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Extended Exhaled Nitric Oxide Analysis in Field Surveys of Schoolchildren: A Pilot Test

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Summary. Extended exhaled nitric oxide (eNO) analysis can distinguish proximal and distal airway contributions to FeNO. Thus, it has the potential to detect effects of different environmental influences, allergic phenotypes, and genetic variants on proximal and distal airways. However, its feasibility in field surveys has not been demonstrated, and models for estimating compartmental NO contributions have not been standardized. In this study we verified that extended NO tests can be performed by children in schools, and assessed different analytical models to estimate bronchial flux and alveolar NO concentration. We tested students at a middle school, using EcoMedics NO analyzers with ambient NO scrubbers, at flows of 50 (conventional), 30, 100, and 300 ml/sec, with 2–3 trials at each flow. Data from 65 children were analyzed by two linear and four nonlinear published models, plus a new empirical nonlinear model. Bronchial NO flux estimates from different models differed in magnitude but were strongly correlated ($r \geq 0.95$), and increased in subjects with allergic asthma. Alveolar concentration estimates differed among models and did not consistently show the same effects of allergy or asthma. A novel index of nonlinear behavior of NO output versus flow was significantly related to asthma status, and not strongly correlated with bronchial flux or alveolar concentration. Field-based extended NO testing of children can yield useful information about NO in different regions of the respiratory tract that is not obtainable from conventional FeNO. Extended NO analysis holds promise for investigating environmental and genetic determinants of regional airway inflammatory states. **Pediatr Pulmonol. 2009; 44:1033–1042.**

Key words: exhaled nitric oxide; epidemiology; children; asthma; allergy.

INTRODUCTION

Exhaled nitric oxide concentration (FeNO) is a useful measure of pulmonary or systemic oxidative/nitrosative stress in research and clinical settings.[1–3] However, mechanistic interpretation of conventional FeNO measured at 50 ml/sec flow) is limited in that the exhaled NO concentration represents a mixture of NO from large proximal airways (influenced by bronchial inflammation) with NO from small distal airways or alveoli (influenced by deep-lung or systemic inflammatory stresses). Extended NO analysis, in which FeNO is measured at several expiratory flows, may overcome this limitation.[4,5] However, extended NO testing is more expensive to perform, and more complex to interpret, than conventional FeNO measurement. This article describes results of a pilot study intended to develop methods and demonstrate feasibility of extended NO testing in large populations of children at schools—to verify that both equipment and children could perform satisfactorily, and to evaluate the suitability of analytical models for identifying both proximal and distal airway NO abnormalities in populations including healthy, allergic, and asthmatic children.

Additional Supporting Information may be found in the online version of this article.

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In most people, exhaled NO concentrations measured at multiple expiratory flows $\geq$50 ml/sec approximately fit a simple linear relationship, which can be represented by:

$$\mathrm{FeNO} = \mathrm{CalvNO} + (\mathrm{JawNO}/\mathrm{Q})$$

Here "CalvNO" represents a steady-state concentration of NO in distal small airways or alveoli, "JawNO" represents a steady-state flux of NO from proximal airway walls diluted in inverse proportion to flow, and "Q" is expiratory flow. Linear regressions of FeNO against $1/Q$[6] or of NO output (the product of Q and FeNO) against Q[7,8] yield estimates of JawNO and CalvNO. Departures from linearity in these relationships result in slightly different estimates of JawNO and CalvNO between the Pietropaoli[6] (P) model and the Tsoukias[7,8] (T) model. Nonlinearity also causes these estimates to vary according to the particular range of flows at which FeNO is measured.[9,10] Increased nonlinearity appears to correlate with increased conventional FeNO and decreased forced expiratory performance.[11] To distinguish estimates from the different analytic models addressed in this study, we use notation that replaces "NO" in the above abbreviations with the first author's initial, for example, JawT, JawP.

Nonlinear extended NO models, with more sophisticated assumptions about NO flow in relation to airway anatomy and physiology, have been published by Silkoff et al.,[12] Hogman and Merilainen,[4] Condorelli et al.,[13] and Kerckx et al.[14] These models, described further in the online supplement, are designated here as S, H, C, and K, respectively. In general, they give higher estimates of JawNO and lower estimates of CalvNO than the simple linear models. Most models have been validated only in small laboratory- or clinic-based populations, and few rigorous comparisons have been made by applying different models to the same data. One comparison in adults with and without chronic obstructive pulmonary disease (COPD) found that results varied by model and by flow range, although shifts in JawNO due to smoking or COPD were detectable consistently.[15]

To optimize and assess the performance of analytical models for extended eNO, studies in larger, more diverse populations are required. Our overarching hypothesis is that estimates of parameters available from extended eNO measurements can differentiate proximal and distal airway effects in populations that reflect health status, genetic susceptibility, and environmental exposures. To begin testing this hypothesis, we conducted a pilot study in a convenience sample drawn from the Asthma Incidence Risk Study (AIRS), a component of the large-scale longitudinal Children's Health Study,[16–18] to assess the feasibility of testing in a field setting to examine the behavior of different model parameters. We performed conventional (50 ml/sec) FeNO measurements in all AIRS participants attending one selected school followed by the Hogman and Merilainen[4] protocol for extended eNO with measurements at 30, 100, and 300 ml/sec. We then analyzed each subject's data using each of the aforementioned published models, plus a new empirical nonlinear model. Finally, we evaluated the consistency of results among different models, and the ability of each model parameter to distinguish healthy, allergic, and asthmatic subjects.

MATERIALS AND METHODS

Test Setting and Subjects

We tested all AIRS population members attending one middle school in an eastern suburb of Los Angeles, subject to high background particulate and oxidant air pollution but away from roads with heavy traffic. The project was reviewed and approved by the Institutional Review Board of the Keck School of Medicine, University of Southern California. Testing took place in an unused classroom on three successive autumn mornings when ambient air pollution was relatively low. All participants had prior experience with conventional FeNO testing, and extensive longitudinal documentation of their clinical status from questionnaires filled out by parents. Height and weight were recorded on the day of testing. Subjects were classified according to the following allergy categories: recent allergy symptoms, past but not recent allergy symptoms, or no history of allergy; and asthma status categories: physician diagnosis of asthma with recent symptoms or medication use, physician diagnosis with past but not recent symptoms, or no lifetime history of asthma.

Protocol

Testing employed two EcoMedics CLD-88-SP analyzers with DeNOx accessories to provide NO-free inhaled air (EcoPhysics, Inc., Ann Arbor, MI/Duernten, Switzerland). Both analyzers were calibrated with a certified span gas (Scott-Marrin, Inc., Riverside, CA) using the manufacturer's procedure; and both were calibration-checked before and after the field visit, using certified span gases and a zero-air filter (Sievers Division, GE Analytical Instruments, Boulder, CO). Differences in concentration readings between analyzers did not exceed 2%, and NO concentrations in air supplied by DeNOx units were consistently below 1 ppb, relative to the zero-air filter. Each DeNOx was modified, using parts supplied by the manufacturer, to allow extended NO testing by the H protocol.[4] Breathing maneuvers followed the manufacturer's instructions based on professional societies' recommendations.[1,19,20] The subject took two or more preliminary tidal breaths and a maximal inspiration from the DeNOx, then exhaled against a calibrated fixed

resistance, controlling flow by observing an analog readout. Testing began with 50 ml/sec blows and continued with the H protocol—30, 100, and 300 ml/sec blows in that order. Two blows were recorded at each flow, or three if either of the first two did not show an acceptable NO concentration plateau according to the system software.

Data Analysis

Digitized records were analyzed using a custom application written in SAS (SAS Institute, Cary, NC) to find the first ATS-acceptable 3-sec plateau.[1] If no ATS-acceptable plateau was found, but the expiratory maneuver appeared technically satisfactory, a visual estimate of plateau concentration was made from a plot. The relationship of FeNO to flow was then modeled for each subject by each analytic method mentioned in the introduction, using commercial software for linear or nonlinear regression. For methods P, T, C, and K, 30 ml/sec data were excluded to minimize bias due to nonlinearity. Method H calculations excluded 50 ml/sec data by design, but gave estimates of FeNO at 50 ml/sec which showed close agreement with measured values. Method S calculations used data from all flows.

Because the published models provide adequate fit to exhaled NO data for most but not all subjects (see the Results Section), we developed and assessed new empirical approaches that were not based on any physiologic assumptions. First, regression slope (distal NO concentration) was estimated by the T method using 100 and 300 ml/sec data only, then a separate estimate was made using 30 and 50 ml/sec data only. The former slope subtracted from the latter provided an index of nonlinearity, referred to here as slope difference (SLPDIF), to supplement the T estimates. Second, data were modeled by plotting FeNO versus 1/flow as in the P method, then applying various empirical curve-fitting techniques available in Microsoft Excel software. A quadratic function provided the best fit. Figure 1 illustrates the SLPDIF calculation and Figure 2 the empirical quadratic (Q) model, applied to observed mean values of FeNO and flow for subjects in each asthma category. In model Q (discussed further in the online supplement), the intercept and linear coefficient were taken as estimates of JawNO and CalvNO as in model P, and the quadratic coefficient was taken as an index of nonlinearity.

Descriptive statistics were computed to compare results from different models, and analyses of variance were performed to determine which parameters varied significantly according to asthma and allergy status.

RESULTS

Performance of Subjects and Equipment

Readings on standard gases were consistent within 2% between the two NO analyzers, and between checks before and after the field testing. Of 76 children tested, one was unable to exhale satisfactorily at 300 ml/sec and was excluded from analysis. Ten others had trouble controlling

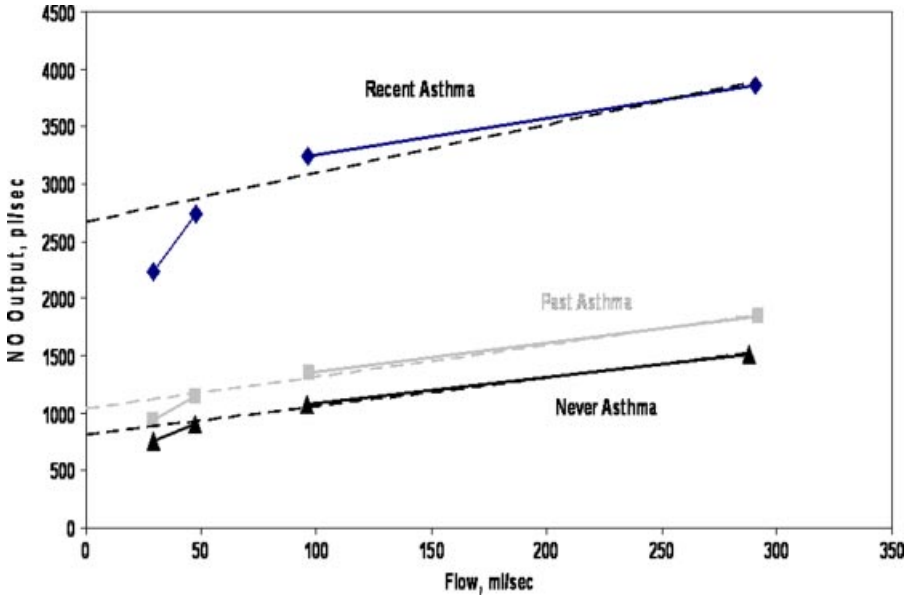

Fig. 1. Mean NO output (product of FeNO and flow) versus mean flow at each level, by asthma category: recent (N = 6), past (N = 9), or never (N = 50). Broken lines indicate usual Tsoukias (T) model fit from 50–300 ml/sec to estimate intercept = JawT and slope = CalvT. Solid lines indicate separate slopes for 30–50 and 100–300 ml/sec segments; their difference (SLPDIF) is taken as an index of nonlinearity.

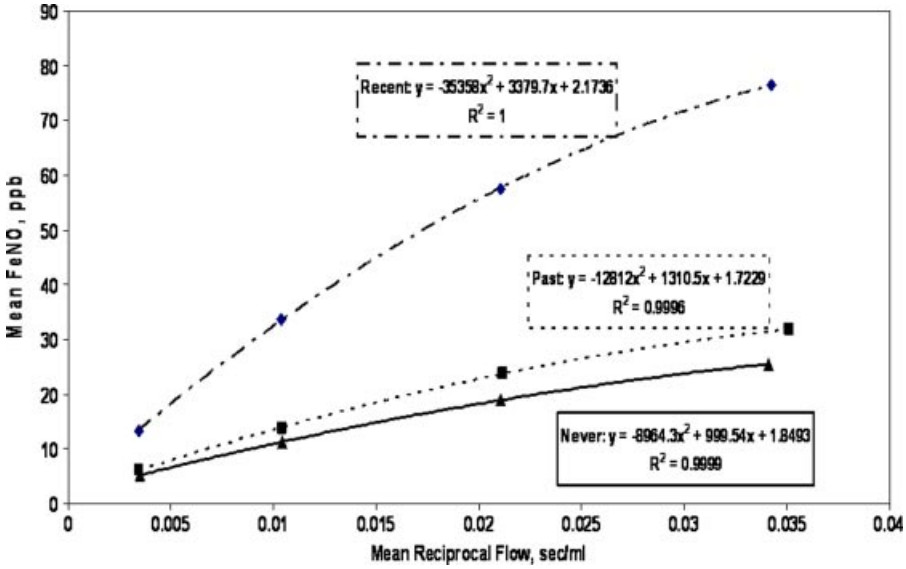

Fig. 2. Mean FeNO and reciprocal flow at each level, and quadratic regression function (model Q), by asthma category: recent (N = 6), past (N = 9), or never (N = 50).

flow at low levels because of an instrument problem, eventually diagnosed as a valve leak in an ambient NO scrubber. Although plateaus were definable in their tracings, some extended NO variables showed significant differences between these children and others, so their data were excluded. Of the remaining 65 children, 6 had no ATS-acceptable tracings at 30 ml/sec and 2 others had none at 300 ml/sec. Their plateau NO concentrations were estimated by visual inspection of plots. Table 1 summarizes characteristics of these 65 subjects included in the analysis.

TABLE 1— Subject Characteristics

	Number	%	
Gender			
Female	28	43.1	
Male	37	56.9	
Ethnicity			
Non-Hispanic white	39	60.0	
Hispanic	21	32.3	
Asian	4	6.2	
Not reported	1	1.5	
Allergy status	Asthma status	Number	%
Recent	Recent	3	4.6
	Past	3	4.6
	Never	18	27.7
Past	Recent	2	3.1
	Past	4	6.2
	Never	18	27.7
Never	Recent	1	1.5
	Past	2	3.1
	Never	14	21.5
	Range	Mean	SD
Age (years)	10.9–13.0	12.0	0.6

Relationships of Extended NO Variables

Table 2 shows summary statistics for JawNO estimates from each model. Estimates varied between models and were higher from nonlinear than from linear models. Despite numerical differences, JawNO estimates from all models were strongly correlated with each other ($r \geq 0.95$). Table 3 presents summary statistics, including correlation coefficients, for CalvNO. The linear estimates CalvP and CalvT were numerically similar and highly correlated ($r = 0.92$). Nonlinear-model estimates generally differed from those based on linear models: the nonlinear estimates were usually lower, and were less than zero for a few individuals. Estimates of CalvNO from different nonlinear models also differed, with some exceptions: the two models that addressed axial diffusion, C and K, were moderately correlated with each other ($r = 0.68$); while the three nonlinear models that did not address axial diffusion, S, H, and Q, also showed moderate correlations among themselves (r between 0.56 and 0.84).

The two alternative indices of nonlinearity, SLPDIF, and the quadratic coefficient from model Q, were highly negatively correlated ($r = -0.95$), and so were considered to give essentially the same information. SLPDIF, being simpler to calculate, was preferred.

Table 4 shows, for each model, pairwise correlations of CalvNO and JawNO estimates with each other, with conventional FeNO at 50 ml/sec, and with SLPDIF. All estimates of JawNO were very highly correlated with conventional FeNO. With the linear models, CalvNO

TABLE 2—Summary Statistics and Correlations for JawNO (Proximal Airway NO Flux in pl/sec) Estimated by Different Models for 65 Subjects With Valid Data

Variable	Mean	Std dev.	Minimum	Maximum		
Linear models						
JawP	969	1,032	204	5,060		
JawT	1,007	1,079	220	5,393		
Nonlinear models						
JawS	1,418	1,619	244	9,656		
JawH	1,292	1,409	174	7,939		
JawC	1,838	1,983	393	10,108		
JawK	969	1,032	204	5,060		
Empirical quadratic model						
JawQ	1,266	1,420	82	7,867		
	JawT	JawS	JawH	JawC	JawK	JawQ
Pearson correlation coefficients (all significant, $P < 0.0001$)						
JawP	0.998	0.957	0.950	0.998	1	0.981
JawT		0.958	0.960	0.999	0.998	0.983
JawS			0.978	0.961	0.957	0.987
JawH				0.961	0.950	0.972
JawC					0.998	0.984
JawK						0.982

correlated positively with FeNO or JawNO; but ~35% (by P model) to 45% (by T model) of the variance in distal NO concentration was independent of proximal airway NO flux. All models indicated that SLPDIF was positively correlated with JawNO and FeNO. That is, subjects with high proximal-airway NO also tended to be less uniform in their patterns of NO excretion with changing flow. However, about 40% of the variance in SLPDIF was independent of JawNO. Linear models also indicated a positive association between SLPDIF and CalvNO. By contrast, nonlinear models suggested that CalvNO was unrelated or negatively related to JawNO or SLPDIF.

Effects of Asthma/Allergy Status on Extended NO Variables

More than 70% of the subjects had some history of respiratory allergy, and more than 20% had received a physician diagnosis of asthma, but about half of those with a positive clinical history had no recent symptoms

TABLE 3—Summary Statistics and Correlations for CalvNO (Distal Airspace NO Concentration in ppb) Estimated by Different Models for 65 Subjects With Valid Data

Variable	Mean	Std dev.	Minimum	Maximum		
Linear models						
CalvP	3.05	1.70	1.30	11.10		
CalvT	2.68	1.22	1.29	8.23		
Nonlinear models						
CalvS	1.45	1.76	−6.15	7.83		
CalvH	1.98	1.16	−0.73	7.63		
CalvC	0.57	1.60	−6.32	2.66		
CalvK	1.36	1.10	−1.78	3.26		
Empirical quadratic model						
CalvQ	1.82	1.30	−2.15	7.84		
	CalvT	CalvS	CalvH	CalvC	CalvK	CalvQ
Pearson correlation coefficients						
CalvP	0.924**	−0.106	0.253	−0.461**	0.235	0.234
CalvT		0.046	0.559**	−0.288	0.218	0.326*
CalvS			0.603**	0.521**	0.370*	0.837**
CalvH				0.273	0.158	0.566**
CalvC					0.681**	0.392*
CalvK						0.528**

*$P < 0.01$.
**$P < 0.0001$.

TABLE 4— Correlations of JawNO and CalvNO From the Same Model With Each Other, With FeNO (50 ml/sec), and With SLPDIF

Model	JawNO vs. FeNO	CalvNO vs. FeNO	JawNO vs. CalvNO	JawNO vs. SLPDIF	CalvNO vs. SLPDIF
P	0.998**	0.826**	0.799**	0.764**	0.638**
T	0.996**	0.771**	0.737**	0.769**	0.530**
S	0.957**	−0.316	−0.547**	0.902**	−0.762**
H	0.955**	0.152	−0.069	0.863**	−0.246
C	0.998**	−0.838**	−0.858**	0.776**	−0.698**
K	0.998**	−0.353*	−0.396*	0.764**	−0.260
Q	0.982**	−0.081	−0.039	0.855**	−0.487**

*$P < 0.01$.
**$P < 0.0001$.

(Table 1). Asthma and allergy status showed consistent effects on JawNO estimated by different models; however, the effects of asthma and allergy status on CalvNO depended on the model used.

Figures 3 and 4 show estimated mean JawNO and CalvNO, respectively, for subjects with and without recent asthma or recent allergy. (The ''healthy'' category in Figures 3 and 4 thus includes individuals with past allergy or asthma). For every model, the main effect of recent asthma was positive and significant for JawNO ($P < 0.01$ by analysis of variance), while recent allergy had no significant main or interactive effect on JawNO. (More details of statistical results are reported in the online supplement, for this and subsequent analyses). For CalvNO, results from different models were inconsistent. Models that yielded a fairly strong correlation (positive or negative) between CalvNO and JawNO, that is, linear models P and T and nonlinear model C based on adjustments of T, again showed a significant effect of recent asthma. The nonlinear S model showed main and interactive effects of recent allergy, while the other nonlinear models showed no significant group differences.

Figures 5 and 6 show estimated mean JawNO and CalvNO, respectively, for subjects with and without a history of asthma or allergy, based on past as well as recent reports. By this classification, the combination of allergy history and asthma history appeared to be the most important positive influence on JawNO. For all models, the effect of asthma was not statistically significant, and the small group with asthma history but no allergy history (N = 3) averaged lower in JawNO than the healthy group. Subjects with history of allergy but no asthma averaged only slightly higher than healthy subjects, while those with history of allergy and asthma averaged markedly higher.

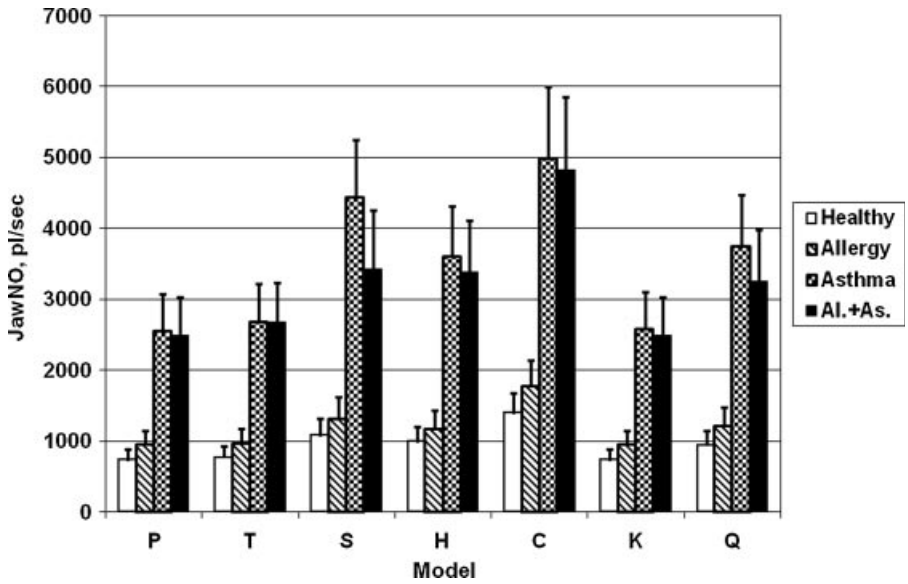

Fig. 3. Proximal-airway (bronchial) NO flux (JawNO) as estimated by each model: results from analysis of variance with grouping by *recent active* allergy or asthma. Column indicates mean, flag indicates standard error.

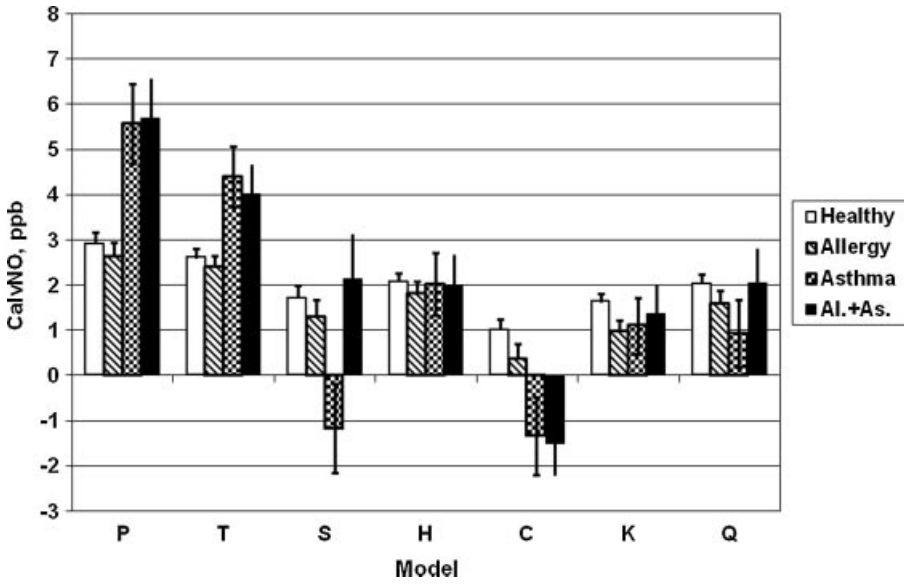

Fig. 4. Distal (alveolar) NO concentration (CalvNO) as estimated by each model, by *recent active* allergy or asthma, plotted in the manner of Figure 3.

By analysis of variance, both the allergy main effect and the allergy–asthma interaction were either significant ($P < 0.05$) or close to significance, depending on the model. Results for CalvNO were again inconsistent across extended eNO models. Most models indicated that the differences between groups defined by asthma/allergy history were not statistically significant. However, CalvC showed a significant ($P = 0.01$) main effect of allergy and a marginally significant ($P \sim 0.05$) allergy–asthma interaction—similar to JawC, but with changes in the opposite direction. That is, CalvNO as estimated by model C was decreased in subjects with allergy history compared to those without, and the estimated decrease was larger in those with a history of both asthma and allergy.

Figure 7 shows estimated mean SLPDIF by allergy/asthma history and by recent allergy/asthma. The pattern of variation in SLPDIF between clinical categories roughly resembled that for JawNO or for linear-model estimates of CalvNO. Recent asthma without recent allergy was associated with unusually high mean SLPDIF, but this difference was based on only three subjects.

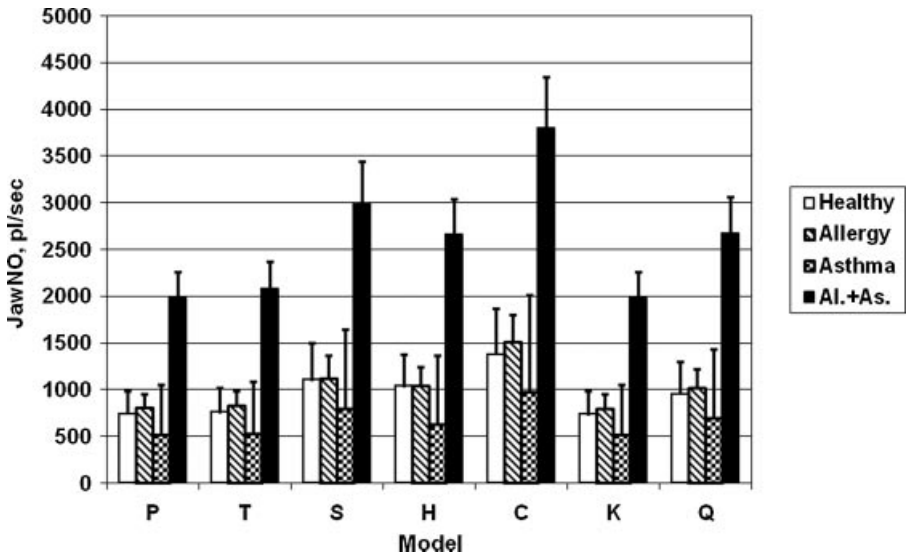

Fig. 5. Proximal-airway NO flux (JawNO) as estimated by each model: results from analysis of variance with grouping by *history of* allergy or asthma. Column indicates mean, flag indicates standard error.

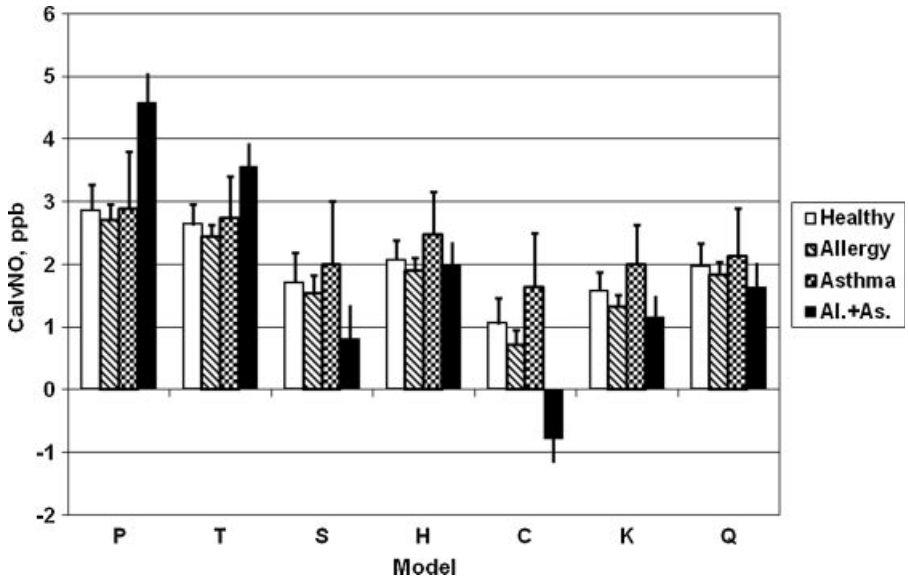

Fig. 6. Distal (alveolar) NO concentration as estimated by each model, by *history of* allergy or asthma, plotted in the manner of Figure 5.

DISCUSSION

We found that field testing using extended eNO protocols was feasible in most children. In children aged 10–11 years, 30–300 ml/sec appears to be the maximum practical flow range for tests. A few performance failures may be expected at either extreme, but most children probably can generate usable data for at least three different flows. The performance of the NO analyzers does not appear to be a limiting factor in large-scale field surveys.

Estimates of JawNO from different models did not agree numerically, but showed similar ability to discriminate among allergy and asthma groupings in our population. The relative importance of asthma and respiratory allergy effects differed depending on whether the classification was based on any history or on recent clinical manifestations. That is not surprising, in that past

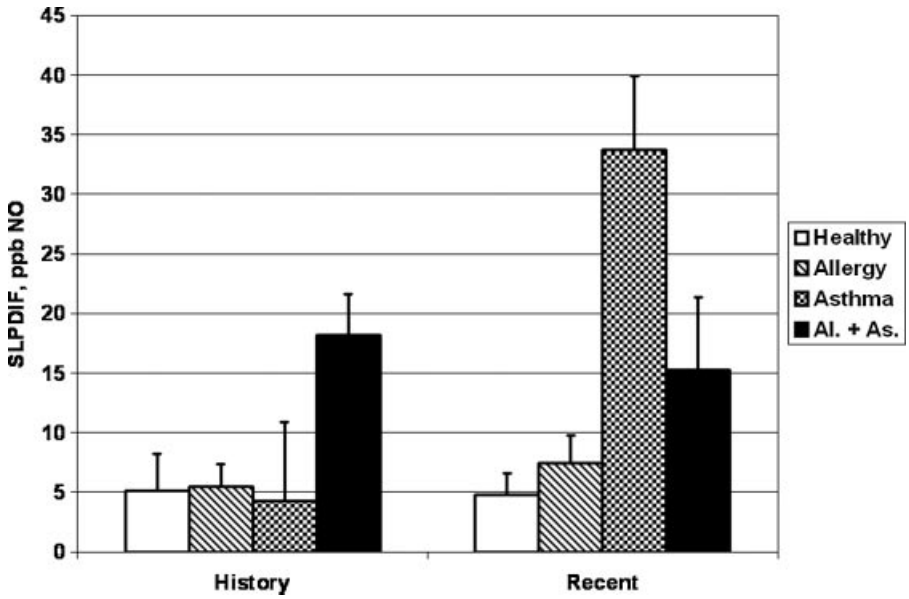

Fig. 7. Variable SLPDIF (difference in T-model slope at 30–50 ml/sec vs. 100–300 ml/sec flow): results from analysis of variance with grouping by history of allergy or asthma (left) or by recent allergy or asthma (right). Column indicates mean, flag indicates standard error.

studies often have disagreed on the relative importance of atopy versus asthma in relationship to increased conventional FeNO, although both are clearly important judging from the overall evidence. All of the models produce highly correlated estimates of JawNO, so the choice of the best model to use to estimate extended eNO parameters should be based on other factors.

CalvNO estimates from different models showed considerable variation. If models were categorized according to similar underlying physiologic assumptions or mathematical properties, results for CalvNO showed some consistency within categories, but large inconsistencies between categories. Individual estimates from the simple linear models—CalvP or CalvT—were the most physiologically plausible, ranging from slightly above 1 ppb in some subjects with low FeNO, to near 10 ppb in some subjects with high FeNO. By contrast, all nonlinear models estimated some individuals' distal NO concentrations as close to or less than zero. Near-zero estimates of distal airspace NO concentration, including negative point estimates with positive upper confidence limits, are not inherently implausible. Pulmonary circulation is an essentially infinite sink for NO,[8,13] which has high affinity for hemoglobin, so NO concentrations near alveolar-capillary interfaces should approach zero. Elsewhere in distal airspaces, concentrations during controlled exhalation would vary according to a complex interaction among in situ NO release, convective flow toward the mouth, and diffusion toward pulmonary capillaries. At low expiratory flows, the diffusion effect should be disproportionately large in subjects with steeper concentration gradients (i.e., higher airway NO concentrations), who might therefore exhibit greater nonlinearity. Different models, with different simplifying assumptions concerning distal and proximal NO behavior, applied to relatively few data measured at the mouth, likely would yield different estimates of a composite distal concentration. Thus, the inconsistency of CalvNO results seen here is not surprising. Assessment of these models using larger data sets will be necessary to determine which models have the best performance. Given the nature of the lung and the various model assumptions, the best performing model may well differ between relatively healthy populations like ours, and clinic-based populations including severe asthmatics.

Another potentially important limitation on the interpretation of any model results is that any differences between laboratories in instrument response, or in flow or NO calibrations, will have relatively large effects at either end of the range, where the lowest flow or the lowest concentration is measured. Inter-laboratory differences are inevitable, given the limits of precision in instrument response and in calibration standards. Thus, instrument-related factors and variation in calibration standards might shift results appreciably in either direction, in one study relative to another.

Based on our results, the parameters currently most useful for describing individual and group extended eNO characteristics are the linear-model parameters CalvT and JawT (estimated from 50, 100, and 300 ml/sec exhalations), plus SLPDIF as an index of nonlinearity, that is, failure to fit the T linear model. In our study, all these variables could detect inter-individual differences relatable to the degree of airway inflammation as inferred from other evidence. Each variable conveyed information about NO at some level of the respiratory tract, not determinable from the other two variables. Based on extensive prior evidence, JawNO reflects the inflammatory state of relatively large proximal airways, and CalvNO that of more distal airways. Specific physiologic interpretation of SLPDIF or alternative indices of nonlinearity must await further investigation. However, the association of nonlinearity with respiratory dysfunction is clear in the present results and in previous findings.[11]

The current protocol with exhalations at 30, 50, 100, and 300 ml/sec is feasible and appears satisfactory for epidemiologic as well as clinical testing. Without requiring excessive subject effort or technician time, it provides a range of data sufficient for extended NO analysis using various analytical models, and allows for comparison of results against those from other published extended NO protocols. Extended NO analysis holds promise for use in investigations of determinants of regional airway inflammatory state and responses to environmental exposures.

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