



A suitable protocol for measuring alveolar nitric oxide in asthma with differing severity to assess peripheral airways inflammation

Zsófia Lázár, Péter Horváth, Rita Puskás, Gabriella Gálffy, György Losonczy, Ildikó Horváth & András Bikov

To cite this article: Zsófia Lázár, Péter Horváth, Rita Puskás, Gabriella Gálffy, György Losonczy, Ildikó Horváth & András Bikov (2019) A suitable protocol for measuring alveolar nitric oxide in asthma with differing severity to assess peripheral airways inflammation, Journal of Asthma, 56:6, 584-593, DOI: [10.1080/02770903.2018.1477957](https://doi.org/10.1080/02770903.2018.1477957)

To link to this article: <https://doi.org/10.1080/02770903.2018.1477957>



Accepted author version posted online: 20 Jun 2018.
Published online: 30 Oct 2018.



Submit your article to this journal [↗](#)



Article views: 71



View Crossmark data [↗](#)

DIAGNOSIS



A suitable protocol for measuring alveolar nitric oxide in asthma with differing severity to assess peripheral airways inflammation

Zsófia Lázár, MD, PhD^a, Péter Horváth, MD, PhD^a, Rita Puskás, MD^a, Gabriella Gálffy, MD, PhD^a, György Losonczy, MD, PhD^a, Ildikó Horváth, MD, PhD^b, and András Bikov, MD, PhD^a

^aDepartment of Pulmonology, Semmelweis University, 1/c Diós árok; ^bNational Korányi Institute of Pulmonology, 1 Pihenő Street, Budapest, Hungary

ABSTRACT

Objective: Extended nitric oxide (NO) analysis offers the partitioned monitoring of inflammation in central and peripheral airways. Different mathematical models are used to estimate pulmonary NO dynamics in asthma with variable results and limitations. We aimed to establish a protocol for extended NO analysis in patients with differing asthma severity. **Methods:** Forty patients with stable asthma and 25 matched control subjects were recruited. Exhaled NO was measured at constant flow rates between 10 and 300 mL/s. Twelve controls performed NO measurements weekly for 4 weeks. **Results:** The proportions of patients with technically acceptable measurements at 10–30–50–100–150–200–250–300 mL/s exhalation flow rates were 8–58–100–98–98–95–90–80%, respectively. Alveolar NO (CANO) and total flux of NO in the conducting airways (JawNO) were calculated with the linear method from NO values measured at 100–150–200–250 mL/s exhalation flows. The mean intrasubject bias for JawNO and CANO in controls was 0.16 nL/s and 0.85 ppb, respectively. Both JawNO (1.31/0.83–2.97/ vs. 0.70/0.54–0.87/nL/s, $p < 0.001$) and CANO (4.08/2.63–7.16/ vs. 2.42/1.83–2.89/ppb, $p < 0.001$) were increased in patients with asthma compared to controls. In patients, CANO correlated with RV/TLC ($r = 0.58$, $p < 0.001$), FEF_{25–75%} ($p = 0.02$, $r = -0.36$) and DL,CO ($r = -0.46$, $p = 0.004$). JawNO was not related to lung function parameters. **Conclusions:** Calculation of alveolar NO concentration with the linear method from values obtained at medium flow rates (100–250 mL/s) is feasible even in asthmatic patients with severe airflow limitation and may provide information on small airways dysfunction in asthma.

ARTICLE HISTORY

Received 23 February 2018
Revised 30 April 2018
Accepted 13 May 2018

KEYWORDS

Airway inflammation;
exhaled biomarker;
extended nitric oxide
analysis; disease
monitoring; severe asthma

Introduction

Bronchial asthma is a heterogeneous disease characterised by accumulation of inflammatory cells in the airways, hypertrophy and hyperplasia of airway smooth muscle layer, increased mucus production, and airway wall remodelling [1]. Inflammatory changes take place simultaneously in central and distal airways and in the alveoli [2–4]. The assessment of airway inflammation at these different localizations can aid better understanding of disease pathomechanism and facilitate the development of more targeted therapies.

Airway inflammation can be studied noninvasively by measuring the exhaled nitric oxide (NO) concentration. The two-compartment model allows the evaluation of NO dynamics in the large central or bronchial and in more distal airways (small airways

and the alveolar/acinar region) [5,6]. For this purpose, measurements are performed at multiple expiratory flow rates (10–500 mL/s) and mathematical models are applied [6]. The recent technical standard task force report of the European Respiratory Society recommends the use of NO plateau values measured at least at three different exhalation flows and proposes several mathematical equations to calculate bronchial and alveolar NO parameters [7]. However, there are currently no standardised method that can reliably be applied to asthmatic patients with varying airflow limitation, smoking status and disease severity.

Therefore, in this study we aimed to establish a feasible method for the partitioned measurement of exhaled NO in asthma. Patients and control subjects carried out expiratory manoeuvres at a broad range of flows. We established a protocol for the calculation of

central bronchial and peripheral airway NO parameters with low week-to-week variation. Furthermore, we compared central and peripheral airway inflammation between patients and control subjects and correlated NO variables to clinical parameters in asthma.

Materials and methods

Subjects

Patients were recruited at the Outpatient Clinic of Department of Pulmonology at Semmelweis University, Budapest, Hungary. They complained of symptoms consistent with the asthma diagnosis, they showed positivity for at least one airway allergen at skin prick testing or serum specific IgE testing. Patients presented documented airflow limitation, and they had airway hyperresponsiveness or were positive for bronchodilator reversibility during prior testing [1]. A change in asthma therapy was not required in 4 weeks prior to the recruitment. Main exclusion criteria were other chronic respiratory diseases, asthma exacerbation in the previous 4 weeks, and signs of acute respiratory infections in the 2 weeks before recruitment. Healthy control subjects were recruited among employees working at the Department. Main exclusion criteria for controls were allergic airway disease or chronic respiratory disease in history, systemic steroid or antibiotic treatment in the previous 4 weeks, and signs of acute respiratory infections in the previous 2 weeks. Patient and control subjects were considered ex-smokers if they had stopped smoking at least 6 months before inclusion. The study was approved by the ethics committee, and all procedures were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study. Measurements were performed between 10 am and 3 pm from 1 December 2015 until 30 November 2017.

Study design

Clinical study

Twenty-five control subjects and 40 patients with asthma were recruited. Medical history was noted, blood leukocyte count and CRP concentration were measured, exhaled nitric oxide concentration was recorded and lung function tests were performed. Exhaled nitric oxide measurements were performed before lung function tests in all cases. Furthermore, patients filled out the Asthma Control Test (ACT) [8].

Repeatability study

Exhaled NO measurements were repeated at a weekly basis for 4 weeks in 12 control subjects, who also participated in the clinical study.

Nitric oxide measurements at multiple constant exhalation flow rates

Subjects were asked not to use inhaled medication, refrain from eating, drinking and smoking 2 h prior to measurement. Exhaled NO concentration was measured during a manoeuvre starting from total lung capacity at the expiratory flows of 10–30–50–100–150–200–250–300 mL/s with a chemiluminescent analyser (Sievers Nitric Oxide Analyzer i280, GE Analytical Instruments, Boulder, CO). Instrument calibration was performed daily according to the manufacturer's instructions. The background NO concentration was <5 ppb. Restrictors, as provided and calibrated by the manufacturer, were applied to generate the required expiratory flows and ensure the closure of the velum during expiration. Manoeuvres at different flows with a duration of ≥ 20 s (10 mL/s exhalations flow), ≥ 10 s (30 mL/s), ≥ 6 s (50 and 100 mL/s) and ≥ 5 s (150, 200, 250 and 300 mL/s) were considered sufficient. Subjects received visual feedback of the expiratory flow during the entire manoeuvre. Plateau values of NO recordings were identified manually. Recordings corresponding to the initial expiratory volume of 150 mL air (i.e. anatomic dead space) were disregarded. We considered a recording technically acceptable and valid if the plateau NO concentration was in a 3-s window with minimal sloping [9] where the actual exhalation flow was $\pm 10\%$ of the target rate in compliance with the recommendations for FENO₅₀ (fractional exhaled nitric oxide concentration at 50 mL/s exhalation flow) analysis. The mean values of two NO recordings with <10% difference were used for further calculations.

Calculation of CANO and JawNO

Data were analysed based on the two-compartment model using the linear method of Tsoukias et al. [5–7], which estimates acinar/alveolar NO (CANO) as the measure of the distal airways and total flux of NO in the conducting airway compartment (JawNO) as a marker of central airways.

Other variables

Leukocyte count (Sysmex XE-2100, Sysmex Corporation, Kobe, Japan) and serum CRP concentration (Beckman

Coulter AU680, Beckman Coulter Inc., Indianapolis, IN) were determined from venous blood samples (asthma: $N=38$, control $N=25$). Measurements for spirometry, body plethysmography and diffusion capacity were performed according to current guidelines (PDT-111, Piston, Budapest, Hungary) [10–12]. Two patients with asthma could not perform the manoeuvre for diffusion capacity measurement. An ACT score ≤ 19 referred to uncontrolled asthma [8].

Statistical analysis

Demographic data were compared with unpaired t -test and expressed as mean \pm standard deviation, categorical variables were compared with the Fisher's exact test. Inhaled corticosteroid (ICS) doses, blood eosinophil percentage, CRP value, $FENO_{50}$, $JawNO$ and $CANO$ data did not show a normal distribution (D'Agostino-Pearson normality test), therefore, these variables were analysed with nonparametric tests (Mann–Whitney, Kruskal–Wallis with Dunn's post hoc and Spearman tests) and expressed as median/interquartile range/. Measurement repeatability was assessed using the Bland–Altman plot [13]. $p < 0.05$ was considered significant (GraphPad Prism 5.0, GraphPad Software, San Diego, CA). Multiple regression analysis with smoking habits as covariates were used to assess relationship between $CANO$ and lung function measures (Statistica 13.2, StatSoft, Tulsa, OK).

The sample size of the clinical study was calculated to reach a statistical power ($1-\beta$) of 0.80 and effect size of 0.75 with respect to the asymptotic relative efficiency of nonparametric tests. This effect size was based on the variability of $JawNO$ and $CANO$ data in the repeatability study.

Results

Subject characteristics

The main clinical characteristics of patients and control subjects are shown in Table 1. Patients were treated at treatment steps of GINA 1 (steroid-naïve, $n=7$), GINA 3–4 (moderate-severe, $n=16$) and GINA 5 (severe on anti-IgE therapy, $n=17$) [1]. Forty percent of patients had uncontrolled asthma according to the ACT scores.

Measurements of exhaled NO at different constant exhalation flow rates

Subjects performed exhalation manoeuvres at various constant flows (10–300 mL/s) both in the repeatability

Table 1. Patient characteristics.

	Control, $N=25$	Asthma, $N=40$	p Value
Male/Female, N (%)	7/18 (28%/72%)	11/29 (27.5%/72.5%)	1.00
Age, years	39 ± 14	44 ± 17	0.17
Smoking status			0.10
Nonsmoker, N (%)	17 (68%)	28 (70%)	
Ex-smoker, N (%)	3 (12%)	10 (25%)	
Current smoker, N (%)	5 (20%)	2 (5%)	
Pack-years	24 ± 7	18 ± 10	0.14
Blood eosinophil, %	1.4/0.7–2.7/	4.2/2.2–5.8/	<0.001
CRP, mg/L	2.0/1.0–4.8/	2.6/1.7–5.1/	0.47
FEV ₁ , %ref.	103 ± 10	79 ± 18	<0.001
FVC, %ref.	107 ± 13	95 ± 14	0.002
FEV ₁ /FVC	0.82 ± 0.06	0.70 ± 0.13	<0.001
FEF _{25–75%} , %ref.	93 ± 18	54 ± 28	<0.001
RV/TLC, %	0.30 ± 0.21	0.33 ± 0.14	0.53
KCO, %ref.	97 ± 19	98 ± 17	0.89
DLCO, %ref.	114 ± 20	112 ± 16	0.65
ICS, N (%)	NA	33 (82.5%)	NA
ICS dose, budesonide equivalent μ g	NA	800/340–800/	NA
LABA, N (%)	NA	31 (77.5%)	NA
LAMA, N (%)	NA	3 (7.5%)	NA
LTRA, N (%)	NA	14 (35%)	NA
Oral theophylline, N (%)	NA	3 (7.5%)	NA
Anti-IgE treatment, N (%)	NA	17 (42.5%)	NA

Data are presented as mean \pm standard deviation or median/interquartile range/and compared with unpaired t -test, Mann–Whitney or chi-square tests (categorical variables).

CRP: C-reactive protein; DLCO: diffusion capacity of the lung for carbon monoxide; ICS: inhaled corticosteroid; IgE: immunoglobulin E; FEF_{25–75%}: forced expiratory flow at 25–75% of vital capacity; FEV₁: forced expiratory volume in 1 s; FVC: forced vital capacity; KCO: transfer coefficient of the lung for carbon monoxide; LABA: long-acting β_2 -agonist; LAMA: long-acting muscarinic antagonist; LTRA: leukotriene receptor antagonist; N : number; NA: not applicable; ref.: reference; RV: residual volume; TLC: total lung capacity.

Table 2. Percentage of technically acceptable exhaled NO manoeuvres with valid recordings at different exhalation flow rates.

Exhalation flow, mL/s	10	30	50	100	150	200	250	300
Control subjects	20%	79%	100%	100%	100%	100%	98%	98%
Patients with asthma	8%	58%	100%	98%	98%	95%	90%	80%

and clinical studies (number of measurements/flow in controls subjects: 61, in patients with asthma: 40; Table 2). Only a fraction of patients could perform a manoeuvre with a technically acceptable recording at very low flow rates (<50 mL/s), while the majority of manoeuvres were technically correct at higher flows (>50 mL/s). Therefore, for the extended NO analysis only the linear model could be applied [7], and NO values obtained at 100, 150, 200 and 250 mL/s expiratory flow rates were used as inputs for the calculation of $JawNO$ and $CANO$ (asthma: $r=0.98 \pm 0.03$, control: $r=0.98 \pm 0.02$). As four patients could not perform the manoeuvre at 250 mL/s exhalation flow, values at 300 mL/s were included in the model in three cases. Using this strategy, 92.5% of all calculations in asthma were executed on data at 4 flow

points (2.5% at 3 flow rates, 5% at 2 flow rates). Data obtained at 4 flow rates were used to calculate *JawNO* and *CANO* in each control volunteer. All subjects could perform valid manoeuvres for *FENO*₅₀. Exhaled NO concentrations were elevated in asthma at all flow rates between 50 and 250 mL/s ($p < 0.001$, Figure 1).

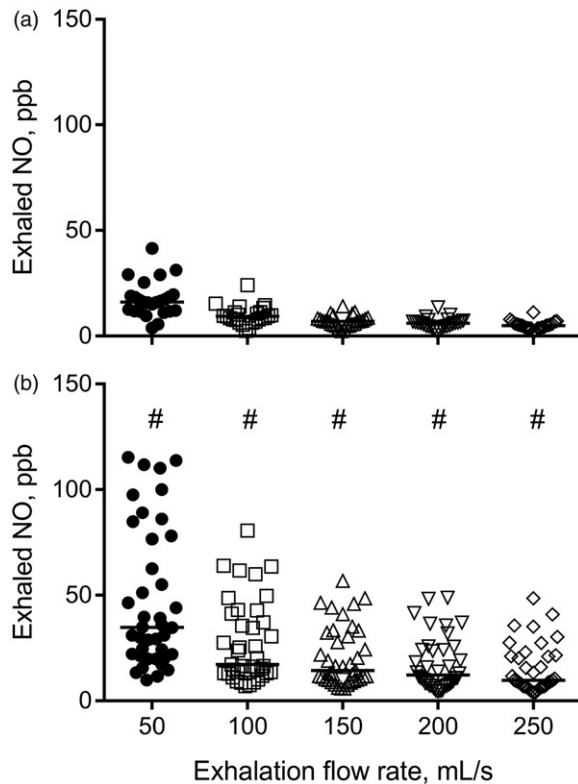


Figure 1. Exhaled NO concentrations at multiple flow rates. Exhaled NO at different constant exhalation flows in controls (a) and patients with asthma (b). Lines show median values. Mann–Whitney test: # $p < 0.001$ compared to corresponding NO concentration in controls.

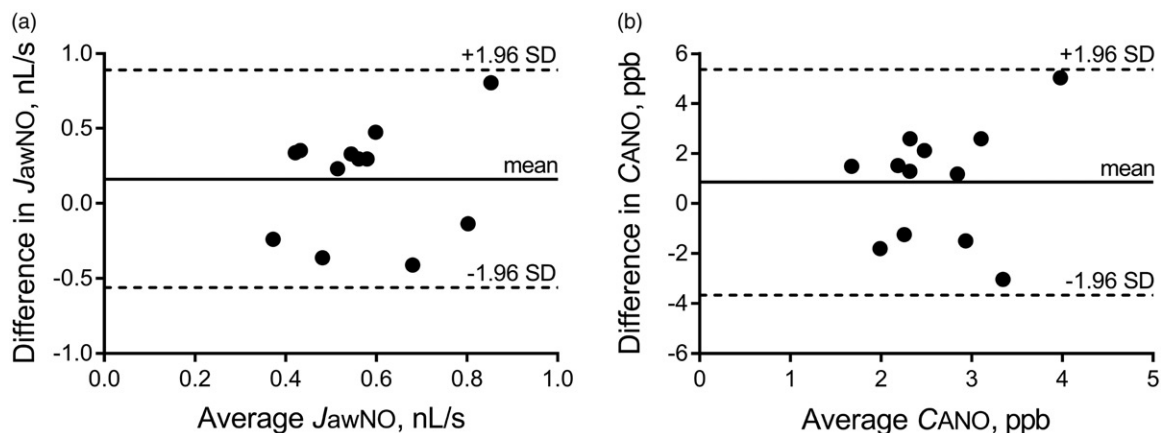


Figure 2. Intrasubject repeatability of *JawNO* and *CANO*. The weekly intrasubject repeatability of total flux of NO in the conducting airway compartment (*JawNO*; a) and alveolar nitric oxide concentration (*CANO*; b) in control subjects as shown by the Bland–Altman plot with mean difference and limits of agreement (mean difference ± 1.96 standard deviation).

Intrasubject repeatability of *JawNO* and *CANO*

Weekly *JawNO* values in control subjects were 0.73/0.59–0.73/, 0.51/0.41–0.67/, 0.58/0.50–0.73/ and 0.50/0.39–0.68/nL/s. The Bland–Altman analysis for the lowest and highest individual values showed a mean difference of 0.16 nL/s (95% limits of agreement: -0.56 – 0.89 nL/s; Figure 2a). *CANO* values at the weekly measurements were 2.64/2.25–3.08/, 3.19/2.30–4.22/, 1.75/1.17–3.12/ and 2.27/1.89–2.86/ppb. The Bland–Altman graph for the lowest and highest individual values demonstrated a mean bias of 0.85 ppb (95% limits of agreement: -3.67 – 5.36 ppb; Figure 2b).

Increased *JawNO* and *CANO* in patients with asthma

JawNO was increased in patients with asthma compared to control subjects (1.31/0.83–2.97/nL/s vs. 0.70/0.54–0.87/nL/s, $p < 0.001$; Figure 3a). In asthma, *JawNO* showed a strong positive correlation with *FENO*₅₀ ($p < 0.001$, $r = 0.94$; Figure 3b) and blood eosinophil percentage ($p = 0.001$, $r = 0.50$), but not with lung function parameters, leukocyte count, CRP or age ($p > 0.05$).

Alveolar NO concentration was higher in patients than in controls (4.08/2.63–7.16/ppb vs. 2.42/1.83–2.89/ppb, $p < 0.001$; Figure 4a). In asthma, *CANO* concentration positively correlated with blood eosinophil percentage ($p = 0.002$, $r = 0.50$), CRP concentration ($p = 0.001$, $r = 0.50$), age ($p = 0.003$, $r = 0.46$), *RV/TLC* ($p < 0.001$, $r = 0.58$; Figure 4c) and airway resistance ($p = 0.02$, $r = 0.38$). It inversely correlated with *FEV*₁% predicted ($p = 0.03$, $r = -0.34$), *FEF*_{25–75%} % reference ($p = 0.02$, $r = -0.36$; Figure 4b)

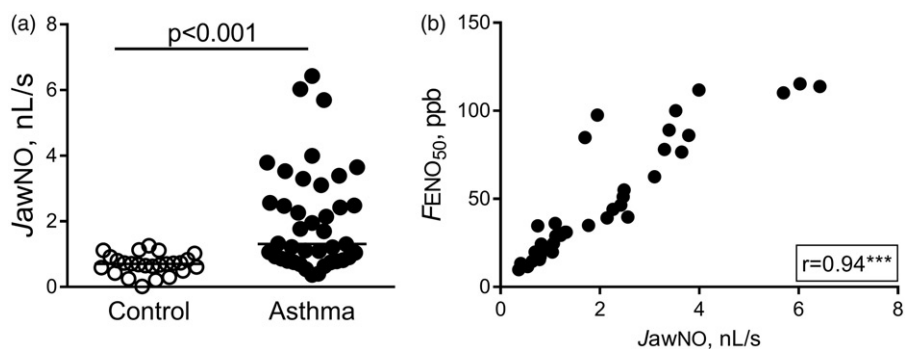


Figure 3. JawNO in patients with asthma. JawNO was increased in asthma (Mann–Whitney test; a), and strongly correlated with FENO₅₀ (b). Spearman correlation: *** $p < 0.001$.

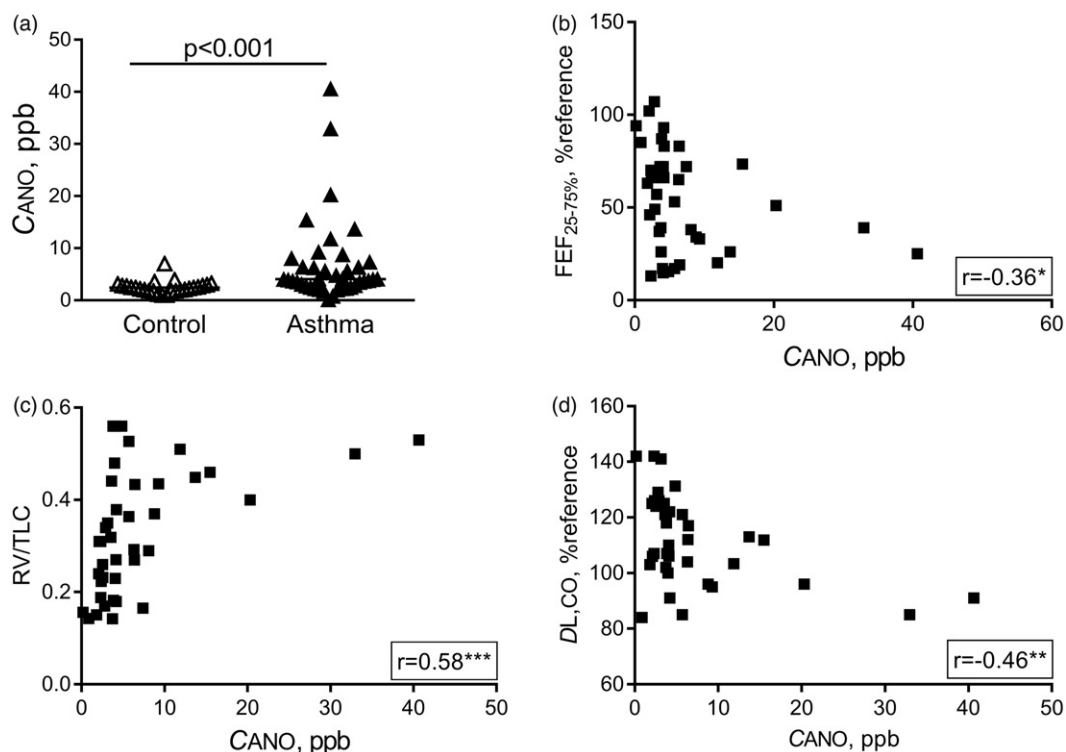


Figure 4. CANO in patients with asthma. CANO was increased in asthma (Mann–Whitney test; a), and correlated with FEF_{25–75%} % reference (b), RV/TLC (c) and DL,CO (d). Spearman correlation: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

and diffusion capacity of the lung for carbon monoxide ($p = 0.004$, $r = -0.46$; Figure 4d). We found a significant positive correlation between CANO and JawNO ($p = 0.005$, $r = 0.44$), and between CANO and FENO₅₀ ($p < 0.001$, $r = 0.60$).

The association of CANO to RV/TLC and DL,CO remained significant in patients with asthma when using multiple regression analysis with smoking status (beta = 0.48, $p = 0.003$ and beta = -0.45, $p = 0.005$) or packyears (beta = 0.46, $p = 0.007$ and beta = -0.43, $p = 0.007$) as a covariate. However, the relationship between CANO and FEF_{25–75%} % reference became statistically insignificant in a model controlled for smoking status ($p = 0.09$) or packyears ($p = 0.14$).

The effect of smoking status on NO parameters

We also analysed nonsmoking and ex-/current smoking control subjects and patients in separate subgroups. There was an increase in JawNO in patients compared to controls with relevant smoking status (control vs. asthma in nonsmokers: 0.68/0.61–0.78/nL/s vs. 1.22/0.79–2.52/nL/s, $p = 0.001$ and in smokers: 0.75/0.26–1.00/nL/s vs. 1.64/0.95–0.3.67/nL/s, $p = 0.009$; Figure 5a). Likewise, CANO was higher in asthma than in control subgroups (control vs. asthma in nonsmokers: 2.48/1.83–3.04/ppb vs. 3.75/2.46–6.35/ppb, $p = 0.04$ and in smokers: 2.04/1.82–2.66/nL/s vs. 5.70/3.86–13.81/ppb, $p = 0.001$; Figure 5b). We found

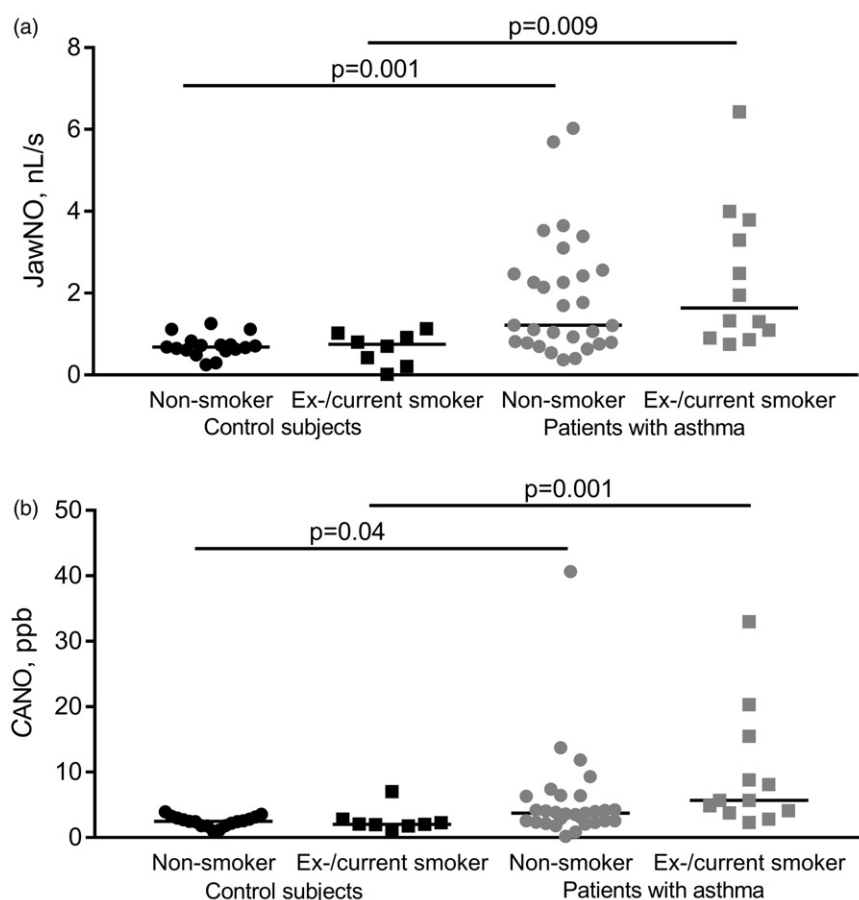


Figure 5. NO parameters in smokers and nonsmokers. JawNO (a) and CANO (b) were increased in ex- and current smoking patients with asthma compared to corresponding control subjects (Kruskal–Wallis with Dunn’s post hoc test).

no difference in JawNO and CANO between non-smokers and smokers within the control ($p=0.99$ and $p=0.99$) and asthmatic ($p=0.99$ and $p=0.23$) groups.

Discussion

The inflammation and dysfunction of small airways are related to important clinical aspects of asthma such as airway hyper-responsiveness [14] or exacerbation risk [15], therefore, targeted anti-inflammatory treatment which can mitigate small airways inflammation can convey clinical benefit. However, the noninvasive assessment of distal lung inflammation is an unmet need in clinical practice. Models using exhaled NO concentration measured at different flow rates allow the partitioned assessment of airway inflammation in central and distal airways. The European Respiratory Society technical standard document provides details for mathematical modelling of pulmonary NO dynamics and highlights the need for further studies [16]. In this study, we presented a feasible protocol for alveolar NO measurement and showed

that inflammation and dysfunction of small airways are related in asthma.

In our study, patients performed exhalation manoeuvres at constant flow rates between 10 and 300 mL/s, but we could measure exhaled NO concentrations at low flow rates only in a minority of patients with asthma. Similarly, Gelb et al. also noted that measurements were not reproducible at low exhalation flows (≤ 50 mL/s) in asthmatics with $FEV_1 < 80\%$ predicted [17], representing a significant number of treated patients in clinical settings. In addition, we also observed some failure in manoeuvre performance at 300 mL/s exhalation flow, which questions the feasibility of applying very high flow rates in this population. Up to 30% of patients with severe asthma had to be excluded from previous studies due to negative CANO values, suggesting inadequate models and the requirement of additional flow rates [18,19]. However, we established a linear model based on exhaled NO values at four flow points (100, 150, 200 and 250 mL/s), which could be successfully and reliably applied to measure alveolar NO concentrations in patients with differing asthma severity and smoking history.

We confirm previous findings that alveolar NO concentration is increased in a mixed group of asthmatic patients with mild-to-severe disease compared to matched control subjects [17]. Other studies also showed that peripheral airway inflammation, as reflected by CANO, is elevated in clinically important phenotypes of asthma: in patients with refractory asthma on high ICS dose compared to mild-to-moderate asthma [20], in patients with steroid-dependent severe asthma compared to severe asthmatics on high dose ICS [19] and in subjects with nocturnal symptoms [21]. Several authors observed that alveolar NO concentration could not be modified by initiating ICS therapy or increasing its dose [22,23]. While a decrease in CANO was reported after oral steroid therapy in some studies [17,20], interestingly, others found no treatment effect [23]. This suggests that despite ongoing anti-inflammatory treatment, increased inflammation in peripheral airways is a distinct disease characteristic in certain asthma phenotypes, which is also steroid-resistant in some cases. Hence, the extended NO analysis might facilitate the identification and better understanding of asthma subgroups, and it can also aid monitoring of novel anti-inflammatory therapies.

We analysed the relationship between alveolar NO concentration and physiological measures of distal lung dysfunction. We reported a moderate correlation between CANO and RV/TLC, which is a known marker of air trapping and hyperinflation in severe and nonsevere asthma [24]. This finding extends the results of a previous study that showed a similar, but stronger correlation between the two parameters in severe asthma [18]. In our study, there was a weak correlation between CANO and $FEF_{25-75\%}$, which was not present when the analysis was controlled for smoking. This lung function parameter is debated to truly reflect peripheral airways dysfunction, partly due to its high measurement variability [25]. Likewise, one study described a correlation with similar strength in mild-to-moderate asthma [26], but others found no relationship between alveolar NO concentration and $FEF_{25-75\%}$ in mild-to-severe asthmatics [19]. Interestingly, in our cohort CANO moderately correlated to pulmonary diffusion capacity, as previously observed in alveolitis [27]. Besides the upregulation of the inducible NO synthase in alveolar epithelial cells, the decreased diffusion of NO might be another mechanism leading to increased CANO in asthma, nonetheless, it must be noted that pulmonary diffusion capacity was within the normal range in patients. Despite of the exploratory nature of these results, they

imply that increased CANO can reflect distal airways dysfunction in asthma.

It was previously shown that alveolar NO concentration is strongly associated with eosinophil percentage in bronchoalveolar lavage fluid in mild asthmatics [20]. However, the weak-to-moderate correlation between CANO and blood eosinophil percentage in our study suggests that inflammation in peripheral airways is not closely related to systemic eosinophilic inflammation, as already shown for $FENO_{50}$ [28].

We reported a positive correlation between age and CANO, which was also observed in another cohort of asthmatic patients [29]. It is known that CANO increases with age in healthy subjects, which could be explained by the reduced pulmonary NO diffusion resulting from decreased capillary blood volume at an older age [30]. It can be speculated that this mechanism might also be present in older patients with asthma.

We found a close correlation between $FENO_{50}$ and $JawNO$ in asthma highlighting that these parameters assess inflammation at similar sites within the airways, and the additional information gained by the calculation of $JawNO$ might be limited as also suggested by others [31].

The weekly repeatability of CANO and $JawNO$ in control subjects was assessed in a 1-month period, relevant to clinical settings for asthma follow-ups. The intrasubject repeatability of these parameters was somewhat better in a previous study using a day-to-day setup [19], nevertheless, the observed difference between controls and patients exceeded the mean intrasubject bias.

Some authors correct alveolar NO for trumpet model and axial NO back-diffusion [32,33]. However, these formulae disregard the effect of central airways constriction on axial back-diffusion, which potentially result in overcorrection [34,35]. According to the recent technical standard document the use of correction factors for axial back diffusion is not recommended [7]. Therefore, we did not apply any correction in our data analysis.

This study has limitations. Cigarette smoking is known to interfere with exhaled nitric oxide concentration [36], and smoking had been previously shown to decrease CANO in asthma [37], which was not confirmed by our findings. Importantly, CANO was elevated in asthma, irrespective of the smoking status. Our cohort reflects realistic asthmatic population in terms of smoking habits, as approximately one third of asthmatics were also reported to be current or former smokers in larger cohorts [38,39]. Cigarette

smoking in asthma results in greater morbidity, uncontrolled and more severe disease, and accelerated decline in lung function [40]. In addition, a recent publication described that one third of patients with severe asthma were active or former smokers, who presented with fixed airflow limitation and clustered into clinical subgroups with either Th2-high or Th2-low signatures, underlining the potential therapeutic relevance of measuring inflammatory markers including exhaled NO in these populations [41]. Furthermore, this is a single-centre observational study, and our results should be validated by future investigations. The cross-sectional nature of the NO measurements does not allow to draw conclusions regarding the repeatability of CANO in asthma, or how it reflects disease course or therapeutic interventions.

Conclusions

The present study describes a feasible protocol for extended NO analysis to calculate alveolar nitric oxide concentration, a marker of distal lung inflammation. Our method can successfully and reliably be applied to patients with asthma of differing severity including those with severe disease. Alveolar NO concentration shows a weak correlation to physiological measures of small airways dysfunction in asthma. The application of our protocol could facilitate understanding the role of CANO in phenotyping asthma.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. This study was funded by the Bolyai Research Grant of the Hungarian Academy of Sciences (BO/00559/16 to Dr. Zsófia Lázár, BO/00262/15 to Dr. András Bikov), the Scientific Grant of the Hungarian Respiratory Society (MPA/2014 to Dr. Zsófia Lázár) and the Hungarian National Research, Development and Innovation Fund's (PuBioRep research project to Prof. György Losonczy).

Acknowledgements

We thank the kind participation of patients and control volunteers in this study. The authors are thankful to Mr. Sándor Nyágúj for measuring lung function. We would like to acknowledge the assistance of Dr. Anikó Bohács and Dr. Ibolya Czaller in patient recruitment.

Funding

This study was funded by the Bolyai Research Grant of the Hungarian Academy of Sciences (BO/00559/16 to Dr. Zsófia Lázár, BO/00262/15 to Dr. András Bikov), the Scientific Grant of the Hungarian Respiratory Society (MPA/2014 to Dr. Zsófia Lázár) and the Hungarian National Research, Development and Innovation Fund's (PuBioRep research project to Prof. György Losonczy).

References

1. Global Strategy for Asthma Management and Prevention. Available from: <http://www.ginaasthma.org> [last accessed 15 Dec 2016].
2. Hamid Q, Song Y, Kotsimbos TC, Minshall E, Bai TR, Hegele RG, Hogg JC. Inflammation of small airways in asthma. *J Allergy Clin Immunol* 1997;100:44–51. doi:10.1016/S0091-6749(97)70193-3
3. Minshall EM, Hogg JC, Hamid QA. Cytokine mRNA expression in asthma is not restricted to the large airways. *J Allergy Clin Immunol* 1998;101:386–390. doi:10.1016/S0091-6749(98)70252-0
4. Kraft M, Djukanovic R, Wilson S, Holgate ST, Martin RJ. Alveolar tissue inflammation in asthma. *Am J Respir Crit Care Med* 1996;154:1505–1510. doi:10.1164/ajrccm.154.5.8912772
5. Tsoukias NM, George SC. A two-compartment model of pulmonary nitric oxide exchange dynamics. *J Appl Physiol* (1985) 1998;85:653–666. doi:10.1152/jappl.1998.85.2.653
6. George SC, Hogman M, Permutt S, Silkoff PE. Modeling pulmonary nitric oxide exchange. *J Appl Physiol* (1985) 2004;96:831–839. doi:10.1152/japplphysiol.00950.2003
7. Horvath I, Barnes PJ, Loukides S, Sterk PJ, Hogman M, Olin AC, Amann A, et al. A European Respiratory Society technical standard: exhaled biomarkers in lung disease. *Eur Respir J* 2017;49:1600965. doi:10.1183/13993003.00965-2016
8. Nathan RA, Sorkness CA, Kosinski M, Schatz M, Li JT, Marcus P, Murray JJ, Pendergraft TB. Development of the asthma control test: a survey for assessing asthma control. *J Allergy Clin Immunol* 2004;113:59–65. doi:10.1016/j.jaci.2003.09.008
9. American Thoracic Society; European Respiratory Society. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. *Am J Respir Crit Care Med* 2005;171:912–930. doi:10.1164/rccm.200406-710ST
10. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, Crapo R, et al. Standardisation of spirometry. *Eur Respir J* 2005;26:319–338. doi:10.1183/09031936.05.00034805
11. Wanger J, Clausen JL, Coates A, Pedersen OF, Brusasco V, Burgos F, Casaburi R, et al. Standardisation of the measurement of lung volumes. *Eur Respir J* 2005;26:511–522. doi:10.1183/09031936.05.00035005
12. Macintyre N, Crapo RO, Viegi G, Johnson DC, van der Grinten CP, Brusasco V, Burgos F, et al. Standardisation of the single-breath determination of

- carbon monoxide uptake in the lung. *Eur Respir J* 2005;26:720–735. doi:10.1183/09031936.05.00034905
13. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;327:307–310. doi:10.1016/S0140-6736(86)90837-8
 14. Zeidler MR, Goldin JG, Kleerup EC, Kim HJ, Truong DA, Gjertson DW, Kennedy NJ, et al. Small airways response to naturalistic cat allergen exposure in subjects with asthma. *J Allergy Clin Immunol* 2006;118:1075–1081. doi:10.1016/j.jaci.2006.06.042
 15. in 't Veen JC, Beekman AJ, Bel EH, Sterk PJ. Recurrent exacerbations in severe asthma are associated with enhanced airway closure during stable episodes. *Am J Respir Crit Care Med* 2000;161:1902–1906. doi:10.1164/ajrccm.161.6.9906075
 16. Roos AB, Mori M, Gronneberg R, Osterlund C, Claesson HE, Wahlstrom J, Grunewald J, et al. Elevated exhaled nitric oxide in allergen-provoked asthma is associated with airway epithelial iNOS. *PLoS One* 2014;9:e90018. doi:10.1371/journal.pone.0090018
 17. Gelb AF, Taylor CF, Nussbaum E, Gutierrez C, Schein A, Shinar CM, Schein MJ, et al. Alveolar and airway sites of nitric oxide inflammation in treated asthma. *Am J Respir Crit Care Med* 2004;170:737–741. doi:10.1164/rccm.200403-408OC
 18. van Veen IH, Sterk PJ, Schot R, Gauw SA, Rabe KF, Bel EH. Alveolar nitric oxide versus measures of peripheral airway dysfunction in severe asthma. *Eur Respir J* 2006;27:951–956. doi:10.1183/09031936.06.00087905
 19. Brindicci C, Ito K, Barnes PJ, Kharitonov SA. Differential flow analysis of exhaled nitric oxide in patients with asthma of differing severity. *Chest* 2007;131:1353–1362. doi:10.1378/chest.06-2531
 20. Berry M, Hargadon B, Morgan A, Shelley M, Richter J, Shaw D, Green RH, et al. Alveolar nitric oxide in adults with asthma: evidence of distal lung inflammation in refractory asthma. *Eur Respir J* 2005;25:986–991. doi:10.1183/09031936.05.00132404
 21. Lehtimäki L, Kankaanranta H, Saarelainen S, Turjanmaa V, Moilanen E. Increased alveolar nitric oxide concentration in asthmatic patients with nocturnal symptoms. *Eur Respir J* 2002;20:841–845. doi:10.1183/09031936.02.00202002
 22. Lehtimäki L, Kankaanranta H, Saarelainen S, Turjanmaa V, Moilanen E. Inhaled fluticasone decreases bronchial but not alveolar nitric oxide output in asthma. *Eur Respir J* 2001;18:635–639. doi:10.1183/09031936.01.00000201
 23. Williamson PA, Short PM, Vaidyanathan S, Lipworth BJ. Inhaled and systemic corticosteroid response in severe asthma assessed by alveolar nitric oxide: a randomized crossover pilot study of add-on therapy. *Br J Clin Pharmacol* 2013;75:93–102. doi:10.1111/j.1365-2125.2012.04319.x
 24. Sorkness RL, Blecker ER, Busse WW, Calhoun WJ, Castro M, Chung KF, Curran-Everett D, et al. Lung function in adults with stable but severe asthma: air trapping and incomplete reversal of obstruction with bronchodilation. *J Appl Physiol* 2008;104:394–403. doi:10.1152/jappphysiol.00329.2007
 25. Burgel PR. The role of small airways in obstructive airway diseases. *Eur Respir Rev* 2011;20:023–033. doi:10.1183/09059180.00010410
 26. Fujisawa T, Yasui H, Akamatsu T, Hashimoto D, Enomoto N, Inui N, Nakamura Y, et al. Alveolar nitric oxide concentration reflects peripheral airway obstruction in stable asthma. *Respirology* 2013;18:522–527. doi:10.1111/resp.12031
 27. Lehtimäki L, Kankaanranta H, Saarelainen S, Hahtola P, Jarvenpää R, Koivula T, Turjanmaa V, Moilanen E. Extended exhaled NO measurement differentiates between alveolar and bronchial inflammation. *Am J Respir Crit Care Med* 2001;163:1557–1561. doi:10.1164/ajrccm.163.7.2010171
 28. Malinowski A, Janson C, Borres M, Alving K. Simultaneously increased fraction of exhaled nitric oxide levels and blood eosinophil counts relate to increased asthma morbidity. *J Allergy Clin Immunol* 2016;138:1301–1308 e2.
 29. Matsumoto H, Niimi A, Jinnai M, Nakaji H, Takeda T, Oguma T, Otsuka K, et al. Association of alveolar nitric oxide levels with pulmonary function and its reversibility in stable asthma. *Respiration* 2011;81:311–317. doi:10.1159/000319566
 30. Gelb AF, George SC, Camacho F, Fraser C, Flynn Taylor C, Shakkottai S. Increased nitric oxide concentrations in the small airway of older normal subjects. Increased nitric oxide concentrations in the small airway of older normal subjects. *Chest* 2011;139:368–375. doi:10.1378/chest.10-1157
 31. Paraskakis E, Brindicci C, Fleming L, Krol R, Kharitonov SA, Wilson NM, Barnes PJ, Bush A. Measurement of bronchial and alveolar nitric oxide production in normal children and children with asthma. *Am J Respir Crit Care Med* 2006;174:260–267. doi:10.1164/rccm.200506-962OC
 32. Kerckx Y, Michils A, Van Muylem A. Airway contribution to alveolar nitric oxide in healthy subjects and stable asthma patients. *J Appl Physiol* (1985) 2008;104:918–924. doi:10.1152/jappphysiol.01032.2007
 33. Gelb AF, George SC, Silkoff PE, Krishnan A, Fraser C, Taylor CF, Shinar CM, Maginot T. Central and peripheral airway/alveolar sites of exhaled nitric oxide in acute asthma. *Thorax* 2010;65:619–625. doi:10.1136/thx.2009.132696
 34. Heijkenskjold-Rentzhog C, Nordvall L, Janson C, Borres MP, Alving K, Malinowski A. Alveolar and exhaled NO in relation to asthma characteristics—effects of correction for axial diffusion. *Allergy* 2014;69:1102–1111. doi:10.1111/all.12430
 35. Verbanck S, Malinowski A, George S, Gelb AF, Vincken W, Van Muylem A. Bronchial and alveolar components of exhaled nitric oxide and their relationship. *Eur Respir J* 2012;39:1258–1261. doi:10.1183/09031936.00105611
 36. Giovannelli J, Cherot-Kornobis N, Hulo S, Ciuchete A, Clement G, Amouyel P, Matran R, Dauchet L. Both exhaled nitric oxide and blood eosinophil count were associated with mild allergic asthma only in non-smokers. *Clin Exp Allergy* 2016;46:543–554. doi:10.1111/cea.12669

37. Spears M, Weir CJ, Smith AD, McSharry C, Chaudhuri R, Johnson M, Cameron E, Thomson NC. Bronchial nitric oxide flux (J_{aw}) is sensitive to oral corticosteroids in smokers with asthma. *Respir Med* 2011;105:1823–1830. doi:[10.1016/j.rmed.2011.06.014](https://doi.org/10.1016/j.rmed.2011.06.014)
38. Orosz M, Tamási L, Gálffy G. Cigarette smoking and asthma control in Hungarian asthmatic patients. *Med Thor* 2009;62:112–119.
39. Chen Y, Mai XM. Smoking and asthma in men and women with normal weight, overweight, and obesity. *J Asthma* 2011;48:490–494. doi:[10.3109/02770903.2011.570404](https://doi.org/10.3109/02770903.2011.570404)
40. Polosa R, Thomson NC. Smoking and asthma: dangerous liaisons. *Eur Respir J* 2013;41:716–726. doi:[10.1183/09031936.00073312](https://doi.org/10.1183/09031936.00073312)
41. Konno S, Taniguchi N, Makita H, Nakamaru Y, Shimizu K, Shijubo N, Fuke S, et al. Distinct phenotypes of smokers with fixed airflow limitation identified by cluster analysis of severe asthma. *Ann Am Thorac Soc* 2018;15:33–41. doi:[10.1513/AnnalsATS.201701-065OC](https://doi.org/10.1513/AnnalsATS.201701-065OC)