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Alveolar nitric oxide *versus* measures of peripheral airway dysfunction in severe asthma

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ABSTRACT: Alveolar nitric oxide (NO) is a measure of peripheral airway inflammation in asthma, potentially associated with disease severity. The relationship between alveolar NO and physiological tests of peripheral airway (dys)function has not been investigated. The present authors hypothesised that peripheral airway inflammation and dysfunction are inter-related and associated with asthma severity.

Alveolar NO was compared between 17 patients with mild-to-moderate asthma and 14 patients with severe asthma and related to total lung capacity (TLC), residual volume (RV)/TLC, thoracic gas volume (FRC), slope of the single breath nitrogen washout curve (dN_2), closing capacity (CC)/TLC and fall in forced vital capacity at the provocative concentration of methacholine causing a 20% fall in forced expiratory volume in one second. In patients with severe asthma, strong correlations were found between alveolar NO and RV/TLC % pred, FRC % pred, dN_2, and CC/TLC. Patients with oral steroid-dependent asthma had higher alveolar NO levels (2.7 ppb) compared with the other patients with severe (0.6 ppb) and mild-to-moderate asthma (0.3 ppb).

The present authors conclude that alveolar nitric oxide is closely related to parameters of peripheral airway dysfunction in patients with severe asthma, and that oral steroid-dependent asthmatics have more peripheral airway disease than nonsteroid-dependent asthmatics. This suggests that patients on chronic oral steroid treatment have more extensive disease and require additional anti-inflammatory treatment to better target the peripheral airways.

KEYWORDS: Asthma, asthma severity, inflammation, nitric oxide, severe asthma, small airways

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The need for a greater understanding of refractory asthma has become increasingly important, since milder forms of the disease can now be well treated [1]. Little is known about the reason why some patients exhibit unstable disease despite maximum therapy with inhaled or even oral steroids. One of the proposed mechanisms is the presence of inflammation in the peripheral airways which seems to be an important feature of patients with asthma, particularly in those with severe disease [2–6]. Inflammation of the distal lung in asthma has been demonstrated in *post mortem* tissue in patients who died from an asthmatic attack [2, 6], in resected lung tissue [3] and in transbronchial biopsies [4, 5], and has been suggested to contribute to instability of the disease [5, 7], therapy resistance [8] and excessive airway narrowing [9].

Recent studies have shown that alveolar nitric oxide (NO) is a potentially useful measurement for investigating the role of peripheral airway inflammation in asthma. Alveolar NO has been related to bronchoalveolar lavage (BAL) eosinophil cationic protein levels in children [10] and to BAL eosinophil counts in adults [11]. However, the relationship between alveolar NO as a measure of peripheral airway inflammation and physiological tests of peripheral airway function has never been investigated.

Over the years, several physiological tests have been proposed to estimate the degree of peripheral airway function [7, 9, 12–15]. A few studies have investigated the relationship between some of these tests and pathological evidence of peripheral airway inflammation. The slope of the nitrogen single breath washout test (dN_2) appeared to be related to the degree of small airway inflammation in lung tissue from smokers in a study by COSIO *et al*. [12], whilst another study showed that thoracic gas volume (FRC) and total lung capacity (TLC) were related to the number of eosinophils in transbronchial biopsies from patients with severe asthma [14].

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In the present study, the current authors hypothesised that the degree of peripheral airway inflammation and dysfunction are inter-related and positively associated with asthma severity. Therefore, the present authors compared alveolar NO between patients with mild-to-moderate and severe asthma, and assessed the relationship between alveolar NO and six different physiological tests proposed to reflect peripheral airway function, including TLC, residual volume (RV)/TLC, FRC, dN_2, closing capacity (CC)/TLC, and the percentage fall in forced vital capacity (FVC) during methacholine challenge (ΔFVC).

METHODS

Subjects

A total of 17 patients with mild-to-moderate asthma and 14 with severe asthma, according to previously published criteria [16], were recruited. All had a pulmonologist diagnosis of asthma and documented reversible airway obstruction [16]. All patients were on inhaled steroid treatment. Patients with mild-to-moderate asthma were using $\leqslant$800 μg beclometasone·day^{-1} (or equivalent) with β-agonists (as necessary). Patients with severe asthma were all using high doses of inhaled corticosteroids ($\geqslant$1,600 μg beclometasone·day^{-1}, or $\geqslant$800 μg in case of chronic oral steroid use) plus long-acting β-agonists, and occasionally additional leukotriene antagonists (n=5). They had at least one exacerbation in the previous year requiring steroid treatment and/or were on chronic oral steroids. Current smokers and patients with a smoking history of >5 pack-yrs were excluded from participation. The patients visited the lung function laboratory on three different days within 1 month. On the first day, alveolar NO measurements were performed before spirometry. Methacholine provocation test was performed on the second day. On the third day, lung volume measurements and the single breath nitrogen washout test were performed. The study was approved by the Ethics Committee of the Leiden University Medical Centre (Leiden, the Netherlands) and all patients gave written informed consent.

Spirometry

Forced expiratory volume in one second (FEV_1) and inspiratory vital capacity were measured before and after bronchodilation (inhalation of 400 μg salbutamol). Reversibility was expressed as:

$$\text{post-bronchodilator } FEV_1\text{-pre-bronchodilator } FEV_1/FEV_1 \text{ per cent predicted} \quad (1)$$

Predicted values were obtained from QUANJER *et al*. [17] and used for the analysis.

Lung volumes

Lung volumes were measured (TLC, RV, FRC) pre-bronchodilation using body plethysmography according to standard methods [17]. Predicted values were obtained from QUANJER *et al*. [17].

Methacholine provocation test and assessment of ΔFVC

Methacholine challenge testing was performed using a standardised tidal breathing method [18], modified according to GIBBONS *et al*. [9]. Patients received doubling doses of methacholine starting with a dose of 0.03 mg·mL^{-1}. After each dose, FEV_1 and FVC were measured until FEV_1 dropped $\geqslant$20% compared with baseline (the provocative concentration causing a 20% fall in FEV_1; PC_{20}). The percentage fall in FVC at the PC_{20} methacholine (ΔFVC) was then calculated using log-linear interpolation.

Single breath nitrogen washout test

Nitrogen single breath washout tests were performed in order to assess ventilation inhomogeneity. The dN_2, closing volume and CC were calculated as previously described [7].

Exhaled NO and calculation of alveolar NO levels

Fractional exhaled NO (F_{ENO}) was measured according to criteria of the American Thoracic Society [19] at three different flow rates: 100, 175 and 370 mL·s^{-1}, with a chemoluminescence analyser (NOA 270B; Sievers, Boulder, CO, USA), mouth pressure 8–10 cmH_2O [20]. At each flow rate, at least three technically adequate measurements were performed. F_{ENO} was measured at a plateau between 5 and 8 s after reaching the correct exhalation flow rate or, in case of insufficient exhalation time, during a plateau phase of $\geqslant$3 s [19]. The average value was then taken for analysis. Measurements were excluded if an adequate NO plateau could not be reached or if NO levels were below the detection limit. The contributions of the bronchi (bronchial NO flux) and the alveoli (alveolar NO concentration) to F_{ENO} were derived from regression analysis, with NO output as the dependent and exhalation flow rate as the independent factor [20]. The slope and intercept of the regression line are approximate values of alveolar NO concentration and bronchial NO flux, respectively.

Analysis

Unpaired t-test, nonparametric tests (Mann-Whitney, Kruskal-Wallis) and Chi-squared analysis were used to analyse differences between groups. Spearman's correlation coefficient was used to assess relationships between different parameters. A p-value <0.05 was considered statistically significant.

RESULTS

Patient characteristics

For patient characteristics, see table 1. Patients with mild-to-moderate and severe asthma did not differ with respect to age and sex. Patients with severe asthma were using higher doses of inhaled steroids as defined in the inclusion criteria, and 43%

TABLE 1 Patient characteristics in mild-to-moderate and severe asthma

	Mild-to-moderate asthma	Severe asthma	p-value
Subjects n	17	14	
Age yrs	35.7±10.9	43.4±16.6	0.13
Sex female %	59	57	0.93
Inhaled steroid μg	445±185	1155±455	0.000
Oral steroid n	0	6	0.004
PbFEV$_1$ % pred	102 (80–120)	89 (40–109)	0.04
Reversibility %	11.0 (1–29.2)	7.2 (0–22.5)	0.003

Data are presented as mean±SD or median (range), unless otherwise stated. Pb: post-bronchodilator; FEV_1: forced expiratory volume in one second; % pred: per cent predicted.

of them were using oral corticosteroids (median (range) dose 7.5 (2.5–15) mg). Post-bronchodilator FEV1 was higher in the patients with mild-to-moderate asthma and these patients had more reversibility in FEV1.

Alveolar NO measurements

Adequate alveolar NO measurements were performed in 16 patients with mild-to-moderate and 10 patients with severe asthma. One patient with mild and four patients with severe asthma were excluded because of the lack of an adequate NO plateau or because of a NO concentration below the detection limit at one or more flow rates (this was the case in one patient with mild and two with severe asthma). The linearity of the relationship between NO output and exhalation flow rate was satisfactory (mean r=0.82). In five patients with mild-to-moderate asthma and two with severe asthma, a negative association between NO output and exhalation flow rate was found, resulting in negative alveolar NO concentrations.

Comparison between mild-to-moderate and severe asthma

Alveolar NO

Alveolar NO was not different between the patients with severe and mild-to-moderate asthma (table 2; fig. 1). However, the subgroup of oral steroid-dependent patients had significantly higher alveolar NO levels (median (range) 2.7 (2.0–9.6) ppb) than the nonsteroid-dependent patients with severe asthma (0.6 (-2.8–8.3) ppb; p=0.05) or those with mild-to-moderate asthma (0.3 (-1.4–3.9) ppb; p=0.01; table 3). F_{ENO} at 100 mL·s^{-1} and bronchial NO flux were similar in patients with mild-to-moderate and (steroid-dependent) severe asthma (p=0.93 and 0.82, respectively)

Functional parameters

In the total group of patients with severe asthma, only dN_2 and ΔFVC were higher than in the patients with mild-to-moderate asthma (table 2; fig. 2). Surprisingly, TLC % pred was higher in the patients with mild-to-moderate asthma compared with severe asthma. In the subgroup of patients with severe steroid-dependent asthma, dN_2, RV/TLC % pred and CC/TLC were significantly higher than in the other patients with severe asthma (table 3).

Relationship between alveolar NO and functional parameters

In the total group of patients with mild-to-moderate and severe asthma, there were no correlations between alveolar NO and

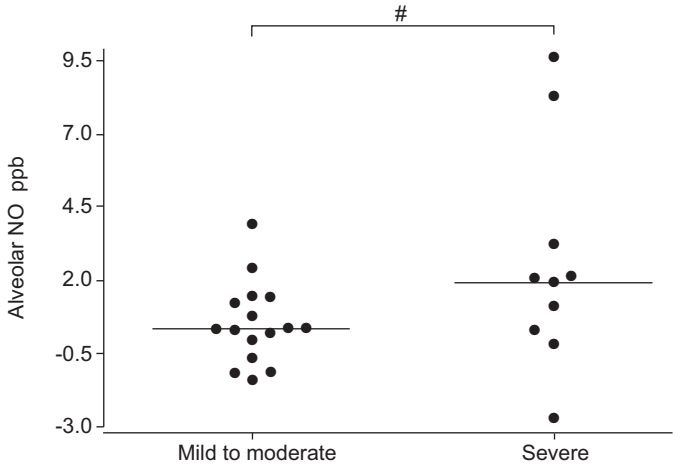

FIGURE 1. Alveolar nitric oxide (NO) levels in mild-to-moderate *versus* severe asthmatic subjects. Horizontal lines represent the median value. #: p=0.11.

TABLE 2 Outcome parameters in mild-to-moderate and severe asthma

	Mild-to-moderate asthma	Severe asthma	p-value
Subjects n	17	14	
Alveolar NO# ppb	0.3 (-1.4–3.9)	1.9 (-2.8–9.6)	0.11
Bronchial NO# nL·s^{-1}	1.9 (0.8–4.3)	2.1 (0.1–5.8)	0.94
FRC % pred	118.8±22.8	101.8±25.2	0.06
TLC % pred	104.0±11.6	94.9±8.0	0.02
RV/TLC % pred	105.5±25.9	113.4±18.2	0.35
dN_2 %·L^{-1}	0.8 (0.3–2.1)	1.6 (0.5–9.1)	0.02
CC/TLC L	39.7±6.3	46.8±12.0	0.06
ΔFVC %	10.8 (4.0–19.1)	13.5 (11.4–21.8)	0.03

Data are presented as mean±SD or median (range), unless otherwise stated. #: Alveolar nitric oxide (NO) and bronchial NO were measured in 16 patients with mild-to-moderate and 10 patients with severe asthma. FRC: functional residual capacity; % pred: per cent predicted; TLC: total lung capacity; RV: residual volume; dN_2: slope of the single breath nitrogen washout curve; CC: closing capacity; ΔFVC: percentage fall in forced vital capacity at the provocative concentration of methacholine causing a 20% fall in forced expiratory volume in one second.

TABLE 3 Comparison of steroid- and nonsteroid-dependent severe asthma

	Severe asthma		p-value
	Oral steroids	No oral steroids	
Subjects n	6	8	
Alveolar NO ppb	2.7 (2.0–9.6)	0.6 (-2.8–8.3)	0.05
Bronchial NO nL·s^{-1}	1.8 (0.1–2.4)	2.1 (0.6–5.9)	0.67
$F_{ENO_{100}}$ ppb	20.7 (5.4–33.4)	19.8 (6.6–57.8)	0.67
FRC % pred	113.5 (89–141)	84.5 (61–131)	0.07
TLC % pred	93.5 (90–104)	96 (81–111)	1.0
RV/TLC % pred	123.5 (113–162)	103.5 (86–117)	0.004
dN_2 %·L^{-1}	3.2 (1.1–9.1)	1.0 (0.5–2.2)	0.02
CC/TLC %	53.8 (44–70)	40.3 (29–53)	0.01
ΔFVC %	12.9 (11.4–14.2)	14.6 (11.7–21.8)	0.26

Data are presented as median (range). #: Alveolar nitric oxide (NO) and bronchial NO were measured in four patients with and six patients without oral steroids; $F_{ENO_{100}}$: fractional exhaled nitric oxide at an exhalation flow rate of 100 mL·s^{-1}; FRC: functional residual capacity; % pred: per cent predicted; TLC: total lung capacity; RV: residual volume; dN_2: slope of the single breath nitrogen washout curve; CC: closing capacity; ΔFVC: percentage fall in forced vital capacity at the provocative concentration of methacholine causing a 20% fall in forced expiratory volume in one second.

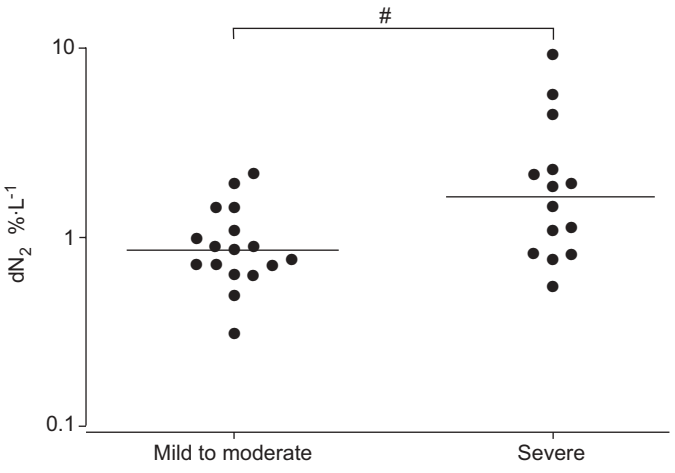

FIGURE 2. Slope of the single breath nitrogen washout test (dN_2) in mild-to-moderate *versus* severe asthmatic subjects. Horizontal lines represent the median value. #: p=0.02.

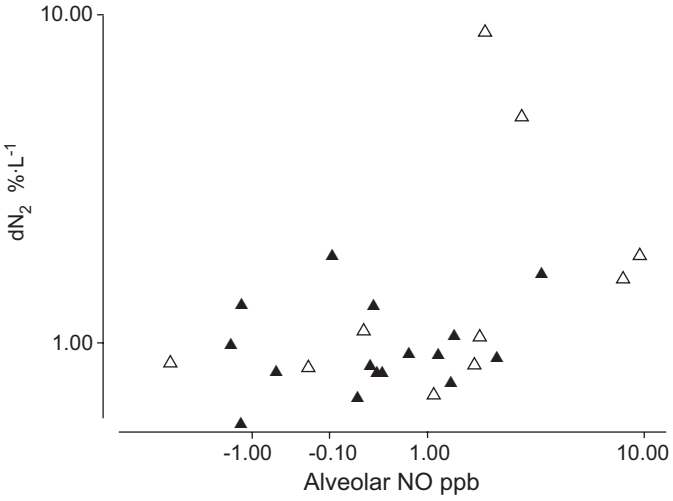

FIGURE 3. Alveolar nitric oxide (NO) levels (in ppb) *versus* slope of the single breath nitrogen washout curve (dN_2) in mild-to-moderate (▲) and severe (△) asthma (r=0.45; p=0.02). Axes are logarithmic.

functional parameters, except for dN_2 (r=0.45; p=0.02; fig. 3). However, within the group of patients with severe asthma, alveolar NO correlated strongly and positively with all functional parameters of airway dysfunction, including FRC % pred (r=0.84; p=0.002), RV/TLC % pred (r=0.83; p=0.003), dN_2 (r=0.72; p=0.02), and CC/TLC (r=0.86; p=0.002), except for TLC % pred (p=0.26) and ΔFVC (p=0.82) (fig. 4). In patients with mild-to-moderate asthma there was no correlation between alveolar NO and any of the functional parameters.

DISCUSSION

The present study shows that alveolar NO is closely related to parameters of peripheral airway dysfunction in patients with severe asthma, but not in patients with mild-to-moderate asthma. Within the group of patients with severe asthma, those patients on continuous oral corticosteroid treatment had more peripheral airway inflammation and dysfunction than patients with severe asthma on inhaled corticosteroids alone, and patients with mild-to-moderate asthma. This suggests that peripheral airway inflammation is not related to asthma severity *per se*, although it seems to be an important characteristic of patients with steroid-dependent asthma.

In the current study, alveolar NO levels could be adequately measured in the majority of adults with asthma of varying severity, including severe asthma. As compared with other investigators who measured alveolar NO in adults with mild-to-moderate persistent asthma [21], severe asthma [11], and in children with mild [22] and refractory asthma [10], the present authors obtained alveolar NO levels in a lower range (3–4-fold lower). This could be due to differences between patient characteristics and/or differences in the technical aspects of the measurements, such as the use of higher flow rates to calculate alveolar NO in the present study. The alveolar NO levels in the current study were comparable to those of LEHTIMAKI and co-workers [23, 24], who used the same flow rates and was the first to measure alveolar NO levels in patients with asthma.

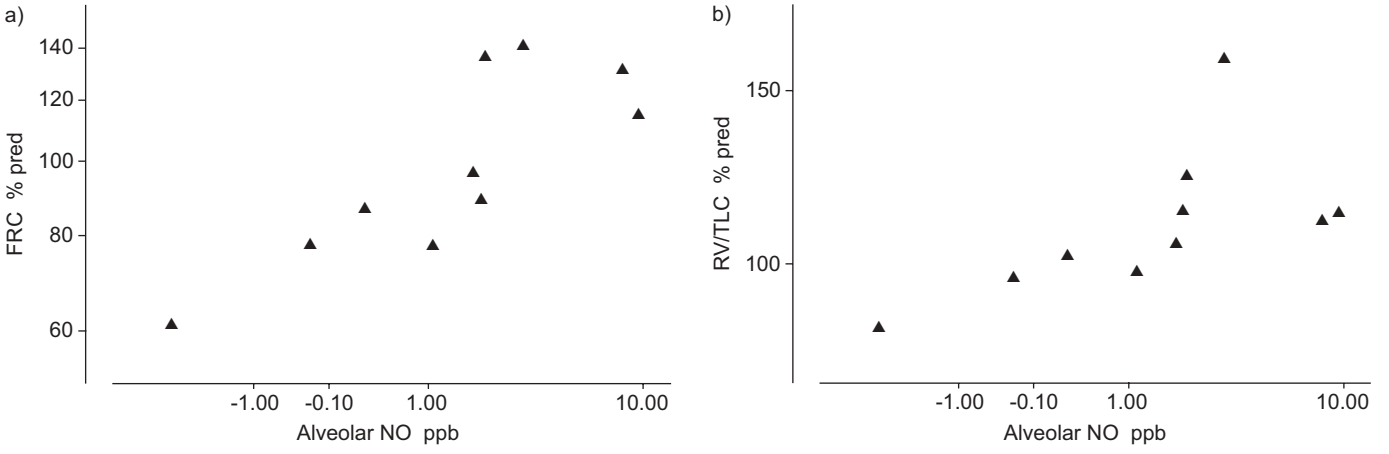

FIGURE 4. Alveolar nitric oxide (NO) levels (in ppb) *versus* functional residual capacity (FRC) per cent predicted (% pred) (r=0.84; p=0.002) and residual volume (RV)/total lung capacity (TLC) % pred (r=0.83; p=0.003) in severe asthma. Axes are logarithmic.

Comparison of alveolar NO levels between patients with mild-to-moderate and severe asthma revealed no significant differences after initial analysis, which was surprising. In studies by other groups, higher levels of alveolar NO were found in symptomatic children *versus* asymptomatic children with asthma [22], in patients with nocturnal asthma compared with non-nocturnal asthma [23], and in patients with severe *versus* mild-to-moderate asthma [11]. An explanation for the lack of a significant difference in alveolar NO levels between patients with mild-to-moderate and severe asthma in the present study could be the large variability of alveolar NO levels amongst the patients with severe asthma. Clearly, these patients are heterogeneous with respect to their degree of peripheral airway inflammation. When considering the subgroup of patients with oral corticosteroid dependency, much higher alveolar NO levels were observed compared with patients on inhaled corticosteroids alone. The same holds true for the parameters of peripheral airway dysfunction. This is remarkable because one could expect that systemic treatment in these patients would reduce the amount of peripheral airway inflammation and dysfunction. It appears that there is extensive peripheral airway disease in these patients which is not fully controlled by such a high level of anti-inflammatory treatment. The heterogeneity of patients with severe asthma is thus an important factor to be taken into account when studying disease mechanisms in these patients.

Alveolar NO and parameters of peripheral airway dysfunction showed strong positive correlations in the patients with severe asthma, but not in patients with mild asthma or in the group as a whole (except for dN_2). Most of these parameters are probably not sensitive enough to detect small changes in patients with milder disease of the peripheral airways. The strong association between alveolar NO and almost all parameters of airway dysfunction suggests that these two entities coexist and are possibly causally related. This fits in with the recently observed relationship between F_{ENO} and dN_2 in patients with mild asthma [25] and in smokers [26]. Additional anti-inflammatory treatment specifically targeting the peripheral airways might improve peripheral airway function and thereby overall lung function in patients with severe asthma.

ΔFVC was not related to alveolar NO, although there was a significant difference in this parameter between mild-to-moderate and severe asthma. ΔFVC is a measurement that is performed after stimulation of the bronchi with the bronchoconstrictor agent methacholine. It is assumed that the more the FVC decreases during bronchoconstriction the more closure of the peripheral airways has occurred. The major difference with the other tests of peripheral airway dysfunction is that ΔFVC is measured in constricted airways, thereby mimicking an acute asthma attack. The discrepancy between ΔFVC and the other tests suggest that airway dysfunction at rest does not predict the dynamics of peripheral airway dysfunction during acute bronchoconstriction. This is compatible with mathematical models of airway function [27]. Thus, peripheral airway disease at rest and peripheral airway dysfunction during bronchoconstriction are two distinct conditions that do not necessarily coexist within the same subject, and may be reflected by different tests of peripheral airways disease.

The present study may have some limitations. First, the power of the study might not have been enough to detect statistical significant differences between the groups of patients with mild-to-moderate and severe asthma. In retrospect, based on the present study data, the power was enough to detect a difference in mean alveolar NO of 5.0 ppb. In order to detect a difference of 2 ppb, 57 patients would have been needed in each group. Secondly, the calculation of alveolar NO is based on a mathematical two-compartment model, which has its limitations. In seven patients, negative alveolar NO values were calculated, while the measurements were technically adequately performed. The choice of expiratory flows rates of 100, 175 and 370 $mL \cdot s^{-1}$ hampered an adequate estimation of the NO concentration–expiratory flow rate curve in these patients. To improve this, the current authors would have needed measurements at extra flow rates, especially in the higher range; however, this is difficult to achieve in patients with flow limitation. This indicates that the model used (with these expiratory flows) was not completely sufficient for all patients. Until now, however, there are no better tests for the assessment of peripheral airway inflammation in patients with asthma except for invasive tests such as transbronchial biopsies.

The results of the present study have implications for the assessment and treatment of patients with severe asthma. Similar to the F_{ENO}, alveolar NO could be successfully used to guide therapy in asthma [28, 29]. The current study shows that patients with similar F_{ENO} can have different alveolar NO levels. Thus, alveolar NO provides additive information for the clinician. The elevated levels of alveolar NO in patients already on continuous oral corticosteroids suggest that the inflammatory process in the peripheral airways is extensive and still relatively undertreated despite the administration of systemic treatment. Therefore, measurement of alveolar NO levels might be used in clinical practice to adjust anti-inflammatory treatment, for example, by the addition of fine-particle inhaled corticosteroids or novel systemic anti-inflammatory therapies. In this way, it might be possible to improve disease severity, avoid excessive airway narrowing and prevent a poor prognosis.

In conclusion, alveolar NO is an easy to perform, noninvasive test to estimate the degree of peripheral airway inflammation. It provides important information on peripheral airway disease and can be used in addition to functional tests, such as total lung capacity, residual volume/total lung capacity, functional residual capacity, slope of the single breath nitrogen washout curve, closing capacity/total lung capacity or percentage fall in forced vital capacity at the provocative concentration of methacholine causing a 20% fall in forced expiratory volume in one second. Patients with severe asthma are heterogeneous with respect to the degree of peripheral airway disease. Those patients who are dependent on oral corticosteroids seem to have the most prominent signs of peripheral airway inflammation and dysfunction. This suggests that these patients might require additional or alternative anti-inflammatory treatment to better target the peripheral airways. Alveolar nitric oxide might become an important new tool for the clinician to detect insufficiencies of asthma treatment, to tailor asthma therapies in individual patients, and to improve asthma control.

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