE_{NO,50}$; however, the P-value for several of the data sets was close to the cut point of $P = 0.05$; hence, Table presents the results of both the paired t-test and the Wilcoxon rank sum test. Regardless of the statistical test employed, there were no differences between the plateau concentrations obtained from time interval and volume interval analyses for the flows of 50 ml/sec ($FE_{NO,50}$) and 100 ml/sec ($FE_{NO,100}$). However,

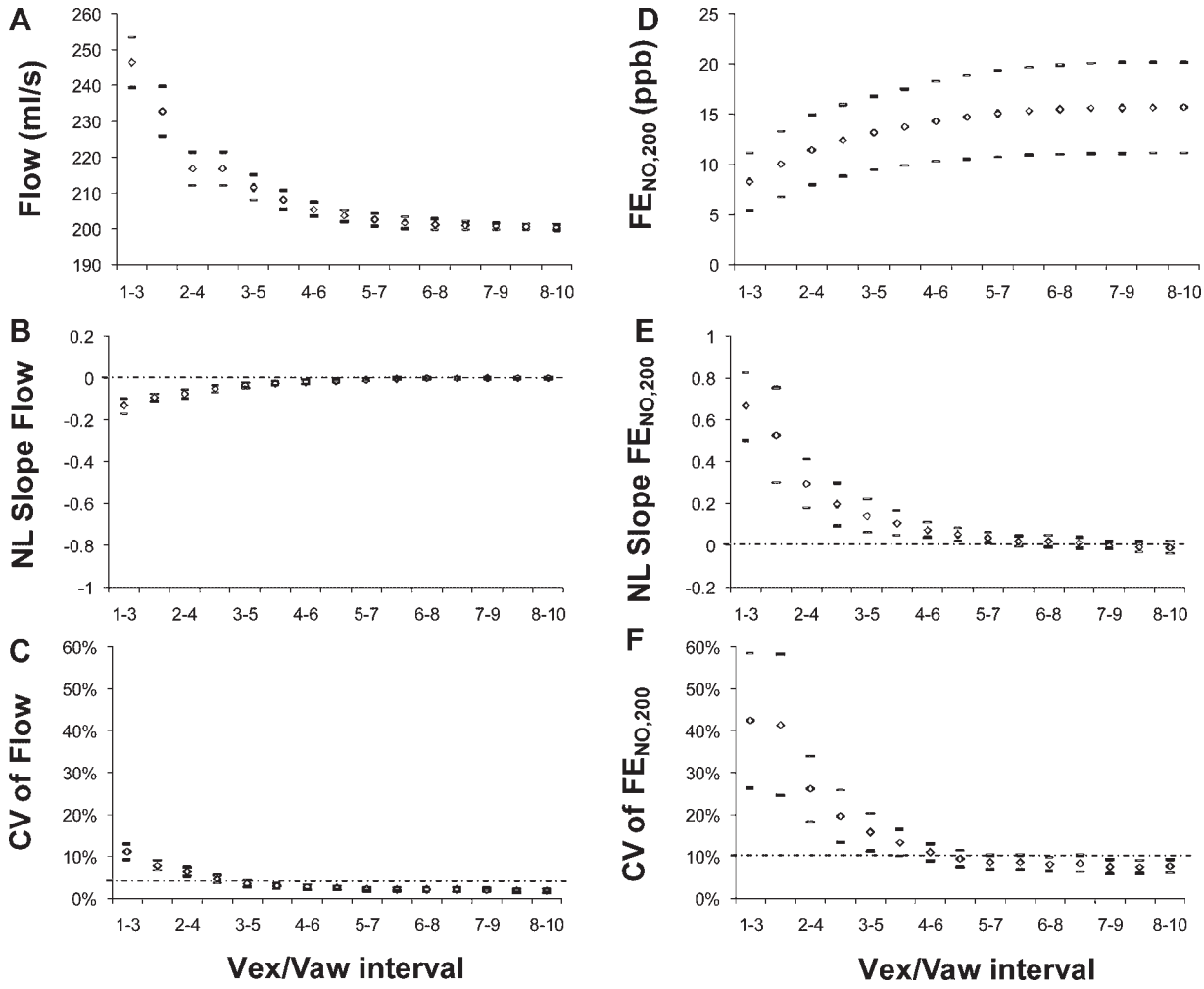

Fig. 3. Critical analysis of exhaled nitric oxide at a flow of 200 ml/sec. A: Flow, (B) normalized (NL) slope of flow, (C) coefficient of variation (CV) of flow, (D) exhaled nitric oxide at flow of 200 ml/sec ($FE_{NO,200}$), (E) NL slope of $FE_{NO,200}$ and (F) CV of $FE_{NO,200}$. Region between each consecutive pair of hash marks represents an increment of 0.5 units on the lower and upper bound of V_{ex}/V_{aw} interval. Criteria for determination of the ideal airway volume turnover were met at 5–7 airway volume turnovers. Data presented as mean, upper and lower 95th confidence intervals.

there was a statistical difference between the plateau concentrations obtained from the different methods of analyses with respect to the highest flow of 200 ml/sec ($FE_{NO,200}$). The mean differences (limits of agreement) [FE_{NO} based on volume interval − FE_{NO} based on time interval] were −0.2 ppb (−3.7 to 3.2 ppb), 0.2 ppb (−1.8 to 2.2 ppb) and 1.0 ppb (−1.4 to 3.4 ppb) for 50, 100, and 200 ml/sec, respectively.

The region specific NO parameters, $J'_{aw_{NO}}$ and CA_{NO}, were determined using the FE_{NO} plateau concentrations calculated from both time interval analyses and volume interval analyses. With respect to $J'_{aw_{NO}}$, there was no statistical difference (regardless of statistical test employed) between time interval and volume interval methods of analyses (Table 2). The mean difference (volume−time) was −0.1 pl/sec with limits of agreement ranging from −0.8 pl/sec below to 0.6 pl/sec above and discrepancies of up to approximately 0.6 pl/sec. With regards to CA_{NO}, a statistical difference was observed between time interval and volume interval methods of analyses (Table 2). The mean difference (volume − time) was 1.4 ppb with limits of agreement ranging from −1.7 ppb below to 4.4 ppb above and discrepancies of up to approximately 6 ppb.

DISCUSSION

This study has examined the impact of the analysis interval (constant time or exhaled volume interval) on FE_{NO} measurements at multiple exhalation flows, and the partitioning of the exhaled NO signal into its proximal airway ($J'_{aw_{NO}}$) and distal airway/alveolar (CA_{NO}) contributions in children with asthma. We have presented a method to analyze the exhaled NO signal based on

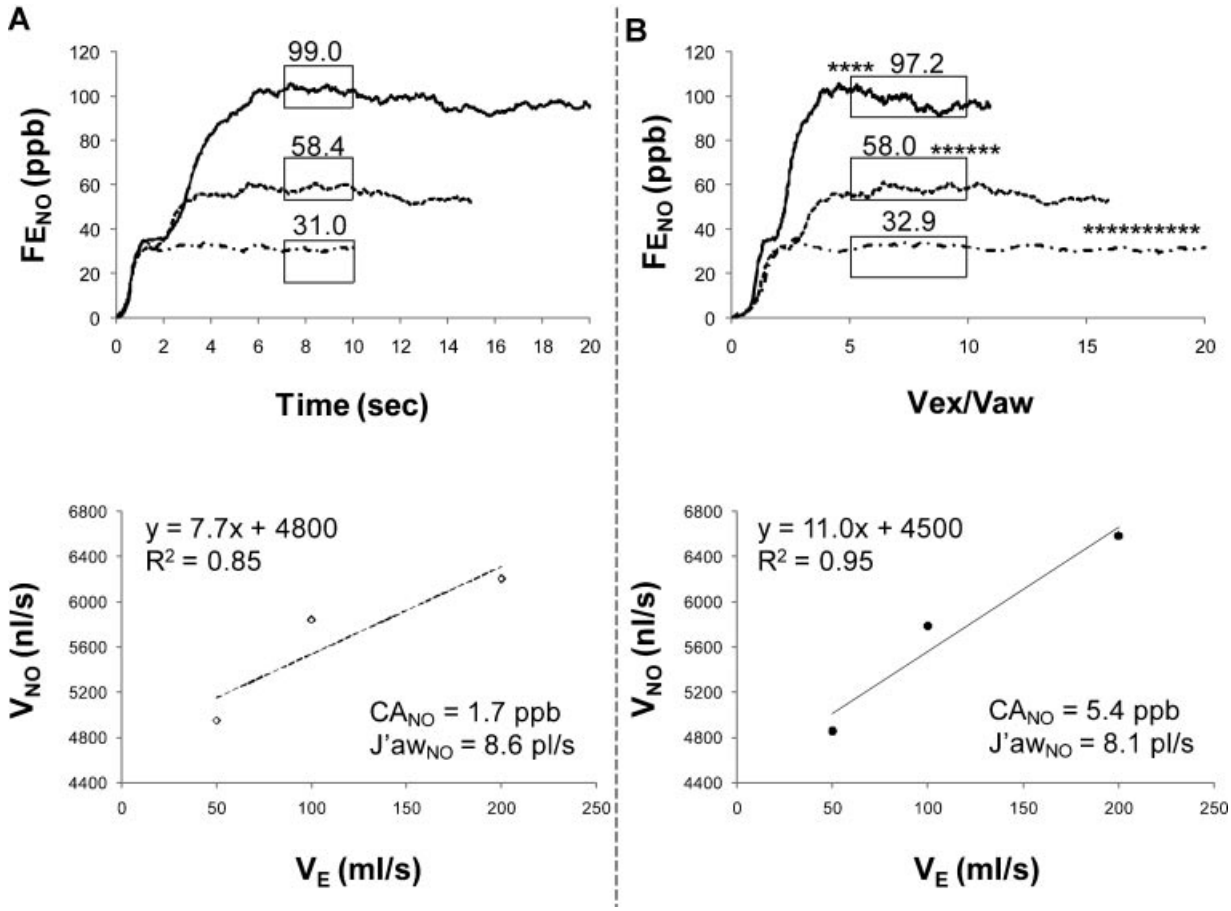

Fig. 4. Example of time interval and volume interval analysis. A: Time interval analysis (i.e., 3 sec) and (B) volume interval analysis (i.e., 5–10 airway volume turnovers) in a steroid naïve subject with asthma. Asterisks (*) represent the 3 sec time interval analysis on the volume interval axis. Exhaled nitric oxide (NO) at 50 ml/sec ($FE_{NO,50}$); exhaled NO at 100 ml/sec ($FE_{NO,100}$); exhaled NO at 200 ml/sec ($FE_{NO,200}$); proximal airway NO ($J'_{aw_{NO}}$); distal airway/alveolar NO (CA_{NO}).

equivalent exhaled airway volumes, and suggest an ideal volume interval (5–10 airway volume turnovers) to analyze and apply the model of NO exchange dynamics. The constant time interval predicts values for $FE_{NO,200}$ that are smaller than the constant exhaled volume interval. The result is a systematic bias for the constant time interval analysis that results in a significant underestimation of CA_{NO}. This finding is particularly relevant given the numerous observations demonstrating the potential clinical utility of CA_{NO} in asthma.[33]

There is mounting evidence supporting $FE_{NO,50}$ as a reproducible,[34] non-invasive measure of inflammation in the asthmatic lung.[35–38] Several studies suggest that $FE_{NO,50}$ can be used to diagnose asthma,[39–41] especially in combination with the results of more traditional measures of lung function such as spirometry.[42] The constant time interval and constant volume interval predict values for $FE_{NO,50}$ that are highly correlated, and not different from each other. This finding is dependent on the choice of the time interval relative to the volume interval. For

TABLE 2— Exhaled NO Measurements Evaluated by Time and Volume Intervals

	Time interval	Volume interval	Kolmogorov–Smirnov	Wilcoxon rank *P*-value	Paired *t*-test *P*-value
$FE_{NO,50}$ (ppb)	46.0 ± 38.7	46.0 ± 38.4	Failed ($P < 0.05$)	0.45	0.49
$FE_{NO,100}$ (ppb)	27.0 ± 22.5	27.2 ± 23.0	Passed ($P = 0.086$)	0.36	0.76
$FE_{NO,200}$ (ppb)	14.5 ± 11.8	15.5 ± 12.4	Passed ($P = 0.10$)	<0.001	<0.001
CA=L> (nl/sec)	3.9 ± 3.4	3.8 ± 3.3	Passed ($P = 0.19$)	0.08	0.17
CA_{NO} (ppb)	1.1 ± 1.0	2.6 ± 2.1	Passed ($P = 0.05$)	<0.001	<0.001

Data are presented as mean ± standard deviation.

example, we chose a time interval consistent with the last 3 sec of the highest flow (e.g., 4–6 sec in 6–9 years old). This corresponds to approximately 4–6 airway volume turnovers (see Fig. 4), which, although slightly earlier in the exhalation profile relative to the constant volume interval method, overlaps with the constant volume interval (5–10 airway volume turnovers), and hence there is no statistical difference.

When examining the impact of a constant time interval versus a constant exhaled volume interval at higher flows, the difference between the regions of analysis depends on the flow (Fig. 4). For an exhalation flow of 100 ml/sec, the time interval analysis (e.g., 4–6 sec for 6–9 years old) corresponds to a larger volume interval (approximately $7 < V_{ex}/V_{aw} < 11$, Fig. 4), but, again, there is significant overlap with the constant volume interval analysis ($5 < V_{ex}/V_{aw} < 10$); hence, the difference in our analysis is negligible. At the highest flow, the region of analysis for the constant time interval is at a much larger V_{ex}/V_{aw} (15–20, Fig. 4), and the result is a smaller estimate in $FE_{NO,200}$ due the slightly negative slope of the exhalation profile at larger exhaled volumes ($V_{ex}/V_{aw} > 8$, Fig. 3E). Therefore, V_{NO} at 200 ml/sec is significantly larger which creates a steeper slope and larger CA_{NO} for the constant volume interval analysis (Fig. 4).

Within a given study, the multiple exhalation flow method to partition exhaled NO has been shown to be a reproducible[43] and reliable method to estimate CA_{NO} in healthy[14,24] and asthmatic[14,26,27] children. In fact, numerous potentially clinically significant findings have been reported, highlighting the biological relevance of CA_{NO} in the context of asthma. For example, increased levels of CA_{NO} have been reported in asthmatics with nocturnal symptoms,[44] asthmatics with poor control[14] and in asthmatics refractory to ICS treatment.[18] Furthermore, oral steroids[18,43] and leukotriene receptor antagonists,[45] but not inhaled corticosteroids,[23,46] have been shown to reduce CA_{NO}. These findings suggest that partitioning exhaled NO to determine CA_{NO} may improve the clinical relevance of the exhaled NO signal, and thus accurate methods to estimate CA_{NO} are needed.

Despite these important findings, there are no specific guidelines on the collection technique or method to determine CA_{NO}, and variations in published CA_{NO} concentrations, in healthy and asthmatic children have been reported. Some of the discrepancy in the asthmatic children may be attributed to heterogeneity in disease severity or symptoms between the studies, or neglecting axial diffusion of NO; however, the reported differences in healthy children are most likely attributable to variable techniques of measurement. For example, in healthy children, Mahut et al.[26] reported an average CA_{NO} of 4.2 ± 2.0 ppb, while Sepponen et al.[24] reported 2.0 ± 0.8 ppb. In our study, the mean concentrations of CA_{NO}, based on time and volume interval analysis were 1.1 and 2.6 ppb, respectively, and were significantly different from each other ($P < 0.001$; Table 2). Our results suggest that differences between studies may stem from ATS and ERS guidelines which do not require a constant exhaled volume interval analysis, and the requirements for a constant exhalation time window are flexible enough to result in significant differences in estimated CA_{NO}.

Two key features of the current ATS guidelines with respect to measuring the FE_{NO} plateau concentration are: (1) prior to analysis, exhalation should occur for at least 4 sec in subjects younger than 12 years old or 6 sec in subjects older than 12 years old and (2) the FE_{NO} concentration is defined as a time-averaged value over a 3 sec window in which the guideline criteria for a stable plateau are met (i.e., the FE_{NO} concentration does not vary by more than 10%). These guidelines are not adequate to address features of FE_{NO} measured at multiple exhalation flows. For example, if the interval of analysis to determine FE_{NO} is a fixed time (i.e., 3 sec), then an exhaled volume of 150 ml is analyzed at a flow of 50 ml/sec, but 600 ml is analyzed at a flow of 200 ml/sec. Furthermore, if the analysis window begins after 6 sec of exhalation in adults and children >12 years, this corresponds to an exhaled volume of 300 ml at a flow of 50 ml/sec and 1,200 ml at a flow of 200 ml/sec. Hence, when using current guidelines to estimate $J'_{aw_{NO}}$ and CA_{NO} by measuring FE_{NO} at multiple flows, the model is applied over different exhaled volumes of air and at different lung volumes. Additionally, the guidelines do not consider variation in the size of the subjects, despite the positive correlation between FE_{NO} and height.[24,47] An algorithm to analyze FE_{NO} based on airway volume turnover intervals is physiologically more accurate than the time interval analysis because the volume interval method considers the height of the individual and permits application of the quantitative model of NO exchange across equivalent exhaled volumes of air and lung volumes.

In conclusion, we have contrasted methods to analyze the exhaled NO profile based on constant exhalation time intervals and volume intervals to determine the effect on both FE_{NO} and the multiple exhalation flow technique to partition exhaled nitric oxide into its proximal airway ($J'_{aw_{NO}}$) and distal airway/alveolar contributions (CA_{NO}). The volume interval analysis method is based on an estimate of the subject's airway volume, which considers the height of the individual and facilitates application of the two-compartment model across equivalent exhaled breath volumes and lung volumes. Analysis of a constant time interval results in a significantly reduced FE_{NO} at higher flows and thus a systematic bias leading to an underestimation of CA_{NO}. The magnitude of the bias will depend on the choice of the time interval. This result is particularly relevant given the recent clinical studies demonstrating the potential of CA_{NO} to characterize distal lung inflammation.[14,25–27,33] An ideal volume interval to

analyze the exhaled NO signal based on achieving relatively steady flows and exhaled NO concentrations is 5–10 airway turnovers. Future studies must address the underlying mechanisms of the negative slope in the exhaled NO profile, and optimal flow ranges for children and adults as we move towards standardizing the methods to assess proximal and distal NO levels in the lungs.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Institutes of Health (R01 HL070645) and the Children's Hospital of Orange County.

REFERENCES

1. Gustafsson LE, Leone AM, Persson MG, Wiklund NP, Moncada S. Endogenous nitric oxide is present in the exhaled air of rabbits, guinea pigs and humans. Biochem Biophys Res Commun 1991;181:852–857.
2. George SC, Hogman M, Permutt S, Silkoff PE. Modeling pulmonary nitric oxide exchange. J Appl Physiol 2004;96:831–839.
3. Silkoff PE, McClean PA, Slutsky AS, Furlott HG, Hoffstein E, Wakita S, Chapman KR, Szalai JP, Zamel N. Marked flow-dependence of exhaled nitric oxide using a new technique to exclude nasal nitric oxide. Am J Respir Crit Care Med 1997;155:260–267.
4. Tsoukias NM, Shin HW, Wilson AF, George SC. A single-breath technique with variable flow rate to characterize nitric oxide exchange dynamics in the lungs. J Appl Physiol 2001;91:477–487.
5. Alving K, Weitzberg E, Lundberg JM. Increased amount of nitric oxide in exhaled air of asthmatics. Eur Respir J 1993;6:1368–1370.
6. Kharitonov SA, Yates D, Robbins RA, Logan-Sinclair R, Shinebourne EA, Barnes PJ. Increased nitric oxide in exhaled air of asthmatic patients. Lancet 1994;343:133–135.
7. Tsoukias NM, Tannous Z, Wilson AF, George SC. Single-exhalation profiles of NO and CO2 in humans: effect of dynamically changing flow rate. J Appl Physiol 1998;85:642–652.
8. Hogman M, Drca N, Ehrstedt C, Merilainen P. Exhaled nitric oxide partitioned into alveolar, lower airways and nasal contributions. Respir Med 2000;94:985–991.
9. Pietropaoli AP, Perillo IB, Torres A, Perkins PT, Frasier LM, Utell MJ, Frampton MW, Hyde RW. Simultaneous measurement of nitric oxide production by conducting and alveolar airways of humans. J Appl Physiol 1999;87:1532–1542.
10. Silkoff PE, Sylvester JT, Zamel N, Permutt S. Airway nitric oxide diffusion in asthma: Role in pulmonary function and bronchial responsiveness. Am J Respir Crit Care Med 2000;161:1218–1228.
11. Tsoukias NM, George SC. Impact of volume-dependent alveolar diffusing capacity on exhaled nitric oxide concentration. Ann Biomed Eng 2001;29:731–739.
12. Recommendations for standardized procedures for the on-line and off-line measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide in adults and children- 1999.This official statement of the American Thoracic Society was adopted by theATS Board of Directors, July 1999. Am J Respir Crit Care Med 1999;160:62104–62117.
13. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. Am J Respir Crit Care Med 2005;171:8912–8930.
14. Paraskakis E, Brindicci C, Fleming L, Krol R, Kharitonov SA, Wilson NM, Barnes PJ, Bush A. Measurement of bronchial and alveolar nitric oxide production in normal children and children with asthma. Am J Respir Crit Care Med 2006;174:260–267.
15. Condorelli P, Shin HW, Aledia AS, Silkoff PE, George SC. A simple technique to characterize proximal and peripheral nitric oxide exchange using constant flow exhalations and an axial diffusion model. J Appl Physiol 2007;102:417–425.
16. Shin HW, Condorelli P, George SC. A new and more accurate technique to characterize airway nitric oxide using different breath-hold times. J Appl Physiol 2005;98:1869–1877.
17. Tsoukias NM, George SC. A two-compartment model of pulmonary nitric oxide exchange dynamics. J Appl Physiol 1998;85:653–666.
18. Berry M, Hargadon B, Morgan A, Shelley M, Richter J, Shaw D, Green RH, Brightling C, Wardlaw AJ, Pavord ID. Alveolar nitric oxide in adults with asthma: evidence of distal lung inflammation in refractory asthma. Eur Respir J 2005;25:986–991.
19. Brindicci C, Ito K, Barnes PJ, Kharitonov SA. Differential flow analysis of exhaled nitric oxide in patients with asthma of differing severity. Chest 2007;131:1353–1362.
20. Girgis RE, Gugnani MK, Abrams J, Mayes MD. Partitioning of alveolar and conducting airway nitric oxide in scleroderma lung disease. Am J Respir Crit Care Med 2002;165:1587–1591.
21. Lehtimaki L, Kankaanranta H, Saarelainen S, Turjanmaa V, Moilanen E. Peripheral inflammation in patients with asthmatic symptoms but normal lung function. J Asthma 2005;42:605–609.
22. Roy K, Borrill ZL, Starkey C, Hazel AL, Morris J, Vestbo J, Singh D. Use of different exhaled nitric oxide multiple flow rate models in COPD. Eur Respir J 2007;29:651–659.
23. Shin HW, Rose-Gottron CM, Cooper DM, Newcomb RL, George SC. Airway diffusing capacity of nitric oxide and steroid therapy in asthma. J Appl Physiol 2004;96:65–75.
24. Sepponen A, Lehtimaki L, Huhtala H, Kaila M, Kankaanranta H, Moilanen E. Alveolar and bronchial nitric oxide output in healthy children. Pediatr Pulmonol 2008;43:1242–1248.
25. Linkosalo L, Lehtimaki L, Laitinen J, Kaila M, Holm K, Moilanen E. Increased bronchial NO output in severe atopic eczema in children and adolescents. Pediatr Allergy Immunol 2008;19:426–432.
26. Mahut B, Delacourt C, Zerah-Lancner F, De Blic J, Harf A, Delclaux C. Increase in alveolar nitric oxide in the presence of symptoms in childhood asthma. Chest 2004;125:1012–1018.
27. Mahut B, Delclaux C, Tillie-Leblond I, Gosset P, Delacourt C, Zerah-Lancner F, Harf A, de Blic J. Both inflammation and remodeling influence nitric oxide output in children with refractory asthma. J Allergy Clin Immunol 2004;113:252–256.
28. Expert Panel Report 3 (EPR-3). Guidelines for the diagnosis and management of asthma-summary report 2007. J Allergy Clin Immunol 2007;120:S94–S138.
29. Silkoff PE, Wakita S, Chatkin J, Ansarin K, Gutierrez C, Caramori M, McClean P, Slutsky AS, Zamel N, Chapman KR. Exhaled nitric oxide after beta2-agonist inhalation and spirometry in asthma. Am J Respir Crit Care Med 1999;159:940–944.
30. Lung function testing. selection of reference values and interpretative strategies. American Thoracic Society. Am Rev Respir Dis 1991;144:1202–1218.
31. Kerr AA. Dead space ventilation in normal children and children with obstructive airways disease. Thorax 1976;31:63–69.
32. Weibel E. Morphometry of the human lung. Berlin: Springler-Verlag; 1963.
33. Puckett JL, George SC. Partitioned exhaled nitric oxide to non-invasively assess asthma. Respir Physiol Neurobiol 2008;163:166–177.

34. Kharitonov SA, Gonio F, Kelly C, Meah S, Barnes PJ. Reproducibility of exhaled nitric oxide measurements in healthy and asthmatic adults and children. Eur Respir J 2003;21:433–438.
35. Mattes J, Storm van's Gravesande K, Reining U, Alving K, Ihorst G, Henschen M, Kuehr J. NO in exhaled air is correlated with markers of eosinophilic airway inflammation in corticosteroid-dependent childhood asthma. Eur Respir J 1999;13:1391–1395.
36. Steerenberg PA, Janssen NA, de Meer G, Fischer PH, Nierkens S, van Loveren H, Opperhuizen A, Brunekreef B, van Amsterdam JG. Relationship between exhaled NO, respiratory symptoms, lung function, bronchial hyperresponsiveness, and blood eosinophilia in school children. Thorax 2003;58:242–245.
37. Barreto M, Villa MP, Monti F, Bohmerova Z, Martella S, Montesano M, Darder MT, Ronchetti R. Additive effect of eosinophilia and atopy on exhaled nitric oxide levels in children with or without a history of respiratory symptoms. Pediatr Allergy Immunol 2005;16:52–58.
38. Thomas PS, Gibson PG, Wang H, Shah S, Henry RL. The relationship of exhaled nitric oxide to airway inflammation and responsiveness in children. J Asthma 2005;42:291–295.
39. Berkman N, Avital A, Breuer R, Bardach E, Springer C, Godfrey S. Exhaled nitric oxide in the diagnosis of asthma: comparison with bronchial provocation tests. Thorax 2005;60:383–388.
40. Dupont LJ, Demedts MG, Verleden GM. Prospective evaluation of the validity of exhaled nitric oxide for the diagnosis of asthma. Chest 2003;123:751–756.
41. Malmberg LP, Pelkonen AS, Haahtela T, Turpeinen M. Exhaled nitric oxide rather than lung function distinguishes preschool children with probable asthma. Thorax 2003;58:494–499.
42. Smith AD, Cowan JO, Filsell S, McLachlan C, Monti-Sheehan G, Jackson P, Taylor DR. Diagnosing asthma: comparisons between exhaled nitric oxide measurements and conventional tests. Am J Respir Crit Care Med 2004;169:473–478.
43. Gelb AF, Taylor CF, Nussbaum E, Gutierrez C, Schein A, Shinar CM, Schein MJ, Epstein JD, Zamel N. Alveolar and airway sites of nitric oxide inflammation in treated asthma. Am J Respir Crit Care Med 2004;170:737–741.
44. Lehtimaki L, Kankaanranta H, Saarelainen S, Turjanmaa V, Moilanen E. Increased alveolar nitric oxide concentration in asthmatic patients with nocturnal symptoms. Eur Respir J 2002;20:841–845.
45. Fritscher LG, Rodrigues MT, Zamel N, Chapman KR. The effect of montelukast on exhaled nitric oxide of alveolar and bronchial origin in inhaled corticosteroid-treated asthma. Respir Med 2009;103:296–300.
46. Lehtimaki L, Kankaanranta H, Saarelainen S, Turjanmaa V, Moilanen E. Inhaled fluticasone decreases bronchial but not alveolar nitric oxide output in asthma. Eur Respir J 2001;18:635–639.
47. Olin AC, Rosengren A, Thelle DS, Lissner L, Bake B, Toren K. Height age, and atopy are associated with fraction of exhaled nitric oxide in a large adult general population sample. Chest 2006;130:1319–1325.