Author

Richard Taylor started working in Professor George's lab in Spring, 2008, which he describes as the opportunity to work "on an innovative approach to diagnosing asthma, on a team bringing their unique talents and experiences together to solve a common problem." He found it particularly rewarding to be able to contribute practical results to a problem that is so widespread and poorly understood. Upon graduating from UCI, Richard hopes to make a career of research, pursuing a Ph.D., and eventually working and teaching at a research institution.

Determining the Optimal Sampling Interval of the Exhaled Nitric Oxide Profile

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Abstract

Nitric oxide (NO) is present in exhaled breath after being produced by cells throughout the lungs. NO is affiliated with inflammation, including pulmonary inflammatory diseases such as asthma. The widespread and growing presence of asthma highlights the need for improved diagnosis and treatment methodologies; the affiliation between NO and inflammation gives rise to the possibility that NO measurements could be used as a clinical tool in the diagnosis and treatment of inflammatory pulmonary disease. As one step toward this goal, this study sought to discover the most reliable interval of the exhaled NO signal for analysis. Exhaled NO measurements of 51 patients aged 7–16 years with mild to moderate asthma were collected at the Breathmobile operated by the Children's Hospital of Orange County. Exhaled volume was measured relative to each subject's airway volume and collected at flows of 50, 100, and 200 ml/s. The volume of exhaled breath was normalized relative to the volume of the airway tree. The data shows that the clearest and most reliable interval of the exhaled breath on which to measure nitric oxide is from four to six airway volumes. This information will enable more reliable use of exhaled nitric oxide, ultimately enabling more accurate asthma diagnosis and treatment decisions for the pediatric population.

Faculty Mentor

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'awNO (nl/s)] and distal airway/alveolar contributions [CANO (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. This project demonstrates that when using the multiple flow technique to partition eNO, the method of analysis (constant time versus constant volume interval) significantly affects the estimation of CANO, and thus potentially the assessment and interpretation of distal lung inflammation.

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Key Terms

- Asthma
- Multi-Compartment Model
- Nitric Oxide
- Pulmonary Inflammation
- Sampling Interval

Introduction and Background

The Problem: Asthma

Asthma is a pulmonary inflammatory disease characterized by episodes of bronchocontriction and airway hyperresponsiveness (Puckett, 2008). Left untreated, asthma can result in airway wall remodeling and ultimately in fixed airflow obstruction. Clinical symptoms include wheezing, dyspnea, chest tightness, and cough, and are triggered primarily by exercise, emotional distress, allergies, air pollutants, and viral infection (Kumar, 2005). At its worst, asthma can culminate in Status Asthmaticus, an attack lasting days to weeks and possibly resulting in death. Also, asthma-induced inflammation is normally treated with inhaled corticosteroids, which have significant problematic side effects, and bronchodilators such as a β2 agonist. Current diagnostic methods are limited to spirometry and symptom analysis; these can be particularly limiting in young children, who may have trouble performing spirometry or describing symptoms. Furthermore, spirometry can be normal in asthmatic children. These methods are particularly unacceptable given the scope of asthma: approximately 20 million Americans suffer from asthma; worldwide, the number of people with asthma is growing, particularly in industrial nations that produce asthma-triggering air-pollutants (Beasley, 2004). Symptoms and spirometry, meanwhile, do not correlate well with airway inflammation (Lious, 2000, Wilson, 2000, and van dem Toom, 2001). This is a problem because patients can subsequently receive under- or over-treatment. Therefore, treatment decisions can be improved by developing a diagnosis that overcomes these shortcomings and is based upon a more reliable marker of inflammation.

A Potential Tool: Nitric Oxide

Nitric oxide (NO) is a mediator of pulmonary inflammatory processes (Kobzik, 1993). Nitric oxide synthase (NOS) produces NO throughout the pulmonary system by the oxidative conversation of L-arginase. There are three known NOS isoforms, which are expressed in the airway epithelial cells and the bronchial and alveolar epithelium. NO functions as an endogenous messenger in the lungs, influencing such systems as smooth muscle tone, ciliary function, and, most relevant to this research project, acting as a natural bronchodilator (Moncada, 1991). NO therefore increases as inflammation increases (*e.g.* during asthma) and decreases when the inflammation decreases. Therefore, the ability to measure and characterize quantity, location, and rate of NO production throughout the pulmonary system may be an effective tool in asthma diagnosis, which may improve treatment decisions and enable an earlier, more accurate diagnosis, particularly in children.

Complications

Exhaled NO was first detected in 1991 (Gustaffson, 1991). Early studies noted an association between asthma and exhaled NO levels, concluding that NO could be used as an indicator of inflammation (Kharitonov, 1994). More recent investigations, two on adults and two on children, attempted appropriate corticosteroid delivery based on exhaled nitric oxide, but perceived only marginal benefits in adults and none in children (Petsky 2008). Still more studies questioned the utility of exhaled NO for asthma diagnosis, concluding that measurements of elevated NO could not be used to distinguish between asthmatic and atopic symptoms (Prasad, 2006). Furthermore, NO measurements have proven difficult to correlate with other analyses. Such challenges demand clarification in order to accurately understand the utility of exhaled NO.

Solutions

The George Lab at UCI has sought a way past these difficulties by considering how exhaled NO measurements can be used to study not only the pulmonary system as a whole, but the distinct airway and alveolar regions. Both regions are subject to inflammation; therefore, both regions produce NO. The relative rate at which each region produces NO associated with asthma-induced inflammation has not been well assessed. Two patients with identical exhaled NO concentrations could have different NO distributions between the alveolar and airway regions. Furthermore, because the majority of NO originates in the airways, alveolar inflammation is particularly obscured. The ability to make the distinction is fundamental to correctly identifying the location of inflammation and, subsequently, to treating its source.

The Multi-Compartment Model

To overcome these challenges, Dr. Steven George developed the "two-compartment model of pulmonary nitric oxide exchange dynamics," which is shown in Figure 1 (Tsoukias, 1998). In essence, the contributions of each region (compartment) are dependent upon the rate of exhalation, that is, on the exhalation flow. Measurements taken at different flows are used to identify the distinct contributions of the airway and alveolar regions. This technique has now seen significant use (Condorelli, 2007; Shin, 2005; Tsoukias, 2001) and revealed "potential physiological and clinical significance" (Berry, 2005; Brindicci, 2007; Girgis, 2002; Lehtimaki, 2005; Roy, 2001; Shin, 2004), especially in children (Linkosalo, 2007; Mahut, 2004; Paraskakis, 2006).

The model has been further improved to consider the trumpet shape of the airway tree, as well as axial diffusion of nitric oxide. The former takes into account increasing

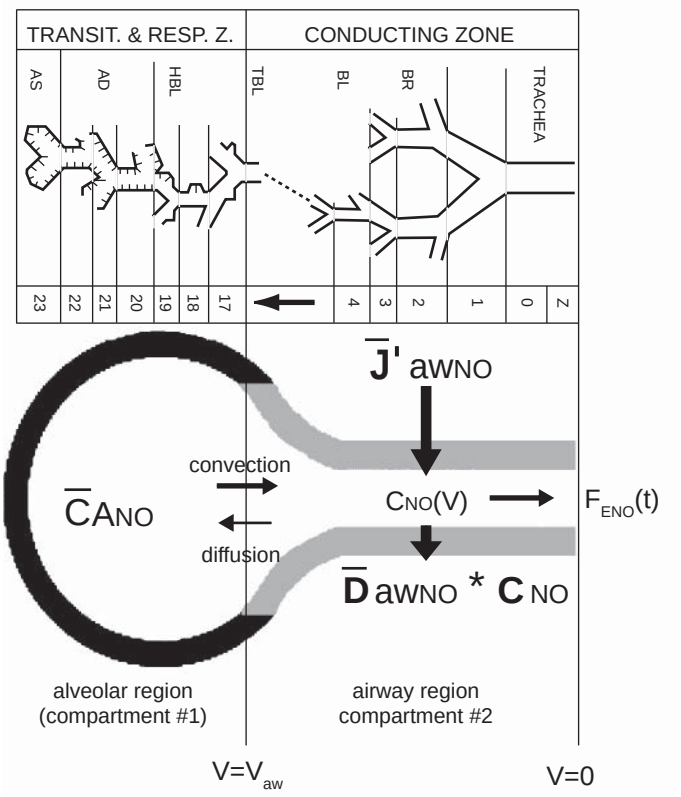

Figure 1

Alveolar and airway regions of the pulmonary system. Nitric oxide originates from both regions.

surface area per unit volume, while the latter considers NO diffusion from the airways back towards the alveoli.

This model influences both what we measure and how we interpret the measurements. The NO signal is simply a plot of time or volume against NO count; the challenge is in gathering the greatest possible amount of useful information. Variation among clinically similar subjects renders the plots particularly difficult to read. Therefore, a next step in overcoming these complications is determining which interval of the exhalation profile yields the most information.

Previous NO Collection Guidelines

Previous studies' frustrations are likely due in part to inadequate NO collection methodologies. Current guidelines regarding NO collection were set by the American Thoracic Society (ATS) and the European Respiratory Society (3) in 2005. The guidelines seek to establish a standard with high reproducibility, yet that very goal may have undermined the utility of NO.

First, exhalation pressure must be greater than five centimeters of water to close the soft palate and avoid contamination of the sample by NO from the nasal cavities, specifically, the paranasal sinuses (Little, 2000). Second, exhalation is to be held constant, sustained at least 4 seconds for children younger than 12 years old, or 6 seconds for subjects older than 12. Given these conditions, NO is recorded as the mean concentration over a three second window for which no point on the interval varies by more than 10% from either endpoint. The result is the fractional concentration of exhaled NO, or FENO. The ATS specifically notes that the first three-second interval meeting this criterion is acceptable and that the exhalation maneuver may subsequently be discontinued.

The ATS recommends taking into account such factors as breath hold, age, eating and drinking, ambient NO, circadian rhythm, and medications taken, but does not provide specific guidance on these issues. Indeed, ATS standards are far from problem-free. First, by measuring at only one flow, it is impossible to differentiate the signal into airway and alveolar components. Furthermore, intrasubject reproducibility of this system is low, with coefficients of variation between 7–26% (5, 8, 12, 20, 26); the number of tests that failed ATS standards—a crucial number when considering the effectiveness of a system—was not reported. Next, the motivation for the 10% standard is unclear in the guidelines as published by the ATS (3). Finally, given that FENO is positively correlated with height (17), ATS guidelines do not sufficiently take into account variation among subject body size, making trends unreliable.

Methods

Subjects

This study sought to determine the optimal sampling interval of the exhaled nitric oxide profile of asthmatic children, not to quantify the subjects' asthma. The conclusions are based upon asthmatic NO profiles, not healthy profiles. The patient population is 51 children with mild to moderate asthma. Measurements were collected on the Breathmobile run by the Children's Hospital of Orange County, directed by Dr. Stanley Galant. The Breathmobile is a vehicle equipped for diagnosis and therapy of asthmatic children, rotating among four free clinics in underserved areas of Orange County. Demographics for this study are presented in Table 1.

Table 1

Demographics

Age (years)	
Min	7
Max	16
Average	10
Ethnicity (%)	
Hispanic	94
Caucasian	6
Gender (%)	
Male	68
Female	32

Experimental Procedures

We measured NO as a function of time and volume. Each measurement is of a vital capacity maneuver, a single exhalation from total lung capacity (TLC) to residual volume (RV). This measurement was repeated three times each at flows of 50, 100, and 200 ml/s. Data was collected with the NIOX analyzer. We also collected background information on each patient, including age, weight, asthma history, current medications, atopic status, symptoms, and spirometry. Patients were excluded from the study if they had cardiopulmonary disease, had greater than five pack-years of smoking, had any smoking in the previous five years, or had taken asthma control medication within the eight weeks prior to the Breathmobile visit.

Analytical Technique

Given the wide demographic range of the population with regard to age and weight, we normalized expiration volume (Vex) relative to each patient's airway volume (Vaw). The latter can be estimated, in milliliters, as the sum of the patient's age in years and weight in pounds (Condorelli, 2007). Thus by graphing NO concentration (ppb) against airway volume intervals (Vex/Vaw, unitless), we obtained a plot for each exhalation maneuver that can be easily compared with other subjects regardless of age or weight. Experience revealed that the upper limit on the interval needed to be six airway volumes, given that many patients in this age bracket could not exhale beyond that cutoff at an exhalation flow of 50 ml/s. Therefore, subjects whose vital capacity maneuvers did not reach six airway volumes were excluded from the study. While the plots vary dramatically from one to the next, a typical plot is characterized by a "washout" region as airway NO is expired, followed by a relative plateau which may have a positive, zero, or negative slope (Figure 2).

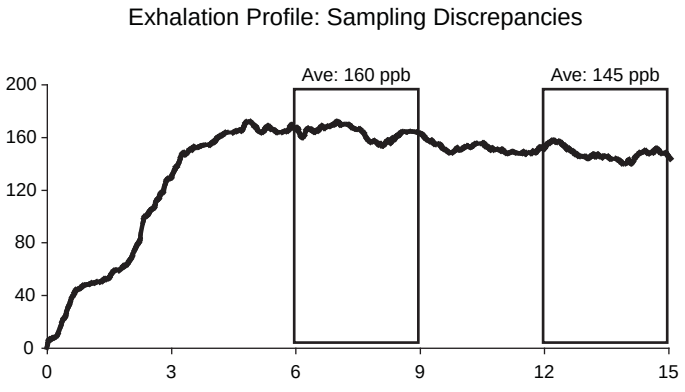

Figure 2

Measured NO quantity is highly dependent upon the sampling interval of the exhaled profile.

Note the difficulty of identifying a true plateau—the ATS standard. Given the shape of the graph, finding a three-second window with less than ten percent variation would be difficult; once achieved, there is little evidence to prove the window to be representative of the subject's NO status. For instance, a sampling between six and nine seconds would have a drastically different average and slope than a sampling between twelve and fifteen seconds.

Volume of nitric oxide was calculated as the product of flow (mL/s) and NO count (ppb, equivalent to nL/L) and is denoted VNO (pl/s), also identified as the "elimination rate" of NO.

To help identify the optimal window of analysis, we calculated the distribution of NO between the alveolar and airway regions using the multi-compartment model previously discussed. This information allows for a more accurate picture of pulmonary NO production; from there we can choose the exhalation interval that best reflects physiological conditions. The slope of VNO plotted against exhalation flow is equivalent to mean alveolar concentration (CANO). The y-intercept is the mean maximum airway flux (J'awNO). These values are then modified to take into account axial diffusion (AD) as follows:

Slope (with AD) = Slope (without AD) – (Intercept / 530)
and
Intercept (with AD) = Intercept (without AD) * 1.9

For detailed determination of these equations, see Condorelli, 2007.

Results

Results vary noticeably on an individual basis, particularly in the slope and variability of the NO plateau at each flow. A sample individual subject is shown in Figure 3.

Note first the sharp initial rise in NO. The first air sampled comes from the mouth, followed by air that has passed through the mouth and airways, followed by air that has passed through the mouth, airways, and alveoli. As volume is normalized relative to the airway volume, it would seem in theory that the signal should plateau at one airway volume; in practice, as seen on the graph, the signal does not peak or stabilize until approximately three to four airway volumes.

Next, note the plateau, which reveals relative stability of the NO signal. As a result, the optimal sampling interval will come from the plateau region. ATS guidelines do not

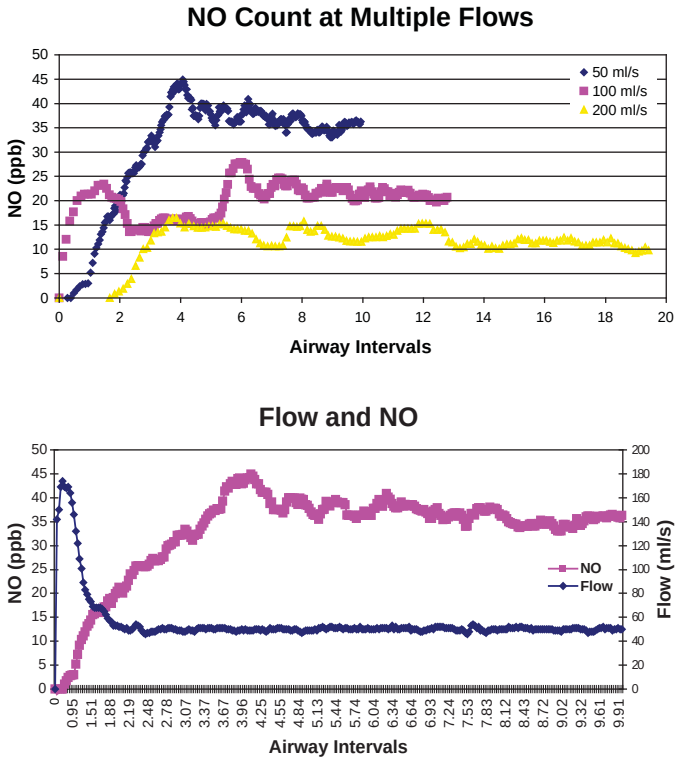

Figure 3

Individual sample. For each flow, note (1) a sharp initial rise, (2) the relative plateau region, (3) the number of aiway intervals exhaled at each flow, given that NO was collected for the same length of time independent of flow, (4) a relatively variable signal (for instance, at 200 ml/s the signal during the "plateau" varies by more than 5 ppb within one exhalation interval), and (5) the flow-dependence of the NO count.

specify where within the plateau is optimal. However, given the variability of the slope of the plateau, the location will greatly impact the output.

Third, while a patient may exhale the same quantity of air for each maneuver, the number of intervals exhaled approximately doubles between the 50 ml/s and 100 ml/s maneuvers. As the multi-compartment model requires measurements of NO at multiple flows at the same exhalation interval, the optimal sampling interval must overlap all three maneuvers. The majority of patients were not able to exhale beyond six intervals at 50 ml/s. Therefore, to maintain use of the multi-compartment model, the optimal sampling interval should not extent beyond six airway volumes.

Note also the variability of the signal. This is a primary challenge in selecting the optimal sampling interval. Given that NO signal variability is greater than flow variability, NO variability is not simply attributable to variations in flow, but rather from other physiological conditions.

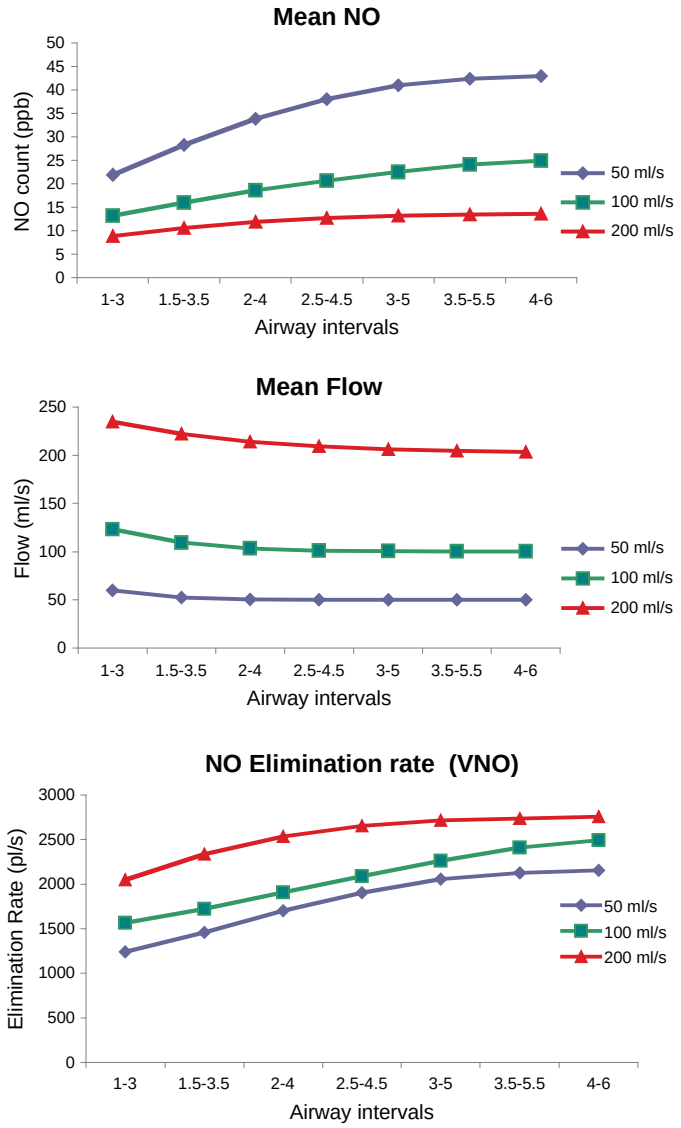

Figure 4

Population averages of NO, flow, and VNO. Note that changes from one airway interval to the next decrease at higher intervals.

Fifth and finally, NO count increases with decreasing flow. This is expected, because NO has more time to accumulate in air that is moving more slowly. Flow-dependence will be used to consider alveolar and airway contributions, which in turn will be considered when selecting the optimal sampling interval.

Thus, several properties of the optimal sampling interval become clear by examining this individual subject. The interval must: (1) begin late enough to establish a plateau, (2) end early enough to include all three flows, and (3) be longer than the NO signal undulations. These traits are next examined for the entire population of this study.

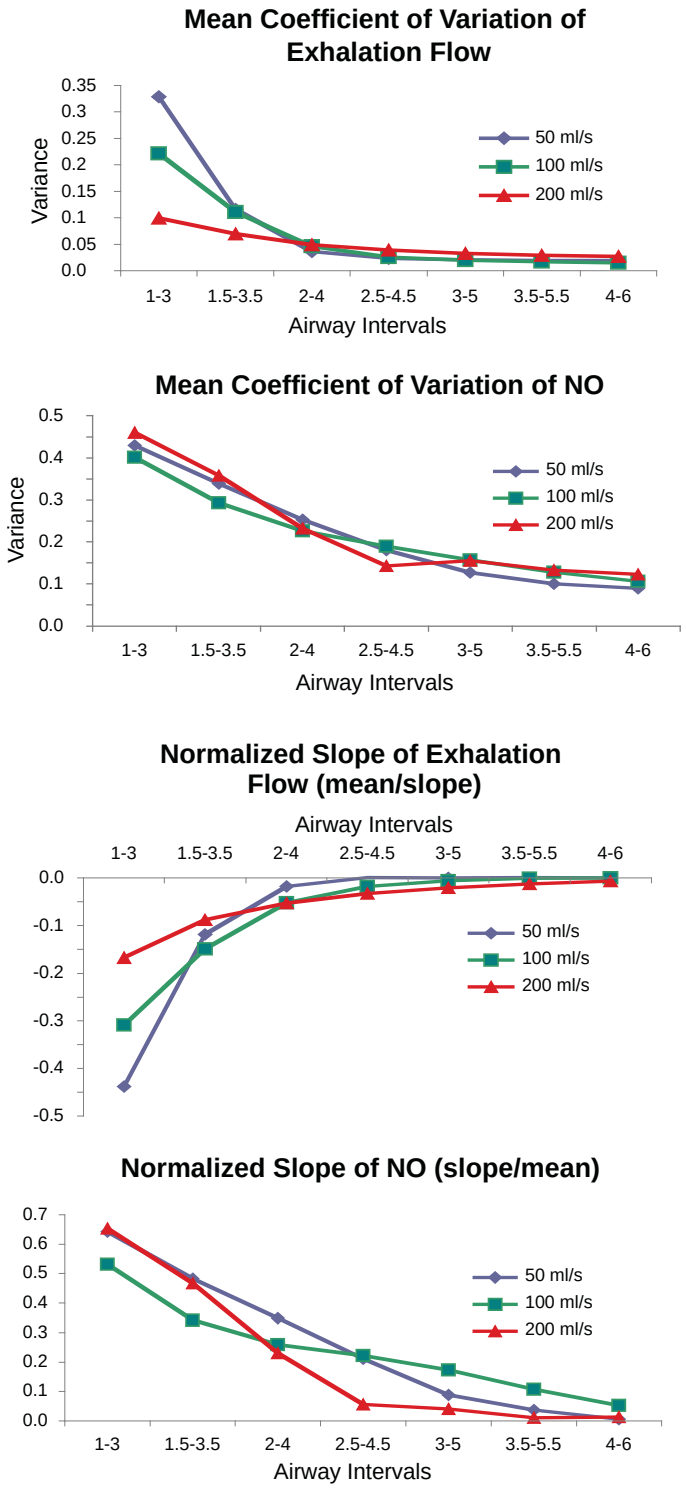

Figure 5

Means and Variance of NO and flow

We chose to sample intervals of two airway volumes each, increasing by half-airway volume increments (1–3, 1.5–3.5, etc.). This decision was based upon the above criteria: an interval long enough to take into account the undulations of the NO signal, but short enough to reflect a specific location on the signal.

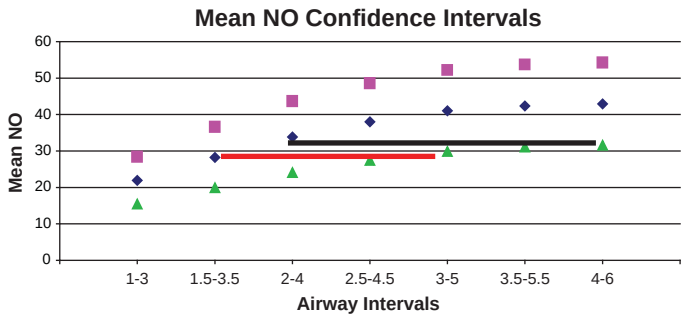

Figure 6

Mean NO confidence intervals. Note that the mean NO at 2.5 airway volumes is outside the confidence intervals of later airway volumes (3 onward).

Figure 4 shows average NO, Flow, and VNO for all 51 subjects. All graphs have sampling interval as the independent variable. Slopes decrease with increasing airway volume. This suggests that the optimal sampling interval is towards the end of the exhalation maneuvers. The VNO graph is particularly indicative of this result, as all three flows show a notable slope change.

Figure 5 provides further evidence that the optimal sampling interval is at the end of the exhalation maneuver. Both exhalation flow and NO signal stabilize, as indicated by the decrease in mean coefficient of variation. Such stabilization is important to the reliability of the measurements, and essential to an accurate diagnosis. Similarly, the slope of both flow and NO approach zero with an increasing number of exhaled airway intervals.

Figure 6 is a statistical analysis based upon mean NO confidence intervals. Crucially, it reveals that the mean NO of the 1–3 and 1.5–3.5 airway intervals is outside one confidence interval of the airway intervals beginning at three airway volumes. A clinically relevant sampling interval cannot have such a discrepancy; therefore, an approved sampling interval must begin no earlier than three airway intervals. Thus, all of the evidence points to using four to six airway volumes as optimal. The ideal sampling interval will begin as late as possible, given the trend for the NO signal to plateau.

Figure 7 demonstrates that the location of the analysis window makes a significant impact on the NO measurement. Figure 7.a shows the elimination rate (VNO) of NO plotted against flow. Each line represents one airway interval. Higher intervals have higher VNOs. The graph provides evidence

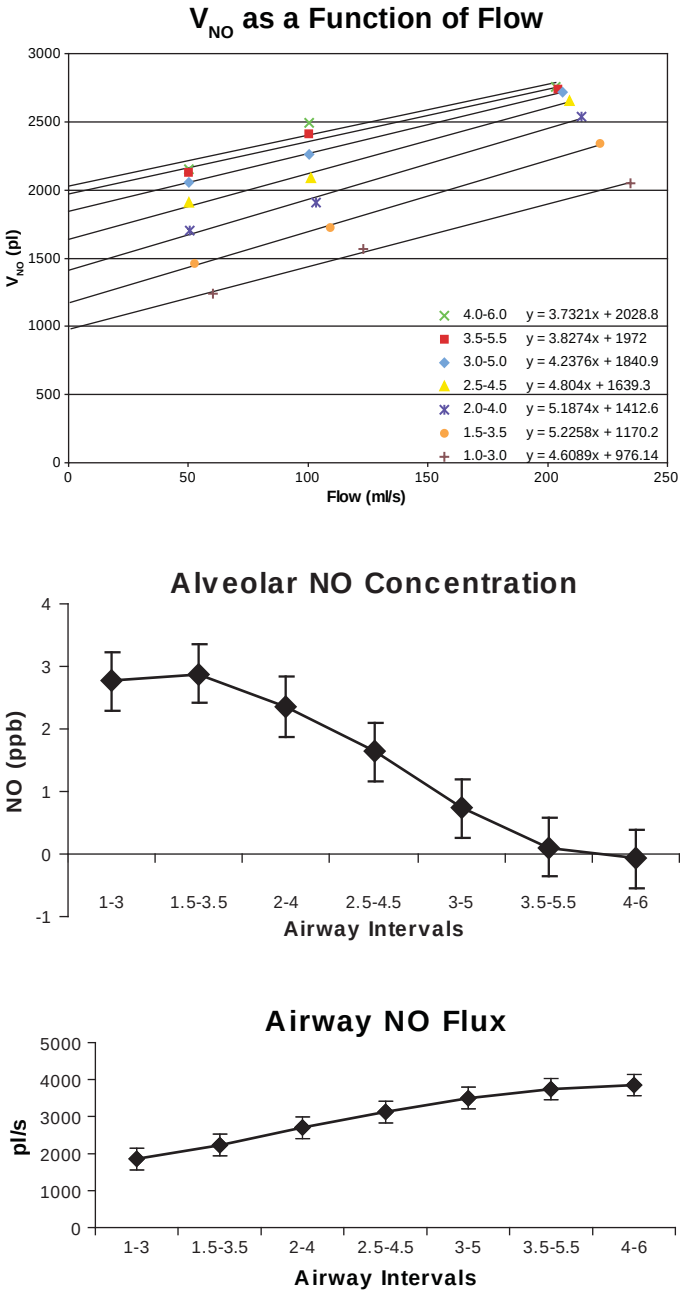

Figure 7

(a) VNO as a function of flow. (b) Mean alveolar concentration, equal to the slope of graph (a). (c) Mean airway NO flux, equivalent to the y-intercept of graph (a).

for increasing stability and reliability with increasing intervals, given that each plot is progressively closer to the previous plot. While the trend on the graph suggests looking towards even higher exhalation intervals, it has already been shown for the population surveyed in this study that the interval must be capped at six airway volumes.

The graph reveals the importance of interval selection for accurate NO analysis. Recall that under this model, when VNO is plotted against flow, the slope is used to estimate alveolar concentration, CaNO, and the y-intercept used to find mean maximum airway flux, J'awNO. Therefore, graphs 7.b and 7.c show alveolar concentration and airway flux plotted against airway interval. They highlight the influence of the sampling interval on the distribution of NO between the alveoli and the airways.

Discussion and Conclusion

Sampling NO on the exhaled breath between four and six airway volumes can provide more accurate characterization of the NO produced by the lungs as compared with results obtained using the previous analytic standards set by the American Thoracic Society. The notable variance of NO and flow for the pediatric population helped reveal the inherent challenges of NO sampling for asthma diagnosis. Nonetheless, using nitric oxide as a marker of inflammation will hopefully provide more accurate diagnoses than those dependent on spirometry and symptoms. This study helped develop a clinically applicable NO-based asthma diagnostic by quantifying NO variability and developing a method to work within this variability. This is only one step, but it is a crucial one, and hopefully will aid future research aimed at treating pulmonary inflammatory disease.

The results of the study can be applied only to persons reflecting the population under study, namely, children ages seven to sixteen with mild to moderate asthma and without such complicating factors as other major health issues. It is possible that patients of a particular physiology or with a particularly severe pulmonary condition may not be able to complete the exhalation maneuvers through six airway volumes. Future studies should therefore include adult patients and those with severe asthma. Similarly, such study would be an excellent opportunity to consider other trends possibly related to asthma such as age, gender, weight, and ethnicity, as well as asthma-specific issues including atopic status and extent of prior treatments such as bronchodilators.

Acknowledgements

The author thanks the George Lab, particularly Dr. Steven George and James "Lee" Puckett of the UC Irvine Biomedical Engineering department, without whom this study would not have been possible, and towards whom he is extremely grateful for their guidance and patience. The author also thanks the Undergraduate Research Opportunities Program. Finally the author is ever grateful to his family and friends for their support.

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