

## Exhaled single-breath nitric oxide measurements are reproducible, repeatable and reflect levels of nitric oxide found in the lower airways

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*Exhaled single-breath nitric oxide measurements are reproducible, repeatable and reflect levels of nitric oxide found in the lower airways. E. Gabbay, A.J. Fisher, T. Small, A.J. Leonard, P.A. Corris. ©ERS Journals Ltd 1998.*

**ABSTRACT:** Measurement of exhaled nitric oxide (NO) may allow noninvasive assessment of inflammatory disease in the lung. We determined immediate and day-to-day reproducibility of single-breath NO measurements at different points on the exhaled test, and whether levels recorded reflect levels of NO in the lower airways.

Using a rapid chemiluminescence analyser, 55 healthy control subjects performed three sequential tests on each of two days. NO levels were compared at the level corresponding with: 1) the time the mouth pressure fell below 4 cmH<sub>2</sub>O (MP); 2) the plateau of end-exhaled CO<sub>2</sub> (CO<sub>2</sub>); and 3) the NO plateau (NO<sub>p</sub>). NO levels were measured directly from the lower airways of 15 lung transplant recipients and compared with NO levels from a single-breath test performed in the same cohort.

For measurements performed at MP, CO<sub>2</sub> and NO<sub>p</sub>, the mean±SD differences between the two closest levels performed on the same day were 0.11±0.18, 0.095±0.16 and 0.094±0.13 parts per billion (ppb), respectively, and between days were 0.18±0.76, 0.19±0.78 and 0.17±0.8 ppb, respectively. End-expiratory levels recorded at the mouth from a single-breath test and in the lower airways were highly correlated (mouth *versus* trachea r<sup>2</sup>=0.95, p<0.0001, mouth *versus* bronchus r<sup>2</sup>=0.92, p<0.0001).

Single-breath exhaled nitric oxide levels are a simple, reproducible and valid measure of nitric oxide production from the lower respiratory tract.

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Since the observations of FURCHGOTT and ZAWADSKI [1] that an endothelium derived relaxant factor (EDRF) is required for acetylcholine to exert its vasodilatory effects, and the subsequent identification of EDRF as nitric oxide (NO) [2], the biological significance of this ubiquitous free radical has become increasingly appreciated. In addition to being a potent vasodilator, NO is involved in the regulation of vascular smooth muscle proliferation [3], acts as a neurotransmitter [4], mediates acute inflammation [5] and is an important factor in host defence [6].

When in gaseous phase, NO is relatively stable and highly diffusible [7], so that NO produced in the lung can be measured in exhaled air. Given its biological importance, many authors have postulated that measurement of exhaled NO may allow noninvasive assessment of inflammatory disease in the lung and bronchial tree [8-10] as well as in pulmonary vascular disease [11]. Exhaled NO concentrations are reported to be increased in asthma [8, 9], bronchiectasis [10] and during upper respiratory tract infections in normals [12]. When asthmatics are treated with inhaled corticosteroids, levels return to normal [13] and it has been advocated that exhaled NO measurements be used to monitor the anti-inflammatory effects of new anti-asthmatic treatments [14].

However, difficulties remain in the interpretation of exhaled NO measurements. Exhaled NO has been measured by several different poorly standardized techniques. The

recent European Respiratory Society (ERS) Task Force guidelines [15] have addressed some of these important issues, yet reproducibility data have been lacking [16]. Much of the difference in reported results may be explained by conflicting methodology [17, 18], the different response times and sensitivities of the chemiluminescent analyser used and, for single-breath measurements, at which point along the expiratory breath measurements are made. Measurements of NO previously reported from the lower airways at bronchoscopy, have not been performed with an analyser which is rapid enough to allow on-line measurements during tidal breathing [19] and arguments persist as to how well measurements at the mouth reflect levels in the lower airways [20].

Therefore, in a group of normal controls, we performed a series of standardized single-breath exhaled NO measurements, using a rapid chemiluminescence analyser, to determine the reproducibility of measurements at different points on the exhaled test. As part of a larger study examining exhaled NO after lung transplantation, we also examined the levels of NO in the lower airways of a group of lung transplant recipients using a rapid analyser which allows on-line recordings during tidal breathing. By comparing these measurements with those obtained in the same cohort during a single-breath manoeuvre, we sought to determine if measurements at the mouth reflect levels in the lower airways.

## Materials and methods

### Patient groups

Healthy, nonsmoking controls without a history of asthma and/or wheeze were taken from hospital staff. To avoid any effects that respiratory infections might have had on NO levels, subjects were free of upper or lower respiratory symptoms on the day of the test and were asked to report the development of any new symptoms within 7 days of their test. In three control patients with unusually high levels, a further follow-up measurement was performed to exclude any change in NO level or the development of respiratory symptoms.

Lower airway measurements were performed on a group of lung transplant recipients who were undergoing surveillance or diagnostic bronchoscopies. To determine the relationship between levels of NO measured at the mouth and in the lower airways, subjects performed a single-breath test just prior to the bronchoscopy. The study was approved by the regional Ethics Committee.

### Nitric oxide analyser

Exhaled NO was measured using a rapid highly sensitive chemiluminescence analyser (LR2000, version 2.2; Logan Research, Rochester, UK) with a resolution of 0.3 parts per billion (ppb) of NO and response time (0–95% rise time) of 0.4 s. The analyser also measured CO<sub>2</sub> (resolution 0.1% CO<sub>2</sub>, response time 0.2 s) by single-beam infra-red absorption, mouth pressure, exhaled flow and volume. The sampling rate was 250 mL·min<sup>-1</sup> for all measurements. At this flow rate the delay time was 1.4 s for the CO<sub>2</sub> analyser and 1.8 s for the NO analyser. The analyser was calibrated daily using medical grade NO at a concentration of 106 ppb in nitrogen (BOC special gases; Surrey Research Park, Guildford, UK) and certified 7.9% CO<sub>2</sub> (Cryoservice; Worcester, UK). Mouth pressure and flow were calibrated using a water manometer and calibration analyser (Timeter RT-200; SLE Ltd., Surrey, UK), respectively. The specifications of the analyser and its maintenance met with the optimum recommendations of the ERS Task Force Guidelines on exhaled NO measurement [15].

### Nitric oxide measurements

**Single breath.** Following a full inspiration, seated subjects were asked to exhale slowly from total lung capacity through a narrow teflon-coated tube and recordings were made of exhaled NO, CO<sub>2</sub>, mouth pressure and exhaled volume (fig. 1). Subjects wore a noseclip and maintained mouth pressure at 4–5 cmH<sub>2</sub>O by using biofeedback visual display. At this pressure, the internal nasal route is closed off by the soft palate, thus eliminating nasal NO contamination of the exhaled gas mixture [21]. A constant expiratory flow (250 mL·s<sup>-1</sup>) was obtained as exhaled NO concentrations are highly flow dependent [22]. The procedure was repeated until three technically acceptable measurements (based on maintenance of a constant expiratory flow and mouth pressure >4 cmH<sub>2</sub>O for at least three quarters of the

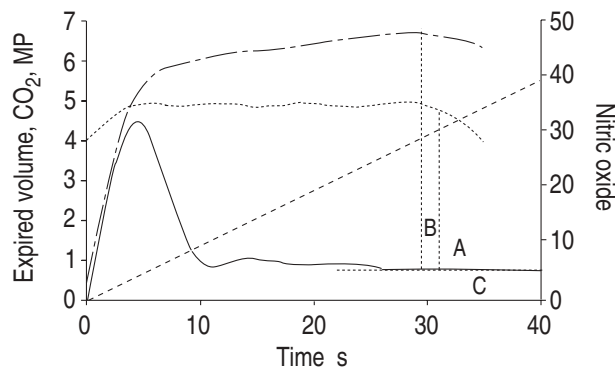


Fig. 1. – Tracings of single-breath exhaled test from total lung capacity showing simultaneous recordings of nitric oxide (parts per billion, —), exhaled CO<sub>2</sub> (%; ---) mouth pressure (MP; cmH<sub>2</sub>O; ..... ) and expired volume (L; -.-.-). Tracing showing typical peak and plateau phase and showing points used to measure NO levels: A) when mouth pressure falls below 4 cmH<sub>2</sub>O; B) when end-exhaled CO<sub>2</sub> reaches plateau; and C) when flattest part of exhaled NO plateau achieved.

exhaled breath) were obtained. These series of measurements were repeated in most subjects the following day but in all subjects within 1 week. Ambient NO concentrations were measured daily.

The single breath test is characterized by a short peak followed by a longer plateau phase. The shape of the "plateau" may vary between individuals so that the NO level may differ depending on where the measurement is taken on the exhaled test. To determine the agreement between NO measurements at different points along the "plateau", we recorded the NO level at three physiologically relevant points (A, B and C; fig. 1). Allowing for the different lag times of the respective analysers, we chose the value corresponding to: A) the time the mouth pressure fell below 4 cmH<sub>2</sub>O (MP); B) the plateau of end-exhaled CO<sub>2</sub> (CO<sub>2</sub>); and C) the NO level corresponding to the longest horizontal line (plateau) of the NO measurement (NO<sub>p</sub>). These points were used because we felt that (B) and (C) were likely to best represent lower airway measurements and because these points have been used by different authors in reporting NO levels. Point (A) was chosen so that we could determine if there was a significant rise in NO levels after mouth pressure and flow rate fell towards the end of exhalation.

**Bronchoscopic.** Measurements of NO and CO<sub>2</sub> were simultaneously made from the lower airways of a group of lung transplant recipients who were undergoing surveillance or diagnostic bronchoscopies. Subjects were premedicated with midazolam and local anaesthesia was obtained with 4% lignocaine. Noseclips were not worn as the nasal route was the chosen route for intubation. Measurements were made at the trachea and in the right middle lobe or lingula after tenacious secretions had been cleared using normal saline. A catheter (internal diameter 1.0 mm) was passed through the working channel of the fiberoptic bronchoscope (Olympus Model B 20D; Keymed Medical Equipment, Southend-on-Sea, UK). Tenacious secretions were removed as these could affect NO measurements by blocking the catheter. To further clear the catheter, 50 mL of air was passed through it by means of a three-way tap prior to on-line recordings. Ambient NO levels were recorded on the day of each bronchoscopy.

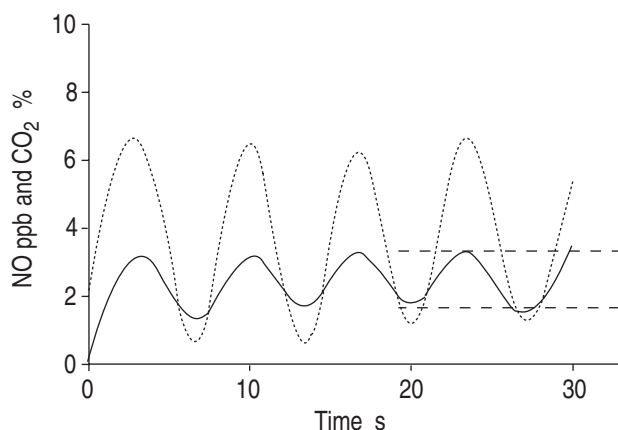


Fig. 2. – Simultaneous tracings of lower airway nitric oxide (NO; —) and CO<sub>2</sub> (.....) recorded at bronchoscopy. Allowing for the greater delay time of the NO analyser, the recordings show typical NO end-inspiratory peak and end-expiratory troughs (indicated schematically by the upper and lower horizontal dashed lines, respectively). ppb: parts per billion.

Due to the rapid response time of the analyser and provided the patient's respiratory frequency was <20 breaths·min<sup>-1</sup>, NO and CO<sub>2</sub> levels could be recorded on-line with peaks and troughs of both NO and CO<sub>2</sub>. Allowing for the different delay times of the NO and CO<sub>2</sub> analysers, the NO cycles correspond to the respiratory cycle (fig. 2). This allowed direct comparisons to be made at end-exhalation between lower airway levels and levels recorded during a single-breath manoeuvre.

#### Reproducibility and repeatability analysis

NO measurements were made at three points on the exhaled test as described previously. At each of these points, three measurements were obtained each day and, as per British Thoracic Society guidelines [23], the two closest NO measurements for each point were compared with each other using the method of BLAND and ALTMAN [24] to determine the coefficient of reproducibility. To determine the day-to-day variability for each measurement, the mean for each measurement on a given day, was similarly compared with the mean measurement obtained on the subsequent day. In a similar way, to determine if there were significant differences in NO levels at the three points chosen, the mean measurement at each point on a single day was compared with the mean measurement for each other point.

To determine whether exhaled NO measurements at the mouth were representative of levels found in the lower airways, measurements corresponding to end-exhaled CO<sub>2</sub> obtained at the trachea and bronchial tree at bronchoscopy were compared to measurements of exhaled NO corresponding to end-tidal CO<sub>2</sub> by univariate regression analysis with NO levels at the mouth as the dependent variable.

## Results

For all single-breath tests performed, NO levels at MP, CO<sub>2</sub> and NOp were (mean (SEM)) 6.56 (0.62), 6.47 (0.61)

and 6.18 (0.633) ppb, respectively. The individual reproducibility of measurements performed sequentially on the same day at each point was determined by calculating the coefficient of reproducibility. The mean±SD of the differences between the measurements was 0.11±0.18 for MP, 0.095±0.16 for CO<sub>2</sub> and 0.094±0.13 for NOp. The coefficients of reproducibility were 0.36; 0.32; and 0.26, ppb respectively.

The individual day-to-day repeatability for each point was also determined. The mean±SD of the differences between NO levels at MP, CO<sub>2</sub> and NOp were 0.18±0.76, 0.19±0.78 and 0.17±0.8 ppb, respectively. Coefficients of reproducibility were 1.52 ppb for MP, 1.56 ppb for CO<sub>2</sub>, and 1.60 ppb at NOp.

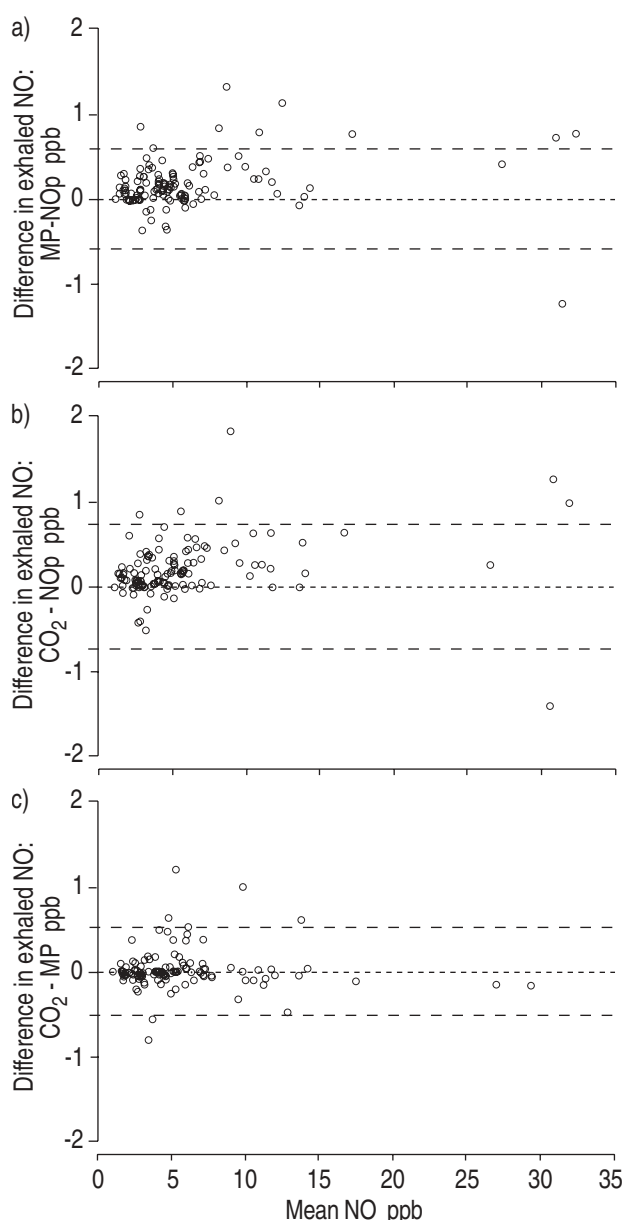


Fig. 3. – Agreement between individual nitric oxide (NO) measurements performed at the level corresponding with: a) time mouth pressure fell to below 4 cmH<sub>2</sub>O (MP) vs the plateau of NO (NOp); b) end-exhaled CO<sub>2</sub> (CO<sub>2</sub>) vs NOp; and c) CO<sub>2</sub> vs MP. .... : mean; - - - - : mean plus or minus 2SD. ppb: parts per billion.

Ambient NO levels were not correlated with any measure of NO recorded during a single-breath manoeuvre ( $r^2=0.14$ ,  $p=0.46$ ) and changes in ambient NO concentration did not influence individual NO levels performed on separate days ( $r^2=0.11$ ,  $p=0.57$ ). Ambient NO levels did not correlate with the peak ( $r^2=0.03$ ,  $p=0.53$ ) or trough ( $r^2=0.01$ ,  $p=0.65$ ) NO levels measured at bronchoscopy.

For the group, NO levels recorded at MP and CO<sub>2</sub> were not significantly different from each other but both were significantly greater than NO levels at NOp (paired t-test; MP vs CO<sub>2</sub>,  $p=0.4$ , MP vs NOp,  $p<0.0001$ , CO<sub>2</sub> vs NOp,  $p<0.0001$ ). The agreement for individuals between each point is shown in figure 3. The mean $\pm$ SD of differences between each point was 0.18 $\pm$ 0.32 ppb for MP and NOp, 0.2 $\pm$ 0.37 ppb for CO<sub>2</sub> and NOp and 0.02 $\pm$ 0.27 ppb for CO<sub>2</sub> and MP. Levels measured at NOp were lower in most individuals than levels measured at either MP and CO<sub>2</sub>.

NO levels recorded at the lower airways of the lung transplant recipients revealed end-inspiratory peaks and end-expiratory troughs. Due to the nature of bronchoscopy in transplant recipients, the inspired oxygen concentration delivered was not the same in all subjects; however, altering the inspired oxygen concentration in an individual did not affect peak or trough levels. There was poor individual correlation between peak and trough levels ( $r^2=0.23$ ,  $p=0.2$ ) and between peak levels and levels corresponding to end-exhaled CO<sub>2</sub> measured at the mouth ( $r^2=0.12$ ,  $p=0.28$ ). However, we demonstrated a clear relationship between NO levels recorded at end-exhaled CO<sub>2</sub> during a single-breath manoeuvre and trough levels recorded in the

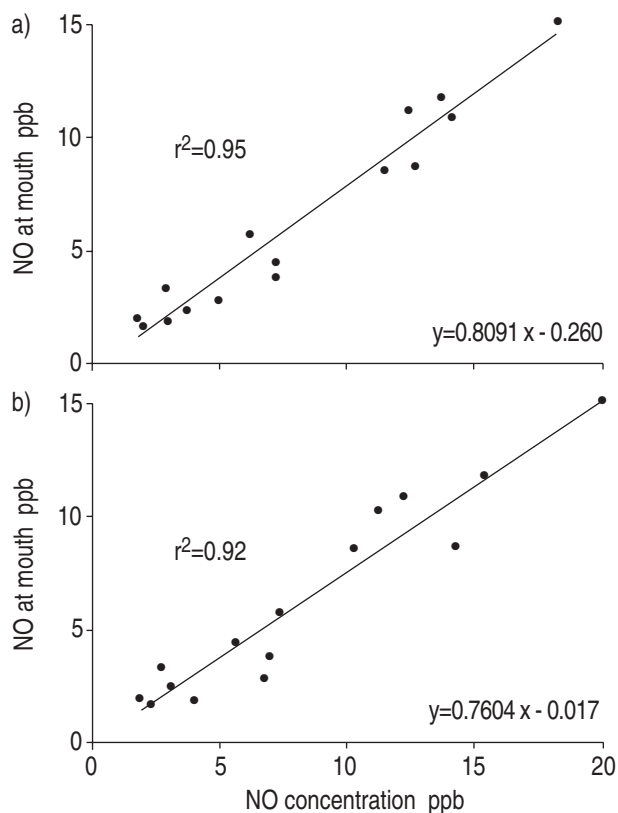


Fig. 4. – Relationship between nitric oxide (NO) levels recorded at end-exhaled CO<sub>2</sub> during a single-breath manoeuvre (mouth) and NO level recorded at end-exhaled CO<sub>2</sub> at: a) the trachea; and b) the bronchus. ppb: parts per billion.

lower airways as shown in figure 4 (mouth vs trachea  $r^2=0.95$ ,  $p<0.0001$ ; mouth vs bronchus  $r^2=0.92$ ,  $p<0.0001$ ). Trough levels recorded at the bronchus and trachea were not significantly different (mean (SEM); 8.47 (1.4) vs 8.46 (1.4),  $p=0.63$  paired t-test) and highly correlated ( $r^2=0.92$ ,  $p<0.0001$ ).

## Discussion

We found that exhaled NO measurements recorded at the mouth during a single-breath manoeuvre with a rapid chemiluminescence analyser are highly reproducible, show little day-to-day variability when subjects are in a stable clinical state and, by comparing end-exhaled levels, that changes in single-breath NO measurements reflect changes in lower airway NO concentrations.

We used a standardized single-breath manoeuvre as recommended in the ERS guidelines [15], ensuring a constant expiratory flow rate of 250 mL·s<sup>-1</sup> and closure of the soft palate and recorded levels along the plateau phase to avoid the influence of nasal contamination and potential influence of breathhold [19, 25] and gender [26] on peak levels. We found NO levels in healthy controls to be in the order of 6–6.5 ppb, in keeping with other studies in which the manoeuvre was performed in a similar way [18, 19, 22]. Although our controls were free of respiratory symptoms or history of respiratory disease, they did not have formal tests of hyperresponsiveness performed prior to the study. Four subjects recorded levels >25 ppb suggesting asymptomatic inflammatory activity within their airways. However, removal of these subjects from the analysis did not significantly alter our findings.

At each point along the "plateau" phase, exhaled NO levels performed sequentially on the same day were highly reproducible. The mean and standard deviation of differences was within the sensitivity of the analyser. The coefficients of reproducibility were <10% as recommended. These findings clear some of the confusion about determination of the exhaled NO value from the single-breath exhaled tracing.

The small day-to-day variability seen in stable subjects was too small to negate the value of sequential NO measurements in the monitoring of lower airway inflammation in an individual.

The individual day-to-day variability that we found was in the order of 0–2 ppb and is significantly smaller than changes seen with disease. Subjects with bronchiectasis and upper respiratory tract infections have exhaled NO levels which are on average four times higher than controls [10, 12] and asthmatic subjects have end-exhaled levels 20–40 ppb higher than controls [19]. The explanation for the variability seen in control subjects is unclear, although it was not explained by changes in ambient NO levels. It remains possible that although our subjects were free of respiratory symptoms, they may have been developing an upper respiratory infection on one of the two days. DILLON *et al.* [17] reported a greater individual variability in five subjects tested on consecutive days. However, they sampled NO which had been breathed into a reservoir bag with resultant variable loss of reactive NO and consequently their results are not applicable to single-breath tests performed with a rapid analyser. We have found that a baseline NO level in health can be defined in

an individual which can be used to monitor changes in disease activity or effectiveness of anti-inflammatory treatment.

The single-breath test is usually characterized by a short peak and longer plateau. The shape of the "plateau" phase varies between individuals yet in most is curvilinear as evidenced by the differences we found at three points. In most subjects, levels were lowest at the NO plateau and slightly higher at MP and CO<sub>2</sub>, most likely as a result of the reduced expiratory flow rate which occurs at the end of exhalation. Although differences were small, our results highlight the need for authors to define precisely from where along the exhaled test the measurement has been taken.

To our knowledge, previously published bronchoscopic recordings of NO have not been performed with a rapid analyser that allows on-line measurements of tidal breathing. As a result, previous comparisons between single-breath measurements and measurements in the lower airways have been inconclusive [17, 19, 20]. We found that lower airways NO concentrations change with tidal breathing, increasing through inspiration and decreasing through expiration. Clearing of tenacious secretions resulted in an increase in recorded NO levels. This observation may explain why, despite the fact that cystic fibrosis (CF) is characterized by airway inflammation, single-breath exhaled levels are paradoxically low [27]. There is disagreement as to the cause of these low exhaled NO measurements in CF. Recent studies have suggested that there is increased nitric oxide synthase activity in the lung of CF patients [28] but it is not detected in exhaled breath. Our results are consistent with the view that it is diffusion of NO from the epithelium which is impaired by thick, tenacious airway secretions.

We found a consistent relationship between end-exhaled NO levels in the lower airways and at the mouth. In individuals, we did not expect nor did we find these levels to be equivalent as the flow rates were different and not easily controlled by the practical limitations of bronchoscopy. For this reason we did not perform a test of agreement as defined by BLAND and ALTMAN [24]. Rather, on univariate analysis with single-breath NO levels as the dependent variable, we found that a relationship between end-exhaled NO levels in the lower airways and at the mouth could be clearly defined. The degree of correlation suggests that changes in NO levels at the mouth are highly representative of changes in NO concentration in the lower airways and lend further support to the contention that the single breath test is a valuable measure of lower airways inflammation.

Although it remains uncertain, our findings give some insight into the source of NO found in the lower airways. KHARITONOV and BARNES [21] have shown that during normal tidal breathing but not during breathing against resistance, argon delivered to the nose could be sampled in the lower airways. If possible, nasal contamination of the lower respiratory tract is excluded by an inflated endotracheal tube [29], then NO is still detected in air sampled directly from the lower respiratory tract. The cyclical levels of NO that we found in the lower airways suggest that during the inspiratory phase of tidal breathing there is auto-inhalation of NO from the nasopharynx. However, we found a poor correlation between peak (end-inspiratory) and trough (end-expiratory) levels, suggesting that auto-inhalation

does not contribute significantly to trough levels. Therefore, we believe that trough levels predominantly reflect lower airway NO production, as has been suggested by others [18, 19].

Our data support the measurement methodology described in the recent ERS guidelines [15], apart from the use of noseclips, which we feel prevents the subject from inhaling *via* the nasal route, thus contaminating the first part of the exhalation. The use of noseclips in our subjects reduced the time taken to reach steady state and made the NO plateau phase longer.

In summary, we have shown that a standardized single-breath test is reproducible and in most healthy controls has little day-to-day variability. Providing that subjects maintain a mouth pressure sufficient to close the soft palate and flow is kept constant throughout the test, then nitric oxide levels recorded at the mouth are closely related to levels in the lower airways. Our findings lend added support to suggestions in the European Respiratory Society guidelines [15] that the single-breath test is a valid noninvasive measure of lower airways inflammation that can be used to monitor disease and effects of therapy.

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