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# Accurate real-time $F_{E}NO$ expirograms using complementary optical sensors

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## Abstract

The fraction of exhaled nitric oxide ( $F_{E}NO$ ) is an important biomarker for the diagnosis and management of asthma and other pulmonary diseases associated with airway inflammation. In this study we report on a novel method for accurate, highly time-resolved, real time detection of  $F_{E}NO$  at the mouth. The experimental arrangement is based on a combination of optical sensors for the determination of the temporal profile of exhaled NO and CO<sub>2</sub> concentrations. Breath CO<sub>2</sub> and exhalation flow are measured at the mouth using diode laser absorption spectroscopy (at 2  $\mu$ m) and differential pressure sensing, respectively. NO is determined in a sidestream configuration using a quantum cascade laser based, cavity-enhanced absorption cell (at 5.2  $\mu$ m) which simultaneously measures sidestream CO<sub>2</sub>. The at-mouth and sidestream CO<sub>2</sub> measurements are used to enable the deconvolution of the sidestream NO measurement back to the at-mouth location. All measurements have a time resolution of 0.1 s, limited by the requirement of a reasonable limit of detection for the NO measurement, which on this timescale is 4.7 ppb (2  $\sigma$ ). Using this methodology, NO expirograms ( $F_{E}NO$ grams) were measured and compared for eight healthy volunteers. The  $F_{E}NO$ grams appear to differ qualitatively between individuals and the hope is that the dynamic information encoded in these  $F_{E}NO$ grams will provide valuable additional insight into the location of the inflammation in the airways and potentially predict a response to therapy. A validation of the measurements at low-time resolution is provided by checking that results from previous studies that used a two-compartment model of NO production can be reproduced using our technology.

## 1. Introduction

Real time monitoring of biomarkers in exhaled breath is a key requirement for optimally determining the location of airway inflammation and to rule out any confounding factors such as sampling manoeuvres, contamination and dilution. As reported in a recent review [1], laser-based sensors allow for fast, real time detection of various volatile compounds, thereby enabling continuous measurement of biomarkers in the different respiratory phases. In particular, the present group of authors has developed a highly accurate in-line breath analyser, termed a molecular flow sensor (MFS), which provides highly precise measurements of gas exchange in the lung [2]. The MFS uses near-IR diode laser absorption of

O<sub>2</sub> within an optical cavity, alongside direct absorption detection of CO<sub>2</sub> and water vapour, to reduce the errors in flow sensing between inspiration and expiration from ~5% to <0.2% and to allow accurate determination of gas exchange on a breath-by-breath basis in a clinical setting. We have also shown that the highly precise time-resolved MFS data afford the opportunity to extract information pertaining to lung inhomogeneity [3]. Key parameters that can be recovered are related to the inhomogeneities in anatomical deadspace, in lung compliance (a measure of the (un)evenness with which the lung expands during inspiration), and in vascular conductance (a measure of the regional variation in blood flow through the lung). Notably, MFS based measures of lung inhomogeneity may have predictive value for disease

progression in patients with COPD and in the case of asthma, have been shown to be useful as a measure of disease control [4]. We emphasise here that it is the combination of accuracy and precision both in airway spectroscopy and flow sensing along with the development of a novel model of lung inhomogeneity that has enabled successful measurement of lung inhomogeneity using MFS technology.

Shortly after the discovery of nitric oxide (NO) in human breath [5], exhaled NO emerged as a promising biomarker associated with asthma and lung inflammation. Specifically, Alving *et al* showed that airway inflammation is associated with increased levels in the fraction of exhaled NO ( $F_{E}NO$ ) [6]. In addition to simply determining  $F_{E}NO$  levels, the respiratory physiology community has been interested in ways of distinguishing the bronchial and alveolar NO components [7, 8]. To this end, theoretical studies to assist in the interpretation of the  $F_{E}NO$  measurements have led to the development of models of pulmonary NO dynamics. The most widely adopted model is the two-compartment model (2CM) [7, 9]. The 2CM is readily implemented and requires as its input  $F_{E}NO$  data collected at different constant flow rates to extract NO-related parameters: the alveolar NO concentration ( $C_{A}NO$ ); the radially averaged NO concentration in the airway tissue ( $C_{aw}NO$ ); the NO flux in the airway compartment ( $J_{aw}NO$ ) and the global airway diffusing capacity, or conductance of NO from the airway wall to the lumen ( $D_{aw}NO$ ) [10, 11]. In general, the 2CM relies on the time-averaged NO concentration in the latter stages of the exhalation (termed phase III)—see later.

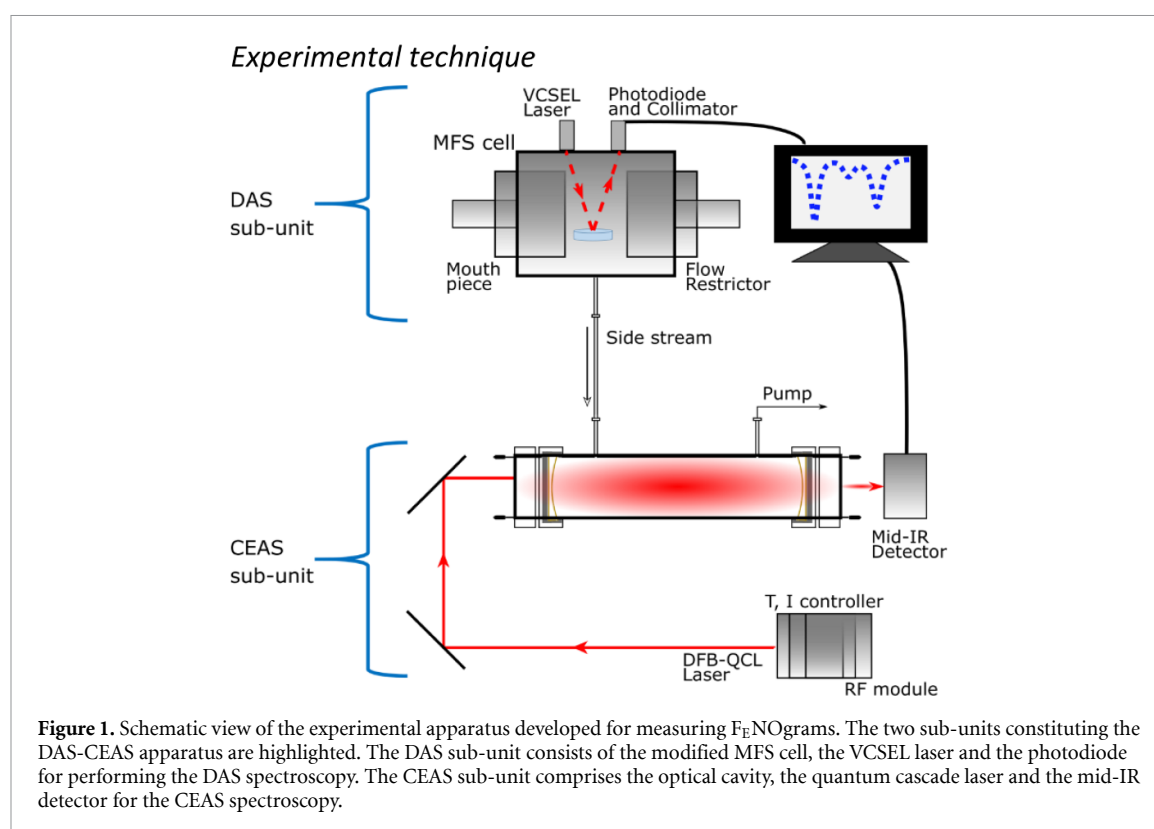
Indeed, it is a mark of the success of the breath analysis field that the measurement of  $F_{E}NO$  has entered mainstream clinical use for the management of asthma. It is used as a non-invasive technique to differentiate between types of inflammation. Specifically, it has been found that the bronchial NO flux,  $J_{aw}NO$ , and the alveolar NO concentration,  $C_{A}NO$ , can be enhanced in subjects with eosinophilic ( $T_{H2}$ -high) asthma or lung inflammation [8]. It has also been proposed that monitoring these NO exchange parameters (in addition to absolute value of the  $F_{E}NO$  levels) would be an ideal way to follow and predict the treatment efficiency of inhaled steroids [12–14].  $F_{E}NO$  is typically high in  $T_{H2}$ -high asthma [15, 16] and in many individuals, this type of asthma can be managed well using inhaled corticosteroids which also suppress the  $F_{E}NO$  production from the epithelial cells that line the lower airways [17]. Although in some individuals with  $T_{H2}$ -high asthma, inhaled corticosteroids do not work well and this can often be observed by their failure to suppress the  $F_{E}NO$  production. If the asthma is severe, then these individuals can then be considered for therapy using more recent biologics [18–21]. It has been reported that  $F_{E}NO$  resulting from pharmacologically induced bronchodilation in asthma sufferers can

show different behaviours depending on the location in the airway tree, where relaxation of the smooth muscle is achieved [22]. This can be used to help identify the site of the airway obstruction, noting that different degrees of airway inflammation may cause different responses in different parts of the airway tree [23, 24].

Significantly, the asthmatic lung is highly inhomogeneous, thus information on homogeneity is an absolute requirement in order to better interpret  $F_{E}NO$  measurements. For example, regularly, some asthmatics may have >50% of the gas in their lungs trapped so as to be inaccessible to the conducting airways, and hence models of  $F_{E}NO$  production must be developed within the context of a *person-specific* lung. MFS based measurements are capable of determining the distribution of deadspace *relative to lung volume* and this vital property of the airway tree is unique to each individual and should be taken into account to optimally interpret time-resolved  $F_{E}NO$  measurements; despite its obvious importance this heterogeneity cannot be measured in standard lung function tests. Intriguingly, the relationship between the form of inhomogeneity (whether deadspace, compliance or conductance) and the site(s) of  $F_{E}NO$  production may help further in classifying asthma.

Previously, MFS technology has allowed measurement of physical biomarkers of ventilation inhomogeneity [2, 3] and by analogy it is hoped that new information about the underlying NO exchange dynamics in lung inflammation-related diseases involving enhanced peripheral NO, such as COPD [11], alveolitis [25], and systemic sclerosis [26], can be obtained by detailed examination of the time-dependent  $F_{E}NO$  concentration throughout a single breath cycle. In order to obtain such a  $F_{E}NO$  expirogram, or  $F_{E}NOgram$ , it is imperative to utilise an instrument capable of fast, accurate, on-line measurements of  $F_{E}NO$  values, time-aligned with critical exhalation parameters such as  $CO_2$  concentration, exhalation flow rate, and exhaled volume. Such a time aligned technique has been recently successfully applied to the detection and modelling of exhaled CO concentrations by Ghorbani *et al* [27, 28]. These researchers evaluated real time breath data by least-squares fitting of complete expirograms using a trumpet-shaped lung model with axial diffusion to simulate the pulmonary gas exchange dynamics. They successfully demonstrated that, in addition to end-tidal CO, maximum CO fluxes, diffusing capacities and equilibrium concentrations in airway and alveolar regions can be extracted from single-exhalation profiles.

Several methods have been developed to measure  $F_{E}NO$  [29–31]. Currently, the gold-standard method is by ozone-chemiluminescence (see for example [32–34]); such sensors have impressive limits of detection of about  $\sim 0.1$  ppb, and have an intrinsically short time response (sub-100 ms). However,



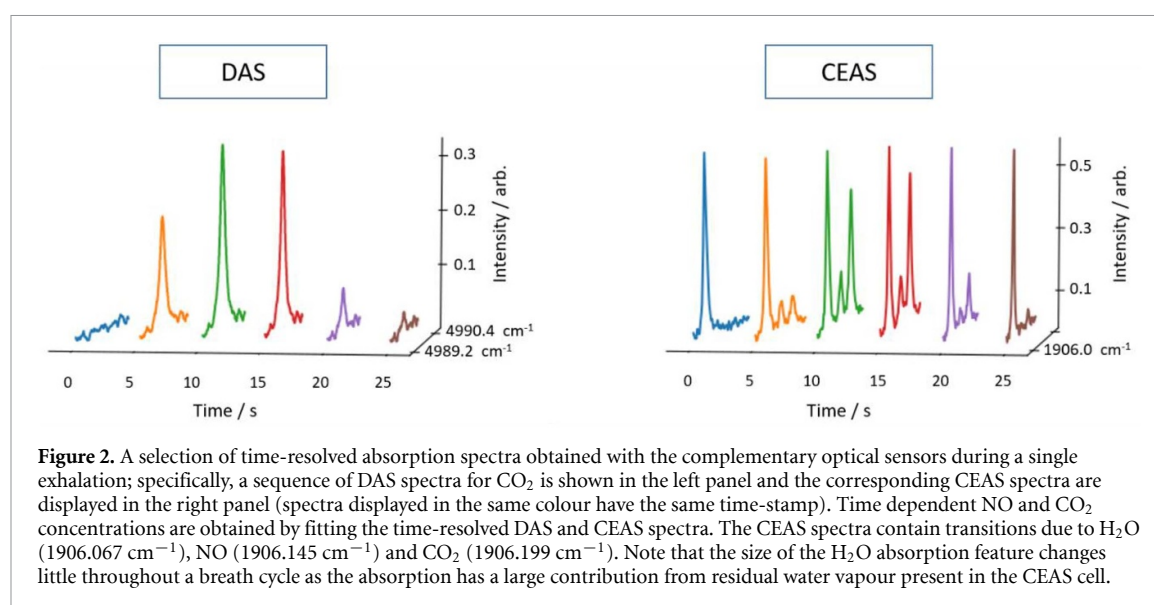
their downstream configuration results in a *ca.* 0.5 s lag which would confound any efforts to time align exhaled NO with other breath gases as required for determination of lung inhomogeneity [3]. Furthermore, other gases present in breath (mainly  $CO_2$  and  $O_2$ ) can affect the chemiluminescence process and lead to significant decreases in NO readings [35, 36]. Other commercially available methods utilise electrochemical sensing. These sensors are typically small enough and easy to operate such that hand-held devices are available both for clinical and domestic use. However, they tend to feature slow response ( $\sim 10$  s) and long averaging times. Difficulties in comparing measurements from different types of electrochemical device have recently been highlighted in comparative studies by Molino *et al* [37]. Further problems in protocol, in terms of data collection techniques, and analytical methods to extract the relevant NO components of a given patient (e.g.  $J_{aw}NO$  and  $C_A NO$ ) have also been highlighted [38].

However, the purpose of the present study is not intended to be a demonstration of new technology for  $F_{E}NO$  measurement. It is to develop a research technology to explore whether time-resolved measurements of  $F_{E}NO$  can also be used to differentiate between individuals who have disease predominantly in their large airways from individuals who have disease predominantly in their lower airways. This report describes the development of laser based methods to achieve this. A validation of the measurements (low time resolution) from our technology is provided by comparison with previous studies that explored

a 2CM of NO production, and we present exemplar data where the ability to make highly time resolved measurements indicate the unexplored potential for  $F_{E}NO$  studies. Future research using this instrument will involve model simulations of production of NO at different depths in the lower airway and determining which depth best reproduces the relation between the rapidly rising phase for  $F_{E}NO$  and expired volume in main gas flow.

## 2. Materials and methods

In order to explore how deep the NO is arising within the airways, it will be necessary to analyse the fast, rising phase of the relationship between  $F_{E}NO$  and exhaled volume, for which the clinically available instruments have not been designed. We choose to explore the use of laser absorption spectroscopy to do this, recognising that such laser methods must be combined with sensitivity enhancing techniques such as cavity ringdown spectroscopy (CRDS and analogues) or Faraday rotation spectroscopy (FRS) to reach the sensitivities required to detect breath NO [39–43]. Both laser spectroscopy and the gold standard  $F_{E}NO$  measurement by chemiluminescence are potentially fast enough, but both have to be conducted using a sidestream flow of gas into the measurement chamber. This sidestream flow is associated with a transport delay, and the volume of the measurement chamber will also introduce a ‘smearing’ of the signals. These features will (significantly) degrade



the time resolution of the relationship between  $F_{\text{E}}\text{NO}$  and exhaled volume.

In the current study, our approach is to use laser absorption spectroscopy, but to choose a wavelength where there are adjacent absorption lines for both NO and for CO<sub>2</sub>. On its own, this does not help because, for both signals, the airway fractions are convolved with the transfer function describing the dynamics of the instrument. However, for CO<sub>2</sub> it is possible simultaneously to measure the concentration very rapidly in the main gas stream close to the mouth using laser absorption spectroscopy and define the dynamics of the sidestream analyser for any given expiration. We can then use these dynamics to de-convolve the sidestream measurement of NO to calculate the concentration time profile for NO in the main airway and so provide the highly time-resolved relation between  $F_{\text{E}}\text{NO}$  and expired volume.

## 2.1. Experimental technique

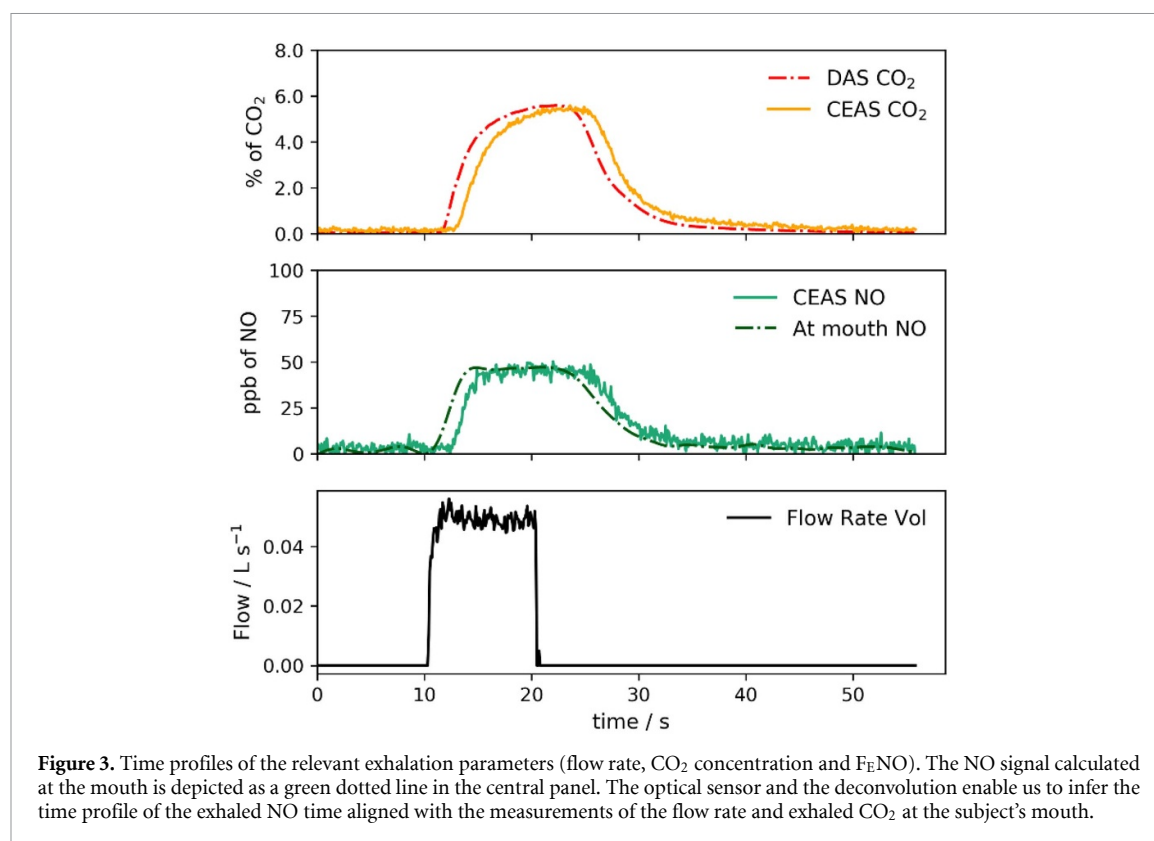
Laser absorption spectroscopy provides a highly selective technique capable of differentiating exhaled NO from other gases present in breath. For NO detection the spectral region of choice is in the mid-infrared (mid-IR) between 5.1 and 5.7  $\mu\text{m}$ , where the strongest rovibrational transitions of NO lie. This spectral region also features pronounced CO<sub>2</sub> and H<sub>2</sub>O transitions which potentially interfere with the NO absorption profile and can significantly hinder the sensitivity of the spectrometer if used at atmospheric pressure. To overcome this issue, and mitigate the effects of pressure broadening, the exhaled gas is admitted to a low-pressure chamber (typically  $\sim 100$  mbar) where measurements are made.

Here, we choose to employ a cavity enhanced absorption spectroscopy (CEAS) based sensor as it presents several operational advantages: it allows continuous fast scanning across the absorption feature; it is relatively easy to align and resistant to vibrations;

it requires little maintenance and does not require magnetic fields to operate (unlike FRS). Additionally, suitable lasers and detectors that do not need bulky cooling systems are now commercially available. There remains however the necessity to use sidestream analysis within a reduced pressure cell. CEAS based measurements of breath NO have been successfully realised previously and we note the work of Marchenko *et al* [44] who developed a CEAS based NO sensor with a 0.7 ppb limit of detection with a time resolution  $\sim 1$  s. The  $F_{\text{E}}\text{NO}$  value was determined remotely from the mouth, within a low-pressure cell. The prominent advantage of the combined CEAS-based method we have developed is that it enables us to obtain accurate real-time *at-mouth*  $F_{\text{E}}\text{NO}$  values with 0.1 s time resolution, necessary data for detailed physiological models aimed at allowing functional location of airway inflammation as already demonstrated in our MFS studies of lung inhomogeneity from measurements of exhaled O<sub>2</sub>, CO<sub>2</sub> and H<sub>2</sub>O.

The experimental apparatus developed for this study relies on two complementary sub-units each of which features its own laser-based sensor. Figure 1 shows a schematic view of the setup. The first sub-unit is a simpler version of the molecular flow sensor (MFS) device previously reported [2, 3]. This unit enables the real time concentration of exhaled CO<sub>2</sub> at the mouth to be measured via direct absorption spectroscopy (DAS) with ms time resolution. The collimated light from a tunable diode laser (Vertilas VCSEL @2004 nm; VL-2004-1-ST-1-14) is sent through the sampling cell in a V-shaped optical path ('V-path'). The light is then collected by a photodiode and signals recorded by an acquisition card (model USB-6351, National Instruments) interfaced to a portable computer.

Additionally, differential pressure ports enable the flow rate to be accurately established as in



**Figure 3.** Time profiles of the relevant exhalation parameters (flow rate,  $\text{CO}_2$  concentration and  $\text{F}_{\text{E}}\text{NO}$ ). The NO signal calculated at the mouth is depicted as a green dotted line in the central panel. The optical sensor and the deconvolution enable us to infer the time profile of the exhaled NO time aligned with the measurements of the flow rate and exhaled  $\text{CO}_2$  at the subject's mouth.

a pneumotachograph, and the unit includes a barometric sensor, and two thermocouples to determine the temperature of the sampling cell and the temperature of the exhaled gas. To prevent condensation, the sampling cell is heated to  $37^\circ\text{C}$  during use. In order to measure accurately the flow rate and the expired volume, the DAS sub-unit was calibrated using a programmable mechanical pump of fixed stroke volume to simulate the range of flow rates expected during breathing [2]. Data from the various sensors are recorded through the acquisition card with a sampling rate of 1 kHz and are averaged over 100 data points (i.e. an averaging time of 0.1 s).

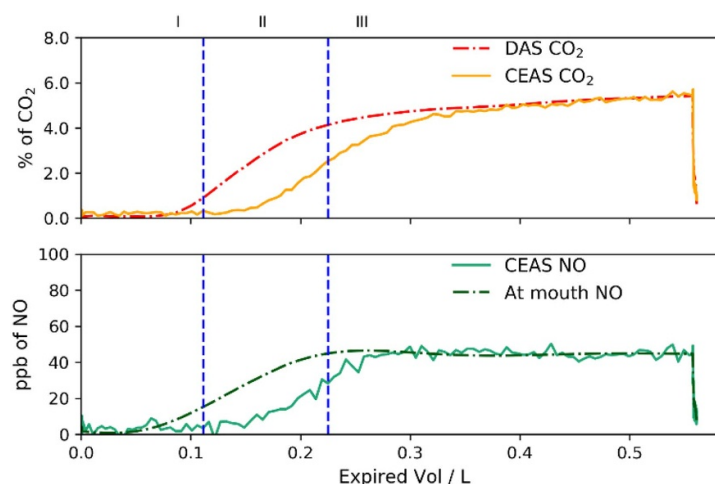
Owing to the wide tuning range of the DAS laser three  $\text{CO}_2$  transitions, R(12), R(14) and R(16) within the  $(2\nu_1 + \nu_3)$  combination band (at 4987.31, 4988.65, and 4989.78  $\text{cm}^{-1}$ , respectively), are observable within the same scan when modulating the laser current input via a 'sawtooth' signal. Probing three transitions at the same time provides a useful check that the laser is operating at the correct wavelength and yields an absolute wavenumber scale.

The DAS sub-unit features a sidestream port that directs gas samples towards the low-pressure cavity of the CEAS sub-unit. The CEAS sub-unit comprises a mid-IR laser coupled with a vacuum compatible optical cavity to perform sensitive spectroscopy of the breath admitted. During breath measurements, exhaled gas is continuously drawn through the cavity by a dry diaphragm pump (KNE, n920 KT 29 18, nominal speed:  $20\text{ l s}^{-1}$ ) connected at the outlet port of the cavity. The pressure in the cavity is

monitored via a pressure gauge (Ceravac CTR 100, Oerlikon Leybold Vacuum). The pressure in the cavity can be readily adjusted over 20–100 Torr via a needle valve present along the sidestream tube that leads to the cavity sub-unit. The cavity consists of a 42.5 cm long metal cylinder and has a volume of 108 ml. Owing to the high reflectivity of the ZnSe concave cavity mirrors (average reflectivity of 99.975%, and radius of curvature of 0.5 m, LohnStar Optics), the effective optical path length inside the cavity is *ca.* 1700 m (cavity finesse  $F \approx 12\,500$ ).

The laser employed in the CEAS sub-unit is a distributed feedback quantum cascade laser (DFB-QCL, Thorlabs QD5250CM1), operating at 5250 nm in continuous wave mode, and it ensures single longitudinal mode emission suitable for high resolution spectroscopy. The laser parameters were chosen to probe a spectral region fulfilling two important requirements; first, the largest possible NO integrated cross-section; second, no significant overlap of the target NO transition with any neighbouring  $\text{CO}_2$  or  $\text{H}_2\text{O}$  transitions (present at high concentration in breath). We note however that the presence of a nearby  $\text{CO}_2$  transition is highly advantageous and provides a key input required for deriving  $\text{F}_{\text{E}}\text{NO}$ grams, as illustrated in the next sections.

The laser is aligned off-axis with respect to the cavity by means of adjustable steering mirrors, a configuration that enables the excitation of multiple transverse electromagnetic modes to reduce residual cavity mode structure, thereby yielding a cavity transmission signal (when no gas is present) which



**Figure 4.** Complete  $F_E\text{NO}$ grams ( $F_E\text{NO}$  expirograms time aligned with  $\text{CO}_2$  expirograms). The top panel shows the  $\text{CO}_2$  expirogram measured both at the cavity (orange trace) and at the mouth (red trace). In the bottom panel, the solid and dotted green traces represent the  $F_E\text{NO}$  expirogram at the cavity and at the mouth, respectively. The vertical lines and the roman numerals indicate the different exhalation phases, as discussed in the text.

is almost independent of the laser wavelength. This is further optimised by the application of radio-frequency noise to the current driving the laser. This technique decreases the residual cavity mode structure by effectively reducing the laser coherence length [45, 46]. The effective optical path length within the cavity is determined using a calibrated mixture of 5%  $\text{CO}_2$  in  $\text{N}_2$  (BOC) and a motorised beam shutter within the beam path allows baseline measurements to be taken. The cavity output is detected by a thermoelectrically cooled mid-IR sensor (VIGO, PVI-4TE-6). This compact sensor is fixed in place to the same 30 mm Thorlab cage system that supports all the other optical elements (including the QCL), thus making the entire CEAS unit resistant to vibrations and mechanical stress. Notably, the entire apparatus is enclosed in a trolley assembly that makes it portable, robust, and convenient for clinical use.

## 2.2. Measurement of the $\text{CO}_2$ and $F_E\text{NO}$ time profiles

The fraction of exhaled  $\text{CO}_2$  at the mouth is measured via DAS on the  $R(16)$  ( $2\nu_1 + \nu_3$ ) rovibronic transition (at  $4989.78\text{ cm}^{-1}$ ). The  $\text{CO}_2$  and NO concentrations in the sidestream cell are measured via CEAS on the  $P(15)$  ( $\nu_1 + \nu_2$ ) and the doublet  $R(8.5)e/f$  fundamental rovibronic transitions (at *ca.*  $1906.199$ ,  $1906.140$ , and  $1906.151\text{ cm}^{-1}$ , respectively). To extract concentrations all data are fitted in real time using a regression of well characterised (for the experimental conditions) reference spectra. We note that in the same frequency region there is also a strong  $\text{H}_2^{17}\text{O}$   $R(15)\nu_2$  transition (at  $1906.067\text{ cm}^{-1}$ ) which must be included in the real time spectral fitting.

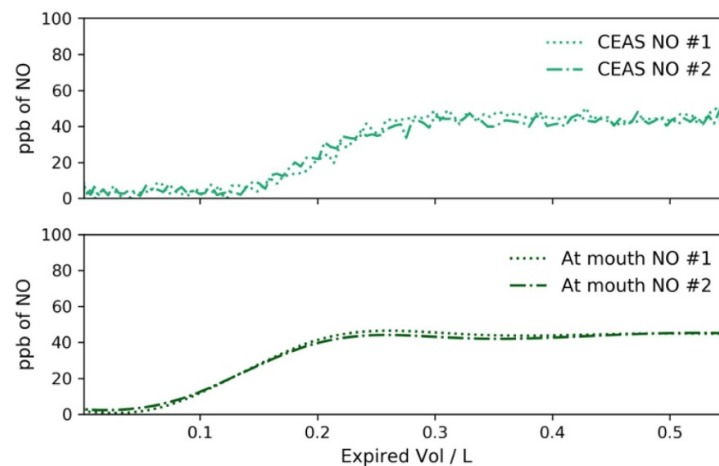
The apparatus is typically operated at a pressure of 50 Torr in order to minimise the effects of pressure broadening which would cause the NO absorption

profile to become overlapped with the more intense absorptions arising from neighbouring  $\text{CO}_2$  and  $\text{H}_2\text{O}$  transitions, and to optimise the refresh time of the gas sample, whilst maximising the NO signal in the absorption spectra. As for the DAS sub-unit, each spectrum from the CEAS sub-unit is the result of the average of 100 scans sampled at 1 kHz, therefore we achieve a time resolution of 0.1 s. This amount of averaging was chosen as a compromise between good signal to noise ratio and sensitivity to fast changes in concentration. On a few occasions, ambient NO ( $>5\text{ ppb}$ ) could be detected with the device: it was chosen not to acquire breath data during these periods.

Figure 2 illustrates an example of the data obtained with the DAS sensor (left panel) and CEAS sensor (right panel). From the progression of the spectra with time, we are able to measure the time profile of the exhaled NO time aligned with the measurements of the flow rate and exhaled  $\text{CO}_2$ , and deduce the at-mouth NO.

## 2.3. Measurement of $F_E\text{NO}$ expirograms

NO is present in breath at trace levels (*ca.* 10–100 ppb, depending on the flow rate at which the breath is sampled), therefore, for its accurate determination, sensitivity enhancing methods such as cavity based spectroscopy are necessary. The additional requirement to operate at reduced pressure to ensure selectivity means that the NO analysis is necessarily a sidestream analysis. As noted in the introduction, any such analysis introduces both temporal delays and temporal broadening of the target signals, and we therefore need a method to transform our time-resolved signals back to a measurement plane located directly after the mouth. Since the complementary optical sensors permit precise measurements of  $\text{CO}_2$  both in the sidestream cavity and at-mouth, the



**Figure 5.** Comparison of two  $F_{E}NO$ grams from the same subject at the  $50 \text{ ml s}^{-1}$  flow rate. The upper panel shows the cavity data that results in the at-mouth  $F_{E}NO$ grams (lower panel). The  $F_{E}NO$ grams are very similar, indicating that the methodology is robust.

instrument transfer function can be obtained that enables the sidestream NO signal to be accurately transformed into the at-mouth  $F_{E}NO$ . Formally, to achieve this, we employ the convolution theorem [47] and calculate the instrument response function for the DAS-CEAS system at each exhalation.

Convolution theory is widely exploited in signal processing to probe and characterise the behaviour of an instrument that takes an input signal  $f(t)_{in}$  and subsequently outputs a signal  $g(t)_{out}$ . Since  $f(t)_{in}$  is known and the signal  $g(t)_{out}$  is measured, by applying the inverse Fourier transform ( $FT^{-1}$ ) to the ratio between the FT of the output function and FT of the input, one can infer the instrument response function of a device. In our method, instead of utilising the same instrument response function for any dataset, a flow-rate edge-detection algorithm determines the beginning and end of each exhalation and the instrument response function is evaluated at each exhalation event. This feature serves to prevent any systematic biases caused by any small variation in the instrument response function over time. After applying the deconvolution to determine the at-mouth  $F_{E}NO$ , the NO cavity absorption signal is smoothed via a 3rd order low pass Butterworth filter (50 Hz). This is necessary to remove artefacts that are sometimes (and seldom) observed from the numerical deconvolution algorithm (FFT), and has been chosen so as to have little influence on the frequency response of the device.

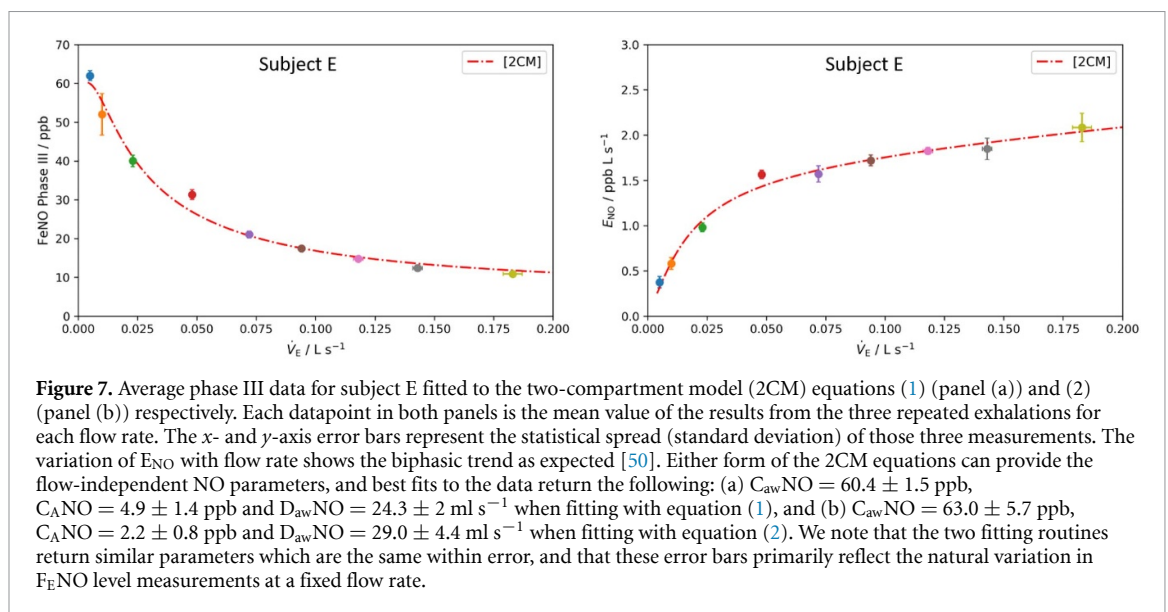
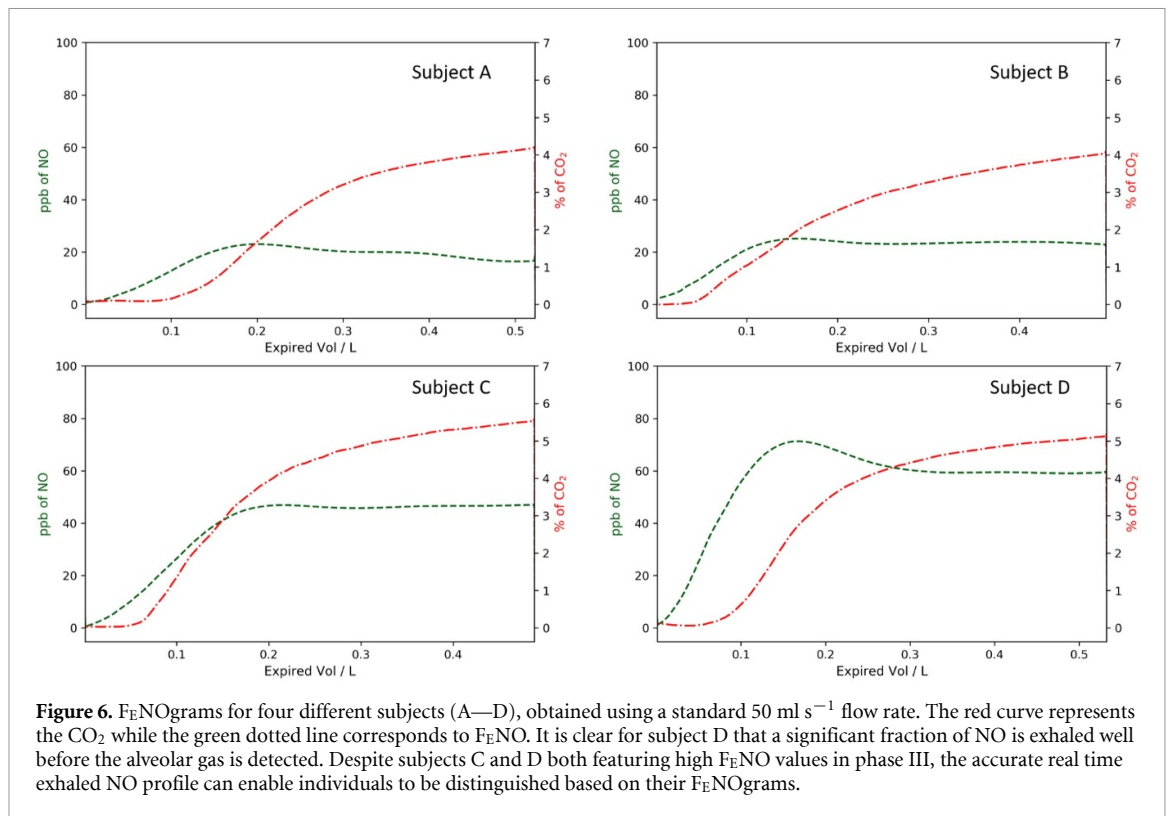
Subjects were examined at various exhalation flow rates; at each flow rate, the volunteers were asked to perform three exhalations into the DAS-CEAS apparatus, always ensuring that the exhaled flow rate was kept at the target value. Each breathing manoeuvre consisted of an inhalation of 3 s followed by an exhalation of 10 s, with no breath-hold or forced exhalation. In this initial study we chose to conduct all measurements using a nose clip to ensure accuracy of the

$F_{E}NO$ gram; without a nose clip we were concerned that there would be a leak through the nose influencing the measured exhaled volumes. We emphasise that these measurements do not closely follow current clinical guidelines but guarantee accurate determination of gas volumes. Measurements were initially conducted using commercial flow restrictors (Eco Medics model M30.8062). Subsequently, custom made flow restrictors (calibrated at 5, 10, 25, 50, 75 100, 125, 150, 175 and  $200 \text{ ml s}^{-1}$ ) were employed in order to probe a larger number of flow rates.

Examples of the exhaled concentrations of NO and  $CO_2$  as a function of time, the measured flow and the transformed at-mouth  $F_{E}NO$ gram are shown in figure 3. In addition, the NO and  $CO_2$  concentrations are plotted with respect to the expired volume (see figure 4) thereby yielding complete  $F_{E}NO$ grams.

In figure 4 we have defined three exhalation phases as in previous studies [9, 28]. I (corresponding to the respiratory deadspace and conducting airways), II (or mixing phase, featuring the  $CO_2$  expiratory upstroke) and III (or alveolar phase) [48]. The phases are determined by the  $CO_2$  levels relative to the maximum end-tidal  $CO_2$  level ( $CO_{2,m}$ ) as follows: phase I ( $0 < CO_2(t)/CO_{2,m} \leq 15\%$ ), phase II ( $15\% < CO_2(t)/CO_{2,m} \leq 75\%$ ), and phase III ( $75\% < CO_2(t)/CO_{2,m} \leq 100\%$ ).

With the aim of establishing the limit of detection and quantifying any losses of either  $CO_2$  or NO, measurements on calibrated samples of known concentrations of each gas (4%  $CO_2$  in  $N_2$  and 100 ppb NO in  $N_2$ , prepared and certified by BOC) were conducted in two different configurations and compared. First, measurements were performed in the normal configuration and then the DAS sub-unit was by-passed and the apparatus was operated utilising only the CEAS unit. No measurable losses occurred in either configuration. This result was corroborated by repeating the comparison test at various flow rates expected during



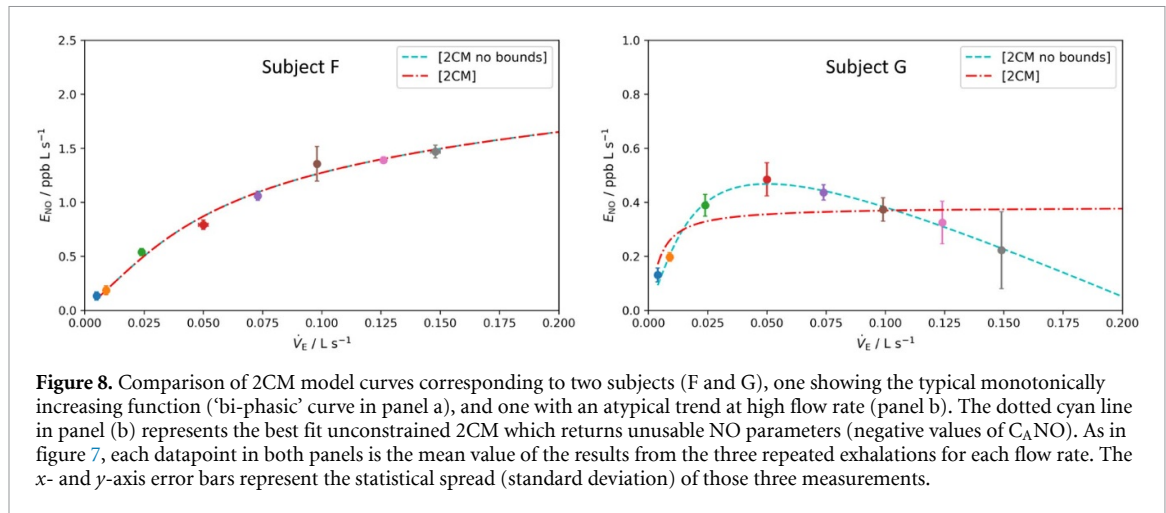
breathing. A sensitivity ( $2\sigma$ ) of 4.7 ppb of NO with 0.1 s of averaging was established by calculating the variations in the absorption of a known mixture containing 100 ppb of NO over a period of 1 min. This level of variability is observed consistently at lower concentrations of NO during normal breath tests.

### 3. Results and discussion

Having developed a method to determine F<sub>E</sub>NOgrams, the applicability of the DAS-CEAS sensor has been validated by examining a small group of volunteers, comprising 8 healthy adult

non-smokers (age range: 20–75 yr), two of whom featured high F<sub>E</sub>NO values. Every scan entailed three tidal breathing manoeuvres to increase the statistical significance of the tests. The robustness of our method ensures the repeatability of the F<sub>E</sub>NOgram test and is illustrated in figure 5 where the F<sub>E</sub>NOgrams of a single subject examined during two distinct exhalations are presented; there is minimal intra-individual variation.

Figure 6 presents an example of four different F<sub>E</sub>NOgrams obtained from four different subjects at a constant flow rate of 50 ml s<sup>−1</sup>. What is apparent here is that although a phase III NO concentration can be



**Figure 8.** Comparison of 2CM model curves corresponding to two subjects (F and G), one showing the typical monotonically increasing function ('bi-phasic' curve in panel a), and one with an atypical trend at high flow rate (panel b). The dotted cyan line in panel (b) represents the best fit unconstrained 2CM which returns unusable NO parameters (negative values of  $C_A\text{NO}$ ). As in figure 7, each datapoint in both panels is the mean value of the results from the three repeated exhalations for each flow rate. The x- and y-axis error bars represent the statistical spread (standard deviation) of those three measurements.

defined at long times, the forms of the  $F_{\text{ENOgrams}}$  vary markedly between participants. The diagrams clearly encode differences, particularly in the behaviour of  $F_{\text{ENO}}$  in phase I. It should be noted that subject D has mild asthma and is clearly a high NO producer.

As a first validation of our technology we now express some of the results in terms of the well-known 2CM. The variation of  $F_{\text{ENO}}$  with flow rate,  $\dot{V}_E$ , is expressed in this model by [14]:

$$F_{\text{ENO}}(\dot{V}_E) = C_{\text{awNO}} + (C_{\text{A}\text{NO}} - C_{\text{awNO}}) e^{-\frac{D_{\text{awNO}}}{\dot{V}_E}} \quad (1)$$

Here  $C_{\text{awNO}}$  and  $C_{\text{A}\text{NO}}$  are the radially averaged bronchial and alveolar NO fractions respectively, and  $D_{\text{awNO}}$  is the conductance from the airway wall to the lumen. The marked inverse relationship between the value of  $F_{\text{ENO}}$  in phase III and flow rate has been noted previously [49], and this is qualitatively predicted by equation (1). Figure 7(a) shows some exemplary data obtained with the current device for a single participant (subject E), and clearly demonstrates the strong variation of phase III  $F_{\text{ENO}}$  as a function of flow rate.

Another parameter often measured in  $F_{\text{ENO}}$  studies is the NO elimination rate,  $E_{\text{NO}}$  (also known as NO output), defined as the product of  $F_{\text{ENO}}$  and the flow-rate,  $\dot{V}_E$ :

$$E_{\text{NO}} = \dot{V}_E \left( C_{\text{awNO}} + (C_{\text{A}\text{NO}} - C_{\text{awNO}}) e^{-\frac{D_{\text{awNO}}}{\dot{V}_E}} \right) \quad (2)$$

The relevant NO output data for the same participant are reported in the graph in figure 7(b), and in this case show an increase in  $E_{\text{NO}}$  with flow rate, again as reported in earlier publications [11, 51]. Previous studies (for example [52]) have also described that the relation between NO elimination rate  $E_{\text{NO}}$  and flow rate becomes linear at high flow rate (when the exponential in equation (2) can be expanded to two terms), with the limiting slope being equal to

the alveolar NO concentration,  $C_{\text{A}\text{NO}}$ . The fits of the data with the 2CM equations are also shown in figures 7(a) and (b), and allows the subject's flow-independent NO parameters to be established. From figure 7(b), this yields reasonable parameters:  $C_{\text{awNO}} = 63.0 \pm 5.7$  ppb,  $C_{\text{A}\text{NO}} = 2.2 \pm 0.8$  ppb and  $D_{\text{awNO}} = 29.0 \pm 4.4$  ml s<sup>-1</sup>.

What also becomes clear from an analysis of this type is just how sensitive the possible outcomes are to the accuracy of the elimination data. This sensitivity is also highlighted in a recent study by Meriläinen and co-workers [50], where it is apparent that small shifts in  $E_{\text{NO}}$  can lead to different interpretations. The same authors question the universality of the method and clearly show examples where unphysical outcomes are possible. Such fragility is obviously a disadvantage, especially considering these measurements are subject to vagaries of intra-individual variations and individual-specific ergonomics of breath acquisition.

Indeed, in this study, for some individuals the elimination data did not follow an increasing trend at high flow rates; rather than follow the expected linear approximation at high flow rates (subject F), the NO elimination rate data featured a decreasing trend (subject G). Such a data set is shown in figure 8. In this case the unconstrained fit to the 2CM returns negative values for the fraction of alveolar NO, an issue that has also been documented in the literature [50, 53, 54], where researchers encountered that a significant fraction of the sampled population exhibited negative  $C_{\text{A}\text{NO}}$  values, which are obviously not interpretable. Also shown in the figure 8 is the best fit from a constrained model in which the alveolar  $F_{\text{ENO}}$  value is set to be positive. Problems of this sort reinforce the notion that the 2CM of NO production in the respiratory system is not universally applicable for assessing the alveolar and the bronchial NO components. Accurate time-resolved  $F_{\text{ENOgrams}}$  however should be able to provide a more complete map of NO exhalation dynamics and provide categorical insight for identifying whether the NO is originating

from a proximal or peripheral part of the lung. In this preliminary study, this is clearly illustrated in figure 6, where two subjects (C and D) with relatively high phase III  $F_{E}NO$  values have markedly different  $F_{E}NO$ grams.

#### 4. Conclusions and outlook

In this paper we discuss the development and testing of a novel complementary optical sensor incorporating an exhalation-specific deconvolution for the accurate deduction of real time at-mouth NO in human breath with a temporal resolution of 0.1 s (for a concomitant 4.7 ppb ( $2\sigma$ ) limit of detection). We report preliminary data on a small set of healthy subjects in order to elucidate the performance of the DAS-CEAS sensor and illustrate the potential of our technique for determining NO in the earliest stages of the breathing cycle and which may in the future allow non-invasive assessment of airway inflammation.

While various  $F_{E}NO$  sensors already exist, they tend to have limited temporal resolution (e.g. electrochemical sensors) or lack the necessary sophistication with which to properly deduce at-mouth expirograms. As highlighted in a recent review and meta-analysis from Karvonen and Lehtimäki [14], all the flow-independent NO parameters are elevated in asthma as compared to healthy subjects. However, they noted that the reported results were highly varied and the evidence on  $C_{aw}NO$  and  $D_{aw}NO$  is still weak due to the limited number of studies reporting them. These researchers advocate that to gain more knowledge on these parameters in asthma, nonlinear methods and standardized study protocols should be used in future studies. It should be emphasized however that, in some cases, the flow rate dependent 2CM analysis may result in unphysical outcomes: in particular, it may return a negative value for  $C_A NO$ . As noted by Horvath *et al* a negative  $C_A NO$  value is more an indication that the model of NO production of the respiratory system is inadequate [54].

It is certainly evident that assessing  $F_{E}NO$  purely based on the plateau level (or just the final seconds of the exhalation as in the majority of the commercial devices) is a restrictive methodology. Long averaging time or lack of any temporal information simply cannot help physicians to understand the depth of the inflammation along the respiratory tract. Coupling gas exchange modelling to real time breath analysis has already proven to be an effective way to determine detailed physiological information from a single exhalation [28], and in our experience, based on the development and utilisation of the MFS, the additional information encoded in the  $F_{E}NO$  expirograms provided by a device such as presented here, can provide a more complete picture of the NO dynamics and highlights the need for an associated mechanistic model.

From a practical point of view, although the DAS-CEAS device is not handheld, it is a versatile, robust and transportable unit, which requires low maintenance and running costs, making it an attractive tool for extensive clinical studies. This work paves the way for establishing the location of the inflammation in the airways, thus broadening our ability to characterise the airway inflammation. Additionally, we anticipate that the combination of the CEAS sub-unit with a standard MFS sensor will enable physicians to obtain for the first time, *quantitative and individualised* measures of lung inhomogeneity and structure with time-resolved measurements of  $F_{E}NO$ .

We are aware that the current clinical guidelines suggest that a nose clip should not be used (under most circumstances) so as to prevent NO accumulation in the nasal cavity which *may* contaminate the measurement [32]. Velum closure is mandatory and achieved by using a positive pressure of 5–20 cm  $H_2O$  against exhalation. Here, we chose to use a nose clip as we were not sure if the nose would self-seal consistently and to the extent required for precise measurement of cumulative expiratory volume: any loss of air through the nose affects our modelling of the lung inhomogeneity and as we are intending to link  $F_{E}NO$  measurements with person specific measures of lung inhomogeneity we chose to make the measurements under the same protocol as we have previously used (see references [3] and [4]). To facilitate velum closure, exhalation into our device occurs against a positive pressure that is in the range 10–20 cm  $H_2O$ , as confirmed from well calibrated flow and pressure measurements within the DAS cell, and as is common practice in clinical  $F_{E}NO$  measurements we also display pressure or expiratory flow rate to the subject, who is requested to maintain these within a certain range.

The current software for interpretation of MFS data does not incorporate any model of mass balance for NO, nor its spatial distribution within the lung. In the 2CM, the production of NO is evenly distributed through the larger conducting airways. In eosinophilic asthma, the production of NO is greatly enhanced from the sites of inflammation, and the lung is also much more inhomogeneous in terms of gas exchange. To address the issue of inhomogeneity, we will refine this simple model by combining a whole set of such 2CMs so that the volume of the conducting airways and the compliance of the expandable part of the lung follow their statistical distributions. This will allow us to study  $F_{E}NO$  production in the context of an inhomogeneous lung. Most importantly, the model will be constructed such that the particular type and degree of inhomogeneity can be estimated from the gas-exchange data from each individual. To identify the site of the excess  $F_{E}NO$  production in eosinophilic asthma, we will initially introduce single point sources of NO at various fractional volume distances along the airway tree and simulate

the resultant  $F_{E}NO$  signals. From this starting point, we will progressively develop a model of  $F_{E}NO$  production that can be incorporated as an objective function into a non-linear least squares algorithm to identify the source(s) of the additional  $F_{E}NO$  in eosinophilic asthma on top of the sources of  $F_{E}NO$  identified in the healthy lung. Once this is complete, it should finally be possible to address the clinical question of whether the responsiveness to inhaled therapy in eosinophilic asthma is related to site of  $F_{E}NO$  production in the airways. High sensitivity spectroscopic measurements of the type presented here, that are suitable for elucidating the time-resolved at-mouth  $F_{E}NO$ , are an essential and integral part of this progression.

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## Ethics

The work conformed with the general principles of the Declaration of Helsinki and was approved by the South Central Oxford A Research Ethics Committee (17/SC/0172).

## Conflicts of interest

The authors declare no competing interests.

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